Scientific Publications on Glutathione

Glutathione Not just a fairness injection

Compiled by Dr L. Prakash

Medical Uses of Intravenous Glutathione: A Comprehensive Overview with selected publications from various Journals

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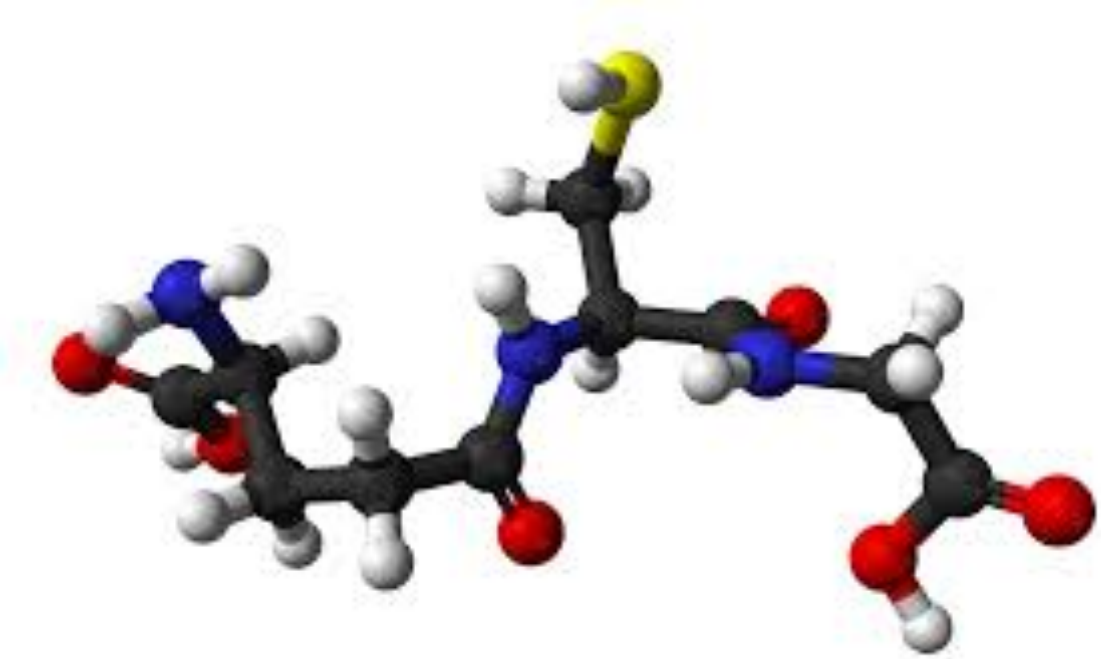
Introduction

Glutathione, often referred to as the "master antioxidant," is a naturally occurring compound in the human body, composed of three amino acids: glutamine, glycine, and cysteine. Its role in maintaining cellular health, detoxification, and immune system function has made it a cornerstone in integrative and alternative medicine. While oral and topical forms of glutathione are available, **intravenous (IV) glutathione therapy** offers enhanced bioavailability and quicker therapeutic effects, making it a popular choice in medical and aesthetic practices.



Glutathione molecule

What is Intravenous Glutathione Therapy?

Intravenous glutathione involves directly administering the antioxidant into the bloodstream, bypassing the digestive system. This method ensures maximum absorption

and immediate availability at the cellular level, where it can exert its protective and

Intra Venous Glutathione injection

regenerative effects.

Medical Uses of IV Glutathione

1. Detoxification and Liver Support

Glutathione is a key player in liver detoxification processes, especially in the breakdown of toxins and heavy metals. IV administration is commonly used in:

- **Chronic Liver Diseases**: Helps manage conditions like hepatitis, fatty liver, and cirrhosis by reducing oxidative stress and inflammation.
- **Drug-Induced Liver Damage**: Protects liver cells from the toxic effects of certain medications, such as acetaminophen overdose.
- **Heavy Metal Detoxification**: Enhances the body's ability to excrete heavy metals like mercury, lead, and arsenic.

2. Antioxidant and Anti-Aging Therapy

As a powerful antioxidant, glutathione neutralises free radicals that contribute to aging and chronic diseases. IV therapy:

- Slows down cellular aging by reducing oxidative damage.
- Improves skin health and elasticity, making it a popular choice in **anti-aging treatments**.
- Enhances overall vitality and energy levels by supporting mitochondrial function.

3. Neurological Protection

Glutathione's neuroprotective properties make it beneficial in managing neurological disorders:

- **Parkinson's Disease**: Helps mitigate symptoms by protecting dopaminergic neurons from oxidative damage.
- **Alzheimer's Disease**: May slow cognitive decline by reducing brain inflammation and oxidative stress.
- **Multiple Sclerosis**: Supports myelin regeneration and immune modulation.

4. Immune System Support

Glutathione plays a critical role in maintaining a balanced immune response. IV therapy is often used in:

- **Autoimmune Conditions**: Reduces inflammation and regulates overactive immune responses in diseases like lupus and rheumatoid arthritis.
- **Chronic Infections**: Boosts immune defense against persistent infections such as Epstein-Barr virus and Lyme disease.

5. Skin Whitening and Brightening

One of the most well-known uses of IV glutathione is in skin aesthetics. By inhibiting melanin production, glutathione:

- Lightens skin tone and reduces hyperpigmentation.
- Promotes an even complexion.
  This application is particularly popular in regions where lighter skin is culturally preferred, although its safety and efficacy for long-term use are still debated.

6. Cardiovascular Health

IV glutathione improves cardiovascular health by reducing oxidative stress and inflammation in blood vessels. Its benefits include:

- Lowering blood pressure in hypertensive individuals.
- Enhancing circulation by preventing arterial plaque buildup.

7. Oncology Support

While glutathione can protect healthy cells from oxidative damage caused by chemotherapy, its use in cancer treatment is nuanced. It is often used to:

- Reduce chemotherapy side effects, such as neuropathy and fatigue.
- Support recovery during and after cancer treatment.
  However, careful consideration is needed as glutathione may also protect cancer cells in certain scenarios.

8. Chronic Fatigue and Fibromyalgia

Patients with chronic fatigue syndrome and fibromyalgia often benefit from IV glutathione therapy, as it:

- Increases energy by optimising mitochondrial function.
- Reduces muscle pain and stiffness through its anti-inflammatory effects.

9. Respiratory Conditions

Glutathione is effective in managing chronic respiratory diseases due to its ability to reduce oxidative damage in lung tissues. IV therapy is used for:

- **Chronic Obstructive Pulmonary Disease (COPD)**: Improves lung function and reduces inflammation.
- **Asthma**: Lowers oxidative stress and inflammation in airway tissues.

How IV Glutathione Therapy Works

- **Oxidative Stress Reduction**: Neutralises free radicals and supports other antioxidants like vitamin C and E.
- **Detox Pathway Activation**: Enhances liver enzymes involved in detoxification (Phase II detoxification).
- **Cellular Repair and Regeneration**: Improves mitochondrial function and DNA repair.

Benefits of IV Glutathione Over Other Forms

1. **Superior Absorption**: IV glutathione bypasses the digestive system, avoiding degradation in the gastrointestinal tract.
2. **Rapid Action**: Immediate availability in the bloodstream allows for quicker therapeutic effects.
3. **Targeted Dosing**: High concentrations can be administered to meet specific clinical needs.

Safety and Precautions

IV glutathione is generally well-tolerated, but potential side effects include:

- Mild nausea or dizziness during infusion.
- Rare allergic reactions, which should be addressed immediately.

Contraindications:

- Patients with certain cancers on chemotherapy may require physician consultation before starting glutathione therapy, as its anti oxidative properties could interfere with treatment goals.

Conclusion

Intravenous glutathione is a versatile and powerful therapy with applications ranging from detoxification and liver support to anti-aging and neurological protection. Its ability to directly address oxidative stress and inflammation makes it invaluable in both chronic disease management and preventive care. As research continues to expand our understanding of its benefits, IV glutathione is likely to remain a cornerstone of integrative and functional medicine.

Patients and practitioners alike should consider this therapy not only for its wide-ranging benefits but also for its potential to enhance overall health and well-being.

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Glutathione: Overview of its protective roles, measurement, and biosynthesis

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Abstract

This review is the introduction to a special issue concerning, glutathione (GSH), the most abundant low molecular weight thiol compound synthesized in cells. GSH plays critical roles in protecting cells from oxidative damage and the toxicity of xenobiotic electrophiles, and maintaining redox homeostasis. Here, the functions and GSH and the sources of oxidants and electrophiles, the elimination of oxidants by reduction and electrophiles by conjugation with GSH are briefly described. Methods of assessing GSH status in the cells are also described. GSH synthesis and its regulation are addressed along with therapeutic approaches for manipulating GSH content that have been proposed. The purpose here is to provide a brief overview of some of the important aspects of glutathione metabolism as part of this special issue that will provide a more comprehensive review of the state of knowledge regarding this essential molecule.

Keywords

Glutathione; Glutamate cysteine ligase; Hydroperoxide; Xenobiotic; Methods

1. Introduction

The tripeptide, γ-L-glutamyl-L-cysteinyl-glycine known as glutathione (GSH) (Fig. 1), is the most important low molecular weight antioxidant synthesized in cells. It is synthesized by the sequential addition of cysteine to glutamate followed by the addition of glycine. The sulfhydryl group (−SH) of the cysteine is involved in reduction and conjugation reactions that are usually considered as the most important functions of GSH. These reactions provide the means for removal of peroxides and many xenobiotic compounds; however, GSH is also involved in regulation of the cell cycle (Meister 1992).

2. Sources of oxidants

GSH plays a major role in removal of many reactive species. But, before addressing those aspects, it is important to understand from where these reactive species come and their pathological consequences that GSH helps avoid. Quinones are a class of redox cycling molecules that includes some drugs and xenobiotic compounds. Redox cycling in this context refers to the ability to cycle between oxidized and reduced forms and in the process, produce reactive oxygen species, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2). In this reaction (Fig. 2), the quinone is reduced by one electron transport reaction to produce a semiquinone, which is a free radical that can react with oxygen to produce O_2^-.

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There are other places in the cells where reactive oxygen species can be generated. In phagocytes, a major part of the mechanism of killing microorganisms involves production of reactive oxygen species (Forman and Thomas, 1986). The first enzyme involved is NADPH oxidase (NOX) that produces O_2^-. That enzyme is now known to be a member of a class of enzymes found in almost all cells (Vignais 2002). Once O_2^- is made, it can be dismuted into H_2O_2 both by a relatively fast non-enzymatic reaction and by very fast reaction catalyzed by one of the superoxide dismutases (SOD). Some phagocytes have the capacity to secrete enzymes called myeleoperoxidases that can catalyze a reaction of H_2O_2 and halides (chloride or bromide) to produce hypochlorous acid (HOCl) or hypobromous acid (HOBr) (Bakkenist et al., 1980). These hypohalous acids kill bacteria but can also damage normal tissue and thereby contribute to an inflammatory reaction.

The H_2O_2 formed can also be potentially hazardous if there are reduced metals present in the cells. H_2O_2 can react with ferrous iron (Fe^{2+}) and produce the hydroxyl radical (OH). This radical has capability to oxidize Teflon or fluorine and any organic molecule at near diffusion limited rates. In other words, OH can react with any molecule next to where it is produced. O_2^- can reduce ferric iron (Fe^{3+}) to Fe^{2+}, which suggests that it can play two roles in producing OH; however, reduction of Fe^{3+}can also occur with other reductants such as ascorbic acid (vitamin C).

One of the dangers of producing OH is when it is produced near a membrane. Lipids can be oxidized by OH and start a free radical chain reaction that will damage the membrane. In the initiation of lipid peroxidation by OH, the reaction with a reduced molecule of the lipid produces a lipid radical (L) and water. The L can react with oxygen to produce hydroperoxide radical (LOO), which then reacts with another lipid molecule, generating a lipid peroxide (LOOH) and another lipid radical L that can continue a chain reaction. One of the dangers from lipid peroxidation besides membrane damage is the production of byproducts such as 4-hydroxy-2-nonenal (HNE). Arachidonic acid is a polyunsaturated fatty acid found in membranes of all cells. When it becomes oxidized, it can break down yielding a large variety of compounds including a, β-unsaturated aldehydes (Poli et al., 1987) These are toxic compounds because they can react with proteins in the cells, particularly at cysteine, lysine or histidine by either Michael addition to the carbon–carbon double bond or by Schiff base formation at the carbon–oxygen double bond (Esterbauer et al., 1991; Eckl, 2003; Schaur, 2003). These reactions can inactivate the function of proteins. For example, reaction with an active site cysteine can destroy the activity of an enzyme.

The final component of oxidative damage considered here is peroxynitrite ($ONOO^-$). This ion is made in a reaction between nitrogen oxide (NO) and O_2^-. These two free radicals react at the fastest rate of any reaction known to occur in biology and is the only reaction that is faster than the dismutation reaction of O_2^- via superoxide dismutases. In its basic form, $ONOO^-$ does not react with organic molecule, it breaks down to form nitrite (NO_2^-) and nitrate (NO_3^-) But when peroxynitrite is protonated it becomes the highly reactive, peroxynitrous acid (ONOOH) that has the reactivity of nitrogen dioxide (NO_2), a very toxic free radical component in smog and cigarette smoke, and OH.

3. Protective functions of glutathione

3.1. Reduction

GSH is found in the cytosol of cells where it is in the range of 1–10 mM (Meister 1988). In most cells the GSH concentration is about 1–2 mM, while in hepatocytes, which export GSH, the concentration can reach about 10 mM. So why do we need GSH outside of the cells? In plasma GSH is in the micromolar range; however, in some extracellular spaces such as the lining fluid of the lung, a thin layer of fluid covering the air spaces where gas exchange occurs,

there is high concentration of GSH that is secreted by epithelial cells (Sutherland et al., 1985; Cantin et al., 1987). In people who smoke or inhale particles or other oxidants, there is potential inflammation that involves invasion of neutrophils from the blood through the endothelial and epithelial cells into the air spaces. As these neutrophils squeeze between the cells, they release HOCl, which can react with GSH secreted from the epithelial cells that normally protects the epithelial cells (Venglarik et al., 2003).

In cystic fibrosis patients, who secrete lower GSH than normal individuals into the lining fluid covering their alveoli, and in smokers, who have exposed their lungs to many oxidants including nitrogen dioxide and H_2O_2, there is both chronic inflammation and lower than normal GSH (Roum et al., 1993). In that case, HOCl can oxidize proteins in the lining fluid or on the surface of the epithelial cells. It can also react with lipid to produce even more dangerous compounds than are produced by lipid peroxidation itself (Pullar et al., 2000). Fig. 3 shows how GSH reacts with HOCl and removes it (Winterbourn and Brennan, 1997). While many studies of GSH in inflammation have been done of the lungs, these reactions can occur in any organ.

Secretion of GSH to the air space in cystic fibrosis is depressed because of a mutation of a protein called cystic fibrosis transport receptor (CFTR) (Roum et al., 1993). The CFT1 cell line, which is derived from a cystic fibrosis patient, has lower GSH secretion to the apical (air space) side. If the wild type CFTR is transfected into the cells, the rate of GSH secretion is increased to the level seen in normal cells (Gao et al., 1999). The generation of HOCl in the surface fluid covering normal epithelial cells to mimic the action of stimulated neutrophils can decrease in the electrical resistance of that epithelial cell layer; however, the presence of GSH at a concentration similar to normal lining fluid protects against the loss of electrical resistance (Venglarik et al., 2003). Similar events occur during inflammation and are exaggerated in cystic fibrosis patients. There is some evidence that other lung diseases, such as idiopathic pulmonary fibrosis, also have a lower GSH concentration (Cantin et al., 1989). Further studies on the potential contribution of GSH deficiency to these pathologies are needed. Understanding the transport of GSH across the plasma membrane is an important issue that is essential to treatment of diseases involving oxidative stress (see reviews by Ballatori et al., 2008 and by Yuan and Kaplowitz, 2008 in this issue).

Compared to the extracellular environment, what happens inside of the cells is quite different. Glutathione plays major roles in the different cellular compartments. In mitochondria it plays a key role in regulating apoptosis versus necrosis (see review by Yuan and Kaplowitz 2008 in this issue). In the nucleus, GSH is a key regulator of cellular division (see review by Pallardó et al., 2008 in this issue.) While lungs are clearly adversely affected by lowered intracellular and extracellular GSH, the majority of studies on the pathologies involving GSH transport and metabolism have been done in liver. Reviews of the involvement of altered intracellular GSH in lung diseases (Biswas and Rahman, 2008), liver diseases (Yuan and Kaplowitz, 2008) and viral diseases (Fraternale et al., 2008) can be found in this issue.

Most of the GSH in antioxidant defense in cells is utilized by three members of glutathione peroxidase (GPx) family (Brigelius-Flohe, 1999) and by one of the peroxiredoxins (Prdx 6). These enzymes catalyze the reduction of H_2O_2 by GSH into H_2O and GSSG. Prdx 6 also requires GSH S transferase Pi in order to be active (Ralat et al., 2006). Phospholipid hydroperoxide glutathione peroxidase (PHGPx or GPx IV) can reduce lipid peroxides to lipid alcohols (Imai and Nakagawa, 2003). GSSG is potentially toxic to the cells but cells normally contain high glutathione reductase activity, which maintain most of the GSH in the reduced form. Some GSSG is also secreted from cells. During oxidative stress, GSSG could react by disulfide exchange with a protein thiol to produce a protein mixed disulfide (PSSG), which can further exchange with another protein thiol to a protein disulfide (Huang and Huang,

2002). These reactions are actually quite slow unless catalyzed by an enzyme such as protein disulfide isomerase (PDI), an important enzyme that is particularly abundant in the endoplasm reticulum where protein folding occurs. In fact, the cysternae of the endoplasm reticulum is the only part of the cell with a relatively high ratio of GSSG/GSH. In the cytosol formation of PSSG is transient except during oxidative stress.

Formation of PSSG with some enzymes may play a role in signal transduction although the exact mechanism of their formation is uncertain. So how might PSSG form during normal metabolism in the cells? While protein disulfide exchange with a thiol can be catalyzed by PDI, some proteins contain a microenvironment in which thiolate ($-S^-$), which is far more reactive than is a thiol in both reaction with H_2O_2 or disulfide exchange, is formed. This requires that the microenvironment be composed in part by basic amino acids in proximity to the cysteine to allow dissociation of the thiol, which normally has a pK_a of around 8.3. GSH peroxidase catalyzes the production of GSSG, which could be potentially exchanged with a thiolate to form mixed disulfide. But in the cytosol, even during oxidative stress, the ratio of GSH/GSSG remains very high, which makes that exchange reaction unfavorable. The enzyme PDI can enhance the rate of that reaction but, like any catalyst, cannot change the equilibrium. Instead, it has been proposed that during physiological signaling when the H_2O_2 is used as the second messenger, some of protein thiolates could potentially react and form sulfenic acid (PSOH) (Fig. 4); however, for most thiolates including that formed by glutathione, the rate of the non-enzymatic reaction is too slow to account for the inactivation of the enzymes (Forman, 2007). We do know that in the active site of peroxiredoxins, where the reaction of H_2O_2 with a thiolate can occur up to six orders of magnitude faster than with glutathione in its thiolate form, the reaction can occur. Regardless, once formed, a protein sulfenate would rapidly react with GSH to produce the mixed disulfide, and this could be the mechanism through which PSSG formed for some proteins in the cytosol during oxidative stress when H_2O_2 is high enough to overcome a slow rate constant.

3.2. Conjugation

The elimination of many xenobiotic compounds can be accomplished through conjugation with GSH followed by secretion of the adduct from the cell (Boyland and Chasseaud 1969). Although the quinone, menadione, can react with GSH to form an adduct non-enzymatically, an enzymatic catalyzed Michael addition by a glutathione-S transferase (GST) is much faster. The glutathione adduct can then be secreted from cells through a membrane transporter such as the multidrug resistant proteins. The product of the addition of GSH can also rearrange into a quinol that are usually considered to be less toxic than the quinone (see above).

GSH is also used in the elimination of electrophiles such as HNE. Almost all these reactions are catalyzed by GSTs, and there is a specific one in human cells that can cause the conjugation of GSH to HNE at about 100 times faster rate than the non-enzymatic reaction. The conjugate, which is a Michael adduct (because the reaction is a Michael addition), can rearrange to form a cyclic hemiacetal (Fig. 5) (Alary et al., 2003). Both of the compounds however, can be excreted from the cells. This is the major route of elimination of HNE and other electrophiles that conjugate with GSH.

3.3. Interaction with other non-enzymatic antioxidants

While GSH is the most important small molecular weight antioxidant produced in the cells, there are other small molecular antioxidants obtained from the diet such as vitamins E (α-tocopherol) and C (ascorbic acid). Vitamin E can reduce lipid hydroxyl radicals and lipid peroxides that are produced from polyunsaturated fatty acids. The oxidized vitamin E is then reduced by vitamin C in a non-enzymatic but rapid reaction. The oxidized vitamin C can then be restored to the reduced form by enzymatic reactions, one of which uses GSH as substrate.

4. Measurement of glutathione

One of the important issues in determining the mechanisms of both oxidative stress and redox signaling is the measurement of the different forms of thiols in cells. The predominant forms are the reduced form of GSH and GSSG. Nitrosoglutathione (GSNO) and protein nitrosothiols (PSNO) are also formed in cells and play a role in NO signaling independent of the cyclic GMP pathway. Cysteine is a precursor amino acid of GSH and cystine is the disulfide form of cysteine. Protein thiols exist as cysteine, mixed disulfides between cysteine and GSH or other thiols, and disulfides between two protein cysteines that may be in the same or different protein molecules. It is important to recognize that an increase in the oxidized forms of these thiols in the cytosol will be transient even during oxidative stress. Therefore it can be very difficult to measure thiol oxidation, particularly that occurring in signal transduction.

GSH reacts with dithionitrobenzoic acid (DTNB) (Akerboom and Sies, 1981) and by reducing GSSG total GSH (GSH + GSSG) can be measured. DTNB reacts with GSH to produce a conjugate and TNB anion that can be detected by fluorescence or absorbance (Fig. 6a). To measure total GSH, a recycling assay is used in which GSH reacts with the conjugate producing GSSG and another molecule of TNB, which can be increases fluorescence or absorbance (Fig. 6b). The enzyme glutathione reductase then reduces the GSSG releasing the GSH that can react with another molecule of DTNB. Therefore, instead of a single determination of how much DTNB reacts with GSH, the rate of TNB production is measured, as that is proportional to the initial amount of GSH. To measure GSSG however, one must first modify the GSH present at the beginning so it is removed from the recycling assay. Modification of GSH is done with N-ethylmaleimide (NEM) or vinylpyridine. To measure protein mixed disulfides, the GSH can be released from the protein mixed disulfide with sodium borohydride ($NaBH_4$), and the GSH is then measured in the recycling assay.

A more commonly used procedure for measuring GSH and GSSG now is high performance liquid chromatography (HPLC) (Fariss and Reed, 1987). In this assay, thiol compounds are first modified by the addition of iodoacetate (Fig. 6c). The amino groups on the compound then are modified by 1-fluoro-2, 4-dinitrobenzene. This then allows separation of many compounds that can be identified by their movement on HPLC.

On method that has been developed to measure nitrosoglutathione involves the production of GSH from it followed by reaction with orthophthaldehyde (OPT) to produce a fluorescent compound (Fig. 6d) (Tsikas et al., 1999) while another method uses a biotinylated fluorescent label in a method called the biotin-switch (Gladwin et al., 2006). First however, as with the measurement of GSSG above, it is necessary to first remove any GSH in the original sample with methyl methanethiosulfonate before reducing GSNO to release GSH. Various reagents have been proposed as best for differentially reducing GSNO as well as PSNO especially as the presence of GSSG or protein mixed disulfides can also yield GSH upon reduction (Gladwin et al., 2006). After reaction with OPT the products are separated by HPLC with a fluorescence detector. There are other methods for measuring GSNO such as using ^{15}N labeling (Kluge et al., 1997), but this is not commonly used and requires mass spectrometry.

5. Glutathione synthesis

The first step in de novo GSH synthesis involves the combination of cysteine with glutamate to produce γ-glutamylcysteine. This reaction is catalyzed by the enzyme glutamate cysteine ligase (GCL), which is also called γ-glutamylcysteine synthetase (Fig. 7). This enzyme requires coupled ATP hydrolysis to form an amide bond between the γ-carboxyl group of glutamate and the amino group of cysteine (Huang et al., 1993). The next step involves the enzyme glutathione synthetase, responsible for adding glycine to the dipeptide to produce GSH (γ-glutamylcysteinylglycine) and also requires coupled ATP hydrolysis (Meister, 1974).

GSH can be transported out of cells. This mechanism is physiologically important as hepatocytes supply GSH found in the plasma, which is used as a source of cysteine for GSH synthesis in other cells (Anderson et al., 1980). In fact, GSH in the plasma is maintained at very low concentration because of the metabolism of GSH by many other cells (Sies and Graf, 1985; Hirota et al., 1986). This process requires two enzymes commonly found on the surfaces of cells. The enzyme γ-glutamyl transpeptidase transfers a glutamate to other amino acids releasing cysteinylglycine, which in turn can be broken down by a dipeptidase to produce cysteine and glycine (Kozak and Tate 1982; Hirota et al., 1986). Cysteine and glycine as well as γ-glutamyl amino acids are moved into cells by specific amino acid transporters and used for GSH biosynthesis (Meister, 1991).

5.1. Regulation of glutamate cysteine ligase activity

GCL is regulated at both the level of its enzymatic activity and the expression of its two subunits. One subunit is the relatively heavy (− 73 kDa) subunit, which has competent but low catalytic activity for production of γ-glutamylcysteine. The catalytic subunit, designated as GCLC, can be feedback inhibited by GSH (Huang et al., 1993). The lower molecular weight (− 28 kDa) subunit regulates the activity of the enzyme by reducing the inhibition by GSH (Huang et al., 1993; Choi et al., 2000) and with purified enzyme has been shown to also decrease the K_M for glutamate (Huang et al., 1993). This subunit, which is designated as GCLM for its modulatory activity can affect the steady state level of GSH found in cells when GCLM/GCLC expression is altered (Richman and Meister, 1975; Choi et al., 2000; Krzywanski et al., 2004). Thus, increased expression of GCLC will tend to elevate GSH while increasing GCLM/GCLC will further increase GSH. An example of when lowering GCLM/GCLC causes decreased GSH is the expression of the HIV-Tat protein, which suppresses GCLM expression (Choi et al., 2000). Finally, the kinetics of GCL seems to be regulated by phosphorylation of both subunits as well (Sun et al., 1996). The functional roles of the two GCL subunits are reviewed in this issue by Franklin et al. (2008)).

5.2. Regulation of glutamate cysteine ligase expression

The expression of GCL is also regulated at many levels. Oxidant species and electrophiles are able to increase the transcription of both the modulatory and catalytic subunits (Shi et al., 1994; Rahman et al., 1996; Tian et al., 1997) (also see review by Lu, 2008 in this issue). This occurs by the activation of signal transduction pathways involved in the control of transcription of GCLC and GCLM genes but also there is some evidence of mRNA stabilization by oxidants and electrophiles (Liu et al., 1998).

It has been known for almost twenty years that sublethal concentrations of electrophiles could increase GSH production (Ogino et al., 1989; Darley-Usmar et al., 1991); however, it was unclear whether the increase was on the kinetic or the transcriptional level or even whether GSSG reduction was increased. Using redox cycling quinones to increase production of hydrogen peroxide and by measuring transcription by nuclear run-on analysis, it was then shown that a sustained increase the amount of GSH in cells could be achieved by increasing the transcription of GCLC (Shi et al., 1994; Shi et al., 1994). Subsequently many labs showed that a variety of other agents, able to generate an oxidative stress throughH_2O_2 generation, increasing concentrations of electrophiles or nitric oxide could also induce GCLC or GCLM subunits or both (Rahman et al., 1996; Tian et al., 1997; Galloway and McLellan, 1998; Liu et al., 1998; Moellering et al., 1999; Wild and Mulcahy, 1999).

The GCLC and GCLM promoter sequences were described first from humans and then they were determined in rodents (Gipp et al., 1992; Gipp et al., 1995; Hudson and Kavanagh, 2000; Yang et al., 2001). The human and rodent promoters have some similar cis elements and appear to be regulated somewhat differently than the human genes (Iles and Liu 2005) (see

review by Lu, 2008 in this issue). For the human GCL genes, the promoter enhancer regions of the two genes contain several elements able to respond to oxidants and electrophiles (Gipp et al., 1992; Gipp et al., 1995; Yang et al., 2001; Dickinson et al., 2002). One of the important oxidant responsive cis elements (transcription factor binding sites) regulating GCL genes is the AP-1 binding site also called the TRE element. TRE binds members of the Jun and Fos family of transcription factors (Ofir et al., 1990; Binetruy et al., 1991). Another important element in human GCL gene promoters that responds to electrophiles in cells and increases gene expression is the EpRE or electrophile response element (Rushmore et al., 1991; Jaiswal, 1994; Vasiliou et al., 1995). EpRE elements are also present in both human GCLC and GCLM promoters (Gipp et al., 1992; Gipp et al., 1995). Initially EpRE was called the antioxidant response element (ARE) because the first compound, shown to activate ARE was a so-called antioxidant that was subsequently shown to generate H_2O_2 through redox cycling (Pinkus et al., 1996). The EpRE elements bind proteins members of the Nrf family, Jun family and small Maf family (Venugopal and Jaiswal, 1998;Kong et al., 2001; Moran et al., 2002; Itoh et al., 2004). One of the transcription factors established as able to bind EpRE is Nrf2, which located in the cytosol through the inhibitory interaction with Keap1 in resting cells. Upon stimulation, Nrf2 is translocated into the nucleus after dissociation from Keap1 (Itoh et al., 1999).

While the redox and electrophilic response cis elements have been identified, less has been done to identify the signaling mechanisms that activate the transcription factors that bind to those elements. We will describe here briefly what is understood regarding the signaling by HNE. Darley–Usmar and coworkers have shown that HNE directly modifies Keap1, which allows Nrf2 to avoid degradation and migrate to the nucleus where it can bind to EpRE elements in the promoters of the human GCLC and GCLM genes (Levonen et al., 2004). But, this cannot be the whole story as there are actually multiple EpRE elements in the promoters and not all of them are involved in regulating transcription (Dickinson et al., 2004). While Nrf2 is critical, EpRE binding also involves a partner protein. For the EpRE element that regulates transcription of GCLC in human bronchial epithelial cells that partner has not yet been firmly identified.

More is understood about the TRE element. Interestingly, the TRE element in the human GCLC promoter appears to bind c-Jun dimers preferentially (Rahman et al., 1999). For HNE induction, the activation of the critical AP-1 binding elements in both human GCL genes can is achieved through the Jun N-terminal kinase (JNK) pathway (Dickinson et al., 2002). JNK phosphorylates c-Jun, which translocates into the nucleus, and binds to the TRE element. Inhibition of JNK completely eliminates GCLC and GCLM gene expression in response to HNE in human bronchial epithelial cells while inhibition of the ERK or $p38^{MAPK}$ pathways had no effect. Recently, the activation of the JNK pathway by HNE has been shown to occur upstream at the protein tyrosine phosphatase SHP-1that is inhibited by HNE, which also appears to accelerate the degradation of the enzyme (Rinna and Forman, 2008).

6. Glutathione therapeutics

As an increase in GSH appears to be a ubiquitous response to oxidants and electrophiles and some diseases appear to be exacerbated by decreasing GSH, increasing GSH by using delivery of permeable esters (Levy et al., 1993) or increasing the availability of cysteine using the non-toxic precursor N-acetylcysteine (Thor et al., 1979) have been proposed. Increasing GSH through synthesis would also seem to be useful therapeutically but as oxidants and most electrophiles would not seem appropriate, natural compounds such as curcumin, a principal ingredient of curry powder (Dickinson et al., 2003), and sulforaphane, a potent Phase II gene-inducing compound in broccoli, (Brooks et al., 2001) have been proposed but none of these natural has actually become a major therapeutic agent.

On the other hand, compounds that decrease GSH and increase the susceptibility of tumors to chemotherapy or radiation have been used. GCL can be inhibited by a buthionine sulphoximine quite specifically making it a useful tool in studying GSH metabolism, and useful in cancer chemotherapy (Martensson et al., 1989; Anderson et al., 1997; Gartenhaus et al., 2002). An inhibitor of γ-glutamyl transpeptidase (GGT), acivicin (AT-125) (Griffith and Meister, 1980) was tried in chemotherapy before it was known to inhibit GGT; however, acivicin also inhibits enzymes in purine and pyrimidine biosynthesis, which may be its actual mode of action (Poster et al., 1981; Elliott and Weber, 1985). Thus, there is still much to be done in understanding how GSH synthesis and metabolism may be manipulated to therapeutic advantage. Further information about the use of GSH and related compounds in therapy for a variety of diseases including viral infection, cystic fibrosis and cancer, can be found in the reviews by Biswas and Rahman (2008)) and by Fraternale et al. (2008) in this issue.

Abbreviations

GSH, glutathione
−SH, sulfhydryl group
O_2^-, superoxide
H_2O_2, hydrogen peroxide
NOX, NADPH oxidase
SOD, superoxide dismutase
HOCl, hypochlorous acid
HOBr, hypobromous acid
Fe^{2+}, ferrous iron
OH, hydroxyl radical
Fe^{3+}, ferric iron
L, lipid radical
LOO, hydroperoxide radical
LOOH, lipid peroxide
HNE, 4-hydroxy-2-nonenal
$ONOO^-$, peroxynitrite
NO, nitrogen oxide
NO_2^-, nitrite
NO_3^-, nitrate
ONOOH, peroxynitrous acid
NO_2, nitrogen dioxide
CFTR, cystic fibrosis transport receptor
GPx, glutathione peroxidase
PHGPx GPx IV, phospholipid hydroperoxide glutathione peroxidase
GSSG, glutathione disulfide
Prdx, peroxiredoxin
PSSG, protein mixed disulfide
PDI, protein disulfide isomerase
$-S^-$, thiolate
PSOH, sulfenic acid
GST, glutathione-S transferase
GSNO, nitrosoglutathione
DTNB, dithionitrobenzoic acid
NEM, N-ethylmaleimide
$NaBH_4$, sodium borohydride
OPT, orthophthaldehyde
GCL, glutamate cysteine ligase

ARE, antioxidant response element
JNK, Jun N-terminal kinase
GGT, γ-glutamyl transpeptidase.

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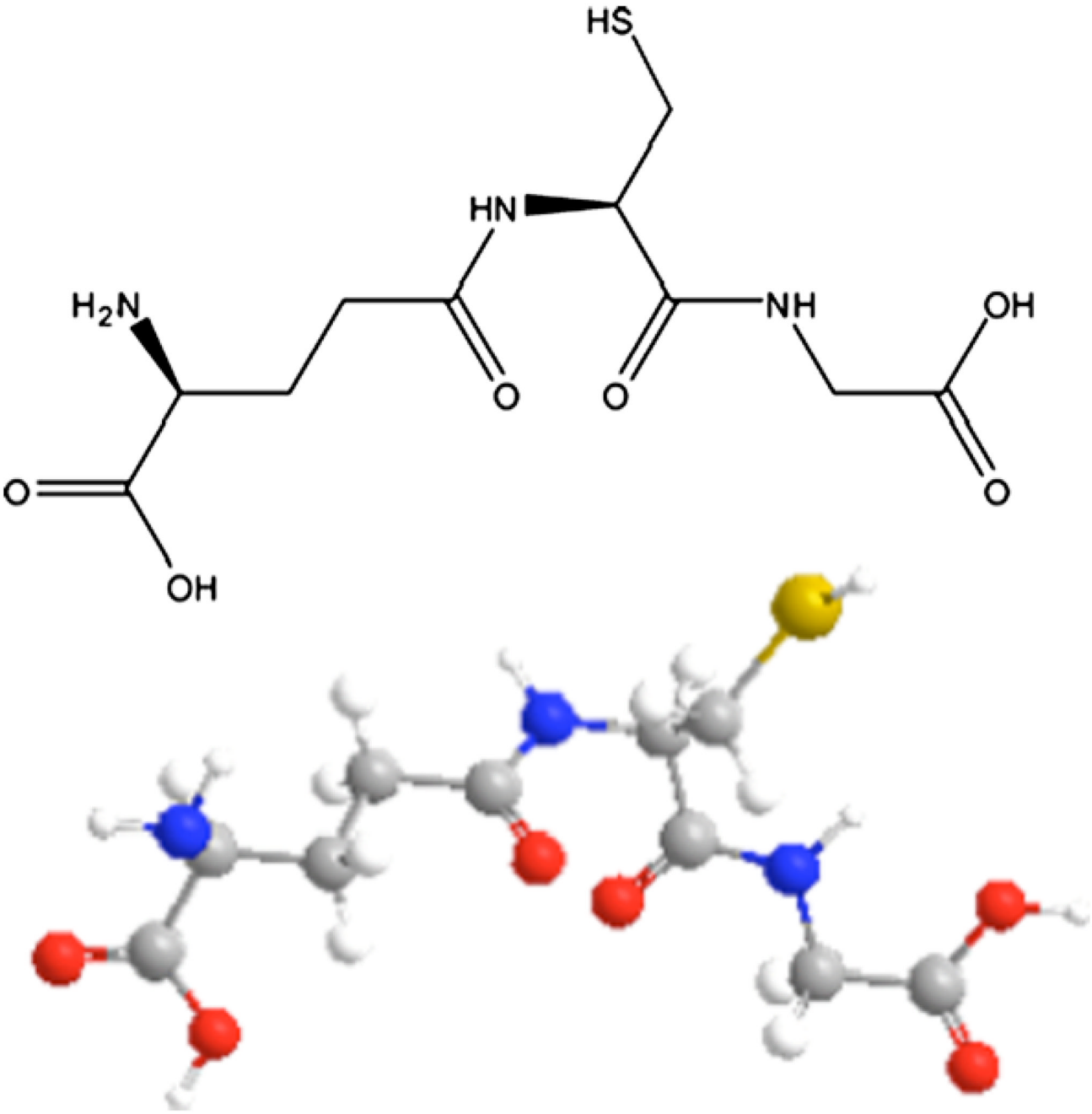


Fig. 1.
Glutathione structure. A stereochemical and ball and stick figure showing γ-glutamyl-cysteinyl-glycine are shown.

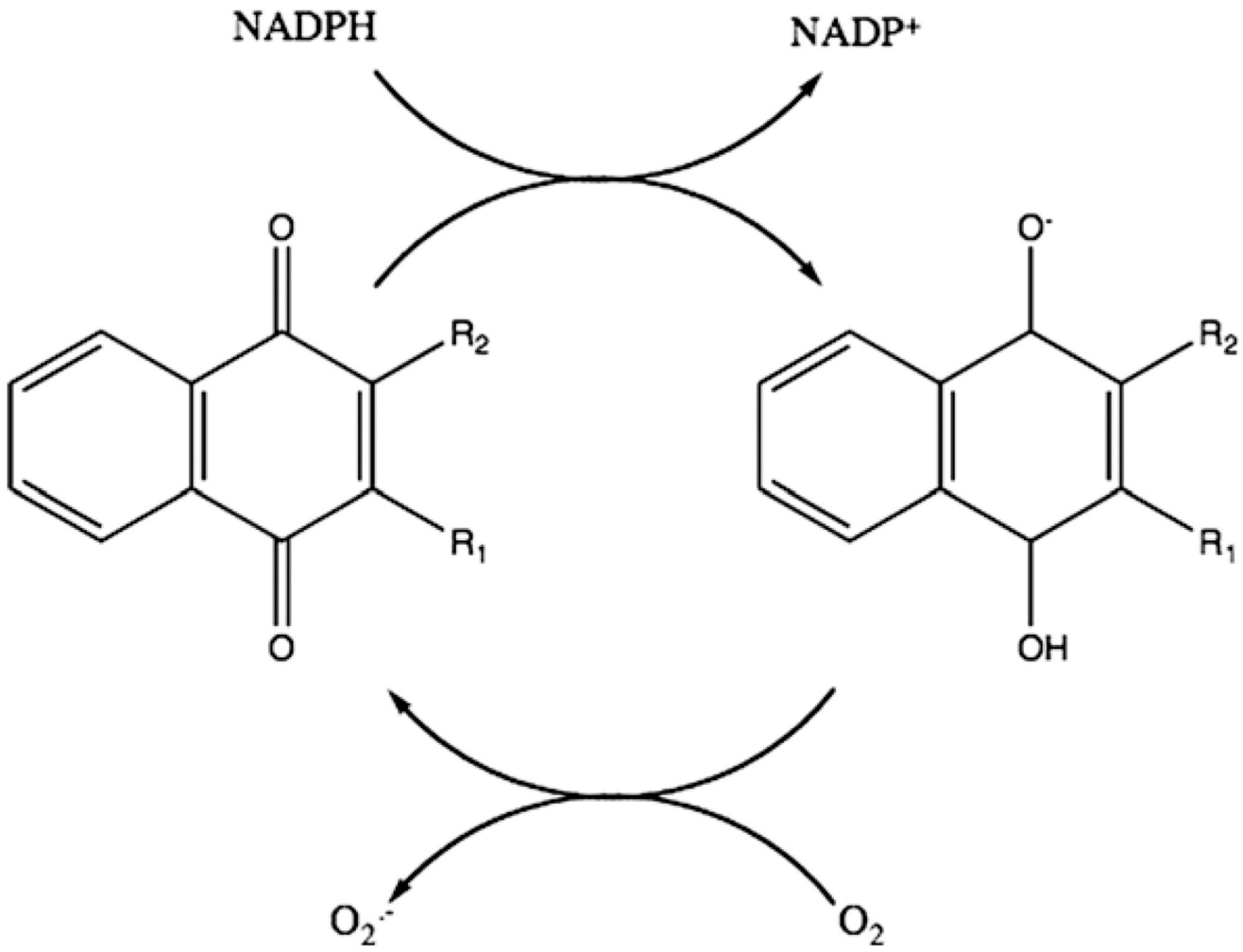


Fig. 2.

Redox cycling of 1,4-naphthoquinones. A naphthoquinone with two variable groups (R) can be reduced by NADPH (or NADH, which is not shown) enzymatically to the semiquinone radical and then will react with oxygen to generate superoxide and restore the naphthoquinone.

$$GSH + HOCl \rightarrow GSCl + H_2O$$

$$GSCl + GSH \rightarrow GSSG + HCl$$

$$GSCl + 2HOCl \rightarrow GSO_2Cl + 2HCl$$

$$GSO_2Cl \rightarrow GSO_2NH\,(\text{formed with an amino group of GSH itself}) + HCl$$

$$GSO_2Cl + GSH \rightarrow GSO_2SG + HCl$$

Fig. 3.
Reactions of glutathione with hypochlorous acid. GSH and HOCl can react to produce several different products.

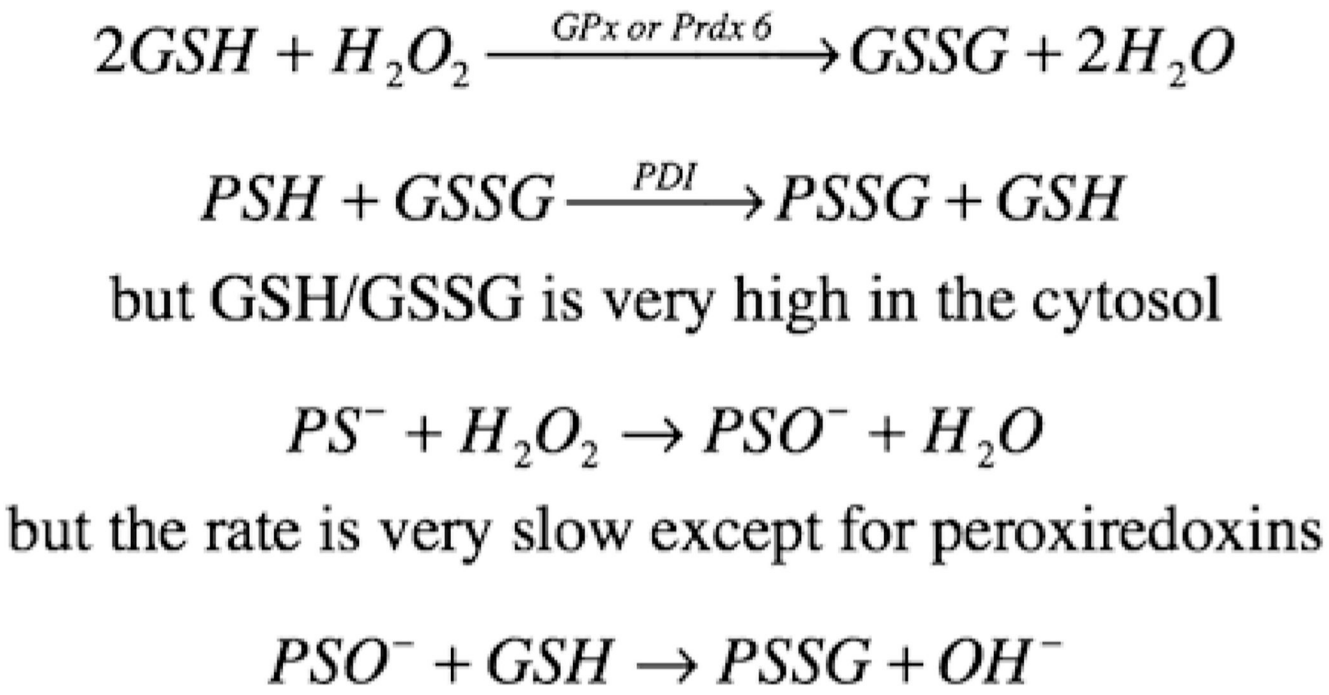


Fig. 4.

Formation of protein mixed disulfide. Both glutathione peroxidases and peroxiredoxin 6 can catalyze the oxidation of glutathione by hydrogen peroxide to glutathione disulfide and water. GSSG can then undergo an exchange reaction with protein sulfhydryl to form PSSG, which is usually catalyzed by a protein disulfide isomerase. An alternative mechanism is the oxidation of a protein thiolate to a sulfenic acid, which then will react with GSH to form PSSG and water.

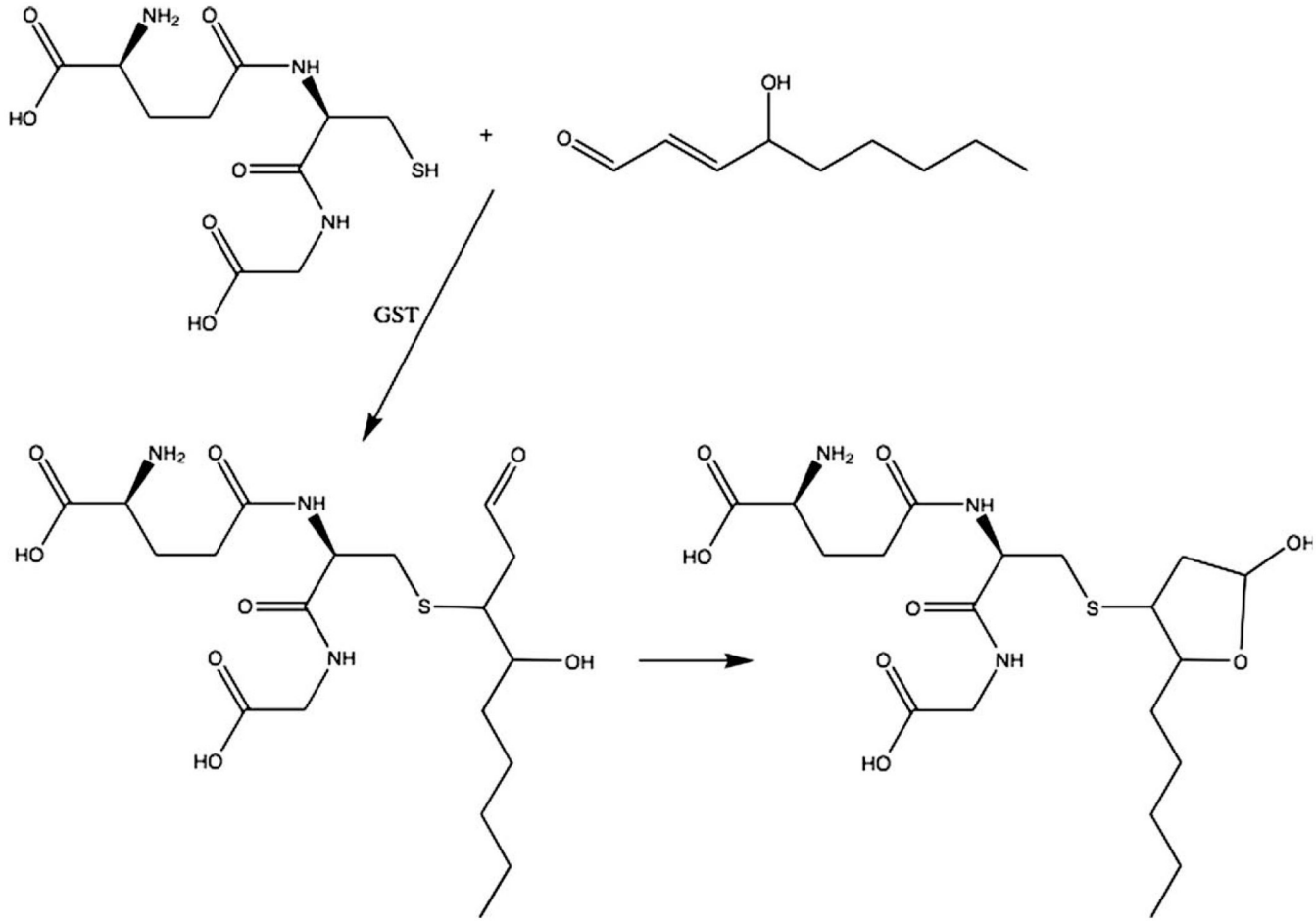
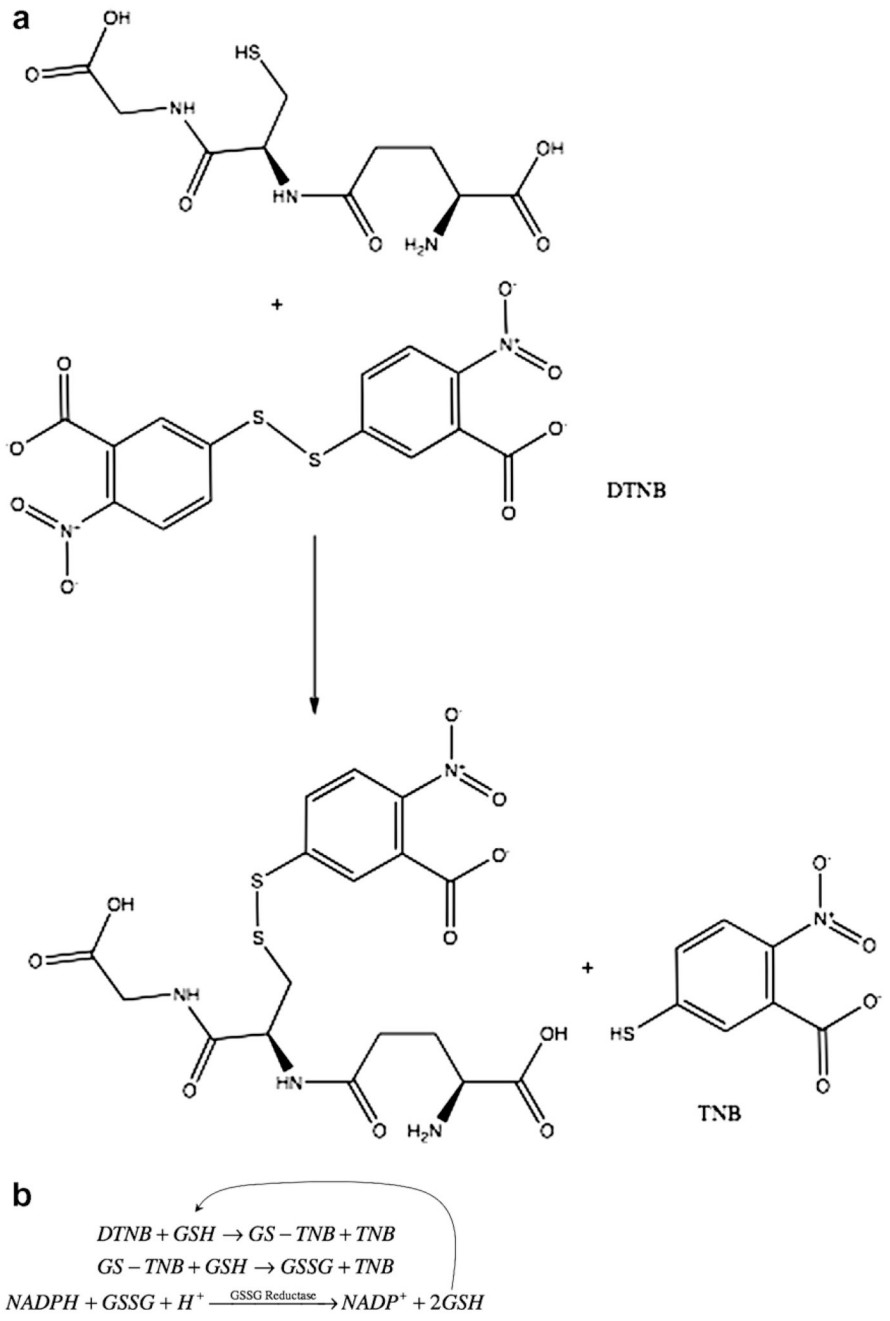


Fig. 5.
Glutathione conjugations with 4-hydroxynonenal. Glutathione S-transferases catalyze the conjugation of GSH with HNE. This is a Michael addition that can slowly occur non-enzymatically.



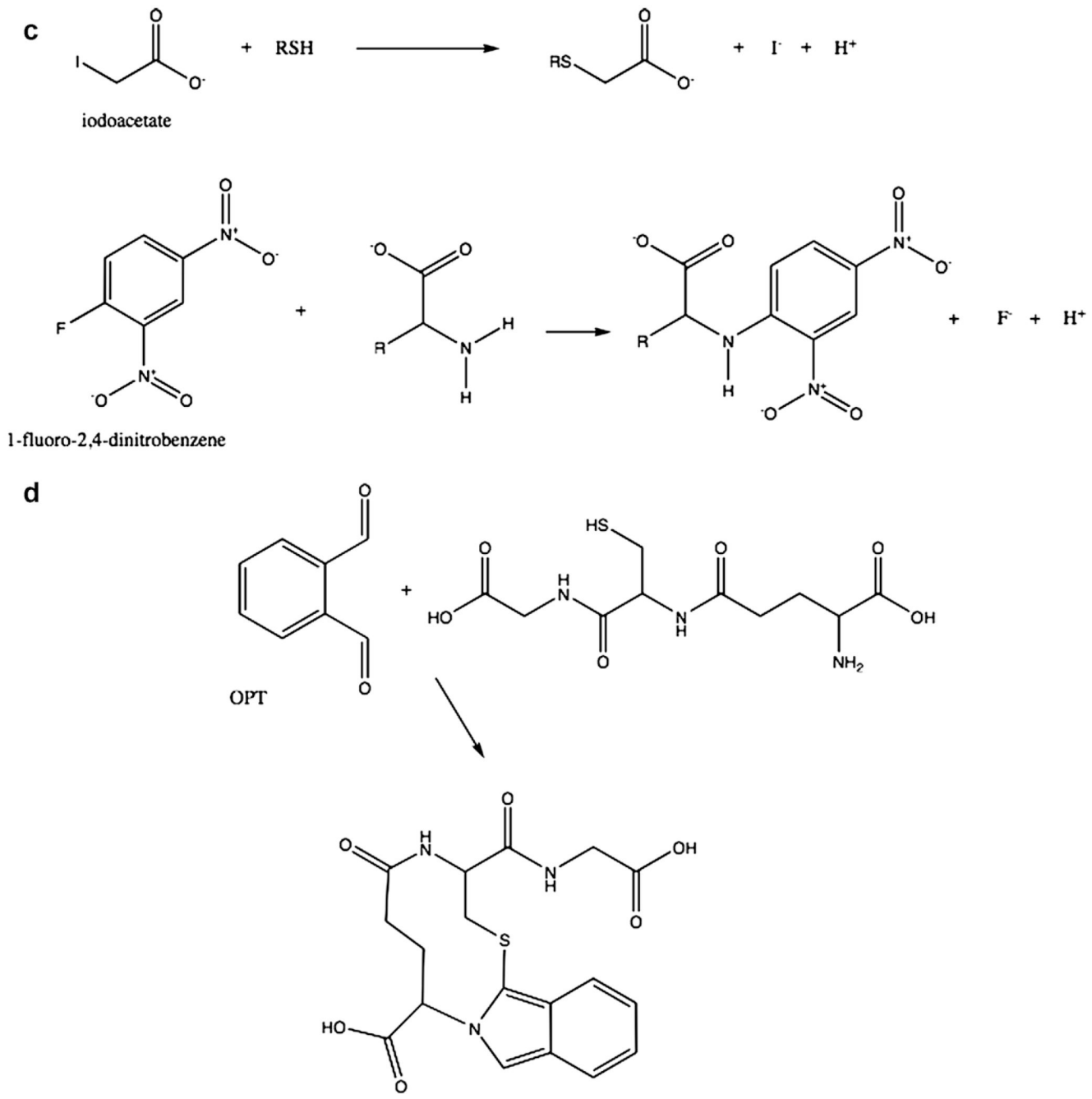


Fig. 6.

Measurements of thiols. (a) Reaction of GSH with DTNB produces an adduct and TNB, which is measured spectrofluorometrically or spectrophometrically; (b) total glutathione can be determined by recycling of GSSG produced in the reaction in (a) and measuring the rate of TNB; (c) Glutathione and related compounds are first derivatized with iodoacetate followed by a second derivatization with 1-fluoro-2,4-dinitrophenol. The second products are then separated by HPLC and measured spectrofluorometrically; (d) Reaction of glutathione with orthophthaldehyde (OPT) yields a product that can be measured spectrofluorometrically.

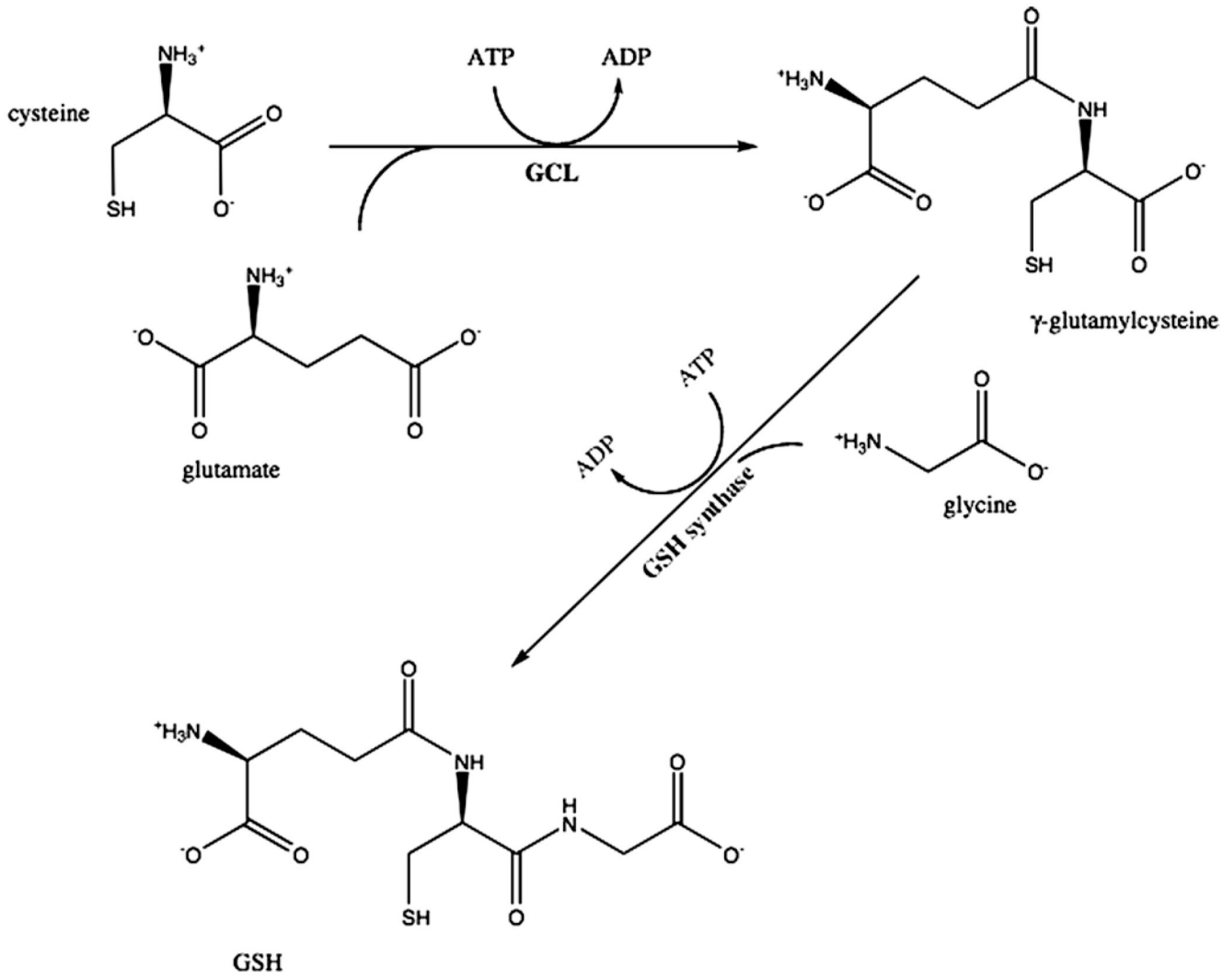


Fig. 7.
Glutathione synthesis. The sequential ATP dependent formation of amide bonds between cysteine and the γ-carboxyl group of glutamate and then between glycine and cysteine are shown.

Glutathione Metabolism and Its Implications for Health[1]

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ABSTRACT Glutathione (γ-glutamyl-cysteinyl-glycine; GSH) is the most abundant low-molecular-weight thiol, and GSH/glutathione disulfide is the major redox couple in animal cells. The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes, γ-glutamylcysteine synthetase and GSH synthetase. Compelling evidence shows that GSH synthesis is regulated primarily by γ-glutamylcysteine synthetase activity, cysteine availability, and GSH feedback inhibition. Animal and human studies demonstrate that adequate protein nutrition is crucial for the maintenance of GSH homeostasis. In addition, enteral or parenteral cystine, methionine, *N*-acetylcysteine, and L-2-oxothiazolidine-4-carboxylate are effective precursors of cysteine for tissue GSH synthesis. Glutathione plays important roles in antioxidant defense, nutrient metabolism, and regulation of cellular events (including gene expression, DNA and protein synthesis, cell proliferation and apoptosis, signal transduction, cytokine production and immune response, and protein glutathionylation). Glutathione deficiency contributes to oxidative stress, which plays a key role in aging and the pathogenesis of many diseases (including kwashiorkor, seizure, Alzheimer's disease, Parkinson's disease, liver disease, cystic fibrosis, sickle cell anemia, HIV, AIDS, cancer, heart attack, stroke, and diabetes). New knowledge of the nutritional regulation of GSH metabolism is critical for the development of effective strategies to improve health and to treat these diseases. J. Nutr. 134: 489–492, 2004.

KEY WORDS: • amino acids • oxidative stress • cysteine • disease

The work with glutathione (γ-glutamyl-cysteinyl-glycine; GSH)[3] has greatly advanced biochemical and nutritional sciences over the past 125 y (1,2). Specifically, these studies have led to the free radical theory of human diseases and to the advancement of nutritional therapies to improve GSH status under various pathological conditions (2,3). Remarkably, the past decade witnessed the discovery of novel roles for GSH in signal transduction, gene expression, apoptosis, protein glutathionylation, and nitric oxide (NO) metabolism (2,4). Most recently, studies of in vivo GSH turnover in humans were initiated to provide much-needed information about quantitative aspects of GSH synthesis and catabolism in the whole body and specific cell types (e.g., erythrocytes) (3,5–7). This article reviews the recent developments in GSH metabolism and its implications for health and disease.

Abundance of GSH in Cells and Plasma. Glutathione is the predominant low-molecular-weight thiol (0.5–10 mmol/L) in animal cells. Most of the cellular GSH (85–90%) is present in the cytosol, with the remainder in many organelles (including the mitochondria, nuclear matrix, and peroxisomes) (8). With the exception of bile acid, which may contain up to 10 mmol/L GSH, extracellular concentrations of GSH are relatively low (e.g., 2–20 μmol/L in plasma) (4,9). Because of the cysteine residue, GSH is readily oxidized nonenzymatically to glutathione disulfide (GSSG) by electrophilic substances (e.g., free radicals and reactive oxygen/nitrogen species). The GSSG efflux from cells contributes to a net loss of intracellular GSH. Cellular GSH concentrations are reduced markedly in response to protein malnutrition, oxidative stress, and many pathological conditions (8,9). The GSH + 2GSSG concentration is usually denoted as total glutathione in cells, a significant amount of which (up to 15%) may be bound to protein (1). The [GSH]:[GSSG] ratio, which is often used as an indicator of the cellular redox state, is >10 under normal physiological conditions (9). GSH/GSSG is the major redox couple that determines the antioxidative capacity of cells, but its value can be affected by other redox couples, including $NADPH/NADP^+$ and $thioredoxin_{red}/thioredoxin_{ox}$ (4).

GSH Synthesis. The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes, γ-glutamylcysteine synthetase (GCS) and GSH synthetase (**Fig. 1**). This pathway occurs in virtually all cell types, with the liver being the major producer and exporter of GSH. In the GCS reaction, the γ-carboxyl group of glutamate reacts with the amino group of cysteine to form a peptidic γ-linkage, which protects GSH from hydrolysis by intracellular peptidases. Although γ-glutamyl-cysteine can be a substrate for γ-glutamylcyclotransferase, GSH synthesis is favored in animal cells because of the much higher affinity and activity of GSH synthetase (9).

Mammalian GCS is a heterodimer consisting of a catalytically active heavy subunit (73 kDa) and a light (regulatory) subunit (31 kDa) (8). The heavy subunit contains all substrate binding sites, whereas the light subunit modulates the affinity of the heavy subunit for substrates and inhibitors. The K_m values of mammalian GCS for glutamate and cysteine are 1.7 and 0.15 mmol/L, respectively, which are similar to the intracellular concentrations of glutamate (2–4 mmol/L) and cysteine (0.15–0.25 mmol/L) in rat liver (9). Mammalian GSH

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[3] Abbreviations used: GCS, γ-glutamylcysteine synthetase; GSH, glutathione; GSSG, glutathione disulfide.

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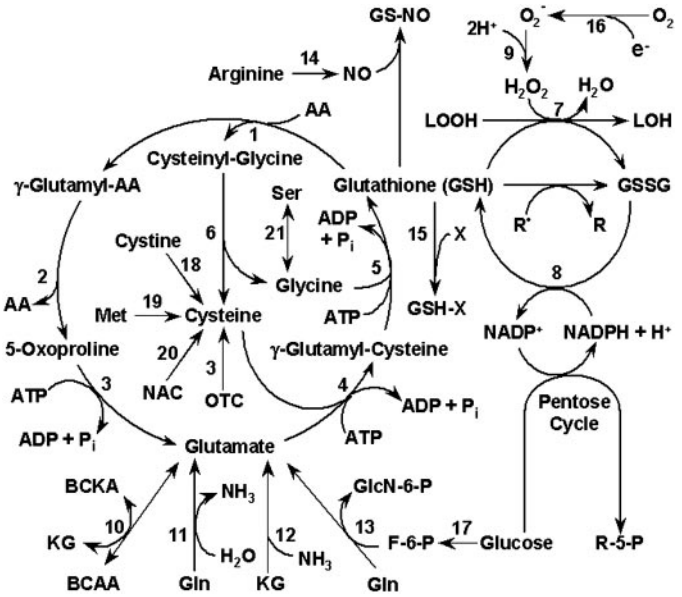

FIGURE 1 Glutathione synthesis and utilization in animals. Enzymes that catalyze the indicated reactions are: *1*) γ-glutamyl transpeptidase, *2*) γ-glutamyl cyclotransferase, *3*) 5-oxoprolinase, *4*) γ-glutamylcysteine synthetase, *5*) glutathione synthetase, *6*) dipeptidase, *7*) glutathione peroxidase, *8*) glutathione reductase, *9*) superoxide dismutase, *10*) BCAA transaminase (cytosolic and mitochondrial), *11*) glutaminase, *12*) glutamate dehydrogenase, *13*) glutamine:fructose-6-phosphate transaminase (cytosolic), *14*) nitric oxide synthase, *15*) glutathione *S*-transferase, *16*) NAD(P)H oxidase and mitochondrial respiratory complexes, *17*) glycolysis, *18*) glutathione-dependent thioldisulfide or thioltransferase or nonenzymatic reaction, *19*) transsulfuration pathway, *20*) deacylase, and *21*) serine hydroxymethyltransferase. Abbreviations: AA, amino acids; BCKA, branched-chain α-ketoacids; GlcN-6-P, glucosamine-6-phosphate; GS-NO, glutathione–nitric oxide adduct; KG, α-ketoglutarate; LOO•, lipid peroxyl radical; LOOH, lipid hydroperoxide; NAC, *N*-acetylcysteine; OTC, L-2-oxothiazolidine-4-carboxylate; R•, radicals; R, nonradicals; R-5-P, ribulose-5-phosphate; X, electrophilic xenobiotics.

synthetase is a homodimer (52 kDa/subunit) and is an allosteric enzyme with cooperative binding for γ-glutamyl substrate (10). The K_m values of mammalian GSH synthetase for ATP and glycine are ~0.04 and 0.9 mmol/L, respectively, which are lower than intracellular concentrations of ATP (2–4 mmol/L) and glycine (1.5–2 mmol/L) in rat liver. Both subunits of rat GCS and GSH synthetase have been cloned and sequenced (9), which facilitates the study of molecular regulation of GSH synthesis. γ-Glutamylcysteine synthetase is the rate-controlling enzyme in de novo synthesis of GSH (8).

Knowledge regarding in vivo GSH synthesis is limited, due in part to the complex compartmentalization of substrates and their metabolism at both the organ and subcellular levels. For example, the source of glutamate for GCS differs between the small intestine and kidney (e.g., diet vs. arterial blood). In addition, liver GSH synthesis occurs predominantly in perivenous hepatocytes and, to a lesser extent, in periportal cells (11). Thus, changes in plasma GSH levels may not necessarily reflect changes in GSH synthesis in specific cell types. However, recent studies involving stable isotopes (5–7) have expanded our understanding of GSH metabolism. In healthy adult humans, the endogenous disappearance rate (utilization rate) of GSH is 25 μmol/(kg · h) (6), which accounts for 65% of whole body cysteine flux [38.3 μmol/(kg · h)]. This finding supports the view that GSH acts as a major transport form of cysteine in the body. On the basis of dietary cysteine intake [9 μmol/(kg · h)] in healthy adult humans (6), it is estimated that most of the cysteine used for endogenous GSH synthesis is derived from intracellular protein degradation and/or endogenous synthesis. Interestingly, among extrahepatic cells, the erythrocyte has a relatively high turnover rate for GSH. For example, the whole-blood fractional synthesis rate of GSH in healthy adult subjects is 65%/d (6), which means that all the GSH is completely replaced in 1.5 d; this value is equivalent to 3 μmol/(kg · h). Thus, whole blood (mainly erythrocytes) may contribute up to 10% of whole-body GSH synthesis in humans (5,6).

Regulation of GSH Synthesis by GCS. Oxidant stress, nitrosative stress, inflammatory cytokines, cancer, cancer chemotherapy, ionizing radiation, heat shock, inhibition of GCS activity, GSH depletion, GSH conjugation, prostaglandin A_2, heavy metals, antioxidants, and insulin increase GCS transcription or activity in a variety of cells (2,8). In contrast, dietary protein deficiency, dexamethasone, erythropoietin, tumor growth factor β, hyperglycemia, and GCS phosphorylation decrease GCS transcription or activity. Nuclear factor κB mediates the upregulation of GCS expression in response to oxidant stress, inflammatory cytokines, and buthionine sulfoximine–induced GSH depletion (2,8). *S*-nitrosation of GCS protein by NO donors (e.g., *S*-nitroso-L-cysteine and *S*-nitroso-L-cysteinylglycine) reduces enzyme activity (8), suggesting a link between NO (a metabolite of L-arginine) and GSH metabolism. Indeed, an increase in NO production by inducible NO synthase causes GCS inhibition and GSH depletion in cytokine-activated macrophages and neurons (12). In this regard, glucosamine, taurine, n-3 PUFAs, phytoestrogens, polyphenols, carotenoids, and zinc, which inhibit the expression of inducible NO synthase and NO production (13), may prevent or attenuate GSH depletion in cells. Conversely, high-fat diet, saturated long-chain fatty acids, low-density lipoproteins, linoleic acid, and iron, which enhance the expression of inducible NO synthase and NO production (13), may exacerbate the loss of GSH from cells.

Regulation of GSH Synthesis by Amino Acids. Cysteine is an essential amino acid in premature and newborn infants and in subjects stressed by disease (14). As noted above, the intracellular pool of cysteine is relatively small, compared with the much larger and often metabolically active pool of GSH in cells (15). Recent studies provide convincing data to support the view that cysteine is generally the limiting amino acid for GSH synthesis in humans, as in rats, pigs, and chickens (6,14,15). Thus, factors (e.g., insulin and growth factors) that stimulate cysteine (cystine) uptake by cells generally increase intracellular GSH concentrations (8). In addition, increasing the supply of cysteine or its precursors (e.g., cystine, *N*-acetylcysteine, and L-2-oxothiazolidine-4-carboxylate) via oral or intravenous administration enhances GSH synthesis and prevents GSH deficiency in humans and animals under various nutritional and pathological conditions (including protein malnutrition, adult respiratory distress syndrome, HIV, and AIDS) (2). Because cysteine generated from methionine catabolism via the transsulfuration pathway (primarily in hepatocytes) serves as a substrate for GCS, dietary methionine can replace cysteine to support GSH synthesis in vivo.

Cysteine is readily oxidized to cystine in oxygenated extracellular solutions. Thus, the plasma concentration of cysteine is low (10–25 μmol/L), compared with that of cystine (50–150 μmol/L). Cysteine and cystine are transported by distinct membrane carriers, and cells typically transport one more

efficiently than the other (8). It is interesting that some cell types (e.g., hepatocytes) have little or no capacity for direct transport of extracellular cystine. However, GSH that effluxes from the liver can reduce cystine to cysteine on the outer cell membrane, and the resulting cysteine is taken up by hepatocytes. Other cell types (e.g., endothelial cells) can take up cystine and reduce it intracellularly to cysteine (Fig. 1); cellular reducing conditions normally favor the presence of cysteine in animal cells.

Extracellular and intracellularly generated glutamate can be used for GSH synthesis (16). Because dietary glutamate is almost completely utilized by the small intestine (16), plasma glutamate is derived primarily from its de novo synthesis and protein degradation. Phosphate-dependent glutaminase, glutamate dehydrogenase, pyrroline-5-carboxylate dehydrogenase, BCAA transaminase, and glutamine:fructose-6-phosphate transaminase may catalyze glutamate formation (Fig. 1), but the relative importance of these enzymes likely varies among cells and tissues. Interestingly, rat erythrocytes do not take up or release glutamate (17), and glutamine and/or BCAAs may be the precursors of glutamate in these cells (Fig. 1). Indeed, glutamine is an effective precursor of the glutamate for GSH synthesis in many cell types, including enterocytes, neural cells, liver cells, and lymphocytes (18). Thus, glutamine supplementation to total parenteral nutrition maintains tissue GSH levels and improves survival after reperfusion injury, ischemia, acetaminophen toxicity, chemotherapy, inflammatory stress, and bone marrow transplantation (19).

Glutamate plays a regulatory role in GSH synthesis through two mechanisms: *1*) the uptake of cystine, and *2*) the prevention of GSH inhibition of GCS. Glutamate and cystine share the system X_c^- amino acid transporter (8). When extracellular glutamate concentrations are high, as in patients with advanced cancer, HIV infection, and spinal cord or brain injury as well as in cell culture medium containing high levels of glutamate, cystine uptake is competitively inhibited by glutamate, resulting in reduced GSH synthesis (20). GSH is a nonallosteric feedback inhibitor of GCS, but the binding of GSH to the enzyme competes with glutamate (9). When intracellular glutamate concentrations are unusually high, as in canine erythrocytes, GSH synthesis is enhanced and its concentration is particularly high (9).

Glycine availability may be reduced in response to protein malnutrition, sepsis, and inflammatory stimuli (21,22). When hepatic glycine oxidation is enhanced in response to high levels of glucagon or diabetes (23), this amino acid may become a limiting factor for GSH synthesis. In vivo studies show that glycine availability limits erythrocyte GSH synthesis in burned patients (7) and in children recovering from severe malnutrition (21). It is important to note that dietary glycine supplementation enhances the hepatic GSH concentration in protein-deficient rats challenged with TNF-α (22).

The evidence indicates that the dietary amino acid balance has an important effect on protein nutrition and therefore on GSH homeostasis (8). In particular, the adequate provision of sulfur-containing amino acids as well as glutamate (glutamine or BCAAs) and glycine (or serine) is critical for the maximization of GSH synthesis. Thus, in the erythrocytes of children with edematous protein-energy malnutrition and piglets with protein deficiency, GSH synthesis is impaired, leading to GSH deficiency (3). An increase in urinary excretion of 5-oxoproline, an intermediate of the γ-glutamyl cycle (Fig. 1), is a useful indicator of reduced availability of cysteine and/or glycine for GSH synthesis in vivo (7,21)

Interorgan GSH Transport. Glutathione can be transported out of cells via a carrier-dependent facilitated mechanism (2). Plasma GSH originates primarily from the liver, but some of the dietary and intestinally derived GSH can enter the portal venous plasma (8). Glutathione molecules leave the liver either intact or as γ-Glu-$(Cys)_2$ owing to γ-glutamyl transpeptidase activity on the outer plasma membrane (Fig. 1). The extreme concentration gradient across the plasma membrane makes the transport of extracellular GSH or GSSG into cells thermodynamically unfavorable. However, γ-Glu-$(Cys)_2$ is readily taken up by extrahepatic cells for GSH synthesis. The kidney, lung, and intestine are major consumers of the liver-derived GSH (8). The interorgan metabolism of GSH functions to transport cysteine in a nontoxic form between tissues, and also helps to maintain intracellular GSH concentrations and redox state (8).

Roles of GSH. Glutathione participates in many cellular reactions. First, GSH effectively scavenges free radicals and other reactive oxygen species (e.g., hydroxyl radical, lipid peroxyl radical, peroxynitrite, and H_2O_2) directly, and indirectly through enzymatic reactions (24). In such reactions, GSH is oxidized to form GSSG, which is then reduced to GSH by the NADPH-dependent glutathione reductase (Fig. 1). In addition, glutathione peroxidase (a selenium-containing enzyme) catalyzes the GSH-dependent reduction of H_2O_2 and other peroxides (25).

Second, GSH reacts with various electrophiles, physiological metabolites (e.g., estrogen, melanins, prostaglandins, and leukotrienes), and xenobiotics (e.g., bromobenzene and acetaminophen) to form mercapturates (24). These reactions are initiated by glutathione-*S*-transferase (a family of Phase II detoxification enzymes).

Third, GSH conjugates with NO to form an *S*-nitrosoglutathione adduct, which is cleaved by the thioredoxin system to release GSH and NO (24). Recent evidence suggests that the targeting of endogenous NO is mediated by intracellular GSH (26). In addition, both NO and GSH are necessary for the hepatic action of insulin-sensitizing agents (27), indicating their critical role in regulating lipid, glucose, and amino acid utilization.

Fourth, GSH serves as a substrate for formaldehyde dehydrogenase, which converts formaldehyde and GSH to *S*-formyl-glutathione (2). The removal of formaldehyde (a carcinogen) is of physiological importance, because it is produced from the metabolism of methionine, choline, methanol (alcohol dehydrogenase), sarcosine (sarcosine oxidase), and xenobiotics (via the cytochrome P450–dependent monooxygenase system of the endoplasmic reticulum).

Fifth, GSH is required for the conversion of prostaglandin H_2 (a metabolite of arachidonic acid) into prostaglandins D_2 and E_2 by endoperoxide isomerase (8).

Sixth, GSH is involved in the glyoxalase system, which converts methylglyoxal to D-lactate, a pathway active in microorganisms. Finally, glutathionylation of proteins (e.g., thioredoxin, ubiquitin-conjugating enzyme, and cytochrome c oxidase) plays an important role in cell physiology (2).

Thus, GSH serves vital functions in animals (**Table 1**). Adequate GSH concentrations are necessary for the proliferation of cells, including lymphocytes and intestinal epithelial cells (28). Glutathione also plays an important role in spermatogenesis and sperm maturation (1). In addition, GSH is essential for the activation of T-lymphocytes and polymorphonuclear leukocytes as well as for cytokine production, and therefore for mounting successful immune responses when the host is immunologically challenged (2). Further, both in vitro and in vivo evidence show that GSH inhibits infection by the

TABLE 1

Roles of glutathione in animals

Antioxidant defense
Scavenging free radicals and other reactive species
Removing hydrogen and lipid peroxides
Preventing oxidation of biomolecules
Metabolism
Synthesis of leukotrienes and prostaglandins
Conversion of formaldehyde to formate
Production of D-lactate from methylglyoxal
Formation of mercapturates from electrophiles
Formation of glutathione-NO adduct
Storage and transport of cysteine
Regulation
Intracellular redox status
Signal transduction and gene expression
DNA and protein synthesis, and proteolysis
Cell proliferation and apoptosis
Cytokine production and immune response
Protein glutathionylation
Mitochondrial function and integrity

influenza virus (29). It is important to note that shifting the GSH/GSSG redox toward the oxidizing state activates several signaling pathways (including protein kinase B, protein phosphatases 1 and 2A, calcineurin, nuclear factor κB, c-Jun N-terminal kinase, apoptosis signal-regulated kinase 1, and mitogen-activated protein kinase), thereby reducing cell proliferation and increasing apoptosis (30). Thus, oxidative stress (a deleterious imbalance between the production and removal of reactive oxygen/nitrogen species) plays a key role in the pathogenesis of many diseases, including cancer, inflammation, kwashiorkor (predominantly protein deficiency), seizure, Alzheimer's disease, Parkinson's disease, sickle cell anemia, liver disease, cystic fibrosis, HIV, AIDS, infection, heart attack, stroke, and diabetes (2,31).

Concluding Remarks and Perspectives. GSH displays remarkable metabolic and regulatory versatility. GSH/GSSG is the most important redox couple and plays crucial roles in antioxidant defense, nutrient metabolism, and the regulation of pathways essential for whole body homeostasis. Glutathione deficiency contributes to oxidative stress, and, therefore, may play a key role in aging and the pathogenesis of many diseases. This presents an emerging challenge to nutritional research. Protein (or amino acid) deficiency remains a significant nutritional problem in the world, owing to inadequate nutritional supply, nausea and vomiting, premature birth, HIV, AIDS, cancer, cancer chemotherapy, alcoholism, burns, and chronic digestive diseases. Thus, new knowledge regarding the efficient utilization of dietary protein or the precursors for GSH synthesis and its nutritional status is critical for the development of effective therapeutic strategies to prevent and treat a wide array of human diseases, including cardiovascular complications, cancer, and severe acute respiratory syndrome.

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EVOLUTIONARY BIOLOGY

A substitution in the glutathione reductase lowers electron leakage and inflammation in modern humans

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Glutathione reductase is a critical enzyme for preventing oxidative stress and maintaining a reduced intracellular environment. Almost all present-day humans carry an amino acid substitution (S232G) in this enzyme relative to apes and Neanderthals. We express the modern human and the ancestral enzymes and show that whereas the activity and stability are unaffected by the amino acid substitution, the ancestral enzyme produces more reactive oxygen species and increases cellular levels of transcripts encoding cytokines. We furthermore show that the ancestral enzyme has been reintroduced into the modern human gene pool by gene flow from Neanderthals and is associated with multiple traits in present-day people, including increased susceptibility for inflammatory-associated disorders and vascular disease.

INTRODUCTION

Aerobic organisms face the challenge of oxidative damage caused by reactive oxygen species produced as metabolic by-products. One evolutionarily conserved mechanism for preventing oxidative damage is glutathione, which, after becoming oxidized by oxygen radicals, is recycled to the reduced form by glutathione reductase (GR). This enzyme is present in the cytosol, in mitochondria (*1*, *2*), and extracellularly (*3*). During the recycling, an electron is transferred from an enzyme cofactor to the oxidized glutathione. However, in the absence of oxidized glutathione (GSSG), GR can paradoxically generate reactive oxygen species by transferring electrons to oxygen instead (*4*). Here, we describe a functionally relevant change in GR on the evolutionary lineage leading to modern humans.

The closest evolutionary relatives of present-day humans, Neanderthals and Denisovans, so-called "archaic" humans, shared an ancestral population with the ancestors of modern humans about half a million years ago (*5*). Three Neanderthal genomes (*5*–*7*) and one Denisovan genome (*8*) have been sequenced to high quality. This makes it possible to identify genetic changes that characterize modern humans. Among the single-nucleotide substitutions on the lineage leading to modern humans, which alter protein sequences, approximately 100 are known to occur among all or almost all humans today but not in the archaic genomes available to date (*9*). One of these affects GR, which, in present-day humans, carries a glycine residue at position 232, whereas Neanderthals, Denisovans, and other primates carry a serine residue at this position. Note that position 232 in the nascent protein sequence corresponds to position 189 after cleavage of the mitochondrial signal peptide.

When modern humans encountered Neanderthals after leaving Africa, the two groups mixed, and as a result, some Neanderthal DNA variants were introduced into the gene pool of modern humans where they persist until today (*10*). Thus, some ancestral genetic variants and some genetic variants unique to Neanderthals exist among present-day people, often at low frequencies. Here, we show that one such variant encodes the ancestral form of GR. We describe the properties of the ancestral and modern human enzymes as well as the effects on the carriers of these variants today.

RESULTS

Enzymology

To characterize the functional consequences of the S232G substitution, we expressed and purified the modern human and Neanderthal (ancestral) forms of GR (fig. S1). GR catalyzes the reduction of GSSG [glutathione disulfide (GSSG)] using NADPH (nicotinamide adenine dinucleotide phosphate) as an electron donor. We measured the specific activity of GR in the presence of GSSG by monitoring NADPH consumption through the decrease of its absorption at 340 nm upon oxidation to $NADP^+$. We find no difference in the kinetics of the Neanderthal and modern human variants of the enzyme (Fig. 1A, table S1, and fig. S2). We further assessed the thermal stability of GR in the absence and presence of NADPH and GSSG, respectively, and found similar stabilities for two forms of the enzyme (Fig. 1, B and C). Moreover, the two variants of the enzyme bound the same amount of flavin adenine dinucleotide (FAD) (table S2).

As mentioned above, GR transfers electrons from NADPH to oxygen in the absence of its physiological substrate, GSSG, thus generating superoxide ($O_2^{\bullet-}$) (*4*, *11*). In an aqueous environment, this leakage of electrons then results in the generation of hydrogen peroxide. In a healthy cell, there are minimal amounts of GSSG, with concentrations of reduced glutathione (GSH) being 100-fold higher than the concentration of GSSG, whereas under oxidative stress, the molar ratio GSH:GSSG is reduced to values of 10:1 and even 1:1 (*12*). To test the extent to which the ancestral and modern human forms of GR may leak electrons, we measured the decrease in absorbance of NADPH at 340 nm in the absence of GSSG. We found that the rate of NADPH consumption for the modern human GR is ~40% of that of the Neanderthal GR ($P = 0.023$; Fig. 1D). Thus, in the absence of GSSG, Neanderthal and Denisovan GRs leak electrons to a higher

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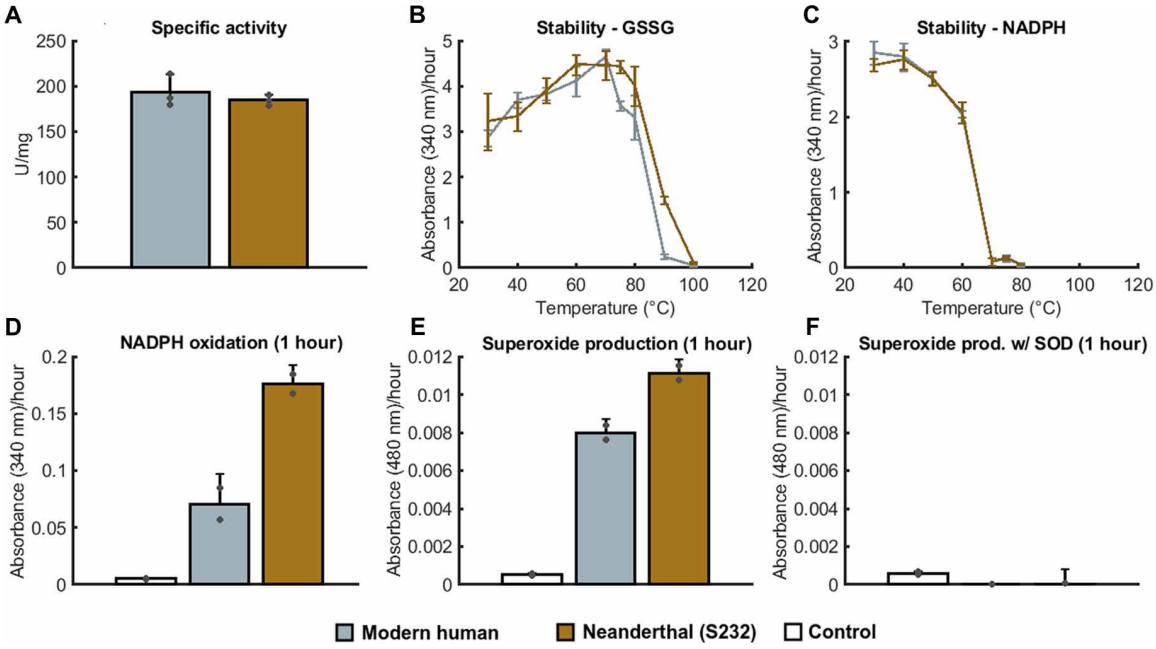

Fig. 1. Characterization of the modern human and Neanderthal variant of GR. (**A**) Specific activity in the presence of NADPH and GSSG. (**B** and **C**) Stability of the enzyme as a function of temperature in the presence of GSSG or NADPH, respectively. (**D**) NADPH oxidation under aerobic conditions, measured as loss of absorbance for NADPH when 1 μM of each enzyme was mixed with 200 μM NADPH. The oxidation of NADPH for the modern human variant is 40% of oxidation for the Neanderthal variant ($P = 0.023$). (**E**) NADPH oxidation resulted in the production of superoxide ($O_2^{\bullet-}$), measured as absorbance for adrenochrome, which is produced when superoxide reacts with epinephrine. The modern human GR variant produces 71% of the superoxide that the Neanderthal variant produces ($P = 0.028$). (**F**) Same as (E), but in the presence of superoxide dismutase. Error bars indicate 95% confidence intervals. Control without GR.

extent than the modern human GR. We verified that the consumption of NADPH leads to the production of superoxide by measuring the conversion of epinephrine to adrenochrome (*13*) and showing that this is abolished by the addition of superoxide dismutase (Fig. 1, E and F).

Neanderthal introgression

The S232G substitution is caused by an A-to-G nucleotide substitution at position 32,888 (RefSeq NG_027719.1) on the coding strand of *GSR*, the gene encoding GR. We next investigated whether this substitution occurs in all present-day humans. Among the individuals in the 1000 Genomes Project (*14*), the ancestral T allele on the noncoding strand (chr8: 30,557,599; hg19) occurs at a carrier frequency of 1.0 to 3.9% in Indian populations, 3.5% in Bangladeshis, 1.0% in Sri Lankan Tamils, and 1.0% in Puerto Ricans, while it is missing in other 1000 Genomes populations (Fig. 2A and table S3).

In the chromosomal region surrounding position 32,888, carriers of the ancestral allele carry nucleotide variants that match the genome of an approximately 45,000-year-old Neanderthal from Vindija Cave in Croatia. This pattern extends 66,705 base pairs (bp) upstream and 222,685 bp downstream (chr8: 30,334,914 to 30,624,304) of the missense variant. Chromosomal segments displaying similarity to Neanderthals may either have entered the modern human genome pool by gene flow when the two groups met between 40,000 and 100,000 years ago (Fig. 2B) or derive from the common ancestors of the two groups that existed about half a million years ago. Given the recombination rate of the region (*15*) and conservative assumptions about generation length of ancient humans, the time of lineage split between modern humans and Neanderthals, and the age of the Neanderthal specimen (*16*), the length of the region is incompatible with this region having existed in the two groups because they diverged from each other ($P = 9.5 \times 10^{-5}$). We thus conclude that the *GSR* variant encoding the ancestral form of the enzyme comes from gene flow from Neanderthals.

Nucleotide variants in the haplotype carrying the ancestral missense variant (Fig. 2C) match the genome of the Neanderthal from the Vindija Cave more closely than two Neanderthals and a Denisovan from southern Siberia (93.8% of the alleles in $r^2 > 0.8$ match the Vindija Neanderthal genome, whereas 75.0, 65.0, and 35.0% match the Altai Neanderthal, Chagyrskaya Neanderthal, and Denisovan genomes, respectively). This is in agreement with gene flow from Neanderthals into modern humans having occurred mainly from Neanderthal populations related closer to the Croatian Neanderthals than to other Neanderthals studied to date (*6*, *7*). In the genomic region harboring the Neanderthal haplotype, we find four human-derived (*17*) intronic variants in *GSR* in addition to the missense variant (table S4).

Phenotypic effects in humans

We found the Neanderthal-derived GR variant in the UK Biobank [~0.6 per mil (‰)], in the FinnGen Biobank (~4.8‰), and in the Michigan Genomics Initiative (~2.2‰) but not in Biobank Japan or the Million Veterans Program and performed a phenome-wide scan for associations in each of the three former biobanks. In the Michigan Genomics Initiative cohort, we detected a single phenome-wide significant ($P < 2.8 \times 10^{-5}$) association with "Disorders involving the immune mechanism" [odds ratio (OR) = 27.1, 95% confidence interval (CI) = 6.2 to 117.9, $P = 1.1 \times 10^{-5}$; Fig. 3A]. This phenotype is not scored in FinnGen, and the number of cases was too low in the UK Biobank ($n = 249$, c.f. $n = 1884$ for Michigan Genomics Initiative) to detect a signal.

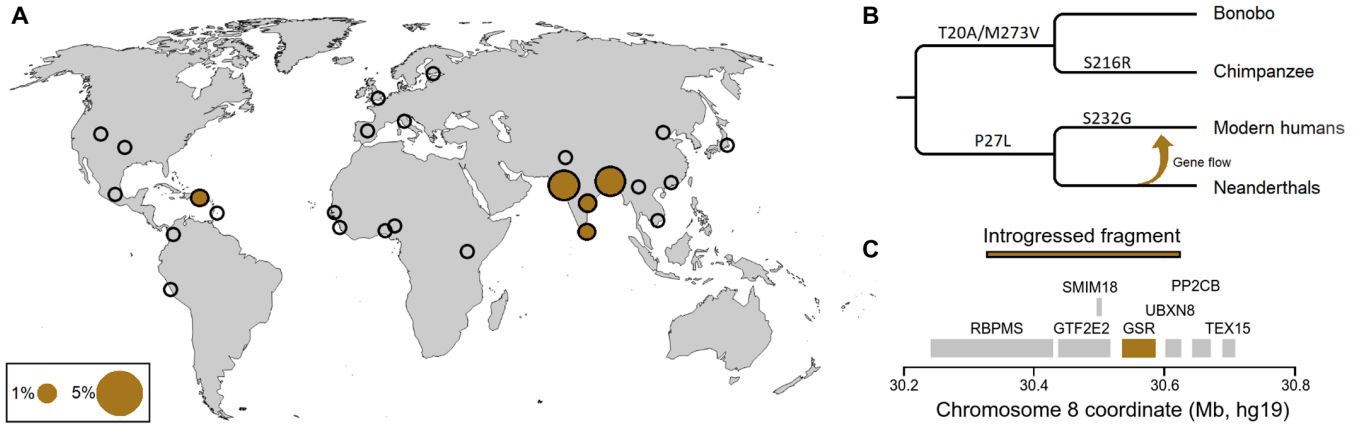

Fig. 2. Geographical distribution, human and ape amino acid replacements in GR, and introgressed fragment. (**A**) Allele frequency of S232 in 26 populations from the 1000 Genomes Project. S232 is mainly present on the Indian subcontinent. Black circle indicates population without the S232 allele. Gold disk indicates the allele frequency with the scale as shown. (**B**) Amino acid replacements in GR among humans and apes. The ancestral S232 was reintroduced into modern humans. (**C**) Genomic region of the introgressed haplotype encoding S232.

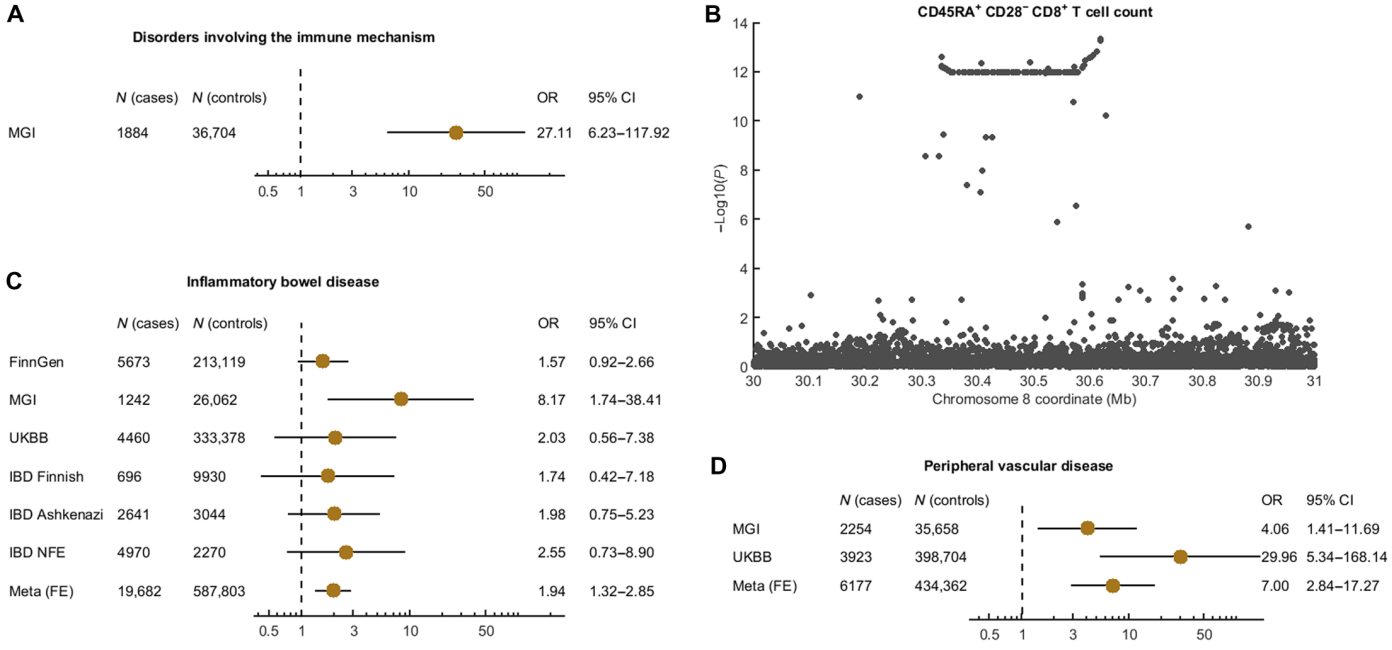

Fig. 3. Phenotypic effects of the Neanderthal GR variant. (**A**) A phenome-wide significant association with the ancestral S232 in Michigan Genomics Initiative (MGI) showing increased susceptibility to disorders involving the immune mechanism ($P = 1.1 \times 10^{-5}$). (**B**) The Neanderthal haplotype is associated with an increased count of TEMRA cells (CD45RA$^+$ CD28$^-$ CD8$^+$). (**C**) Carriers of S232 have an increased risk of IBD in three biobanks and three study populations from the IBD Exomes Portal ($P = 7.0 \times 10^{-4}$). "NFE" denotes Non-Finnish Europeans, and "Meta (FE)" denotes a fixed-effect meta-analysis by inverse-variance weighting. (**D**) S232 is a genetic risk factor for peripheral vascular disease in two cohorts ($P = 2.3 \times 10^{-5}$).

To shed light on the physiological connection between the S232 residue in GR and the immune system, we search for associations in a recent dataset of 731 immune cell traits in 3757 Sardinians (*18*). We find that carriers of the Neanderthal allele have ~4 times higher numbers of CD45RA$^+$ CD28$^-$ CD8$^+$ T cells in the blood (β = 301, 95% CI = 219 to 383, $P = 1.0 \times 10^{-12}$; Fig. 3B), an association that is significant on the genome- and phenome-wide level ($P < 6.8 \times 10^{-11}$). These "terminally differentiated effector memory cells reexpressing CD45RA" (TEMRA) (*19*) are involved in chronic immunological disorders such as graft-versus-host disease (*20*), chronic hepatitis C infection (*21*), and inflammatory bowel disease (IBD) (*22*). It is plausible that TEMRA cells may be increased in carriers of the Neanderthal GR due to increased oxidative stress.

Because oxidative stress is involved in multiple diseases (*23*), we investigated other associations with disease in the biobanks where the S232 residue occurs. The most significant association of S232G with diseases in the UK Biobank is with "Peripheral vascular disease" (OR = 29.96, 95% CI = 5.34 to 168.14, $P = 1.4 \times 10^{-4}$), a disease

known to be associated with oxidative stress (*24*). This association is seen also in the Michigan Genomic Initiative biobank (OR = 4.06, 95% CI = 1.41 to 11.69, $P = 7.5 \times 10^{-3}$), and a combination of the two cohorts results in phenome-wide significant ($P < 3.7 \times 10^{-5}$) risk increase for carriers of the ancestral allele (Fig. 3D, OR = 7.01, 95% CI = 2.84–17.27, $P = 2.3 \times 10^{-5}$) (this phenotype was not scored in FinnGen).

Oxidative stress is also part of the pathophysiology of IBD (*25*, *26*) and the ileum mucosa of IBD patients has markedly low GSH:GSSG ratios (*26*). We therefore investigated the association between S232 and IBD in the three biobanks where it occurs. The Neanderthal-like ancestral allele increases the risk for IBD (Fig. 3C), and a meta-analysis shows that carriers of the Neanderthal allele are almost twice as likely to suffer from IBD relative to noncarriers (OR = 1.89, 95% CI = 1.18 to 3.01, $P = 7.8 \times 10^{-3}$). In the IBD Exomes Portal that combines data from three populations where IBD has been studied, a similar increase in risk is seen (OR = 1.87, 95% CI = 1.33 to 2.64, $P = 0.049$). A meta-analysis of the six study populations strengthens the association (OR = 1.94, 95% CI = 1.32 to 2.85, $P = 7.0 \times 10^{-4}$).

Phenotypic effects in cells

A possible functional link between the higher oxidative potential of the ancestral GR and inflammatory conditions is the production of cytokines, which mediate inflammatory responses and are induced by oxidative stress (*23*). We examined whether the ancestral GR, which produces more reactive oxygen species, may induce more inflammatory cytokines and chemokines compared to the modern human GR, when coincubated with either of two cell lines: Jurkat (human T lymphocytes) and CHME3 (human microglia). We added the two forms of GR to the culture medium at a physiological concentration (2.5 U/ml) for 24 hours and measured the levels of mRNAs encoding nuclear factor κB (NF-κB), which is involved in the transcription of many cytokines, and tumor necrosis factor–α (TNF-α), interleukin-1β (IL-1β), and IL-6, three mediators of the inflammatory response, by reverse transcription and quantitative polymerase chain reactions (RT-PCR). As expected, adding Neanderthal GR to the medium resulted in the production of more hydrogen peroxide than adding modern human GR ($P = 3.5 \times 10^{-4}$ and $P = 1.0 \times 10^{-4}$ for Jurkat and CHEM3, respectively) (Fig. 4, A and B). In Jurkat T cells, transcript levels of TNF-α were 47% higher ($P = 0.017$) when the medium was supplemented with the ancestral, Neanderthal-like GR compared to when it was supplemented with the modern human GR (Fig. 4C). In microglial CHME3 cells, levels of mRNAs encoding NF-κB were 57% higher in the presence of the ancestral GR than in the presence of the modern variant of GR ($P = 0.036$; Fig. 4F). A joint analysis of TNF-α and NF-κB in both Jurkat T cells and microglia CHME3 cells revealed a general effect of the Neanderthal variant ($P = 0.036$) compared to the modern human GR. This suggests that the higher oxidative stress caused by Neanderthal GR may have direct effects on cytokine production in immune cells, which may contribute to its association with diseases with an inflammatory component.

DISCUSSION

The S232G amino acid substitution in GR is present in almost all humans today. We here show that it causes the enzyme to leak fewer electrons, resulting in lower levels of oxidative stress. We note that in the quaternary structure of the protein, the S232G amino acid substitution is in the proximity of the NADPH-binding site (positions 232 to 243), compatible with an effect on electron leakage.

The expression of *GSR* could also potentially differ between Neanderthals and modern humans. One of the human-derived intronic variants in *GSR* (rs8191007; table S4), which falls between the 9th and the 10th exon of *GSR*, has some regulatory potential based on chromatin accessibility and a predicted transcription factor

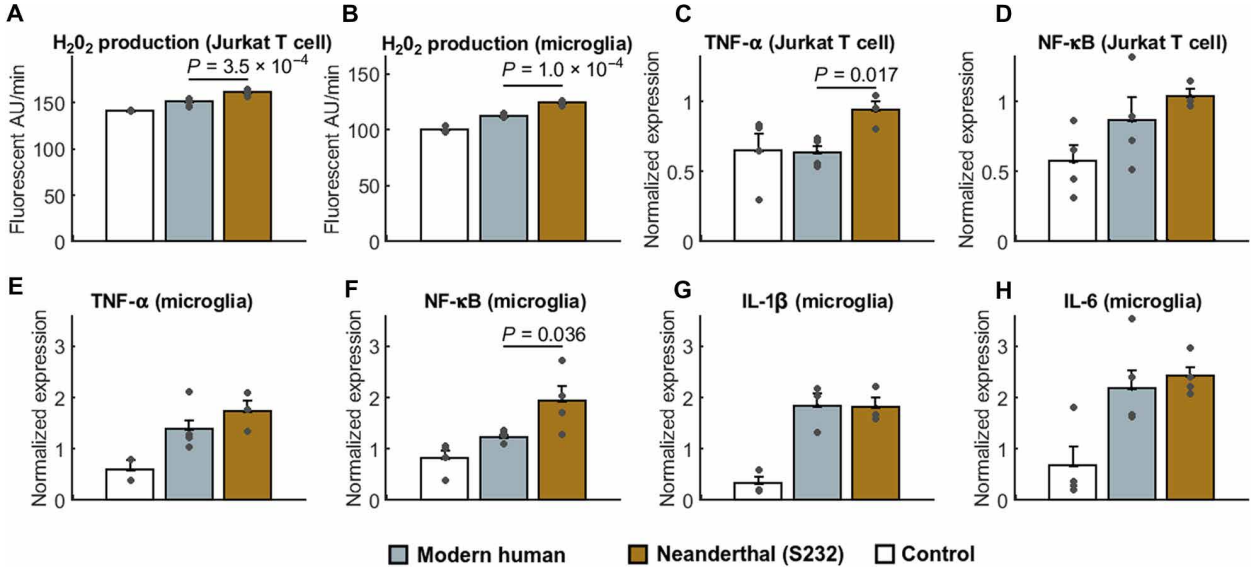

Fig. 4. Extracellular production of reactive oxygen species and transcriptional consequences. (**A** and **B**) Production of reactive oxygen species in the extracellular medium of Jurkat T cells or microglia cells (*CHME3*) in medium supplemented with the modern human or Neanderthal GR variants (2.5 U/ml) or in medium without GR (control). AU, arbitrary units. (**C** to **H**) mRNA expression in Jurkat or CHME3 cells after 24-hour exposure to modern human or Neanderthal GR variants. Normalized to expression in cells treated with lipopolysaccharide (10 ng/ml) for 4 hours. Error bars indicate SEM.

binding site (*27*). However, because the expression of *GSR* does not differ significantly between humans and apes or macaques (*28*), there is little reason to assume that expression of *GSR* would differ between modern humans and Neanderthals.

As a result of gene flow from Neanderthals, the ancestral GR version occurs on the Indian subcontinent at an allele frequency of 1 to 2% and at very low levels in Europe. By analyzing phenotypes with high prevalence in multiple large cohorts, we detect associations between the ancestral variant and vascular disease and IBD, despite its low allele frequency. Both these disorders have well-established links to oxidative stress and the immune response. In vascular disease, overproduction of reactive oxygen species causes vascular inflammation (*24*), and GSH deficiency characterizes the intestinal mucosal of patients with IBD (*26*, *29*). However, given that GR is ubiquitously expressed and that oxidative stress is involved in many diseases, the S232G amino acid replacement might have protective effects in addition to those described here. It may also affect traits such as aging. We can only speculate about any putative evolutionary advantage conferred by this variant upon modern humans in the past.

MATERIALS AND METHODS

Experimental design

Recombinant GR expression and purification

The plasmid pD441-H6SUMO-hGR expressing wild-type human GR with an N-terminal His-6-SUMO tag was obtained from Q. Cheng and E. S. J. Arnér (Karolinska Institutet) and was mutagenized at position 189 in the inferred protein using the primers GTAGGTCTCCTGCCTAGCCGTAGCGTTATTGTTGGTGCAG-3′ and GTAGGTCTCAGGCAGTTCTTCCAGCTGAAAAAAACC-3′. Initial denaturation at 95°C for 30 s was followed by 30 cycles of 10 s at 95°C, 30 s at 54°C, and 3 min at 72°C. The PCR product was digested with Eco31 I and Dpn I at 37°C for 1 hour. T4 DNA ligase was used to ligate the digested PCR product, and the ligation mixture was transformed into competent *Escherichia coli* DH5α cells and grown on kanamycin plates. The presence of the desired mutation was confirmed by DNA sequencing.

Plasmids were transformed into *E. coli* (Turbo Competent, New England Biolabs) and a single colony grown overnight in 10 ml of Terrific Broth media, containing kanamycin (100 μg/ml) and glycerol (8 g/liter) and was grown under agitation to A_{600} (absorbance at 600 nm) of 0.6 to 0.8. Then, 0.5 mM isopropyl-1-thio-β-D-galactopyranoside was added followed by incubation overnight at 24°C. Cells were pelleted; lysed in 100 mM Hepes (pH 7.5), 10% glycerol, 300 mM NaCl, and cOmplete EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich) at 4°C; sonicated using a Sonics Vibra Cell CV33; and centrifuged at 16,000 RPM for 30 min at 4°C. The lysate was loaded onto an immobilized metal affinity chromatography (IMAC) Nickel Sepharose column and eluted by Hepes buffer containing 100 mM imidazole. The His6-SUMO-tag was cleaved by ULP1 (SUMO protease), and the protein was collected in the flow-through from a second IMAC column, concentrated on 30-kDa Amicon centrifugal filters and stored in TE buffer containing 20% glycerol at −20°C.

Enzyme characterization

The apoenzyme precipitation and reconstitution followed (*30*), and concentration was measured by absorbance at A_{278} and 57,000 M^{-1} cm^{-1} as extinction coefficient. Protein concentrations were determined spectrophotometrically by FAD absorption at 460 nm (ϵ = 11,300 M^{-1} cm^{-1}; one FAD assumed to correspond to one GR subunit) in phosphate-buffered saline (PBS) (Thermo Fisher Scientific), 2 mM EDTA, and BSA (bovine serum albumin; 0.05 mg/ml; Sigma-Aldrich). Absorbance spectra between 300 and 700 nm were recorded in 1-ml quartz cuvettes in a Tecan Infinite M200 Pro plate reader. To measure the specific activity, 1 nM modern human GR or 1 nM Neanderthal GR was dissolved in the assay buffer [PBS, 2 mM EDTA, and BSA (0.05 mg/ml)] with 300 μM NADPH. NADPH absorbance was standardized by the absorbance at 340 nm before each sample. The reaction was started by adding GSSG to a final concentration of 1 mM, and the reduction of GSSG was determined indirectly by the consumption of NADPH. One unit of enzyme activity is defined as the reduction of 1 μmol of GSSG per minute per milligram of GR at 25°C. We tested which incubation temperature between 30° and 90°C leads to 50% enzyme inactivation in 10 min after the addition of 200 μM NADPH or 1 mM GSSG and recording of the absorbance at 340 nm every 20 s for 5 min. To test the oxidation of NADPH by GRs in the absence of GSSG, 1 μM enzyme was mixed with 200 μM NADPH at 37°C, and the absorbance at 340 nm was recorded every 30 s for 1 hour. Superoxide production was also assessed in reactions containing 2 mM epinephrine, and adrenochrome production was measured by absorbance at 480 nm. Addition of an excess of superoxide dismutase (6 U per well) completely abolished the adrenochrome formation, confirming that superoxide was formed. K_M and V_{max} for NADPH were estimated in 200-μl volumes containing 1 nM enzyme and 7.5 to 50 μM NADPH concentrations, starting the reactions by the addition of 1 mM GSSG. To determine K_M and V_{max} for GSSG, reactions contained 200 μM NADPH, 1 nM enzyme, and 10 to 640 μM GSSG concentrations and were started by adding 200 μM NADPH.

Cell culture experiments

CHME3 and Jurkat cells were maintained in a DMEM (Dulbecco's modified Eagle's medium; Lonza) and RPMI 1640 (Merck) medium, respectively, supplemented with 10% fetal bovine serum, at 37°C and 5% CO_2. Viability was measured in 96-well culture plates (7000 cells per well) with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich) and absorbance at 570 nm as previously described (*31*). For mRNA isolation, 400,000 CHME3 cells or 200,000 Jurkat cells were plated in six-well plates. The next day, modern human GR or Neanderthal GR (2.5 U/ml) was added. Each well was washed twice with PBS, and cells were collected in 200 μl of TRIzol (Life Technologies) and reverse-transcribed using Maxima First Strand cDNA synthesis kit (Thermo Fisher Scientific). Quantification of IL-1 and IL-6, TNF-α, and NF-κB mRNA levels was assessed by real-time RT-PCR using the primers in table S5. Relative expression was calculated using the ΔΔCT (delta-delta cycle threshold) method after normalizing to actin expression and normalized to lipopolysaccharide (10 ng/ml) treatment.

Measurement of H_2O_2 in the cell medium was carried out as previously described (*32*). Briefly, final concentrations of 50 μM Amplex Red and horseradish peroxidase (0.1 U/ml) were added to Jurkat or CHME3 cell medium, respectively, and fluorescence was monitored (560 nm excitation/590 nm emission) for 1 hour.

Evidence for introgression

The S232G polymorphism sits on a 289.4-kb haplotype (r^2 > 0.8 in all 1000 Genomes individuals) defined by 46 private single-nucleotide polymorphisms on the Neanderthal lineage (i.e., the Neanderthal allele is missing in 108 Yoruba individuals, and Yoruba carry the ancestral allele) with coordinates chr8: 30,334,914 to 30,624,304 (hg19). Using this length, a local recombination rate of 0.34 cM/Mb

(deCODE) (*15*), the parameters from (*33*), and the formula derived by Huerta-Sánchez *et al.* (*16*) yields a probability of $P = 9.5 \times 10^{-5}$ for incomplete lineage sorting.

Phenotypic consequences of S232

We searched for carriers of the S232 allele (T at rs8190976) in the UK Biobank, FinnGen, the Michigan Genomics Initiative, the Million Veterans' Program, and Biobank Japan. We found carriers of the minor allele at rs8190976 in the UK BioBank and in the Michigan Genomics Initiative. In FinnGen, we found carriers of the minor allele of rs376850387, which is in perfect linkage disequilibrium ($r^2 = 1$) with rs8190976 in the 1000 Genomes Project (*14*) and was used as a proxy, whereas rs8190976 was present in IBD Exomes Portal.

Statistical analysis

Comparisons between groups were made by two-tailed unpaired *t* tests. The joint analysis of TNF-α and NF-κB in both Jurkat T cells and microglia CHME3 cells was performed using a two-way analysis of variance (ANOVA). The Michaelis-Menten equations were calculated using GraphPad Prism Software (GraphPad Software Inc., La Jolla, CA), as well as the thermal stabilities. The expected length of a shared ancestral sequence was assumed to follow a Gamma distribution, as previously described (*16*). Meta-analyses of phenotypic effects were done using the inverse variance method.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abm1148

View/request a protocol for this paper from *Bio-protocol*.

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Glutathione: A Samsonian life-sustaining small molecule that protects against oxidative stress, ageing and damaging inflammation

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Many local and systemic diseases especially diseases that are leading causes of death globally like chronic obstructive pulmonary disease, atherosclerosis with ischemic heart disease and stroke, cancer and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causing coronavirus disease 19 (COVID-19), involve both, (1) oxidative stress with excessive production of reactive oxygen species (ROS) that lower glutathione (GSH) levels, and (2) inflammation. The GSH tripeptide (γ- L-glutamyl-L-cysteinyl-glycine), the most abundant water-soluble non-protein thiol in the cell (1–10 mM) is fundamental for life by (a) sustaining the adequate redox cell signaling needed to maintain physiologic levels of oxidative stress fundamental to control life processes, and (b) limiting excessive oxidative stress that causes cell and tissue damage. GSH activity is facilitated by activation of the Kelch-like ECH-associated protein 1 (Keap1)-Nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) redox regulator pathway, releasing Nrf2 that regulates expression of genes controlling antioxidant, inflammatory and immune system responses. GSH exists in the thiol-reduced (>98% of total GSH) and disulfide-oxidized (GSSG) forms, and the concentrations of GSH and GSSG and their molar ratio are indicators of the functionality of the cell. GSH depletion may play a central role in inflammatory diseases and COVID-19 pathophysiology, host immune response and disease severity and mortality. Therapies enhancing GSH could become a cornerstone to reduce severity and fatal outcomes of inflammatory diseases and COVID-19 and increasing GSH levels may prevent and subdue these diseases. The life value of GSH makes for a paramount research field in biology and medicine and may be key against systemic inflammation and SARS-CoV-2 infection and COVID-19 disease. In this review, we emphasize on (1) GSH depletion as a fundamental risk factor for diseases like chronic obstructive pulmonary disease and atherosclerosis (ischemic heart disease and stroke), (2) importance of oxidative stress and antioxidants in SARS-CoV-2 infection and COVID-19 disease, (3) significance of GSH to counteract persistent damaging inflammation, inflammaging and early (premature) inflammaging associated

with cell and tissue damage caused by excessive oxidative stress and lack of adequate antioxidant defenses in younger individuals, and (4) new therapies that include antioxidant defenses restoration.

KEYWORDS

glutathione, oxidative stress, reactive oxygen species, nuclear factor erythroid 2-related factor 2, inflammaging, chronic obstructive pulmonary disease, atherosclerosis, COVID-19

Introduction

Glutathione (GSH) is a unique molecule essential for life that participates in key aspects of cellular homeostasis, having a paramount role in defense against the oxidative damage that occurs during all different diseases including coronavirus disease 19 (COVID-19) disease. GSH has a central participation in *trans*-hydrogenation reactions needed to maintain a reduced state of sulfhydryl groups of other molecules, proteins and enzymes, as well as formation of deoxyribonucleotides and vitamin reduction (1–5). GSH has the function of "master antioxidant" in all tissues and is involved in antioxidant defense, detoxication of xenobiotics, intracellular redox homeostasis, cysteine carrier/storage, cell signaling, protein folding and function, gene expression, cell differentiation/proliferation, immune response and antiviral defense, that make it a "Samsonian (mighty) little molecule" (1, 2). The high (millimolar) concentration of the reduced form highlights its central role in the control of those processes (5–8). The central role of GSH in oxidative stress and inflammation, in the pathophysiology of inflammatory diseases and COVID-19, and in host immune response and disease severity and mortality, makes GSH a little but powerful player in maintaining health and avoiding disease. In this review we will focus on (a) GSH depletion as a fundamental risk factor for diseases like chronic obstructive pulmonary disease and atherosclerosis (ischemic heart disease and stroke), (b) importance of oxidative stress and antioxidants in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and COVID-19 disease, (c) significance of GSH to counteract persistent damaging inflammation, inflammaging and early (premature) inflammaging associated with cell and tissue damage caused by excessive oxidative stress and lack of adequate antioxidant defenses in younger individuals, and (d) new therapies that include antioxidant defenses restoration.

Glutathione brief history

Glutathione was discovered in 1888 by de Rey-Pailhade and initially named "philothion" (from the Greek words meaning "love" and "sulfur") because of its reactivity with sulfur to form hydrogen sulfide (4, 9). Subsequently, Hopkins reported this substance as a dipeptide containing glutamate and cysteine, and he named it "glutathione" (10), which is a tripeptide consisting of glutamate, cysteine, and glycine (11, 12). Harington and Mead finally described the correct chemical structure of the tripeptide in 1935 (13). GSH was virtually forgotten for 40 years until in 1969, Kosower and Kosower (14) emphasized the scant GSH research in those days. GSH research had a great momentum especially in the 1980s, with studies carried out by Meister and his collaborators who contributed to understanding the physiological functions and the metabolism (4).

Glutathione composition and synthesis

The GSH (γ- L-glutamyl-L-cysteinyl-glycine) is a water-soluble tripeptide formed by the amino-acids glutamic acid, cysteine and glycine (**Figure 1**) present in the cytoplasm of all cells. GSH is found in all mammalian tissues as the most abundant non-protein thiol that defends against oxidative stress and possess a distinctive stability provided by a γ-carboxyl bond within the molecule (**Figure 1A**). GSH exists in the thiol-reduced and disulfide-oxidized (GSSG) forms (1, 2); and it's free and bound to proteins (**Figure 1**). The reduced form GSH is the active form of the molecule, it is the most abundant and it is found inside the cells in millimolar concentrations in the range of 1–10 mM (highest concentration in liver) (5–8), while extracellularly they are found in micromolar (GSH in plasma: 10–30 μM) levels (5, 15, 16). The active group of the molecule is represented by the thiol group (-SH) of the cysteine residue (**Figure 1**) which provides its reductive power. The millimolar GSH intracellular concentrations, the low plasma micromolar concentrations and the low GSH redox potential ($E'_0 = -240$ mV) make GSH an ideal and perfect cellular redox buffer (5, 16–18). GSH accounts for >98% of total GSH (3, 19–23). Eukaryotic cells have three major reservoirs of GSH. Most (80–85%) of the cellular GSH are in the cytosol, 10–15% is in the mitochondria and a small percentage is in the endoplasmic reticulum (**Figure 2**) (3, 24–28). GSSG is found mainly extracellularly. The redox state of

the GSH/GSSG couple can serve as an important indicator of redox environment (29–31), and changes in this couple correlate with multiple cellular processes, including cell differentiation (32–37), cell proliferation (32–37), and cell apoptosis (38–42). GSH deficiency as evidenced by a decrease in the GSH/GSSG ratio manifests itself largely through an increased susceptibility to oxidative stress, and the resulting damage is thought to be involved in SARS-CoV-2 infection leading to COVID-19 disease (6, 7, 39, 43–53). In addition, imbalances in GSH levels affect immune system function, and are thought to play a role in the aging process and the diseases of aging, one of the principal risk factors for the development and progression of COVID-19 disease.

Glutathione is synthesized in the cytosol of all cells from their precursor amino acids: glutamic acid, cysteine and glycine by consecutive action of two enzymes: γ-glutamyl-cysteine (γ-GluCys) synthetase (also known as glutamate cysteine ligase, GCL) that in a first step uses glutamate and cysteine as a substrate to form the dipeptide γ-glutamyl-cysteine; and glutathione synthetase that in a second step combines γ-glutamyl-cysteine with glycine for forming GSH (54, 55) (**Figure 3**). ATP (adenosine triphosphate) acts as a co-substrate for both enzymes (**Figures 2**, **3**). Under normal physiological conditions, the rate of synthesis of GSH is determined to a large extent by two factors: (a) the *activity of GCL* and (b) the *availability of the cysteine substrate*. Therefore, the intracellular levels of GSH are regulated by the negative feedback of GSH itself on the GCL enzyme (1, 4, 55–57) and by the availability of the amino acid L-cysteine (1, 4, 58). The GCL enzyme is a heterodimer formed by two subunits: the heavy subunit or glutamate cysteine ligase catalytic subunit (GCLC, 73 kDa) and the light subunit or glutamate cysteine ligase modulating subunit (GCLM, 30 kDa). The heavy subunit has the active site responsible for the union between the amino group of the cysteine and the γ-carboxyl group of glutamate. The GCLM subunit has no enzymatic activity but has an important regulatory function increasing the efficiency of the GCLC subunit. This subunit is required for optimal activity and feedback inhibition by GSH (59). GSH inhibits GCL by competing with glutamate in the active site of GCLC (1, 57–60). The enzyme glutathione synthetase (GS) is formed by two identical subunits (52 kDa/subunit) and is not regulated by intracellular levels of GSH. The active site of the enzyme that binds glycine to the dipeptide γ-L-glutamyl-L-cysteine is highly specific (57). GCL is considered the speed limiting enzyme of synthesis since overexpression of GS does not increase GSH levels while overexpression of GCL increases the synthesis of GSH (61) (**Figure 3**). ATP is the energy donor for both enzymes. As mentioned above, GSH cellular concentrations are regulated by GSH-mediated GCL inhibition (**Figures 2**, **3**). Thus, the biological control of intracellular GSH homeostasis through consumption and supply is an intricately balanced process that prevents oxidative stress. Cellular GSH (cytosol, mitochondria, endoplasmic reticulum, nucleus; **Figures 2**, **4**) availability is maintained by *de novo* synthesis from precursor amino acids, (glutamate, cysteine, and glycine), reduction of GSSG by glutathione reductase (GR), and uptake from exogenous GSH sources across plasma membranes (**Figure 4**) (62, 63). The three amino acids are adsorbed by transporters. Additionally, intestinal epithelial cells can import intact GSH from the lumen *via* specific plasma membrane transporters (7).

Glutathione cellular distribution

Glutathione is found in almost all cellular compartments, including the nucleus (5, 54, 64–68) (**Figures 2**, **4**). The GSH transport between the various cell compartments is vital to buffer reactive oxygen species (ROS) and facilitate redox signaling in order to control cell growth, development and defense, as well as regulate cell proliferation. GSH is predominantly in its thiol-reduced form inside the cells, except in the lumen of the endoplasmic reticulum where it exists only in its GSSG form (**Figures 2**, **4**). The GSH content existing in millimolar concentrations varies among different organs; liver being among organs with the highest content (56). GSH content also varies among different areas of the same tissues; periportal hepatocytes may contain nearly twice the centrilobular concentration, enterocytes at the villus tip have a higher content than the crypts, and renal proximal tubular cells have more GSH than other parts of the nephron (56). Mitochondria contain 10–15% of the intracellular GSH reaching a concentration of 10–12 mM (54) while in the cytosol the concentration is 7 mM (54, 56). This difference in concentration is associated with the absence of catalase inside the mitochondria, what leaves GSH in charge of all inactivation of the hydrogen peroxide generated during the oxidative processes that occur in the mitochondrial matrix (57).

The concentration of GSH in the mitochondrial compartment is more important for cell survival than the GSH found in the cytosol. Since mitochondria do not have the enzymes involved in the synthesis of GSH, all the GSH found in the mitochondrial compartment comes from the cytosol. A system transport present in the inner mitochondrial membrane, that involves dicarboxylate and 2-oxoglutarate anion transporters, allows the passage of negatively charged GSH from the cytosol to the mitochondria. The first incorporates GSH into the mitochondria by inorganic phosphate exchange and the second by exchange of 2-oxoglutarate (27, 28, 64) (**Figures 2**, **4**). While the greater amount of cellular reduced GSH is found in the cytosol and mitochondria, the endoplasmic reticulum becomes a reservoir of small concentrations of the oxidized form of GSH (GSSG). The ratio of reduced GSH to the disulfide form (GSH/GSSG)

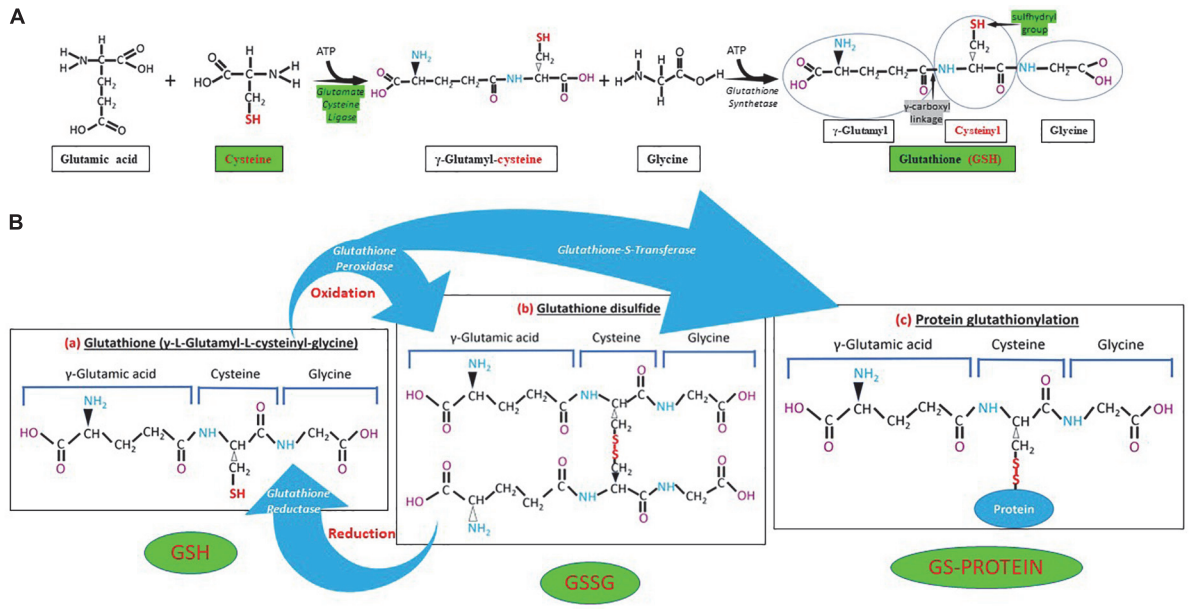

FIGURE 1
Glutathione (GSH) synthesis, chemical structure and different forms of GSH. **(A)** GSH is synthesized in the cytosol in two steps. The first step is the formation of γ-glutamylcysteine from glutamate and cysteine by the enzyme γ-glutamylcysteine synthetase (glutamate cysteine ligase). The second step in GSH synthesis is regulated by glutathione synthetase. Glutathione cysteine ligase and cysteine (green) are the limiting factors in GSH synthesis. The γ-carboxyl linkage (gray) and the sulfhydryl group (green) provide stability and reductive power to the molecule, respectively. **(B)** Chemical structure of reduced (GSH), oxidized (GSSG) glutathione and GS-protein generated by protein glutathionylation. Glutathione peroxidase oxidizes GSH and glutathione reductase reduces GSSG, while glutathione-S-transferase participates in protein glutathionylation.

within the endoplasmic reticulum ranges from 1:1 to 4:1, whereas the overall cellular GSH/GSSG ratio ranges from 30:1 to 100:1 (26) (**Figure 2**). There is a preferential transport of GSSG from the cytosol to the endoplasmic reticulum to maintain an adequate environment for protein disulfide bond formation and protein folding (69–71). There is little data about the concentrations of GSH in the nucleus and endoplasmic reticulum largely because of a lack of adequate techniques to accurately determine the GSH pool at those locations (15, 69, 72, 73). There are great variations in nuclear GSH concentration and its regulation mechanisms during the cell cycle since cells starting the proliferation phase have high levels of nuclear GSH, while resting cells have similar or lower GSH levels in the nucleus than in the cytoplasm (68, 72, 73). High nuclear GSH concentrations are vital since increase in total GSH is necessary for the cells to progress from the G1- (with low GSH levels) to the S-phase; addition of GSSG causes the cell cycle to arrest at G1; and excessive and prolonged oxidation arrest cell cycle triggering cell death (68, 72, 73). GSH behaves as "redox sensor" at the DNA synthesis onset by maintaining nuclear architecture providing the appropriate redox environment for DNA replication and safeguarding DNA integrity (72) and is a key regulator of epigenetic events critical in cell proliferation regulation and cellular resistance to apoptosis (73).

Glutathione and the γ-glutamyl cycle

The synthesis, transport and catabolism of GSH occur in a series of enzymatic steps and transports of membrane that are collectively called *γ-glutamyl cycle* (**Figure 5**) (1, 74, 75). The γ-glutamyl cycle was postulated by Meister (76) and it accounts for the GSH biosynthesis and degradation. The GSH biosynthesis has been described previously. After its synthesis, GSH is transported to the intracellular compartments, mitochondria, endoplasmic reticulum and nucleus, but most of it is released through transporters toward the extracellular space. In contrast to the synthesis, that occurs only intracellularly, the degradation or catabolic part of the GSH cycle, takes place partially extracellularly and partially inside cells. The extracellular degradation of GSH occurs on the surface of the cells that express the enzyme γ-glutamyl transpeptidase and the dipeptidases found in the external plasma membrane (1) (**Figure 5**). After the plasma membrane carrier-mediated GSH release from the cell, GSH becomes accessible to the active site of γ-glutamyl transpeptidase, which catalyzes the breakdown of the GSH γ-glutamyl bond forming two fractions: The γ-glutamyl fraction and the cysteinyl-glycine by transferring the γ-glutamyl fraction to an amino acid acceptor, forming γ-glutamyl-amino acid. Once inside the cell, the γ-glutamyl-amino acid can be metabolized to release the amino acid and 5-oxoproline, which

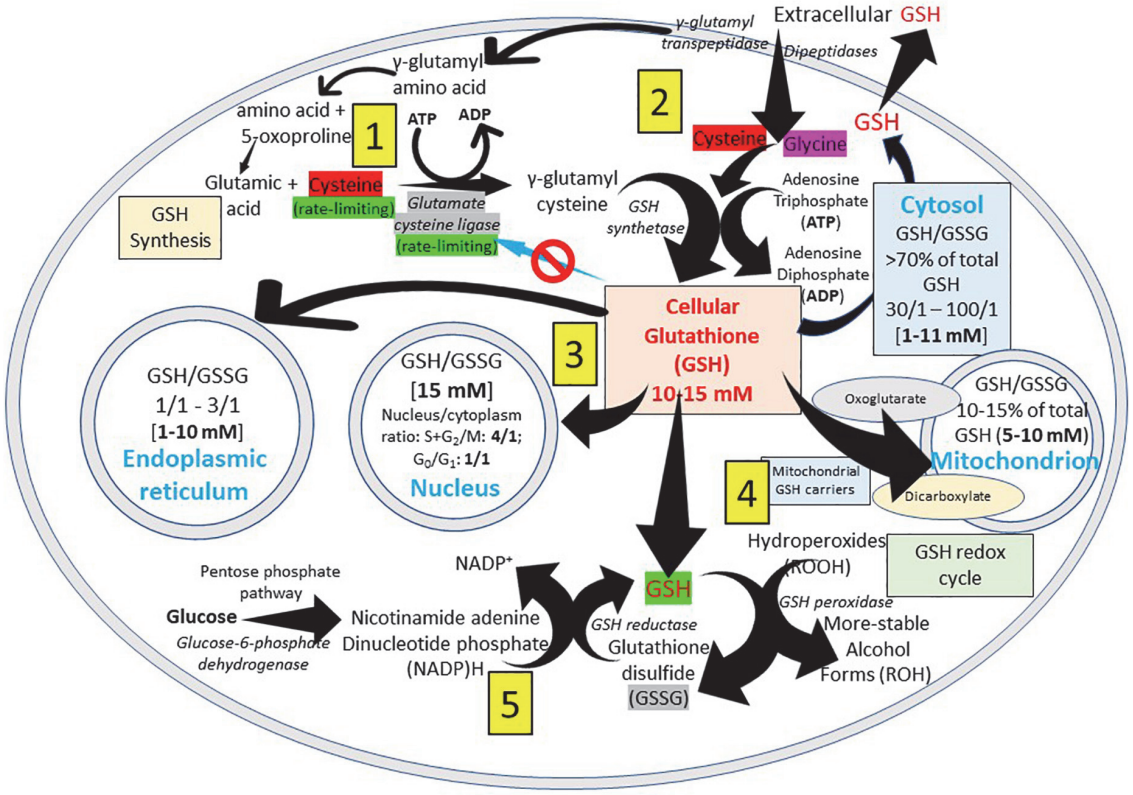

FIGURE 2
Glutathione distribution in subcellular compartments. GSH (γ- L-glutamyl-L-cysteinyl-glycine), a water-soluble tripeptide formed by the amino-acids glutamic acid, cysteine and glycine, is considered the major non-protein low molecular weight modulator of redox processes and the most important thiol reducing agent of the cell. (1) ATP-dependent GSH biosynthesis occurs in the cytosol of the cell and cysteine (red) and glutamate cysteine ligase (gray) are rate-limiting factors for its production. (2) Extracellular GSH is enzymatically degraded on the surface of the cells by γ-glutamyl transpeptidase generating the γ-glutamyl fraction (taken into the cell as γ-glutamyl-amino acid that can be metabolized to release the amino acid and 5-oxoproline, which can then be converted into glutamate to be used in the synthesis of GSH) and the cysteinyl-glycine fraction; and by dipeptidases splitting cysteinyl-glycine generating cysteine and glycine that are taken into the cell. (3) To allow normal cell function, it is essential to maintain an optimal GSH: GSSG ratio throughout all cell compartments. (4) The inner mitochondrial membrane system transport, that involves dicarboxylate and 2-oxoglutarate anion transporters, allows the passage of negatively charged GSH from the cytosol to the mitochondria. (5) GSH is present in both reduced (GSH) and oxidized (GSSG) states, and reduced GSH is maintained by GSH reductase, a cytosolic NADPH-dependent enzyme. GSSG returns to the reduced state by the NADPH-dependent activity of glutathione reductase. NADPH is rapidly regenerated from NADP + using electrons derived from catabolism of substrate molecules, such as glucose or isocitric and malic acid (pentose phosphate pathway). Reduced GSH neutralizes cellular hydroperoxides through GSH peroxidase activity.

can then be converted into glutamate to be used in the synthesis of GSH. On the other hand, also in the extracellular space, the cysteinyl-glycine fraction is split by the enzyme dipeptidase generating cysteine and glycine. The cells incorporate cysteine and most of the intracellular cysteine is incorporated into the synthesis of GSH. Depending on the metabolic needs of the cell, the cysteine can be used for protein synthesis and part can be degraded to sulfate and taurine. The cycle γ-glutamyl allows GSH to be used as a continuous source of cysteine. The γ-glutamyl amino acid is taken up by cells through a specific transport mechanism. Cysteinyl glycine is also taken up by cells. Inside the cell, the γ-glutamyl amino acid is hydrolyzed by γ-glutamyl cyclo-transferase and converted into oxoproline and a free amino acid. Oxoproline is a cyclic form of glutamate and is converted into glutamate *via* oxoprolinase (**Figure 5**).

The γ-glutamyl cycle was initially postulated by Meister as a mechanism for amino acid transport (76). However, this presents major problems. The most important is the energetic one. The γ-glutamyl cycle requires the use of three ATP molecules per turn of the cycle. Thus, the uptake of an amino acid would require the use of three high-energy phosphate bonds. In favor of the cycle was the fact that addition of γ-glutamyl transpeptidase inhibitors *in vivo* caused a decrease in amino acid transfer into cells. The gamma-glutamyl cycle should be considered not as a mechanism for amino acid transport but rather a generator of extracellular signals, gamma-glutamyl amino acids, that are converted intracellularly to 5-oxoproline, which activates uptake and/or metabolism of amino acids (1, 74, 75). The γ-glutamyl amino acids or oxoproline could be signaling molecules to activate the transport of amino

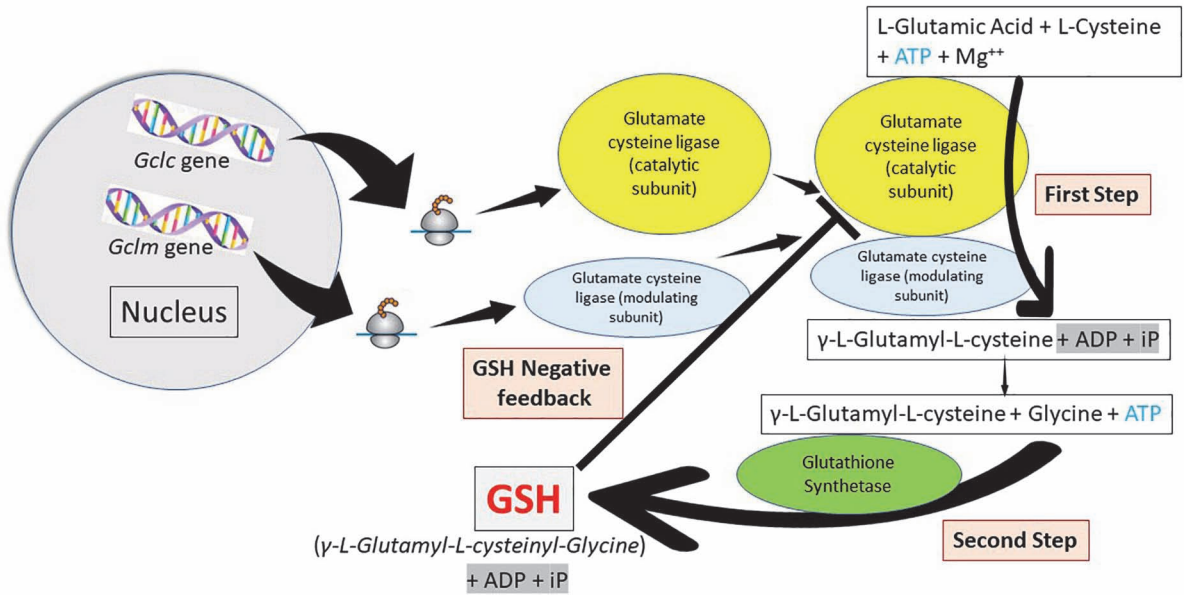

FIGURE 3
Glutathione synthesis: A two-step pathway. Homeostasis of cellular glutathione. Synthesis and regulation of the cell concentrations. Glutamate cysteine ligase (γ-glutamyl cysteine synthetase) constitute the first step in the synthesis of glutathione (GSH) forming γ-L-glutamyl-L-cysteine using adenosine triphosphate (ATP). Glutathione synthetase constitute the second step forming GSH, also using ATP. Cellular GSH concentration regulates the function of glutamate cysteine ligase.

acids through membranes. Oxoproline catalytically activates the uptake of amino acids through the placental barrier, and the transfer of amino acids through the blood–brain barrier is activated by oxoproline (2, 75). Thus, the γ-glutamyl cycle, apart from explaining the synthesis and degradation of glutathione, may serve as a generator of signals to activate amino acid transport into cells (2, 75). GSH turnover may be considered as a multi-organ process. In fact, in liver, an organ in which glutathione synthesis is most active, the degradation is very slow due to the very low activity of γ-glutamyl transpeptidase. In the kidney, however, γ-glutamyl transpeptidase is very high. Thus, the γ-glutamyl cycle may be considered as a multi-organ cycle in which the synthetic part occurs in liver and the catabolic part occurs in kidney amongst other tissues.

Glutathione and damaging inflammation in lower respiratory diseases

Glutathione Samsonian (mighty) power is centered in the thiol (sulfhydryl) group of the cysteine amino acid. GSH participates in numerous key processes where the thiol reducing potential is utilized. Several lung disorders are believed to be characterized by an increase in alveolar oxidant burden, potentially depleting alveolar and lung GSH. Low GSH has been linked to abnormalities in the lung surfactant system and the interaction between GSH and antiproteases in the epithelial lining fluid of patients. Normal levels of intracellular GSH may exert a critical negative control on the elaboration of proinflammatory cytokines. The increase of intracellular ROS is believed to correlate with the activation of nuclear factor (NF)-kappa B, a transcription activator linked to the elaboration of several cytokines (**Figure 6**). There is now sufficient data to strongly implicate free radical injury in the genesis and maintenance of several lung disorders in humans. This information is substantial and will help the development of clinical studies examining a variety of inflammatory lung disorders.

Oxidative stress and inflammation are considered fundamental mediators of chronic obstructive pulmonary disease (COPD) pathophysiology (77–91). The lungs are directly exposed to tobacco smoke and air pollutants that are main sources of ROS. ROS directly cause lung damage as a result of DNA, lipid, carbohydrate, and protein alterations, and activate local inflammatory responses that contribute to COPD development and progression (79–83). ROS can further activate epithelial cells and macrophages facilitating neutrophil, monocyte, and lymphocyte recruitment, and the recruited activated inflammatory cells subsequently enhance additional ROS generation, increasing the pro-oxidant burden (80–85). ROS and RNS production are facilitated by pattern recognition receptors [C-reactive protein (CRP), toll-like receptors] capable of recognizing pathogen (bacterial/viral)-associated molecular patterns and/or damage-associated molecular patterns in apoptotic or damaged cells (92–100) (**Figure 6**). The

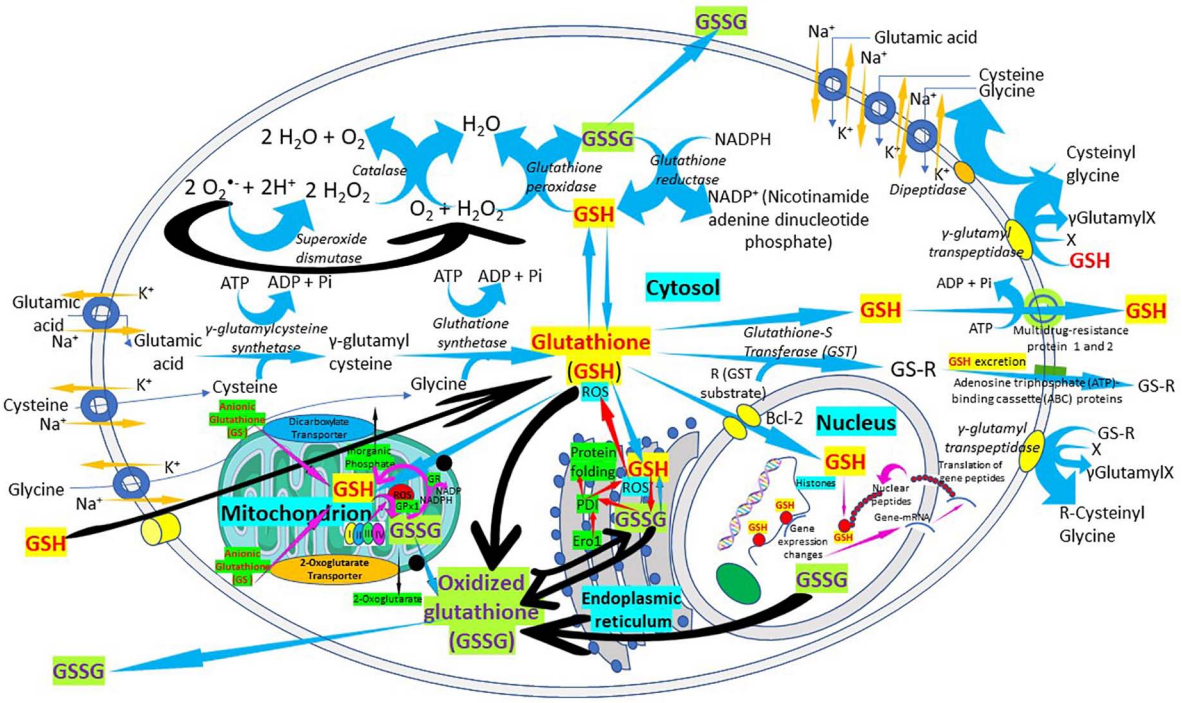

FIGURE 4
The "Glutathione Pathway." *Glutathione synthesis, γ-glutamyl pathway, cellular distribution, antioxidant properties, catabolism of xenobiotics,* and *glutathione recycling in the cell*. The figure shows a schematic representation of the "glutathione pathway." Glutathione (GSH) is synthesized from glutamate, cysteine, and glycine by γ-glutamyl-cysteine synthetase (glutamate cysteine ligase) and glutathione synthetase. Glutathione redox state is regulated, in part, by glutathione peroxidases, forming oxidized glutathione (GSSG), and by a reaction catalyzed by glutathione reductase. Glutathione is conjugated to substrates both through the action of the glutathione S-transferases and through non-enzymatic reactions. Glutathione conjugates can be excreted from the cells by members of the ATP-binding cassette (ABC) transporter family.

phosphocholine head group in phospholipids of normal healthy cell membranes is not accessible but, when cells are damaged and die, enhanced availability of lysophosphatidylcholine and disruption of the lipid bilayer expose phosphocholine residues to which CRP avidly binds (99). These events lead to a state of persistent inflammation and chronic oxidative stress (82–85), characterized by increased ROS production, reduced GSH peroxidase activity, selenium deficiency and reduced GSH levels (80–91). Asymptomatic smokers and stable COPD patients showed increased GSH levels in bronchoalveolar lavage, while patients with severe/very severe exacerbation periods had significantly decreased levels (101). Patients with decreased GSH and increased oxidative stress also showed increased neutrophil influx and IL-8 levels (101). Alveolar macrophages derived from circulating monocytes recruited into the lungs by monocyte chemotactic factors produced by lung cells are increased 20-fold in COPD patients and release ROS as superoxide anions and hydrogen peroxide (102). Antioxidant therapies should be effective in preventing COPD disease progression and exacerbations. Although prolonged treatment with oral N-acetylcysteine (NAC) prevents acute exacerbations of chronic bronchitis, it remains controversial for the treatment of COPD (91, 103–105). A combination of antioxidants including thiol-based antioxidants, mitochondria-targeted antioxidants and Nrf2 activators should be more effective in the treatment of COPD patients (91).

In acute respiratory distress syndrome (ARDS), there is extensive overproduction of free radicals and reduced extracellular and intracellular GSH leading to oxidative cell damage (106). ROS such as hydrogen peroxide and hypochlorous acid may play a key role in the pathogenesis of the acute lung injury (107). It has been shown that alveolar epithelial lining fluid of patients with ARDS is deficient in total GSH compared to normal subjects (107), and neutrophil-mediated oxidants release leads to GSH deficiency and lung cell injury (107). The global antioxidant capacity of the epithelial lining fluid, despite an increase in single antioxidant compounds, seems unable to fully counterbalance the increased oxidative burden (108). NAC benefited ARDS patients as evidenced by intracellular (inside red blood cells) and extracellular (plasma) antioxidant defense biomarkers and outcome. NAC treatment increased extracellular total antioxidant power and total thiol molecules and enhanced intracellular GSH and patients' outcome (106). NAC treatment improved oxygenation and decreased mortality in ARDS patients; and patients with glutathione-S-transferase M1 (proinflammatory-cytokine

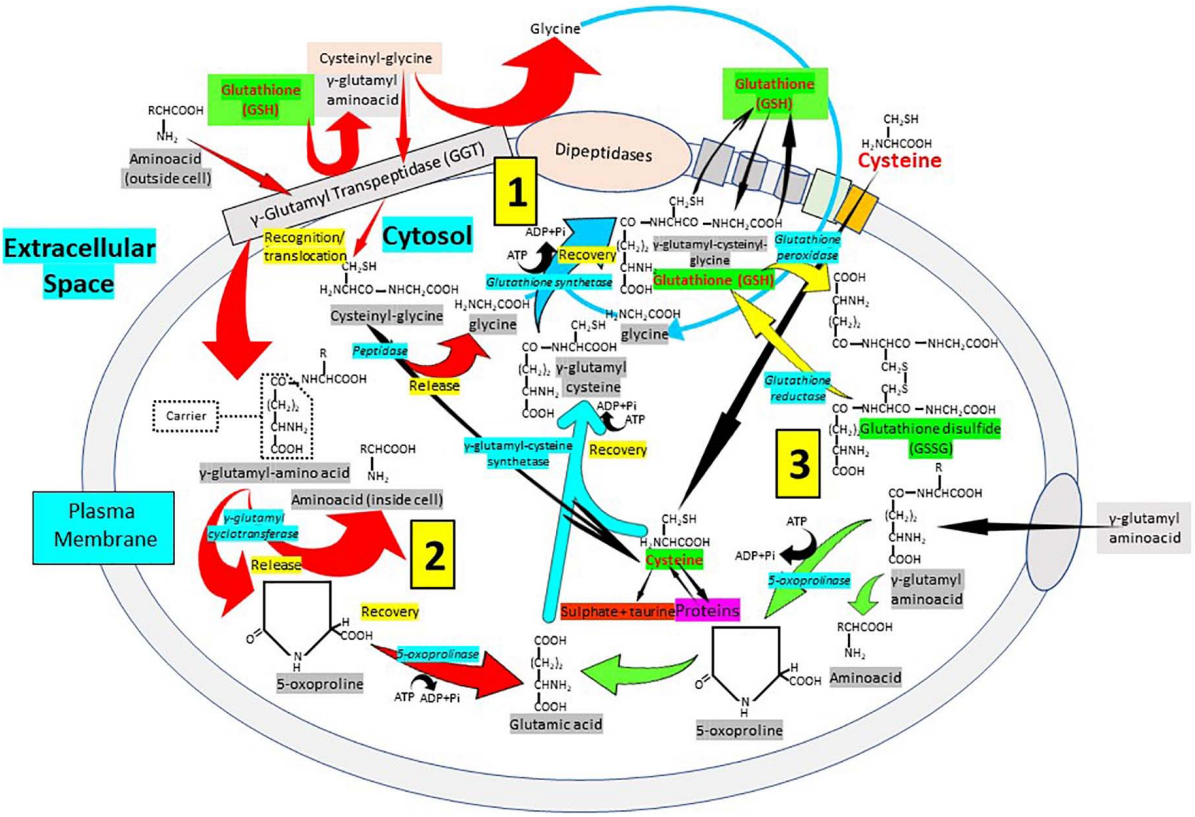

FIGURE 5
Cellular glutathione synthesis and recycling: The importance of the γ-glutamyl pathway. The degradation or catabolic part of the GSH cycle, takes place partially extracellularly and partially inside cells. (1) The extracellular degradation of GSH occurs on the surface of the cells that express the enzyme γ-glutamyl transpeptidase (GGT) and the dipeptidases found in the external plasma membrane. Following plasma membrane carrier-mediated GSH release from the cell, GSH becomes accessible to the active site of γ-glutamyl transpeptidase, which catalyzes GSH breakdown into γ-glutamyl fraction and cysteinyl-glycine by transferring the γ-glutamyl fraction to an amino acid acceptor, forming γ-glutamyl-amino acid. The cysteinyl-glycine fraction is split by the enzyme dipeptidase generating cysteine and glycine. (2) The γ-glutamyl-amino acid can be metabolized to release the amino acid and 5-oxoproline, which can then be converted into glutamate to be used in the synthesis of GSH. (3) The cells incorporate cysteine and most of the intracellular cysteine is used for the synthesis of GSH. Cysteine can be used for protein synthesis and part can be degraded to sulfate and taurine. The cycle γ-glutamyl allows GSH to be used as a continuous source of cysteine. The γ-glutamyl amino acid is taken up by cells through a specific transport mechanism. Cysteinyl glycine is also taken up by cells. Inside the cell, the γ-glutamyl amino acid is hydrolyzed by γ-glutamyl cyclo-transferase and converted into oxoproline, a cyclic form of glutamate converted into glutamate *via* oxoprolinase, and a free amino acid.

producer macrophages) null and M1 and T1 (Type 1 helper CD4 + lymphocytes) double null polymorphisms had increased mortality suggesting that antioxidant therapy becomes fundamental for those patients (109). A depressed antioxidant defense and dysfunctional iron regulation in ARDS might cause greater inflammation and anemia (110).

Glutathione is an important antioxidant in the lungs, but its concentration is low in the airways of patients with cystic fibrosis, since GSH is transported into the airways by the cystic fibrosis transmembrane conductance regulator, which is mutated in cystic fibrosis patients (111). The concentration of GSH that is normally about 400 μM in the epithelial lining fluid, over a 100-fold higher than in plasma, is low in the airways of patients with cystic fibrosis from an early age (112–114). Extracellular glutathione S-transferase omega-1, a cytosolic enzyme that modulates the S-thiolation status of intracellular factors involved in the inflammatory response, and its polymorphisms have been associated with an increased risk to develop COPD and could have a biological and clinical significance in cystic fibrosis (115). Low GSH, neutrophil infiltration, myeloperoxidase activity and inflammation increase oxidative stress overwhelming the antioxidant defense, and hypochlorous acid mediated GSH oxidation and its attachment to proteins contribute to further GSH deficiency (114). The lack of efficacy of inhaled GSH in patients with cystic fibrosis could be explained by the high concentrations of the GSH-degrading enzyme γ-glutamyltransferase present in lung fluids of those patients (116–123), and then, the use of precursors of GSH synthesis like NAC and cystine could be more effective in

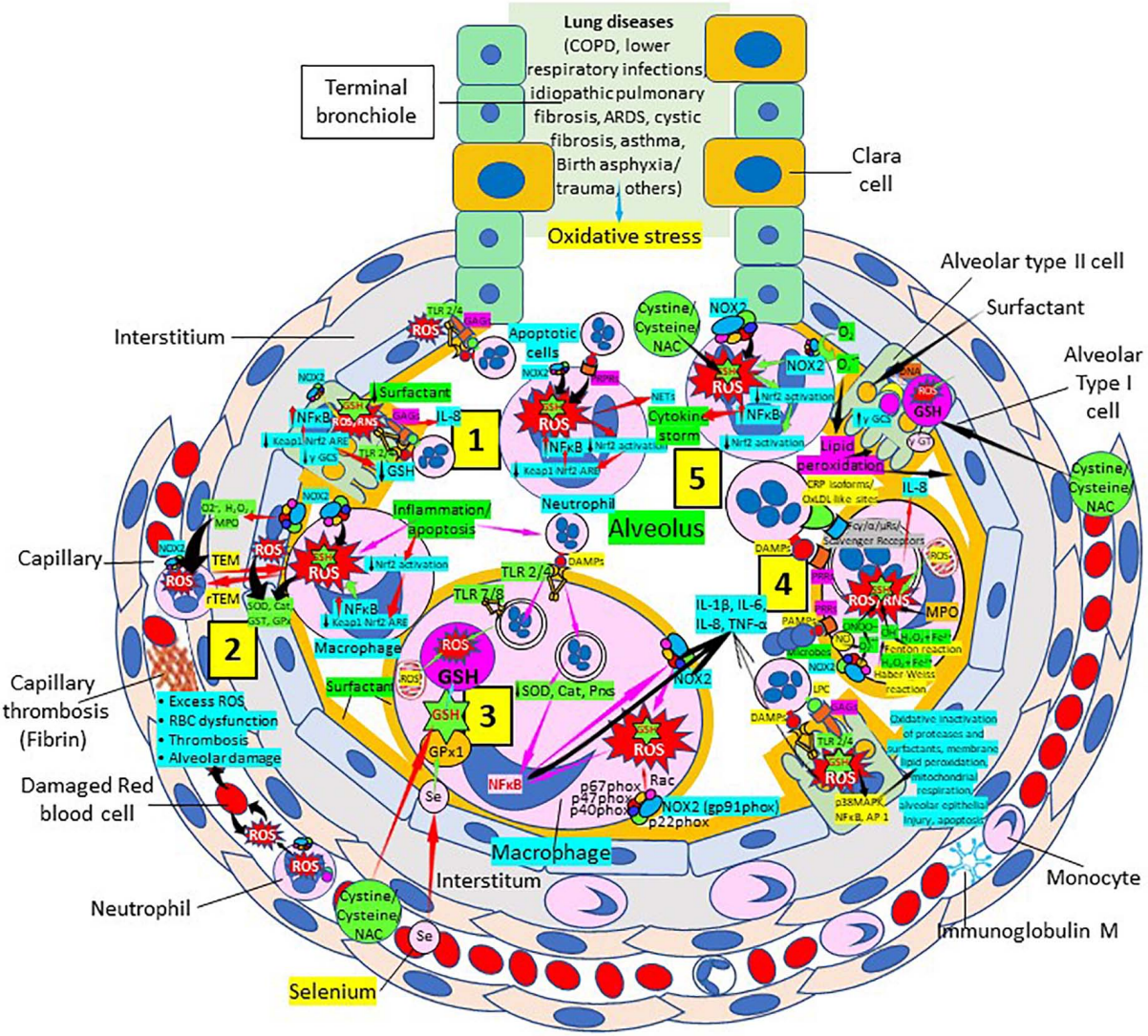

FIGURE 6
Oxidative stress, reduced glutathione (GSH) and lung diseases. (1) Lung diseases affect alveolar cells increasing reactive oxygen species (ROS) production, reduce Kelch-like ECH-associated protein 1 (Keap1)-Nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) redox regulator pathway and become defective for surfactant production. Damaged/apoptotic cells cause alveolar cell activation of nuclear factor (NF)-κB and release cytokines like interleukin (IL)-8. Alveolar type I cells augment ROS production *via* toll-like receptors (TLRs) 1 and 2. Inflammation enhances neutrophil extracellular trap (NET) release and increases ROS production. (2) Inflammation associated to lung diseases augments macrophage's ROS production, inhibiting Nrf2 activation and enhancing NF-κB upregulation. ROS are counterbalanced by enzymes like superoxide dismutase (SOD), catalase (Cat), glutathione S-transferase (GST), and glutathione peroxidase (GPx) to protect cells from oxidative damage caused by nicotinamide adenine-dinucleotide phosphate (NADPH) oxidase 2 (NOX2), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and myeloperoxidase (MPO). Capillary neutrophils migrate to and from alveoli by *trans*-endothelial (TEM) and reverse transmigration (rTEM), respectively. Inflammation can cause excessive ROS production in capillaries, red blood cell (RBC) dysfunction, thrombosis and alveolar damage. (3) Activated alveolar macrophages release increased levels of IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α. Inflammation-associated activated macrophages (*via* TLRs) reduce enzymes like SOD and Cat, among others, and activate NF-κB. NOX2 activation increases ROS production that enhance NF-κB activation. Glutathione (GSH) precursors (Cystine, cysteine, N-acetyl cysteine, NAC), and selenium (Se) restore GSH and GPx, respectively, to counteract the effects of ROS. (4) Alveolar macrophages engulf microbes and apoptotic cells *via* Fc (γ/α/μ) and scavenger receptors and/or pattern recognition protein receptors (PRPRs) leading to increased ROS production and cytokine release. MPO, nitric oxide (NO), O_2^-, and H_2O_2 through the Fenton and Haber-Weiss reactions that generate hydroxyl radicals, participate in ROS and RNS generation. Lung disease-associated inflammation and apoptosis [*via* TLRs and glycosaminoglycans (GAGs)] enhance alveolar cell ROS production that *via* p38MAPK, NF- κB, and AP-1 activation, contribute to epithelial injury and further inflammation. (5) Neutrophils contribute to O_2^- production, lipid peroxidation and increased oxidative stress to promote a cytokine storm (249). Administration of GSH precursors [cystine, cysteine, NAC; see (3), (4), and (5)] facilitate GSH formation to reduce oxidative stress. Abbreviations: PRRs, pattern recognition receptors; ɣ-GCS, ɣ-glutamyl cysteine synthetase; DAMPs, damage associated molecular patterns; Prxs, peroxiredoxins; NAC, N-acetyl cysteine; ɣ-GT, ɣ-glutamyl transpeptidase; PAMPs, pathogen associated molecular patterns; LPC, lysophosphatidylcholine.

the synthesis of GSH (124). Lack of oral GSH supplementation effects upon growth or changes in serum or fecal inflammatory markers in children with cystic fibrosis with pancreatic insufficiency (125) could be probably explained by the inability of the cells to uptake extracellular GSH to be used inside the cells. Decreased GSH content in the apical fluid in cystic fibrosis could be the result of abnormal GSH transport associated with a defective cystic fibrosis transmembrane conductance regulator as mentioned previously (126).

An oxidant/antioxidant imbalance characterized by oxidative stress and low GSH levels is involved in the pathogenesis of idiopathic pulmonary fibrosis, since data show marked GSH deficiency in the lower respiratory tract of those patients (127). Glutathione-S-transferase π (GSTP) that participates in the conjugation of GSH to reactive cysteines (S-glutathionylation) seems to play an important role in idiopathic pulmonary fibrosis lung fibrogenesis, since GSTP immunoreactivity is increased in the lungs of idiopathic pulmonary fibrosis patients, notably within type II epithelial cells (128, 129). GSTP inhibition *via* the airways may be a novel therapeutic strategy for the treatment of idiopathic pulmonary fibrosis (128, 129). The use of GSH precursors like N-acetyl cysteine, enhancers of nuclear factor erythroid 2-related factor 2 (Nrf2) like sulforaphane, melatonin, and many more molecules involved in antioxidant defense were proposed as supplementation of other idiopathic pulmonary fibrosis therapies (130). Inhaled (nebulized or aerosolized) reduced GSH to augment the deficient GSH levels of the lower respiratory tract has been used effectively in numerous pulmonary diseases and respiratory conditions like HIV seropositive individuals, cystic fibrosis and idiopathic pulmonary fibrosis, among others (131–133). GSH has clearly a regulatory role in inflammation and immunity (134). GSH acts as an inhibitor of extended inflammation directing components of innate immunity like polymorphonuclear neutrophils specifically to the site of infection/damage allowing a proper response to infection. GSH then directs the migration of inflammatory polymorphonuclear neutrophils away from the lung, where they cause ARDS, and toward the site of infection, where they kill microorganisms. As a result, it develops more immunity and less inflammation, with the concomitant increased survival; in addition, GSH becomes not just an inhibitor of inflammation but a regulator of innate immunity in a direction that benefits the host (134).

Glutathione and atherosclerosis

Cardiovascular diseases are the leading causes of death in the US compared to any other cause (135). Cardiovascular complications are thought to result from increased free radical levels that impair redox homeostasis, that represents the interaction between oxidative stress and reductive stress. A prolonged oxidative or reductive stress will alter the homeostatic redox mechanism to cause cardiovascular complications. GSH, the most abundant antioxidant in the heart, plays a fundamental role in normalizing a redox homeostatic mechanism that was shifted toward oxidative or reductive stress. This may lead to impairment of cellular signaling mechanisms and accumulation of misfolded proteins causing proteotoxicity associated with cardiac dysfunction (136–143). Oxidative stress is crucial in atherogenesis (144–151), suggesting that a specific antioxidant/prooxidant imbalance, characterized by a weak GSH-related enzymatic antioxidant shield present in human atherosclerotic lesions, may be involved in atherogenic processes in humans (152). A higher level of oxidative stress as evidenced by elevated plasma malondialdehyde levels and low levels of GSH, α-tocotrienol and GSH peroxidase activity in patients under 45 years old may play a role in the development of premature coronary artery disease and be potential biomarkers for premature coronary artery disease (153). Similarly, coronary artery disease patients with single, double, or triple-vessel stenosis and patients with acute coronary syndrome had a significant increase in malondialdehyde levels and the percentage of malondialdehyde release, associated with a marked decrease in GSH concentration, total antioxidant capacity and erythrocyte GSH peroxidase activity compared with controls (154). Interestingly, differences in prooxidative parameters were more profound in acute coronary syndrome patients compared with coronary artery disease patients indicating that the acute form of coronary artery disease is more susceptible to oxidative damage, suggesting that use of antioxidant therapy may be warranted to reduce oxidative stress in this disorder (154).

Glutathione might inhibit the effects of cerebral infarction and enhance antiapoptotic signaling after ischemic stroke, suggesting that GSH may be a potent therapeutic antioxidant that can attenuate severe pathologies after ischemic stroke, and stimulating GSH synthesis through administration of GSH precursors and micronutrients like selenium can optimize GSH and GSH peroxidase for optimal antioxidant defense in cerebral ischemia (155, 156). Low total GSH and high homocysteine levels are considered as novel risk markers for acute stroke severity, and low total and reduced GSH levels may be potential risk markers for stroke severity and insufficient functional independence in large-artery atherosclerosis (157, 158). Since GSH is the final product of the homocysteine metabolism in the transsulfuration pathway by transferring sulfur from homocysteine to cysteine, a deficiency in transsulfuration pathway leads to excessive homocysteine production (hyperhomocysteinemia) and reduced GSH synthesis (159, 160). Homocysteine is a sulfur-containing amino acid tightly involved in methionine metabolism. Indeed, if there is a methionine deficit, homocysteine can be re-methylated to form methionine, and if there is an adequate amount of methionine, homocysteine is used to produce cysteine (161). Hyperhomocysteinemia decreases

GSH peroxidase activity leading to the prevalence of GSSG on GSH with the GSH/GSSG impaired ratio causing some common cardiovascular and neurodegenerative disorders (159). N-acetyl-cysteine administration supplies the cysteine necessary for GSH synthesis and concomitantly reduces hyperhomocysteinemia, improving GSH peroxidase activity and reducing oxidative stress (159). Furthermore, the well documented efficacy of combined folic acid, B6, and B12-vitamin supplementation to reduce hyperhomocysteinemia could enhance GSH activity and reduce oxidative stress (161). Recently, it was shown that cysteine uptake *via* excitatory amino acid carrier 1 suppresses ischemia-induced neuronal death through promotion of hippocampal GSH synthesis in ischemic animal models (162). Alterations in the normal function of excitatory amino acid carrier 1 affect cysteine transport and GSH synthesis impairing zinc homeostasis (the thiol group of GSH can function as a principal Zn^{2+} chelator for the maintenance of Zn^{2+} homeostasis in neurons) and oxidative stress, enhancing susceptibility to ischemia-induced neuronal cell death in the hippocampus (162–164). Increased GSH synthesis neutralizes reactive oxygen and nitrogen species and regulates zinc homeostasis promoting neuroprotection after ischemia/reperfusion (162).

Atherosclerosis represents a state of intense oxidative stress characterized by vascular wall lipid and protein oxidation that contributes to chronic inflammation within the arterial wall, in which CRP is a major player (**Figure 7**). The balance of the different CRP isoforms, monomeric (mCRP) or native pentameric (nCRP) within the plaque determines the preponderance of a proinflammatory or anti-inflammatory effect, respectively (165). CRP is synthesized in smooth muscle cells of atherosclerotic lesions with active disease, foam cells, macrophages, lymphocytes, monocytes, and endothelial cells within the atherosclerotic plaque (166–170). CRP binds and aggregates oxidized low-density lipoprotein (ox-LDL) and enhances macrophage oxLDL uptake, promoting mitogen-activated protein kinase activation (171) required for foam cell formation (172). OxLDL enhances toll like receptor 4 expression further facilitating foam cell formation and development and progression of atherosclerosis (173, 174). CRP binding to oxLDL and apoptotic cells occurs through phosphorylcholine, and binding to this ligand starts phagocytosis (100, 170, 175–181). The different CRP isoforms, nCRP, non-native pentameric CRP (nnCRP) and mCRP (175–178, 181–184), may explain their protective and destructive effects, with nCRP being primarily antiinflammatory inducing Th2/M2 responses, while mCRP being typically proinflammatory inducing Th1/M1 responses (170, 185–189). Pentameric nCRP and CRP peptides 77–82, 174–185, and 201–206 can control the inflammatory response resolving inflammation by reducing inflammatory cell endothelial adhesion and tissue migration, and the described CRP-mediated enhanced monocyte chemotaxis could be explained by local generation of mCRP (190, 191). Pro-inflammatory and proatherogenic mCRP, but not nCRP, induces ROS monocyte/macrophage production and facilitates macrophage uptake of necrotic cells (170, 192) contributing to foam cell formation, atherosclerotic plaque formation and plaque rupture or destabilization (190, 191). Foam cell formation during atherogenesis could be also explained in part by uptake of CRP-opsonized native LDL (193). The dissociation/relaxation of nCRP into nnCRP occurs on necrotic, apoptotic, and ischemic cells, membranes of activated platelets, monocytes, and endothelial cells, and on the surface of microparticles, *via* phosphorylcholine binding and seems to be, as mCRP, proinflammatory (194–196). Pentameric nCRP does not possess intrinsic proinflammatory properties, while nnCRP and mCRP do (170, 196). The mCRP isoform, unlike nCRP, has a stimulatory effect on platelets, facilitates thrombus growth through platelet stimulation, and is the more potent reagent, both increasing monocyte activation and ROS production, generated through myeloperoxidase-mediated respiratory burst and raft-associated reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase during oxLDL-mediated foam cell formation (100, 170, 197–204). ROS activity in the vessel wall contributes to the formation of oxidized LDL, a major contributor to the pathogenesis of atherosclerosis (205, 206). Thrombus formation and the subsequent activation of the coagulation cascade with final generation of fibrin is facilitated by the mCRP-mediated enhancement of tissue factor on the endothelial cell surface, platelet aggregation and thrombus growth (207, 208) (**Figure 7**). OxLDL components and their interaction with toll-like receptors (TLRs) 2 and 4, CD36 and other cellular receptors further mediate thromboinflammation enhancing tissue and organ damage culminating in organ failure, i.e., myocardial infarction, stroke, and pulmonary artery embolism (174, 209–212).

The strong role of severe oxidative stress, reduced antioxidant defenses like GSH with increased lipid peroxidation and malondialdehyde generation (213), lipid, protein and DNA oxidation with increased apoptosis and necrosis in atherosclerosis as a major cause of cardiovascular diseases and stroke, supports the use of complementary and alternative medicines, dietary supplements, and antioxidants with hardly any adverse effect, able to restore homeostasis reversing oxidative stress (214). Enhancing GSH synthesis, selenium levels and redox-active selenoproteins, and activating Nrf2 and other antioxidant enzymes will strengthen the cardiovascular antioxidant defense. Phenolic compounds like phenolic acids, flavonoids, lignans and tannins can limit LDL oxidation and foam cell formation (215). Selenium is an essential micronutrient that modulates cardiovascular functions *via* its incorporation into selenoproteins as the amino acid selenocysteine (216, 217). Intravenous reduced GSH supplementation reverses endothelial dysfunction in patients with atherosclerosis enhancing NO activity and NO-mediated

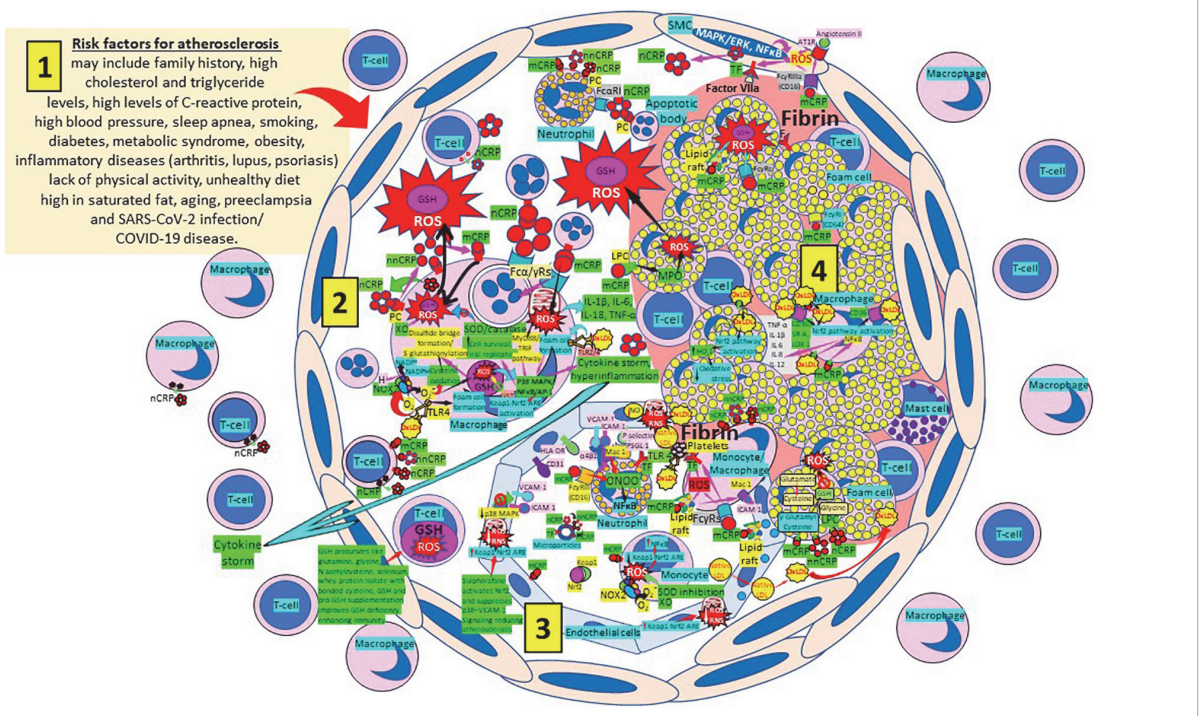

FIGURE 7
Oxidative stress, reduced glutathione (GSH) and atherosclerosis. (1) Risk factors for atherosclerosis. (2) Atherosclerosis risk factors facilitate oxidative stress and inflammation in the arterial intima. Native C-reactive protein (nCRP), a pattern recognition receptor produced in the liver, macrophages, lymphocytes, smooth muscle cells (SMC), and other cells, promotes inflammation through monomeric CRP (mCRP) enhancing intimal oxidative stress. Oxidized (ox) LDL binds macrophage toll-like receptor (TLR) 4 and facilitates nicotinamide adenine dinucleotide phosphate (NADP)H oxidase 2 (Nox2) activity and superoxide (O_2^-) production causing cysteine oxidation, disulfide bridge formation and S-glutathionylation. Xanthine oxidase (XO) and inhibition of superoxide dismutase (SOD)/catalase further facilitate O_2^- cellular activity. OxLDL bound to TLRs 2 and 4 promotes foam cell formation and activates transcription factors like nuclear factor (NF)-κB facilitating cytokine storm and hyperinflammation. Excessive mitochondrial reactive oxygen species (ROS) generation further enhances cytokine production. CRP (nCRP, mCRP) can facilitate macrophage and neutrophil uptake of apoptotic cells through Fcγ and Fcα receptors, respectively (FcRs). Oxidative stress also activates the Kelch-like ECH-associated protein 1 (Keap1)-Nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) redox regulator pathway in monocytes [see (3) and macrophages (2)], releasing Nrf2 to regulate the expression of genes that control antioxidant enzymes like glutathione S-transferase (GST), facilitating glutathione (GSH) activity. Macrophages, T-lymphocytes, neutrophils and SMCs can generate mCRP increasing inflammation. (3) Monocytes, macrophages, neutrophils, endothelial cells and microparticles can generate mCRP, increase $O_2^-$and ROS formation and reactive nitrogen species like peroxinitrite ($ONOO^-$), and tissue factor (TF) expression enhancing oxidation, inflammation and thrombosis. TLR 4-mediated oxLDL-binding to platelets promotes thrombosis; mCRP binding to lipid rafts and FcγRs enhances inflammation; and endothelial activation allows intimal cell migration. GSH enhancement and Nrf2 activation augment immunity and reduce atherosclerosis. (4) Foam cells and smooth muscle cells associated with atherosclerotic plaques enhance ROS formation, cytokine release and tissue factor (TF)-mediated fibrin deposition. Abbreviations: MAPK/ERK, mitogen-activated protein kinases/extracellular signal-regulated kinases; AT1R, angiotensin II type 1 receptor; PC, phosphorylcholine; LPC, lysophosphatidylcholine; MPO, myeloperoxidase; nnCRP, non-native CRP; TNF, tumor necrosis factor; IL, interleukin; ACE, angiotensin converting enzyme; MyD88/TRIF, myeloid differentiation primary response 88/TIR-domain-containing adapter-inducing interferon-β; PI3K/Akt, phosphatidylinositol-3-kinase/protein kinase B; MAPK, mitogen-activated protein kinase; AP-1, activator protein 1; CD31, cluster of differentiation 31; ICAM-1, intercellular adhesion molecule-1; Mac-1, macrophage-1 antigen; PSGL-1, P-selectin glycoprotein ligand-1; HLA-DR, human leukocyte antigen–DR isotype.

vasodilation (218). GSH stores and transports cysteine, and cysteine forms less diffusion-limited NO adducts that may transport NO to reach sites within vascular smooth muscle cells and platelets (218, 219). Since GSH is not carried inside the cell, exogenously administered GSH is most likely to act by increasing plasma GSH levels reducing luminal oxidative stress and increasing NO bioavailability in patients with endothelial dysfunction (220).

Administration of GSH precursors like cysteine/N-acetylcysteine, glycine and/or glutamic acid will facilitate the synthesis of GSH within each cell of the body including the atherosclerotic plaque, reducing ROS and LDL oxidation, enhancing NO production, and mitigating atherosclerosis and its complications (221–229). Considering the paramount importance of oxLDL in the pathogenesis of atherosclerosis, it is reasonable to evaluate the role of antioxidants in the treatment of the disease as adjuvant strategies to lipid-lowering or anti-inflammatory therapies designed to reduce the risk of cardiovascular disease (230). Since oxidation participates as an essential messenger of cellular signaling pathways, treatment of oxidative stress needs to consider maintaining that physiologic threshold (230, 231). The lack of standardized methods to

evaluate total antioxidant capacity and the oxidation state and the use of inadequate antioxidants and/or improper concentrations of antioxidants lead to failure of numerous clinical trials directed to prevent or mitigate progression of atherosclerosis (230–233).

Nuclear factor erythroid 2-related factor 2 plays a fundamental role in the response to oxidative stress and xenobiotic metabolism and detoxification, and the Nrf2 signaling pathway is intimately associated with development of atherosclerosis. During development and progression of atherosclerosis, Nrf2 signaling modulates many physiological and pathophysiological processes, like regulation of lipid homeostasis, CD36 gene expression regulation, foam cell formation, macrophage polarization, immunity regulation (Th2 differentiation and inhibition of pro-inflammatory gene expression through NFκB down-regulation), redox regulation and inflammation, improvement of endothelial dysfunction, as well as GSH synthesis and utilization (234–245). Antioxidant pathways induced by NRF2 include enzymes for the reduced GSH synthesis, utilization, and regeneration. Glutamate-cysteine ligase catalytic and modulator subunits as well as GSH synthetase are the three NRF2 targets involved in the GSH synthesis (242). The redox cycling enzymes thioredoxin, thioredoxin reductase, sulfiredoxin, peroxiredoxin, GSH peroxidase, superoxide dismutase 1, and catalase, and several GSH S-transferases, which are the enzymes mediating the elimination of ROS, are all Nrf2 targets (242). Nrf2 displays both pro- and anti-atherogenic effects in experimental animal models, and the Nrf2 pathway becomes a promising target for atherosclerosis prevention (234). Macrophage Nrf2 activates genes encoding CD36, heme oxygenase-1 and other stress proteins in response to oxLDLs and other byproducts of lipid peroxidation (240). Nrf2 depletion in macrophages leads to increased foam cell formation, increases the M1 inflammatory phenotype with enhanced expression of pro-inflammatory monocyte chemoattractant protein-1 and interleukin-6, and aggravates atherosclerosis (244, 245). Nrf2 improves endothelial function by resisting oxidative stress and mitochondrial damage, thereby delaying atherosclerosis (245); and treatment with sulforaphane, a dietary antioxidant, activates Nrf2 and suppresses p38–VCAM-1 signaling, and may provide a novel therapeutic strategy to prevent or reduce atherosclerosis (237).

Glutathione and severe acute respiratory syndrome coronavirus 2

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection targets primarily the respiratory and cardiovascular systems causing COVID-19 disease identified largely by a respiratory tract infection (246, 247); sadly, many patients develop severe fatal outcomes because of overwhelming inflammation known as "cytokine storm" (248, 249), that leads to ROS-mediated cell death and tissue damage typical of RNA viruses (250). This intense inflammation is associated with damaging systemic events like oxidative stress, dysregulation of iron homeostasis, hypercoagulability and thrombus formation, acute respiratory distress syndrome, uncontrolled inflammation and organ failure (251–257) (**Figure 8**). Several viral infections, and the progression of virus-induced diseases, especially those associated with COVID-19, are characterized by an alteration in the intracellular redox balance (6). This imbalance disallows reactive intermediate detoxification by the cell biological systems. ROS production and associated inflammation are closely related to aging and numerous chronic diseases as diabetes, cardiovascular atherosclerosis-related diseases (144, 145) and respiratory diseases, known risk factors for developing severe illness and death in patients with SARS-CoV-2 and COVID-19 disease.

Atherosclerosis, a chronic inflammatory disease, may be an ideal environment for the high viral replication capabilities of SARS-CoV-2 in human cells, enhancing hyper-inflammation secondary to immune system dysregulation (**Figure 9**) that leads to adverse outcomes, as shown in patients with cardiovascular risk factors (258, 259). In a vicious circle, feeding itself, SARS-CoV-2 may aggravate the evolution of atherosclerosis as a result of excessive and aberrant plasmatic concentration of cytokines (258–260). Atherosclerosis progression, as a chronic inflammatory mechanism, is characterized by immune system dysregulation associated with increased pro-inflammatory cytokine production, including interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), and IL-1β, as well as pattern recognition receptor proteins like CRP (170, 261–267). CRP, an active regulator of host innate immunity, is a biomarker of chronic inflammatory conditions and severe COVID-19 disease, including lung and atherosclerotic disease progression; strongly predicts the need for mechanical ventilation; and may guide intensification of treatment of COVID-19-associated uncontrolled inflammation (99, 183, 261, 262, 265, 267–269). Macrophage activation and foam cell formation may explain the elevated CRP serum levels and contribute to disease progression (**Figure 9**). CRP-mediated inflammation in atherosclerosis during SARS-CoV-2 infection may be related to the presence of mCRP in the lesions (188, 198, 204, 263, 267–269). The affinity of SARS-CoV-2 for ACE2 receptors makes the virus prone to cause vascular infection that could explain atherosclerosis progression and arterial and venous thrombosis (270, 271). Endothelial injury generated directly by intracellular viral replication and by ACE2 downregulation, exposing cells to angiotensin II in the absence of the modulator effects of angiotensin 1–7 (270, 271), and vascular chronic inflammation promoting the development of tissue macrophages overloaded by cholesterol (foam cells), both increase the possibility of acquiring a severe COVID-19 infection (170, 209, 258, 259, 272, 273).

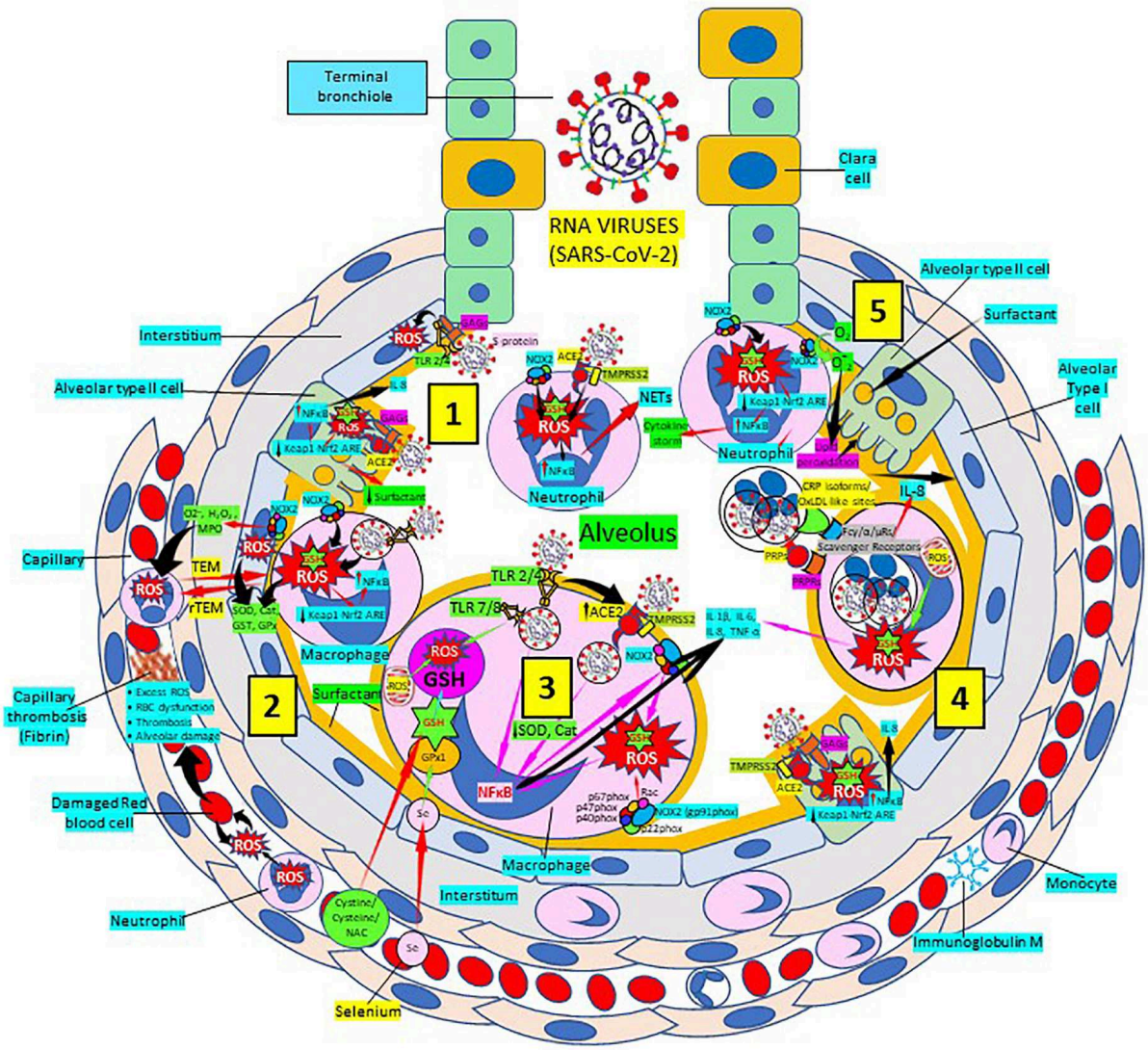

FIGURE 8
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pulmonary infection, oxidative stress and antioxidant defenses. (1) After entry of SARS-CoV-2 into the alveolus, viruses invade type II alveolar cells through angiotensin-converting enzyme 2 receptors (ACE2) and glycosaminoglycans (GAGs) [see (4)], and infected cells increase reactive oxygen species (ROS) production, reduce Kelch-like ECH-associated protein 1 (Keap1)-Nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) redox regulator pathway and become defective for surfactant production. Infected cells activate nuclear factor (NF)-κB and release cytokines like interleukin (IL)-8. Alveolar type I cells augment ROS production *via* toll-like receptors (TLRs) 1 and 2. SARS-CoV-2 enhances neutrophil extracellular trap (NET) release and increases ROS production (2) SARS-CoV-2 augments macrophage's ROS production, inhibiting Nrf2 activation and enhancing NF-κB upregulation. ROS are counterbalanced by enzymes like superoxide dismutase (SOD), catalase (Cat), glutathione S-transferase (GST), and glutathione peroxidase (GPx) to protect cells from oxidative damage caused by nicotinamide adenine-dinucleotide phosphate (NADPH) oxidase 2 (NOX2), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and myeloperoxidase (MPO). Capillary neutrophils migrate to and from alveoli by *trans*-endothelial (TEM) and reverse transmigration (rTEM), respectively. SARS-CoV-2 infection can cause excessive ROS production in capillaries, red blood cell (RBC) dysfunction, thrombosis and alveolar damage. (3) Activated alveolar macrophages release increased levels of IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α. SARS-CoV-2-infected macrophages (*via* ACE2 and TLRs) reduce enzymes like SOD and Cat, among others, and activate NF-κB. NOX2 activation increases ROS production that enhance NF-κB activation. Glutathione (GSH) precursors (Cystine, cysteine, N-acetyl cysteine, NAC), and selenium (Se) restore GSH and GPx, respectively, to counteract the effects of ROS. (4) Alveolar macrophages engulf SARS-CoV-2-infected apoptotic cells *via* Fc (γ/α/μ) and scavenger receptors and/or pattern recognition protein receptors (PRPRs) leading to increased ROS production and cytokine release. (5) Neutrophils contribute to O_2^- production, lipid peroxidation and increased oxidative stress to promote the cytokine storm. Abbreviations: TMPRSS2, Transmembrane protease Serine 2; PRPs, pattern recognition proteins. Reprinted from Labarrere and Kassab (335).

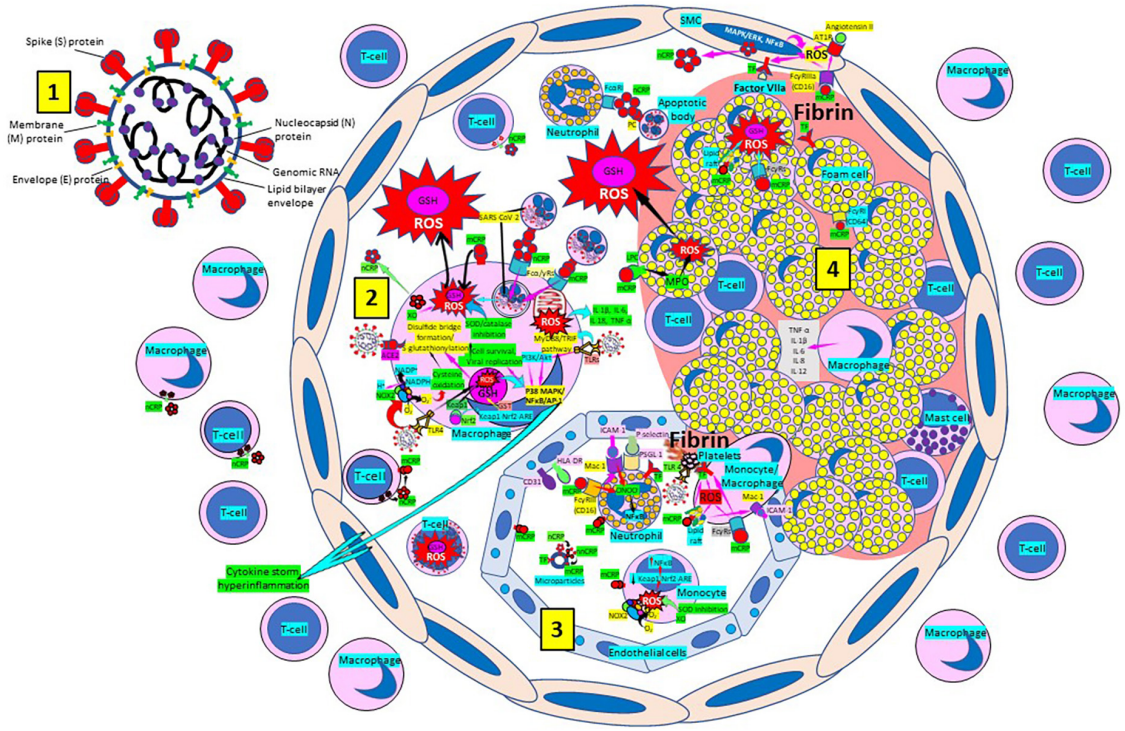

FIGURE 9
Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) enhances oxidative stress and atherosclerosis progression. (1) SARS-CoV-2 structure. (2) SARS-CoV-2 viruses facilitate oxidative stress and inflammation in the arterial intima. Native C-reactive protein (nCRP), a marker of severe SARS-CoV-2 produced in liver, macrophages, lymphocytes, smooth muscle cells (SMC) and other cells, promotes inflammation through monomeric CRP (mCRP) enhancing intimal oxidative stress. SARS-CoV-2 binds macrophage toll-like receptor (TLR) 4 and facilitates nicotinamide adenine dinucleotide phosphate (NADP)H oxidase 2 (Nox2) activity and superoxide (O_2^-) production causing cysteine oxidation, disulfide bridge formation and S-glutathionylation. Xanthine oxidase (XO) and inhibition of superoxide dismutase (SOD)/catalase further facilitate O_2^- cellular activity. SARS-CoV-2 can bind TLRs 2 and 4 and activate transcription factors like nuclear factor (NF)-κB facilitating cytokine storm and hyperinflammation. Excessive mitochondrial reactive oxygen species (ROS) generation further enhances cytokine production. CRP (nCRP, mCRP) can facilitate macrophage and neutrophil uptake of SARS-CoV-2-infected apoptotic cells through Fcγ and Fcα receptors, respectively (FcRs). Oxidative stress also activates the Kelch-like ECH-associated protein 1 (Keap1)-Nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) redox regulator pathway in monocytes [see (3) and macrophages (2)], releasing Nrf2 to regulate the expression of genes that control antioxidant enzymes like glutathione S-transferase (GST), facilitating glutathione (GSH) activity. Macrophages, T-lymphocytes, neutrophils and SMCs can generate mCRP increasing inflammation. (3) Monocytes, macrophages, neutrophils, endothelial cells and microparticles can generate mCRP, increase superoxide (O_2^-) and ROS formation and reactive nitrogen species like peroxinitrite (ONOO−), and tissue factor (TF) expression enhancing oxidation, inflammation and thrombosis. TLR 4-mediated SARS-CoV-2-binding to platelets promotes thrombosis, mCRP binding to lipid rafts and FcγRs enhances inflammation and endothelial activation allows intimal cell migration. (4) Foam cells and smooth muscle cells associated with atherosclerotic plaques enhance ROS formation, cytokine release and tissue factor (TF)-mediated fibrin deposition. Abbreviations: MAPK/ERK, mitogen-activated protein kinases/extracellular signal-regulated kinases; AT1R, angiotensin II type 1 receptor; PC, phosphorylcholine; LPC, lysophosphatidylcholine; MPO, myeloperoxidase; nnCRP, non-native CRP; TNF, tumor necrosis factor; IL, interleukin; ACE, angiotensin converting enzyme; MyD88/TRIF, myeloid differentiation primary response 88/TIR-domain-containing adapter-inducing interferon-β; PI3K/Akt: phosphatidylinositol-3-kinase/protein kinase B; MAPK, mitogen-activated protein kinase; AP-1, activator protein 1; CD31, cluster of differentiation 31; ICAM-1, intercellular adhesion molecule-1; Mac-1, macrophage-1 antigen; PSGL-1, P-selectin glycoprotein ligand-1; HLA-DR, human leukocyte antigen–DR isotype. Reprinted from Labarrere and Kassab (335).

Summary and conclusions: Glutathione and early (premature) inflammaging

Chronic inflammatory diseases especially those compromising the lower respiratory system (chronic obstructive pulmonary disease, lower respiratory infections, cystic fibrosis, idiopathic pulmonary fibrosis, acute respiratory distress syndrome), diseases compromising the cardiovascular system (atherosclerosis, ischemic heart disease, stroke, and others), many other systemic inflammatory diseases like diabetes (274, 275); and actually SARS-CoV-2 causing COVID-19, all are characterized by persistent inflammation, continuous production of reactive oxygen/nitrogen species and oxidative stress that predominate over the antioxidant defenses (GSH and free radical scavenger enzymes) resulting in cell/tissue/organ aging associated with early (premature) chronic inflammation/"inflammaging." We propose that, although inflammaging was introduced for the aging process

(276–289), it could also apply to early (premature) tissue and organ aging associated with cell and tissue damage caused by excessive oxidative stress and lack of adequate antioxidant defenses, especially low GSH levels, in younger individuals. Indeed, inflammaging is associated with cell ROS over-production leading to oxidation/damage of cellular components, enhanced inflammation, and activation of cell death pathways; and oxidative stress and reduced antioxidant defenses contribute to progression of practically all diseases (290–297). In most diseases, ROS appear to have a direct connection with inflammaging and cell senescence, and oxidative stress and inflammaging increase the aging-related phenotype, and induce and aggravate the inflammatory response, creating a chronic state of systemic inflammation (290–297). Then, as proposed above, inflammaging can also be involved in aged cell/tissue processes in younger individuals. All chronic diseases, including COVID-19 with the long-COVID-19-syndrome (271), are characterized by the presence of persistent chronic inflammation and sustained generation of reactive oxygen and nitrogen species that when confronted with inadequate antioxidant defenses (likely leading components of anti-inflammaging) precipitate excessive oxidative stress. The demand for detailed analysis of the pathogenesis and clinical course of chronic diseases and viral diseases like COVID-19, as well as the use of efficacious therapies with minimal or no side effects are paramount. Here we present the antioxidant GSH as a potential unexplored way for further investigation as intervention to counteract inflammaging, premature inflammaging, inflammatory diseases and long-COVID-19-syndrome, since GSH levels are correlated with tissue and organ damage, disease severity and progression, and disease outcome (294–296, 298, 299). Enhancing GSH, mainly through NAC, GSH precursors rich in cysteine (whey protein, whey protein isolate rich in cysteine) or pro-GSH compound administration, becomes a potential treatment option for inflammatory diseases by reducing oxidative stress and cytokine expression especially in diabetic patients that also are at risk of more severe COVID-19 disease (299). GSH dysregulation might cause global immune cell autophagy decline with increased generation of proinflammatory cytokines in aging, further provoked by mitochondrial ROS signaling (293). Whey protein concentrate ameliorates lung damage and inhibits lung furin activity targeting SARS-CoV-2 S1/S2 site cleavage and SARS CoV-2 spike protein-angiotensin converting enzyme binding and could be used to protect against COVID-19 inhibiting SARS-CoV-2 cell entry (300). Glutamine, glycine, N-acetylcysteine, selenium, whey protein isolates with bonded cysteine, GSH and pro-GSH supplementation improves GSH deficiency, oxidative stress, mitochondrial dysfunction, inflammation, insulin resistance, endothelial dysfunction, genotoxicity, muscle strength, cognition and surfactant regeneration (301–307). A combination of vitamin D and L-cysteine administration significantly augmented GSH levels and lowered oxidative stress and inflammation (308, 309). Maintaining an adequate GSH redox status and 25-hydroxy-vitamin D levels will have the potential to reduce oxidative stress, enhance immunity and diminish the adverse clinical consequences of COVID-19 especially in African American communities having glucose-6-phosphate dehydrogenase (G6PD) deficiency, enzyme necessary to prevent GSH exhaustion and depletion (6, 213, 310). In normal red blood cells, pentose phosphate pathway and glycolysis are enhanced and G6PD is sufficient to produce NADPH efficiently for GSSG reduction and maintenance of GSH pool (311). G6PD-deficient cells are unable to generate enough NADPH under the condition of severe thiol depletion and GSH biosynthesis and methionine cycle are upregulated at the expense of ATP but fail to compensate for GSH depletion (311).

Severe acute respiratory syndrome coronavirus 2 can sequester mitochondria and replicate within them aging those vital organelles weakening immunity; facilitating over-stimulated or sustained inflammatory responses with interferon and cytokine release, influencing ROS production, iron storage, platelet coagulability, cytokine production stimulation, regulation of fission and fusion, mitochondrial biogenesis, and interference of apoptosis and mitophagy (312–319). By affecting all these cellular functions already impaired in aging individuals it could explain why older, comorbid patients have the most severe outcomes with COVID-19 (312) and stimulate the use of GSH and Nrf2 enhancers as well as develop new therapies to protect mitochondria. We propose that enhancement of the reduced form of GSH will reduce the body's oxidation and inflammation associated with chronic inflammatory diseases and SARS-CoV-2 infection and COVID-19 disease (320–324). Maintaining GSH levels using therapies that do not deplete the body's GSH (324) would be the best choice. In a patient that is overloaded with cytokine storm, the best way to fortify the immune system would be to supply it with reduced GSH, since reduced GSH is already able to provide reducing equivalents from its thiol group. This is particularly relevant when we consider GSH pathways, as well as their transcriptional regulator Nrf2, for proliferation, survival and function of T cells, B cells and macrophages (325, 326). The value of GSH and nutritional strategies like amino acids, vitamins, minerals, phytochemicals, sulforaphane to enhance cellular Nrf2, and other supplements used to restore GSH levels (327–330) as adjunct treatments for all inflammatory diseases including SARS-CoV-2 infection needs to be further emphasized. Reducing the levels of proinflammatory molecules like mCRP and nnCRP (331, 332) will further reduce the detrimental effects of inflammaging. Reestablishing the cellular metabolic homeostasis in inflammatory diseases as well as SARS-CoV-2 infection and COVID-19 disease especially in the lungs and cardiovascular system, could become paramount to balance altered innate and adaptive immunity and cell function and reduce morbimortality (333–335). Treatment of

chronic inflammatory diseases and now COVID-19 appears to be complex and may resist finding a single silver bullet intervention (247) supporting the use of combination therapies (170); especially in COVID-19 bearing in mind that "no one is safe until everyone is safe" (336).

Author contributions

CL and GK participated in the design, writing, and final corrections of the manuscript. Both authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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METABOLISM

Autoregulatory control of mitochondrial glutathione homeostasis

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Mitochondria must maintain adequate amounts of metabolites for protective and biosynthetic functions. However, how mitochondria sense the abundance of metabolites and regulate metabolic homeostasis is not well understood. In this work, we focused on glutathione (GSH), a critical redox metabolite in mitochondria, and identified a feedback mechanism that controls its abundance through the mitochondrial GSH transporter, SLC25A39. Under physiological conditions, SLC25A39 is rapidly degraded by mitochondrial protease AFG3L2. Depletion of GSH dissociates AFG3L2 from SLC25A39, causing a compensatory increase in mitochondrial GSH uptake. Genetic and proteomic analyses identified a putative iron-sulfur cluster in the matrix-facing loop of SLC25A39 as essential for this regulation, coupling mitochondrial iron homeostasis to GSH import. Altogether, our work revealed a paradigm for the autoregulatory control of metabolic homeostasis in organelles.

Cells require the ability to sense changes in the abundance of nutrients to ensure their efficient use for survival and growth under environmental perturbations (*1*). Although several nutrient sensing mechanisms have been described for cytosolic metabolites such as amino acids and cholesterol (*2*), whether organelles sense and regulate metabolite availability is not fully understood. Mitochondria are semiautonomous compartments with endosymbiotic origins. As a source of oxidative reactions and with an independent genetic system, mitochondria must maintain optimal concentrations of nucleotides, amino acids, and antioxidant molecules to perform critical protective and biosynthetic functions. Indeed, previous work provided evidence for such homeostatic mechanisms for a subset of redox-active molecules (*3*, *4*). Among these, glutathione (GSH) is a small-molecule thiol that is highly abundant in mitochondria and is required for antioxidant defense and iron homeostasis (*5*). Mitochondrial GSH availability is largely controlled by its import through the SLC25A39 transporter on the mitochondrial inner membrane (*6*, *7*). SLC25A39 protein accumulates upon GSH depletion, which strongly indicates a potential feedback mechanism to control the availability of mitochondrial GSH (*6*). In line with this observation, cells treated with buthionine sulfoximine (BSO; a GSH synthesis inhibitor) alone or in combination with erastin (an antagonist of uptake of the GSH precursor cystine) increased the abundance of SLC25A39 commensurate with the intensity of GSH depletion (fig. S1, A and B). However, how GSH levels are sensed and maintained through SLC25A39 in mitochondria is unknown.

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SLC25A39 is a short–half-life protein regulated by mitochondrial GSH availability

To understand how SLC25A39 is regulated in mitochondria, we compared global mRNA and protein abundance using the OpenCell database (*8*). This revealed a small subset of post-transcriptionally regulated proteins with low protein-to-mRNA ratios, such as Hypoxia Inducible Factor 1 Subunit Alpha (HIF1A), DAP3 binding cell death enhancer 1 (DELE1), Activating Transcription Factor 4 (ATF4), ornithine decarboxylase antizyme (OAZ1), and F-box and leucine-rich repeat protein 5 (FBXL5). These proteins are rapidly degraded or translationally repressed under basal conditions but accumulate upon diverse environmental stimuli, which enables their dynamic response to cellular stress. Among these proteins, SLC25A39 was the only mitochondrial transporter, indicating that a posttranscriptional mechanism may underlie its regulation (Fig. 1A). To determine the mode of SLC25A39 regulation in response to GSH depletion, we generated a reporter construct in which a 3xFLAG-tagged *SLC25A39* cDNA was cotranslated with an internal control (RFP), separated by a 2A self-cleaving peptide from porcine teschovirus-1 (P2A). GSH depletion in human embryonic kidney HEK293T cells expressing this reporter strongly induced the accumulation of the 3xFLAG-SLC25A39 protein relative to the abundance of RFP (Fig. 1B). This effect was independent of oxidative stress, as only supplementation of GSH, but not other antioxidants, suppressed SLC25A39 accumulation (Fig. 1B and fig. S1C). Because these observations point to a protein-level control, we measured the half-life of SLC25A39 in cycloheximide chase assays. SLC25A39 protein was unstable, with an estimated half-life of 15 min (Fig. 1C). GSH depletion substantially extended the half-life of SLC25A39 (>300 min), and supplementing cells with GSH restored this rapid degradation (Fig. 1C). These data indicate that SLC25A39 has a short half-life and becomes stabilized when GSH is depleted.

Given that the mitochondrial matrix harbors a distinct GSH pool (*9*), we sought to determine whether SLC25A39 stability is regulated specifically by changes in the availability of local mitochondrial GSH, thus forming a feedback loop in which the substrate controls its own uptake. To test this possibility, we engineered the human GSH-specific gamma-glutamylcyclotransferase (CHAC1), a cytosolic enzyme that catalyzes the conversion of GSH into 5-oxoproline and L-cysteinylglycine (*10*, *11*), to be expressed in mitochondria (hereby referred to as MitoCHAC1) (Fig. 1, D and E, and fig. S1D). This engineered enzyme allowed us to specifically deplete mitochondrial GSH without significantly altering whole-cell GSH levels (Fig. 1F and fig. S1, E to G). Confirming the robust enzymatic activity of MitoCHAC1, we observed 5-oxoproline accumulation in the mitochondria (fig. S1H). Depletion of mitochondrial GSH with MitoCHAC1 expression was sufficient to induce SLC25A39 accumulation (Fig. 1G and fig. S1I). Expression of MitoCHAC1 did not affect the abundance of SLC25A39 mRNA and only slightly decreased cell proliferation (fig. S1, J and K). Moreover, expression of MitoCHAC1 did not impact the abundance of the master regulator of the antioxidant response, nuclear factor erythroid 2–related factor 2 (NRF2), or that of its downstream transcriptional targets (*12*), indicating that NRF2 activity is not controlled by mitochondrial GSH availability (fig. S1L). To formally test whether SLC25A39 stability can be regulated independently of any signaling input from the cytosol, we developed a cell-free system with immunopurified mitochondria (Fig. 1H). Although isolated mitochondria from GSH-depleted cells displayed highly stable SLC25A39, direct supplementation with exogenous glutathione led to rapid degradation of SLC25A39, indicating that signaling input from cytosol or nucleus is dispensable for such regulation. Thus, a compartmentalized feedback mechanism appears to enable mitochondria to rapidly respond to the changes in the

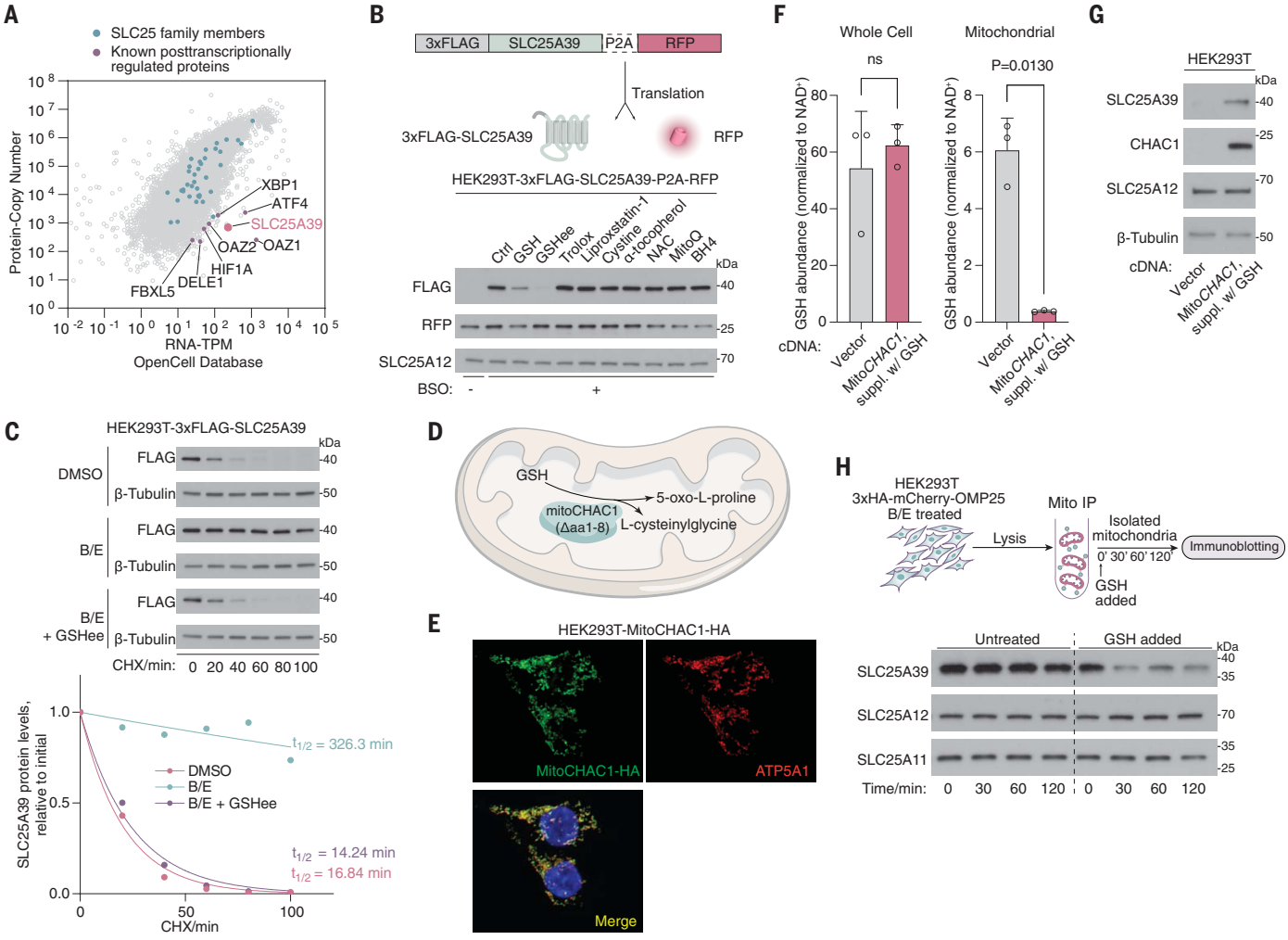

Fig. 1. SLC25A39 is a short–half life protein regulated by mitochondrial GSH availability. (**A**) Scatter plot showing the protein copy number versus mRNA abundance (TPM) for all genes in HEK293T cells detectable across the proteome. Original data were retrieved from the OpenCell database. Green dots denote SLC25 family proteins, and purple dots denote representative proteins known to be regulated post transcriptionally. (**B**) (Top) Schematic showing the construct for cotranslational expression of 3xFLAG-tagged SLC25A39 and RFP, separated by a self-cleaving P2A peptide. (Bottom) Immunoblots of the indicated proteins in HEK293T cells expressing the aforementioned construct. Cells were treated with BSO (1 mM) for 48 hours and were then treated with GSH (10 mM), GSH ethyl ester (GSHee, 10 mM), Trolox (50 μM), Liproxstatin-1 (1 μM), cystine (200 μM), N-acetylcysteine (NAC, 1 mM), α-tocopherol (5 μM), MitoQ (30 nM), or BH4 (4 μM) for 8 hours. RFP was used as an internal control for the translational levels of the construct, and SLC25A12 was used as a loading control. (**C**) (Top) Immunoblots of the indicated proteins in HEK293T cells expressing 3xFLAG-*SLC25A39* cDNA treated with cycloheximide (CHX, 50 μg/ml) for the indicated times. Prior to CHX treatment, cells were treated with BSO (1 mM) and erastin (5 μM) for 24 hours and GSH ethyl ester (GSHee, 10 mM) for 8 hours. DMSO was used as the control. β-tubulin was used as a loading control. (Bottom) Quantification of FLAG band signal intensity from the immunoblots above. Half-life ($t_{1/2}$) was calculated by the nonlinear fitting of FLAG band signal intensity versus time to one phase decay exponential model. (**D**) Schematic showing the localization and the catalytic reaction of engineered MitoCHAC1 protein. (**E**) Immunofluorescence images of MitoCHAC1 (HA, green), ATP5A1 (red), and DAPI (blue) in HEK293T cells. (**F**) Whole-cell and mitochondrial abundance of GSH (normalized to NAD^+ abundance) in HEK293T cells expressing empty vector or MitoCHAC1 (in the presence of exogenous GSH). Data are mean ± SD representing three biologically independent samples. *P* values were calculated from Welch's *t* test. (**G**) Immunoblots showing the amounts of the indicated proteins in HEK293T cells that express an empty vector or MitoCHAC1 (in the presence of exogenous GSH). SLC25A12 and β-tubulin were used as loading controls. (**H**) (Top) The schematic of the cell-free assay that uses immunopurified mitochondria (mito IP) from HEK293T cells to analyze SLC25A39 stability. (Bottom) Immunoblots of the indicated proteins from purified mitochondria after treating them with GSH (20 mM) for the indicated times. SLC25A12 and SLC25A11 were used as loading controls.

cncentration of GSH in the matrix by tuning SLC25A39 stability.

AFG3L2 degrades SLC25A39 in a GSH-dependent manner

SLC25A39, unlike other SLC25 family members, has an exceptionally low protein copy number to mRNA-TPM ratio and is distinctly sensitive to GSH availability. We therefore considered that a particular structural feature on SLC25A39 might allow this regulation. We aligned AlphaFold (*13*)–predicted structures of SLC25A39 with those of highly expressed SLC25 family members as well as that of SLC25A40, a paralog of SLC25A39 that is not sensitive to GSH availability (Fig. 2A and fig. S2, A and B). This analysis revealed a protruding loop on the matrix side of the SLC25A39 protein that corresponded to amino acids 42 to 106 (aa42–106) (Fig. 2A and fig. S2A). Although this domain is not critical for GSH transport activity (fig. S2C),

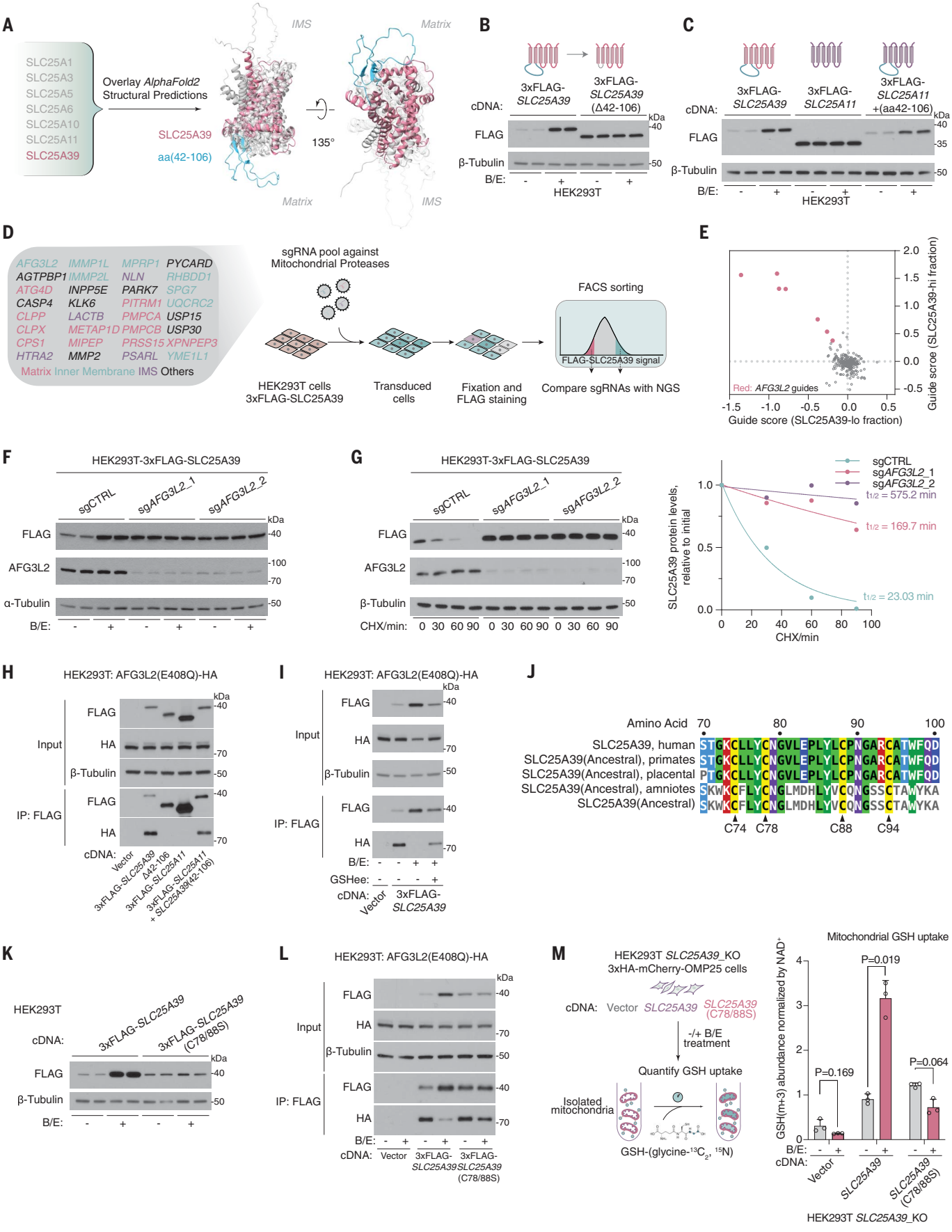

Fig. 2. AFG3L2 binds and degrades SLC25A39 through a matrix loop domain in a GSH-dependent manner. (**A**) Schematic showing the alignment of AlphaFold2-predicted structural models of the indicated proteins. SLC25A39 is highlighted in pink, with aa42–106 highlighted in green. (**B**) Immunoblots of the indicated proteins in HEK293T cells expressing 3xFLAG-SLC25A39 or 3xFLAG-SLC25A39 without the matrix-facing loop (Δaa42–106) after 24-hour treatment with BSO (1 mM) and erastin (5 μM) or DMSO as the control. (**C**) Immunoblots of the indicated proteins in HEK293T cells expressing 3xFLAG-SLC25A39, 3xFLAG-SLC25A11, or a chimeric protein in which aa42–106 of SLC25A39 is spliced into SLC25A11 after 24-hour treatment with BSO (1 mM) and erastin (5 μM) or DMSO as the control. (**D**) Schematic showing the library design of the mitochondrial peptidase sgRNA library and the workflow of the FACS-based CRISPR screen for 3xFLAG-SLC25A39 stability. (**E**) Scatter plot showing the enrichment of sgRNAs targeting mitochondrial proteases in the SLC25A39-lo cell fraction (*x* axis) and SLC25A39-hi cell fraction (*y* axis). Red dots represent sgRNAs targeting *AFG3L2*. (**F**) Immunoblots of the indicated proteins in HEK293T cells expressing 3xFLAG-SLC25A39 and sgRNAs targeting control or *AFG3L2* after 24-hour treatment with BSO (1 mM) and erastin (5 μM) or DMSO as the control. (**G**) (Left) Immunoblots of the indicated proteins in HEK293T cells expressing 3xFLAG-SLC25A39 and sgRNAs targeting *AFG3L2* or control upon treatment with cycloheximide (CHX, 50 μg/ml) for the indicated times. β-tubulin was used as a loading control. (Right) Quantification of FLAG bands signal intensity from the immunoblots. Half-life ($t_{1/2}$) was calculated by the nonlinear fitting of FLAG band signal intensity versus time to one phase decay exponential model. (**H**) Immunoblots of the indicated proteins from whole-cell lysates or FLAG-immunoprecipitation from HEK293T cells stably expressing cDNAs for vector, 3xFLAG-*SLC25A39*, 3xFLAG-*SLC25A39* lacking matrix-facing loop (Δaa42–106), 3xFLAG-*SLC25A11*, or a chimeric protein in which aa42–106 of SLC25A39 is spliced into SLC25A11 and transiently transfected with *AFG3L2* (E408Q)-HA cDNA. (**I**) Immunoblots of the indicated proteins from whole-cell lysates or FLAG immunoprecipitation from HEK293T cells stably expressing cDNA for empty vector or 3xFLAG-*SLC25A39* and transiently transfected with *AFG3L2*(E408Q)-HA cDNA. Cells were treated for 24 hours with BSO (1 mM) and erastin (5 μM) or DMSO as the control. Indicated cells were then treated for 8 hours with GSHee (10 mM). (**J**) Multiple sequence alignment between SLC25A39 and the inferred ancestral sequence of SLC25A39 reconstructed from amino acid sequences of SLC25A39 homologs in the indicated taxa. Four conserved cysteines in the matrix-facing loop are highlighted. (**K**) Immunoblots for the indicated proteins in HEK293T cells that express 3xFLAG-SLC25A39 or 3xFLAG-SLC25A39(C78/88S). Cells were treated for 24 hours with BSO (1 mM) and erastin (5 μM) or DMSO as the control. (**L**) Immunoblot of the indicated proteins from whole-cell lysates or FLAG immunoprecipitation from HEK293T cells stably expressing cDNAs for vector, 3xFLAG-*SLC25A39*, or 3xFLAG-*SLC25A39*(C78/88S) and transiently transfected with *AFG3L2*(E408Q)-HA cDNA. Cells were treated for 24 hours with BSO (1 mM) and erastin (5 μM) or DMSO as the control. (**M**) (Left) Schematic showing the GSH uptake assay that uses immunopurified mitochondria from HEK293T-*SLC25A39*_KO cells expressing cDNAs for empty vector, *SLC25A39*, or *SLC25A39*(C78/88S). (Right) Abundance of GSH-(glycine-$^{13}C_2$,^{15}N) taken up by isolated mitochondria. Data are mean ± SD representing three biologically independent samples. *P* values were calculated from Welch's multiple *t* test with the Holm-Šídák method.

its deletion completely abrogated GSH sensitivity and extended protein half-life (>250 min), thereby uncoupling the GSH-mediated regulation of SLC25A39 from its transport function (Fig. 2B and fig. S2D, lanes 1 to 4). Splicing this fragment into a distant SLC25 family member (SLC25A11) that does not respond to perturbations in GSH levels rendered it GSH responsive (Fig. 2C). The differential responses of SLC25A39 and its close paralog, SLC25A40, to GSH availability raised the possibility that the matrix loop may be the critical attribute for the diversification of these two genes. Indeed, a significant portion of the SLC25A39 loop domain responsible for GSH sensitivity is evolutionarily new, with no homologs outside vertebrates (data S1). Using a maximum likelihood method, we reconstructed the ancestral sequence of SLC25A39 (fig. S2, E to G). When spliced into SLC25A11, the loop domain in the ancestral SLC25A39 sequence conferred GSH responsiveness, indicating that this feature arose early in the evolution of SLC25A39 and might be the key driving force behind the diversification of mitochondrial GSH transporters (fig. S2H). Thus, the conserved matrix-facing loop domain is necessary and sufficient for the GSH-mediated regulation of SLC25A39.

We next sought to determine the proteolytic machinery involved in SLC25A39 degradation. Inhibition of proteasomal or lysosomal function did not impact SLC25A39 stability (fig. S3A). Given these results and the mitochondria-localized regulation of SLC25A39 (Fig. 1H), we focused on proteolysis systems in the mitochondria. We designed a fluorescence-activated cell sorting (FACS)–based CRISPR screen for 3xFLAG-SLC25A39 stability with a single guide RNA (sgRNA) library targeting all annotated mitochondrial proteins with peptidase activity (34 genes; 7 sgRNAs per gene) (Fig. 2D and fig. S3, B and C). Transduced cells were stained with a FLAG antibody, and sgRNA sequences from cells with the highest (SLC25A39-hi) and the lowest (SLC25A39-lo) FLAG signal were quantified by next-generation sequencing. Among all candidates, AFG3L2, a mitochondrially localized protease, was the only target whose loss significantly altered the abundance of SLC25A39 (*q* value = 0.009) (Fig. 2E, fig. S3D, and data S2). Consistent with the screen results, the loss of AFG3L2 completely abolished its GSH-mediated regulation (Fig. 2F and fig. S3E) and stabilized SLC25A39 even at normal GSH concentrations (Fig. 2G). SPG7, the paralog of AFG3L2, was dispensable for this process (fig. S3, F to H). Furthermore, immunoprecipitation experiments with an ATP triphosphatase–mutant AFG3L2(E408Q), which allows the detection of transient protease-target interactions (*14*), revealed that SLC25A39 associated with AFG3L2 through its loop domain (aa42–106) (Fig. 2H). When this loop was removed from SLC25A39, the loss of AFG3L2 did not further stabilize the protein (fig. S2D). This interaction was highly sensitive to changes in GSH availability and was abrogated by GSH depletion (Fig. 2I). Unbiased proteomic profiling further confirmed that SLC25A39, but not other mitochondrial membrane proteins, was specifically targeted for degradation by AFG3L2 in a GSH-dependent manner (fig. S4, A to F, and data S3). GSH availability therefore determines the turnover of SLC25A39 protein by enabling the recruitment of AFG3L2 through the matrix-facing loop domain of SLC25A39.

CRISPR screen identifies [2Fe-2S] cluster assembly as essential for SLC25A39 stability

To further dissect the function of the loop domain in the GSH-mediated regulation of SLC25A39, we used two orthogonal approaches. We used a reporter assay for mitochondrial protein stability (fig. S5A) to identify a short fragment on the GSH-responsive loop of SLC25A39 (aa72–86) necessary for its recruitment to AFG3L2 and proteolysis (fig. S5, B to E). Additionally, a detailed conservation analysis revealed four highly conserved cysteines—Cys74, Cys78, Cys88, and Cys94—within the vicinity of this fragment (Fig. 2J and fig. S5F). Introducing individual cysteine-to-serine mutations, particularly on Cys88 and Cys94, partially abrogated SLC25A39 stabilization upon GSH depletion (Fig. 2J and fig. S6A). More notably, mutating two cysteines in the loop domain was sufficient to completely abolish the regulation (Fig. 2K and fig. S6, B and C). The absence of these conserved cysteines led to the constitutive association of SLC25A39 and AFG3L2, independent of GSH abundance (Fig. 2L). To investigate the functional role of these cysteines in maintaining GSH homeostasis in the mitochondria, we performed uptake assays with isotope-labeled GSH [GSH-(glycine-$^{13}C_2$,^{15}N)] in mitochondria isolated from control cells or cells in which GSH was depleted. Consistent with the strong feedback mechanism, in the presence of wildtype SLC25A39,

GSH depletion led to a 3.5-fold compensatory increase in mitochondrial GSH uptake (Fig. 2M). By contrast, mutating cysteine residues (C78/88S) completely abolished this homeostatic mechanism (Fig. 2M and fig. S6D), confirming the essential role of conserved cysteines in the feedback regulation of SLC25A39-mediated GSH uptake.

Cysteine residues on proteins have critical roles in redox sensing, posttranslational modifications, and cofactor binding (*15*). However, our initial interrogation showed that disulfide bond formation and relay or hypoxic response did not impact SLC25A39 regulation (fig. S7, A to E). To identify the precise mechanism by which cysteines in the loop domain enable GSH-mediated regulation of SLC25A39 stability, we performed a FACS-based CRISPR screen for SLC25A39 stability under GSH depletion in HEK293T cells expressing a 3xFLAG-tagged *SLC25A39* cDNA (Fig. 3A). Given that regulation of SLC25A39 can occur independently of any cytosolic machinery (Fig. 1H), for these screens, we generated an sgRNA library that contained all annotated mitochondrial proteins (MITO-sgRNA) (*16*, *17*). After immunostaining with a FLAG antibody, transduced cells with the highest (SLC25A39-hi) and lowest (SLC25A39-lo) FLAG signal intensity were isolated and their sgRNA abundances were quantified. Among the genes whose disruption led to a lower amount of SLC25A39 were many assembly factors for [2Fe-2S] clusters such as

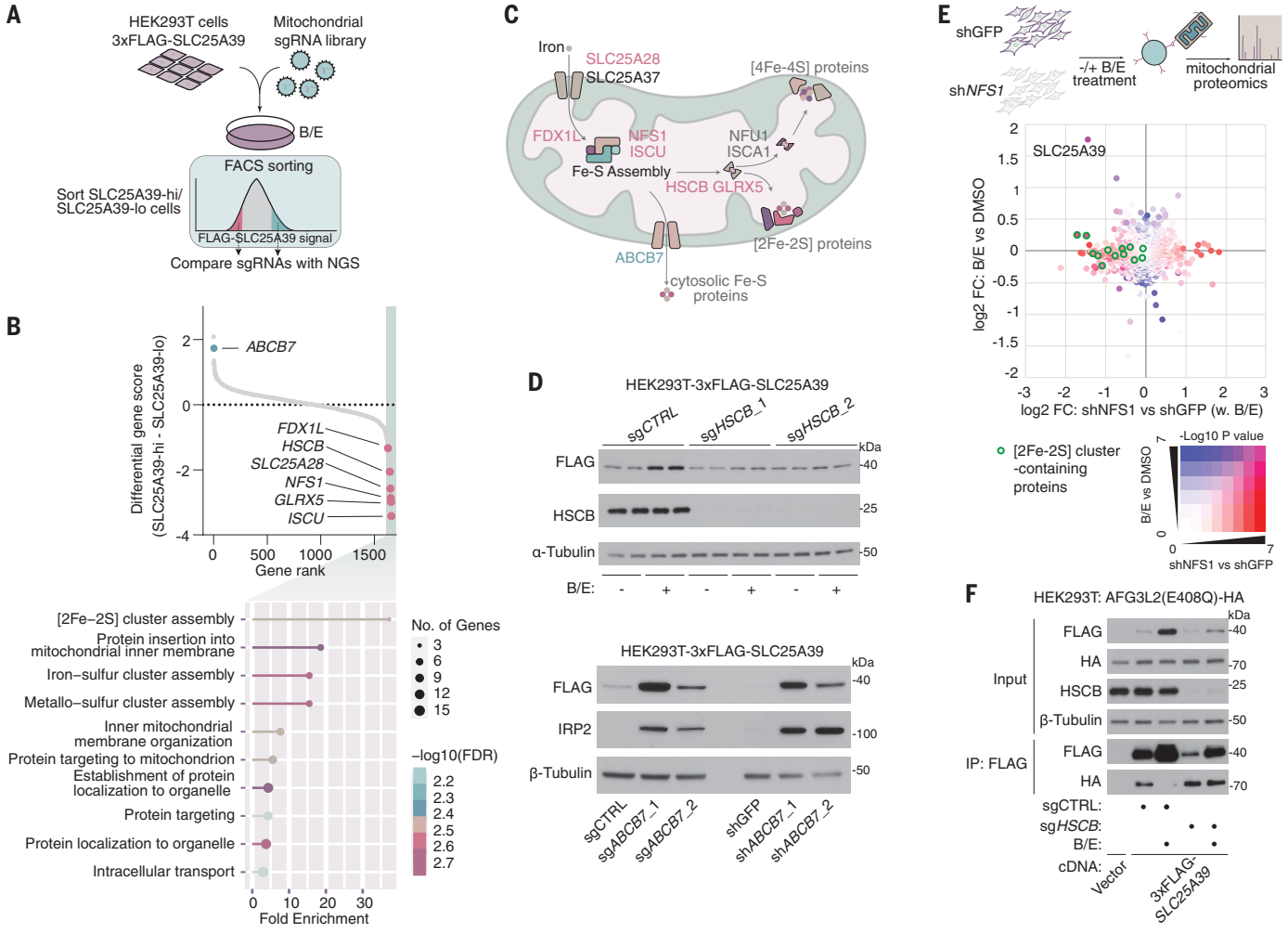

Fig. 3. CRISPR screen identifies [2Fe-2S] cluster assembly as essential for the regulation of SLC25A39 stability. (**A**) Schematic of the CRISPR screen workflow with mitochondrial sgRNA library for SLC25A39 stability under GSH depletion. (**B**) (Top) Dot plot showing the distribution of differential CRISPR gene score calculated as (median guide enrichment in the SLC25A39-hi fraction) − (median guide enrichment in the SLC25A39-lo fraction). Pink dots indicate genes in the [2Fe-2S] cluster assembly pathway. The green dot indicates the putative mitochondrial [2Fe-2S] cluster exporter *ABCB7*. (Bottom) Gene Ontology enrichment analysis of genes with a differential CRISPR gene score lower than −1. (**C**) Schematic of the mitochondrial Fe-S cluster assembly pathways that highlights the genes that scored (pink and green) in the CRISPR screen. (**D**) Immunoblots of the indicated proteins in HEK293T cells that express 3xFLAG-SLC25A39. (Top) Cells were transduced with lentivirus expressing Cas9 and control sgRNA or sgRNAs that target iron-sulfur cluster assembly factor HSCB. Cells were treated for 24 hours with BSO (1 mM) and erastin (5 μM) or DMSO as the control. (Bottom) Cells were transduced with lentivirus expressing control sgRNA or sgRNAs targeting *ABCB7* (left), or shRNAs targeting GFP or *ABCB7* (right). (**E**) Scatter plot showing log2 fold change and −log10(*P* value) of proteomics analysis from immunopurified mitochondria of the indicated samples. The *x* axis represents log2 protein fold change (FC) in isolated mitochondria from HEK293T cells that express shRNA targeting GFP or cysteine desulfurase *NFS1* after 24-hour treatment with BSO (1 mM) and erastin (5 μM). The *y* axis represents log2 fold change (FC) in mitochondrial protein abundance after treating cells with BSO (1 mM) and Erastin (5 μM) versus DMSO as the control. The color grid indicates −log10(*P* values) and green circles represent [2Fe-2S] cluster–containing proteins. (**F**) Immunoblots of the indicated proteins from the whole-cell lysates or FLAG-immunoprecipitation from HEK293T cells stably expressing cDNAs for vector or 3xFLAG-*SLC25A39*, are infected with lentivirus-expressing control sgRNA or sgRNAs for *HSCB*, and transiently transfected with *AFG3L2*(E408Q)-HA cDNA. Cells were treated for 24 hours with BSO (1 mM) and erastin (5 μM) or DMSO as the control.

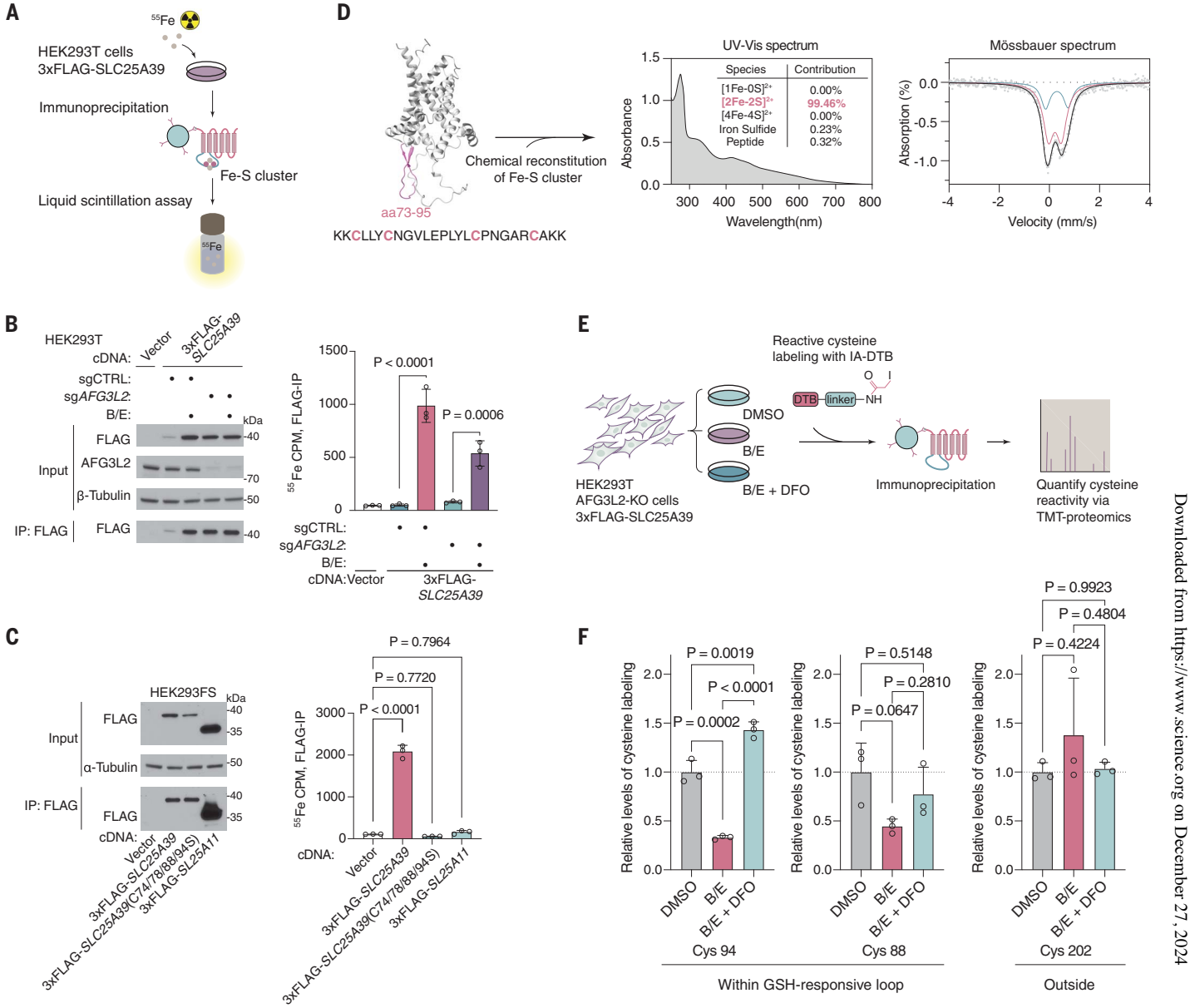

Fig. 4. A GSH-sensitive iron-sulfur cluster associates with SLC25A39 and mediates its regulation. (**A**) Schematic showing the method of tracking iron-sulfur cluster bound to SLC25A39 by means of ^{55}Fe tracing. (**B**) (Left) Immunoblots of the indicated proteins from whole-cell lysates or FLAG immunoprecipitation from HEK293T cells stably expressing cDNAs for empty vector or 3xFLAG-*SLC25A39* and infected with lentivirus-expressing control sgRNA or sgRNA targeting *AFG3L2*. Cells were labeled with $^{55}FeCl_3$ in the culture media and treated for 24 hours with BSO (1 mM) and erastin (5 μM) or DMSO as the control. (Right) The amount of ^{55}Fe bound to FLAG immunoprecipitant, from the identical cells as those in the immunoblot, quantified by liquid scintillation assay. (**C**) (Left) Immunoblots of the indicated proteins from whole-cell lysates or FLAG immunoprecipitation from HEK293FS cells stably expressing cDNAs for empty vector, 3xFLAG-*SLC25A39*, 3xFLAG-*SLC25A39*(C74/78/88/94S), or 3xFLAG-*SLC25A11*. Cells were labeled with $^{55}FeCl_3$ in the culture media and treated for 24 hours with BSO (1 mM) and erastin (5 μM). (Right) The amount of ^{55}Fe bound to FLAG immunoprecipitant, from the identical cells as those used for the immunoblot, quantified by liquid scintillation assay. (**D**) (Left) Schematic showing the location and peptide sequence of SLC25A39(aa73–95) used to reconstitute peptide-[2Fe-2S] cluster complex in vitro. (Right) UV-visible spectrum (with the calculated contribution of the indicated species) and Mössbauer spectrum of the reconstituted peptide-[2Fe-2S] complex. The Mössbauer spectrum was least-squares fit to extract hyperfine parameters: isomer shift (δ), quadrupole splitting (Δ), full width at half-maximum (FWHM), and intensities (I). The red and green curves of the Mössbauer spectrum plot represent two Fe^{3+} doublets with slightly different shifts and quadrupole splitting fitted to the spectral data. Red: δ = 0.23 mm/s, Δ = 0.51 mm/s, and I = 70%. Green: δ = 0.30 mm/s, Δ = 0.90 mm/s, and I = 30%. (**E**) Schematic for cysteine reactivity profiling for SLC25A39 with an iodoacetamide-desthiobiotin (IA-DTB) probe. (**F**) Reactivity of SLC25A39-Cys94, Cys88, and Cys202 after a 24-hour treatment with indicated reagents, quantified by mass spectrometry as the intensity ratio between IA-DTB–labeled versus unlabeled peptide containing SLC25A39-Cys94 and normalized to DMSO-treated samples. [(B), (C), and (F)] Data are mean ± SD and represent three biologically independent samples. *P* values were calculated from one-way analysis of variance.

NFS1, *ISCU*, *HSCB*, and *GLRX5* (Fig. 3, B and C, fig. S8A, and data S2). Additionally, one of the genes whose loss most strongly enhanced SLC25A39 stability was *ABCB7*, a putative transporter involved in the export of iron-sulfur clusters from mitochondria (*18*–*20*). A gene ontology analysis further confirmed [2Fe-2S] cluster assembly as the most enriched pathway for the scoring genes in the SLC25A39-lo fraction (FDR = 1.8×10^{-5}) (Fig. 3B and fig. S8A). Consistent with the screen results, acute loss of Fe-S cluster assembly factors (ISCU, GLRX5, or HSCB) abolished SLC25A39 stabilization under GSH depletion (Fig. 3D and fig. S8, B to F), whereas ABCB7 loss constitutively stabilized SLC25A39, likely owing to the mitochondrial accumulation of [2Fe-2S] clusters (Fig. 3D and fig. S8G) (*21*). Unbiased proteomics experiments under GSH-depleted conditions also revealed SLC25A39 as the only mitochondrial transporter whose abundance decreased upon blocking synthesis of [2Fe-2S] clusters (Fig. 3E, fig. S8H, and data S4). The loss of [2Fe-2S] assembly factor HSCB (*22*) led to the constitutive association of SLC25A39 with AFG3L2 (Fig. 3F). Altogether, these results suggest that [2Fe-2S] cluster synthesis is necessary for stabilizing SLC25A39 when cells are depleted of GSH.

Iron-sulfur clusters are typically coordinated by cysteines and are essential for the stability of the holoprotein. Because iron-sulfur cluster assembly and conserved cysteines are indispensable for SLC25A39 stability, we considered the possibility that SLC25A39 might be associated with a [2Fe-2S] prosthetic group. To test the presence of potential Fe-containing cofactors associated with SLC25A39, we labeled HEK293T cells expressing 3xFLAG-tagged *SLC25A39* cDNA with radioactive $^{55}FeCl_3$ (Fig. 4A). Although we did not observe evidence of increased iron-sulfur cluster synthesis or accumulated mitochondrial iron (fig. S9, A to C), the amount of ^{55}Fe immunoprecipitated with SLC25A39 was increased upon GSH depletion (Fig. 4B). This increase of bound iron appeared to precede the stabilization of SLC25A39 because similar results were observed in *AFG3L2*-knockout cells with a comparable amount of immunoprecipitated SLC25A39 protein (Fig. 4B). This factor is unlikely to exist as heme iron or [4Fe-4S] clusters as depleting cells of ferrochelatase (FECH), a critical enzyme of heme synthesis or [4Fe-4S] cluster-assembly factor NFU1 had no effect on SLC25A39 regulation (fig. S9, D and E). By contrast, the loss of [2Fe-2S] assembly factor HSCB almost completely blocked iron binding to SLC25A39 (fig. S9F). The iron-binding activity of SLC25A39 lies within its glutathione-sensing matrix loop. Deleting this loop abolished the iron-binding of SLC25A39, and splicing the loop domain of SLC25A39 to SLC25A11, which normally does not bind iron, enabled it to associate with iron (fig. S9G). Mutating the critical cysteines in the loop domain (Cys74/78/88/94) prevented the association of ^{55}Fe to SLC25A39 upon glutathione depletion (Fig. 4C). Furthermore, with a synthetic peptide encompassing the GSH-responsive cysteines of SLC25A39 (aa73–95), we were able to reconstitute the peptide-[2Fe-2S] cluster complex in vitro that displayed characteristic spectral features of a [2Fe-2S] cluster (Fig. 4D) in which all four cysteines could engage in metal coordination (fig. S9H). We conclude that SLC25A39 associates with a GSH-sensitive iron-sulfur cluster through its cysteine residues.

As a small-molecule thiol, GSH could exchange with the cysteine ligands of a [2Fe-2S] cluster, resulting in partial or complete dissociation of the cluster from the holoprotein (*21*, *23*, *24*) (fig. S9I). In line with these previous observations, GSH supplementation induced a notable change in the spectral feature of the SLC25A39 (aa73–95)–[2Fe-2S] cluster complex (fig. S9, J and K), indicating the displacement of one or more cysteine residues in the loop domain. Additionally, supplementation of cell-permeable GSH to GSH-depleted cells significantly reduced the amount of iron bound to SLC25A39 (fig. S9L). To further support these findings, we used chemical proteomics to assess the reactivity of cysteines with an iodoacetamide-desthiobiotin probe, given that coordination with iron-sulfur clusters could limit cysteine reactivity (Fig. 4E) (*25*). Cys94, a conserved cysteine in the SLC25A39 matrix loop that was most reliably detected by mass spectrometry, displayed a strong decrease in reactivity upon GSH depletion, which was restored by iron chelation (Fig. 4F and data S5). We observed a similar trend for Cys88; by contrast, Cys202, a cysteine residue that lies outside of the matrix-facing loop, showed no significant change in reactivity. These observations support a model in which GSH depletion leads to the tight association of a [2Fe-2S] cluster to SLC25A39 through cysteines (C74/78/88/94) in the loop domain, which in turn prevents the recruitment of protease AFG3L2, thus stabilizing SLC25A39.

SLC25A39-mediated GSH import maintains mitochondrial Fe-GSH balance

We considered why such a feedback mechanism might have evolved and how it might contribute to mitochondrial function. In addition to its antioxidant role, GSH is a major endogenous iron ligand in cells (*26*). In yeast, the absence or an excess of GSH can both lead to abnormal iron metabolism and the activation of cellular stress responses (*27*). Given the essential role of iron-sulfur clusters in SLC25A39 stability, this feedback mechanism might help maintain a proper balance between the availability of iron and GSH in mitochondria. Consistent with this idea, when we increased the mitochondrial iron/GSH ratio by overexpressing Mitoferrin 1 (SLC25A37) or Mitoferrin 2 (SLC25A28), the two major mitochondrial iron importers, SLC25A39 protein levels increased (Fig. 5A and fig. S10A). This regulation occurs through the GSH-responsive cysteines on SLC25A39 because mutating these cysteines in situ dampened the response of SLC25A39 to iron overload (fig. S10B). The increase in mitochondrial iron accompanied a compensatory increase in the abundance of GSH in the mitochondria (fig. S10C). By contrast, iron chelation, which decreases the mitochondrial iron/GSH ratio, abolished the stabilization of SLC25A39 upon GSH depletion (Fig. 5B and fig. S10D). We used multiple orthogonal approaches to perturb mitochondrial iron and GSH pools, and the abundance of SLC25A39 responded in accordance with the change in iron/GSH ratio, indicating that a feedback mechanism maintains iron/GSH balance (Fig. 5, C and D). We tested whether GSH limitation and iron overload imposed similar stresses on mitochondrial function by comparing mitochondrial proteomes from cells depleted of GSH depletion (*6*) or exposed to excess mitochondrial iron after the overexpression of Mitoferrin 2 (fig. S11A). These conditions led to the depletion of similar proteins, particularly those that function in the mitochondrial translation machinery, ETC components, and iron-sulfur proteins (fig. S11, B and C, and data S6). Decreases in the abundance of mitochondrial proteins induced by mitochondrial iron overload could be largely restored by boosting mitochondrial GSH uptake, supporting a critical buffering role of GSH in iron overload (Fig. 5, E and F; fig. S11, D and E; and data S7). SLC25A39 appeared to protect mitochondrial function at least partially through its adaptive response to iron/GSH balance because defects in the GSH-sensing function of SLC25A39 led to suboptimal mitochondrial function and impaired viability under GSH limitation (fig. S12, A to D). Thus, a feedback mechanism for SLC25A39 stability enables cells to maintain optimal mitochondrial functions by restoring iron/GSH balance.

Discussion

Metabolic homeostasis is maintained by negative feedback regulation at both the cellular and organismal levels. We provide evidence that organelles use similar principles to control their internal metabolite pools. In mitochondria, autoregulatory control of GSH availability occurs by coupling GSH sensing in the matrix to the degradation of its transporter, SLC25A39. This regulation pattern mirrors the homeostatic mechanisms for redox potential (*28*) or pH (*29*) in other organelles. Conceivably, other cellular compartments, the internal chemical environments of which differ from that of cytosol, may harbor similar mechanisms for sensing and regulating metabolites abundance (*30*).

Concentrations of metabolites are often maintained within strict limits. Deviations from the optimal range, either through deficiency or excess, can damage cellular functions. We propose

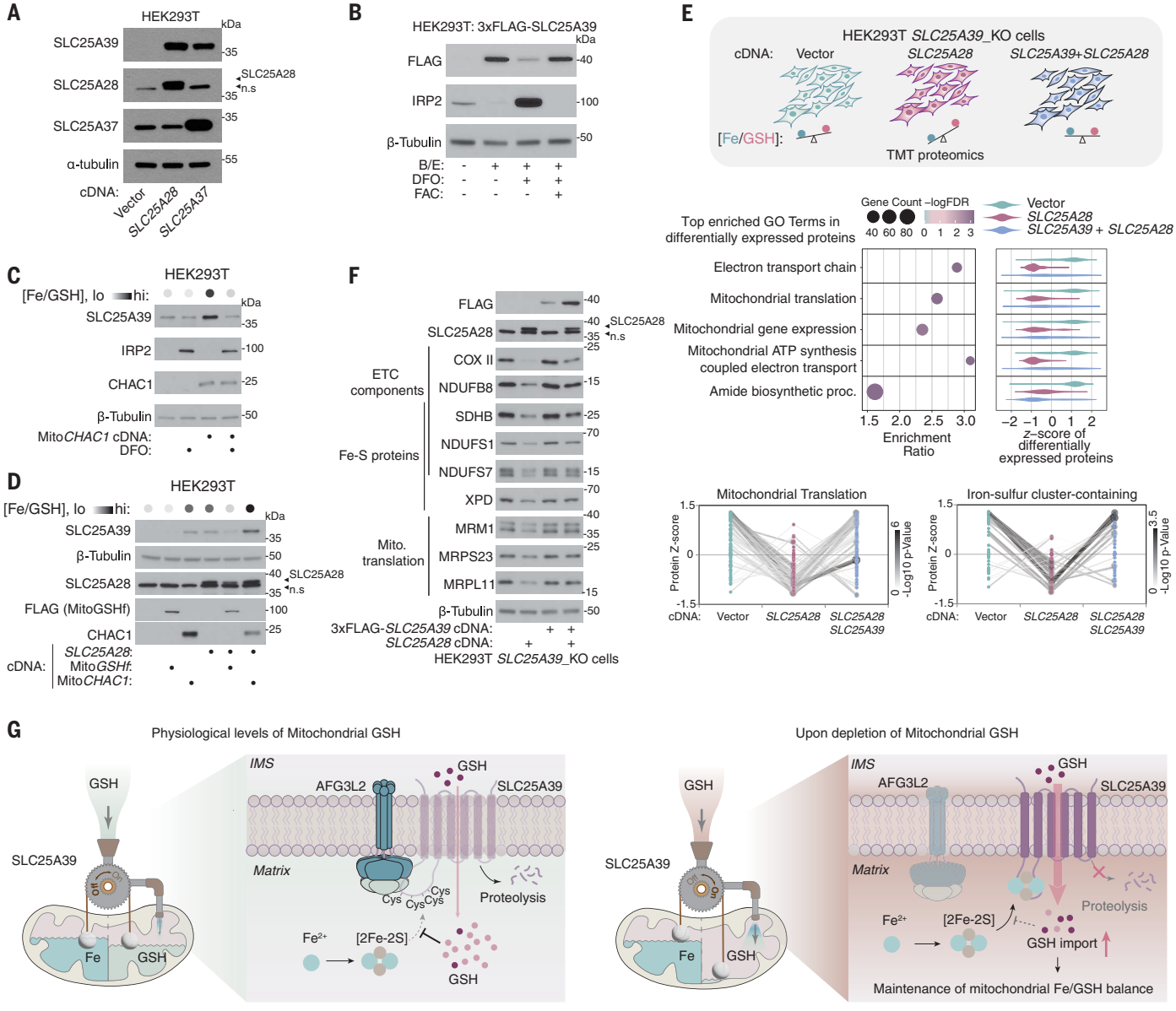

Fig. 5. SLC25A39-mediated GSH import maintains iron/GSH balance in mitochondria. (**A**) Immunoblots of the indicated proteins from HEK293T cells overexpressing cDNAs of Mitoferrin 1 (*SLC25A37*), Mitoferrin 2 (*SLC25A28*), or an empty vector. (**B**) Immunoblot of the indicated proteins in HEK293T cells expressing 3xFLAG-*SLC25A39* cDNA after 24-hours treatment with BSO (1 mM) and Erastin (5 μM); BSO, Erastin, and deferoxamine (50 μM); or BSO, Erastin, deferoxamine (50 μM), and Ferric Ammonium Citrate (FAC, 10 μg/ml). (**C**) Immunoblots of the indicated proteins from HEK293T cells overexpressing cDNAs of Mito*CHAC1* or empty vector after 4 hours of treatment with 50-μM iron chelator deferoxamine (DFO) or control. (**D**) Immunoblots of the indicated proteins from HEK293T cells overexpressing cDNAs of *SLC25A28*, Mito*CHAC1*, or FLAG-tagged Mito*GSHf*, an engineered bacterial GSH synthase localized to the mitochondria. (**E**) (Top) Schematic of the experiment setup of TMT proteomics for cells with different iron/GSH ratios. Three biologically independent samples per condition from HEK293T-*SLC25A39*_KO cells expressing the indicated cDNAs were used. (Middle) Gene ontology enrichment analysis of the most differentially expressed proteins across the three conditions that shows the top five most significantly enriched biological processes. The violin plots indicate the relative protein abundance (*z*-scores) of the differentially expressed proteins in the indicated biological processes. (Bottom) Dot plot representing protein levels (*z*-scores) of the mitochondrial translation machinery and iron-sulfur cluster–containing proteins. The darkness of the lines represents the statistical significance of the changes in protein abundance. (**F**) Immunoblots of the indicated proteins in HEK293T-*SLC25A39*_KO cells overexpressing empty vector, *SLC25A39* cDNA, Mitoferrin 2 (*SLC25A28*) cDNA, or both. (**G**) Schematic for the model describing the autoregulatory control of mitochondrial iron/GSH balance by SLC25A39.

that feedback regulation of mitochondrial GSH may primarily serve to maintain an appropriate amount of GSH to accompany free iron. This is consistent with the chemical properties of GSH, which is predicted to be the major iron ligand in cells (*26*). Labile iron in mitochondria is essential for enzymatic activities and macromolecular complex assembly in many pathways. By contrast, excess iron can cause oxidative damage to proteins, DNA, and membrane lipids (*31*). This dual effect of free iron implies that its concentration in the mitochondria must be properly buffered. Feedback regulation of the availability of mitochondrial GSH is absent in lower organisms and appears to arise more recently in

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Glutathione and mitochondria

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Glutathione (GSH) is the main non-protein thiol in cells whose functions are dependent on the redox-active thiol of its cysteine moiety that serves as a cofactor for a number of antioxidant and detoxifying enzymes. While synthesized exclusively in the cytosol from its constituent amino acids, GSH is distributed in different compartments, including mitochondria where its concentration in the matrix equals that of the cytosol. This feature and its negative charge at physiological pH imply the existence of specific carriers to import GSH from the cytosol to the mitochondrial matrix, where it plays a key role in defense against respiration-induced reactive oxygen species and in the detoxification of lipid hydroperoxides and electrophiles. Moreover, as mitochondria play a central strategic role in the activation and mode of cell death, mitochondrial GSH has been shown to critically regulate the level of sensitization to secondary hits that induce mitochondrial membrane permeabilization and release of proteins confined in the intermembrane space that once in the cytosol engage the molecular machinery of cell death. In this review, we summarize recent data on the regulation of mitochondrial GSH and its role in cell death and prevalent human diseases, such as cancer, fatty liver disease, and Alzheimer's disease.

Keywords: glutathione, mitochondria, cholesterol, reactive oxygen species, steatohepatitis, Alzheimer disease

INTRODUCTION

Glutathione (GSH), the major intracellular thiol compound, is a ubiquitous tripeptide produced by most mammalian cells and it is the main mechanism of antioxidant defense against reactive oxygen species (ROS) and electrophiles. GSH (γ-glutamyl-cysteinyl-glycine) is synthesized *de novo* in two sequential enzymatic ATP-dependent reactions. In the first step, cysteine and glutamate are linked in a reaction catalyzed by the γ-glutamylcysteine synthase (γ-GCS) to form γ-glutamylcysteine. This first reaction is the rate-limiting step in the synthesis of GSH and is regulated by cysteine availability. The completion of GSH synthesis is catalyzed by glutathione synthetase (GS), in a reaction in which γ-glutamyl-cysteine is covalently linked to glycine (**Figure 1**). The antioxidant function of GSH is determined by the redox-active thiol (-SH) of cysteine that becomes oxidized when GSH reduces target molecules (Pompella et al., 2003). Upon reaction with ROS or electrophiles, GSH becomes oxidized to GSSG, which can be reduced to GSH by the GSSG reductase (GR). Thus, the GSH/GSSG ratio reflects the oxidative state and can interact with redox couples to maintain appropriate redox balance in the cell.

The synthesis of GSH from its constituent amino acids occurs exclusively in cytosol, where γ-GCS and GS reside. However, GSH is found in intracellular organelles including endoplasmic reticulum (ER), nucleus, and mitochondria to control compartment-specific needs and functions (Mari et al., 2009, 2010). Except for the ER, intracellular GSH is mainly found in its reduced form. While the percentage of the total cell GSH content found in mitochondria is minor (10–15%), the mitochondrial glutathione (mGSH) concentration is similar to that found in the cytosol. As GSH has a net negative charge at physiological pH, the high concentration of mGSH implies the existence of specific transport systems that work against an electrochemical gradient (Griffith and Meister, 1985; Garcia-Ruiz et al., 1994; Mari et al., 2009, 2010). As discussed below, despite being a small fraction of total intracellular GSH, mGSH plays a critical function in the maintenance of mitochondrial function and cell survival (Lash, 2006; Mari et al., 2013).

Mitochondria in mammalian cells generate most of the cellular energy by means of the oxidative phosphorylation (OXPHOS) that is essential for myriad cellular functions. OXPHOS provides an efficient mechanism to couple electron transport to synthesize ATP from ADP. Mitochondria are also involved in key cellular functions such as Ca^{2+} homeostasis, heme biosynthesis, nutrient metabolism (Cheng and Ristow, 2013), steroid hormone biosynthesis, removal of ammonia, integration of metabolic and signaling pathways for cell death and autophagy (Hammerman et al., 2004; Renault and Chipuk, 2013). Emerging evidence indicates a central role of mitochondria in initiating

Abbreviations: AD, Alzheimer disease; APP, amyloid precursor protein; ASH, alcoholic steatohepatitis; ETC, electron transport chain; Gpx, glutathione peroxidase; Grx, glutaredoxin; GSH, glutathione; GSSG, oxidized glutathione; GST, glutathione-*S*-transferase; IMM, inner mitochondrial membrane; mGSH, mitochondrial glutathione; MPT, mitochondrial permeability transition; NASH, non-alcoholic steatohepatitis; NO, nitric oxide; NPC, Niemann–Pick type C disease; OMM, outer mitochondrial membrane; OXPHOS, oxidative phosphorylation; Prx, peroxiredoxin; ROS, reactive oxygen species; SOD, superoxide dismutase; StARD1, steroidogenic acute regulatory domain 1; Trx, thioredoxin.

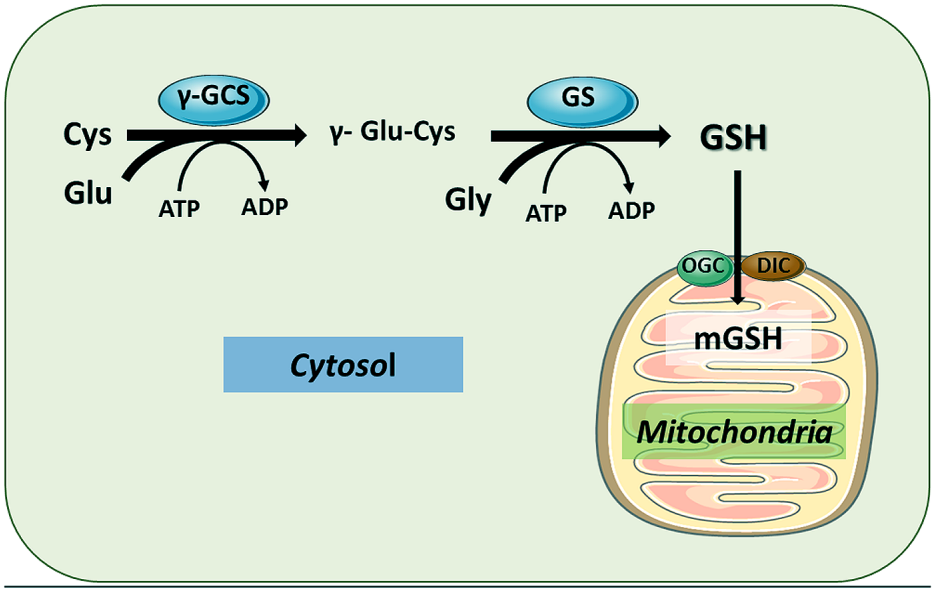

FIGURE 1 | Glutathione synthesis in cytosol and compartimentalization in mitochondria. GSH is synthesized from its constituent amino acids in the cytosol by the sequential action of γ-glutamylcysteine synthase (γ-GCS) and GSH synthase (GS). The functions of GSH are determined largely by the –SH of cysteine as by its role as a cofactor for antioxidant enzymes. Once synthesized in the cytosol, GSH can be transported to mitochondrial matrix by different carriers, particularly the 2-oxoglutarate carrier (OGC) and the dicarboxylate carrier (DIC), located in the mitochondrial inner membrane. The function the OGC has been shown to be dependent on changes in mitochondrial membrane dynamics.

signals in response to metabolic and genetic stress which affects nuclear gene expression, causing changes in cell function (Raimundo, 2014). Mitochondria contain multiple copies of their own genome, mitochondrial DNA (mtDNA), which encodes for 13 polypeptides of the OXPHOS and respiratory chain, as well as two ribosomal RNAs and 22 transfer RNAs necessary for translation of polypeptides inside mitochondria. As a consequence, the main mitochondrial proteome (∼1500 proteins) is encoded by the nucleus, translated in the cytosol and imported into the mitochondria through specific translocator complexes (TIM and TOM) of the inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM), respectively.

Oxidative phosphorylation is organized in a series of subsequent steps involving several redox centers distributed in five protein complexes embedded in the IMM (Sun et al., 2013; Venditti et al., 2013). Complex I obtain the electrons from NADH (NADH-coenzyme Q oxidoreductase) and complex II (succinate-coQ oxidoreductase) from succinate. Both these two complexes, independently of each other, use the lipid soluble carrier located into the IMM, ubiquinone (coenzyme Q) to form ubiquinol. From ubiquinol, the electrons pass down the redox gradient through complex III (coenzyme Q-cytochrome *c* oxidoreductase) to cytochrome *c*, then to complex IV (cytochrome *c* oxidase) and to the final acceptor, oxygen (O_2) to produce water. The fall in electron potential energy through this electron transport chain (ETC) is used to pump protons out the mitochondrial matrix to the intermembrane space (IMS). This proton pumping creates a proton-motive force consisting of electrical and proton gradients. This force is used by the fifth protein complex (Complex V, ATP synthase) to regenerate ATP from ADP. The proton-motive force created by the ETC is also used for many additional mitochondrial processes, especially those related with transport across the IMM (Kulawiak et al., 2013).

Although the primary function of mitochondria is to generate ATP as an energy molecule required for countless cell functions, a small fraction of electrons from the ETC are transferred directly to O_2, resulting in the generation of the superoxide anion, which can give rise to other ROS as well as reactive nitrogen species (RNS). Mitochondria are the primary intracellular site of oxygen consumption and the major source of ROS, most of them originating from the ETC. In accordance with this, it has been estimated that the steady-state concentration of superoxide in the mitochondrial matrix is 5- to 10- fold higher than in the cytosol (Cadenas and Davies, 2000). Associated with this constant flow of ROS generation, mitochondria are also a target for the damaging effects of oxygen radicals (Fernandez-Checa and Kaplowitz, 2005; Kaelin, 2005; Orrenius et al., 2007).

Although ROS generated under physiological conditions are not harmful, and likely play a signaling role, toxic or pathological conditions that lead to an impairment of mitochondrial function can increase the release of ROS. Mitochondrial ROS are increased under hypoxia, ischemia/reperfusion injury, chemical stress, drug treatment, and under many pathophysiological conditions (Srinivasan and Avadhani, 2012). Despite that mitochondria are exposed to the generation of oxidant species, the existence of an efficient antioxidant defense system, of which mGSH is a critical component, prevents or repairs oxidative damage generated during normal aerobic metabolism (Mari et al., 2013). In the following sections, we summarize some of the most important aspects of mGSH physiology, its role in mitochondrial function and release

of mitochondrial apoptotic factors and the impact of its depletion in disease.

MITOCHONDRIAL ROS GENERATION AND DEFENSE

Reactive oxygen species can be generated in several intracellular sites, including cytosol, peroxisomes, plasma membrane, and ER. However, mitochondrial ETC is the main cellular process of ROS generation in most cell types in physiological circumstances (Venditti et al., 2013). Although normal electron transport in mitochondria involves four-electron reduction of O_2 to water, partial reduction reactions occur under physiological conditions, causing release of superoxide anion and hydrogen peroxide (H_2O_2). Although ROS can be generated at several sites of the ETC (**Figure 2**; Brand, 2010; Quinlan et al., 2013), complex I and complex III (Venditti et al., 2013) have been shown to be the most important sources of mitochondrial superoxide generation, although significant production of ROS in complex II has recently also been reported (Quinlan et al., 2012).

The primary ROS produced by the ETC is superoxide, a free radical with moderate reactivity, whose generation can lead to more reactive or secondary ROS derivatives. Indeed, superoxide can undergo dismutation to H_2O_2, a mild oxidant that can be converted to the highly reactive hydroxyl radical in the presence of transition metals (Fe^{2+}, Cu^{+}) by means of the Fenton reaction. H_2O_2 has a longer half-life and can cross membranes (Cadenas and Davies, 2000), consequently it has been identified as a suitable second messenger molecule, in part because of its reactions with specific oxidation-prone protein cysteinyl residues (Sies, 2014), which confers properties to H_2O_2 as a mitochondrial signal (Raimundo, 2014).

Reactive oxygen species can attack biomembranes, enzymes, proteins, and nucleic acids (Venditti et al., 2013). These oxidative effects can be neutralized by antioxidant systems, engaging in a delicate balance that determines the fate and impact of ROS in cells. Although oxidative stress was defined originally as a balance between oxidants and antioxidants systems, an equilibrium among antioxidant strategies is needed to avoid the generation of oxidants and ROS (Mari et al., 2010). For instance, if the activity of superoxide scavenging by SOD2 exceeds the capacity to remove the H2O2 generated, this oxidant can cause oxidative damage or be converted to other ROS.

SUPEROXIDE, HYDROGEN PEROXIDE, AND PEROXYNITRITE GENERATION

Despite the fact that superoxide can be generated in extramitochondrial reactions, in most cell types mitochondria appear as the main source of superoxide generation. From the several sites that can generate superoxide in the mitochondrial matrix, only the superoxide produced at complex III appears to be released

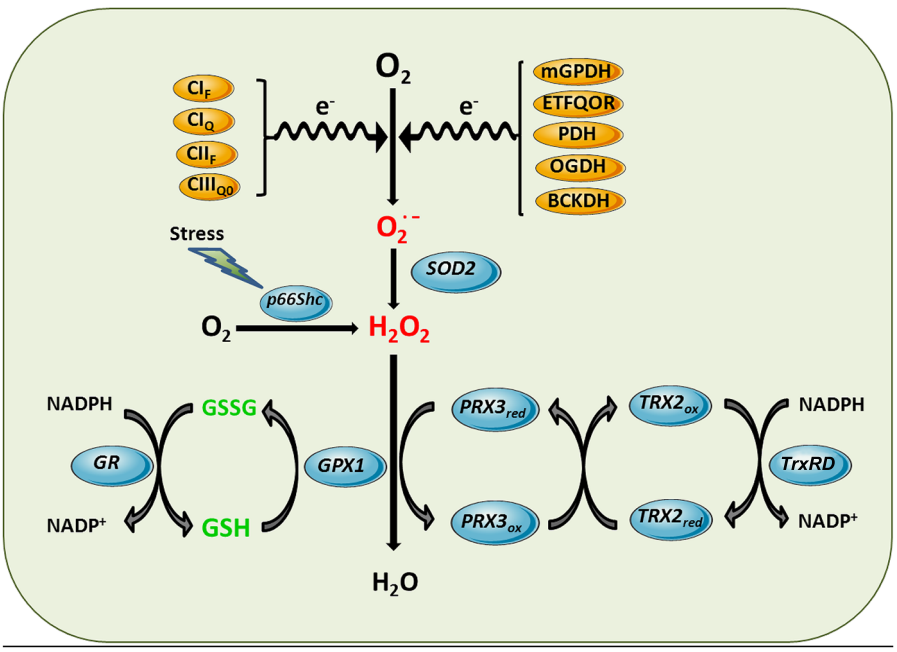

FIGURE 2 | Mitochondrial ROS generation and antioxidant defense systems. Complex I flavin site (CI_F), Complex I ubiquinone site (CI_Q), Complex II flavin site (CII_F), and Complex III_{Qo} ($CIII_{Q0}$) are sites of the ETC components shown to generate superoxide anion. Other sources of superoxide can be enzymatic reactions that transfer electrons to the ETC such as mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH), and the last step of β-oxidation, electron-trasferring flavoprotein ubiquinone oxidoreductase (ETFQOR) or dehydrogenases such as pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (OGDH) and branched-chain 2-oxoacid dehydrogenase (BCKDH). Superoxide generated in the mitochondrial matrix by these sites is dismutated to hydrogen peroxide by SOD2. Moreover, in response to stress p66Shc translocates to mitochondria to directly stimulate hydrogen peroxide generation by transferring electrons to cytochrome *c*. Hydrogen peroxide is further inactivated using the reducing equivalents of NADPH by mGSH/Gpx or Prx3/Trx2 antioxidant systems, yielding water. Mn-dependent superoxide dismutase 2 (SOD2), GSH peroxidase (GPX1), GSSG-reductase (GR), peroxiredoxin 3 (PRX3), thioredoxin-2 (TRX2), thioredoxin reductase (TrxRD).

both into the matrix and the IMS (Quinlan et al., 2013). This spatial difference (matrix vs. IMS) may determine whether mitochondrial superoxide reaches the cytosol or not. The anionic nature of superoxide and the fact that it is mostly produced in the mitochondrial matrix determine that the bulk of antioxidant defenses to neutralize superoxide and other ROS reside in the matrix. The first line of defense against superoxide is the presence of a specific member of the family of metalloenzymes called superoxide dismutases (SODs), MnSOD or SOD2, specifically located in the mitochondrial matrix, which catalyzes the dismutation of superoxide anion into H_2O_2 as shown in **Figure 2**. The dismutation of superoxide can also occur spontaneously, but such reaction is 10^4 times slower than the enzymatic dismutation by SOD2. The relevance of this enzyme is illustrated by the fact that global SOD2 deficiency leads to neonatal death in mice (Huang et al., 1997). Superoxide released into the IMS can be eliminated by a different SOD isoenzyme (Cu, Zn-SOD, or SOD1), which is found in the cytoplasm of eukaryotic cells, or scavenged by cytochrome *c* plus cytochrome *c* oxidase system (Okado-Matsumoto and Fridovich, 2001). It has been proposed that α-tocopherol can also scavenge superoxide, as suggested by experiments with submitochondrial particles isolated from mice fed with vitamin-E supplemented diet (Chow et al., 1999).

Although the dismutation of superoxide by SOD2 is a predominant source of H_2O_2, there are other reactions that directly generate H_2O_2 in mitochondria. For example, the redox activitiy of p66Shc within mitochondria has been shown to generate H_2O_2 in the absence of superoxide through oxidation of cytochrome *c* (Giorgio et al., 2005). P66Shc normally resides in the cytosol where it is involved in signaling from tyrosine kinases to Ras. However, in response to stress p66Shc translocates to mitochondria to contribute to the generation of H_2O_2. Due to the lack of unpaired electrons, H_2O_2 is not a free radical but a potent oxidant that can oxidize mitochondrial components (proteins, lipids, DNA). Besides being a potential source of more reactive free radicals via Fenton reaction, physiological generation of H_2O_2 fulfills a second messenger role and can be transported across membranes by aquaporins, a family of proteins that act as peroxiporins (Sies, 2014). The detoxification against H_2O_2 in mitochondria occurs mainly through the GSH redox system, including the glutathione peroxidases (Gpxs) and GSH reductases, as well as the presence of peroxiredoxins (Prxs; **Figure 2**) using the reducing equivalents of NADPH. Besides these antioxidant defenses that ensure H_2O_2 elimination, aquaporins have been shown to modulate mitochondrial ROS generation. In this paradigm, aquaporin 8 silencing, which is specifically expressed in IMM, enhances mitochondrial ROS generation and results in mitochondrial depolarization and cell death (Marchissio et al., 2012). In addition to these conventional sites of mitochondrial ROS generation, it has been recently reported that the branched-chain 2-oxoacid dehydrogenase (BCKDH) complex in mitochondria can produce superoxide and H_2O_2 at higher rates than complex I from mitochondria (Quinlan et al., 2014).

Peroxynitrite is a potent oxidant that is generated upon the reaction of superoxide with nitric oxide (NO). Its impact on inactivation of mitochondrial proteins depends on the level of generation in mitochondria. While ETC is the source of superoxide, the existence of mitochondrial NO synthase (mtNOS) that provides the NO required to form peroxynitrite is controversial. Although the existence of mtNOS has been described in mitochondrial fractions from different organs, recent evidence in rat liver mitochondria has questioned the existence of mtNOS, minimizing the contribution of *in situ* NO generation within mitochondrial to the formation of peroxynitrite (Venkatakrishnan et al., 2009). However, since NO is freely diffusible across membranes, it is possible that the mitochondrial production of peroxynitrite may derive from extramitochondrial NO diffusing into mitochondria to react with superoxide generated by ETC.

GLUTATHIONE REDOX CYCLE

Hydrogen peroxide is rapidly reduced to water mostly by Gpx, which utilizes the reducing equivalents from its substrate GSH. In this enzymatic reaction, GSH becomes oxidized to GSSG, which is recycled back to GSH by the NADPH-dependent GSSG reductase as shown in **Figure 2**. Since GSSG is not readily exported out of mitochondria (Olafsdottir and Reed, 1988; Yin et al., 2012), the activity of GR is an important mechanism to control the level of GSSG in mitochondria. The uncontrolled generation of GSSG during oxidative stress can contribute to mitochondrial dysfunction by glutathionylation of target proteins, as described below. The supply of NADPH is essential to regenerate GSH and dictates the rate of H_2O_2 reduction by Gpx while keeping the reduced status of mitochondria.

So far, eight isoforms of Gpx have been identified in humans, which vary in cellular location and substrate specificity (Brigelius-Flohe and Maiorino, 2013). Gpx1 is the major isoform localized in various cellular compartments, including the mitochondrial matrix and IMS (Legault et al., 2000; Mari et al., 2009), which in the liver account for about one third of the total Gpx activity (Chance et al., 1979). This selenium-containing homotetramer protein has substrate specificity for H_2O_2 and has been classically believed to be the major H_2O_2 reducing enzyme. It also has been described that γ-glutamylcysteine, the intermediate of GSH biosynthesis, is able to act as a Gpx1 cofactor in mitochondrial H_2O_2 detoxification, mimicking the physiological properties of GSH (Quintana-Cabrera et al., 2012). Surprisingly, mice with specific genetic deletion of Gpx1 appear phenotypically normal and with normal life span (Ho et al., 1997), suggesting that there are alternative compensatory mechanisms for H_2O_2 scavenging in Gpx1 deficiency. However, another report demonstrated mitochondrial stress and bioenergetics defects in GPx1 null mice (Esposito et al., 2000). Besides Gpx1, Gpx4 displays preference for lipid hydroperoxides (**Figure 3**), and hence plays a key role in protecting phospholipids, cholesteryl esters and cardiolipin and defense against apoptosis and maintenance of ETC and OXPHOS (Cole-Ezea et al., 2012). In line with this vital role in mitochondrial defense, Gpx4 null mice die during early embryonic development, while $Gpx4^{+/-}$ cells are sensitive to oxidative stress triggers (Legault et al., 2000; Muller et al., 2007). Moreover, Gpx4 has been shown recently to modulate ferroptotic

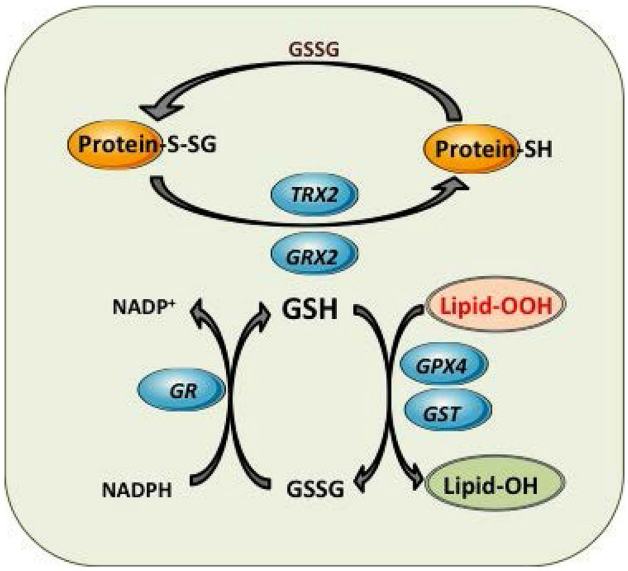

FIGURE 3 | Mitochondrial GSH redox cycle and interaction with other antioxidant defenses. Detoxification of harmful lipid peroxides (Lipid-OOH) to their corresponding hydroxides (lipid-OH) by glutathione peroxidase 4 (GPX4) and glutathione-*S*-transferases (GST). Control of mitochondrial protein glutathionylation (Prot-S-SG, Prot -SH) by glutaredoxin (GRX2) and thioredoxin 2 (TRX2).

cancer cell death, a specific form of cell death characterized by the production of iron-dependent ROS generation (Yang et al., 2014). This process involved metabolic dysfunction that results in increased production of cytosolic and lipid ROS, independently of mitochondria.

PEROXIREDOXIN–THIOREDOXIN REDOX CYCLE

Peroxiredoxins constitute a family of thiol-specific peroxidases that rely on thioredoxins (Trxs) as the hydrogen donor for the reduction of H_2O_2 and lipid hydroperoxides (Chae et al., 1999). Prx3 is the Prx isoform exclusively located in mitochondria, suggesting that it plays a primary line of defense against H_2O_2 produced by the mitochondrial respiratory chain. Prx3 homodimer has a redox-sensitive cysteine that upon reaction with H_2O_2 is oxidized to Cys-SOH, which then reacts with neighboring Cys-SH of the other subunit to form an intermolecular disulfide that can be readily reduced by thioredoxin reductase 2 (TrxRD2; Chang et al., 2004; Orrenius et al., 2007). The fact that the oxidation state of the active site cysteine of Prx can be transferred to other proteins allows Prx to function as a sensor of H_2O_2 (Rhee et al., 2012). Likewise, Prx5, the last identified member of the six mammalian Prxs (Knoops et al., 2011), is widely expressed in tissues but it is not exclusively located in mitochondria. In human cells, it has been shown that Prx5 can be targeted to mitochondria, peroxisomes, cytosol, and nucleus. The targeting of Prx5 to mitochondria is highly conserved among species (Van der Eecken et al., 2011), and it has been associated with the protection of mtDNA from oxidative attacks (Banmeyer et al., 2005). Prx5 is a peroxidase that can use cytosolic or mitochondrial Trx to reduce alkyl hydroperoxides or peroxynitrite with high rate constants, whereas its reaction with H_2O_2 is modest (Knoops et al., 2011). Therefore, as opposed to Prx3, Prx5 has been viewed mainly as a cytoprotective antioxidant enzyme rather than as a redox sensor and appears to be a unique Prx exhibiting specific functional and structural feature (Knoops et al., 2011; Zhu et al., 2012).

As noted above, Trxs are responsible for reducing Prx back to their reduced, oxidant-scavenging state, while thioredoxin reductases (TrxRDs) keep the reduced state of Trxs using NAPDH reducing equivalents, as depicted in **Figure 2**. Either Trx and TrxRD are expressed as isoforms for both predominantly cytosolic (Trx1 and TrxRD1) or mitochondrial (Trx2 and TrxRD2) localization (Enoksson et al., 2005). There are direct links between the Trx system and protein glutathionylation (Casagrande et al., 2002); and the direct reduction of mitochondrial glutaredoxin 2 (Grx2) by TrxR is also of important physiological relevance (Johansson et al., 2004; Enoksson et al., 2005). However, the reduction of the intermolecular disulfide of Prx is specific to Trx and cannot be achieved by GSH or Grx (Chang et al., 2004; Orrenius et al., 2007).

Due to their high rate constant and high abundance, Prx are thought to be responsible for scavenging nanomolar concentrations of H_2O_2 associated with redox signaling, while Gpx are likely important at higher intracellular concentrations, buffering high ROS levels to avoid cell damage and stress signaling response (Sena and Chandel, 2012). In addition there is emerging evidence indicating that both antioxidant systems (mGSH and Prx3) are mutually regulated. For instance, depletion of mGSH results in Trx2 oxidation (Zhang et al., 2007), while hypercholesterolemic pigs with selective depletion of mGSH in heart mitochondria, exhibit decreased levels of the mitochondria-specific antioxidant enzymes such as SOD2, Trx2, and Prx3 (McCommis et al., 2011). Collectively, these data highlight a key role of mGSH in maintaining a healthy antioxidant system in both systems and on H_2O_2 homeostasis.

DEFENSE AGAINST ELECTROPHILES AND PROTEIN GLUTATHIONYLATION

In addition to the defense against oxidants and ROS, GSH plays also an important role in the protection against electrophiles by glutathione-*S*-transferases (GSTs). Electrophiles can be generated as a consequence of metabolic processes involving both endogenous compounds and xenobiotics. GSTs exhibit a wide intracellular distribution, being localized in mitochondria (GSTA1), cytosol (alpha, mu, pi, and zeta) and membrane-bound (MGST1) isoforms (Aniya and Imaizumi, 2011; Li et al., 2011). Mitochondrial GSTs display both GSH transferase and peroxidase activities that detoxify harmful byproducts through GSH conjugation or GSH-mediated peroxide reduction (**Figure 3**; Hayes et al., 2005; Aniya and Imaizumi, 2011). Among human mitochondrial GSTs, the isoforms hGSTA4-4, hGSTA1, hGSTA2, and hGSTP1 showed peroxidase activity, with hGSTA4-4 exhibiting the highest activity (Gardner and Gallagher, 2001; Gallagher et al., 2006). Moreover, recent studies have shown that GSTA4 expression is selectively downregulated in adipose tissue of obese insulin-resistant C57BL/6J mice and in human obesity-linked insulin resistance (Curtis et al., 2010). Mitochondrial function

in adipocytes of lean or obese GSTA4-null mice was significantly compromised compared with wild-type controls and was accompanied by an increase in superoxide anion production.

Glutathionylation, a key mechanism of post-translational modification of proteins, involves the formation of a disulfide bridge between GSH and an available protein cysteine thiol. Non-enzymatic glutathionylation occur mostly during oxidative stress when GSH/GSSG is ∼1 and levels of ROS are high. This process is non-specific and can lead to the hyper-glutathionylation of proteins, altering their activity. Enzymatic glutathionylation reactions are tightly controlled and highly specific and are considered a major post-translational modification that occurs in response to fluctuations in local redox environments. The Grx family of proteins plays a key role in the regulation of glutathionylation reactions (**Figure 3**; Lillig et al., 2008). Although Grx are mainly responsible for deglutathionylation reactions, recent evidence has indicated a role for Grx in protein glutathionylation, mediated by the stabilization of a GSH thiyl radical which is then subsequently transferred to an available protein thiol (Starke et al., 2003). In most cases, Grx catalyze the deglutathionylation of proteins GSH disulfide mixtures (PSSGs). Grx exhibit site-specific distribution with Grx1 being specifically located in cytosol while Grx2 localizes in mitochondria. Both Grx catalyze the deglutathionylation of protein targets in two steps; first, the N-terminal cysteine on Grx deglutathionylates PSSG via a thiol disulfide exchange reaction yielding PSH and a Grx–SSG intermediate; second, Grx–SSG binds GSH and the glutathionyl moiety is removed regenerating Grx and producing GSSG. Grx2 has close to 34% homology to Grx1 and was recently identified as the enzyme required for deglutathionylation reactions in mitochondria (Gladyshev et al., 2001; Lundberg et al., 2001; Gallogly et al., 2009; Stroher and Millar, 2012). The catalytic cycle of Grx2 is also quite similar to Grx1 except that the Grx2–SSG intermediate can be reduced by NADPH and TrxRD. It is also important to point out that unlike Grx1, Grx2 complexes iron (Fe), which is required to modulate its activity (Johansson et al., 2004; Lillig et al., 2005). Interestingly, Grx2 has been shown catalyze both the deglutathionylation and glutathionylation of target proteins in mitochondria. The reversible nature of Grx2 is associated with its sensitivity to changes in GSH/GSSG; a high GSH/GSSG promotes protein deglutathionylation and a low GSH/GSSG activates Grx2 glutathionylase activity (Beer et al., 2004; Hurd et al., 2008). The main target for Grx2 in mitochondria is Complex I, although UCP3 and the 2-oxoglutarate dehydrogenase (OGDH) have been also shown to be deglutathionylated by Grx2. The role of Grx2 in maintaining mitochondrial function has been recently shown in heart from Grx2 null mice. Grx2 deletion decreased ATP production by complex I-linked substrates (Mailloux et al., 2014). $Grx2^{-/-}$ hearts also developed left ventricular hypertrophy and fibrosis and mice developed hypertension.

MITOCHONDRIA AND CELL DEATH

Besides their fundamental role in energy generation, mitochondria also play a strategic role in the regulation of cell death, including apoptosis (caspase-dependent and independent) and necrosis. Apoptosis describes a programmed mode of cell death that is characterized by a series of biochemical events that ultimately lead to cell fragmentation into compact membrane-enclosed structures, called "apoptotic bodies" that are taken up by neighboring cells and phagocytes, preventing inflammation, and tissue damage (Taylor et al., 2008). Apoptosis is induced via two main routes involving either the mitochondria (the intrinsic pathway) or the activation of death receptors (the extrinsic pathway). Both pathways are linked in some cell types by the cleavage of BID, a proapoptotic member of the Bcl-2 family of proteins, generating tBID in a process catalyzed by caspase-8 activated by the extrinsic pathways (Scaffidi et al., 1998). The intrinsic pathway of apoptosis is activated by stimuli that lead to the permeabilization of the OMM and the subsequent release of proteins from the mitochondrial IMS, such as cytochrome *c* (Martinou and Green, 2001; Kroemer et al., 2007). Cytochrome *c* normally resides within the cristae of the IMM and is sequestered by narrow cristae junctions. As mentioned above, within the IMM, cytochrome *c* participates in the mitochondrial ETC, using its heme group as a redox intermediate to shuttle electrons between complex III and complex IV. However, when the cell detects an apoptotic stimulus, such as DNA damage or metabolic stress, the intrinsic apoptotic pathway is triggered and mitochondrial cytochrome *c* is released into the cytosol (Kroemer et al., 2007). This process is thought to occur in two phases, first the mobilization of cytochrome *c* and then its translocation through permeabilized OMM. In addition to cytochrome *c*, other IMS proteins are also mobilized and released into the cytosol where they promote or counteract caspase activation and hence cell death (Li et al., 2001; Munoz-Pinedo et al., 2006). For instance, the release of Smac/Diablo into the cytosol ensures the efficiency of caspase 3 in proteolyzing target proteins through inhibition of inhibitor of apoptosis proteins (IAPs). Moreover, other specialized mitochondria-residing proteins, such as the apoptosis inducing factor (AIF) and endonuclease G, are translocated to the nuclei following their release from mitochondria and promote peripheral chromatin condensation and high molecular weight DNA fragmentation. While the above evidence indicates that the mitochondrial apoptotic pathway promotes cell death, recent provocative evidence has shown that the intrinsic apoptosis pathway mediates the pro-longevity response to mitochondrial ROS in *Caenorhabditis elegans* by triggering a unique pattern of gene expression that modulates stress sensitivity and promotes survival (Yee et al., 2014). Whether this newly described pathway has implication in mammals needs further verification.

MITOCHONDRIAL MEMBRANE PERMEABILIZATION AND RELEASE OF PROAPOPTOTIC PROTEINS

While mitochondrial proteins are normally secured in the IMS, understanding the mechanism of release may be of relevance to control cell death. The rupture of the physical barrier (OMM) that limits their release into the cytosol constitutes a point-of-no-return in cell death (Martinou and Green, 2001; Kroemer et al., 2007). Current evidence supports the existence of two compatible mechanisms leading to the breakage of OMM: the mitochondrial permeability transition (MPT), and the permeabilization of OMM without disruption of the inner membrane. The former

is a process characterized by mitochondrial swelling, IMM permeabilization and OMM rupture as a secondary event. On the other hand, there is evidence indicating the selective permeabilization of OMM in the absence of disrupted inner membrane. The relative prevalence of these pathways in the regulation of cell death is not definitively established. One important feature of mitochondrial permeabilization is the loss of function resulting in the inability of mitochondria to synthesize ATP through the OXPHOS. However, while the final outcome of mitochondrial dysfunction is cell death, the phenotype of death (apoptosis and/or necrosis) depends on the level of cellular ATP, as ATP is required for the efficient assembly of the apoptosome. Alternatively to MPT in the control of OMM permeabilization, Bcl-2 family members are also known to play a major function. Bcl2-family death agonists induce OMM permeabilization, thereby promoting cytochrome *c* release, whereas Bcl2-family death antagonists prevent it. Thus, Bcl2-family proteins control mitochondrial integrity, regulate cytochrome *c* release and intrinsic apoptosis (Youle and Strasser, 2008). Under non-apoptotic conditions, Bax is inactive and present in the cytosol as a monomer. Following an apoptotic stimulus, Bax is activated and translocates to the mitochondria, where it undergoes a conformational change and inserts into the OMM. Bax oligomerization is associated with the formation of openings in the OMM to allow the release of cytochrome *c* and other IMS proteins into the cytosol, and hence Bax oligomerization is considered a critical regulatory point in cell death (Youle and Strasser, 2008). Further understanding the mechanisms underlying OMM permeabilization may provide novel strategies to regulate cytochrome *c* and control apoptosis.

REGULATION OF CELL DEATH BY mGSH

As opposed to apoptosis, necrosis is a morphologically distinct form of cell death responsible for irreversible tissue destruction due to bioenergetic failure and oxidative damage. The fundamental difference relative to apoptosis is the rapid loss of cellular membrane potentials due to energy depletion and ion pump/channel failures, leading to swelling, rupture, and cytolysis. MPT is a regulated non-selective water and solute-passing protein complex whose molecular characterization remains elusive. Available evidence suggests a role for voltage-dependent anion channel (VDAC), located in the OMM, and adenine nucleotide translocase (ANT) across the IMM (Kroemer et al., 2007; Baines, 2010) and the translocator protein TSPO [previously called peripheral benzodiazepine receptor (PBR)] as components of MPT. However, liver mitochondria from mice lacking ANT1 and ANT2 can still undergo Ca^{2+}-induced swelling and MPT, although at a higher threshold, which has been interpreted as evidence against a role for ANT in MPT (Kokoszka et al., 2004). However, recent evidence has demonstrated that TSPO is dispensable for MPT (Sileikyte et al., 2014). In particular, heart mitochondria from mice with selective TSPO deletion in hearts undergo MPT and are as sensitive to ischemia–reperfusion injury as hearts from control mice. In contrast, the prolyl isomerase cyclophilin D in the mitochondrial matrix is an essential regulator of MPT and the only genetically proven indispensable MPT component (Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). Upon oxidative stress, sudden MPT causes massive ion influx that dissipates mitochondrial membrane potential and shuts down OXPHOS, ATP production and ROS overgeneration. Concomitantly, water influx causes matrix swelling, rupture of the rigid OMM and release of apoptogenic proteins sequestered in IMS although, apoptotic cell death under MPT is inhibited due to energetic failure and ATP exhaustion and oxidative stress-mediated caspase inactivation.

A critical step in mitochondrial apoptosis is the mobilization of cytochrome *c* from IMS. It has been proposed that during mobilization cytochrome *c* detaches from the IMM and dissociates from the membrane phospholipid cardiolipin. A significant proportion of the cytochrome *c* in the mitochondria seems to be associated with cardiolipin, involving two major mechanisms. At physiological pH, cytochrome *c* has a net positive charge (+8), establishing an electrostatic bond with the anionic cardiolipin (Gonzalvez and Gottlieb, 2007). In addition, cytochrome *c* has a hydrophobic channel through which one of the four-acyl chains of cardiolipin inserts. The other chains of cardiolipin remain in the membrane, thereby anchoring cytochrome *c* to the IMM. One mechanism that contributes to cytochrome *c* detachment from IMM involves cardiolipin oxidation because oxidized cardiolipin has a much lower affinity for cytochrome *c* than the reduced form. Cardiolipin can be oxidized by ROS or by the cardiolipin–cytochrome *c* complex (Kagan et al., 2005). Detachment of cytochrome *c* from cardiolipin might also be triggered by increased cytosolic calcium, which weakens the electrostatic interaction between cytochrome *c* and cardiolipin and further generates ROS via MPT.

In addition, it has been described that oxidized cardiolipin modulates the biophysical properties of OMM to allow oligomerized Bax to insert and permeabilize the OMM (Mari et al., 2008; Montero et al., 2010; Landeta et al., 2011). Since mitochondrial ROS contribute to cardiolipin oxidation and are controlled by antioxidants (Mari et al., 2008, 2009), mGSH arises as an important modulator of apoptotic cell death by indirectly controlling the redox state of cardiolipin (Mari et al., 2008; Montero et al., 2010). mGSH not only regulates cell death susceptibility but the outcome of cell death (necrosis or apoptosis). Thiol redox status regulate MPT and enhanced ROS generation can target critical cysteine residues in cyclophilin D, implying that mGSH depletion would favor MPT via redox pathways targeting MPT components. In addition, through modulation of cardiolipin redox state, mGSH can also regulate OMM permeabilization via MPT and the release of apoptogenic proteins.

GLUTATHIONE IMPORT TO MITOCHONDRIA

As indicated above, despite the fact that the concentration of mGSH is high, GSH is not synthesized *de novo* in the mitochondrial matrix, as this organelle lacks the enzymes required for GSH synthesis. Furthermore, GSH has an overall negative charge at physiological pH and mitochondria exhibit a large negative membrane potential. Moreover, although GSH can cross OMM, its transport into mitochondrial matrix cannot be explained by simple diffusion. Therefore, mGSH arises from the cytosol GSH by the activity of specific carriers (**Figure 1**; Griffith and Meister, 1985).

Accordingly, recent findings using dynamic oxidant recovery assays and GSH-specific fluorescent reporters, established that free communication of GSH pools exists between cytosol and IMS. In contrast, no appreciable communication was observed between the GSH of the IMS and matrix (Kojer et al., 2012). Based on substrate specificity, potential candidates to transport GSH into mitochondria have been identified, including the 2-oxoglutarate carrier (OGC; SLC25A11) and the dicarboxylate carrier (DIC; SLC25A10; Chen and Lash, 1998; Chen et al., 2000; Coll et al., 2003; Wilkins et al., 2012), mainly in kidney and liver, and tricarboxylate carrier (TTC, SLC25A1) in brain mitochondria and astrocytes (Wadey et al., 2009). OGC imports cytosolic GSH into mitochondria in exchange for 2-oxoglutarate (2-OG) and other dicarboxylates. Instead, DIC mediates electro-neutral exchange of dicarboxylates or GSH for inorganic phosphate (Mari et al., 2009). The relative contribution of each system is different depending on the cell type, as discussed in the following section.

TISSUE-SPECIFIC FEATURES OF GLUTATHIONE IMPORT TO MITOCHONDRIA

Previous studies in a renal proximal tubular cell line, NRK-52E, indicated that overexpression of OGC and DIC increased mGSH levels and protected against oxidant-mediated cell death (Lash et al., 2002; Xu et al., 2006). Similar findings were recently reported in primary renal proximal tubular cells from uninephrotectomized rats (Benipal and Lash, 2013). The findings with DIC overexpression in kidney cells indicated a role for this carrier in the mitochondrial transport of GSH in exchange with inorganic phosphate. In contrast to kidney, no clear evidence for DIC in the transport of mitochondrial GSH was found in rat liver (Coll et al., 2003). The functional expression in *Xenopus laevis* oocytes microinjected with the DIC cRNA from rat liver did not result in significant GSH transport activity (Coll et al., 2003). Moreover, in contrast to rat kidney mitochondria, the import of GSH in rat liver mitochondria showed both a high affinity and low affinity transport component (Martensson et al., 1990). Likewise, kinetic analyses of 2-oxoglutarate transport in rat liver mitochondria indicated the presence of a single Michaelis–Menten component with kinetic parameters in the range of those reported previously for kidney mitochondria (Chen et al., 2000; Coll et al., 2003). These findings suggest that the OGC accounts for the low-affinity high capacity of GSH transport in liver mitochondria, and imply that the nature of the high affinity GSH transporter remains to be identified. Also, OGC and DIC together accounted for only an apparent 45–50% of the total GSH uptake in liver mitochondria, in contrast to 70–80% described in kidney mitochondria (Zhong et al., 2008).

Interestingly, it has been suggested that Bcl-2 participates as a regulator of mGSH transport by modulating the affinity of OGC for GSH (Wilkins et al., 2012). Bcl-2 and OGC appear to act in a coordinated manner to increase the mGSH pool and to enhance the resistance of neurons to mitochondrial oxidative stress. In line with this outcome, stable motoneuron-like cell lines overexpressing OGC displayed an increased expression of Bcl-2 protein, an effect that was dependent on the mGSH increase. Conversely, a knockdown of Bcl-2 provoked a decrease in mGSH and a concomitant oxidative stress sensitization (Wilkins et al., 2014). Therefore, the antioxidant-like and antiapoptotic function attributed to Bcl-2 could, at least in part, depend on its potential to regulate the mGSH transport and status.

In brain, the properties of GSH transport in isolated rat brain mitochondria seemed to be different from those reported previously for kidney mitochondria, as they were influenced most by inhibitors of the tricarboxylate carrier, citrate, isocitrate, and benzenyl-1,2,3-tricarboxylate (Wadey et al., 2009). Moreover, in mouse brain mitochondria another study showed that OGC and DIC are both expressed in cortical neurons and astrocytes (Kamga et al., 2010). In addition, butylmalonate, an inhibitor of DIC, significantly decreased mGSH, suggesting DIC as the major GSH transporter in mouse cerebral cortical mitochondria (Kamga et al., 2010). It has been shown that pharmacological inhibition or knockdown of a single mGSH transporter significantly sensitized neurons to oxidative and nitrosative stress (Wilkins et al., 2013). Interestingly, a role for UCP2 in the transport of mGSH has been described in neurons, suggesting that the transport of protons back into the matrix by UCP2 may favor the movement of GSH (de Bilbao et al., 2004). These studies suggest that multiple IMM anion transporters might be involved in mGSH transport and that they might differ in different cell populations within the brain. These findings indicate that mGSH levels and its transport are major determinants in brain cell susceptibility to oxidative stress, although little is known about the regulation of the mGSH transport in brain.

MITOCHONDRIAL MEMBRANE PROPERTIES AND IMPACT ON GLUTATHIONE IMPORT

Previous studies in liver mitochondria have revealed that membrane dynamics regulate the transport of mGSH. Membrane physical properties are mainly regulated by fatty acid composition and the cholesterol/phospholipid molar ratio (Coll et al., 2003; Lluis et al., 2003; Ikonen, 2008). Parallel to the findings from rat liver mitochondria, it has been recently reported that mitochondrial cholesterol enrichment, resulting in mGSH depletion, is a major mechanism of anthrax lethal toxin-induced macrophage cell death (Ha et al., 2012). Mitochondria are cholesterol-poor organelles compared to plasma membrane, and this regulated transport of cholesterol in mitochondria plays physiological role in the synthesis of bile acids in liver and steroidogenic hormones in other tissues. (Garcia-Ruiz et al., 2009; Montero et al., 2010). Consistent with the role of cholesterol in the regulation of membrane dynamics, cholesterol loading in mitochondrial membrane results in increased membrane order parameter and in the reduction in the activity of specific membrane carriers, i.e., GSH transport system without effect on other transporters, indicating that the impact of changes in membrane dynamics on carrier function is not universal (Fernandez et al., 2009a; Ha et al., 2012). Moreover, functional expression studies in *X. laevis* oocytes demonstrated that the OGC is sensitive to increased membrane order caused by cholesterol loading (Coll et al., 2003). Thus, cholesterol regulates the transport of mGSH, which in turn, modulates susceptibility to oxidative stress and cell death, therefore emerging as an important target in pathophysiology of diverse diseases such as steatohepatitis (SH) or Alzheimer's disease (AD;

Figure 4; Krahenbuhl et al., 1995; Armstrong and Jones, 2002; Garcia-Ruiz et al., 2002; Fernandez-Checa and Kaplowitz, 2005; Lluis et al., 2005, 2007; Mari et al., 2006, 2008, 2009; Lu and Armstrong, 2007; Fernandez et al., 2009b; Fernandez-Checa et al., 2010).

Based on the above findings, understanding the regulation of mitochondrial cholesterol trafficking may be of potential relevance in cell death regulation and disease progression. Given its lipophilic properties and water insolubility, non-vesicular transport by specific carriers stands as the major mechanism of cholesterol transport between organelles. In particular, mitochondrial cholesterol transport is preferentially regulated by the steroidogenic acute regulatory domain 1 (StARD1), the founding member of a family of lipid transporting proteins that contain StAR-related lipid transfer (START) domains (Miller, 2013). StARD1 is an OMM protein which was first described and best characterized in steroidogenic cells where it plays an essential role in cholesterol transfer to the IMM for metabolism by cholesterol side chain cleavage enzyme (CYP11A1) to generate pregnenolone, the precursor of steroids. Pregnenolone synthesis in mitochondria is limited by the availability of cholesterol in the IMM (Clark, 2012). Despite similar properties with StARD1, other StART members cannot replace StARD1, as germline StARD1 deficiency is lethal due to adrenocortical lipoid hyperplasia (Caron et al., 1997). For instance, targeted mutations in MLN64 (StARD3), another START member with wide tissue distribution, have been shown to cause minor alterations in metabolism and intracellular distribution of cholesterol, questioning its contribution to intramitochondrial cholesterol trafficking (Kishida et al., 2004; Miller, 2007). StARD1 activation and regulation is complex and poorly understood. Its activation is regulated at the transcriptional and post-translational levels, as StARD1 phosphorylation at serine194 has been shown to enhance the trafficking of cholesterol to IMM in murine steroidogenic cells, resulting in increased steroidogenesis (Arakane et al., 1997; Kil et al., 2012). Moreover, the role of ER stress in the regulation of StART family members has been limited to StARD5 with conflicting results reported for StARD4. However, recent data have provided evidence that ER

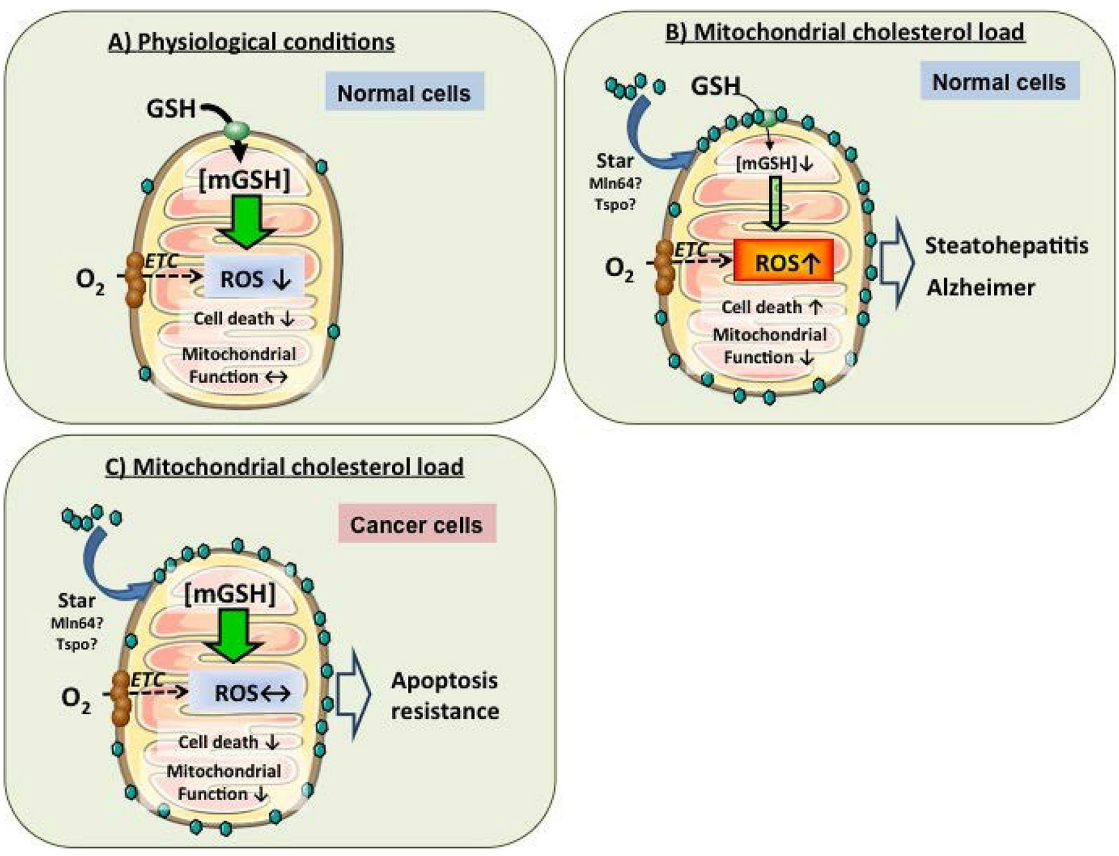

FIGURE 4 | Regulation of mGSH by mitochondrial cholesterol loading in health and disease. (A) In healthy mitochondria, mGSH levels can cope with the ROS generated in physiological conditions, avoiding cell death induction and maintenance of mitochondrial functions. **(B)** In cholesterol-enriched mitochondria, the mGSH transport is impaired resulting in mGSH depletion. mGSH levels below a certain threshold compromise ROS detoxification, leading to oxidative stress and resulting ultimately in higher susceptibility to cell death. **(C)** However, cancer cells exhibit increased cholesterol enrichment of mitochondrial membrane but paradoxically maintain mGSH levels despite changes in membrane physical properties by an as yet uncharacterized mechanism. Increased mitochondrial cholesterol and mGSH protect against mitochondrial membrane permeabilization and cell death. Thus, targeting mitochondrial cholesterol or mGSH may be a novel approach in the treatment of cancer.

stress induces the transcriptional upregulation of StARD1 independently of SREBP regulation (Fernandez et al., 2013). High cholesterol feeding caused the repression of SREBP-2 regulated genes, HMG-CoA reductase, but not that of StARD1. Similar findings have been reported in brain mitochondria in a murine model of AD (Barbero-Camps et al., 2014). Furthermore, the increase in mitochondrial cholesterol in brain mitochondria of AD was not accompanied by a selective increase in mitochondrial-associated membranes (MAMs), corresponding to the contact between ER and mitochondria, suggesting that StARD1-mediated cholesterol trafficking to mitochondria is independent of MAM, a specific membrane domain made of ER and mitochondria bilayers, which is thought to be of relevance in the traffic of lipids. TSPO, a protein particularly abundant in steroidogenic tissues and primarily localized in the OMM, has been suggested to play an important role in steroidogenesis via the transport of cholesterol to the IMM (Papadopoulos and Miller, 2012; Miller, 2013). However, quite interestingly, recent studies using tissue-specific genetic deletion of TSPO demonstrated that TSPO is dispensable for steroidogenesis in Leydig cells (Morohaku et al., 2014), questioning the relevance of previous findings on TSPO using pharmacological ligands and inhibitors. These data underscore that TSPO does not play a significant role in the trafficking of cholesterol to IMM, and highlights the relevance of StARD1 in this process.

Finally, a role for caveolin-1 (CAV1) in mitochondrial cholesterol has been recently reported. CAV1 is a key component of caveolae, specialized membrane domains particularly enriched in cholesterol and sphingolipids, and CAV is known to bind cholesterol with high affinity (Murata et al., 1995; Pol et al., 2005; Boscher and Nabi, 2012). CAV's ability to move between cell compartments, mitochondria–ER and plasma membrane, might contribute to regulation of cholesterol fluxes and distributions within cells (Pol et al., 2001, 2005; Parton and Simons, 2007; Bosch et al., 2011). In line with these features, CAV1 deficiency has been shown to increase mitochondrial cholesterol in hepatocytes causing perturbations in mitochondrial membrane dynamics and function, and as expected, mGSH depletion (Bosch et al., 2011). The mitochondrial dysfunction sensitizes CAV1 null mice to SH and neurodegeneration. Whether the trafficking of mitochondrial cholesterol in the absence of caveolin-1 occurs via MAM or StARD1 remains to be further investigated.

ROLE OF MITOCHONDRIA AND MITOCHONDRIAL GSH IN DISEASE

Given the role of mitochondria in oxygen consumption, metabolism and cell death regulation, alterations in mitochondrial function or dysregulation in cell death pathways contribute to many diseases such as cancer, SH, or neurodegeneration. Consistent with its role in regulating mGSH, mitochondrial cholesterol accumulation emerges as a key factor regulating ROS and electrophile detoxification, and hence disease progression by sensitizing to secondary hits such as TNF, hypoxia or toxic amyloid peptides. In the following sections we will briefly cover examples of diseases where mitochondria cholesterol, oxidative stress, and mGSH depletion have been shown to play a role, such as cancer, fatty liver disease, and AD.

CANCER BIOLOGY AND THERAPEUTICS

Cancer cells exhibit critical metabolic transformations induced by mutations in oncogenes (gain-of-function) and tumor suppressor genes (loss-of-function) that result in cell deregulation associated with enhanced cellular stress. Adaptation to this stress phenotype is required for cancer cells to survive and involves the participation of genes that regulate generation and sensitization to ROS-mediated cell death. In this context, small molecules that selectively kill cancer cells are a promising approach for the treatment of cancer. Experiments using a cell-based small-molecule screening and quantitative proteomics, revealed the potential of piperlongumine, a natural product isolated from the plant species *Piper longum L*, as a cytotoxic agent triggering apoptosis and necrosis in leukemia cells (Bezerra et al., 2007). Moreover, piperlongumine induces ROS generation resulting in the killing of transformed cells *in vitro* and *in vivo* but not primary normal cells (Raj et al., 2011). Piperlongumine leads to decreased GSH and increased GSSG levels in cancer cells without effects in non-transformed cells, and these effects paralleled the ability of piperlongumine to cause alterations in mitochondrial morphology and function. Consequently, co-treatment with piperlongumine and *N*-acetyl-L-cysteine (NAC) prevented piperlongumine-mediated GSH depletion and cell death in cancer cells. These findings support the concept that normal cells have low basal levels of ROS and a diminished reliance on the ROS stress-response, while cancer cells have high levels of ROS, and hence, are expected to have a strong reliance on the ROS stress-response pathway. In line with the relationship between ROS and cancer it has been suggested that antioxidants may protect against cancer. However, randomized clinical trials have produced inconsistent results and some studies indicated that antioxidants increase cancer risk (van Zandwijk et al., 2000; Klein et al., 2011; Watson, 2013). A recent study in oncogene-induced lung cancer demonstrated that treatment with NAC and vitamin E accelerate cancer progression, stimulating cell proliferation by reducing ROS, DNA damage, and p53 expression (Sayin et al., 2014). The use of small molecules that alter the levels of ROS such as β-phenethyl isothiocyanate (PEITC), buthionine sulphoximine, curcumin, or 2-cyano-3,12-dioxooleana-1,9-diene-28-oic acid (CDDO) derivatives, has been suggested for the treatment of cancer by promoting ROS generation and GSH depletion in cancer cells (Schumacker, 2006; Trachootham et al., 2006; Yue et al., 2006; Ravindran et al., 2009). Interestingly, mGSH depletion has also been associated with apoptosis or autophagy induced by chemotherapeutic drugs. For instance, the novel triterpenoid methyl CDDO derivative (CDDO-Me) potently induced cytotoxicity in imatinib-resistant myeloid leukemia cells, accompanied by a rapid and selective depletion of mGSH resulting in increased generation of ROS and mitochondrial dysfunction (Samudio et al., 2005, 2008). Moreover, PEITC caused a rapid depletion of mGSH and a significant elevation of ROS and NO, induced a disruption of the mitochondrial electron transport complex I, and a significant suppression of mitochondrial respiration that resulted in cytotoxicity in leukemia cells (Chen et al., 2011).

As mGSH is regulated by cholesterol, as described above, the trafficking of mitochondrial cholesterol may modulate cancer cell biology. Cholesterol metabolism is deregulated in tumors,

which exhibit a paradoxical stimulation in *de novo* cholesterol synthesis despite hypoxia-mediated downregulation of HMG-CoA reductase by hypoxia (Nguyen et al., 2007; Garcia-Ruiz et al., 2009). In addition to its continued synthesis, cholesterol trafficking to mitochondria has been reported in tumor cells, including in mitochondria from hepatocellular carcinoma (HCC) due to overexpression of StARD1 (Montero et al., 2008). Mitochondrial cholesterol loading in cancer cells may actually account for the recognized mitochondrial dysfunction and resistance to Bax-mediated cell death induced by chemotherapy agents. In line with this hypothesis, treatments that resulted in mitochondrial cholesterol loading in tumor cells impaired stress-induced apoptosis (Lucken-Ardjomande et al., 2008; Montero et al., 2008), while StARD1 knockdown or treatments that resulted in downregulation of cholesterol loading sensitized HCC cells to chemotherapy. These findings identify the mitochondrial cholesterol loading in cancer cells, particularly HCC, as a mechanism contributing to chemotherapy resistance and evasion of Bax-mediated apoptosis (**Figure 4**). While mitochondrial cholesterol depletes mGSH due to impaired transport via OGC in primary hepatocytes and in SH, cancer cells paradoxically maintain mGSH homeostasis by a still ill-defined mechanism that is under investigation.

ALCOHOLIC AND NON-ALCOHOLIC FATTY LIVER DISEASE

Fatty liver disease represents a spectrum of liver disorders that begins with simple steatosis. This intial stage can progress to SH and culminate in cirrhosis and liver cancer. SH is an intermediate stage of fatty liver disease and one of the most common causes of chronic liver disease worldwide that may progress to cirrhosis and liver cancer. SH is characterized by steatosis, oxidative stress, hepatocellular death, inflammation and fibrosis and encompasses alcoholic (ASH) and non-alcoholic steatohepatitis (NASH). Unfortunately, there is no approved therapy for ASH/NASH, which reflects our incomplete understanding of the underlying mechanisms (Tilg and Diehl, 2000; Angulo and Lindor, 2002; Brunt, 2004). The development of steatosis in ASH/NASH is secondary to the metabolic disturbances in ASH and NASH, including insulin resistance, adipose tissue lipolysis, stimulation of *de novo* lipid synthesis and impaired mitochondrial fatty acid oxidation (Garcia-Ruiz and Fernandez-Checa, 2006; Garcia-Ruiz et al., 2011, 2013a,b). A key concept in SH pathogenesis is the two-hit hypothesis, which posits that hepatic steatosis sensitizes fatty liver to secondary hits, such as inflammatory cytokines and oxidative stress. However, recent evidence has shown that the type rather than the amount of fat plays a critical role in the transition from steatosis to ASH/NASH. In line with this hypothesis, previous studies have shown that chronic alcohol feeding in various models results in the depletion of mGSH due to cholesterol loading in mitochondria (Garcia-Ruiz et al., 1994; Colell et al., 1997, 1998, 2001; Zhao et al., 2002; Garcia-Ruiz and Fernandez-Checa, 2006) and that strategies aimed to correct the loss of mitochondrial membrane fluidity restore the mitochondrial transport of GSH and replenish the mGSH pool in alcohol-fed models. Moreover, recent evidence in rats fed an ethanol-polyunsaturated fatty acid treatment confirmed the mitochondrial cholesterol accumulation and GSH depletion, leading to SH, and these effects were prevented by betaine treatment (Varatharajalu et al., 2014). Altered alcohol-induced ER stress involves alterations in the methionine cycle and hyperhomocysteinemia, and treatment with betaine prevents alcohol-induced ER stress, steatosis, and liver injury (Ji and Kaplowitz, 2003, 2004). Moreover, tauroursodeoxycholic acid, a chemical chaperone shown to prevent ER stress (Ozcan et al., 2006), restored the mGSH pool in alcohol fed rats (Colell et al., 2001) and blocked alcohol-induced ER stress (Fernandez et al., 2013). The mechanisms of alcohol-induced mitochondrial cholesterol trafficking, mediated by alcohol-induced upregulation of StARD1, requires alcohol-induced acid sphingomyelinase activation (Fernandez et al., 2013).

Increased cholesterol synthesis and levels have been reported in liver biopsies from patients with NASH (Puri et al., 2007; Caballero et al., 2009) and mGSH depletion has been observed in models and patients with NASH (Serviddio et al., 2008). This outcome is consistent with the increased expression of StARD1 in patients with NASH but not with simple steatosis (Caballero et al., 2009). In line with these findings, recent data reported that the inhibition of microsomal triglyceride transfer protein, a model of liver steatosis, induced the increase in free cholesterol in mitochondria resulting in mGSH depletion (Josekutty et al., 2013). In addition to mGSH depletion, NASH is also characterized by impaired SOD2 activity, which may contribute the increased generation of mitochondrial superoxide and subsequent peroxynitrite levels that target mitochondrial proteins causing their inactivation. In principle, strategies such as SOD mimetics aimed to improve SOD2 activity may be of relevance in NASH. However, the use of SOD mimetics in parallel with the reported mGSH depletion can cause increased H_2O_2 and overall oxidant-dependent liver injury (Montfort et al., 2012). This scenario implies that the combination of SOD mimetics and mGSH replenishment may more efficient in NASH treatment.

ALZHEIMER DISEASE

Alzheimer disease is a major neurodegenerative disorder and the main cause of adult dementia. The main risk factor for AD is aging and therefore the number of people worldwide facing AD development increases every year. AD is characterized by progressive memory loss, cognitive impairment and disruption of synaptic plasticity. Although there are recommended therapies for AD, such as acetylcholinesterase inhibitors and the *N*-methyl-D-aspartate receptor antagonists, they are inefficient and do not prevent disease progression, reflecting our incomplete understanding of AD pathogenesis. Experimental models and human data established two main theories underlying AD, the accumulation of toxic amyloid β (Aβ) peptides, characteristic of senile plaques, and the aggregation of tau protein, a microtubule-associated protein expressed in neurons that is involved in the stabilization of microtubules in the cytoskeleton. The pathogenic processing of the amyloid precursor protein (APP) leads to toxic Aβ generation and is considered a critical mechanism of AD. Accordingly, a coding mutation (A673T) in APP has been recently shown to protect against AD and age-related cognitive decline in elderly Icelanders (Jonsson et al., 2012). This substitution, which is close to the aspartyl protease

β-site in APP, reduces the formation of amyloidogenic peptides *in vitro* by 40%. The protective effect of the A673T substitution against AD provides strong evidence for the hypothesis that reducing the β-cleavage of APP may protect against the disease. Amiloidogenic processing of APP yields toxic Aβ peptides. In this pathway, the β- and γ-secretases cleave APP at the N- and C-termini of the Aβ peptide, respectively. β-Secretase has been characterized as a membrane-bound aspartic protease termed beta-site APP-cleaving enzyme 1 (BACE1), while γ-secretase is a complex comprised of presenilin-1 or -2, nicastrin, anterior pharynx-defective 1 (Aph-1) and presenilin enhancer 2 (Pen-2; Haass, 2004). Another novel member of the γ-secretase complex has been identified. β-arrestin 2 physically associates with the Aph-1α subunit of the γ-secretase complex and redistributes the complex toward detergent-resistant membranes, increasing the catalytic activity of the complex (Thathiah et al., 2013). Moreover, β-arrestin 2 expression is elevated in individuals with AD and its overexpression leads to an increase in Aβ peptide generation, whereas genetic silencing of Arrb2 (encoding β-arrestin 2) reduces generation of Aβ in cell cultures and in $Arrb2^{-/-}$ mice. In addition to its amyloidogenic processing by β- and γ-secretases, APP can be cleaved within the Aβ domain by α-secretase. This non-amyloidogenic processing prevents the deposition of intact Aβ peptide and results in the release of a large soluble ectodomain, sAPPα, from the cell, which has neuroprotective and memory-enhancing effects. Members of the ADAMs, a disintegrin and metalloprotease family of proteases, have been shown to possess α-secretase activity (Hooper and Turner, 2002). The pathogenic processing of APP into toxic Aβ fragments occurs in cholesterol-enriched membrane domains of the plasma membrane, known as lipid rafts, consistent with the recognized role of cholesterol in AD pathogenesis based upon experimental and epidemiological evidence linking plasma cholesterol levels and AD development (Notkola et al., 1998; Wolozin et al., 2000; Anstey et al., 2008). High cholesterol levels correlated with Aβ deposition and the risk of developing AD, while patients taking the cholesterol-lowering drug statins were found to have a lower incidence of the disease (Notkola et al., 1998; Wolozin et al., 2000). Exploiting the relative detergent insolubility of lipid rafts, there has been evidence indicating the localization of APP, the α-, β- and γ-secretases in rafts (Wahrle et al., 2002; Vetrivel et al., 2005). In addition, the activities of BACE1 and γ-secretase are stimulated by lipid components of rafts, such as glycosphingolipids and cholesterol (Sawamura et al., 2004; Kalvodova et al., 2005; Ariga et al., 2008; Osenkowski et al., 2008). Besides its extracellular deposition, current evidence indicates the processing and targeting of APP and Aβ to intracellular sites, including mitochondria (Lin and Beal, 2006). Moreover, levels of mitochondrial APP are higher in affected brain areas and in subjects with advanced disease symptons (Devi et al., 2006). Immunoelectron microscopy analyses indicated the association of APP with mitochondrial protein translocation components, TOM40 and TIM23, which correlated with decreased import of respiratory chain subunits *in vitro*, decreased cytochrome oxidase activity, increased ROS generation and impaired mitochondrial reducing capacity (Devi et al., 2006). Although the molecular mechanisms of mitochondrial Aβ targeting remains poorly understood, Aβ stimulates mitochondrial ROS generation, contributing to Aβ toxicity in neurons (Behl et al., 1994; Casley et al., 2002; Lustbader et al., 2004). In addition to the amyloidogenic effect of cholesterol by fostering Aβ generation from APP, recent data has provided evidence that mitochondrial cholesterol accumulation sensitizes neurons to Aβ-induced neuroinflammation and neurotoxicity by depleting mGSH, effects that are prevented by mGSH replenishment (Fernandez et al., 2009b). The mechanism of mitochondrial cholesterol accumulation involves the upregulation of StARD1 induced by Aβ *via* ER stress (Barbero-Camps et al., 2014), confirming previous findings in hepatocytes (Fernandez et al., 2013). Although not reported in patients, the trafficking of cholesterol to mitochondria may be of clinical relevance to human AD due to the described enhanced expression of StARD1 in pyramidal hippocampal neurons of AD-affected patients (Webber et al., 2006). Moreover, a novel mouse model engineered to have enhanced cholesterol synthesis by SREBP-2 overexpression superimposed to APP/PS1 mutations triggered Aβ accumulation and tau pathology (Barbero-Camps et al., 2013). This triple transgenic model exhibited increased mitochondrial cholesterol loading and mGSH depletion and accelerated Aβ generation by β-secretase activation compared to APP/PS1 mice (Barbero-Camps et al., 2013). Moreover, SREBP-2/APP/PS1 mice displayed synaptotoxicity, cognitive decline, tau hyperphosphorylation and neurofibrillary tangle formation in the absence of mutated tau, indicating that cholesterol, particularly mitochondrial cholesterol, can precipitate Aβ accumulation and tau pathology. Importantly, *in vivo* replenishment of mGSH with cell- permeable GSH monoethyl ester (GSH–EE) attenuated neuropathological features of AD in SREBP-2/APP/PS1 mice.

In addition to the proteolytic processing by secretases, APP and its corresponding C-terminal fragments are also metabolized by lysosomal proteases. SORLA/SORL1 is a unique neuronal sorting receptor for APP that has been causally implicated in sporadic and autosomal dominant familial AD. Brain concentrations of SORLA are inversely correlated with Aβ in mouse models and AD patients. Indeed, transgenic mice overexpressing SORLA exhibit decreased Aβ concentrations in brain (Caglayan et al., 2014). Mechanistically, Aβ binds to the amino-terminal VP10P domain of SORLA and this binding is impaired by a familial AD mutation in SORL1. Moreover, sphingosine-1-phosphate (S1P) accumulation by S1P lyase deficiency has recently been shown to impair lysosomal APP metabolism, resulting in increased Aβ accumulation (Karaca et al., 2014). The intracellular accumulation of S1P interferes with the maturation of cathepsin D and degradation of Lamp2, suggesting a general impairment of lysosomal function and autophagy. As sphingolipids have strong affinity to bind cholesterol (Slotte, 1999; Ridgway, 2000), it is conceivable that increased lysosomal cholesterol may contribute to impaired lysosomal Aβ degradation in the S1P lyase knockout mice. However, this aspect remains to be investigated, raising the question of whether lysosomal cholesterol plays a role in lysosomal Aβ degradation and hence has any relevance in AD. Quite intriguingly, recent findings have reported increased expression of the lysosomal cholesterol transporter Niemann–Pick type C disease 1 (NPC1) in AD (Kagedal et al., 2010). NPC is an endolysosomal

protein essential for the intracellular regulation of cholesterol and its mutation and loss-of-function elicits the lysosomal storage disease NPC disease, characterized by the accumulation of lysosomal cholesterol and sphingolipids. NPC1 expression was described to be upregulated at both mRNA and protein levels in the hippocampus and frontal cortex of AD patients compared to controls subjects. However, no difference in NPC1 expression was detected in the cerebellum, a brain region that is relatively spared in AD. Moreover, murine NPC1 mRNA levels increased in the hippocampus of 12-month-old APP/PS1 mice compared to wild-type mice. These findings strongly suggest the lack of lysosomal cholesterol accumulation in AD, and imply that lysosomal impairment and subsequent contribution to decreased Aβ degradation in AD may occur through mechanisms independent of cholesterol accumulation in lysosomes. Although several similarities exist between NPC disease and AD, including altered intracelular cholesterol homeostasis, changes in the lysosomal function, neurofibrillary tangles, and increased Aβ generation and neurodegeneration, the likely common nexus between these diseases is mitochondrial cholesterol loading, rather than lysosomal cholesterol accumulation, as reported both in AD and NPC disease (Yu et al., 2005; Colell et al., 2009; Fernandez et al., 2009b). Thus, targeting mitochondrial cholesterol may be of relevance not only for AD but also for other neurodegenerative and lysosomal storage diseases, including NPC.

CONCLUSION AND FUTURE APPROACHES

Mitochondria play an essential role in providing the energy needed for multiple signaling cascades and cellular functions. The consumption of molecular oxygen in the respiratory chain not only is the driving force for the ATP synthesis required for cell viability, but also the source of ROS that target mitochondrial and extramitochondrial targets. As described above, mitochondrial oxidative stress and the mGSH depletion are central events of many pathological conditions. However, a challenge to counteract mitochondrial oxidative stress is to recover mGSH pool when GSH transport is defective due to alterations in membrane dynamics triggered by increased mitochondrial cholesterol accumulation. In addition to the ability of mitochondrial-permeable GSH-EE to directly increase mGSH levels bypassing the mitochondrial transport defect, it has been recently described additional strategies that supply mitochondria with GSH, including parental molecules that generate GSH once inside the mitochondrial matrix. This approach has been recently illustrated with the use of *S*-D-lactoylglutathione (Armeni et al., 2014). This compound is an intermediate of the glyoxalase system, which is hydrolyzed in the mitochondrial matrix yielding lactate and GSH; hence showing the ability to replenish mGSH resulting in recovery of mitochondrial function and antioxidant defense. Unlike these permeable GSH prodrugs that directly boost mGSH, strategies aimed to increase cytosol GSH (e.g., NAC) may not be an optimal approach for boosting mGSH and therefore for treatment of SH or AD, as it would result in mainly increasing cytosol GSH without replenishing mGSH levels. Another strategy to combat ROS generation would be the supply of antioxidants that are targeted selectively to mitochondria. Since lipophilic cations accumulate in mitochondria, the covalent attachment of a neutral bioactive compound to a lipophilic cation should lead to its selective delivery to mitochondria. In this regard, alkyl-triphenylphosphonium (TPP) cations are excellent tools for the delivery of compounds to mitochondria as they preferentially accumulate quite efficiently within mitochondria in cells, making it possible to deliver a wide range of mitochondria-targeted lipophilic TPP-labeled cations (Ross et al., 2005; Sheu et al., 2006). This approach has been exploited recently in several contexts by the development of a series of cationic antioxidants targeted to mitochondria, including derivatives of the endogenous antioxidants ubiquinol (MitoQ), alpha-tocopherol (MitoVit E), and of the synthetic spin trap PBN (MitoPBN; Ross et al., 2005). These compounds have been found to block oxidative damage in isolated mitochondria and cells more effectively than untargeted antioxidant analogs due to their concentration within mitochondria. More importantly, oral administration of these compounds leads to their accumulation in the brain, heart, muscle and liver mitochondria (Ross et al., 2005). In fact, MitoQ has been used in a range of *in vivo* studies, in rats and mice, and in two phase II human trials demonstrating that it can be safely delivered to patients with promising results, lending further support that mitochondria-targeted antioxidants may be applicable to a wide range of human pathologies that involve mitochondrial oxidative damage (Smith and Murphy, 2010).

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Review Article

Glutathione as a skin whitening agent: Facts, myths, evidence and controversies

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ABSTRACT

Glutathione is a low molecular weight thiol-tripeptide that plays a prominent role in maintaining intracellular redox balance. In addition to its remarkable antioxidant properties, the discovery of its antimelanogenic properties has led to its promotion as a skin-lightening agent. It is widely used for this indication in some ethnic populations. However, there is a dichotomy between evidence to support its efficacy and safety. The hype around its depigmentary properties may be a marketing gimmick of pharma-cosmeceutical companies. This review focuses on the various aspects of glutathione: its metabolism, mechanism of action and the scientific evidence to evaluate its efficacy as a systemic skin-lightening agent. Glutathione is present intracellularly in its reduced form and plays an important role in various physiological functions. Its skin-lightening effects result from direct as well as indirect inhibition of the tyrosinase enzyme and switching from eumelanin to phaeomelanin production. It is available in oral, parenteral and topical forms. Although the use of intravenous glutathione injections is popular, there is no evidence to prove its efficacy. In fact, the adverse effects caused by intravenous glutathione have led the Food and Drug Administration of Philippines to issue a public warning condemning its use for off-label indications such as skin lightening. Currently, there are three randomized controlled trials that support the skin-lightening effect and good safety profile of topical and oral glutathione. However, key questions such as the duration of treatment, longevity of skin-lightening effect and maintenance protocols remain unanswered. More randomized, double-blind, placebo-controlled trials with larger sample size, long-term follow-up and well-defined efficacy outcomes are warranted to establish the relevance of this molecule in disorders of hyperpigmentation and skin lightening.

Key words: Depigmenting, glutathione, hyperpigmentation, skin lightening, skin whitening

INTRODUCTION

A lighter skin tone has been considered a superior trait in most races, especially in women of Asian or African descent who have Fitzpatrick skin types IV–VI. The higher prevalence of pigmentary disorders in these skin types adds to the woes of the patients. In relatively conservative societies such as India, many people are obsessed with the desire for a fair complexion for themselves as well as their spouse. Such traditions motivate the patient to desire fair complexion and sometimes seek it even against their will.

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Realizing this growing need for fair skin, many pharmaceutical companies are developing different molecules for skin lightening. A lot is already known about topical depigmenting agents such as hydroquinone, glycolic acid, arbutin, kojic acid, vitamin C, vitamin E and niacinamide, all of which are readily available over-the-counter. The advent of newer depigmenting molecules such as pycnogenol, orchid and marine algae extracts, cinnamic acid, soy, aloesin and *Boswellia* has offered more topical options. Apart from the local adverse effects of these agents, the

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important limitation is the localization of their effect to the site of application alone. The quest for systemic skin lightening logically ensued. Agents that have been promoted for this purpose include glutathione, tranexamic acid, l-cysteine peptide, vitamin C, different plant extracts and their combinations.[1]

This review focuses on glutathione as a skin-lightening agent. Aggressive media campaigns about its exaggerated effects as a "skin lightening" agent and over-the-counter availability of this drug have resulted in consumption of improper doses and schedules. These consumers, as well as dermatologists who prescribe oral glutathione for general skin lightening or as an adjuvant for disorders of hyperpigmentation, are often oblivious about its efficacy, dosing and adverse effects. Dermatologists frequently encounter patients who are inclined to self-medicate with glutathione, enticed by the manufacturers' claims. We are expected to intelligently answer queries regarding the efficacy and safety of this drug.

Oral and intravenous glutathione have been available in some countries such as the Philippines for many years. This drug has recently made inroads in other countries including India. Most of the patients who desperately seek fair complexion or a new treatment modality for their refractory facial melanosis are typically internet and social media savvy. They are rich enough to afford expensive treatment. Pharmaceutical companies that manufacture intravenous glutathione have a marketing agenda and pursue dermatologists to administer this drug to such patients. Not surprisingly, the trend of recommending and administering intravenous glutathione has increased within months of it becoming available, despite the potential adverse effects and lack of evidence.

It is important that dermatologists know about glutathione: its efficacy, the mechanism of hypopigmentary effects, pharmacokinetics, evidence-level and safety profile. In this review, we attempt to crystallize these concepts and analyze the current evidence supporting the efficacy of glutathione as an inhibitor of melanization.

MOLECULAR STRUCTURE AND FUNCTION OF GLUTATHIONE

Glutathione (γ-glutamyl-cysteinylglycine) is a small, low-molecular weight, water-soluble thiol-tripeptide formed by three amino acids (glutamate, cysteine and glycine).[1] It is a ubiquitous compound with a biologically active sulfhydryl group contributed by the cysteine moiety that acts as the active part of the molecule.[2] This sulfhydryl group allows for interaction with a variety of biochemical systems, hence the abbreviation "GSH" for its active form. Glutathione is one of the most active antioxidant systems in human physiology.[3]

Biological activity: The glutathione redox cycle

Glutathione exists in two interconvertible forms, reduced glutathione (GSH) and oxidized glutathione (GSSG). GSH is the predominant intracellular form, which acts as a strong antioxidant and defends against toxic compounds and xenobiotics. In this process, GSH is constantly oxidized to GSSG by the enzyme glutathione peroxidase [Figure 1]. To maintain the intracellular redox balance, GSH is replenished through the reduction of GSSG by glutathione reductase enzyme.

Biological functions of glutathione

Glutathione plays a key role in multiple biological functions. The most important ones have been enumerated in Box 1.[4]

GLUTATHIONE DEPLETION AND SUPPLEMENTATION IN MEDICAL CONDITIONS

Extensive research in various specialties has shown that many human diseases are associated with low glutathione levels. These conditions and causes include emphysema, asthma, allergic disorders, drug

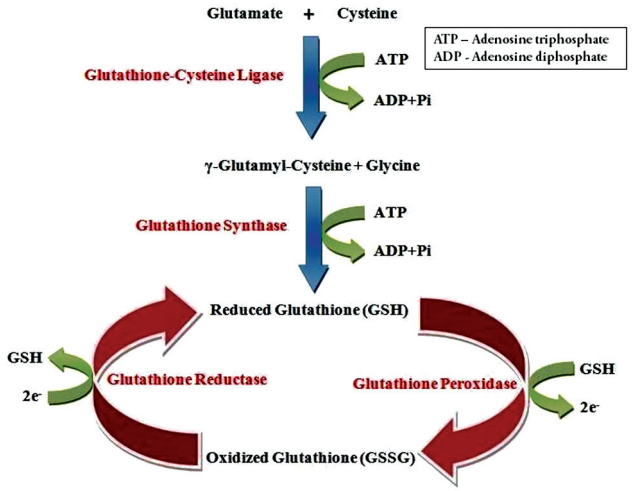

Figure 1: The glutathione redox cycle, demonstrating the inter-conversion of oxidized and reduced glutathione

Box 1: Important physiological functions of glutathione

Maintenance of the sulfhydryl groups of proteins and other molecules

Catalysis of exchange reactions

Scavenging of free radicals, most importantly hydrogen peroxide

Translocation of amino acids across cell membranes

Detoxification of xenobiotics

Participation as a coenzyme in certain important processes of cellular metabolism

toxicity, metabolic disorders, cancer, chemotherapy and human immunodeficiency virus-acquired immune deficiency syndrome, among others.[5,6] Research on the role of glutathione supplementation in these diseases is limited. Most of the studies have been done for autism and cystic fibrosis.[7,8]

GLUTATHIONE AND HUMAN PIGMENTATION

Melanin in human skin is a polymer of various indole compounds synthesized from L-tyrosine by the Raper–Mason pathway of melanogenesis [Figure 2] with tyrosinase being the rate limiting enzyme. The ratio of the two different types of melanin found in skin, black-brown colored eumelanin and yellow-red pheomelanin, determines the skin colour.[9] An increased proportion of pheomelanin is associated with lighter skin colour.

Exposure to ultraviolet radiation is the most important factor that causes undesirable hyperpigmentation. The crucial cellular event is enhanced tyrosinase activity. Exposure to ultraviolet radiation results in generation of excessive amounts of reactive oxygen and nitrogen species within the cells.[10,11] Oral antioxidants partially reduce melanogenesis by suppressing these free radicals.

One of the earliest pieces of evidence of the association between thiols and skin came from the effect of an extract of human skin that contained an active sulfhydryl-containing compound. It prevented melanin formation by tyrosinase inhibition. Hyperpigmentation was observed when this compound got oxidized and inactivated by factors such as heat, radiation and inflammation with consequent loss of the inhibitory effect on tyrosinase. Halprin and Ohkawara provided physical and biochemical evidence that this "sulfhydryl compound" was glutathione![12]

Postulated effects of glutathione on pigmentation

The role of glutathione as a skin-lightening agent was an accidental discovery when skin lightening was noticed as a side effect of large doses of glutathione.[1] Various mechanisms for the hypopigmentary effect of glutathione have been proposed, with inhibition of tyrosinase being the most important [Box 2]. Glutathione can reduce tyrosinase activity in three different ways.[13] Tyrosinase is directly inhibited through chelation of the copper site by the thiol group. Secondly, glutathione interferes with the cellular transfer of tyrosinase to premelanosomes, a prerequisite for melanin synthesis.[13] Thirdly, tyrosinase inhibition is effected indirectly via its antioxidant effect. Glutathione shifts melanogenesis from eumelanin to phaeomelanin synthesis by reactions between thiol groups and dopaquinone leading to the formation of sulfhydryl-dopa conjugates.[14]

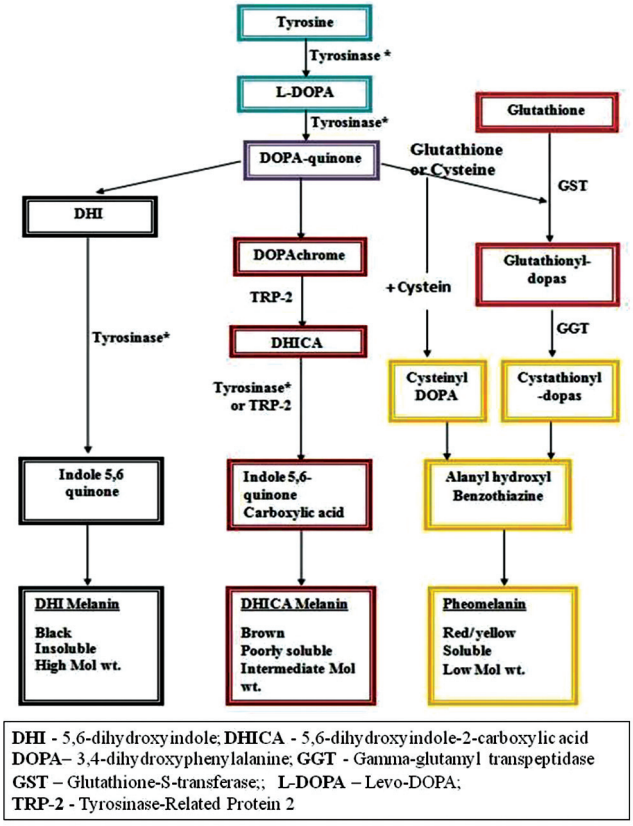

Figure 2: The Raper–Mason pathway, depicting the steps in melanin synthesis. Notice how the presence of glutathione/ cysteine can induce a switch towards higher pheomelanin production as compared to eumelanin

Glutathione has potent antioxidant properties. The free radical scavenging effect of glutathione blocks the induction of tyrosinase activity caused by peroxides.[14] Glutathione has been shown to scavenge ultraviolet radiation induced reactive oxygen species generated in epidermal cells.[15] A recent study on

Box 2: Summary of proposed mechanisms of action of glutathione (GSH) in disorders of hyperpigmentation

Direct inactivation of tyrosinase (the key enzyme of melanogenesis) by binding with the copper-containing active site of the enzyme

Indirect inactivation of tyrosinase via antioxidant effect which leads to quenching of free radicals and peroxides

Switching production of eumelanin to phaeomelanin

Modulation of the depigmenting abilities of other melanocytotoxic agents

melasma patients noted significantly higher levels of glutathione-peroxidase enzyme in patients compared to controls, confirming the role of oxidative stress in melasma.[16] Based on these observations, the potential of glutathione in management of melasma and hyperpigmentation seems plausible.[17]

Natural dietary sources of glutathione

Fresh fruits, vegetables, and nuts are natural sources of glutathione. Tomatoes, avocados, oranges, walnuts and asparagus are some of the most common edibles that help to increase levels of glutathione in the body. Whey protein is another rich source of glutathione and has been used to enhance systemic glutathione levels in cystic fibrosis.[8]

ADMINISTRATION OF GLUTATHIONE: PHARMACEUTICAL FORMULATIONS

Glutathione is primarily available as oral formulations (pills, solutions, sublingual tablets, syrups and sprays) and parenteral formulations (intravenous and intramuscular). It has been administered by intranasal and intrabronchial routes as well. The three major routes of administration used for skin lightening are topical (creams, face washes), oral (capsules and sublingual/buccal tablets) and intravenous injections.

Topical glutathione

Glutathione is commercially available as face washes and creams. A randomized, double-blind, placebo-controlled clinical trial conducted in 30 healthy Filipino women aged 30–50 years has provided some evidence favouring the efficacy of topical 2% GSSG lotion in temporary skin lightening. Patients were randomized to apply glutathione as 2% GSSG lotion and a placebo lotion in a split-face protocol, twice daily for ten weeks. GSSG was preferred over GSH, as GSH is unstable in aqueous solutions. GSSG eventually generates GSH after cutaneous absorption. The changes in the melanin index, moisture content of the stratum corneum, skin smoothness, skin elasticity and wrinkle formation were objectively assessed. The reduction of the melanin index with glutathione was statistically significant when compared to placebo [Table 1].[10] Glutathione treated areas had significant improvement in other parameters as well. No adverse drug effects were reported. Glutathione has also become available in the form of soaps, face washes and creams.[18] Recently, a glutathione based chemical peel has been launched. Although evidence of efficacy is lacking, the manufacturers claim improvement of melasma, hyperpigmentation and skin ageing.[19]

Glutathione mesotherapy

Despite the lack of published literature on the efficacy and methodology of using glutathione solution as mesotherapy, it is widely practiced by dermatologists for the treatment of melasma and other facial melanoses. It is used as monotherapy, or in combination with ascorbic acid, vitamin E, tranexamic acid, etc.[20] Although the results are claimed to be very good, use of glutathione as mesotherapy needs more evidence and published data.

Oral glutathione: Pharmacokinetics and metabolism of orally administered glutathione

Oral glutathione is derived from torula yeast (*Candida utilis*). It is marketed as a food or dietary supplement, either alone, or in combination with vitamin C, alpha lipoic acid and other antioxidants.

The fate of orally administered glutathione has been studied in animal models and human volunteers. The principal site of absorption is the upper jejunum. Circulating glutathione is primarily cleared by the kidney.[21] Older studies have suggested that glutathione is absorbed intact from the gut. This is based on the observation of lack of similar increase in plasma glutathione levels after the administration of the constituent amino acids of glutathione when compared to the administration of glutathione capsules.[22] After absorption into plasma, glutathione needs to be broken down into amino acids and re-synthesized intracellularly. The administration of cysteine-rich glutathione precursors, especially N-acetyl cysteine, has been shown to increase intracellular glutathione levels.[23]

The bioavailability of oral glutathione in humans is a controversial subject. A single-dose study conducted

by Witschi *et al.* in seven healthy volunteers reported no significant increase in plasma glutathione levels for up to 270 min. However, Hagen and Jones reported an increase in plasma glutathione levels in four out of five subjects after a single oral dose of 15 mg/kg body weight. In that study, the plasma glutathione levels increased to 300% of baseline levels after one hour, followed by a decrease to approximately 200% of baseline levels within the next three hours.[24,25] The inadequate absorption of glutathione in humans compared to that in rats has been attributed to a higher hepatic gamma-glutamyl transferase activity in humans. This results in increased hydrolysis of glutathione with resultant low serum levels.[21]

A randomized, double-blind, placebo-controlled study on oral glutathione supplementation (500 mg twice daily for four weeks) in 40 healthy adult volunteers failed to show any significant change in serum glutathione levels.[26] Another randomized, double-blinded, placebo-controlled trial was conducted in 54 adults which administered oral glutathione for six months, either in a dose of 250 mg or 1000 mg per day. Results showed a steady increase in glutathione levels when compared to the baseline. There were higher levels in the high-dose group (30–35% increase vs 17% increase in the low-dose group). The raised levels returned to baseline after a one-month washout period.[27] In another study, glutathione administered at a single dose of 50 mg/kg body weight led to a considerable increase of protein-bound glutathione levels in plasma but not of the deproteinized fraction, measured after two hours of supplementation.[28] Since intracellular glutathione levels can increase only after its amino acid components are transported through the cell membrane after deproteinization, the results of this study remain ambivalent.

In summary, human trials performed before 2013 have shown that over-the-counter oral glutathione supplementation has a negligible effect on raising plasma levels in humans. The only trials that support the concept of oral supplementation to raise glutathione levels in healthy adults have been conducted by Richie *et al.* and Park *et al.* It is important to take note of the fact that both studies used a specific brand of glutathione, manufactured by the trial funding company.[27,28] Thus, the evidence for the clinically efficacious bioavailability of oral glutathione in humans remains scarce and controversial.

Oral formulations of glutathione: Manufacturing and processing issues

Manufacturing high dose glutathione pills is technically difficult as GSH has a very high electrostatic charge which makes processing and encapsulating higher strengths of glutathione very difficult.[29] Addition of crystalline ascorbic acid dissipates this electrostatic charge and allows packaging of pills with up to 750 mg of the drug.[29] However, oral formulations may have a combination of vitamin C, vitamin E, alpha-lipoic acid, N-acetyl cysteine, grape seed extract, etc. Alpha lipoic acid is a glutathione replenishing disulfide that increases whole blood and intracellular GSH levels.[30] The dosage and duration of oral glutathione has not been standardized with different dosages having been "recommended" by different manufacturers [Box 3].[31] These manufacturer specific guidelines have no clear scientific basis. Oral glutathione is also available as sublingual tablets and solutions. While sublingual preparations contain very low doses (50–100 mg), oral suspensions and solutions have a foul sulfurous taste and need to be freshly prepared.[29] Thus, the controversies regarding the effectiveness of oral glutathione continues to pose a challenge to its prescribers [Box 4].

Statutory approval status of oral glutathione supplements

Glutathione based oral dietary supplements have been granted the status of "Generally recognized as safe",

Box 3: Recommended dosage of glutathione capsules/tablets for skin lightening effects

Dose: 20-40 mg/kg body weight per day (i.e. 1-2 grams GSH per day) divided into two doses, for skin lightening effects

Time duration required for the skin lightening effects: May become visible within four weeks; although a significant effect may need 1-3 months, 3-6 months, 6-12 months, and 2 years (or more) in medium brown skin, dark brown skin, very dark skin, and black skin, respectively

Maintenance dose: After attaining the 'desired' skin colour, a maintenance dose of 500mg/day for an indefinite duration has been suggested

Box 4: Controversies regarding the effectiveness of glutathione as an oral therapy

Discordance between plasma levels achieved after oral supplementation with high dose glutathione in animal models e.g. rats and mice (where high plasma levels have been documented) versus humans

Contradictory results of plasma levels attained in different studies conducted in human volunteers

Short term maintenance of effect with normalization of plasma levels within a month of stopping oral supplementation

Beneficial effects anticipated in patients with documented depletion of glutathione, with no defined role in otherwise healthy volunteers

Table 1: Evidence of Glutathione as a skin lightening agent: A summary of studies conducted till date

Glutathione formulation	Topical (GSSG cream)	Oral (Capsules)	Oral lozenges for buccal mucosal absorption	Intravenous
Authors	Watanabe *et al*[10]	Arjinpathana and Asawanonda[33]	Handog *et al*[34]	NA
Year of publication	2014	2010	2015	NA
Study subjects	30 healthy Filipino women aged 30-50 years with baseline facial melanin index value of 200-350	60 healthy medical students aged 19-22 years	30 healthy women (aged 22-42 years) with Fitzpatrick skin types IV or V, with melanin indices of ≥20 (maximum value = 99)	NA
Study design	Randomized, double-blind, placebo -controlled, matched-pair study	Randomized, double-blind, placebo controlled study	Open-label, single-arm pilot study	NA
Methodology	2% (w/w) GSSG lotion and placebo lotion, randomly assigned to either the right or the left side of the face of each subject, was spread evenly to the designated site twice daily for 10 weeks	Subjects were block-randomized to receive either glutathione (500 mg) or placebo capsules daily, in two divided doses on an empty stomach for 4 weeks	One lozenge (500mg) per day, to be kept in the mouth against the inner cheek (buccal mucosa). Clinical evaluation was performed at baseline and every two weeks over a period of eight weeks by a portable Mexameter	NA
Parameters evaluated objectively	Frequency of evaluation At baseline and then weekly for 10 weeks Parameters evaluated 1) Skin lightening (over cheek bones) - by Mexameter® MX18 2) Others: Skin moisture, skin smoothening, wrinkles and skin firmness	Frequency of evaluation At baseline and at end of study (4 weeks) Parameters evaluated 1) Melanin index - by Mexameter. Measurements were done in triplicate at six sites : *Sun-exposed areas*: Face – left and right sides. Extensor surfaces of the forearms, left and right sides. *Sun protected areas*: Upper, inner arms - left and right. 2) Standardized digital photographs – to quantitatively evaluate UV spots, pores and evenness on the left and right sides of the face.	Frequency of evaluation At baseline and then twice weekly for 8 weeks. Parameters evaluated 1) Melanin index - by Mexameter. Measurements were done in triplicate and the mean was taken at two sites: *Sun-exposed area*: Extensor surface of the right wrist *Sun-protected area*: Mid-sternum	
Subjective evaluation	Frequency of evaluation: At baseline and then on alternate weeks for 10 weeks Parameters evaluated (by investigators as well as subjects): Skin lightening Wrinkle reduction and skin smoothening Scoring pattern used: −3=Marked deterioration −2=Moderate, visibly uneven deterioration −1=Slight deterioration 0=No perceptible change or improvement 1=Slight change or improvement 2=Moderate change or improvement (For lightening: perceptible and visible change, with <50% lightening of skin color) 3=Marked improvement or remarkable change or improvement (For lightening: very visible change with even and uniform skin lightening covering >80% of the contact area)	Global evaluation by subjects for the overall response done with the help of a 4-point rating scale: 4 = very satisfactory 3 = moderately satisfactory 2 = minimally satisfactory 1 = not satisfactory	Global evaluation by subjects for the overall response done with the help of a 5-point rating scale: 0 = None 1 = Mild change 2 = Moderate 3 = Obvious 4 = Very marked	
Follow-up	Not mentioned beyond the study duration (10 weeks)	Not mentioned beyond the study duration (4 weeks)	Not mentioned beyond the study duration (8 weeks)	NA

Contd...

Table 1: Contd...

Glutathione formulation	Topical (GSSG cream)	Oral (Capsules)	Oral Lozenges for buccal mucosal absorption	Intravenous
Results	Skin lightening Progressive and significant reduction in melanin index values over 10 weeks at the GSSG application sites as compared to placebo sites. Skin moisture - significantly higher at GSSG sites than placebo in weeks 8 and 9 Curvature index values - significantly lower at GSSG sites than at placebo sites in weeks 6 and 10. Keratin index values - significantly lower at GSSG sites than at placebo sites from weeks 6 to 10 Elasticity index values – no significant difference between the two sites	Melanin Index In subjects receiving glutathione - consistent and statistically significant reduction at all six sites. In placebo group - increased in facial areas and reduced at other sites. On comparison of both groups, greater reduction in glutathione group especially for the right side of the face and the left forearm. UV spots Minimally increased in number in subjects who received oral glutathione. Statistically significant increase in the number of UV spots on the face in the placebo group. Skin evenness - increased in glutathione group (not statistically significant) Pore size - decreased in glutathione group (not statistically significant)	Melanin Index At both sun-exposed and sun-protected sites, all the subjects (100%) showed a significant reduction in melanin index (P<0.0001) Global Assessment: Twenty-seven of the subjects (90%) noted moderate skin lightening and three noted mild skin lightening (10%)	NA
Tolerance and Safety	Very well tolerated, with no untoward symptoms reported. Adverse effect: Only one subject experienced erythema of the entire face on day 1 that subsided in 2 days without stopping use of either lotion	Very well tolerated. Adverse effects: Flatulence reported by one subject in the glutathione group during initial days and constipation reported by one subject receiving placebo	Very well tolerated in those who completed the study. One drop out complained of sour taste and chalky texture. Adverse effects: None amongst those who completed the study. One drop out complained of soreness of gums due to the lozenges	NA
Limitations	Small sample size and a short duration of study involving Filipino women. The results cannot be extrapolated for other ethnic groups	Short study period and on a small sample size that comprised of healthy young adults, follow up for evaluation of persistence of efficacy is lacking. Serum GSH levels were not measured	Short duration and small sample size that comprised of healthy young women, follow up for evaluation of persistence of efficacy is lacking. Serum GSH levels were not measured	NA
Level of evidence	Ib	Ib	2b	None

NA: Not available

consistent with Section 201(s) of the federal food, drug and cosmetic act of the United States Food and Drug Administration.[32] There is no restriction on its availability in United States, Philippines and Japan. This has recently become available over-the-counter in India as well.

Evidence-based efficacy of glutathione as an oral-lightening agent

On review of literature, we could find only two studies that evaluated the efficacy of oral glutathione as a skin-lightening agent. A randomized, double-blind, two-arm, placebo-controlled study conducted in the Thai population studied the effect of orally administered glutathione on the skin melanin index in sixty healthy medical students [Table 1]. The subjects were randomized to receive glutathione capsules in a dose of 500 mg/day in two divided doses, or placebo for four weeks. The primary end-point studied was the reduction of melanin indices at six different sites. At four weeks, the melanin indices decreased consistently at all six sites in the glutathione group. There was a statistically significant reduction at two sites in the placebo group, namely the right side of the face and the sun-exposed left forearm. The tolerance to glutathione was excellent. The limitations of this study include a short study period, lack of follow-up, lack of measurement of serum glutathione levels and the choice of cohort, which consisted of a young and healthy population. Despite these shortcomings, this study was the first to demonstrate the beneficial effects of oral glutathione in skin lightening.[33] Another

open-label study that used glutathione containing lozenges reported improvement in the skin melanin index, as measured by Mexameter [Table 1].[34] They used buccal lozenges instead of capsules to enhance and ensure steady bioavailability. In our opinion, the sublingual or buccal route is likely to increase the bioavailability of glutathione better than oral tablets or capsules. A comparative study between these two routes of administration is the only way to provide reliable evidence in this regard.

Intravenous glutathione

Due to the low bioavailability of oral glutathione, intravenous injections are being promoted to provide desired therapeutic levels in the blood and skin and to produce "instant" skin-lightening. Interestingly, intravenous injections of glutathione have been used for years but there is not even a single clinical trial evaluating its efficacy. Manufacturers of intravenous glutathione injections recommend a dose of 600–1200 mg for skin lightening, to be injected once to twice weekly. The duration for which they should be continued is not specified. Intravenous administration is expected to deliver 100% bioavailability of glutathione, much more compared to that achieved by oral administration. However, there are no studies to support this hypothesis. Although intravenous glutathione delivers a much higher therapeutic dose that enhances its efficacy, it also provides a narrower margin of safety due to the possibility of overdose toxicity.

There is no available data on the efficacy of intravenous glutathione for skin lightening. The data on safety are available, but scarce. In an animal-based study, no significant adverse effects were reported in dogs, who were administered up to 300 mg of glutathione per kg body weight every day for 26 weeks.[35] Human studies in which parenteral glutathione was administered for male infertility (600 mg/day glutathione intramuscularly for two months), or given to enhance insulin secretion in people with impaired glucose tolerance, did not report any significant adverse effects.[36,37] However, the adverse effects of intravenous glutathione have been documented from the Philippines, one of the leading consumers of glutathione. The Food and Drug Administration of Philippines have issued a position paper with a public warning regarding the safety of off-label use of glutathione injection [Box 5] and the adverse drug reactions reported from the use of intravenous glutathione for skin lightening [Box 6].[38] Proponents of intravenous glutathione suggest that these adverse effects may be attributed to other additives present in the glutathione injection vials and the risk is minimized if pure glutathione is used instead.

Another issue pertaining to pure and high-quality intravenous glutathione solution is the extremely high cost. The cheaper versions may be counterfeit, with the risk of life-threatening events. Considering the many limitations of intravenous glutathione, it is prudent that dermatologists refrain from administering such injections for skin lightening until further trials and high quality studies establish a favourable benefit versus risk ratio that justify its use [Box 7]. The recent surge of intravenous glutathione in India has prompted the media and health authorities to spread awareness about its potential complications, although a statutory ban remains elusive.

Other potential adverse effects of glutathione

Since glutathione is a component of human cellular metabolism, the adverse effects seen with oral supplementation are expected to be mild, akin to high-dose vitamin supplements. The adverse effects of

Box 5: Public warning issued by the Food and Drug Administration of the Philippines (12 May 2011)

"The alarming increase in the unapproved use of glutathione administered intravenously as a skin-lightening agent at very high doses is unsafe and may result in serious consequences to the health of users. There is inadequate safety documentation on the use of high doses of glutathione administered at 600 mg to 1.2 grams once weekly and even up to twice weekly. The only approved indication of the intravenous format of glutathione is an adjunctive treatment to reduce neurotoxicity associated with cisplatin chemotherapy"

Box 6: Adverse effects reported with intravenous glutathione injection by the Food and Drug Administration of the Philippines

Adverse cutaneous eruptions ranging from skin rashes, to serious and potentially fatal Stevens Johnson Syndrome (SJS) and toxic epidermal necrolysis (TEN)

Thyroid dysfunction

Kidney dysfunction with potential of development of renal failure; possibly due to high doses of intravenous glutathione overloading the renal circulation

Severe abdominal pain in a patient receiving twice-weekly IV glutathione

Apart from the adverse effects from the molecule, incorrect injection technique by untrained staff may lead to lethal complications such as air embolism, or potentially fatal sepsis. The usage of unsterile or used needles can lead to blood borne infections. Counterfeit intravenous glutathione may lead to infections

Box 7: Limitations of intravenous glutathione

Lack of any published or reliable source of evidence supporting the efficacy of intravenous glutathione in skin lightening
Undefined dose and duration of intravenous injections, excepting the recommendations of manufacturers, which have no apparent scientific basis
Need for indefinite, perhaps lifelong maintenance with either oral or intravenous GSH, even after the 'desirable' skin lightening has been attained
Multiple adverse effects reported
Lack of approval from US-FDA, and warning against the use of intravenous glutathione by the FDA of Philippines
High cost of injectable glutathione vials

US: United States, FDA: Food and Drug Administration

intravenous glutathione speculatively arise from the direct delivery of huge amounts of the molecule in the blood circulation. Other potential adverse effects of high dose and long-term glutathione supplementation include:

- Lightening of hair colour: A logically expected effect since hair colour is dependent on the amount and type of melanin which may be altered by glutathione supplementation. This adverse effect has not yet been clinically reported
- Hypopigmented patches, especially on sun-exposed areas have been observed after 10–12 doses of intravenous injection by practitioners (unpublished observations). Their experience suggested that the patchy hypopigmentation tended to resolve after 30-40 doses due to the evolution of a uniform skin-lightening effect
- Depletion of natural hepatic stores of glutathione: Hypothetically, long-term supplementation with any external synthetic compound may signal the body to stop its own production resulting in dependence on synthetic supplements.[39] Depletion of liver glutathione levels (the site of glutathione storage) may be devastating to health. This hypothetical adverse effect, although not clinically reported until now, is analogous to the hypothalamic-pituitary axis suppression seen with long-term use of systemic corticosteroids
- Exacerbation of *Helicobacter pylori* associated peptic ulcers: *Helicobacter pylori* is known to feed on macrophages and neutrophils abundant at the site of inflammation caused by the ulcer. As glutathione can improve the numbers and activity of macrophages, peptic ulcers may be exacerbated[40]
- Increased susceptibility to melanoma: Theoretically, long-term administration of systemic glutathione switches eumelanin to pheomelanin, and may increase the susceptibility towards development of melanoma in the long run.[28]

Summary of the role of glutathione as a skin-lightening agent

While there is no published data for intravenous glutathione, the results of the three randomized controlled trials mentioned above have provided grade Ib and 2b evidence in favour of the skin-lightening effects of topical and oral glutathione, with no significant adverse effects [Table 1]. However, larger and long-term studies are warranted to generate more evidence.

Role of glutathione in disorders of hyperpigmentation

At present, there are no publications that document improvement in any specific hyperpigmentation disorder with the use of topical or oral glutathione. The new-fangled concept of recommending glutathione as an adjuvant (orally, topically or as mesotherapy) for melasma, freckles and postinflammatory hyperpigmentation is based on its depigmenting properties detailed in Box 2. In a study that was conducted to evaluate the role of oxidative stress in melasma, the levels of glutathione peroxidase enzyme activity and other pro-oxidant parameters were significantly higher in the blood of patients compared to controls. This confirmed the role of oxidative stress in the pathogenesis of melasma.[16] Glutathione-peroxidase depletes the serum levels and cellular levels of glutathione. Thus, supplementation of glutathione is logically expected to downregulate melanogenesis and improve melasma. Based on the current level of evidence, other authors have also suggested the use of oral or topical glutathione as an adjunctive therapy for facial melanosis.[1,41] Further, topical compositions containing S-acyl glutathione (about 0.1–10% by weight) or S-palmitoyl glutathione (about 3–9% by weight) admixed with other depigmenting and anti-oxidant substances have been prepared. They are awaiting grant of a patent by the US Food and Drug Administration to be used for the treatment of melasma, freckles, lentigines and postinflammatory hyperpigmentation.[42] However, one should note that glutathione mainly affects the melanin indices and ultraviolet spots in the

sun-exposed areas. It only affects new melanogenesis and not pre-existing pigmentation.[28]

Role of glutathione in skin disorders other than hyperpigmentation

A decrease in the cellular and serum levels of glutathione has been speculated to be associated with the pathogenesis of autoimmune and inflammatory dermatoses that include psoriasis, vitiligo, alopecia areata, polymorphic light eruption, acne vulgaris, etc.[43-47] In addition, there is sufficient evidence demonstrating the importance of glutathione levels in the genesis of melanoma and related skin tumors.[48]

Future developments

Liposomal glutathione consists of the molecule encapsulated in water inside a fat ball with the intention of "tricking" the digestive system to interpret it as a fat cell. This prevents it from being hydrolysed thereby allowing it to enter the bloodstream. However, the lack of human trials, quick degradability of liposomes and safety concerns of soy lecithin (a liposomal component) are barriers against its current use.

S-acetyl-glutathione consists of oral glutathione attached to a sulfur atom. It is taken up intact by chylomicrons in the gut. The acetyl group prevents its oxidation and increases its plasma stability. Studies conducted in mice and human foreskin fibroblasts have revealed that S-acetyl-glutathione molecules are taken up directly by cells with subsequent conversion to glutathione by cleavage of the acetyl bond within the cell. This results in higher levels of intracellular glutathione.[49] S-acetyl-glutathione is also known to have antiviral and immunomodulatory properties.[50] However, there is no human data available to prove the superiority of S-acetyl-glutathione over plain glutathione for skin-lightening effects.

CONCLUSION

As of now, there is a lack of robust evidence in favour of glutathione for the treatment of hyperpigmentation. The mechanism of action favours its potential as a skin lightening agent. Only three randomized controlled trials have been conducted so far but with short term follow-up periods. These studies support some skin lightening effects of topical, as well as oral glutathione. The safety profile of topical and oral glutathione seems to be reasonable. The use of intravenous glutathione finds no evidence to support it and is further marred by its potential complications. The need of the hour is to have more randomized, double-blind, placebo-controlled trials with a larger sample size, long term follow-up period, with well defined primary and secondary outcomes, targeted to evaluate the efficacy and safety of the skin-lightening effects of topical, oral and parenteral glutathione. In addition, the role of glutathione in specific disorders of hyperpigmentation needs to be elucidated.

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Conflicts of interest

There are no conflicts of interest.

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The Role of Glutathione Metabolism in Chronic Illness Development and Its Potential Use as a Novel Therapeutic Target

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Abstract

Glutathione (GSH) is the most abundant thiol antioxidant in the human body and serves many important biochemical functions, including the regulation of vitamins, such as vitamins D, E, and C, and detoxification of drugs and toxins. As a powerful antioxidant, GSH is particularly important as a regulator of mitochondrial metabolism and a free radical scavenger that limits oxidative damage to cellular components. Low GSH levels have been associated with many chronic pro-inflammatory conditions, such as metabolic syndrome, cardiovascular, renal, and hepatic disease, as well as neurodegenerative conditions and autoimmune diseases. Given GSH's known direct protective role in mitochondrial metabolism and its association with chronic diseases of highly metabolically active tissues, this review aims to examine the literature for evidence that low GSH levels may be an important causative factor in the development of chronic illnesses. Because no large prospective human trials have been conducted using direct measurements of GSH, this review focused on the more common biomarker gamma-glutamyl transferase (GGT) which is directly correlated to low GSH levels. Several large prospective studies support this hypothesis by demonstrating that higher GGT levels are correlated with the risk of developing metabolic syndrome and cardiovascular disease in a dose-dependent fashion. Furthermore, as a corollary to this hypothesis, human and animal trials utilizing GSH augmentation using precursor supplementation in chronic conditions, including metabolic syndrome, cardiovascular disease, hepatic disease, renal disease, and neurodegenerative conditions, were also reviewed. While many of these trials were preliminary and small, there is strong evidence that GSH supplementation leads to improved outcomes in all of these chronic conditions. This review seeks to highlight these studies as preliminary evidence demonstrating the contributory role of GSH in chronic disease progression because a simple and cost-effective strategy can be created to screen for, track, and intervene in susceptible patients in the primary care setting at the earliest possible time in the disease process. Such a novel strategy would impact the majority of chronic diseases contributing to the bulk of morbidity and mortality in the Western world, and, thus, even minor benefits across many conditions may substantially impact population-wide health and longevity.

Categories: Endocrinology/Diabetes/Metabolism, Preventive Medicine, Public Health
Keywords: atherosclerotic cardiovascular disease, neurodegenerative disesase, chronic liver disease (cld), chronic renal disease, metabolic syndrome, ggt, n-acetylcysteine, glutathione

Introduction And Background

Glutathione (GSH) is a tripeptide molecule consisting of the amino acids glutamate, cysteine, and glycine. It is the most abundant thiol-containing antioxidant in the human body and exists in both a reduced and oxidized state in cells, with over 95% of GSH existing in its reduced state [1]. GSH was initially isolated from yeast in 1888 by J. de Rey-Paihade and subsequently found in animal tissues by Frederick Gowland Hopkins in 1921. Hopkins was also the first to identify the constituent amino acids of GSH in 1929 and was corroborated independently by Edward Calvin Kendall.

Subsequent research on the biological role of GSH has shown that it serves a critical role in protecting cells from oxidative damage by neutralizing damaging reactive oxidative species (ROS) in cells by reducing them before they can damage critical cellular components such as DNA. This antioxidant function plays an especially important role in mitochondria which generate ROS through the natural function of the electron transport chain, particularly during periods of metabolic stress. Human studies support the clinical importance of this relationship by demonstrating that an increased ratio of oxidized to reduced GSH is closely correlated with global levels of oxidative stress and inflammation [2]. Similarly, this is also seen in the setting of many chronic diseases such as metabolic syndrome and diabetes [3,4], atherosclerosis and cardiovascular disease [5], chronic kidney disease [6], neurodegenerative conditions such as Alzheimer's disease and Parkinson's disease [7-9], as well as autoimmune diseases [10], cancer [11] and chronic infections such as human immunodeficiency virus [12].

GSH also plays an important role in the detoxification of toxins, xenobiotics, and drugs. Decreased levels of GSH have been noted in alcoholics, smokers, and patients with chronic liver disease [13]. It is particularly important in acetaminophen overdoses where increasing GSH levels by supplementing the precursor N-

acetylcysteine (NAC) is the treatment of choice to clear the toxic intermediate metabolite N-acetyl-p-benzoquinone imine (NAPQI) via conjugation [14]. Unconjugated NAPQI which overwhelms the capability of endogenous GSH to reduce it can bind to cellular proteins damaging them in the process and inducing cell death [15]. Other important functions attributed to GSH include the regulation of immunity, the synthesis of leukotrienes and prostaglandins [16,17], and the regulation of vitamin D, E, and C metabolism [18,19]. Moreover, it plays an important role in glutamate neurotransmitter metabolism and neural regulation [20]. Because GSH is implicated in several biochemical processes and regulates other antioxidants, it is often referred to as the "master antioxidant."

Review

The biochemical role of glutathione as a driver of chronic illness

Low total GSH levels and elevated ratios of oxidized to reduced GSH are common in chronic illnesses as well as advanced age. While these relationships have been known for years, most literature has overlooked these findings as the predictable result of increased inflammation and oxidative stress similar to other biomarkers such as C-reactive protein (CRP). Few, if any, studies have explored the possibility of low GSH levels as a potentially important causative driver of disease pathology in itself [21]. However, due to the important biochemical role of GSH in protecting mitochondria from oxidative damage, this link should be re-examined. GSH protects mitochondria via the glutathione-ascorbate cycle, also known as the Asada-Halliwell pathway [22], which is the main pathway to neutralize the hydrogen peroxide radicals created as a byproduct of normal metabolism. Through this pathway, electrons are channeled from nicotinamide adenine dinucleotide phosphate (NADPH) to reduce hydrogen peroxide, with GSH as the rate-limiting step for regenerating NADPH [23,24].

Without adequate GSH to replenish NADPH, low NADPH levels result directly in increased ROS which can damage mitochondria, DNA, and other important cellular components [24,25]. This process is most pronounced in metabolically stressed cells, and the accelerated damage to these organelles can lead to premature dysfunction and early cell senescence, culminating in cell death [24]. Thus, in the most metabolically active tissues such as renal cells, hepatocytes, endocrine cells, endothelial cells, and neurons, low GSH levels should be expected to be directly correlated with worse cellular function and early cell death. In essence, the biochemical role of GSH in mitochondria is analogous to motor oil in a gasoline combustion engine, and it is a key protector of the cellular powerhouses from the damage caused by everyday metabolic demands. Without its protection, one can reasonably expect a diminished useful lifespan in many of the critical tissues which are necessary to support normal homeostasis.

The underlying biochemistry implicates low GSH as an important contributing factor in the development of several chronic illnesses that have been associated with increased oxidative stress such as metabolic syndrome, kidney disease, neurodegenerative diseases, liver disease, and cardiovascular diseases. Fundamentally, all of these conditions can be at least partially attributed to the dysfunction and failure of mitochondria in different highly metabolic tissues. This implies that GSH levels may be used as a useful predictive marker to identify patients who have an elevated risk of developing these chronic conditions and may also offer a novel way to delay or even outright arrest disease progression by replenishing endogenous GSH using simple dietary modifications and lifestyle changes at the earliest possible stage when such interventions are most beneficial. Interestingly, there is evidence in the literature that both of these hypotheses are true.

Evidence that glutathione metabolism can predict chronic disease development and mortality

Currently, there is very limited population-wide data on the potential predictive value of GSH levels because it is not a common clinical test and measuring the ratio of oxidized to reduced GSH in the body is time-consuming and expensive. The evidence for the possible predictive nature of GSH is seen in large studies utilizing the more common biomarker gamma-glutamyl transferase (GGT) and its correlation with increased all-cause mortality. GGT is a well-known biomarker typically associated with hepatocyte damage. Biochemically, GGT is associated directly with GSH regulation and maintenance of its normal redox status in cells by breaking down extracellular GSH which cannot cross the cellular membrane to release cysteine and glycine building blocks which can more easily cross into the cell for de-novo synthesis of GSH during periods of oxidative stress [26].

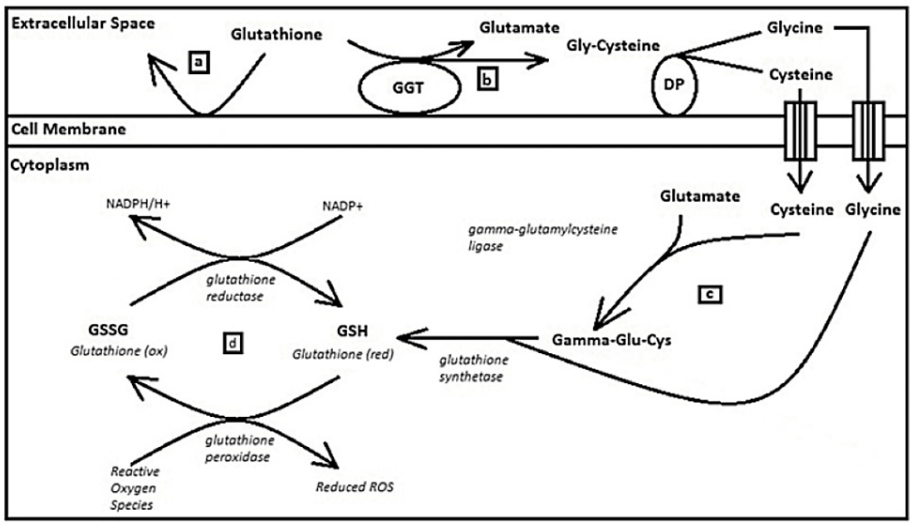

FIGURE 1: Extracellular glutathione is unable to cross the cell membrane as it is not lipophilic (a). In times of increased oxidative stress which may outstrip the ability of intracellular stores of glutathione to reduce all reactive oxygen species produced during metabolism, the cell will increase expression of gamma-glutamyl transferase bound to the cell membrane (b) as this enzyme will readily break down extracellular glutathione in unison with membrane-bound dipeptidases to its constituent amino acids glutamate, cysteine, and glycine. These amino acids readily cross the cell membrane and serve as building blocks for glutathione synthesis (c). Once new glutathione is synthesized, it can be used to reduce the damaging reactive oxygen species (d).

GSH: glutathione; GGT: gamma-glutamyl transferase

Thus, elevations in GGT on the external cellular membrane are correlated directly to relative GSH deficiency in the cellular cytoplasm of the host cell. Furthermore, while this marker is traditionally associated with hepatocytes, studies have shown that this marker is elevated in cardiovascular disease and chronic kidney disease [27-29]. Experimentally, this relationship has been verified in breast cancer patients with an r^2 of 0.6262 [30], supporting its use as a surrogate marker of low global GSH states in general.

Examining numerous liver function tests collected in the life insurance industry, Pinkham et al. showed that GGT levels had one of the best predictive dose-dependent negative correlations with all-cause mortality among a group of 560,000 life insurance applicants whose primary cause of death was cardiovascular disease [31]. In another study of life insurance data, Palmier et al. looked specifically at all-cause cancer mortality using a multivariate analysis of biochemical markers from life insurance applicants and found that liver function tests, specifically GGT and alkaline phosphatase (ALP) were most predictive of all-cause cancer mortality [32]. In their analysis, the hazard ratio (HR) for cancer mortality in 40-49-year-old males nearly doubled from 0.75 in the population with GGT levels in the 15-20 U/L range to about 1.45 in the patients with GGT levels in the 45-85 U/L range. While both of these studies were retrospective, the predictive utility of GGT has been demonstrated in prospective trials as well. In a trial of 3,124 subjects free of liver disease who were followed for an average of 40 months, Monami et al. showed that elevations in GGT over 40 U/L were associated with increased incidence of diabetes diagnosis (HR = 2.54 (1.26-5.11); p = 0.05) and cardiovascular disease diagnosis (HR = 2.21 (0.98-5.43); p = 0.10) [33]. Lee et al. corroborated these findings in more detail in the Framingham Heart Study which examined 3,451 participants over 19 years who were stratified into four groups based on GGT level. The lowest quartile of patients had GGT values between 1 U/L and 11 U/L in men and 1 U/L and 6 U/L in women, and the highest quartile included men with GGT values from 25 U/L to 99 U/L and women with GGT values from 14 U/L to 88 U/L. In this study, groups with higher GGT levels showed dose-dependent elevations in all-cause mortality and cardiovascular disease when adjusted for age, sex, body mass index (BMI), blood pressure, alcohol consumption, and smoking. The quartile cohort with the highest GGT elevation had a 23.8% incidence of cardiovascular disease compared to the lowest quartile which had a 10.5% incidence (HR = 2.11). For all-cause mortality, the incidence was noted to be 16.1% in the highest quartile group compared to 6.3% in the lowest quartile group (HR = 2.21)

[27]. Because GGT elevations correlated in a relatively tight and dose-dependent relationship with the outcomes tracked in both of these studies, these findings are suggestive of an underlying mechanism of causation.

Evidence that glutathione augmentation can delay or reverse chronic illness progression

Whereas population-wide studies tracking GGT illustrate the predictive potential for chronic diseases and mortality of decreased GSH levels, other studies utilizing NAC to increase cellular GSH stores have demonstrated that it is possible to intervene and have meaningful improvements in the same conditions. Oral GSH has extremely poor oral availability and is not lipophilic to easily cross the cell membrane and thus cannot be used to effectively increase systemic intracellular GSH levels. Instead, precursor supplementation with the rate-limiting precursor amino acid building block NAC is typically used in studies to increase endogenous GSH levels. NAC has been validated experimentally to increase GSH levels and redox homeostasis in patients with GSH deficiency [34] and is often combined with glycine (another GSH precursor amino acid) in some study protocols for enhanced efficacy.

In liver disease, NAC administration was first utilized and proven efficacious in acute conditions. It was first suggested as a possible treatment for acetaminophen toxicity in 1974 [35], and its efficacy was demonstrated by Smilkstein et al. in 1988 in a landmark trial that used oral NAC in 2,540 patients suffering from acetaminophen poisoning [36]. The study showed zero mortality, improved hepatic function, and faster recovery in patients who began treatment within 16 hours of acetaminophen ingestion. In more recent studies, the benefits of NAC have been demonstrated in acute liver failure from non-acetaminophen causes, including viral infection and other drugs [37]. Furthermore, NAC administration has been associated with improved transplant-free survival in liver transplant patients [38], possibly by attenuating the damage from ischemia and reperfusion of the grafts.

The benefits of NAC administration in heterogeneous causes of acute liver failure demonstrate that GSH augmentation can improve clinical outcomes in diverse conditions that lead to hepatic oxidative stress and not just acute acetaminophen toxicity. Analogously, improved outcomes should extend to more chronic inflammatory conditions as well. Indeed, using a rat model, Ozaras et al. demonstrated that supplementation with NAC in animals undergoing oxidative stress from ethanol infusions led to decreased inflammatory markers such as aspartate aminotransferase (AST), alanine transaminase (ALT), and GGT dramatically [39]. In this study, the decreases in the markers approached the levels of the control group without any alcohol infusion. Human trials of NAC in chronic liver disease are sparse. However, two small studies in patients with liver disease from chronic hepatitis B infection and non-alcoholic fatty liver disease reported some benefits. In 90 patients with acute-on-chronic hepatitis B infections, Wang et al. were able to show decreases in bilirubin, GGT, and ALP and improved coagulation profiles as well as decreased intrahepatic cholestasis in the treatment group [40]. Furthermore, Khoshbaten et al. followed 15 patients with non-alcoholic fatty liver disease for three months and showed a decrease in ALT in the treatment group and, more importantly, a significant decrease in spleen size which was correlated with the degree of fatty infiltration of the liver [41].

Metabolic syndrome is another chronic condition in which NAC administration has been shown to be beneficial. An open-label pilot study by Rani et al. utilized a six-month course of NAC supplementation in diabetic patients and showed beneficial effects, including improved glucose control via decreased HbA1C, decreased blood pressure, decreased CRP, decreased triglycerides, and increase in high-density lipoprotein compared to control group patients [42]. Another pilot study by Kumar et al. using NAC and glycine supplementation for 24 weeks in eight geriatric patients reported decreased insulin resistance, body fat, and waist circumference [43]. This study also demonstrated decreases in oxidative stress levels and endothelial dysfunction, exercise strength, and cognition in the intervention group. Some of the biochemical mechanisms underlying these improvements were experimentally shown by Alnahdi et al. in vitro to be due to the minimization of the glucolipotoxicity effects and mitochondrial dysfunction caused by hyperglycemia in pancreatic beta cells [44].

Although no large human studies have been undertaken to examine the effects of NAC on the cardiovascular system long term, there are human randomized trials showing a decrease in ischemic damage by 60% after myocardial infarction in patients undergoing percutaneous coronary intervention for acute ST-elevation myocardial infarction in a randomized control trial by Pasupathy et al. [45]. A small pilot randomized control study by Marian et al. showed small improvements in measures of cardiac function in 24 patients with hypertrophic cardiomyopathy who were given NAC for 12 months compared to a control group of 11 patients [46]. However, the results of this study were not statistically significant as the study was underpowered. Other studies in animals have provided strong evidence of the potential benefits of NAC on heart disease. In a rabbit model of heart failure induced by doxorubicin administration of NAC and glycine, Wu et al. demonstrated increased GSH levels as well as decreased nuclear factor kappa B activity corresponding with overall decreased oxidative stress on the cardiomyocytes and decreased myocyte apoptosis [47]. More importantly, these findings corresponded clinically with improved cardiac function in vivo, as assessed by echocardiography. In a mice study looking at NAC supplementation in atherosclerosis, Cui et al were able to show decreased in-vivo oxidation of low-density lipoprotein in the experimental group which clinically

corresponded with atherosclerotic plaque formation [48].

In renal disease, most trials utilizing NAC have typically focused on minimizing acute kidney injury due to contrast-induced nephropathy (CIN). Overall, these studies have reported mixed results, with a recent meta-analysis of 86 randomized controlled trials by Subramanian et al. showing that there may be a small benefit of low-dose NAC administration on CIN [49]. In chronic hemodialysis patients, NAC administration has been shown to decrease circulating homocysteine levels [50,51] as well as decrease other inflammatory markers, including asymmetric dimethylarginine and malondialdehyde. Other benefits noted in these studies include improved renal anemia [51,52]. Interestingly, Tsai et al. showed that NAC administration decreased homocysteine levels the greatest in patients with some preserved renal function and had minimal effects in completely anuric patients, which supports that the beneficial effects seen are due to improvement in nephron function [50]. This was corroborated by Ahmadi et al. who showed improvements in residual glomerular filtration rate in a separate randomized control trial [53]. In animal studies, NAC administration also has shown beneficial immune-modulatory effects on lymphocytes [54] and decreased interstitial fibrosis and reperfusion injury in mice models [55].

Neurodegenerative conditions such as Alzheimer's and Parkinson's disease are associated with neurofibrillary tangles and associated inflammatory oxidative stress. Small randomized controlled studies have shown benefits in both of these diseases. In patients with Parkinson's disease, small randomized controlled trials of NAC and cysteine supplementation have shown an increase in dopamine binding in the caudate and putamen, as measured by ioflupane imaging [56]. In patients with Alzheimer's disease, supplementation with NAC has been correlated with improved measures of cognitive function after three and six months on standardized assessments [57]. The neuroprotective effect of NAC has been also illustrated in rat models showing that it downregulates inflammatory markers and upregulates expression of the protective gene *Sirtuin 1* [58].

Interestingly, the neuroprotective effects of NAC are also associated with notable benefits in some chronic psychiatric conditions which are known to be associated with low GSH levels such as schizophrenia and autism. In patients with schizophrenia, randomized trials of NAC administration have shown decreases in the Positive and Negative Syndrome Scale total score, negative symptom factor, and disorganized thought factor compared to the placebo group [59]. In autism spectrum disorders, a meta-analysis by Lee et al. of four trials showed overall decreases in hyperactivity and irritability and increases in social awareness in children treated with NAC [60].

Overall, studies utilizing NAC supplementation for chronic illnesses are few and tend to be smaller pilot trials. Nevertheless, when taken together, this body of literature provides the strongest evidence that GSH plays a key role in many disparate conditions which may seem unrelated at first glance. More importantly, the evidence supports that augmenting GSH levels can lead to improved outcomes and presents a novel adjunct treatment modality that is currently not being utilized.

Potential use of glutathione metabolism in patient care

Although GSH metabolism is not directly monitored in primary care as a marker for health, the biochemical function of this molecule combined with supporting evidence from NAC trials suggests that it should. The best study supporting this assertion is by Kumar et al. [43]. This small randomized trial of elderly patients who were supplemented with NAC and glycine showed improvements in baseline insulin resistance, cognitive performance, endothelial dysfunction, muscle strength, and mitochondrial oxidative stress. While this study demonstrated the potential benefits of GSH augmentation in an elderly cohort, a future study design utilizing direct or indirect GSH level measurement in larger and more heterogeneous populations could be undertaken to more precisely identify which patients may benefit from such therapy while sparing others who may have sufficient levels of GSH from unnecessary intervention. Similar randomized controlled trials looking at vitamin D supplementation in critically ill patients have demonstrated a survival benefit in select patients who were deficient on admission, but not in patients with normal levels at the time of admission [61].

Ultimately, additional studies would need to be undertaken to identify how to optimize screening and interventions for patients as well as to provide larger population-wide data on total GSH levels, reduced to oxidized GSH levels, as well as GGT. This would no doubt necessitate many longer-term, population-wide studies. However, by examining GSH metabolism in a new way, we may be able to identify chronic diseases at the earliest possible stage of development and intervene with low-cost strategies when there is the greatest potential impact to slow down their progression. Because the chronic diseases associated with GSH deficiency are also the leading causes of death today, it serves to reason that even small potential health benefits across many chronic illnesses would have a meaningful impact in decreasing population-wide morbidity and mortality.

Conclusions

GSH is a key thiol antioxidant in the human body which, among its many functions, serves as a major mitochondrial protector, and through this function is linked to many chronic illnesses which make up the

bulk of the healthcare burden in Western societies today. Studies presented in this review show that low GSH levels have a demonstrable correlation to the faster onset of these chronic diseases and increased mortality. Fortunately, other studies have reported that GSH levels can be easily augmented in patients with elevated metabolic stress states by supplementing with its precursor-building amino acids NAC and glycine. These studies have also shown measurable clinical benefits in the study participants across many different and unrelated conditions. This review aimed to present this literature because GSH metabolism can present a novel target to identify and treat high-risk patients at the very earliest stage of the disease development process with a simple, safe, and cheap intervention. If large future randomized controlled trials can confirm these findings and a population-wide algorithm could be created and applied, then the cumulative benefits across many conditions could be dramatic even if the measurable benefits of treatment are relatively small.

Additional Information

Disclosures

Conflicts of interest: In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

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ORIGINAL RESEARCH

Glutathione and its antiaging and antimelanogenic effects

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Background: Previous studies showed that supplementation of reduced form of glutathione (GSH, 500 mg/d) has a skin-lightening efficacy in humans. This study was designed to evaluate the influences of both GSH and oxidized form (GSSG), at doses lower than 500 mg/d, on improving skin properties.

Patients and methods: A randomized, double-blind, placebo-controlled, parallel, three-arm study was conducted. Healthy female subjects were equally randomized into three groups and took GSH (250 mg/d), GSSG (250 mg/d), or placebo orally for 12 weeks. At each visit at baseline and for 12 weeks, skin features including melanin index, wrinkles, and other relevant biophysical properties were measured. Blood samples were collected for safety monitoring.

Results: In generalized estimating equation analyses, melanin index and ultraviolet spots of all sites including face and arm when given GSH and GSSG tended to be lower than placebo. At some sites evaluated, subjects who received GSH showed a significant reduction in wrinkles compared with those taking placebo. A tendency toward increased skin elasticity was observed in GSH and GSSG compared with placebo. There were no serious adverse effects throughout the study.

Conclusion: We showed that oral glutathione, 250 mg/d, in both reduced and oxidized forms effectively influences skin properties. Overall, glutathione in both forms are well tolerated.

Keywords: glutathione, melanin, pigment, aging, wrinkle, whitening

Introduction

The quest for means to alter skin color is endless. Caucasians seek ways to tan their skin, while many darker skin-type individuals are always in search of whitening or lightening agents.

Numerous topical agents available for melasma treatment are also used to lighten the skin color. However, as many people would prefer their skin to be thoroughly fairer, oral or even intravenous agents are administered to obtain these results. One of the widely used, systemic agents is glutathione, a thiol compound and one of the regulators of melanogenic pathway in the human system.

Glutathione is an antioxidant present in almost every cell in the body, playing a role in the detoxification of drugs and xenobiotics.[1] Furthermore, reduced glutathione (GSH) acts as a hydrogen donor in the detoxification of hydrogen peroxide.[2] As a dietary supplement, GSH possesses various systemic effects such as improvement of liver abnormalities,[3,4] improvement of diabetic complication,[5] protection from viral infection,[6] and antitumor activity.[7,8] It is even used to treat autism.[9]

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In vitro experiments have demonstrated that glutathione is related to melanogenesis.[10–13] Its antimelanogenic properties result from a variety of mechanisms including stimulation of pheomelanin synthesis rather than darker eumelanin, its antioxidant effects,[14] and interference with intracellular trafficking of melanogenic enzymes.[15] Glutathione also possesses certain antiaging properties.[16]

Glutathione is generally a safe ingredient for use as a dietary supplement. An oral acute toxicity study of GSH in mice found that the lethal dose 50 (LD50) was more than 5 g/kg, indicating that glutathione is nontoxic. In many clinical trials, no serious adverse reactions have been observed.[9,17–19] On the contrary, it can even reverse the toxic effects following excessive intake of other amino acids.[20]

In the human body, glutathione exists in two forms, reduced and oxidized (GSSG), which can be readily converted to each other. However, it is not clear whether the two forms are physiologically similar, especially when melanogenesis is concerned. Moreover, efficacy and long-term safety of either form have not been examined systematically.

Glutathione is regarded as food or health supplements in several countries including the Philippines, Malaysia, Taiwan, and Thailand, while it is considered a pharmaceutical agent in Korea, Japan, and People's Republic of China. Our group previously reported that oral GSH administration (500 mg/d) resulted in lightening of skin color, when given for 4 weeks.[21] The main objective of this study was to find out whether glutathione, in the reduced and oxidized forms, maintains its skin-lightening efficacy when given at a dose of 250 mg/d for 12 weeks, a dosage allowed by the Thai and Taiwanese Food and Drug Administrations.

Patients and methods

Study design

The study protocol was conducted in accordance with the Declaration of Helsinki, in compliance with the International Conference on Harmonization - Good Clinical Practice and reviewed and approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University. Clinical study was conducted at Chula Clinical Research Center, Faculty of Medicine, Chulalongkorn University. A randomized, double-blind, placebo-controlled, parallel, three-arm study was applied. The subjects were equally block-randomized into three groups: GSSG (250 mg/d), GSH (250 mg/d), or placebo. Each subject received their assigned capsules in identical packages at weeks 0, 4, and 8, with 30 capsules per visit (two extra capsules per package). The subjects were informed to take the study capsules once before bedtime. Subjects returned for evaluations at weeks 4, 8, and 12.

Subjects

Sixty healthy volunteers, aged between 20 and 50 years, were eligible for this study. They were residents of Bangkok and recruited through the dermatology clinic at King Chulalongkorn Memorial Hospital. Only female volunteers were enrolled to reduce gender variability and also because females are by far the majority of individuals who seek skin-whitening agents. All volunteers were found to be healthy based on their medical history and physical and clinical laboratory examination including serology, hematology, and biochemistry tests. All volunteers had to abstain from other medications, supplementary vitamins, and alcohol intake for 2 weeks prior to enrollment and throughout the study. The methods and conditions of the study were clearly explained to all volunteers. Signed written informed consent was obtained from each volunteer before screening processes for this study. However, each subject had the right to withdraw their consent at any time.

Study medications

Daily doses of GSSG (AquaGluta™; 250 mg/d), GSH (Setria®; 250 mg/d), or dibasic calcium phosphate as placebo in identical capsules and packages were provided by Kyowa Hakko Bio Co., Ltd (Tokyo, Japan). The total weight of the three capsules for each group was the same.

Objective evaluation of skin properties

At each visit, subjects rested in a room controlled at a temperature of 21 ± 3°C for 20 minutes before assessments. Prior to the evaluation, they washed their face with soap and water, pat-dried with paper towel, and waited 5–10 minutes for air drying.

Melanin index

For objective evaluation of skin color, melanin index as determined by Mexameter (Courage-Khazaka Electronic, Koln, Germany) was used as the primary outcome. All measurements were done in triplicate at six sites to represent the skin of the sun-exposed and sun-protected areas as follows:

1. Sun-exposed areas
   - Face: left and right; 2.5 cm caudally from the lateral canthi.
   - Extensor surfaces of the forearms, left, and right; 7 cm above the ulnar styloid processes.
2. Sun-protected areas
   - Upper, inner arms, left, and right; 10 cm from the axillary vault.

VISIA™ CR system

Standardized digital photographs were taken by the VISIA™ CR system (Canfield Scientific, Fairfield, NJ, USA), a software which was also used to quantitatively evaluate ultraviolet (UV) spots, pores, and evenness on the left and right sides of the face.

Transepidermal water loss (TEWL)

TEWL was measured using Tewameter® TM300 (Courage+Khazaka Electronic GmbH, Köln, Germany). All measurements were done in triplicate at sites designated for melanin index to represent the skin of the sun-exposed and sun-protected areas.

Water contents (Corneometer)

Water contents were measured by Corneometer® CM825 (Courage+Khazaka) in triplicate at sites as mentioned earlier.

Elasticity

Elasticity was measured by Cutometer MPA580® (Courage+Khazaka) in triplicate at the sites mentioned previously.

Wrinkle

Wrinkle formation was objectively measured by Visioscan® (Courage+Khazaka).

Subjective evaluation of skin properties

For global evaluation, at each visit, subjects were asked to grade the overall response using a 4-point rating scale: 4 = very satisfactory, 3 = moderately satisfactory, 2 = minimally satisfactory, and 1 = not satisfactory. In the questionnaires, the following features/items were addressed: skin lightening, facial skin evenness, pigmented spots lightening, pore size improvement, skin smoothness, wrinkle reduction – crow's feet, wrinkle reduction – nasolabial folds, wrinkle reduction – forehead, general skin condition, fatigability, sleep (e.g., quality and length)

Safety

To evaluate the safety of subjects, vital signs were determined at each visit. Also, at all visits, a blood aliquot of 7 mL was drawn for complete blood cell counts, chemistry, and lactate dehydrogenase.

Statistical analysis

Paired *t*-test was used to compare baseline values with those of the final visit. The generalized estimating equation (GEE) was performed to investigate how the efficacies of GSSG, GSH and placebo varied over time before and after treatment. Subjective evaluations of the two glutathione preparations and placebo were compared by analysis of covariance (ANCOVA), with the baseline values as covariates. Statistically significant level was defined as *P*-value <0.05 (two-tailed). Analyses were performed using STATA software version 11.0 (StataCorp, College Station, TX, USA).

Results

Sixty volunteers were enrolled in the study. Three volunteers had to terminate, two due to elevation in liver function tests and one due to unanticipated start of oral contraceptive pills. Fifty-seven volunteers were included for final analysis. The majority of subjects had skin phototype IV (96.4%). Subjects' demographic data are summarized in Table 1.

A total of 18 subjects received GSSG, 20 received GSH, and 19 received placebo. Mean baseline measurements (Mexameter, VISIA, Tewameter, Corneometer, Cutometer, Visioscan) in the three groups at six sites were not significantly different (ANCOVA, $P > 0.05$).

The GEE was performed to investigate how the efficacies of GSSG, GSH, and placebo varied over time during treatment (Table 2). As our subjects were recruited from a broad age range, we also decided to categorize the age groups into those younger and older than 40 years.

Table 1 Baseline demographics

Characteristics	Number (%) of patients
Age distribution (year)	
20–30	17 (29.8%)
31–40	20 (35.1%)
41–50	20 (35.1%)
Age (year)	
Mean ± SD	36.1 ± 8.1
Median	36
Min/max	21/48
Hair color	
Black	48 (84.2%)
Brown	9 (15.8%)
Skin phototype	
III	1 (1.8%)
IV	55 (96.4%)
V	1 (1.8%)
Homogeneity of facial skin color	
Homogenous	26 (45.6%)
Not homogenous	31 (54.4%)
Treatment	
GSSG	18 (31.6%)
GSH	20 (35.1%)
Placebo	19 (33.3%)

Abbreviations: SD, standard deviation; GSSG, oxidized glutathione; GSH, reduced glutathione.

Table 2 GEE analysis

Variables	Placebo vs. GSSG		Placebo vs. GSH	
	Mean difference	*P*-value	Mean difference	*P*-value
Mexameter				
Sun-exposed face, right	18.4241	N.S.	23.7472	N.S.
Sun-exposed face, left	18.9250	N.S.	18.8436	N.S.
Sun-exposed arm, right	15.7356	N.S.	37.0054	0.072
Sun-exposed arm, left	8.4700	N.S.	31.9076	0.091
Sun-protected arm, right	9.4779	N.S.	18.8066	N.S.
Sun-protected arm, left	7.4993	N.S.	15.1403	N.S.
Mexameter (age >40 years)				
Sun-exposed face, right	−10.6149	N.S.	−18.8602	N.S.
Sun-exposed face, left	−9.4868	N.S.	−23.2070	N.S.
Sun-exposed arm, right	−38.3149	N.S.	60.4821	0.031*
Sun-exposed arm, left	−43.4550	N.S.	53.7161	0.057
Sun-protected arm, right	−18.9622	N.S.	31.2671	0.078
Sun-protected arm, left	−25.8103	N.S.	17.7876	N.S.
VISIA				
Sun-exposed face, right	23.1060	N.S.	5.2296	N.S.
Sun-exposed face, left	21.1206	N.S.	7.9414	N.S.
VISIA (age >40 years)				
Sun-exposed face, right	−0.8889	N.S.	−6.8889	N.S.
Sun-exposed face, left	−18.5556	N.S.	−10.0198	N.S.
Tewameter				
Sun-exposed face, right	0.2515	N.S.	−0.3893	N.S.
Sun-exposed face, left	−0.0641	N.S.	−0.0011	N.S.
Sun-exposed arm, right	−0.5992	N.S.	0.0108	N.S.
Sun-exposed arm, left	−0.4294	N.S.	−0.1032	N.S.
Sun-protected arm, right	−0.6957	N.S.	−0.3497	N.S.
Sun-protected arm, left	−0.1941	N.S.	0.1534	N.S.
Tewameter (age >40 years)				
Sun-exposed face, right	−0.8032	N.S.	−0.5919	N.S.
Sun-exposed face, left	−1.4715	N.S.	−0.3575	N.S.
Sun-exposed arm, right	−0.2515	N.S.	−0.0403	N.S.
Sun-exposed arm, left	−0.0561	N.S.	−0.4224	N.S.
Sun-protected arm, right	−0.1284	N.S.	−0.3981	N.S.
Sun-protected arm, left	0.5856	N.S.	−0.0566	N.S.
Corneometer				
Sun-exposed face, right	0.3321	N.S.	3.6350	N.S.
Sun-exposed face, left	−0.2904	N.S.	2.4277	N.S.
Sun-exposed arm, right	3.0026	N.S.	3.9812	N.S.
Sun-exposed arm, left	2.8878	N.S.	3.0428	N.S.
Sun-protected arm, right	1.2492	N.S.	2.1739	N.S.
Sun-protected arm, left	1.3142	N.S.	3.8116	0.031*
Corneometer (age >40 years)				
Sun-exposed face, right	5.4455	N.S.	9.9433	0.048*
Sun-exposed face, left	4.6751	N.S.	7.9991	0.090
Sun-exposed arm, right	6.0388	N.S.	1.5504	N.S.
Sun-exposed arm, left	5.6142	N.S.	0.4824	N.S.
Sun-protected arm, right	3.5240	N.S.	0.1463	N.S.
Sun-protected arm, left	5.4652	0.052	0.1840	N.S.
Cutometer				
Sun-exposed face, right	−0.0372	0.082	0.0036	N.S.
Sun-exposed face, left	−0.0283	N.S.	−0.0116	N.S.
Sun-exposed arm, right	−0.0031	N.S.	−0.0088	N.S.
Sun-exposed arm, left	0.0001	N.S.	−0.0029	N.S.
Sun-protected arm, right	−0.0006	N.S.	−0.0052	N.S.
Sun-protected arm, left	0.0007	N.S.	−0.0010	N.S.
Cutometer (age >40 years)				
Sun-exposed face, right	−0.0395	N.S.	0.0089	N.S.
Sun-exposed face, left	0.0288	N.S.	−0.0052	N.S.
Sun-exposed arm, right	−0.0060	N.S.	−0.0008	N.S.
Sun-exposed arm, left	−0.0095	N.S.	−0.0096	N.S.
Sun-protected arm, right	−0.0097	N.S.	−0.0105	N.S.
Sun-protected arm, left	−0.0086	N.S.	−0.0029	N.S.
VISIO				
Sun-exposed face, right	−0.0542	N.S.	0.4993	N.S.
Sun-exposed face, left	0.0601	N.S.	1.1137	N.S.
Sun-exposed arm, right	0.5420	N.S.	0.7263	N.S.
Sun-exposed arm, left	0.7505	N.S.	0.8105	N.S.
Sun-protected arm, right	0.6234	N.S.	0.2277	N.S.
Sun-protected arm, left	0.7887	N.S.	2.3124	0.006*

Table 2 (*Continued*)

Variables	Placebo vs. GSSG		Placebo vs. GSH	
	Mean difference	*P*-value	Mean difference	*P*-value
VISIO (age >40 years)				
Sun-exposed face, right	−0.8735	N.S.	0.5056	N.S.
Sun-exposed face, left	0.1897	N.S.	1.3635	N.S.
Sun-exposed arm, right	−0.1998	N.S.	1.3337	N.S.
Sun-exposed arm, left	0.4910	N.S.	2.1052	0.043*
Sun-protected arm, right	1.6827	N.S.	0.8387	N.S.
Sun-protected arm, left	0.2899	N.S.	2.5568	0.066

Note: *$P<0.05$.
Abbreviations: GEE, generalized estimating equation; N.S., not significant; GSSG, oxidized glutathione; GSH, reduced glutathione.

Melanin index

In all subjects with an age range between 20 and 50 years, GEE model showed that melanin index and UV spots of all sites including face and arm from GSSG and GSH groups tended to be lower than placebo group (Table 2) but were not statistically significant ($P > 0.05$). There were no significant differences between GSSG and GSH groups. The subgroup analysis of middle-aged individuals showed that the melanin index of sun-exposed right forearm of subjects aged >40 years who received GSH (N = 7) was significantly lower than the index of those who received placebo (N = 10, $P = 0.031$). Melanin index measured at the sun-exposed left forearm from those receiving GSH was also lower than those receiving placebo. However, this did not reach statistically significant level ($P = 0.057$).

TEWL and water content

TEWL measurement of sun-exposed right forearm of the GSH group was significantly lower than that of GSSG group ($P = 0.044$). However, the water contents of sun-protected left arm of those who received GSH were lower than that of those who received placebo ($P = 0.031$). This was also true for subjects aged >40 years, for the sun-exposed right face ($P = 0.048$).

Wrinkles

Visioscan measurements of sun-protected left arm of subjects in the GSH group were significantly lower than those of the placebo group ($P = 0.006$). This was also true for those aged >40 years when measurements were taken at the sun-exposed left forearm ($P = 0.043$). A similar trend was seen for GSH vs. placebo for the sun-protected left arm, in advanced age group ($P = 0.066$).

Elasticity

Although statistically significant differences could not be demonstrated, GSSG and GSH supplementation tended to increase skin elasticity. Especially, the elasticity of sun-exposed right face of those who received GSSG was notably higher than the elasticity of those who received placebo ($P = 0.082$).

Subjects were asked to fill the questionnaires to subjectively evaluate skin properties at each visit. Satisfaction was scored as rating scale. There were no statistically significant differences in any of the ratings among the three groups ($P > 0.05$, ANCOVA).

Compliance

Compliance was not an issue in the present study. All subjects took the capsules as directed and assessed by the protocol throughout the study.

Adverse events

Adverse reactions included pruritus, macular erythema, transient minute red spots on the skin, and tiredness. In the treatment groups combined (GSSG, GSH), these occurred in five patients (13.15%), which included three with pruritus (7.89%), one with erythema (2.63%), three with red spots (7.89%), and one with tiredness (2.63%). In the placebo group, there were two incidents of pruritus (10.52%), one erythema (5.26%), and three red spots (15.79%) (Table 3). No serious adverse events took place. The two incidents of transaminitis were temporary and the liver function tests returned to normal within a few weeks. Detailed blood parameters are described in Table 4.

Discussion

Our group has previously demonstrated that oral glutathione, 500 mg/d, can reduce skin pigmentation after 4 weeks'

Table 3 Adverse events

Treatment	n	AE (%)	Pruritus (%)	Erythema (%)	Red spot (%)	Tiredness (%)
GSSG	18	2 (11.11)	1 (5.56)	0	2 (11.11)	0
GSH	20	3 (15.0)	2 (10.0)	1 (5.0)	1 (5.0)	1 (5.0)
Placebo	19	4 (21.05)	2 (10.53)	1 (5.26)	3 (15.79)	0
Total	57	9 (15.79)	5 (8.77)	2 (3.51)	6 (10.53)	1 (1.75)

Abbreviations: AE, adverse event; GSSG, oxidized glutathione; GSH, reduced glutathione.

Table 4 Blood parameters

Laboratory parameters		Baseline	EOT
WBC	Missing – n (%)	0	0
	Mean ± standard error	6.5 ± 1.5	6.4 ± 1.5
RBC	Missing – n (%)	0	0
	Mean ± standard error	5.0 ± 1.4	4.6 ± 0.4
Hb	Missing – n (%)	0	0
	Mean ± standard error	13.3 ± 2.2	12.6 ± 1.2
Platelets	Missing – n (%)	0	0
	Mean ± standard error	283.8 ± 66.2	280.4 ± 66.7
AST	Missing – n (%)	0	0
	Mean ± standard error	17.7 ± 7.5	18.1 ± 7.6
ALT	Missing – n (%)	0	0
	Mean ± standard error	16.6 ± 11.6	16.3 ± 11.1
GGT	Missing – n (%)	0	0
	Mean ± standard error	19.5 ± 11.0	21.9 ± 14.2
TP	Missing – n (%)	0	0
	Mean ± standard error	1.3 ± 0.4	1.4± 0.4
TC	Missing – n (%)	0	0
	Mean ± standard error	206.0 ± 35.2	204.1 ± 32.2
TG	Missing – n (%)	0	0
	Mean ± standard error	88.8 ± 41.5	87.7 ± 40.5
LDH	Missing – n (%)	0	0
	Mean ± standard error	154.0 ± 26.3	158.3 ± 23.2
BUN	Missing – n (%)	0	0
	Mean ± standard error	11.1 ± 2.7	10.5 ± 2.8
Cr	Missing – n (%)	0	0
	Mean ± standard error	0.8 ± 0.1	0.8 ± 0.1

Abbreviations: EOT, end of treatment; WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; AST, aspartate transaminase; ALT, alanine transaminase; GGT, gamma-glutamyl transferase; TP, total protein; TC, total cholesterol; TG, triglyceride; LDH, lactate dehydrogenase; BUN, blood urea nitrogen; Cr, creatinine.

administration in young, otherwise-healthy medical students.[21] Watanabe et al also demonstrated that topically applied GSSG can significantly reduce melanin indices.[22] Recently, Handog et al investigated the use of intraoral lozenge containing 500 mg of glutathione in an open-label study and demonstrated significant skin lightening after 8 weeks of administration.[23]

It is well established that glutathione can be transported across the intestinal epithelium after oral supplementation,[9,19] yet the fate of orally administered GSH is to be resolved as it is readily oxidized within the human body. On the contrary, its oxidized counterpart is much more stable. Until very recently, most studies were not able to detect blood or plasma glutathione, despite large doses of oral intake.[17] However, Park et al demonstrated that although no glutathione could be measured in the whole plasma compartment, GSH could be detected in the protein-bound fraction of the human blood between 60 and 120 minutes after oral intake.[24]

In this study, we have demonstrated that both GSSG and GSH exerted their effects on melanin indices, which reached statistically significant levels at specific site and higher age group. This is in agreement, yet to some degree dissimilar, to our earlier study, the explanations for which are several-fold. First, the dose of glutathione used in this study is half of that used in the prior study. This is to comply with the daily dosage of L-glutathione allowed in some countries including Thailand. Second, the subjects recruited in this study are all females and of more advanced age. Interestingly, with subgroup analysis, the changes in subjects aged more than 40 were even more pronounced than when the whole group was analyzed. Being affected with more photodamage can definitely affect the final outcomes measured, especially when pigment is concerned.

Our results also showed that GSH was significantly superior to placebo in its ability to improve wrinkles, at least at some anatomic locations. This is an extremely interesting and novel finding as cutaneous aging is a significant problem faced by the majority of people of any age. As the world's populations are rapidly heading toward an aging society and human life spans are increasing, this problem will certainly be of greater magnitude in the foreseeable future. Having to apply topical antiwrinkle preparation to the entire skin is both costly and in many circumstances impractical for the elderly, especially when compared with popping a pill.

Also of importance are the findings that both forms of glutathione showed trends in increased skin elasticity at various sites, both sun-exposed and sun-protected skin. These findings have never been reported before and deserve further investigations in larger populations.

Because glutathione has regulatory properties on melanogenesis and antioxidants in general are protective against aging process, the "dual" antimelanogenic and antiaging properties demonstrated in Watanabe's and our studies are not surprising. In fact, the link between melanization and aging has been studied in animal models.[25]

The strengths of our study are, first, objective and well-standardized measurements. Second, a randomized, double-blind study that analyzes the effect of glutathione in both reduced and oxidized forms in comparison with placebo has never been conducted. Limitations are that our subjects are all female, Asian, and of certain age range.

Overall, glutathione in both forms are well tolerated. No major adverse events took place during the study period. Increases in transaminases occurred in two subjects highlighting the fact that blood chemistry should be performed even when individuals are taking over-the-counter supplements. Nonetheless, these adverse events were transient and the blood parameters promptly returned to their normal values upon cessation of consumption.

Conclusion

In summary, we have shown that oral glutathione, 250 mg/d, in both reduced and oxidized forms have various beneficial effects on skin properties and is possibly an antiaging agent, at least in middle-aged female subjects. Further studies in larger and more diverse populations are warranted.

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Disclosure

The authors report no conflicts of interest in this work.

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Review

Glutathione in the Brain

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Abstract: Glutathione (GSH) is the most abundant non-protein thiol, and plays crucial roles in the antioxidant defense system and the maintenance of redox homeostasis in neurons. GSH depletion in the brain is a common finding in patients with neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, and can cause neurodegeneration prior to disease onset. Excitatory amino acid carrier 1 (EAAC1), a sodium-dependent glutamate/cysteine transporter that is selectively present in neurons, plays a central role in the regulation of neuronal GSH production. The expression of EAAC1 is posttranslationally controlled by the glutamate transporter-associated protein 3–18 (GTRAP3-18) or miR-96-5p in neurons. The regulatory mechanism of neuronal GSH production mediated by EAAC1 may be a new target in therapeutic strategies for these neurodegenerative diseases. This review describes the regulatory mechanism of neuronal GSH production and its potential therapeutic application in the treatment of neurodegenerative diseases.

Keywords: glutathione; cysteine; excitatory amino acid carrier 1; glutamate transporter-associated protein 3-18; miR-96-5p; neurodegeneration

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1. Introduction

In 1888, de Rey-Pailhade described a substance with the property of reducing sulfur to hydrogen sulfide in extracts from yeast; he named this molecule '*philothion*' [1], meaning "love of sulfur" in Greek [2,3]. Heffter suggested that cysteine (Cys) was involved in this molecule [4], although the structure of this substance was not revealed until the 1920s. Since that time, many researchers have been engaged in the extraction and synthesis of glutathione (GSH), including three Nobel laureates. In 1921, Frederick G. Hopkins isolated a dipeptide containing glutamate (Glu) and Cys from yeast and animal tissues, and named it 'glutathione' [5]. However, de Rey-Pailhade thought that glutathione was a side chain of philothion, which was speculated to be a protein [3]. Other researchers isolated GSH from yeast, blood, and liver, and suggested that GSH was not a simple dipeptide composed of Glu and Cys [6]. Ultimately, in 1929, Hopkins redetermined that GSH was a tripeptide containing Glu, Cys, and glycine (Gly) [7]. In the same year, Hopkins was awarded the Nobel Prize in Physiology or Medicine for his work on vitamins, not GSH. Also in 1929, Edward C. Kendall crystallized GSH and identified its chemical structure [8]; Kendall went on to be awarded the Nobel Prize in Physiology or Medicine for his work on corticosteroids in 1950. Finally, Vincent du Vigneaud first reported the synthesis of GSH in 1936 [9]; he was awarded the Nobel Prize in Chemistry in 1955 for his work on biochemically important sulfur compounds, especially for the first synthesis of oxytocin. As evident from this thumbnail history, GSH inspired the interest of some of the most prominent researchers of the early twentieth century. More than 130 years after its discovery, GSH is still a promising therapeutic target for the treatment of neurodegenerative diseases. In this review, I will discuss the functions and regulatory mechanisms of GSH, with a special focus on its neuroprotective role against oxidative stress in the brain.

2. GSH Function

GSH is a major antioxidant that maintains the homeostasis of redox states in cells, and plays important roles in maintaining the physiological functions of all cells in vivo. The thiol (sulfhydryl, SH) residues play an important role in maintaining the redox state homeostasis intracellularly. In mammals, Cys and methionine (Met) are particularly important as thiol-containing amino acids [10], but GSH is the most abundant thiol-containing substance (derived from a non-protein) in all kinds of cells. The functions of GSH in living cells are diverse, and include roles in maintenance of the intracellular antioxidant system, redox balance, Cys transport/storage, cell signaling, regulation of some enzyme activities, gene expressions, and cell differentiation/proliferation [11]. GSH is especially abundant in the liver and kidney [12], both of which utilize the transsulfuration pathway to produce Cys from Met via homocysteine [13], while it is present at lower levels in the brain, where the regulatory system for GSH synthesis is independent of that in peripheral tissues. Therefore, the molecular mechanisms underlying GSH dysfunction in the brain differ from those in peripheral tissues.

All of the major biological processes of GSH involve the redox state of the thiol residue within the GSH molecule. Two molecules of GSH are oxidized to produce one molecule of GSH disulfide (GSSG) in order to eliminate reactive oxygen species (ROS)/reactive nitrogen species (RNS), or to maintain intracellular redox homeostasis, and GSSG can be reduced back to two GSH molecules via reaction with GSH reductase (GR). The intracellular GSH/GSSG ratio is 100 or more in the steady state, but decreases to 10 or less under oxidative stress conditions [14]. Proteins oxidized by ROS/RNS are reduced by glutaredoxins (Grxs) or thioredoxins (Trxs) [15] (Figure 1). Functionally, Grxs and Trxs share many common features; however, Grxs are more versatile than Trxs in terms of their substrate selectivity and reaction mechanisms [16]. The isoforms of Grxs and Trxs are known as cytosolic Grx1 and Trx1, and as mitochondrial Grx2 and Trx2 in mammals [16,17]. These isoforms are involved in controlling intracellular redox signaling for the cellular processes of apoptosis and proliferation [17,18]. Grx2 and Trx1 are also found in the nucleus. Many transcription factors are known to undergo reduction by Grxs and Trxs. In order for a transcription factor to bind to DNA, the thiol groups of Cys residues in the DNA-binding site should be in the reduced form. The thiol groups mainly exist in their oxidized forms, and are reduced in the process of becoming activated to enable DNA binding. Subsequently, the oxidized Grxs and Trxs are reduced back via reaction with GSH and Trx reductase (TrxR), respectively. GSH functions as an enzyme cofactor for Grxs, which are low-molecular-weight redox enzymes that are also known as thiol transferase, to maintain cellular redox homeostasis, and also acts as the primary reductant of the disulfide bonds of oxidized proteins [15] (Figure 1). However, excessive oxidative stress causes irreversible oxidation of the thiol residues and impairs cellular protein function [19,20]. In particular, Cys residues in active sites or functional motifs of intracellular proteins are important for their protein functions. Oxidative stress by ROS/RNS on the Cys residues in proteins can cause irreversible modifications that lead to critical dysfunction of the proteins [21]. GSH can also react with intracellular protein thiol residues to protect protein functions related to enzyme activities, DNA binding by transcription factors, and protein stability [22–24]. Therefore, under such oxidative stress conditions, GSH reversibly binds to the thiol residues to prevent irreversible changes in proteins due to oxidative stress. This post-translational modification, called "glutathionylation", is reversible and protects the intracellular signal transduction system against oxidative stress [19] (Figure 1). The caspase family of Cys proteases, which induces cell apoptosis, would be a potential target for glutathionylation. Caspase-3, an important regulator of apoptotic responses, can undergo glutathionylation, leading to the inactivation of caspase-3 by GSSG in a dose- and time-dependent manner [25], suggesting that apoptosis can be regulated by glutathionylation. Once the cellular environment is free from oxidative stress, the disulfide bonds in the proteins are reduced back by Grx to function normally under physiological conditions [15]. Thus, the regulation of the redox state by intracellular GSH is extremely important for maintaining cellular

functions under both physiological and pathological conditions. Especially in the brain, the regulatory mechanism of GSH function is more fragile in neurons than in glial cells, and intracellular GSH levels are also lower in neurons than in glial cells [26]. Under some pathological conditions, decreased GSH levels could have critical influences on neuronal activities, leading to neurodegeneration.

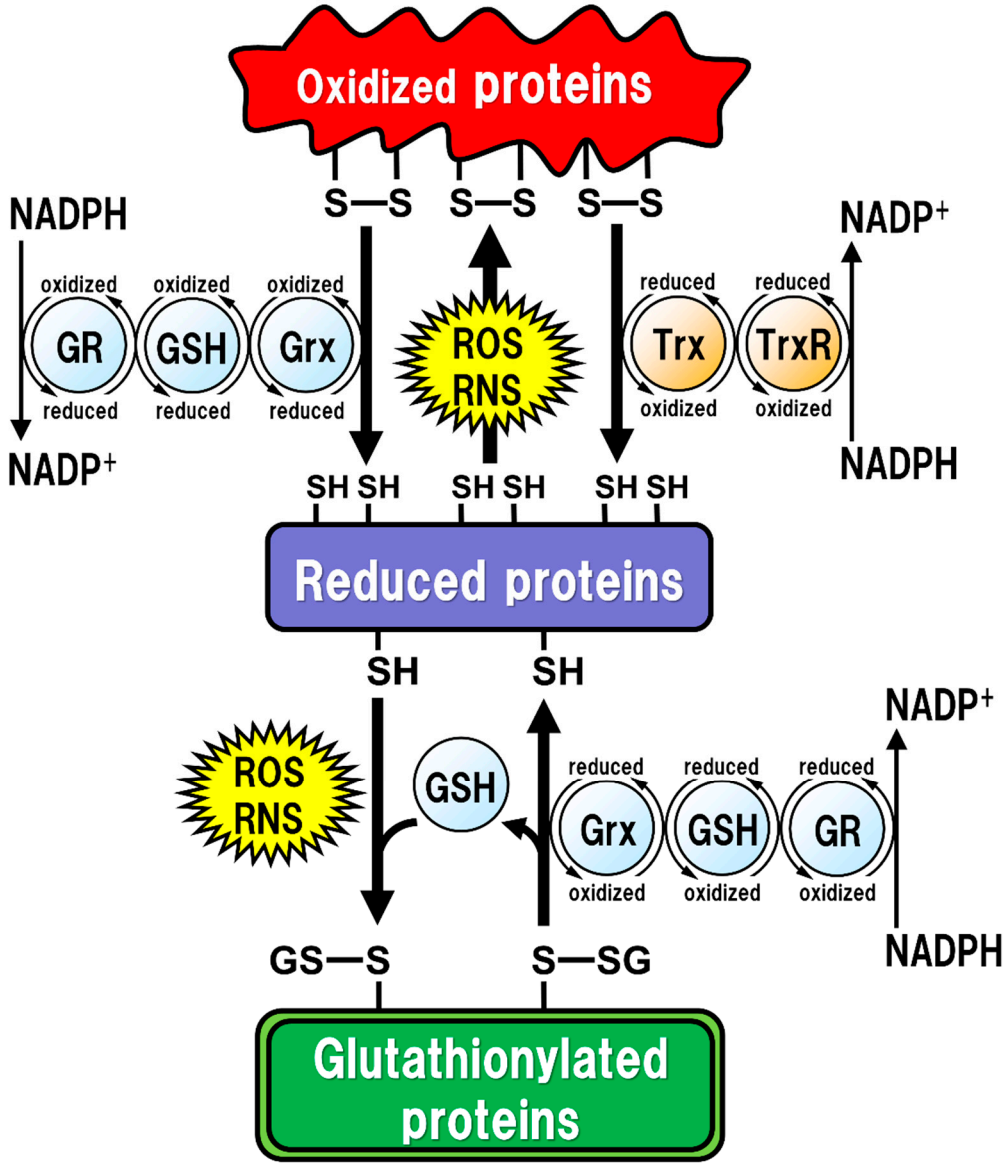

Figure 1. Regulation of the intracellular protein redox state by glutathione (GSH), glutaredoxin (Grx), and thioredoxin (Trx). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) cause protein dysfunction, which is induced by the oxidation of thiol (SH) residues to form disulfide (S-S) bonds in the active site. Grx and Trx regulate protein function by reducing the S-S bonds of the substrate proteins. Consequently, Grx and Trx themselves result in the oxidized forms, which are reduced back by GSH and Trx reductase (TrxR), respectively. Oxidized GSH (GSSG) is reduced back to GSH by GSH reductase (GR). Both oxidized TrxR and GR are reduced by receiving electrons from nicotinamide adenine dinucleotide phosphate (NADPH). Under oxidative stress conditions, GSH can bind to cysteine residues (GS-S) in a process known as '*S*-glutathionylation' to prevent the irreversible dysfunction of the proteins. Grx also functions in the deglutathionylation of the GS-S containing proteins to resume protein functions under physiological conditions.

3. Oxidative Stress in the Brain

GSH occupies approximately 95% of non-protein thiol groups in vivo and is ubiquitously present in mammalian cells at concentrations of 0.5 to 10 mM, depending on the tissues [27,28]; these concentrations are 10 to 100 times higher than the concentrations of Cys in mammalian cells [29]. GSH is the major intracellular thiol compound, and is made from Glu, Cys, and Gly by two-step enzymatic reactions requiring ATP. The first step of these reactions is mediated by Glu-Cys ligase (GCL), and the second by GSH synthetase (GSS). In intracellular GSH synthesis, GCL can be the rate-limiting enzyme under the condition that all substrates are sufficiently present for the reactions, but the intracellular Cys concentration is much lower than those of Glu or Gly under physiological conditions, suggesting that the Cys availability is limiting for GSH synthesis [30,31]. GCL is comprised of both a catalytic (GCLc) and a modulatory (GCLm) subunit. GCLc is responsible for all of the enzyme activity of GCL, which is regulated via feedback inhibition by GSH [32]. Most GSH is present in the cytoplasm, where it is synthesized in mammalian cells [33]. Although mitochondria contain about 5–15% of all the GSH in the cell [34], they cannot synthesize GSH by themselves because they lack GCL [33]. The finding that GCLc- or GSS-deficient mice are non-viable in the embryonic period [35,36], while GCLm-deficient mice are viable and fertile with decreased GSH levels in the tissues compared to those of wild-type mice [37], suggest that GSH is essential for embryogenesis.

The highest GSH concentration in the body is in the liver (about 5 to 10 mM) [12], but hepatocytes can also produce Cys for GSH synthesis from Met via the transsulfuration pathway [38]. GSH in the liver is then released systemically, but it is decomposed in the blood, with the result that the blood GSH concentrations (approximately 2 to 20 μM) are hundreds to thousands of times lower than those in the liver [28]. In addition, GSH cannot directly enter the brain due to the existence of the blood-brain barrier (BBB). Moreover, extracellular GSH cannot be directly transported into the cells, and thus the three amino acids used as substrates for GSH synthesis should be taken up into the cells via transporters.

The brain tissue is generally rich in unsaturated fatty acids, which are targets of oxidative stress, and has relatively low levels of antioxidants or antioxidant enzymes. ROS, such as singlet oxygen ($^1O_2$), superoxide ($O_2{\cdot}^-$), and hydroxyl radicals (·OH), are endogenously produced by mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation. Mitochondria generate most ROS, including $O_2{\cdot}^-$, into the matrix and the intermembrane space via the electron transport chain. The steady-state concentration of $O_2{\cdot}^-$ is about 5–10 times higher in the mitochondrial matrix than in the cytoplasm or nucleus [39], but the mitochondrial matrix contains Mn-superoxide dismutase (SOD), which can react with $O_2{\cdot}^-$ to form hydrogen peroxide (H_2O_2) (Figure 2). In addition, $O_2{\cdot}^-$ leaked into the cytoplasm reacts with Cu/Zn-SOD (SOD1) to form H_2O_2. H_2O_2 is toxic to eukaryotic cells at concentrations of 0.1 to 1×10^{-3} M, but the reaction with catalase or GSH peroxidase (GPx) can decompose H_2O_2 to oxygen and water. As a result, the concentrations of H_2O_2 in mitochondria are maintained in the range of 10^{-9} to 10^{-8} M [15]. Such high concentrations of H_2O_2 are unlikely to occur under physiological conditions in vivo. However, overproductions of both $O_2{\cdot}^-$ and H_2O_2 can be induced by mitochondrial dysfunction [40]. The increased H_2O_2 produces ·OH, which possesses the highest reactivity and the strongest oxidizing power among ROS, via the Fenton reaction (Figure 2). In addition, $O_2{\cdot}^-$ reacts with nitric oxide (NO) to generate peroxynitrite ($ONOO^-$) (Figure 2), which targets DNA, proteins, and lipids, causing DNA damage, dysfunction of enzymes, receptors, transporters, and membrane channels, as well as protein aggregation, mitochondrial dysfunction, and lipid peroxidation [15]. $ONOO^-$ is produced approximately one million times faster, and can spread approximately 10,000 times farther over cells, than ·OH [41]. $ONOO^-$ is more globally toxic within tissues than ·OH, whose toxicities are limited to the local area inside the cells [42]. GSH acts protectively against oxidative stress by reacting directly with NO, $O_2{\cdot}^-$, H_2O_2, ·OH, and $ONOO^-$ (Figure 2). GSH also acts as an enzyme cofactor for GPx to degrade H_2O_2 and hydroperoxides (ROOH), and is involved in detoxifying electrophilic xenobiotics via GSH-*S*-transferase (GST) [43]

(Figure 2). From these protective functions, GSH is considered to play an important role not only under physiological conditions but also under pathological conditions induced by oxidative stress in order to maintain the homeostasis of cell functions.

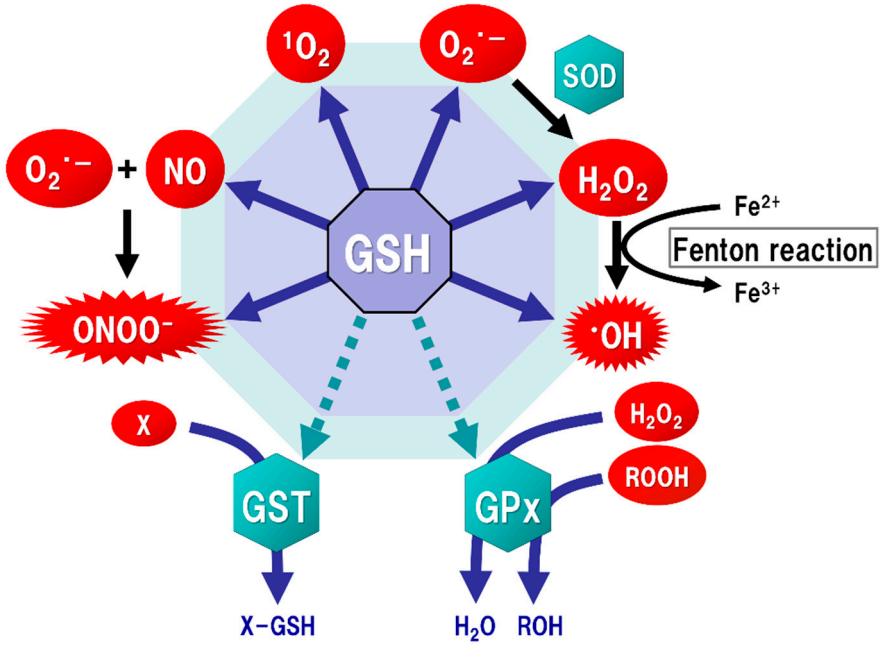

Figure 2. Function of glutathione (GSH) as an antioxidant. Mitochondria generate superoxide ($O_2\cdot^-$), which reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), a typical reactive nitrogen species (RNS) that is a potent inducer of cell death. $O_2\cdot^-$ is catalyzed to hydrogen peroxide (H_2O_2) by the reaction of superoxide with superoxide dismutase (SOD). H_2O_2 reacts with Fe^{2+} (Fenton reaction) to form a highly oxidizing radical, hydroxyl radical (·OH). GSH can directly act as an antioxidant (solid arrows) by non-enzymatically reacting with NO, singlet oxygen ($^1O_2$), $O_2\cdot^-$, H_2O_2, ·OH, and $ONOO^-$. GSH can also indirectly serve as an enzyme cofactor for detoxification (dotted arrows). H_2O_2 is catalyzed to water and oxygen by GSH peroxidase (GPx), which requires GSH as an electron donor to react with H_2O_2 and hydroperoxides (ROOH). GSH-S-transferase (GST) can detoxify various xenobiotics (X) via GSH conjugation to excrete toxic compounds from the cell.

4. GSH Synthesis in Neurons

In in vitro studies, GSH levels in neurons are lower than those in astrocytes [26], and are increased when the neurons are co-incubated with astrocytes [44]. Neuronal GSH synthesis is supported by astrocytes, which supply GSH precursors to neurons. Notably, neuronal GSH levels in vitro are increased by the administration of Cys, but not Glu, Gly, or cystine, the latter of which is formed by two Cys molecules with a disulfide linkage [44,45]. Both Cys and Met are major sources of mammalian thiols [10], and Cys is an important substrate for GSH synthesis in neurons [46], while astrocytes can utilize both Cys and cystine for their GSH synthesis [45]. The activity of GCL, the rate-limiting enzyme for GSH synthesis, was upregulated in neurons co-cultured with GSH-depleted astrocytes, but the neuronal GSH levels were not increased [47]. These findings suggest that not only neuronal GCL activity, but also the astroglial supply system with Cys-containing precursors, is important in maintaining neuronal GSH levels.

The uptake of Cys into neurons is mainly mediated by excitatory amino acid carrier 1 (EAAC1, in rodents), also known as excitatory amino acid transporter type 3 (EAAT3, in humans) (Figure 3). Five types of EAAT have been reported so far, and their expressions differ depending on the cell type. In the brain, GLAST (also known as EAAT1) and GLT-1 (also known as EAAT2) are primarily distributed in astrocytes, whereas EAAC1 is exclusively expressed in neurons. EAAT4 and EAAT5 are distributed in cerebellar Purkinje cells and neurons of the retina, respectively [48]. All of these transporters can

take up extracellular Glu into the cells, but unlike GLAST and GLT-1, EAAC1 can also transport Cys with the same efficiency as Glu [49]. Based on the experimental results using a mutation model of EAAC1, it has been considered that the mechanisms of Glu and Cys uptake by EAAC1 are independent of each other [50]. There were no significant changes in extracellular Glu concentrations in an EAAC1-knockdown animal model [51]. GLAST and GLT-1 act as Glu transporters in glial cells in vivo and are involved in the regulation of Glu concentration in synaptic clefts, whereas EAAC1 is not involved in the regulation of extracellular Glu levels in synaptic clefts, but rather in the regulation of GSH production via extracellular Cys uptake. Moreover, EAAC1-deficient mice exhibit decreased brain GSH levels, vulnerability to oxidative stress in the hippocampus, and age-related learning dysfunction [52]. EAAC1-deficient mice also showed age-dependent loss of dopaminergic neurons in the substantia nigra pars compacta accompanied by increased oxidative stress [53]. EAAC1 is responsible for approximately 70–80% of Cys uptake in neurons [54], and can transport 10- to 20-fold greater amounts of Cys than can GLAST or GLT-1 [49]. Based on these results, the physiological roles of EAAC1 in the central nervous system (CNS) would be involved in the neuroprotective roles mediated by GSH production [55].

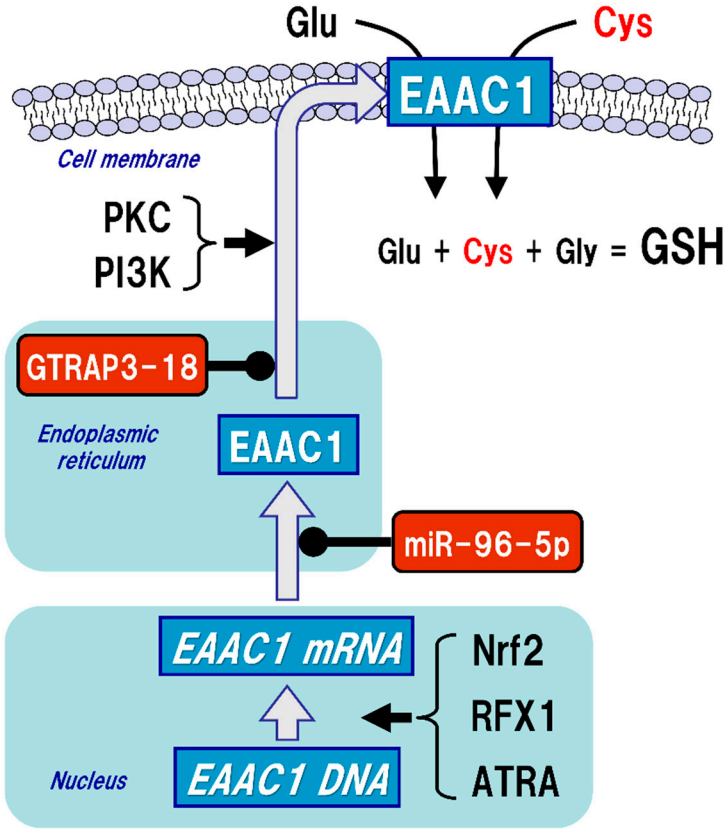

Figure 3. Regulation of excitatory amino acid carrier 1 (EAAC1) expression. Glutathione (GSH) is a tripeptide synthesized from glutamate (Glu), cysteine (Cys), and glycine (Gly). Neuronal GSH synthesis relies on intracellular Cys but not Glu or Gly level. Cys uptake (red font) is subjected to the regulation of both gene expression and post-translational modifications of EAAC1 under facilitative (arrow) and suppressive (black circles) controls. EAAC1 gene expressions are promoted by nuclear factor erythroid 2-related factor 2 (Nrf2), regulatory factor X1 (RFX1), and all-trans-retinoic acid (ATRA). Protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K) activations increase the EAAC1 expression on the plasma membrane. Glu transporter-associated protein 3-18 (GTRAP3-18) and miR-96-5p post-translationally suppress the protein expression of EAAC1, leading to decreased Cys uptake and subsequently decreased GSH synthesis in neurons.

5. Regulatory Mechanism of EAAC1 Expression in Neurons

While GLAST and GLT-1 are constitutively expressed on the cell membrane of glial cells, the membrane expression levels of EAAC1 are approximately 20% of the total under normal conditions, while protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K) activations increase the EAAC1 expression on the plasma membrane [56] (Figure 3). On the other hand, Glu transporter-associated protein 3-18 (GTRAP3-18), which is an endoplasmic reticulum protein, binds to EAAC1 in the endoplasmic reticulum and suppresses the membrane trafficking of EAAC1 [57,58]. In our experiments both in vitro and in vivo, increased expression of GTRAP3-18 resulted in decreased GSH levels due to increased interaction with EAAC1 [59]. Subsequent experiments using antisense oligonucleotides and small interfering RNAs demonstrated that silencing the expression of GTRAP3-18 increased the GSH levels in neurons [59]. Indeed, in GTRAP3-18-deficient mice, the expression of EAAC1 on the cell membrane in neurons and both Cys and GSH levels in the brain tissues were also increased, leading to the resistance to oxidative stress [60]. These results suggest that suppression of GTRAP3-18 in neurons leads to resistance to neurodegeneration by promoting the function of EAAC1 to increase neuronal GSH synthesis. GTRAP3-18 hinders neurite outgrowth in vitro [61], while GTRAP3-18-deficient mice showed enhanced neurogenesis in the hippocampus [62] and spatial cognitive potentiation as assessed by the Morris water maze test [60,62]. Hippocampal neurons require GSH to sustain dendrite integrity and cognitive function [63]. Regulation of GTRAP3-18 would be a promising strategy to modulate neuronal GSH synthesis and thereby confer neuroprotection.

EAAC1 gene expression is promoted by nuclear factor erythroid 2-related factor 2 (Nrf2) [64], regulatory factor X1 (RFX1) [65], and all-trans-retinoic acid (ATRA) [66], while miR-96-5p, which is one of the microRNAs (miRNAs), has a target sequence in the 3′-UTR of EAAC1 and suppresses the protein expression of EAAC1 posttranslationally, leading to decreased GSH levels in the brain [67,68] (Figure 3). The function of EAAC1 is also promoted by the mammalian target of rapamycin (mTOR) [69] and Janus-activated tyrosine kinase-2 (JAK-2) [70], which are involved in cell growth, differentiation, and proliferation. On the other hand, activation of AMP-activated protein kinase (AMPK) reduces the expression of EAAC1 on the cell surface and suppresses its function [71]. AMPK is a serine-threonine kinase that is activated by cellular ATP depletion and is known to be involved in the maintenance of energy homeostasis by inhibiting anabolic action while promoting catabolism in cells. However, it is not clear how membrane translocation of EAAC1 is regulated by the activity of AMPK. Thus, it is quite probable that expression of EAAC1 is subject to pre- and post-translational regulations in neurons.

6. GSH Synthesis in Astrocytes

For the last 50 years, evidence has suggested that astrocytes outnumber neurons 10-fold and make up 25–50% of the brain volume [72,73], while recent papers have reported a glia:neuron ratio of less than 1:1 in the human brain [73]. In the brain, astrocytes play several important roles in maintaining physiological neuronal activity. Astrocyte-neuron interactions have been suggested to be crucial for neuronal survival [74,75]. Astrocytes promote the growth of neurites by releasing neurotrophic factors and reducing neurotoxicity by Glu uptake after brain injuries [76], while also protecting neurons from oxidative stress via a GSH-dependent mechanism [74,75]. Notably, GSH metabolic genes and GSH production in astrocytes can be up-regulated in neuronal co-culture through the modulation of astrocytic Nrf2 [77]. On the other hand, decreased GSH levels in astrocytes enhance neurotoxicity due to oxidative stress [75], indicating that neurons are more vulnerable to oxidative stress without a supply of Cys from astrocytes.

The BBB prevents direct entry of GSH into the brain. GSH is oxidized to GSSG or decomposed to amino acids in blood, and the Cys in blood is easily oxidized to cystine. Astrocytes can take cystine into cells via a sodium-independent Glu/cystine antiporter named system xc^- [78], which can exchange extracellular cystine for intracellular Glu and then intracellularly reduce cystine back to two Cys molecules that can be utilized as a

substrate for GSH synthesis. Moreover, astrocytes can utilize the dipeptides γGluCys and CysGly for GSH synthesis, or convert Met to Cys via the transsulfuration pathway [79] to maintain high intracellular stores of GSH (approximately 8 mM) [29]. Astrocytes supply Cys-containing peptides to neurons in order to maintain GSH synthesis [80]. Astrocytes can release about 10% of their intracellular GSH per hour [81] to the extracellular space via multidrug resistance protein 1 (MRP1) [82]. Extracellular GSH is decomposed into CysGly by the astroglial ectoenzyme γ-glutamyl transpeptidase (GGT) [82]. Since CysGly is not directly taken up into neurons [81], CysGly is hydrolyzed by neuronal ectopeptidase into Cys and Gly [44,83], both of which are transported into neurons. Therefore, neuronal GSH synthesis depends on the system xc^- and the GSH-supply mechanisms in astrocytes and is maintained by the mechanism of astrocyte-neuron interactions.

7. GSH Synthesis in Microglia

In the brain, microglia occupy about 5–12% of all cells and are more abundant in gray matter than white matter [84]. Microglia are activated in response to various injuries, such as ischemia, infection, inflammatory diseases, brain trauma, and neurodegenerative diseases. In contrast to astrocyte GSH synthesis, which plays a role in supporting neurons, microglial GSH synthesis appears to be exclusively focused on eliminating ROS generated under pathological conditions. GSH levels and their related enzyme activities, such as the activities of GPx and GR, are higher in cultured microglia than in cultured astrocytes and neurons, especially under oxidative stress conditions [85,86]. In addition, the microglia themselves, which are involved in the phagocytosis of dead cells and pathogens, produce $O_2\cdot^-$ and NO when activated [87,88]. Therefore, microglia must have a sufficient defense mechanism against oxidative stress. GSH can suppress $ONOO^-$ production by directly reacting with $O_2\cdot^-$ and NO, and can eliminate the cytotoxicity of H_2O_2 and peroxides by promoting the action of GPx. Microglia also express system xc^- for GSH synthesis [89]. System xc^- can take up extracellular cystine into the cell instead of excreting intracellular Glu out of the cell. The activated microglia express GLT-1 [90] and promote reuptake of the excreted Glu by system xc^- for use in GSH synthesis [91]. An in vitro study demonstrated that the increase in microglial Glu uptake capacity was accompanied by an increase in intracellular GSH contents [89]. The results indicated that GLT-1 expressed in the vicinity of the system xc^- was closely coupled to GSH production in microglia [89]. Since the microglia are exposed to large amounts of RON/RNS, especially under pathological conditions, the coupling between the system xc^- and GLT-1 plays a critical role in microglial GSH synthesis against oxidative stress.

8. Brain GSH Levels in Neurodegenerative Diseases

Ageing is one of the risk factors involved in neurodegeneration, and both increased oxidative stress and decreased GSH levels are important risk factors for age-related neurodegeneration in the CNS [29]. Tissue GSH measurements in human autopsy brains have revealed that the total GSH pool is predominantly (>98.8%) in the reduced form, and the GSH levels of the gray matter (~0.83 mM) are lower than those of the white matter (~1.18 mM) [92]. Lower GSH levels in the gray matter, where neurons are rich, compared to those in the white matter could plausibly result in susceptibility to neurodegeneration due to oxidative stress. Moreover, hippocampal GSH levels in human postmortem brain samples have been reported to decrease with age [93], suggesting that they are implicated in the pathogenesis of Alzheimer's disease (AD). However, GSH levels in postmortem brain samples are likely not fully representative of the original GSH levels in living patients, since they may change with time after death.

Proton magnetic resonance spectroscopy (^{1}H-MRS) is a non-invasive technique for the detection of various neurochemicals, including GSH [94]. In normal adult volunteers, brain GSH levels are around 1–2 mM, with large variations depending on the region, gender, and age differences [95]. Brain GSH levels appear to be decreased in age-related neurodegenerative diseases such as AD, Parkinson's disease (PD), and amyotrophic lateral

sclerosis (ALS). Several studies have used ^{1}H-MRS to investigate brain GSH measurements in patients with these neurodegenerative diseases.

For example, in the temporal and parietal lobes of healthy older adults, GSH levels measured by ^{1}H-MRS were negatively correlated with amyloid β (Aβ) levels, as assessed by positron-emission tomography with the amyloid tracer Pittsburg compound-B (PiB) [96]. AD is the most common cause of dementia in the world. It is pathologically characterized by Aβ deposition and neurofibrillary tangles in the brain [97]. In the brains of patients with AD, increased oxidative stress due to abnormal aggregation of Aβ is considered to play a critical role in the onset of disease. Indeed, Aβ impairs EAAC1 function and suppresses Cys uptake [98]. Aberrant EAAC1 accumulation has been observed in degenerating neurons in AD brains, and is considered a specific feature of AD in the hippocampus [99]. In patients with AD or mild cognitive impairment, hippocampal GSH levels measured by ^{1}H-MRS were significantly decreased compared to those of healthy older-age controls [100]. GSH levels were also found to be decreased in the frontal cortex of patients with AD, and the GSH reductions in these regions were correlated with the decline in cognitive functions [100].

PD is the second most common aging-related neurodegenerative disease after AD. PD is pathologically characterized by insolubilized α-synuclein accumulation in neurons and dopaminergic neurodegeneration in the substantia nigra of the midbrain. An initial study in the postmortem brains of PD patients reported decreased GSH levels in the substantia nigra of the midbrain [101], suggesting that the decrease in neuronal GSH levels may be a critical change prior to the onset of PD [102]. Exposure to certain neurotoxins has been suggested to be a risk factor for PD [103,104]. One of these neurotoxins, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is commonly used in an experimental PD model in vivo [105]. Our previous study using the MPTP mouse model of PD showed GSH depletions with increased oxidative stress and EAAC1 dysfunction in the midbrain [106]. These MPTP-induced neurotoxicities were prevented by pre-administration of n-acetylcysteine (NAC), a membrane-permeable Cys precursor [106]. A recent study using ^{1}H-MRS demonstrated that intranasal administration of 200 mg of GSH significantly increased GSH levels in the dorsal putamen of patients with PD [107]. Many studies suggest that small polar molecules may be able to 'bypass' the BBB by nasal administration, indicating that the interface between the nasal cavity and the brain may be a more vulnerable part of the BBB [108]. Intranasal administration of reduced GSH could thus be an effective approach for delivery of GSH to the CNS.

ALS is also a neurodegenerative disease associated with oxidative stress [109]. The brains of ALS patients showed a 90% decrease of GLT-1 and a 20% decrease of EAAC1 compared to those of controls [110]. Recent clinical studies using ^{1}H-MRS showed that GSH levels in the brains of ALS patients were decreased compared to those of age-matched healthy volunteers [111], and the decreased GSH levels in the motor cortex and corticospinal tract were inversely correlated with the time after diagnosis [112]. The decrease of GSH levels was more prominent in the motor cortex than in the white matter in ALS patients [112]. These results suggest that the brains of patients with ALS have limited antioxidant capacity.

Mutations in SOD1 cause ALS in humans [113], and the overexpression of the ALS-linked mutant hSOD1 also causes an ALS-like phenotype in rodents [114]. Hemizygous mice over-expressing wild-type hSOD1 (hSOD1WT) did not show the ALS-like phenotype, but did show it when crossed with GCLm-knockout mice, with a 70–80% decrease in total GSH levels [115]. These results indicate that GSH depletion enhances neurodegeneration in ALS models in vivo.

Transactive response DNA-binding protein 43 kDa (TDP-43) is an RNA-binding protein that abnormally accumulates in the motor neurons of ALS patients [116]. Mutations in the gene for TDP-43 cause familial ALS in humans and the ALS-like phenotype in transgenic animals [117]. Expression of the A315T mutant TDP-43 in vitro decreased GSH levels and increased both ROS and cell death, while the restoration of GSH levels

by treatment with GSH monoethyl ester prevented cell death and TDP-43 pathological changes in motor neurons [118]. These results indicate that restoring GSH levels could be a promising strategy for the treatment of TDP-43-mediated ALS.

Multiple system atrophy (MSA) is an adult-onset neurodegenerative disease characterized by progressive cerebellar ataxia, autonomic symptoms, and parkinsonism. No radical treatment is available to prevent the onset or progression of MSA. Although the etiology has not been fully elucidated yet, the involvement of oxidative stress has been suggested as an important causative factor in recent years [119–121]. Recent papers in the field of neurodegenerative diseases have examined the posttranscriptional regulation of proteins by microRNA (miRNA) [68,122], and one of the miRNAs, named miR-96-5p, was particularly upregulated in the brains of MSA patients [123]. Our experimental results also showed that the increase in miR-96-5p causes a decrease in EAAC1 protein levels, leading to reduced GSH levels in neurons, while a treatment with anti-miR-96-5p restored the EAAC1 levels and increased GSH levels, leading to neuroprotective effects against oxidative stress in vitro and in vivo [67]. Moreover, anti-miR-96-5p indirectly decreased GTRAP3-18 protein levels [124]. For more details regarding the non-coding RNA-mediated regulatory mechanism of GSH synthesis, see our review article entitled "The role of non-coding RNAs in the neuroprotective effects of glutathione" by Kinoshita C. et al. in this special issue.

9. GSH Treatment for Neurodegenerative Diseases

The number of cases of age-related neurodegenerative diseases such as AD and PD are estimated to increase exponentially worldwide, and these diseases threaten to become a major clinical problem in the future. In recent years, many studies have been conducted with the goal of actively developing therapeutic agents for patients with these neurodegenerative diseases. In particular, there is a need for the development of "disease-modifying drugs" that suppress neurodegeneration, since none of the medicines clinically used at present provide radical therapeutic effects against the progression of these neurodegenerative diseases.

Since the 1990s, along with continued elucidation of the mechanism of neurodegeneration induced by oxidative stress in the CNS, GSH depletion in the brains of patients with neurodegenerative diseases has been increasingly reported. Subsequently, basic research on GSH in the CNS has been focused on therapeutic strategies aimed at reducing neurodegeneration, and a drug increasing GSH levels in the brain would be promising as a 'disease-modifying drug' characterized by neuroprotective effects. Since GSH hardly crosses the BBB [125], the clinical effects of direct GSH replacement therapy could not be expected to be neuroprotective. Orally administered GSH is not directly absorbed by the body because of its degradation by gastrointestinal peptidase. In addition, most of the intravenously administered GSH is also metabolized by GGT in the blood, so that the elimination half-life is as short as about 7 min [126], which is not sufficient for clinically effective administration. GSH in the blood is predominantly oxidized to GSSG under aerobic conditions, so that the administered GSH concentrations are lowered in the blood. It is difficult to increase the brain GSH levels directly by peripheral administration. Further studies on drug delivery technology will be needed in the future.

In addition to GSH, some other antioxidants, such as ascorbic acid (vitamin C) and α-tocopherol (vitamin E), are also present in the brain. The concentrations of ascorbic acid in the brain are similar to those of GSH (about 1–2 mM) [127], but the reactivity of ascorbic acid to $ONOO^-$ is too low to provide neuroprotection [128]. The concentrations of α-tocopherol in the brain are lower than those of GSH or ascorbic acid, so that α-tocopherol is unlikely to play a central role among antioxidants [129]. Moreover, ascorbic acid and α-tocopherol, like GSH, hardly cross the BBB, so their brain concentrations cannot be increased by peripheral administration. In fact, the effectiveness of these antioxidants has not been clear in clinical studies of AD [130,131] and PD [132] patients. Indeed, no significant decreases in ascorbic acid or α-tocopherol levels were observed in the brains of

AD and PD patients [129,133,134]. In addition, a clinical study applying coenzyme Q10, a mitochondrial antioxidant, did not demonstrate clinical efficacy in patients with early PD [135]. Although administration of some antioxidants may suppress neurodegeneration, no clinically apparent efficacy has been demonstrated yet. Among the antioxidants, GSH remains a promising agent because it is selectively decreased in the brains of patients with these neurodegenerative diseases.

NAC is a membrane-permeable Cys precursor for GSH synthesis. NAC can diffuse into neurons without EAAC1 to supply Cys via intracellular deacetylation [52,136]. NAC also acts as an antioxidant [137] and stimulates GR, leading to a reduction of GSSG to GSH [138,139]. These results suggest a promising clinical application of NAC to increase neuronal GSH levels in the brains of patients with neurodegenerative diseases. A recent clinical trial with oral administration of NAC did not demonstrate increased GSH levels in some brain regions measured by ^{1}H-MRS [140]. However, intravenous administration of NAC increased brain GSH levels by 55% in patients with PD [141]. These results indicate that an improvement of the drug-delivery system is necessary for treatment with NAC.

The BBB is a strict barrier in terms of protecting the CNS against toxic xenobiotics. Our paper published in 2021 [124] introduced a drug-delivery system that overcame the issue of the BBB by means of ultrasound combined with microbubbles containing anti-miR-96-5p; this system was recently shown to successfully realize neuroprotective effects by increasing brain GSH levels in vivo [124]. In our previous study, we found that the GTRAP3-18 levels were increased by miR-96-5p, which decreases EAAC1 levels in the brain [67]. We also found that intra-arterial injection of anti-miR-96-5p into mice using microbubbles and an ultrasound system decreased GTRAP3-18 levels, leading to increased EAAC1 and GSH levels in the hippocampus [124]. Recently, this drug-delivery system has received much attention as a new technology [142,143], and it might be useful for clinical application in the future. In combination with the development of drug-delivery systems, neuron-specific GSH replacement therapy holds promise for the future treatment of patients with neurodegenerative diseases.

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GLUTATHIONE SYNTHESIS

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Abstract

BACKGROUND—Glutathione (GSH) is present in all mammalian tissues as the most abundant non-protein thiol that defends against oxidative stress. GSH is also a key determinant of redox signaling, vital in detoxification of xenobiotics, regulates cell proliferation, apoptosis, immune function, and fibrogenesis. Biosynthesis of GSH occurs in the cytosol in a tightly regulated manner. Key determinants of GSH synthesis are the availability of the sulfur amino acid precursor, cysteine, and the activity of the rate-limiting enzyme, glutamate cysteine ligase (GCL), which is composed of a catalytic (GCLC) and a modifier (GCLM) subunit. The second enzyme of GSH synthesis is GSH synthetase (GS).

SCOPE OF REVIEW—This review summarizes key functions of GSH and focuses on factors that regulate the biosynthesis of GSH, including pathological conditions where GSH synthesis is dysregulated.

MAJOR CONCLUSIONS—GCL subunits and GS are regulated at multiple levels and often in a coordinated manner. Key transcription factors that regulate the expression of these genes include NF-E2 related factor 2 (Nrf2) via the antioxidant response element (ARE), AP-1, and nuclear factor kappa B (NFκB). There is increasing evidence that dysregulation of GSH synthesis contributes to the pathogenesis of many pathological conditions. These include diabetes mellitus, pulmonary and liver fibrosis, alcoholic liver disease, cholestatic liver injury, endotoxemia and drug-resistant tumor cells.

GENERAL SIGNIFICANCE—GSH is a key antioxidant that also modulates diverse cellular processes. A better understanding of how its synthesis is regulated and dysregulated in disease states may lead to improvement in the treatment of these disorders.

Keywords

GSH; glutamate-cysteine ligase; GSH synthase; Nrf2; MafG; c-Maf; antioxidant response element

1. Introduction

Glutathione (GSH) is a tripeptide, γ-L-glutamyl-L-cysteinylglycine, present in all mammalian tissues at 1–10 mM concentrations (highest concentration in liver) as the most abundant non-protein thiol that defends against oxidative stress. GSH is also a key

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determinant of redox signaling, vital in detoxification of xenobiotics, modulates cell proliferation, apoptosis, immune function, and fibrogenesis. This review is focused on factors that determine GSH synthesis and pathologies where dysregulation in GSH synthesis may play an important role with emphasis on the liver. This is because the liver plays a central role in the interorgan GSH homeostasis [1].

2. Structure and functions of GSH

GSH exists in the thiol-reduced and disulfide-oxidized (GSSG) forms [2]. GSH is the predominant form and accounts for >98% of total GSH [3–5]. Eukaryotic cells have three major reservoirs of GSH. Most (80–85%) of the cellular GSH are in the cytosol, 10–15% is in the mitochondria and a small percentage is in the endoplasmic reticulum [6–8]. Rat liver cytosolic GSH turns over rapidly with a half-life of 2–3 hours. The structure of GSH is unique in that the peptide bond linking glutamate and cysteine of GSH is through the γ-carboxyl group of glutamate rather than the conventional α-carboxyl group. The only enzyme that can hydrolyze this unusual bond is γ-glutamyltranspeptidase (GGT), which is only present on the external surfaces of certain cell types [9]. As a consequence, GSH is resistant to intracelluar degradation and is only metabolized extracellularly by cells that express GGT. This allows for released GSH to be broken down and its constituent amino acids taken up by cells and reincorporated into GSH (so called γ-glutamyl cycle, see below). The bulk of plasma GSH originates from the liver, which plays a central role in the interorgan homeostasis of GSH by exporting nearly all of the GSH it synthesizes into plasma and bile [1,10,11]. Thus, dysregulation of hepatic GSH synthesis has impact on GSH homeostasis systemically.

GSH serves several vital functions including antioxidant defense, detoxification of xenobiotics and/or their metabolites, regulation of cell cycle progression and apoptosis, storage of cysteine, maintenance of redox potential, modulation of immune function and fibrogenesis [4, 5,9,12–15]. Some of these key functions, namely antioxidant defense, redox signaling, storage of cysteine via the γ-glutamyl cycle, regulation of growth and death are described in more detail below.

2.1 Antioxidant function of GSH

The antioxidant function of GSH is accomplished largely by GSH peroxidase (GPx)-catalyzed reactions, which reduce hydrogen peroxide and lipid peroxide as GSH is oxidized to GSSG. GSSG in turn is reduced back to GSH by GSSG reductase at the expense of NADPH, forming a redox cycle [13]. Organic peroxides can also be reduced by GPx and GSH S-transferase. Catalase can also reduce hydrogen peroxide but it is present only in peroxisome. This makes GSH particularly important in the mitochondria in defending against both physiologically and pathologically generated oxidative stress [16,17]. As GSH to GSSG ratio largely determines the intracellular redox potential (proportional to the log of $[GSH]^2/[GSSG]$) [5], to prevent a major shift in the redox equilibrium when oxidative stress overcomes the ability of the cell to reduce GSSG to GSH, GSSG can be actively exported out of the cell or react with a protein sulfhydryl group leading to the formation of a mixed disulfide. Thus, severe oxidative stress depletes cellular GSH [13] (Figure 2).

2.2 GSH in redox signaling

GSH regulates redox-dependent cell signaling. This is largely accomplished by modifying the oxidation state of critical protein cysteine residues [5,18]. GSH can be reversibly bound to the –SH of protein cysteinyl residues (Prot-SH) by a process called glutathionylation, generating glutathionylated proteins (Prot-SSG), which can either activate or inactivate the protein [18]. This is a mechanism to protect sensitive protein thiols from irreversible

oxidation and may also serve to prevent loss of GSH under oxidative conditions (Figure 2). Deglutathionylation can then occur through glutaredoxin and sulfiredoxin-catalyzed reactions using GSH as a reductant [15]. Many transcription factors and signaling molecules have critical cysteine residues that can be oxidized and this is an important mechanism whereby reactive oxygen and nitrogen species (ROS and RNS) regulate protein function and cell signaling that can be modulated by GSH [13,15].

2.3 GSH and the γ-glutamyl cycle

Alton Meister first described the γ-glutamyl cycle in the early 1970's, which allows GSH to serve as a continuous source of cysteine [19] (Figure 3). This is an important function as cysteine is extremely unstable and rapidly auto-oxidizes to cystine extracellularly, which can generate potentially toxic oxygen free radicals [19]. In the γ-glutamyl cycle, GSH is released from the cell and the ecto-enzyme GGT transfers the γ-glutamyl moiety of GSH to an amino acid (the best acceptor being cystine), forming γ-glutamyl amino acid and cysteinylglycine. The γ-glutamyl amino acid can be transported back into the cell and once inside, the γ-glutamyl amino acid can be further metabolized to release the amino acid and 5-oxoproline, which can be converted to glutamate and used for GSH synthesis. Cysteinylglycine is broken down by dipeptidase to generate cysteine and glycine. Most cells readily take up cysteine. Once taken up, the majority of cysteine is incorporated into GSH, some is incorporated into protein, and some is degraded into sulfate and taurine [19].

2.4 GSH regulates growth and death

In many normal and malignant cell types, increased GSH level is associated with a proliferative response and is essential for cell cycle progression [20–26]. In normal hepatocytes, GSH level increases when cells shift from G_0 to G_1 phase of the cell cycle *in vitro* [25], and after 2/3 partial hepatectomy prior to the onset of increased DNA synthesis [27]. If this increase in GSH was blocked, DNA synthesis following partial hepatectomy was reduced by 33% [26]. In liver cancer and metastatic melanoma cells, GSH status also correlated with growth [26,28]. Interestingly, hepatocyte growth factor (HGF) induces the expression of GSH synthetic enzymes and acts as a mitogen in liver cancer cells only under subconfluent cell density condition and the mitogenic effect required increased GSH level [29]. A key mechanism for GSH's role in DNA synthesis relates to maintenance of reduced glutaredoxin or thioredoxin, which are required for the activity of ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis [30]. In addition, the GSH redox status can affect the expression and activity of many factors important for cell cycle progression. Of particular interest is the finding that GSH co-localizes to the nucleus at the onset of proliferation, which through redox changes can affect the activity of many nuclear proteins including histones [14,31]. These recent studies show that a reducing condition in the nucleus is necessary for cell cycle progression [14].

GSH also modulates cell death. Apoptosis, characterized by chromatin condensation, fragmentation and internucleosomal DNA cleavage, and necrosis, characterized by rupture or fragmentation of the plasma membrane and ATP depletion [32] can coexist and share common pathways, such as involvement of the mitochondria [33]. GSH modulates both types of cell death. GSH levels influence the expression/activity of caspases and other signaling molecules important in cell death [4,32]. GSH levels fall during apoptosis in many different cell types, due to ROS, enhanced GSH efflux, and decreased GCL activity (see section on post-translational regulation of GCLC) [34,35]. Although GSH efflux may be a mechanism to circumvent the normally protective role of GSH, it appears essential for apoptosis to occur in many cell types [4, 36]. However, profound GSH depletion can convert apoptotic to necrotic cell death [34], suggesting very high levels of ROS may overwhelm the apoptotic machinery. Consistently, severe mitochondrial GSH depletion leads to increased

levels of ROS and RNS, mitochondrial dysfunction and ATP depletion, converting apoptotic to necrotic cell death [32].

3. Synthesis of GSH

The synthesis of GSH from its constituent amino acids involves two ATP-requiring enzymatic steps: formation of γ-glutamylcysteine from glutamate and cysteine and formation of GSH from γ-glutamylcysteine and glycine (Figure 1). The first step of GSH biosynthesis is rate limiting and catalyzed by GCL (EC 6.3.2.2; formerly γ-glutamylcysteine synthetase), which is composed of a heavy or catalytic (GCLC, Mr ~ 73 kDa) and a light or modifier (GCLM, Mr ~ 31 kDa) subunit, which are encoded by different genes in fruit flies, rodents and humans [37–41]. In contrast, GCL in yeast and bacteria have only a single polypeptide [41]. GCLC exhibits all of the catalytic activity of the isolated enzyme and feedback inhibition by GSH [42]. GCLM is enzymatically inactive but plays an important regulatory function by lowering the K_m of GCL for glutamate and raising the K_i for GSH [38,43]. Thus, the holoenzyme is catalytically more efficient and less subject to inhibition by GSH than GCLC. However, GCLC alone does have enzymatic activity as *Gclm* knockout mice are viable but have markedly reduced tissue GSH levels (reduced by about 85 to 90%) [44]. Redox status can influence GCL activity via formation of the holoenzyme [45]. Most of the GCL holoenzyme can be reversibly dissociated by treatment with dithiothreitol [42], while oxidative stress may enhance holoenzyme formation as it increases GCL activity in the absence of any change in the expression of GCL subunits [45].

Under physiological conditions GCL is regulated by: (a) nonallosteric feedback competitive inhibition (with glutamate) by GSH (K_i=2.3mM) [46] and (b) availability of L-cysteine [9]. The K_m values of GCL for glutamate and cysteine are 1.8 and 0.1–0.3 mM, respectively [46]. The intracellular glutamate concentration is 10-fold higher than the K_m value but cysteine concentration approximates the apparent K_m value [47].

The second step in GSH synthesis is catalyzed by GSH synthetase (GS, EC 6.3.2.3, also known as GSH synthase). GS is composed of two identical subunits (Mr ~ 118 kDa) and is not subject to feedback inhibition by GSH [48]. Since the product of GCL, γ-glutamylcysteine, is present at exceedingly low concentrations when GS is present, GCL is considered rate limiting [41]. In support of this is the finding that overexpression of GS failed to increase GSH level whereas overexpression of GCL increased GSH level [49]. Although GS is generally thought not to be important in the regulation of GSH synthesis, there is accumulating evidence that GS is important in determining overall GSH synthetic capacity in certain tissues and/or under stressful conditions [13]. Surgical trauma decreased GSH levels and GS activity in skeletal muscle while GCL activity was unchanged [50]. In rat hepatocytes, increased GS expression further enhanced GSH synthesis above that observed with increased GCLC expression alone [51].

3.1 Factors that determine cysteine availability

Cysteine is derived normally from the diet, protein breakdown and in the liver, from methionine via transsulfuration (see below). Cysteine is unstable extracellularly where it readily autoxidizes to cystine, which is taken up by some cells and is rapidly reduced to cysteine intracellularly [47]. In hepatocytes, the key factors that regulate cysteine availability include membrane transport of cysteine (via the ASC system), cystine (via the X_c^- system which is induced under oxidative stress), methionine (via the L system) and the activity of the transsulfuration pathway [47,52,53].

3.2 Transsulfuration pathway

The transsulfuration pathway (also called the cystathionine pathway) allows the utilization of methionine for GSH synthesis [54] (Figure 4). Liver plays a central role in methionine metabolism as up to half of the daily intake of methionine is catabolized in the liver. The first step in methionine catabolism is the generation of S-adenosylmethionine (SAMe), the principal biological methyl donor, in a reaction catalyzed by methionine adenosyltransferase (MAT) [55]. Under normal conditions, most of the SAMe generated is used in transmethylation reactions [56]. SAMe donates its methyl group to a large variety of acceptor molecules in reactions catalyzed by methyltransferases (MTs), generating S-adenosylhomocysteine (SAH), which is in turn hydrolyzed to homocysteine (Hcy) and adenosine through a reversible reaction catalyzed by SAH hydrolase. SAH is a potent competitive inhibitor of methylation reactions so that prompt removal of Hcy and adenosine is required to prevent SAH accumulation. In hepatocytes, Hcy can be remethylated to generate methionine by methionine synthase (MS), which requires normal levels of folate and vitamin B_{12}, and betaine homocysteine methyltransferase (BHMT), which requires betaine. Hcy can also be converted to cysteine via the transsulfuration pathway by a two-enzyme process. First, Hcy condenses with serine to form cystathionine in a reaction catalyzed by cystathionine β synthase (CBS), which requires vitamin B_6. The second step cleaves cystathionine, catalyzed by another vitamin B_6-dependent enzyme γ-cystathionase, and releases free cysteine for GSH synthesis [56]. The transsulfuration pathway is particularly active in hepatocytes but outside of the liver, it is either absent or present at very low levels [56]. The hepatic transsulfuration pathway activity is markedly impaired or absent in the fetus and newborn infant and in cirrhotic patients [13]. Part of the mechanism relates to decreased cofactor availability (such as B vitamins). In addition, cirrhotic patients also have decreased MAT activity and diminished SAMe biosynthesis, which further contribute to decreased GSH levels [55].

4. Regulation of glutamate-cysteine ligase (GCL)

Changes in GCL activity can result from regulation at multiple levels affecting only GCLC or both GCLC and GCLM.

4.1 GCLC pre-translational regulation

Many conditions are known to affect GCLC pre-translationally. Drug-resistant tumor cell lines and oxidative stress are associated with increased cell GSH levels, GCL activity, GCLC mRNA levels and GCLC gene transcription [57–63]. While many of these treatments induced both GCLC and GCLM expression, selective transcriptional induction of only GCLC occurred when cultured rat hepatocytes were treated with insulin or hydrocortisone [64–66]. The physiologic significance of the hormonal effect was confirmed using insulin-deficient diabetic or adrenalectomized rats. Both exhibited lower hepatic GSH levels and GCL activity, which were prevented with hormone replacement [64]. Importantly, lower levels of GSH in the erythrocytes of diabetic patients and increased susceptibility to oxidative stress of these cells have been reported [67]. Kim et al reported that the effect of insulin on GSH levels and GCLC expression in rat hepatocytes involve PI3K/Akt/p70S6K but not ERK, JNK and p38 MAPK [68]. However, Li et al reported that insulin's effect on GSH synthesis in cardiac myocytes required PI3K, MEK and p38 MAPK [69]. More recently, Langston et al reported that in human brain endothelial cell line, insulin activated GCLC promoter activity under altered glycemic condition (both low and high) that required PI3K/Akt/mTOR signaling [70]. Thus, while the PI3K signaling pathway appears to be a central player in mediating insulin's effect in many cell types, other signaling pathways activated by insulin may act more in a tissue-specific manner in the up-regulation of GCLC expression and GSH level.

Another condition where GCLC is induced transcriptionally while GCLM is unchanged is during rapid liver growth. Plating hepatocytes under low-density and liver regeneration following partial hepatectomy are such examples [25,27,65]. Increased GSH levels and GCLC transcription and mRNA levels (GCLM expression was unchanged) also occur in human hepatocellular carcinoma (HCC) [26]. Thus, hormones and increased growth selectively regulate hepatic GCLC expression in most circumstances. Since an increase in GCLC expression alone led to an increase in GSH level, we speculated that GCLC might be limiting in hepatocytes. However, this point remains controversial, as others have reported the opposite, namely GCLM is limiting [13,45].

Transforming growth factor-β1 (TGF-β1), a pleiotropic cytokine implicated in the pathogenesis of idiopathic pulmonary fibrosis and in liver fibrosis, has also been shown to regulate GSH synthesis at the level of GCLC [71–73]. In type II alveolar epithelial cells, TGF-β1 lowered the transcriptional activity of GCLC [72]. Similarly, in rat hepatic stellate cells (HSCs) TGF-β1 suppressed the expression of GCLC (no effect on GCLM) and lowered GSH levels [73]. This was a key mechanism for TGF-β1-mediated profibrogenic effect in HSCs that is targeted by (−)-epigallocatechin-3-gallate, the major constituent of green tea that exerts antioxidant effect [73].

Other conditions known to influence expression of GCLC at the transcriptional level include treatments with antioxidants such as butylated hydroxyanisole [74,75], 5,10-dihydroindeno [1,2-b]indole and tert-butyl hydroquinone (TBH) [44,76,77], inducers of Phase II detoxifying enzymes such as β-naphthoflavone (β-NF) [78], formation of Michael reaction acceptors (containing an electrophilic electron-deficient center that is susceptible to nucleophilic attack) by treatment with diethyl maleate (DEM) to produce GSH conjugates [75], heat shock [79], zinc [80], melatonin [81], curcumin [82], and lipid peroxidation products such as 4-hydroxynonenal (4-HNE), 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) [83–86], trivalent arsenite (As^{3+}) [87], and ajoene, a major compound in garlic extracts [88]. 4-HNE is a major end product of lipid peroxidation that is present in normal human plasma at concentrations ranging from 0.1μM to 1.4μM and this can increase more than 10 folds during oxidative stress *in vivo* [84]. 4-HNE was shown to induce GCLC with concentrations found in human plasma, suggesting that the "basal" GCLC expression is under regulation by products of lipid peroxidation [84]. In endothelial cells, nitric oxide (NO) was found to protect against H_2O_2-induced toxicity via induction of GCLC that required the participation of zinc and Nrf2 [89]. Thus, GCLC gene expression is up-regulated when increased cellular defense is needed. However, if the toxic or injurious insult persists, GCLC expression may become dysregulated. An example is treatment with the toxic bile acid lithocholic acid, which caused initial GCLC induction, followed later by suppression at the transcriptional level in hepatocytes [90]. A similar pattern also occurred during bile duct ligation (BDL) [90] (see below under GSH synthesis dysregulation during cholestatic liver injury).

There have been numerous studies that examined the molecular mechanism(s) of GCLC transcriptional regulation [13]. Rodents and human GCLC promoter regions share similar regulatory mechanisms. The promoter region of GCL subunits of human, rat, and mouse has been cloned [78,91–97]. Consensus NFκB, Sp-1, activator protein-1 (AP-1), AP-2, metal response (MRE), and ARE/EpRE elements have been identified in the human GCLC promoter. A proximal AP-1 element (−263 to −269) was found to be critical in mediating the effect of oxidative stress-induced increase in human GCLC transcription [98–103]. However, a distal ARE element located ~ 3.1kb upstream of the transcriptional start site of human GCLC was found to mediate constitutive and β-NF inducible expression in HepG2 cells (a human hepatoma cell line) [78]. The transcription factor Nrf2, possibly in complexes with other Jun or Maf proteins, was found to be responsible for *trans*-activating the human GCLC promoter via binding to ARE4 in response to numerous treatments including β-NF,

pyrrolidine dithiocarbamate, TBH [104,105], and insulin [106]. The ARE4 is also the site of TGF-β1 action in type II alveolar cells, as TGF-β1 induced the binding of Fra-1/c-Jun dimer to the internal AP-1 sequence of the ARE4 site that led to suppression of GCLC transcriptional activity [72]. Thus, depending on the make up of the transcription factors bound to the ARE, opposite effects may occur. Indeed, this is further illustrated in cholestatic liver injury (see below). Thus, ARE appears to be the key element for various inducers of GCLC. However, As^{3+}, despite being an inducer of oxidative stress, induced GCLC transcriptional activity by a mechanism that is independent of Nrf1 or Nrf2 [87]. The transcription factor(s) responsible for As^{3+}-mediated GCLC induction is unknown.

Nrf2 is the key transcription factor required for activation of ARE. Nrf1 and Nrf2 are members of the cap 'n' collar-basic leucine zipper proteins (CNC-bZIP) and both can *trans*-activate ARE [107–109]. Under non-stressful physiological condition, Nrf2 is kept in the cytosol by Keap1, a component of an E3 ubiquitin ligase complex that targets Nrf2 for proteasomal degradation [110,111]. Oxidative stress and treatment with many agents that activate Nrf2 act by modifying either Keap1 or Nrf2 posttranslationally to cause their dissociation [88,110]. Once Nrf2 is released from Keap1, it escapes proteasomal degradation and translocates to the nucleus to induce genes involved in antioxidant defense [110]. NO's activation of Nrf2 in endothelial cells required an increase in free zinc level intracellularly but how zinc activates Nrf2 is not clear [89]. Once Nrf2 translocates to the nucleus, it forms heterodimers with small Maf (MafG, MafK and MafF) and Jun (c-Jun, Jun-D, and Jun-B) proteins to bind to ARE [108]. Nrf2/MafG heterodimer generally activates ARE-dependent gene transcription and has also been reported to enhance Nrf2 nuclear retention [108,112]. Another key mechanism that controls Nrf2 nuclear level is importin α7-mediated Keap1 nuclear import [111]. Thus, Keap1 shuttles between the nucleus and the cytoplasm and the nuclear import requires the interaction of importin α7 with the C-terminal Kelch domain of Keap1 [111]. Keap1 contains a strong nuclear export signal, which facilitates the nuclear export of Keap1-Nrf2 complex to keep Nrf2 activation and ARE-dependent gene induction under control [111]. Keap1 does not control Nrf1's activity; instead Nrf1 is primarily localized to the membrane of the endoplasmic reticulum and is released and translocates to the nucleus during endoplasmic reticulum stress [113]. Nrf1 knockout mice die *in utero* but fetal hepatocytes and embryonal fibroblasts have lower GSH levels and are more susceptible to oxidative stress [107,114]. Nrf2 knockout mice also exhibit lower GSH levels and are more susceptible to acetaminophen-induced liver injury [115]. Both Nrf1 and Nrf2 knockout mice have lower GCLC expression [114,115] and overexpression of Nrf1 and Nrf2 can induce the human GCLC promoter activity [104,116]. Nrf1 and Nrf2 may induce GCLC promoter directly and indirectly. Although the rat GCLC promoter also contain a distal ARE 4 kb upstream [117], the 1.8 kb 5′-flanking region of the rat GCLC does not contain any consensus ARE element (5′-G/ATG/TAG/CNNNGCA/G-3′) [105] and yet TBH treatment can induce the reporter activity driven by this 1.8 kb construct [118]. The explanation lies in cross talks between Nrf1/Nrf2 and AP-1 and NF B family members. The basal expression and nuclear binding activities of c-Jun, c-Fos, p50 and p65 are lower in fibroblast cells lacking Nrf1 or Nrf2. Other AP-1 and NF B family members are either unaffected (JunB, JunD) or increased (Fra-1, JAB1, and c-Rel). Overexpression of Nrf1 and Nrf2 restored the rat 1.8 kb-GCLC promoter activity and response to TBH by enhancing the expression of key NFκB and AP-1 family members [118]. However, this was blocked if the AP-1 and NFκB binding sites were mutated, proving the importance of these *cis*-acting elements at least in the rat GCLC. Interactions between NF B and AP-1 also occur [97]. Tumor necrosis factor α (TNFα) induces NFκB and AP-1 nuclear-binding activities and both are required for normal expression of both GCL subunits and GS in rat. While all three genes have multiple AP-1-binding sites, only GCLC has an NFκB-binding site. The explanation for the ability of NFκB to induce rat GCLM and GS promoter activity is that NFκB can increase AP-1 expression and nuclear binding activity. Thus, both c-Jun and NFκB are required for basal

and TNFα-mediated induction of GSH synthetic enzymes in H4IIE cells (a rat hepatoma cell line). While NFκB may exert a direct effect on the GCLC promoter, it induces the GCLM and GS promoters indirectly via c-Jun. These findings further illustrate the complex cross talks among the different families of transcription factors. In mouse, both GCLC and GCLM have NFκB binding sites and the basal expression of these two genes requires NFκB [119].

In addition to ARE/EpRE, AP-1 and NFκB, c-Myc has been identified to also contribute to the basal expression and induction of human GCL under oxidative stress [120]. Thus, down-regulation of c-Myc lowered GSH while overexpression of c-Myc increased GSH. Two noncanonical c-Myc binding sites (CACATG, E box) are present in the human GCLC promoter at –559/–554 and –500/–495 and together with ARE4, are responsible for H_2O_2-induced GCLC promoter activity [120]. The signaling pathway activated by H_2O_2 involves ERK-mediated phosphorylation and activation of c-Myc [120].

Recently, c-AMP-response element binding protein (CREB) was found to be the key transcription factor rather than Nrf1 or Nrf2, in binding to ARE4 and induction of GCLC expression in response to anthocyanin (a flavonoid antioxidant) treatment in HepG2 cells [121]. The induction occurred when Nrf1 or Nrf2 was silenced using siRNA but whether CREB binds to the ARE4 alone or requires the participation of other transcription factors is unknown.

In addition to increased gene transcription, DEM and 4-HNE have also been found to stabilize the GCLC mRNA [102,122]. However, the mechanism of this effect remains unknown.

4.2 GCLC post-translational regulation

GCL is also regulated post-translationally. GSH synthesis was inhibited by hormone-mediated activation of various signal transduction pathways [123,124]. These hormones are secreted under stressful conditions, many of which have associated lower hepatic GSH levels [10,13]. The fall in hepatic GSH level occurs by both an increase in sinusoidal GSH efflux [125,126] and an inhibition of GSH synthesis [123]. This may represent the hepatic stress response by increasing the systemic delivery of GSH and cysteine and channeling cysteine to synthesis of stress proteins [127]. We showed that GCLC is phosphorylated directly by activation of protein kinase A (PKA), protein kinase C (PKC) or Ca^{2+}-calmodulin kinase II (CMK) [128]. Cultured hepatocytes exhibit basal GCLC phosphorylation, which increased when treated with DBcAMP or phenylephrine, suggesting GCLC may be under a basal inhibitory tone [128]. Thus, phosphorylation-dephosphorylation may be an important physiologic regulator of GCL. Since many pathologic and toxic conditions can lead to activation of CMK and phosphorylation of GCLC, inhibition of GCL may further contribute to toxicity. Consistent with this notion is the report that toxic doses of acetaminophen suppressed hepatic GSH synthesis in rats [129].

GCLC can be cleaved by a caspase 3-dependent mechanism from the full-length 73 kDa to a 60 kDa form during apoptosis induced by TGF-β1, TNFα and α-Fas [45,130]. Cleavage of GCLC occurs at Asp^{499} within the sequence $AVVD^{499}G$, which is located upstream of Cys^{553} thought to be important for disulfide bond formation with GCLM [35,45]. Theoretically, this would result in decreased GCL activity but this was not observed during apoptotic cell death [45]. Cleavage of GCLC at Asp^{499} generates a 13 kDa-C-terminal fragment with a N-terminal glycine residue that is predicted to be a myristoylation site [45]. While myristoylation was demonstrated when a GCLC fragment was overexpressed [131], whether this actually occurs during apoptotic cell death remains unclear.

Recently the lipid peroxidation product 4-HNE was shown to directly adduct GCLC Cys553 (and GCLM Cys35) *in vitro* [132]. Formation of 4-HNE GCLC adduct increased the activity of monomeric GCLC but inhibited formation of GCL holoenzyme and lowered GCL holoenzyme activity [132]. In cells where GCLC predominates, this mechanism may allow increased GSH synthesis by increasing the enzymatic activity of monomeric GCLC. However, whether this occurs *in vivo* remains to be examined.

4.3 Regulation of GCLM

GCLM plays a critical regulatory role on the overall function of GCL [38,43]. The two subunits of GCL are often coordinately induced by oxidative stress but as described above, hormones (insulin, hydrocortisone, TGF-β1) and rapid growth induce GCLC selectively in the liver. The exception is HGF, which induced the expression of both GCL subunits in hepatocytes under low-density condition [29]. GCLM is induced by xenobiotics such as β-NF and TBH [92,93,104,105,133,134]. Similar to the human GCLC, up-regulation of the human GCLM by β-NF involved binding of transcription factor Nrf2 (possibly in complexes with other Jun or Maf proteins as in GCLC) to a functional ARE/EpRE site located at −302 of the human GCLM [104,134]. Also, there is a critical c-Myc-binding E-box at −1609/−1604 of the human GCLM promoter that in conjunction with the proximal ARE, mediate the full induction of the GCLM promoter under H_2O_2-induced stress [120]. Nrf2 is also required for GCLM induction by 15d-PGJ_2 and physiological 4-HNE concentrations [84–85]. Transcription factors and *cis*-acting elements important for mouse and rat GCLM genes are similar to the human gene. Fibroblast cells derived from Nrf1 and Nrf2 knockout mice have lower GSH levels and reduced basal expression of GCLM [107,118]. The rat GCLM promoter also has a functional ARE element (−295 to −285) [97]. This ARE element is important for basal expression and TNFα-mediated induction of rat GCLM [97]. AP-1, NFκB and Nrf2 are positive regulators of the rat GCLM gene and are induced by TNFα treatment [97]. While AP-1 and Nrf2 have direct effects on the rat GCLM promoter, NFκB activates it indirectly via AP-1 [97]. Not all inducers of oxidative stress induce GCLM. Ethanol and TGF-β1 treatments do not affect rat GCLM expression [73,135]. The reason for this discordance is not clear. Finally, GCLM expression also changes in a biphasic manner during BDL and when hepatocytes are treated with lithocholic acid [90,136 - see below under cholestatic liver injury].

Post-transcriptional regulation of GCLM also occurs with 4-HNE treatment, which increased the stability of GCLM mRNA by an unknown mechanism that required *de novo* protein synthesis [122]. This is in contrast to the effect of 4-HNE on GCLC mRNA stability, which did not require *de novo* protein synthesis. As^{3+} increased GCLM mRNA stability in addition to GCLM transcription but the mechanism is unclear [87].

5. Regulation of GSH synthase (GS)

GS has received relatively little attention in the field of GSH biosynthesis. GS is composed of two identical subunits and is not subject to feedback inhibition by GSH [48]. GS deficiency in humans can result in dramatic metabolic consequences because the accumulated γ-glutamylcysteine is converted to 5-oxoproline, which can cause severe metabolic acidosis, hemolytic anemia and central nervous system damage [137,138]. Choi et al described decreased hepatic GSH levels, which correlated with reduced GS activity in Tat transgenic mice [139]. A decrease in GS activity alone without a change in GCL and a fall in GSH levels occurred after surgical trauma in human skeletal muscle [50]. These findings seem to contradict the notion that GCL is rate-limiting. Although the specific activity of GS is normally 2 to 4 times that of GCL activity in normal liver [64,140], this may not be the case in other tissues and under stressful conditions. In fact, in normal human skeletal muscle, the specific activity of GS is only 36% higher than that of GCL [50]. Surgical

trauma selectively reduced GS activity, which probably became rate limiting [50]. Recently, all-trans retinoic acid (ATRA), was shown to induce the expression of GS selectively (no effect on GCLC or GCLM) and GSH levels in myeloid-derived suppressor cells [141]. Taken together, these results suggest regulation of GS may also be important in determining the overall GSH synthetic capacity under certain conditions and especially in non-hepatic tissues.

We found treatments that increase the expression of both GCL subunits, such as DEM, buthionine sulfoximine (BSO), TBH, TNFα and HGF treatment of cultured rat hepatocytes and thioacetamide (TAA) treatment of rats, also increased the expression of GS [26,29,97]. In contrast, treatments that increase the expression of GCLC alone such as insulin, hydrocortisone in cultured hepatocytes and ethanol feeding *in vivo*, had no influence on GS expression. There are exceptions, one is liver regeneration after partial hepatectomy, another is HCC, and a third is liver-specific retinoid X receptor α (RXRα) knockout mice. GS mRNA levels changed in parallel to that of GCLC in all three conditions while GCLM mRNA levels were unchanged [26,51,142]. We speculated that when GCL is induced tremendously, the step catalyzed by GS might become limiting. A coordinated induction in the activity of both enzymes would further enhance GSH synthesis capacity. Consistent with this hypothesis, treatments that induced only GCLC increased the GSH synthesis capacity by 50 to 100% [64,135], whereas treatments that induced both GCLC and GS expression increased the GSH synthesis capacity by 161–200% [27,140].

Given the coordinated regulation of GCL and GS, it is not surprising that transcriptional regulation of these genes is quite similar. For the rat GS promoter, AP-1 serves as an enhancer directly while NF-1 acts as a repressor [143]. NFκB can also activate the rat GS promoter, albeit indirectly via AP-1 [97]. Both Nrf1 and Nrf2 overexpression induced the human GS promoter activity [144]. The human GS promoter contains two regions with homology to the NFE2 (nuclear factor erythroid 2) motif that are required for basal activity [144]. ATRA, which works via RXR, induced the expression of GS selectively and GSH levels in myeloid-derived suppressor cells [141]. ATRA treatment for 48 hours also increased GSH levels in mononuclear cells isolated from patients with metastatic renal cell carcinoma, but GS expression was not examined [141]. The mechanism of ATRA's inductive effect on GS expression required ERK1/2 signaling but the mechanism is not clear, as ATRA treatment did not influence the expression of Nrf2 or NFκB and it had no effect on the GS promoter activity [141]. This suggests the possibility of post-transcriptional regulation of GS by ATRA but this remains to be examined. Post-translational regulation of GS has not been reported.

6. Dysregulation of GSH synthesis

There is accumulating data that reduced GSH levels occur in many human diseases and they contribute to worsening of the condition [4]. While oxidative injury plays a dominant role in GSH depletion in many of these disorders, some are causally related to reduced expression of GSH synthetic enzymes [13]. In the most severe cases, polymorphisms of GCLC and/or GCLM that result in significantly reduced GCL expression and activity can present with severe phenotype including hemolytic anemia, aminoaciduria and spinocerebellar degeneration [reviewed in 45]. GCLC and GCLM polymorphisms have been reported in many disorders, including schizophrenia, cardiovascular diseases, stroke, and asthma [45,145,146]. Outside of polymorphism, decreased GSH synthesis occurs during aging, diabetes mellitus, fibrotic diseases (including cystic fibrosis and pulmonary fibrosis), endotoxemia, and several hepatic disorders such as cholestatic and alcoholic liver injury [13,15]. The opposite situation, namely increased GSH synthesis, plays an important role in conferring drug and/or radiation resistance to many different cancers [13]. Targeting this

increase in GCL activity using BSO (the irreversible GCL inhibitor) is now often used as an adjuvant chemotherapeutic agent in cancer treatment [13]. Given the central role of hepatic GSH in systemic GSH homeostasis, four liver disorders (cholestasis, endotoxemia, alcohol and fibrosis) where decreased GSH synthesis may participate in the pathogenesis of liver injury are described in more detail.

6.1. Cholestasis

Cholestasis is the underlying mechanism for many chronic liver diseases. The underlying mechanism for cell toxicity is thought to be retention of toxic bile acids, which can cause oxidative stress, apoptosis, fibrosis leading to cirrhosis [147,148]. Recently we used the BDL model in mice and showed that hepatic expression of GSH synthetic enzymes increased early on likely as an adaptive response to oxidative stress but decreased markedly along with GSH levels during later stages of BDL [136]. A key observation from this study was the fall in Nrf2 nuclear binding to the ARE two weeks after BDL. A similar pattern of early induction followed by fall in GSH synthetic enzymes also occurred when Huh-7 cells (a human hepatoma cell line) was treated with lithocholic acid [90]. In both BDL and lithocholic acid-treated Huh-7 cells, the fall in expression of GSH synthetic enzymes coincided with an increase in the expression of several Maf proteins (c-Maf, MafG and MafK) as well as increased c-Maf and MafG nuclear binding to ARE. MafG and MafK are small Mafs that have been reported to heterodimerize with Nrf2 to either activate or repress ARE-dependent genes [108,149]. Small Mafs lack transcriptional activation domain and can form homodimers to repress ARE-mediated gene expression [150]. In addition, large Maf protein such as c-Maf can bind to ARE as homodimers and heterodimers with small Mafs (but not Nrfs) to repress ARE-mediated gene expression [151]. Given these known effects of small Mafs and c-Maf, we speculated that the induction in Mafs and displacement of Nrf2 from nuclear binding to ARE during cholestasis might have caused the fall in the expression of GSH synthetic enzymes. Consistent with this, blocking either c-Maf or MafG induction during BDL protected against the fall in expression of GSH synthetic enzymes, GSH levels and BDL-induced liver injury [90]. Interestingly, ursodeoxycholic acid (UDCA), the only medication approved by the FDA for the treatment of primary biliary cirrhosis [152], a chronic cholestatic disorder, and SAMe were able to raise nuclear Nrf2 level, block the increase in MafG and c-Maf expression, protect against the fall in expression of GSH synthetic enzymes and GSH levels in these models [90,136]. Combining UDCA and SAMe exerted additional benefit, suggesting they have different mechanisms. Murine cholestatic liver injury is the first example that illustrates the importance of Maf proteins on ARE-dependent gene expression in liver pathology.

6.2 Endotoxemia

Lipopolysaccharide (LPS, synonymous as endotoxin) is a major constituent of the outer cell wall of all gram-negative bacteria that can trigger the synthesis and release of pro-inflammatory cytokines and inducible nitric oxide synthase (iNOS) [153,154]. Liver clears gut-derived LPS [154]. This explains why endotoxemia occurs in cirrhotic patients and the degree of endotoxemia correlates with the degree of liver failure [154]. Endotoxemia also participates in worsening of alcoholic liver disease and non-alcoholic steatohepatitis [154,155]. Endotoxemia lowers GSH levels in the liver [156,157], peritoneal macrophages and lymphocytes [158]. Septic patients have lower blood GSH:GSSG ratios [159]. Exogenous GSH treatment suppressed LPS-induced systemic inflammatory response and reduced mortality [160]. GSH level is an important variable that determines susceptibility to LPS-induced injury in multiple tissues [157,160,161]. This may be related to GSH's ability to influence toll like receptor 4 (TLR4) signaling. Specifically, LPS-induced mortality and TNFα secretion were higher when GSH level was reduced [162]. The fall in GSH is multifactorial. In liver, increased GSH efflux and increased oxidative stress both contribute

[153,156]. One study showed that a major mechanism of hepatic GSH depletion during endotoxemia is a fall in GCLC mRNA level and GCL activity [157]. Consistent with this, we found that hepatic GSH level fell more than 50% following LPS, coinciding with a comparable fall in the mRNA and protein levels of GCLC and GCLM (50–60%) [163]. GS expression fell to a lesser extent (40% fall). SAMe pretreatment protected against liver injury and prevented the fall in GCLC and GCLM expression and GSH level. Nearly maximum inhibition in the expression of GSH synthetic enzymes occurred as early as six hours after LPS administration [163]. The molecular mechanisms remain to be elucidated.

6.3 Alcohol

Alcoholic liver disease patients have low hepatic and plasma GSH levels due to multiple mechanisms such as oxidative stress, nutritional deficiency and abnormalities in the methionine metabolic pathway that impairs cysteine availability [164–166]. In addition, we found a 50% fall in the mRNA levels of GCLC and GS (GCLM was unchanged) in patients hospitalized for alcoholic hepatitis [166]. The mechanism for this is unclear but these abnormalities may contribute to the high morbidity and mortality associated with this disorder.

6.4 Fibrogenesis

HSCs are the key effectors in hepatic fibrogenesis [167]. HSCs reside in the space of Disse and in normal liver are the major storage sites of vitamin A. Following chronic liver injury, HSCs proliferate, lose their vitamin A and undergo a major phenotypical transformation to α-smooth muscle actin (α-SMA) positive activated HSCs, which produce a wide variety of collagenous and non-collagenous extracellular matrix (ECM) proteins [168]. The profibrogenic potential of activated HSCs is due to their capacity to synthesize fibrotic matrix proteins and components that inhibit fibrosis degradation. Pro-fibrogenic factors include TGF-β [169], connective tissue growth factor [170], leptin [171] and platelet derived growth factor [172]. Activation of HSC is mediated by various cytokines and ROS released from damaged hepatocytes and activated Kupffer cells [173]. Hence, inhibition of HSC activation and its related events such as ECM formation and cellular proliferation are important targets for therapeutic intervention. In both hepatic and pulmonary fibrosis, TGF-β1 has been shown to target GSH synthesis (see above under GCLC regulation) [15,73,82]. EGCG and curcumin, two agents that exert anti-fibrotic effect in hepatic HSCs, require de novo GSH synthesis to exert this effect [73,82]. In BDL, preventing the fall in hepatic GSH also resulted in amelioration of hepatic fibrosis [90]. Consistent with the importance of GSH in hepatic fibrogenesis, we found that a lower hepatic GSH level greatly potentiated BDL-induced fibrosis and if induction in GCLC expression was blocked (by using RNAi), the therapeutic efficacy of UDCA and SAMe was nearly lost [174]. We also established that GCLC expression is a critical factor in determining the phenotype of rat HSCs (GCLM and GS were unchanged at the protein level) [174]. Specifically, GCLC expression fell during HSC activation and increased as activated HSCs revert to quiescence. Blocking the increase in GCLC expression kept HSCs in an activated state. Although activated HSCs have increased nuclear MafG level, formation of Nrf2/MafG heterodimer and binding to ARE is greatly diminished. In contrast, quiescent HSCs have markedly lower total nuclear MafG level but increased Nrf2/MafG heterodimerization and binding to ARE. This is due to enhanced sumoylation of Nrf2 and MafG by SUMO-1 in the quiescent state, which facilitated heterodimerization and binding to ARE [174]. Thus, a key mechanism that controls Nrf2/MafG trans-activation of GCLC ARE is sumoylation by SUMO-1 in HSCs. Taken together, a fall in GSH facilitates activation of HSCs and fibrosis to proceed. Targeting this is an attractive therapeutic strategy that yielded promising results in animal models of pulmonary and hepatic fibrosis [15, 90] but human trials have not been as positive [15].

7. Concluding remarks

Up until recently, most of the literature on GSH synthesis has focused on understanding how the enzymes are regulated transcriptionally and post-transcriptionally. There are now increasing evidence that show dysregulation of GSH synthesis in multiple conditions, such as aging, diabetes, pulmonary and hepatic fibrosis, alcoholic and cholestatic liver injuries. Some of these have been confirmed to occur also in humans. GCLC and GCLM polymorphisms have also gained attention as another determinant of chronic oxidative injury to various organs. While the status of screening for these polymorphisms remains to be established, uncovering the molecular mechanisms responsible for the dysregulation in GSH synthesis may provide novel therapeutic approaches.

Acknowledgments

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Abbreviations (in alphabetical order)

4-HNE	4-hydroxynonenal
15d-PGJ$_2$	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
AP-1	activator protein-1
As^{3+}	trivalent arsenite
α-SMA	a-smooth muscle actin
ARE	antioxidant response element
ATRA	all-trans retinoic acid
BDL	bile duct ligation
BHMT	betaine homocysteine methyltransferase
β-NF	β-naphthoflavone
BSO	buthionine sulfoximine
CBS	cystathionine β synthase
CMK	Ca^{2+}-calmodulin kinase II
CNC-bZIP	cap 'n' collar-basic leucine zipper proteins
CREB	c-AMP-response element binding protein
DEM	diethyl maleate
ECM	extracellular matrix
EpRE	electrophile response element
GCL	glutamate-cysteine ligase
GCLC	GCL-catalytic subunit
GCLM	GCL-modifier subunit
GGT	γ-glutamyltranspeptidase
GPx	GSH peroxidase
GS	GSH synthase

GSH	glutathione
GSSG	oxidized GSH
HCC	hepatocellular carcinoma
Hcy	homocysteine
HGF	hepatocyte growth factor
HSC	hepatic stellate cell
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
MAT	methionine adenosyltransferase
MRE	metal response element
MS	methionine synthase
MT	methyltransferase
NFE2	nuclear factor erythroid 2
NO	nitric oxide
Nrf2	nuclear factor-erythroid 2 related factor 2
PKA	protein kinase A
PKC	protein kinase C
RNS	reactive nitrogen species
RXRα	retinoid X receptor α
ROS	reactive oxygen species
SAH	S-adenosylhomocysteine
SAMe	S-adenosylmethionine
TAA	thioacetamide
TBH	tert-butyl hydroquinone
TGF-β1	transforming growth factor-β1
TLR4	toll like receptor 4
TNFα	tumor necrosis factor α
UDCA	ursodeoxycholic acid

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Highlights

- GSH regulates antioxidant defense, growth, death, immune function, and fibrogenesis.
- GSH is synthesized via two enzymatic steps that are regulated at multiple levels.
- GSH synthesis is dysregulated in multiple human diseases.

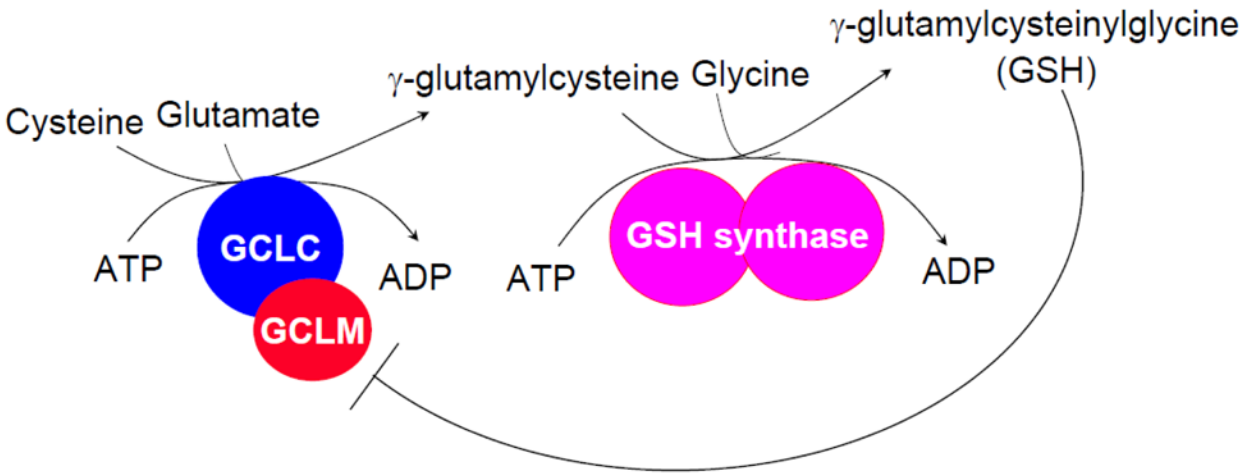

Fig. 1. GSH synthesis

Synthesis of GSH occurs via a two-step ATP-requiring enzymatic process. The first step is catalyzed by glutamate-cysteine ligase (GCL), which is composed of catalytic and modifier subunits (GCLC and GCLM). This step conjugates cysteine with glutamate, generating γ-glutamylcysteine. The second step is catalyzed by GSH synthase, which adds glycine to γ-glutamylcysteine to form γ-glutamylcysteinylglycine or GSH. GSH exerts a negative feedback inhibition on GCL.

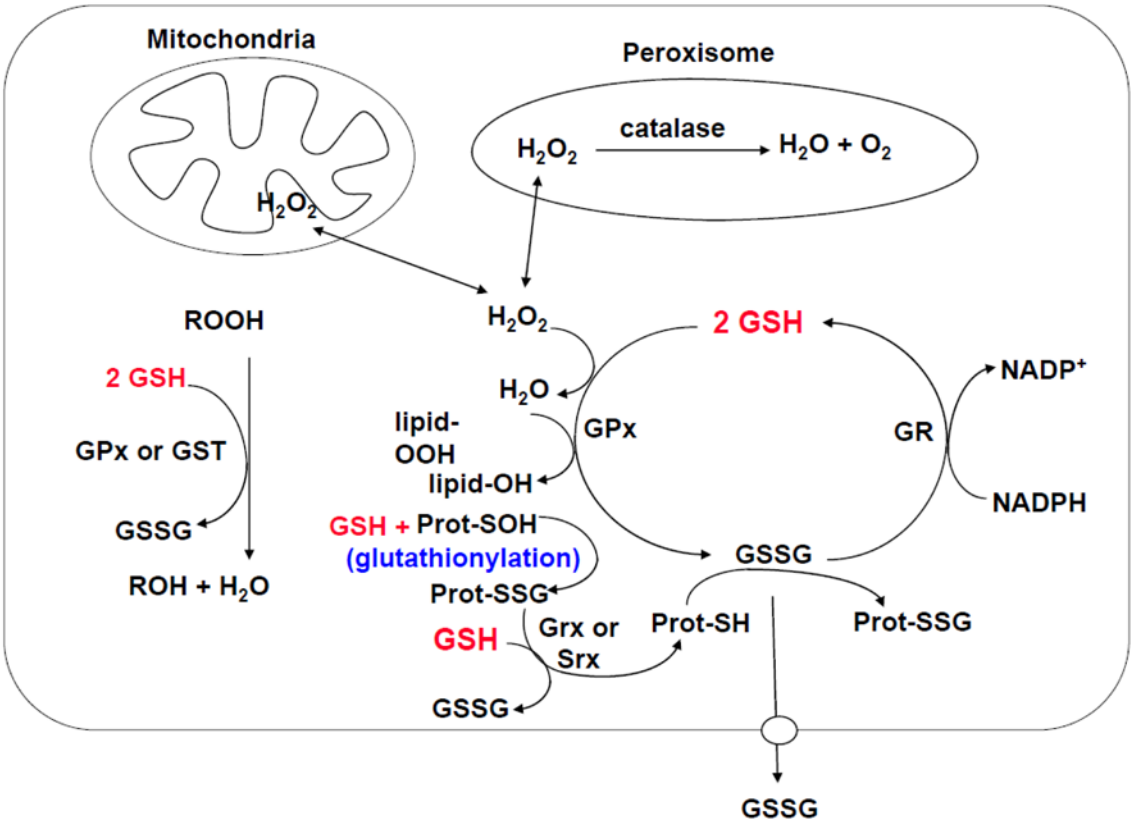

Fig. 2. Antioxidant function of GSH

Aerobic metabolism generates hydrogen peroxide (H_2O_2), which can be metabolized by GSH peroxidase (GPx) in the cytosol and mitochondria, and by catalase in the peroxisome. GSSG can be reduced back to GSH by GSSG reductase (GR) at the expense of NADPH, thereby forming a redox cycle. Organic peroxides (ROOH) can be reduced by either GPx or GSH S-transferase (GST). GSH also plays a key role in protein redox signaling. During oxidative stress, protein cysteine residues can be oxidized to sulfenic acid (Prot-SOH), which can react with GSH to form protein mixed disulfides Prot-SSG (glutathionylation), which in turn can be reduced back to Prot-SH via glutaredoxin (Grx) or sulfiredoxin (Srx). This is a mechanism to protect sensitive protein thiols from irreversible oxidation and may also serve to prevent loss of GSH under oxidative conditions. The ability of the cell to reduce GSSG to GSH may be overcome during severe oxidative injury, leading to an accumulation of GSSG. To prevent a shift in the redox equilibrium, GSSG can either be actively transported out of the cell or react with a protein sulfhydryl (Prot-SH) to form a mixed disulfide (Prot-SSG).

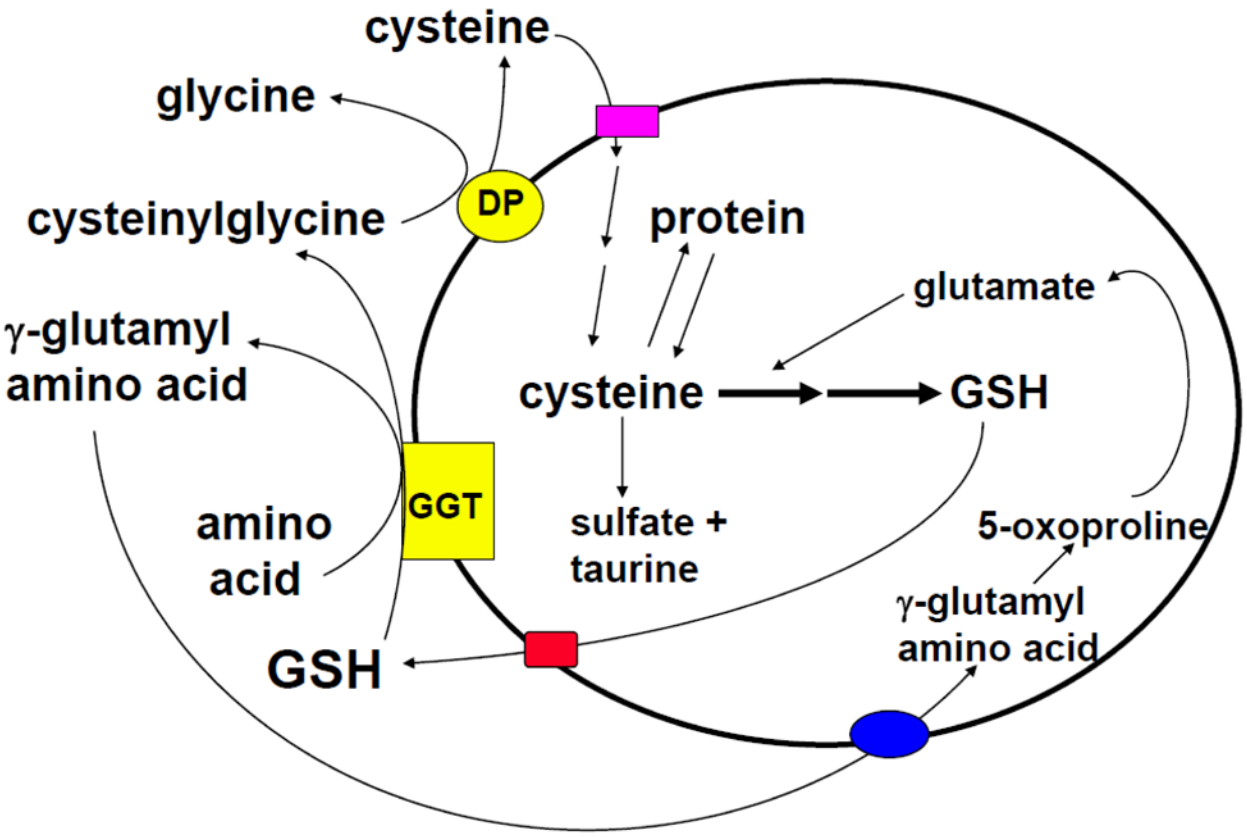

Fig. 3. GSH is a continuous source of cysteine via the γ-glutamyl cycle

GSH is transported out of the cell where the ecto-enzyme γ-glutamylpeptidase (GGT) transfers the γ-glutamyl moiety of GSH to an amino acid (the best acceptor being cystine), forming γ-glutamyl amino acid and cysteinylglycine. The γ-glutamyl amino acid can then be transported back into the cell and once inside, the γ-glutamyl amino acid can be further metabolized to release the amino acid and 5-oxoproline, which can be converted to glutamate and reincorporated into GSH. Cysteinylglycine is broken down by dipeptidase (DP) to generate cysteine and glycine, which are also transported back into the cell to be reincorporated into GSH. Most of the cysteine taken up is incorporated into GSH while the rest is incorporated into newly synthesized proteins and/or broken down into sulfate and taurine.

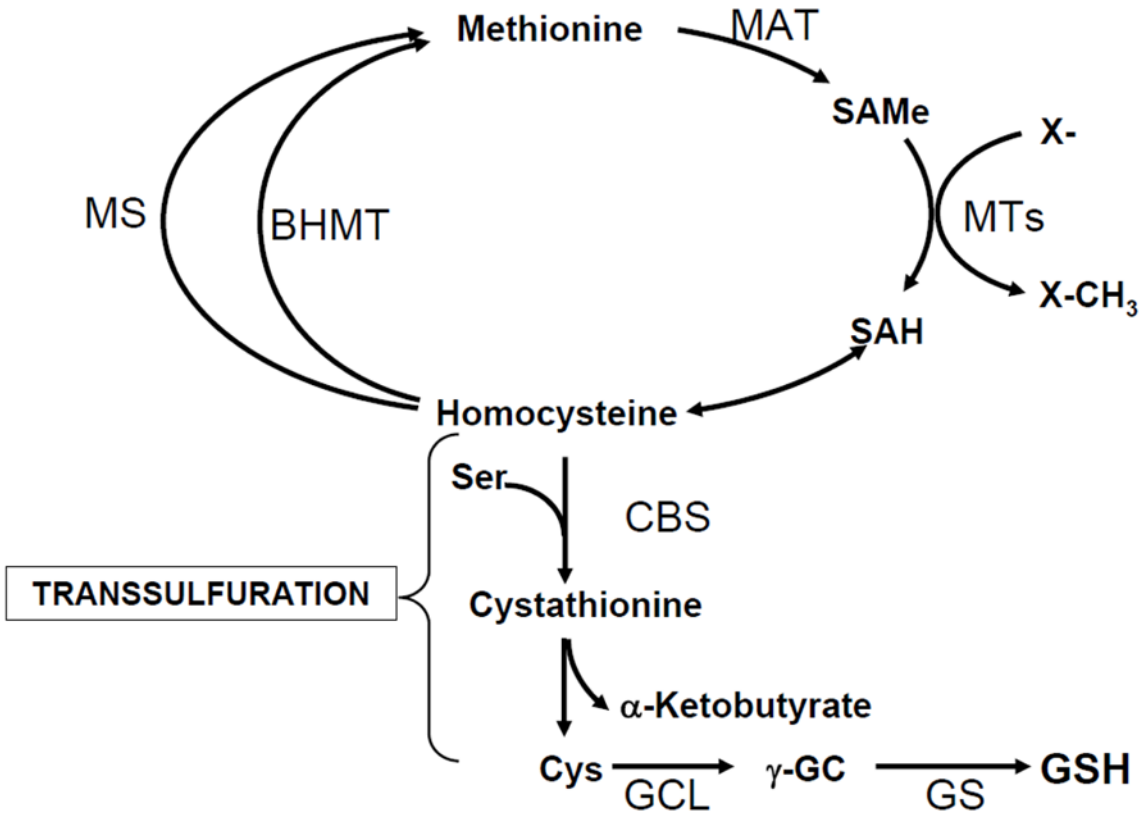

Fig. 4. Hepatic methionine metabolism

Liver plays a central role in methionine catabolism as up to half of the daily intake of methionine is catabolized to S-adenosylmethionine (SAMe) in the liver in a reaction catalyzed by methionine adenosyltransferase (MAT). SAMe is the principal biological methyl donor and donates its methyl group to a large variety of acceptor molecules in reactions catalyzed by methyltransferases (MTs). S-adenosylhomocysteine (SAH), generated as a result of transmethylation, is a potent inhibitor of all transmethylation reactions. To prevent SAH accumulation, it is hydrolyzed to homocysteine and adenosine is through a reversible reaction catalyzed by SAH hydrolase, whose thermodynamics favors biosynthesis rather than hydrolysis. Prompt removal of homocysteine and adenosine ensures SAH is hydrolyzed. Homcysteine can be remethylated to form methionine via methionine synthase (MS), which requires folate and vitamin B_{12} and betaine homocysteine methyltransferase (BHMT), which requires betaine. In hepatocytes, homocysteine can also undergo conversion to cysteine (Cys) via the transsulfuration pathway, a two-step enzymatic process catalyzed by cystathionine β-synthase (CBS) and cystathionase, both requiring vitamin B_6. Liver has the highest activity of transsulfuration, which allows methionine and SAMe to be effectively utilized as GSH precursor.

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REVIEW

Oxidative stress and regulation of glutathione in lung inflammation

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ABSTRACT: Inflammatory lung diseases are characterized by chronic inflammation and oxidant/antioxidant imbalance, a major cause of cell damage. The development of an oxidant/antioxidant imbalance in lung inflammation may activate redox-sensitive transcription factors such as nuclear factor-κB, and activator protein-1 (AP-1), which regulate the genes for pro-inflammatory mediators and protective antioxidant genes. Glutathione (GSH), a ubiquitous tripeptide thiol, is a vital intra- and extracellular protective antioxidant against oxidative/nitrosative stresses, which plays a key role in the control of pro-inflammatory processes in the lungs. Recent findings have suggested that GSH is important in immune modulation, remodelling of the extracellular matrix, apoptosis and mitochondrial respiration. The rate-limiting enzyme in GSH synthesis is γ-glutamylcysteine synthetase (γ-GCS). The human γ-GCS heavy and light subunits are regulated by AP-1 and antioxidant response elements and are modulated by oxidants, phenolic antioxidants, growth factors, and inflammatory and anti-inflammatory agents in lung cells.

Alterations in alveolar and lung GSH metabolism are widely recognized as a central feature of many inflammatory lung diseases such as idiopathic pulmonary fibrosis, acute respiratory distress syndrome, cystic fibrosis and asthma. The imbalance and/or genetic variation in antioxidant γ-GCS and pro-inflammatory *versus* antioxidant genes in response to oxidative stress and inflammation in some individuals may render them more susceptible to lung inflammation. Knowledge of the mechanisms of GSH regulation and balance between the release and expression of pro- and anti-inflammatory mediators could lead to the development of novel therapies based on the pharmacological manipulation of the production as well as gene transfer of this important antioxidant in lung inflammation and injury.

This review describes the redox control and involvement of nuclear factor-κB and activator protein-1 in the regulation of cellular glutathione and γ-glutamylcysteine synthetase under conditions of oxidative stress and inflammation, the role of glutathione in oxidant-mediated susceptibility/tolerance, γ-glutamylcysteine synthetase genetic susceptibility and the potential therapeutic role of glutathione and its precursors in protecting against lung oxidant stress, inflammation and injury.
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Inflammation is an important protective response to cellular/tissue injury. The purpose of this process is to destroy and remove the injurious agent and injured tissues, thereby promoting tissue repair. When this crucial and normally beneficial response occurs in an uncontrolled manner, the result is excessive cellular/tissue damage that results in chronic inflammation and destruction of normal tissue. Reactive oxygen species (ROS), such as the superoxide anion liberated by phagocytes recruited to sites of inflammation, are proposed to be a major cause of the cell and tissue damage, including apoptosis, associated with many chronic inflammatory diseases [1–3]. Lung cells, in particular alveolar epithelial type II cells, are susceptible to the injurious effects of oxidants. It has been shown that lung cells release inflammatory mediators and cytokines/chemokines such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1 and IL-8 in response to oxidative/nitrosative stress. The release of cytokines/chemokines induces neutrophil recruitment and the activation of key transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), thereby augmenting the inflammatory response and tissue damage [4, 5]. As a result, the acute and chronic alveolar and/or bronchial inflammatory response is a fundamental process involved in the pathogenesis of many lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis (IPF) and cystic fibrosis (CF). The site and specific characteristics of the inflammatory responses may differ in each of these diseases, but all are characterized by the recruitment to the lungs and activation of inflammatory cells leading to an oxidant/antioxidant imbalance.

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) containing a thiol (sulphhydryl) group. GSH

is an important protective antioxidant against free radicals and other oxidants and has been implicated in immune modulation and inflammatory responses [6–8]. These events include modulation of redox-regulated signal transduction, regulation of cell proliferation, remodelling of the extracellular matrix (ECM), and antiprotease screen, apoptosis and mitochondrial respiration [7–12]. The antioxidant GSH has been shown to be critical to the lungs' antioxidant defences, particularly in protecting airspace epithelium (membrane integrity) from oxidative/free radical (cigarette smoke/air particulates)-mediated injury and inflammation [13–15]. Alterations in the levels of GSH in the lung lining fluid have been shown in various inflammatory conditions. For example GSH levels are decreased in the epithelial lining fluid (ELF) in IPF [16, 17], ARDS [18], CF [19], lung allograft [20] and human immunodeficiency virus (HIV)-positive patients [21]. In contrast, total glutathione concentrations, including the oxidized form (GSSG), are higher in the bronchial and alveolar fluid in patients with mild asthma (table 1) [22]. Glutathione is present in increased concentrations in the ELF of chronic smokers, whereas this is not the case in the ELF of acute smokers [23, 24]. However, GSH levels were not decreased in the ELF of IPF and HIV-positive patients who were smokers [25, 26]. A low GSH concentration in the ELF may contribute to an imbalance between oxidants and antioxidants in the lungs and may amplify inflammatory responses and potentiate lung damage.

It has been suggested that oxidants, antioxidants, and inflammatory and anti-inflammatory agents modulate the activation of redox-sensitive AP-1 and NF-κB [5]. AP-1 and AP-1-like antioxidant response element (ARE) have also been reported to modulate the expression of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in *de novo* GSH synthesis. γ-GCS consists of a catalytic heavy subunit (γ-GCS-HS) and a regulatory light subunit (γ-GCS-LS). It has recently been shown that the promoter (5′-flanking) region of both the human catalytic γ-GCS-HS and regulatory γ-GCS-LS genes contain putative AP-1 and ARE response elements which are necessary for γ-GCS expression in response to diverse stimuli [27–30]. It is possible that differences in ELF glutathione levels in various inflammatory lung diseases are due to changes in the molecular regulation of GSH synthesis by AP-1 and ARE activation by oxidants and inflammatory and anti-inflammatory agents, its turnover/breakdown and/or transport in lung cells. However, the molecular mechanism of glutathione synthesis in lungs of patients with inflammatory lung diseases has not been studied. The imbalance and genetic variability of γ-GCS and pro-inflammatory gene expression in response to oxidative stress and inflammatory response may be a determinant of susceptibility to lung disease. The aims of this review are: 1) to describe the sources of oxidative stress in inflammation and the redox control of the transcription factors NF-κB and AP-1; 2) review the regulation, transport and metabolism of lung cell glutathione and γ-GCS gene expression in inflammation and oxidative stress; 3) discuss the possible role of γ-GCS *versus* pro-inflammatory gene imbalance in susceptibility/tolerance; and 4) assess the potential protective and therapeutic role of glutathione and other related thiols in oxidant-induced lung injury and inflammation.

Table 1. – Epithelial lining fluid reduced glutathione (GSH) concentration in inflammatory lung diseases

	GSH μM		[Ref.]
	Controls	Patients	
Controls (nonsmokers)	339±112	–	[24]
Smokers	544±97.6	–	[24]
Idiopathic pulmonary fibrosis	429±34	97±18	[16]
Acute respiratory distress syndrome	651±103.1	31.5±8.4	[18]
Lung allograft	302.6±40.8	94.0±9.7	[20]
Cystic fibrosis	257±21	78±13	[19]
HIV-seropositive	245±12	170±23	[21]
Asthma	23.3±3*	36.5±9.4*	[22]

*: GSH plus oxidized glutathione (in μM·mg protein^{-1}). HIV: human immunodeficiency virus.

Role of cell-derived oxidants in inflammation

The presence of oxidative stress in the airspaces and the blood initiates a number of early events during pulmonary inflammation. Inflammatory cells are sequestered into the pulmonary microcirculation and recruited to the airspaces as a result of the generation of mediators such as IL-8. Once recruited, inflammatory cells become activated and generate ROS in response to a sufficient level of a secretagogue stimulus (threshold concentration). The mechanism for this may involve neutrophil adhesion to endothelium and upregulation of CD18 integrins [31, 32], which is known to upregulate the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase hydrogen peroxide-generating system [1]. Activation of macrophages, neutrophils and eosinophils generates $O_2\cdot^-$, which is rapidly converted to H_2O_2 by superoxide dismutase (SOD), and hydroxyl radicals, formed nonenzymatically in the presence of Fe^{2+} as a secondary reaction. In neutrophils, myeloperoxidase also catalyses the formation of the potent oxidant hypochlorous acid from H_2O_2 in the presence of chloride ions. ROS, which may also be released by lung epithelial cells [33, 34] may also stimulate inflammatory cells directly, thereby amplifying lung inflammatory and oxidant events (fig. 1).

ROS are highly reactive and, when generated close to cell membranes, deplete intracellular GSH and oxidize membrane phospholipids (lipid peroxidation), which may continue in a chain reaction. Thus, a single ·OH can result in the formation of many molecules of lipid hydroperoxide in the cell membrane, which may severely disrupt its function and may lead to cell death, or to damage of deoxyribonucleic acid (DNA) in alveolar epithelial cells [35]. ROS and reactive nitrogen species (RNS) also act on certain amino acids in proteins (*e.g.* enzymes, kinases) such as methionine, tyrosine and cysteine, profoundly altering the function of these proteins in inflammatory lung diseases [36]. Many of the effects of oxidants in airways may be mediated by the secondary release of inflammatory lipid mediators such as 4-hydroxy-2-nonenal, which is known to induce various cellular events

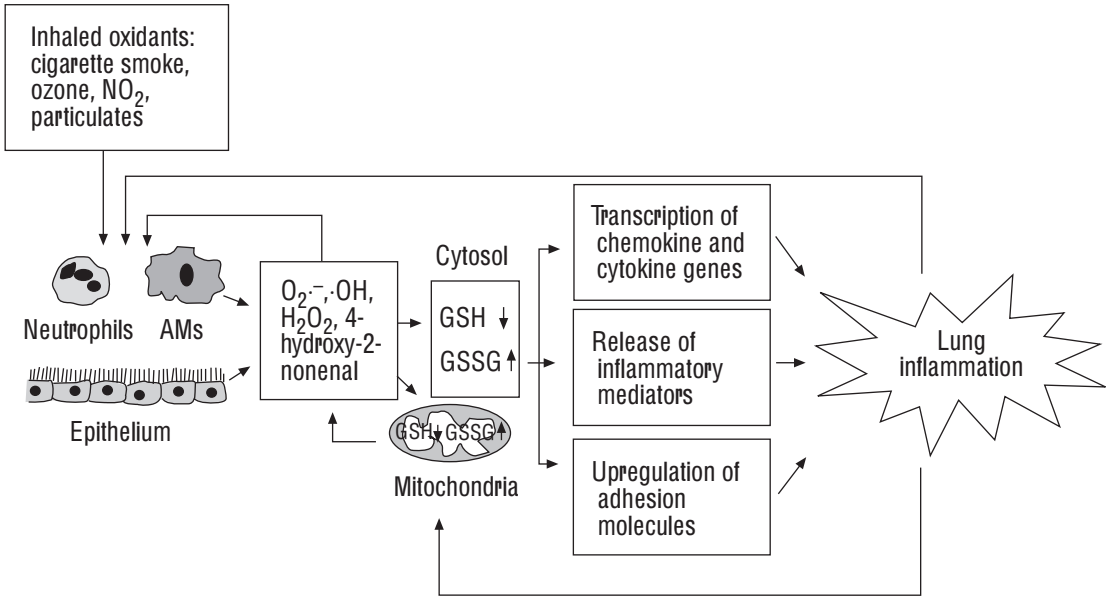

Fig. 1. – Mechanisms of oxidant-mediated lung inflammation. The inflammatory response is mediated by oxidants which are inhaled and/or released by the activated neutrophils, alveolar macrophages (AMs) and epithelial cells leading to depletion of the antioxidant reduced glutathione (GSH). Activation of transcription of the pro-inflammatory cytokine and chemokine genes, upregulation of adhesion molecules and increased release of pro-inflammatory mediators are involved in the inflammatory responses. GSSG: oxidized glutathione; ↓ : decrease; ↑ : increase.

such as proliferation and activation of signalling pathways [37].

Inhaled oxidants in lung inflammation

Inhaled environmental oxidants exacerbate the underlying inflammation in inflammatory lung diseases. Ozone is a potent oxidant, which causes cellular damage by lipid peroxidation as well as loss of functional groups on biomolecules. Inhalation of ozone may lead to an increase in neutrophil numbers, increased airway responsiveness and reduced pulmonary function in normal subjects [38]. This has been linked to neutrophil infiltration into the airway epithelium [39]. Cigarette smoking, another environmental hazard, also delivers oxidants and free radicals to the lungs. Cigarette smoke contains many oxidants and free radicals, both in the gas and the tar phase [40], and causes sequestration of neutrophils into the pulmonary microcirculation and accumulation of macrophages in respiratory bronchioles [41], with the potential to release oxidants [1, 41, 42]. It has been shown recently that the effect of cigarette smoke on acute increases in airway resistance and constriction occurs *via* a direct oxidant-mediated mechanism [43]. The release of ROS from activated neutrophils in the pulmonary microcirculation has been implicated as a contributor to the inflammatory responses in lung diseases [1, 31]. Nitrogen dioxide and sulphur dioxide are other inhaled air pollutant oxidants, which may alter lung function by the release of reactive electrophiles and the generation of oxidants [44–47]. Inhaled oxidants generated from air pollution particulates (particles with a 50% cut-off aerodynamic diameter of 10 μm (PM_{10})) are also associated with the release of inflammatory cytokines by airway epithelial cells [48].

Activation of redox-sensitive transcription factors

Nuclear factor-κB

Oxidants, either inhaled or produced by inflammatory cells, are directly implicated in the inflammatory responses in lung cells *via* signalling mechanisms. Transcription factors such as NF-κB and AP-1, which are redox-sensitive [25, 49], have been shown to be activated in epithelial cells and inflammatory cells during oxidative stress/inflammation, leading to the upregulation of a number of pro-inflammatory genes [25]. Maintenance of a high intracellular GSH/GSSG ratio (>90%) minimizes accumulation of disulphides and provides a reducing environment within the cell. However, if oxidant or other environmental stress alters this ratio, this shift in the GSH/GSSG redox buffer influences a variety of cellular signalling processes, such as activation of the transcription factors AP-1 and NF-κB. Oxidative stress including the presence of lipid peroxidation products [50] or depletion of GSH and subsequent increases in cytosolic GSSG in response to oxidative stress causes rapid ubiquitination and phosphorylation and thus subsequent degradation of the inhibitor of NF-κB (IκB), which is a critical step for NF-κB activation [51, 52]. Under reducing conditions, such as an increase in intracellular GSH following treatment with *N*-acetyl-L-cysteine (NAC), the phosphorylation of serine groups on IκB-α following TNF-α treatment is inhibited, leading to the down-regulation of NF-κB in endothelial cells (fig. 2) [53].

NF-κB regulates the expression of many genes involved in inflammation whose products mediate inflammatory responses in the lungs such as inducible nitric oxide synthase, the pro-inflammatory cytokines IL-1β, TNF-α and IL-6, the chemokine IL-8, E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and granulocyte-macrophage colony-stimulating factor [4, 54, 55]. In many inflammatory

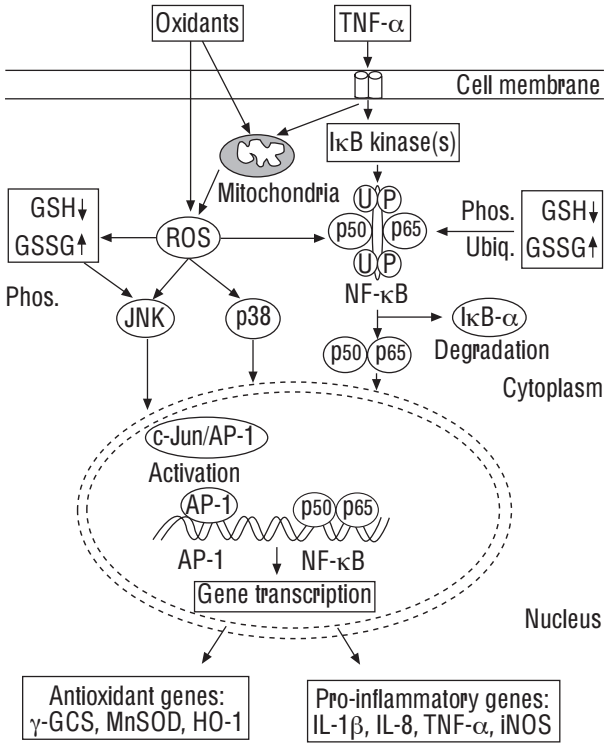

Fig. 2. – Model of the mechanism of nuclear factor-κB (NF-κB) and activation protein-1 (AP-1) activation leading to gene transcription in lung epithelial cells. Tumour necrosis factor-α (TNF-α)/oxidants act on mitochondria to generate reactive oxygen species (ROS), which are involved in the activation of NF-κB and AP-1. Activation of NF-κB involves the phosphorylation, ubiquitination and subsequent proteolytic degradation of the inhibitor of NF-κB (IκB). Free NF-κB then translocates into the nucleus and binds to its consensus sites. The intracellular ratio of reduced glutathione (GSH)/oxidized glutathione (GSSG) can modulate AP-1 and NF-κB activation. Similarly, either c-Jun/c-Jun (homodimer) of c-Fos/c-Jun (heterodimer) is activated by phosphorylation of the c-Jun N-terminal protein kinase (JNK), leading to the activation AP-1, which binds to its tetradecanoylphorbol-13-acetate response element (TRE) consensus regions. Activation of NF-κB/AP-1 leads to the co-ordinate expression of antioxidant protective and pro-inflammatory genes. γ-GCS: γ-glutamylcysteine synthetase; MnSOD: manganese superoxide dismutase; HO-1: haemoxygenase-1; IL: interleukin; iNOS: inducible nitric oxide synthase; ↓ : decrease; ↑ : increase. Phos/P: phosphorylation; Ubiq/U: ubiquitination; p38: p38 kinase; p30, p65: NF-κB subunits.

lung diseases such as IPF, ARDS, CF and HIV, depletion of intracellular GSH levels or increased GSSG levels are present concomitant with the induction of inflammatory mediators and chemotactic cytokines [8]. This suggests that the intracellular redox state (GSH/GSSG levels) of the cell may play a key role in the regulation and potentiation of the inflammatory responses in lung cells.

Activator protein-1

AP-1 is composed mainly of the Jun and Fos gene products, which form homodimeric (Jun/Jun) or heterodimeric (Jun/Fos) complexes. DNA-binding of the Fos/Jun heterodimer is increased by the reduction of a single conserved cysteine in the DNA-binding domain of each of the proteins [56]. Antioxidants such as NAC increase unstimulated and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-stimulated AP-1 DNA-binding and transactivation in HeLa cells [57]. This may be due to conservation of the cysteine (sulphydryl) residue required for the nuclear AP-1 DNA binding. The DNA-binding of AP-1 can be enhanced by thioredoxin as well as the nuclear redox protein, Ref-1, and inhibited by GSSG in many cell types, suggesting that disulphide bond formation by cysteine residues inhibits AP-1 DNA-binding [58, 59]. By contrast, oxidative stress imposed by H_2O_2 treatment, depletion of intracellular GSH using DL-buthionine-(*S,R*)-sulphoximine (BSO) or an increase in the ratio GSH/GSSG by diamide treatment of the liver cell line HepG2 also stimulates AP-1 binding [60]. This suggests that another mechanism besides direct protein/DNA binding such as redox-sensitive signalling pathways are involved in the regulation of AP-1 activation. This supports work by WILHELM *et al*. [61] demonstrating that perturbation of cellular thiol redox status provides a signal for AP-1 activation by the induction of stress-activated signal transduction pathways by c-Jun N-terminal protein kinase (JNK) and p38 kinase. Moreover, as both oxidants and antioxidants stimulate AP-1, differences in biological responses to these agents are likely to be related to the extent of AP-1 activation and the distinct AP-1 subunits which are upregulated, and hence the response which is provided, since different AP-1 dimers can either stimulate or repress gene expression. In addition, activation of redox-sensitive JNK and p38 by pro-inflammatory cytokines, such as TNF-α and IL-1, leads to the induction of genes for cytokines, chemokines and various pro-inflammatory mediators, which play an important role in the inflammatory response [5, 62, 63].

Glutathione synthesis and its redox recycling

The synthesis of glutathione requires the presence of two enzymes and the amino acids, glycine, cysteine and glutamic acid, with cysteine being the rate-limiting substrate. The tripeptide GSH is formed by the consecutive actions of γ-GCS and glutathione synthetase [64].

In general, the activity of γ-GCS determines the rate of glutathione synthesis. The reaction, catalysed by γ-GCS is feedback-inhibited by GSH [64]. The mammalian γ-GCS holoenzyme is a heterodimer consisting of γ-GCS-HS (73 kDa) and γ-GCS-LS (30 kDa) [65]. Although γ-GCS-HS contains all of the catalytic activity, γ-GCS activity can be modulated by the association of γ-GCS-HS with the regulatory γ-GCS-LS [65]. The regulatory properties of γ-GCS-LS have been proposed to be mediated by a disulphide bridge between the subunits that would allow conformational changes in the active site depending on the oxidative state of the cell [65]. This implies that potential for increasing the rate of GSH synthesis exists under conditions of GSH depletion.

The GSH redox system is crucial in maintaining intracellular GSH/GSSG homeostasis, which is critical to normal cellular physiological processes, and represents one of the most important antioxidant defence systems in lung cells [64]. This system uses GSH as a substrate in the detoxification of peroxides such as H_2O_2 and lipid peroxides, a reaction which involves glutathione peroxidase. This reaction generates GSSG which is then reduced to

GSH by glutathione reductase in a reaction requiring the hexose monophosphate shunt pathway utilizing NADPH.

Regulation of glutathione and γ-glutamylcysteine synthetase

At transcriptional level

Identification and characterization of the types of diverse stimuli that act as potent inducers of γ-GCS should aid in the development of effective pharmacological strategies for antioxidant treatment involving GSH regulation in inflammatory lung diseases. To this end, several studies have been directed towards understanding and elucidating the molecular mechanisms of GSH synthesis and regulation in type II alveolar epithelial cells in response to various environmental oxidants, antioxidants and inflammatory stimuli (table 2). The authors′ group and other investigators have reported that the promoter (5′-flanking) region of the human γ-GCS-HS gene is regulated by a putative c-Jun homodimer (AP-1) binding site [30, 66–69]. The sequence for this binding site is located in the proximal region of the γ-GCS-HS TATA box in various cell lines, including human alveolar epi-thelial cells [30, 67, 68].

Table 2. – Inducers of glutathione and γ-glutamylcysteine synthetase

Agent	[Ref.]
Oxidants	
Hydrogen peroxide	[30, 70]
Menadione	[30, 70–72]
Cigarette smoke	[13, 29]
Dimethyl naphthoquinone	[73, 74]
Xanthine/xanthine oxidase	[71]
t-butyl hydroperoxide and hydroquinone	[27, 67, 68, 75]
4-Hydroxy-2-nonenal	[37, 76]
Ozone	[77]
Hyperoxia	[71]
Phenolic antioxidants	
β-Naphthoflavone	[28, 78]
Apocynin	[79]
Butylated hydroxyanisole	[80]
Butylated hydroxytoluene	[81]
Pyrrolidinedithiocarbamate	[82, 83]
5, 10 dihydroindeno(1,2-*b*) indole	[84]
Cytokines	
TNF-α	[85, 86]
IL-1β	[87]
Nitric oxide donors	
Nitric oxide	[88]
DETA NONOate	[89]
S-nitrosopenicillamine	[90]
Radiation	
Ionizing radiations	[91, 92]
Metals	
Selenium	[93]
Iron	[94]
Cadmium	[95]
Mercury	[96]
Chemotherapeutic agents	
Cisplatin	[66, 97]
Melphalan	[98]
Growth factors	
Nerve growth factor	[99]
Acid fibroblast growth factor	[100]
Others	
Heat shock	[101]
Oxidized low density lipoprotein	[69]

TNF-α: tumour necrosis factor-α IL: interleukin.

MULCAHY and coworkers [28, 78], however, have reported a distal ARE containing an embedded TPA-responsive element (TRE) and an electrophile responsive element (or its functional equivalent ARE), which play a key role in the regulation of the γ-GCS-HS and γ-GCS-LS, respectively, in response to a planar aromatic xenobiotic, the phenolic antioxidant β-naphthoflavone, specifically in HepG2 cells. They also showed that the internal AP-1 site is important for the constitutive expression of the γ-GCS-LS gene [78]. However, recently, GALLOWAY and coworkers [27, 75] were unable to demonstrate a role for ARE in the induction of γ-GCS-LS by oxidants such as *t*-butyl hydroquinone in HepG2 cells. They suggested that an AP-1 site was the critical element for the basal regulation of this subunit. Therefore, it is likely that the expression of the γ-GCS subunit genes is regulated by different regulatory signals in response to diverse stimuli in specific cells.

Exposure to phenolic antioxidants such as dietary 2(3)-*t*-butyl-4-hydroxyanisole and butylated hydroxytoluene as well as the synthetic indolic antioxidant 5,10-dihydroindeno(1,2-*b*) indole and pyrrolidine dithiocarbamate (PDTC), a sulphydryl-modifying antioxidant compound, upregulate γ-GCS-HS and γ-GCS-LS in human endothelial cells and other cell lines [75, 80–84]. The plant-derived phenolic antioxidant apocynin (4-hydroxy-3-methoxyacetophenone) also induces GSH synthesis in human alveolar epithelial cells [79]. These effects of phenolic antioxidants are associated with AP-1 transactivation [57, 60, 102]. Therefore, in addition to their scavenging abilities, phenolic antioxidants may provide additional protection from oxidant-induced injury by upregulating the expression of γ-GCS and increasing GSH levels.

A role for NF-κB in the modulation of γ-GCS-HS gene expression has also been suggested [91, 103, 104]. It has been shown that blocking the activation of NF-κB, which is present at the transcriptional site of the γ-GCS-HS promoter, by various strategies prevented oxidant/cytokine-induced increase in γ-GCS-HS transcription in mouse endothelial cells and hepatocytes [91, 103]. However, mutation and deletion techniques applied to the γ-GCS-HS promoter region have ruled out the possible involvement of NF-κB in the transcriptional upregulation of the γ-GCS-HS gene in alveolar epithelial cells and other cell lines in response to TNF-α and oxidative stress [29, 30, 68, 85–87].

At post-transcriptional and translational levels

Regulation of GSH and γ-GCS has also been described at the post-transcriptional and pretranslational levels in rat liver *in vivo* and in other cells [105, 106]. Various inflammatory agents such as cyclic adenosine monophosphate (cAMP) and intracellular calcium, which are released during inflammation, may inhibit GSH synthesis at the translational level (table 3) [107]. It has been shown that γ-GCS activity is inhibited by agonists of various signal transduction pathways in rat hepatocytes [107], suggesting a role for signalling mechanisms in the

Table 3. – Inhibitors of glutathione synthesis

Agent	[Ref.]
Glucocorticoid (dexamethasone)	[71, 86]
Transforming growth factor-β	[109, 110]
Cyclic adenosine monophosphate	[107, 108]
Cytosolic free calcium	[107]
Insulin and hydrocortisone	[107]
Prostaglandin E_2	[111]

regulation of GSH levels. Lu *et al*. [107] reported that hepatic GSH synthesis is downregulated in response to hormones known to mediate their effects through the activation of distinct signal transduction pathways. Using various specific inhibitors of signalling pathways, these investigators determined that hormone-specific inhibition of GSH synthesis was mediated by the activation of protein kinase A, protein kinase C and Ca^{2+}/calmodulin-dependent kinase II. This inhibition of GSH synthesis correlated with the direct phosphorylation of γ-GCS-HS on serine and threonine residues which was dependent on the concentration of Mg^{2+}. Phosphorylation of γ-GCS-HS was also detected in rat hepatocytes treated with dibutyryl cAMP, resulting in the inhibition of γ-GCS activity *in vivo* [108]. Thus, phosphorylation/dephosphorylation may regulate γ-GCS activity [108] and may provide a mechanism for altering GSH levels in lung cells during oxidative stress.

Role of γ-glutamyl transpeptidase in the regulation of glutathione levels in lungs

Modulation of γ-glutamyl transpeptidase (γ-GT) may be another avenue for the regulation of intracellular GSH levels in lung cells. γ-GT cleaves extracellular GSH into its constituent amino acids and leads to the resynthesis of intracellular GSH rather than direct intact cellular GSH uptake [112]. The enzyme γ-GT is a plasma membrane enzyme, with its active site directed toward the outside of the cell, and is present in lung epithelial cells. This enzyme breaks the γ-glutamyl bond of γ-glutamyl-cysteinyl-glycine [113]. The glutamyl moiety is then transferred to an amino acid, a dipeptide or GSH itself, producing its γ-glutamyl derivative. Thus γ-GT acts as a salvage enzyme for cellular GSH synthesis. The lung epithelium has been shown to contain high levels of γ-GT activity and utilizes extracellular GSH from the alveolar lining fluid [114, 115]. Hence most plasma GSH is catabolized by the enzyme γ-GT in lungs [114, 115]. As a result, γ-GT may be important in determining the levels of GSH in lung ELF. Endothelial cells, alveolar macrophages and fibroblasts have lower γ-GT levels, and, therefore, less easily use extracellular GSH for intracellular GSH synthesis [113, 115, 116].

In an animal model, rats exposed to hyperoxia exhibited low γ-GT activity in ELF; this was associated with low ELF GSH levels [117]. γ-GT expression is increased in rat lung epithelial cells by oxidants such as menadione and *t*-butyl hydroquinone [118], suggesting that γ-GT might play a role in protection against oxidative stress. However, cigarette smoke condensate and oxidative stress had no effect on γ-GT activity in a human type II alveolar epithelial cell lines (A549 cells) [71]. A possible explanation for the differential regulation of γ-GT activity in response to oxidants may be due to differential expression of the γ-GT gene in different cell lines and organs and in different species. However, the role of the direct involvement of γ-GT in the lungs of smokers remains to be proven.

Cystine/cysteine transport and regulation of glutathione levels in lung cells

The rate-limiting step in the biosynthesis of GSH is the availability of cysteine as a substrate within the cell [64]. Cystine, an oxidized form of cysteine, is efficiently transported in cells by the specific inducible Na^+-independent anionic amino acid transport X_c^- mechanism and subsequently reduced for use in various metabolic processes including GSH synthesis in lungs [119–122]. Intracellular transport of cystine is accompanied by the extracellular release of glutamate. Cysteine is also transported into cells by sodium-dependent amino acid transport systems (labelled as A or ASC) shared with glutamine and serine [123]. It has been reported that isolated rat alveolar type II cells have a constitutive noninducible Na^+-dependent active uptake system that transports exogenous GSH and its γ-glutamyl analogues into cells against a concentration gradient [124–126]. These transport systems may increase intracellular GSH levels in lung cells, and might be one of the alternative mechanisms modulating intracellular GSH levels in lungs.

Various forms of oxidant stress and NO also increase the activity of membrane cystine and glutamate transport leading to increased GSH synthesis in lung cells [90, 122, 127, 128]. It has been clearly shown that the cystine uptake is the rate-limiting step for GSH synthesis in cultured lung cells, especially under conditions of oxidative stress [66, 129]. Oxidants (hyperoxia and H_2O_2), and agents such as sodium arsenite, cadmium, electrophilic compounds and diethyl maleate, also induce cystine transport in various lung cells, macrophages and erythrocytes that is analogous to the X_c^--transport system, a sodium-independent inducible system specific to the intracellular transport of cystine and glutamate [130–133]. It has been shown that exposure of rats to hyperoxia results in increases in total lung GSH within 24 h [6, 134]. It is, therefore, possible that induction of cystine or cysteine transport could contribute to the increased GSH levels in lungs after exposure to hyperoxia [6, 134].

The regulation of cystine/glutamate transport is governed by the availability of extracellular cysteine or cystine as well as the extracellular redox state (which is, in part determined by extracellular GSH levels) [120, 135]. Treatment with reducing agents such as NAC or GSH increases intracellular GSH levels by making intracellular cysteine available and reducing cystine to cysteine in bovine pulmonary artery endothelial cells [121]. Furthermore, NAC increases intracellular GSH levels in bovine pulmonary artery endothelial cells even in the absence of cystine in the medium [135]. This suggests that a different transport mechanism independent of the X_c^- system may be involved in type II epithelial cells in increasing GSH levels in response to various stresses [125]. Nevertheless, this is one of the mechanisms whereby lung cells increase

intracellular GSH levels under various stresses (either oxidant stress or GSH depletion).

The levels of intracellular GSH are regulated, in part, by the rate of the bidirectional membrane transport system present in lung and liver cells [136, 137]. It is likely that this membrane transport system causes GSH to efflux to the lung ELF. The function of such a GSH transport system is influenced by the redox/thiol status of the cell, the membrane potential and presence of cations in the extracellular environment [138, 139]. GSH-related structural compounds, such as glutathione-*S*-conjugates and GSH ethyl ester, inhibit cellular GSH uptake or influx [137, 138]. Furthermore, a more oxidized extracellular environment stimulates cells to retain GSH, whereas a more reduced extracellular state facilitates GSH efflux [137, 140]. However, these effects are in direct contrast with the situation in lungs *in vivo*, since the increased oxidant burden imposed by smoking and endogenous oxidative stress during inflammation could cause lung cells to retain GSH, rather than release it into the ELF. This mechanism is difficult to explain in the presence of such a bidirectional GSH transporter in the lung. Thus the mechanisms that determine the levels of GSH in the lung ELF using the bidirectional transporter are not fully understood.

Regulation of glutathione in oxidant-mediated susceptibility/tolerance

Effect of pro- and anti-inflammatory mediators

TNF-α is a ubiquitous pro-inflammatory cytokine and is recognized as an important mediator of inflammatory events in the lungs. It induces chronic inflammatory changes associated with an increase in a variety of defence mechanisms including antioxidants levels [141]. TNF-α is an important inflammatory mediator in COPD and ARDS and is present in increased amounts in the bronchoalveolar lavage fluid (BALF) and sputum of COPD patients [142]. Recently, various investigators [86, 143] have shown rapid depletion of intracellular GSH by TNF-α exposure in epithelial and endothelial cells *in vitro*, due to oxidation of GSH to GSSG. This is followed by a rebound increase in GSH levels in epithelial and endothelial cells as an adaptive response to oxidant stress, occurring as a result of upregulation of the γ-GCS-HS and activation of AP-1 [85, 87]. Similarly, exposure of fibroblasts to prostaglandin E_2, an inflammatory mediator capable of regulating fibroblast cell proliferation and matrix protein production, resulted in decreased GSH synthesis [111]. Furthermore, GSH concentrations in peripheral blood lymphocytes may be decreased as a result of lung inflammation and may be inversely correlated with lung function in patients with CF [144]. Thus oxidative stress imposed by inflammatory mediators may acutely deplete GSH during inflammation and render cells susceptible to the amplification of inflammatory responses.

Glucocorticoids, such as dexamethasone, are widely used as anti-inflammatory agents in various inflammatory lung diseases. Airway epithelium is one of the most important targets for inhaled glucocorticoids in lung diseases [145]. Exposure of lung epithelial A549 cells to dexamethasone decreases both basal and stimulated GSH levels (TNF-α-treated) in these cells [71, 86]. Dexamethasone also decreases γ-GCS-HS gene expression in alveolar epithelial cells *in vitro* by a transcriptional mechanism involving inhibition of the AP-1 transcription factor [86]. Thus it is possible that the use of dexamethasone in patients with inflammatory lung diseases may prevent synthesis of the protective antioxidant GSH.

Effects of oxidants

Exposure of alveolar epithelial cells *in vitro* to oxidants, such as H_2O_2, hyperoxia and redox recycling compounds like menadione, causes an initial depletion of GSH, associated with increased formation of GSSG, followed by a rebound increase in GSH levels at 24 h [29, 70–72]. This is concomitant with increased expression of messenger ribonucleic acid (mRNA) for the γ-GCS gene [30, 70, 72]. Epithelial cells also take up glutathione by a redox-dependent mechanism [137]. This transport system may act to regulate intracellular glutathione depending on the presence of reduced thiols or disulphides in the extracellular environment [138]. Thus, the short-term effects of various oxidants and oxidant-generating systems appear to be to upregulate the gene for glutathione synthesis, possibly providing a protective/adaptive mechanism against subsequent oxidative stress (table 2).

Oxidative stress produced by ozone [77], xanthine/xanthine oxidase [71], lipid peroxidation products (4-hydroxy-2-nonenal) [76], ionizing radiation [91, 92], hypoxia [146] and heat shock [101] all lead to short-term falls in intracellular GSH levels, followed by increases in GSH levels or upregulation of γ-GCS-HS mRNA in alveolar epithelial cells, endothelial cells *in vitro* and other cell types as well as *in vivo* in rats. NO and its donors, such as *S*-nitrosopenicillamine or DetaNONOate, also cause transient depletion of GSH followed by induction of GSH synthesis by enhanced expression of the γ-GCS-HS and γ-GCS-LS in rat aortic vascular smooth muscle cells [90], pulmonary fibroblasts [89] and bovine aortic endothelial cells [88]. The increase in GSH levels caused by NO donors is a further potential mechanism for protecting cells against oxidative stress and subsequent inflammation. Oxidative stress imposed by heavy metals such as selenium [93], iron [94], methyl mercury [96], and cadmium [95] also induce GSH synthesis in various organs in both rats and mice. Other cytotoxic compounds that act through the generation of ROS such as the chemotherapeutic agents cisplatin [66, 97] and melphalan [98] also increase GSH synthesis in cancer cell lines. These drugs may also coinduce the multidrug resistance-associated protein (MRP) gene and the adenosine triphosphate-dependent transporter, glutathione-conjugate (GS-X) pump, in lung cells [147–150]. The functional role of MRP coexpression in lung cells is currently unknown. It is possible that the coexpression of MRP and GS-X is related to protection against the inflammatory response and detoxification of xenobiotics and endogenous cysteinyl leukotrienes [151]. The induction of GSH synthesis may be associated with activation of mitogen-activated protein kinases, particularly c-JNK, in response to oxidants, heavy metals and NO [37]. γ-GCS-LS is also concomitantly induced in

response to oxidants and phenolic antioxidants in rat lung epithelial L2 cells and liver HepG2 cells, suggesting that concomitant induction of both subunits may be a potential mechanism for enhancing cellular GSH synthesis, and so developing cellular tolerance to oxidative stress [28, 73]. Exposure to sublethal doses of oxidants and oxidant-generating systems may initiate an adaptive intracellular antioxidant response, thus protecting cells from subsequent exposures to oxidant stresses [74, 152]. It is possible that the GSH synthesis and tolerance mechanism which occurs in response to various stimuli described in various cells may differ in lung cells.

Effect of growth factors

Transforming growth factor (TGF)-β1 is a multifunctional growth factor that modulates cellular proliferation and induces differentiation and synthesis of extracellular matrix proteins, including collagens and fibronectin, in many types of lung cell [153]. Recent studies have shown increased expression of TGF-β1 in bronchiolar and alveolar epithelium in IPF and COPD patients, and higher levels in the BALF of atopic asthmatics as compared to healthy subjects [154, 155]. TGF-β1 also downregulates γ-GCS-HS mRNA and glutathione synthesis in human alveolar epithelial cells and pulmonary artery endothelial cells *in vitro* [109, 110]. Interestingly, recent studies by FACTOR *et al*. [156] showed decreased glutathione synthesis in a TGF transgenic (overexpression) mouse model and increased susceptibility to oxidant-mediated injury. Various workers have shown that γ-GCS-HS mRNA expression is under the control of the AP-1 transcription factor [31, 67, 68, 86], and that TGF-β1 may decrease γ-GCS-HS gene expression *via* an AP-1 mechanism [157]. Thus higher levels of TGF-β1 may downregulate glutathione synthesis in the lungs of patients with inflammatory diseases such as IPF and COPD. Moreover, decreased GSH levels may also have direct functional consequences. *In vitro* studies showed that GSH (in the concentration range normally found in ELF) suppressed fibroblast proliferation [158]. The relevance of GSH regulation and subsequent tolerance/susceptibility in lung epithelial cells in response to pro-/anti-inflammatory mediators and/or oxidants under conditions of chronic inflammation *in vivo* is not known.

Differential regulation of glutathione and γ-GCS gene expression have been demonstrated by other growth factors such as nerve growth factor [99] and plating cells at a low cell density [103, 159, 160]. Intracellular GSH levels have also been shown to be elevated in response to mitogenic stimulation as cells exit from their quiescent state and conditions that lead to cellular transformation [161–165]. Extracellular acid fibroblast growth factor (FGF-1) has been demonstrated to cause transformation and aggressive cell growth in murine embryonic fibroblasts. Recent data from CHOI *et al*. [100] have shown that expression of a chimeric human FGF-1 gene containing a signal peptide sequence for secretion (hst/KS0FGF-1) in an embryonic fibroblast cell line caused increased gene expression of both γ-GCS subunits associated with increased γ-GCS activity without elevation of intracellular GSH levels [100]. They suggested that an increase in GSH content *per se* is not required for altered cell growth though increased expression of γ-GCS and γ-GCS activity is associated with a common response to growth factors.

Role of γ-glutamylcysteine synthetase in genetic susceptibility to disease

Recent studies have shown that GSH regulation/the GSH redox system might be one of the factors involved in genetic susceptibility to oxidant-/pollutant-mediated lung cell damage. For example genetic susceptibility to cigarette smoke has been suggested as a risk factor for the development of COPD [166, 167]. Furthermore, it has been shown that polymorphic expression of several different xenogenes, including those encoding cytochrome P-450 1A1 (CYP1A1), glutathione-*S*-transferases (GSTs) (GST-M1 and GST-pi) and microsomal epoxide hydrolase, as well as pro-inflammatory genes such as TNF-α, is associated with an increased risk of chronic inflammatory lung diseases [168–172] and inflammatory response [173, 174]. The expression of these genes is directly or indirectly related to the modulation of enzymes of the glutathione redox system and γ-GCS [85–87, 102, 166]. Variations in the expression of the γ-GCS gene in humans may represent a new susceptibility factor in oxidant-induced injury, which is thought to occur as part of the pathogenesis in COPD. It has been proposed that a GAG trinucleotide repeat polymorphism occurs in the 5′-coding and noncoding regions of the γ-GCS-HS gene [175]. Genetic analysis of 50 unrelated Caucasians identified three alleles as follows: A1 (nine repeats, 35% frequency), A2 (eight repeats, 11% frequency), and A3 (seven repeats, 54% frequency). Although certain trinucleotide repeats have been associated with recombinatory events, the functional significance of this particular allelic polymorphism, if any, is unknown. Depletion of GSH levels has been associated with a genetic polymorphism and deficiency of γ-GCS activity in a patient with haemolytic anaemia [176]. Genetic analysis revealed that the γ-GCS polymorphism was associated with an A to T mutation/transversion at nucleotide 1109 that predicted substitution of histidine with leucine at amino acid 370, a diallelic polymorphism in nucleotide +206 of an intron and another polymorphism that consisted of a duplication of CAGC at complementary DNA (cDNA) nucleotides 1972–1975 in the 3′-untranslated region of the γ-GCS catalytic subunit. This mutation was not present in the γ-GCS DNA of 38 healthy subjects [176]. The presence of such mutation and possibly associated genetic polymorphism in the coding and/or noncoding region of the γ-GCS catalytic/regulatory subunit might be associated with the depletion of GSH in the inflammatory response and susceptibility to chronic inflammatory diseases.

The γ-GCS subunit genes are located on separate chromosomes and expression of their mRNA varies considerably between different tissues [175, 177, 178]. Human γ-GCS-HS is located on chromosome 6 (6p12) and γ-GCS-LS on chromosome 1 (1p21) [177–179]. Genetic analysis reveals that a frequent deletion of the γ-GCS-LS chromosome, 1p22→p21, occurs in human malignant mesothelioma. This gene deletion is considered to predispose an individual to the development of mesothelioma [180]. Recent data have demonstrated that AP-1 and ARE, which are present in the promoter region of the

γ-GCS-HS and γ-GCS-LS genes, may be directly involved in the regulation of GSH in human cells [28, 30, 75, 78]. Within a population, it is likely that there will be variation (gene deletion or mutation) in the 5′-coding/noncoding regions of γ-GCS-HS and γ-GCS-LS genes. Future studies need to be directed towards understanding the nature of any polymorphisms that exist and whether any association exists between these polymorphisms and susceptibility to the development of inflammatory lung diseases such as COPD and IPF.

Role of glutathione in the regulation of pro-inflammatory and antioxidant protective genes

There is increasing evidence to suggest that many inflammatory lung diseases are associated with airway/airspace inflammation and/or oxidant/antioxidant imbalance leading to alteration in glutathione levels in the ELF. It is also well documented that many pro-inflammatory and antioxidant genes are regulated by a redox-dependent signalling mechanism in lung cells. Hence critical regulation of intracellular glutathione levels under oxidative stress and inflammation might determine the expression of pro-inflammatory and antioxidant genes. Therefore, it is likely that both redox GSH levels and the various forms of oxidative stress (ROS)/nitrosative stress would determine the regulation of specific genes for pro-inflammatory mediators and antioxidant enzymes.

Pro-inflammatory genes

Inflammatory mediators play a crucial role in chronic inflammatory processes and appear to determine the nature of the inflammatory response by directing the selective recruitment and activation of inflammatory cells and their perpetuation within the lungs. In *in vitro* studies, using macrophages and alveolar and bronchial epithelial cells, oxidants (ROS) have been shown to cause both the release of inflammatory mediators such as IL-8, IL-1 and NO and increased expression of pro-inflammatory genes *via* alteration in redox GSH-dependent mechanisms [181–183]. The genes for these inflammatory mediators are regulated by transcription factors such as NF-κB. This critical transcription factor in the inflammatory response is redox-sensitive. It is also known that various forms of nitrosative stress (reactive nitrogen intermediates/RNS or NO donors) have an effect on intracellular lung GSH levels leading to the expression of various pro-inflammatory mediators such as TGF-β1 and activation of nuclear enzymes such as poly (adenosine diphosphate-ribose) synthetase, which is involved in inflammation [184–186].

The modulation of intracellular thiol status not only quenches oxidants/free radicals but also buffers the antioxidant potential of the cell and detoxifies electrophilic compounds. Thiol antioxidants such as NAC and *N*-acystelyn (lysine salt of *N*-acetyl-L-cysteine), which have potential as therapies in inflammatory diseases, have been shown, in *in vitro* and *in vivo* experiments, to block the release of these inflammatory mediators from epithelial cells and macrophages by a mechanism involving increasing intracellular GSH levels and decreasing NF-κB activation [182, 183, 187].

Antioxidant protective genes

An important effect of oxidative stress and inflammation is the upregulation of protective antioxidant genes (fig. 3). Among the antioxidant enzymes, GSH and its redox enzymes appear to play an important protective role in the airspaces and intracellularly in epithelial cells. The protective role of GSH against the effects of cigarette smoke/oxidants has been demonstrated both *in vivo* in the rat and *in vitro* using monolayer cultures of alveolar epithelial cells [13, 14, 188]. Acute intratracheal instillation of cigarette smoke condensate in the rat and exposure of epithelial cell monolayers to cigarette smoke *in vitro* [13] lead to a profound decrease in GSH levels in BALF, and in the lungs of rats and epithelial cells. This is followed by a rebound adaptive increase in GSH levels and γ-GCS-HS mRNA expression in both rat lungs and epithelial cell lines [13, 189]. This finding is mirrored in humans, in whom GSH levels are elevated in ELF associated with increased expression of γ-GCS mRNA in the lungs of chronic cigarette smokers, whereas this is not the case in acute smoking compared to nonsmokers [23, 24, 190]. Thus oxidative stress, including that produced by cigarette smoking, causes upregulation of an important gene involved in the synthesis of GSH as an adaptive mechanism against subsequent oxidative stress. However, this adaptive response may not counteract the potential burden of pro-inflammatory mediators and oxidants released during inflammation.

A recent study has shown that expression of γ-GCS mRNA is elevated in smokers′ lungs and that this is even more pronounced in smokers with COPD [190]. This implies that GSH synthesis might be upregulated (GSH levels were not studied) in the lungs of smokers with and without COPD. Similarly, rats exposed to cigarette smoke have shown increased expression of genes encoding manganese SOD (MnSOD), metallothionein and glutathione peroxidase (GPx) in bronchial epithelial cells, suggesting the importance of the antioxidant gene adaptive response against the injurious effects of cigarette smoke [191]. Important protective antioxidant genes such as those encoding MnSOD, γ-GCS-HS, haem oxygenase-1 (HO-1), GPx, thioredoxin reductase and metallothionein are induced by modulation of cellular GSH/GSSG

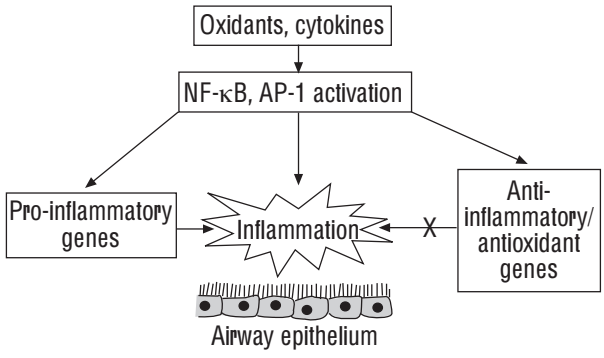

Fig. 3. – Activation of the transcription factors nuclear factor-κB (NF-κB) and activation protein-1 (AP-1) by oxidants and/or pro-inflammatory cytokines leads to the induction of both pro-inflammatory and anti-inflammatory/antioxidant genes in lung cells. Products of pro-inflammatory genes cause airway inflammation, which is inhibited (X) by anti-inflammatory and/or antioxidant genes.

levels in response to various oxidative stresses including hyperoxia and inflammatory mediators such as TNF-α and lipopolysaccharide in lung cells [85, 86, 141, 192, 193].

Thus oxidative/nitrosative stresses, including redox modulation, cause increased gene expression of pro-inflammatory genes *via* oxidant-mediated activation of transcription factors such as AP-1 and NF-κB and also activation of stress response protective genes such as γ-GCS-HS, HO-1 and MnSOD in lungs. A balance may therefore exist between pro- and anti-inflammatory gene expression and the levels of GSH in response to oxidative stress and during inflammation, which may be critical to whether this leads to cell injury or protection against the injurious effects of inflammation (fig. 4). Knowledge of the molecular mechanisms that sequentially regulate this battery of genes in relation to GSH levels in lung cells may open new therapeutic avenues in the modulation of inflammatory responses in lung diseases.

Protective role of glutathione in oxidant and free radical-mediated lung injury

Alveolar epithelial cells are important in maintaining the integrity and fluid balance of the lungs. The epithelium lining the airways and alveoli has a protective barrier function. The lower respiratory tract is sensitive to injury from inhaled and locally produced oxidants. In response to injury, the epithelium loses its selective permeability and becomes more permeable to the movement of water, ions and macromolecules. Increased epithelial permeability is one of the earliest events in lung injury and may enhance the inflammatory process by allowing easier access for inflammatory and injurious mediators between the blood, interstitium and alveolar space.

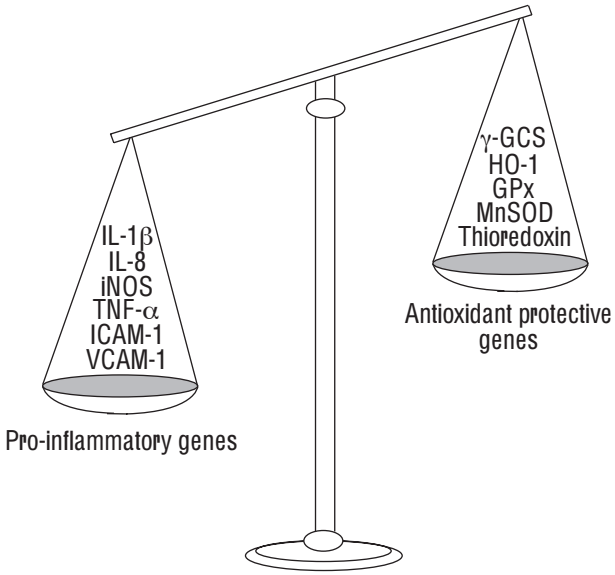

Fig. 4. – Pro-inflammatory and antioxidant protective gene imbalance in inflammation. In inflammation, the balance appears to be tipped in favour of increased pro-inflammatory mediators, due to either release of inflammatory mediators or amplification of the pro-inflammatory effects. Induction of antioxidant protective genes may be a delayed response and decline sharply. IL: interleukin; iNOS: inducible nitric oxide synthase; TNF-α: tumour necrosis factor-α; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1; γ-GCS: γ-glutamylcysteine synthetase; HO-1: haem oxygenase-1; GPx: glutathione peroxidase; MnSOD: manganese superoxide dismutase.

Cigarette smoke/air particulate-mediated lung injury

Alveolar cells are normally covered in a thin protective layer of epithelial fluid, which is rich in antioxidants such as GSH [194]. It has been reported that incubation with extracellular GSH and increasing intracellular GSH levels protect against oxidant stress in alveolar type II cells [7, 195]. In addition, extracellular GPx, which has been described recently [196], is secreted into ELF by alveolar epithelial cells and macrophages and may provide further defence against oxidants [196]. Following acute inflammation and oxidative stress, the ELF may become depleted of antioxidants such as GSH, increasing the potential for damage to the underlying epithelial cells. Both *in vivo* and *in vitro* in monolayers of cultured epithelial cells, this decrease in GSH was associated with an increase in airspace epithelial permeability [13, 14, 188]. Decreasing GSH levels in both these *in vivo* and *in vitro* models using the γ-GCS inhibitor BSO produces increased epithelial permeability [13]. Nishikawa *et al*. [197] recently demonstrated that acute cigarette smoke exposure, in guinea-pigs, produced neutrophil influx into the airways associated with NF-κB activation and IL-8 mRNA expression in alveolar macrophages. This may be due to GSH depletion of lung and alveolar macrophages by cigarette smoke. Furthermore, Li *et al*. [198] reported that instillation of air particulate matter (PM_{10}) into the lungs of rats caused inflammation, decreases in lung GSH levels and increases in epithelial permeability. Oxidative stress and inflammation in response to air particulates have been shown to be prevented by NAC treatment in alveolar epithelial cells [48, 199]. Similarly, other air pollution gases such as SO_2 and NO_2 produce an inflammatory response and alveolar permeability *via* depletion of lung GSH levels in rats and A549 epithelial cells [44, 200, 201]. The decrease in GSH levels was associated with inhibition of several enzymes involved in the GSH redox system and glutathione synthesis, and the production of lipid peroxidation products in rat lungs and human alveolar epithelial cells [46, 200, 202, 203]. These studies suggest that GSH plays a critical role in maintaining epithelial membrane integrity and may protect epithelial cells against the inflammatory response produced by either inhaled or endogenous oxidants/free radicals. Furthermore, Linden and coworkers [204, 205] demonstrated that airway obstruction, measured by means of the forced expiratory volume in one second (FEV_1) in patients with COPD, correlated significantly with the concentration of GSH in BALF; the higher the BALF GSH, the lower the FEV_1. It may be that the BALF GSH levels were influenced by the recent smoking history of these patients [24].

Against inflammatory events in lungs

Neutrophil/endothelial interactions are events necessary for the progression of inflammatory responses in lung diseases. Recently, it has been shown that changes in endothelial cell GSH/GSSG ratio produce expression of different adhesion molecules on the cell surface, which are

associated with enhanced neutrophil/endothelial adhesion [206]. Agents that cause oxidation of GSH led to increases in neutrophil adhesion to endothelial cells *via* upregulation of ICAM-1 and VCAM-1 [207, 208], and increasing intracellular thiol concentrations with NAC attenuated the oxidant- or cytokine-mediated neutrophil adhesion to endothelial cells [208]. Therefore, a change in intracellular GSH redox balance may be an important mechanism in neutrophil adhesion, which is involved in chronic lung inflammation.

Modulation of growth factor receptors and altered cellular signalling is proposed to occur through a redox-mediated mechanism in inflammatory and lung cells. Tyrosine phosphorylation of the epidermal growth factor (EGF) receptor in lung epithelial cells by H_2O_2 is thought to influence inflammatory processes in lungs [209]. H_2O_2 is known to induce apoptosis in many cells including epithelial cells, and this response is inhibited by glutathione [3, 9, 210, 211]. In addition, a decrease in intracellular GSH levels in alveolar macrophages caused by oxidants, hyperoxia and cigarette smoke produces downregulation of vascular endothelial growth factor (VEGF) and its functional receptor [212, 213]. Downregulation of VEGF and its receptor may be associated with apoptosis, which may be linked in the pathogenesis of inflammatory lung diseases such as emphysema and COPD. GSH and other thiols such as NAC inhibit TNF-α-induced sphingomyelin hydrolysis, ceramide generation and programmed cell death (apoptosis), suggesting that GSH has antiapoptotic properties through its ability to detoxify oxidants and free radicals [214].

HO-1 is a member of the heat shock family of proteins, which plays an important role in inflammation. A role for GSH in the regulation of heat shock factor and activation of heat shock protein has been suggested [215]. The intracellular levels of GSH in fibroblasts modulate oxidant-induced expression of HO-1 [216]. This effect was due to the direct involvement of AP-1 (Jun/Jun) binding [192]. Similarly, metal-induced expression of the heat shock protein gene *hsp*72 is attenuated by glutathione, implying a protective role of GSH in acute inflammation [217]. Hence, it is clear that maintenance of intracellular GSH levels is important in the control of inflammatory responses in lungs involving heat shock proteins.

Oxidant-mediated mitochondrial damage

Mitochondria normally produce a substantial quantity of ROS (*e.g.* H_2O_2 and $O_2\cdot^-$), which are normally broken down by GSH-dependent peroxidase-catalysed reactions. Mitochondria contain 15–20% of the total cellular GSH. The mitochondrial GSH pool is derived solely from the activity of a mitochondrial transporter that translocates GSH from the cytosol into the mitochondrial matrix, as mitochondria do not possess the enzymes γ-GCS or γ-GT [218]. Mitochondrial GSH may also be susceptible to the oxidative stress imposed by TNF-α, and inhaled or endogenous oxidants in human lungs [10, 219]. TNF-α is known to deplete cytosolic GSH levels transiently in lung epithelial and endothelial cells [86, 143, 220]. This depletion by TNF-α is thought to be due to oxidative stress from mitochondrial leakage of $O_2\cdot^-$ *via* the electron transport chain [143, 221]. Oxidation of GSH is associated with activation of NF-κB and damage to mitochondrial DNA leading to apoptosis in fibroblasts *in vitro* and a decline in lung function in smokers [219, 221, 222]. It is likely that mitochondrial GSH plays a key role in maintaining cellular antioxidant defence systems, and thus cell integrity and function, under various conditions of oxidative stress [221, 223]. CHEN *et al*. [220] have recently demonstrated that depletion of mitochondrial GSH in human umbilical vein endothelial cells (HUVECs) increased TNF-α-induced adhesion molecule (VCAM-1) expression but not ICAM-1 expression and mononuclear leukocyte adhesion in HUVECs, suggesting that mitochondrial GSH is involved in endothelial cell function [220]. Recent studies have shown that gene delivery of glutathione reductase to mitochondria and overexpression of GPx in various cell lines provided protection against oxidative stress [224, 225]. This finding demonstrates the importance of mitochondrial GSH homeostasis in the regulation of cell function. It is possible that an imbalance in mitochondrial GSH redox status may help to perpetuate inflammation in lung cells.

Oxidant-mediated extracellular matrix remodelling

Intracellular redox GSH levels have been shown to be involved in the remodelling of ECM during oxidant-mediated lung injury [226, 227]. This is supported by two observations: 1) oxidant-induced lung injury was attenuated by the synthetic matrix metalloproteinase (MMP) inhibitor British Biotech (BB)-3103 [228]; and 2) depletion of intracellular GSH was associated with activation of MMPs, thereby increasing degradation of the alveolar ECM in lungs [227]. This breakdown of lung ECM by MMP-9 and MMP-2 activation was blocked by increasing lung glutathione levels [227]. It has been shown that oxidative stress imposed by ozone decreases lung glutathione levels associated with altered ECM protein type I collagen gene expression [229]. Other forms of oxidative stress which are derived from *t*-butyl hydroperoxide and iron could also modify collagen synthesis, by a mechanism presumably involving a redox sensor/receptor [226, 230]. TYAGI and coworkers [11, 226] demonstrated that *in vitro* exposure of normal myocardium to GSSG leads to the activation of MMPs, which was associated with oxidation of the active cysteine residue present on MMPs. GSSG also inhibited tissue inhibitor of metalloproteinase (TIMP) in fibroblasts [226]. Thiol antioxidants such as GSH and NAC and the phenolic antioxidant PDTC have been shown to inhibit the activation of MMP-2 and MMP-1 associated with increased TIMP levels in cultured fibroblast cells [220].

Immune effector response

The recent findings of PETERSON *et al*. [231] regarding the importance of GSH levels in antigen-presenting cells in modulating T-helper (Th) cell 1 *versus* Th2 cytokine response patterns in immune responses to the nature of the antigen, and the decreased intracellular levels of GSH in peripheral blood lymphocytes of CF and HIV-seropositive patients [144, 232], led to the assumption that this important tripeptide thiol may be involved in the

functional regulation of the immune response. However, the impact of chronic GSH depletion in T-cells, B-cells, macrophages and neutrophils in immune/inflammatory lung diseases have not been studied so far. Chronic depletion of GSH may be coupled with immunodeficiency and poor survival, as evidenced in the CD4 T-cells of HIV-seropositive patients [8, 21, 232, 233]. Intracellular redox GSH levels in these immune/inflammatory cells may also effect signal transduction and activation of transcription factors and lead to elevated gene expression (*e.g.* of IL-8 and IL-4) [8, 234]. GSH deficiency also leads to T-cell inactivation and apoptosis [235, 236]. More research is necessary to understand the mechanism/involvement of redox GSH levels and the regulation of glutathione synthesis in the regulation of the immune response.

Protective role of thiol compounds in inflammation

NAC, a cysteine-donating compound, acts as a cellular precursor of GSH and on deacetylation becomes cysteine. It reduces disulphide bonds, but also has the potential to interact directly with oxidants. NAC is also used as a mucolytic agent (to reduce mucus viscosity and improve mucociliary clearance) [237]. NAC has been used in an attempt to enhance lung GSH levels and reduce inflammation in patients with COPD and IPF with variable success [238–241]. Oral treatment with NAC (200 mg three times daily for 8 weeks) in healthy chronic cigarette smokers reduced inflammation and lowered BALF levels of eosinophilic cationic protein, lactoferrin, antichymotrypsin and chemotactic activity for neutrophils [242].

In some cases, NAC might cause elevation of the antioxidant screen in lung ELF, as shown in patients with IPF, in whom NAC caused significantly elevated GSH levels in alveolar lavage fluid [241]. This may provide therapeutic effects on the rate and extent of the development of fibrotic lesions in these patients. Indeed, oral administration of 600 mg NAC three times daily for 12 weeks to patients with IPF improved lung function in these patients [243]. Intravenous NAC treatment for 72 h improved systemic oxygenation and reduced the need for ventilatory support in patients with mild-to-moderate acute lung injury but failed to influence the development of the condition, or its mortality [244]. In a prospective randomized double-blind placebo-controlled study, administration of NAC (150 $mg \cdot kg^{-1}$ bolus, followed by a continuous infusion of 50 $mg \cdot kg^{-1}$ over 4 h) led to decreased IL-8 levels without any change in plasma TNF-α, IL-6 or IL-10 levels in patients with septic shock [245]. This anti-inflammatory effect of NAC was associated with improved oxygenation and static lung compliance in these patients, suggesting that NAC may be used as an adjunctive treatment in patients with septic shock [245]. In an *in vitro* study, NAC was shown to inhibit neutrophil and monocyte chemotaxis and the respiratory burst [246]. Animal studies have suggested that NAC produces deleterious effects on the lung epithelium in response to hyperoxia exposure [247]. Furthermore, a direct link between these clinical effects (*i.e.* reductions in the number of exacerbations and in the decline in lung function and inflammation) and the efficacy of NAC as an *in vivo* antioxidant has not been convincingly established to date [1, 13].

NAL, a lysine salt of *N*-acetyl-L-cysteine, is a mucolytic and antioxidant thiol compound. The advantage of NAL over NAC is that it has a neutral pH in solution, whereas NAC is acidic. NAL can be aerosolized into the lung without causing significant side-effects [248]. GILLISSEN *et al*. [248] compared the effects of NAL and NAC and found that both drugs enhanced intracellular glutathione levels in alveolar epithelial cells and inhibited H_2O_2 and $O_2\cdot^-$ release from human blood-derived polymorphonuclear neutrophils (PMNs) from smokers with COPD. NAL also inhibited ROS generation induced by serum-opsonized zymosan by human PMNs. This inhibitory response was comparable to the effects of NAC [249]. Therefore, NAL may represent an interesting alternative approach to augmenting the antioxidant screen in the lungs.

Certain other thiol-releasing agents such as glutathione ethyl ester and L-thiozolidine-4-carboxylate are potentially useful compounds for cysteine/glutathione delivery [250, 251]. However, studies are needed to validate the bioavailability of these compounds in lung inflammation.

Glutathione therapeutic perspectives in inflammatory lung diseases

The evidence is overwhelming that glutathione plays a vital role in cellular modulation of the inflammatory response, antioxidant capability, antiprotease system, immune effectiveness, remodelling of the ECM, surfactant/phosphatidylcholine and mucolysis. It is clear that ELF GSH levels are decreased in various inflammatory lung diseases. Thus increasing lung cellular levels of the GSH/antioxidant screen would be a logical approach in inflammatory lung diseases. Extracellular augmentation of GSH has been attempted through intravenous administration of GSH, oral ingestion of GSH and inhalation of nebulized GSH [252–260] in an attempt to reduce inflammation in lung diseases [261] such as in IPF [253], mild asthmatics [254] and CF [255, 256]. GSH aerosol therapy normalized low GSH levels in the lungs of these patients [253–256]; however, nebulized GSH also had a detrimental effect in asthmatic patients by producing bronchoconstriction, presumably due to the formation of GSSG [254]. This suggests that GSH aerosol therapy may not be an appropriate means of increasing GSH levels in lung ELF and cells in patients with asthma. GSH aerosols also increased the formation of GSSG in patients with IPF [253] but suppressed lung epithelial surface inflammatory cell-derived oxidants in patients with CF. Recent data have indicated that the low levels of ELF/apical fluid GSH in patients with CF is attributable to abnormal GSH transport (inability to bring out GSH efflux), which might be associated with a missing or defective CF transmembrane conductance regulator channel, through which efflux of organic anions normally occurs [262, 263]. The depletion of lung GSH may be reflected in peripheral blood lymphocytes in these patients [144]. It may be feasible to use a GSH aerosol to restore the oxidant/antioxidant imbalance in these patients [256, 259]. In all of these studies, questions were raised as to the bioavailability of GSH, the pH and osmolality at the site of

the microenvironment and the resultant formation of deleterious products (GSSG). It seems rational to suggest that neutralizing the pH, providing GSH in salt form, using liposome-entrapped GSH delivery and maintenance of isotonicity would be useful in designing any GSH inhalation therapy in inflammatory lung diseases.

Increasing the activity of γ-GCS and glutathione synthetase by gene transfer techniques may increase cellular GSH levels. Transfection of cDNAs for the heavy and light subunits of human γ-GCS-HS resulted in elevation of intracellular glutathione levels in COS-7 cells [264]. These cells were thereafter resistant to chemotherapeutic drugs. Similarly, MANNA *et al*. [265] have recently demonstrated that overexpression of γ-GCS in rat hepatoma cells completely protected against the TNF-α-induced activation of NF-κB, AP-1, stress-activated protein kinase/JNK and apoptosis. They highlighted the importance of glutathione and γ-GCS in protecting against the cytotoxic effects of various agents and that most of the actions of TNF-α are regulated by the glutathione-controlled redox status of the cell. Modulation of GSH synthesis in organs such as the skeletal muscle of critically ill patients, in whom GSH synthesis is markedly affected, would be another means of targeting GSH synthesis by gene transfer [259, 266]. Thus the induction of γ-GCS by molecular means to increase cellular GSH levels or γ-GCS gene therapy holds great promise in protection against chronic inflammation and oxidant-mediated injury in various inflammatory diseases.

Conclusions and future directions

ROS and RNS are generated by several inflammatory and immune and various structural cells of the airways. An imbalance of oxidant/antioxidant in favour of oxidants contributes to the pathogenesis of several inflammatory lung diseases. GSH is an important protective antioxidant in the lungs, the levels of which are altered in the ELF in several of these conditions. Glutathione and γ-GCS are regulated by oxidants, phenolic antioxidants, pro-inflammatory mediators and anti-inflammatory agents and growth factors in lung cells. In addition, intracellular and extracellular GSH levels are also regulated by cystine/cysteine transport mechanisms and by γ-GT. γ-GCS is regulated at both transcriptional (AP-1, ARE and NF-κB) and translational levels. Although the molecular mechanism of γ-GCS activity is well characterized, its regulation in response to specific stimuli in particular inflammatory lung diseases has not been studied. More work should be carried out to address this regulation using human tissue.

Regulation of intracellular redox GSH levels in response to ROS/RNS and in inflammation should have critical effects, in different lung cells, on the activation of redox sensor/signal transduction pathways and various transcription factors such as NF-κB and AP-1. The regulation of intracellular glutathione levels may determine the balance between expression of pro-inflammatory mediators and antioxidant genes. Imbalance of redox-regulated pro-inflammatory and antioxidant genes in response to inflammatory mediators, oxidants and growth factors might be associated with susceptibility/tolerance to disease. Modern technologies such as DNA array and differential display might be utilized in detecting and assessing the novel target genes involved in disease susceptibility. The polymorphism in the γ-GCS gene may be associated with initiation/exacerbation of inflammation as a result of a mutation in this critical antioxidant gene. Hence, studies must be directed at detecting the existence, if any, of such genetic polymorphisms in susceptible/nonsusceptible populations and patients with inflammatory lung diseases.

Study of the protective role of reduced glutathione/thiol compounds in inhibition of the inflammatory response (activation of nuclear factor-κB/inhibitor of nuclear factor-κB kinases) and correcting the fundamental oxidant/antioxidant imbalance in patients with chronic inflammatory diseases are important areas of further research. Augmentation of intra- and extracellular levels of glutathione and related thiols *via* aerosolization/inhalation and γ-glutamylcysteine synthetase by gene transfer in lungs will not only enhance the protective antioxidant potential but may also inhibit oxidant-mediated acute and chronic inflammatory responses. Understanding of the cellular and molecular redox-regulating mechanisms in inflammation is needed to design antioxidant therapeutic strategies for the treatment of various inflammatory lung conditions.

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ARTICLE

CINP

Decreased levels of glutathione, the major brain antioxidant, in post-mortem prefrontal cortex from patients with psychiatric disorders

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Abstract

Accruing data suggest that oxidative stress may be a factor underlying the pathophysiology of bipolar disorder (BD), major depressive disorder (MDD), and schizophrenia (SCZ). Glutathione (GSH) is the major free radical scavenger in the brain. Diminished GSH levels elevate cellular vulnerability towards oxidative stress; characterized by accumulating reactive oxygen species. The aim of this study was to determine if mood disorders and SCZ are associated with abnormal GSH and its functionally related enzymes. Post-mortem prefrontal cortex from patients with BD, MDD, SCZ, and from non-psychiatric comparison controls were provided by the Stanley Foundation Neuropathology Consortium. Spectrophotometric analysis was utilized for the quantitative determination of GSH, while immunoblotting analyses were used to examine expression of glutamyl-cysteine ligase (GCL), GSH reductase (GR), and GSH peroxidase (GPx). We found that the levels of reduced, oxidized, and total GSH were significantly decreased in all psychiatric conditions compared to the control group. Although GCL and GR levels did not differ between groups, the levels of GPx were reduced in MDD and SCZ compared to control subjects. Since oxidative damage has been demonstrated in MDD, BD, and SCZ, our finding that GSH levels are reduced in post-mortem prefrontal cortex suggests that these patient groups may be more susceptible to oxidative stress.

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Key words: Bipolar disorder, depression, glutathione, oxidative stress, schizophrenia.

Introduction

A growing body of evidence implicates that mitochondrial dysfunction is associated with mood disorders. Mitochondria are the major source for production of reactive oxygen species (ROS) that cause oxidative damage. Many studies have reported increased oxidative damage in peripheral blood samples from subjects with mood disorders. For example, investigators demonstrated increased total oxidant status in serum from patients with major depressive disorder (MDD) (Cumcurcu *et al*. 2009). Malondialdehyde (MDA), a marker for lipid peroxidation, was elevated in plasma and erythrocytes in MDD (Bilici *et al*. 2001; Sarandol *et al*. 2007). A meta-analysis conducted by our group on oxidative markers in bipolar disorder (BD) patients indicated increased lipid peroxidation (Andreazza *et al*. 2008). Most recently, studies have identified oxidative damage to mitochondrial proteins in prefrontal cortex (Andreazza *et al*. 2010), increased lipid peroxidation in cingulate cortex and increased RNA oxidation in hippocampus from patients with BD and schizophrenia (SCZ) (Che *et al*. in press; Wang *et al*. 2009). Similar findings have also been reported in prefrontal cortex from SCZ patients (Prabakaran *et al*. 2004). All of these studies together suggest that oxidative stress may play a significant role in the pathophysiology of psychiatric illness (Ng *et al*. 2008).

Glutathione (GSH) is the brain's major antioxidant system (Dringen, 2000) and plays a key role against oxidative stress. GSH is biologically synthesized by two enzymatic reactions: (i) L-cysteine and glutamate are combined to form γ-glutamyl-cysteine, via the rate-limiting enzyme glutamyl-cysteine ligase (GCL); (ii) glycine is added via GSH synthetase. GSH exists in

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either a reduced GSH (GSH_R) or oxidized GSH (GSSG) state and it is the reduced state that detoxifies ROS through the donation of a reducing equivalent. Hydrogen peroxide is detoxified by GSH through GSH peroxidase (GPx), thus forming GSSG that is recycled back to GSH_R by GSH reductase (GR) (Meister, 1983, 1988). Hence, maintenance of adequate levels of GSH is essential for preventing oxidative damage to the brain.

ROS are detoxified by specific antioxidant defences. Oxidative stress is an imbalance between the oxidant and antioxidant systems, where the production of free radicals outweighs a system's ability to detoxify reactive intermediates (Halliwell, 2001). Therefore, deficiency of the antioxidant defence system also results in oxidative stress. Reduced total antioxidant capacity in serum has been reported in MDD (Cumcurcu *et al*. 2009; Sarandol *et al*. 2007; Yanik *et al*. 2004). In BD patients, Benes *et al*. (2006) demonstrated lowered gene expression of several antioxidant enzymes in hippocampus, including catalase, GSH, GPx, GSH S-transferase (GST), and superoxide dismutase (SOD). Taken together, psychiatric patients, especially those with SCZ, MDD, and BD have increased levels of oxidative stress; however, to our knowledge no studies have examined GSH and its associated enzyme levels in MDD and BD. Thus, in this study we examined the GSH antioxidant system in post-mortem brain from patients with MDD, BD, and SCZ, and report diminished levels of GSH_R in all patient groups studied.

Methodology

Sample

Post-mortem brain tissues were donated by the Stanley Medical Research Institute's brain collection courtesy of Drs Michael B. Knable, E. Fuller Torrey, Maree J. Webster, and Robert H. Yolken. Subjects were divided into four groups including BD, MDD, SCZ, and non-psychiatric, non-neurological control groups. Detailed information was available on all subjects through medical records that included demographic data, medical history, substance abuse history, psychotropic drug treatment history, cause of death, and medication at time of death (Dowlatshahi *et al*. 1999; Knable *et al*. 2004; Torrey *et al*. 2000). Diagnoses were established according to DSM-IV criteria by two senior psychiatrists after reviewing the medical records and interviewing family members. Similar review of control subjects confirmed lack of psychiatric illness and substance abuse. All groups were matched for age, sex and post-mortem delay interval. In our studies we used area BA 10 of prefrontal cerebral cortex, a region in which abnormalities have been demonstrated in subjects with mood disorders. For GSH oxidized and reduced measurements, one control subject was not included and for protein level measurements of GCL, GR, and GPx one MDD subject was not included as there was not sufficient tissue for respective analyses.

Assay for GSH

Post-mortem prefrontal cortical tissue was homogenized on ice at 25 mg/ml in a 0.1 M potassium phosphate buffer with 8.8 mM EDTA disodium salt (pH 7.5) containing 0.6% sulfosalicylic acid and 0.1% Triton-X (Sigma-Aldrich, USA). Brain homogenates were centrifuged at 8000 *g* for 10 min at 4 °C before the supernatants were collected and stored at −20 °C. For determination of total glutathione (GSH_T), 20 μl brain homogenate was added to each well of 96-well microplate followed by 120 μl of 1.68 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and 3.3 units/ml GR prepared in 0.1 M potassium phosphate buffer with 8.8 mM EDTA disodium salt (pH 7.5). After 30 s, 60 μl of 0.8 mM β-NADPH prepared in potassium phosphate buffer with 8.8 mM EDTA disodium salt (pH 7.5) was added to each well. Absorbance was immediately measured at 405 nm by plate reader (Multiskan Ascent, Thermo Labsystems, USA) every 30 s for 5 min. The rate of 2-nitro-5-thiobenzoic acid formation was calculated from the change in absorbance/min and we determined the GSH_T concentration in our samples by using linear regression to calculate these values from the standard curve. For determination of GSSG, 50 μl brain homogenate was mixed well with 1 μl 2-vinylpyridine for 1 h at room temperature. To this mixture, 3 μl triethanolamine was added to the solution for 10 min. The resulting sample solution was run according to the aforementioned method of GSH_T. All solutions were prepared fresh before use and were protected from light exposure. Reduced GSH (GSH_R) levels were calculated by subtracting GSSG levels from GSH_T levels. Samples were run in triplicate and were blind to diagnosis.

Immunoblotting analyses

The levels of GCL (1:1000; Rb-1679-P0; Thermo Scientific), GPx (1:1000; ab16798; Abcam, USA), GR (1:3000; ab55075; Abcam), and β-actin (1:3000; ab8226; Abcam) were measured by immunoblotting in post-mortem prefrontal cortex as previously described (Tian *et al*. 2007). Briefly, protein extracts were subjected to SDS–PAGE electrophoresis with a 12% acrylamide gel at 100 V for 90 min at 4 °C. Proteins were

Table 1. Demographic data for post-mortem brain tissue

	Con	SCZ	MDD	BD	*F*	*p*
Age, yr (mean ± SD)	49.3 ± 11.0	44.6 ± 13.6	46.7 ± 9.6	41.5 ± 11.7	1.051	0.378
Range (yr)	29–68	25–62	30–65	25–61		
n	12	14	14	14		
Gender	7M, 5F	8M, 6F	9M, 5F	8M, 6F		
PMI (h) (mean ± SD)	23.2 ± 11.1	32.5 ± 14.4	27.1 ± 11.1	32.1 ± 16.6	1.37	0.263
Range (h)	8–42	12–61	7–47	13–62		
pH	6.2 ± 0.2	6.2 ± 0.2	6.2 ± 0.2	6.2 ± 0.2	0.41	0.75
Range	5.8–6.5	5.8–6.6	5.8–6.5	5.8–6.5		
Cause of death						
Suicide	0	4	6	8		
Cardiopulmonary	10	7	7	5		
Accident	2	2	0	0		
Other	0	1	1	1		
Current alcohol/drug abuse	0	3	3	4		
Past alcohol/drug abuse	2	3	1	3		
Medication at time of death						
Antipsychotic	0	11	0	7		
Antidepressant	0	5	10	8		
Mood stabilizer	0	2	2	9		

Con, Controls; SCZ, schizophrenia; MDD, major depressive disorder; BD, bipolar disorder.
n, Number of individuals; M, male; F, female; PMI, post-mortem interval.

transferred to polyvinylidene difluoride membranes for 1 h at 100 V and 4 °C. Membranes were dried at room temperature overnight prior to blocking in 5% milk-Tris-buffered saline with 0.01% Tween-20 for 1 h at room temperature. Blots were washed and incubated with secondary antibody goat anti-rabbit, rabbit anti-goat, or goat anti-mouse immunoglobulin G (IgG; Abcam) conjugated to horseradish peroxidase diluted to 1:2000 in blocking buffer for 1 h at room temperature and immunoreactive bands detected with the enhanced chemiluminescence system. Each gel contained a pre-stained broad range protein ladder (Fermentas International, Canada) to measure molecular weights of individual bands. Samples were run in duplicate and blind to diagnosis.

Data analyses

Statistical analyses were computed with SPSS version 17.0 for Windows (SPSS Inc., USA). Normal distribution of data was determined by the Kolmogorov–Smirnov test. Parametric tests were used as data presented a normal distribution. One-way analyses of variance (ANOVA) were employed to analyse data between groups followed by least squares derivation *post-hoc* comparisons. The influence of age, post-mortem interval, and pH were determined by analysis of covariance (ANCOVA). We also examined if our measures were affected by the presence or absence of substance abuse and suicide by independent-samples *t* tests. Correlations were analysed by Pearson correlation test. Data are presented as means and standard deviations (S.D.). Significance was set at $p \leq 0.05$. Outliers were defined as data-points that fall more than 2 S.D. from the mean and were subsequently removed from a particular analysis. For the BD group, we found a single outlier in each of the GSSG, GCL, and GPx measurements.

Results

Demographic data is presented in Table 1. Factors such as age, post-mortem interval, and pH can affect protein levels and enzymatic reactions (Halliwell & Gutteridge, 2000). We therefore assessed the potential influence of these factors by analysing their correlation and covariance (ANCOVA) with our data. We found no influence of any of these factors on the GSH measures in all subjects (Fig. 1*a–c*) nor in any individual diagnostic group. However, we found a negative correlation between age and GR in all subjects

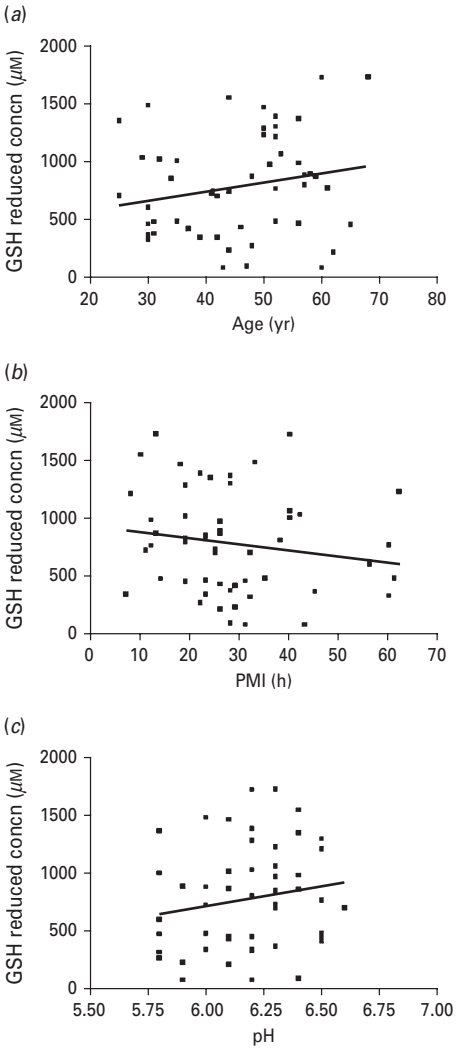

Fig. 1. Relationship between reduced glutathione (GSH) with (*a*) age, (*b*) post-mortem interval (PMI) and (*c*) pH.

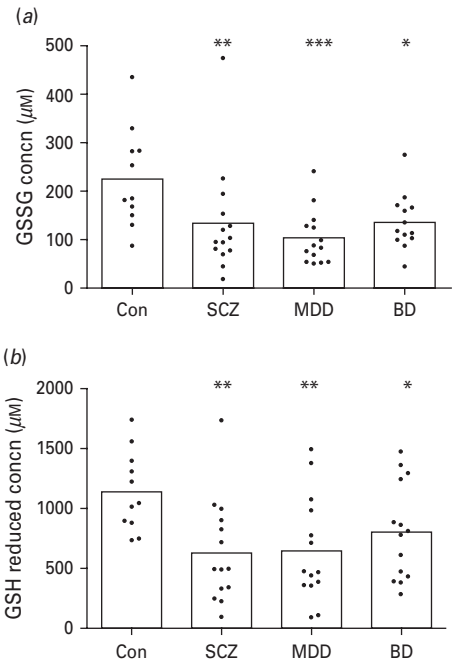

Fig. 2. Glutathione (GSH) levels were measured by a chromophoric enzymatic recycling method in controls (Con), schizophrenia (SCZ), major depressive disorder (MDD), and bipolar disorder (BD) patients. (*a*) Concentration levels for oxidized GSH (GSSG) are significantly reduced in SCZ, MDD, and BD compared to Con. (*b*) Concentration levels for reduced GSH are significantly diminished in SCZ, MDD, and BD compared to Con. Bar results are the means of individual data-points. Significance was measured by a one-way ANOVA: * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$ when specific psychiatric groups were compared to the least significant difference *post-hoc* test against control.

($r = -0.322$, $p = 0.019$) and in BD ($r = -0.593$, $p = 0.026$). A positive correlation was found between post-mortem interval and GR ($r = 0.599$, $p = 0.039$) in all subjects. To examine the influence of alcohol/drug abuse, we divided subjects with psychiatric illness based on this factor. GSH_R was not different between individuals who abused alcohol/drugs and those who did not [$t(40) = 0.159$, $p = 0.874$]. Moreover, the levels of GSH_R were not different in subjects who died by suicide compared to those who did not [$t(40) = 0.217$, $p = 0.829$]. All of the GSH system measurements evaluated in our study did not significantly differ between individuals who abused alcohol/drugs and those who did not. Further, these measurements did not differ between individuals who committed suicide and those who did not. Tobacco-use information was not available for these patients and therefore we could not analyse the influence of smoking on our data.

Significantly reduced levels of GSSG were found in all three patient groups compared to non-psychiatric controls [Fig. 2*a*; $F(3,48) = 4.601$, $p = 0.007$], GSH_R [Fig. 2*b*; $F(3,49) = 3.888$, $p = 0.014$]. The ratio of GSH_R/GSSG was not different between subject groups [Con: 5.8 ± 2.7; SCZ: 7.7 ± 11.6; MDD: 6.3 ± 3.2; BD: 5.9 ± 3.0; $F(3,48) = 0.7113$, $p = 0.550$]. To determine whether GSH_R levels were due to altered GSH synthesis, we measured the levels of the rate-limiting enzyme, GCL's catalytic subunit, and found no difference between groups [Fig. 3*a*; $F(3,48) = 0.705$, $p = 0.554$]. Next, we examined enzymes involved in GSH metabolism, the recycling enzymes, GR and GPx. Although GR levels did not differ between subject groups [Fig. 3*b*; $F(3,49) = 0.125$, $p = 0.945$], there was a trend towards significantly decreased GPx levels [Fig. 3*c*;

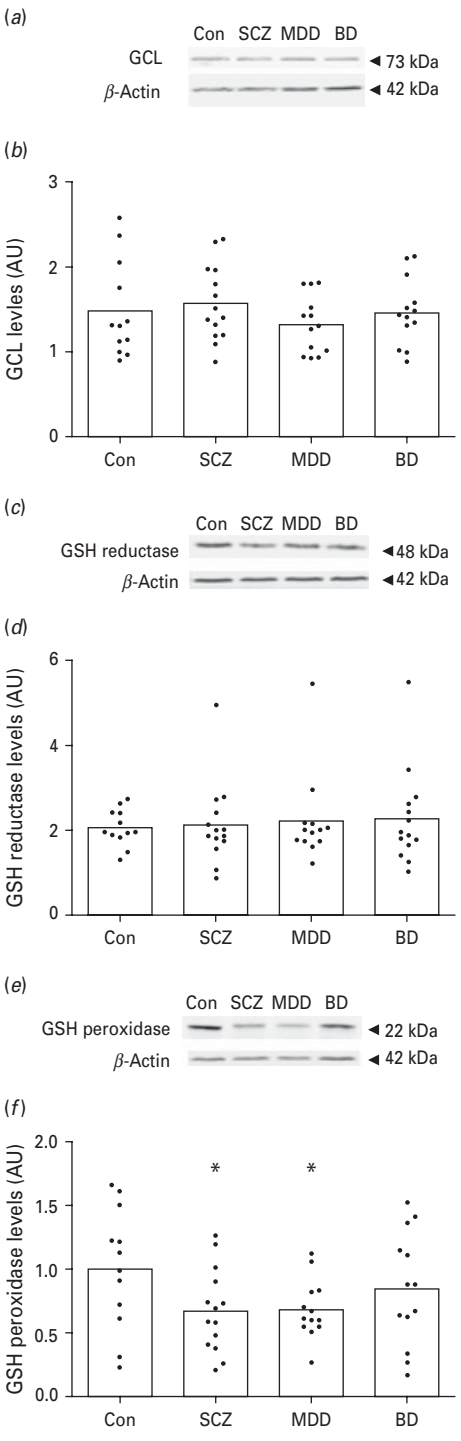

Fig. 3. Glutamyl-cysteine ligase (GCL) and glutathione (GSH) recycling enzymes, GSH reductase (GR), and GSH peroxidase (GPx), levels were measured via immunoblotting analyses in controls (Con), schizophrenia (SCZ), major depressive disorder (MDD), and bipolar disorder (BD) patients. (*a*) Representative blot for GCL. (*b*) Levels of GCL were not significantly different between SCZ, MDD, and BD compared to Con. GSH recycling enzymes, GR and GPx, were measured via immunoblotting analyses in Con, SCZ, MDD, and BD patients. (*c*) Representative blot for GR. (*d*) Levels of GR were not significantly different between SCZ, MDD, and BD compared to Con. (*e*) Representative blot for GPx. (*f*) Levels of GPx were significantly reduced in SCZ and MDD compared to Con whereas levels in BD were not significantly different from Con. Bar results are the means of individual data-points normalized to β-actin levels that were standardized to human prefrontal cortical control tissue that is expressed as arbitrary units (AU). Significance was measured by a one-way ANOVA: * $p \leq 0.05$ when specific psychiatric groups were compared to the least significant difference *post-hoc* test against control.

$F(3, 48) = 2.221$, $p = 0.098$]. In *post-hoc* analysis, SCZ ($p = 0.028$) and MDD ($p = 0.037$) patients were significantly decreased compared to controls.

Discussion

We report here decreased, reduced and GSSG levels in post-mortem prefrontal cortex from individuals with SCZ, MDD, and BD compared to age- and sex-matched healthy non-psychiatric controls. We found no changes in the levels of the rate-limiting enzyme for GSH synthesis, GCL, however, we did find that patients with MDD and SCZ had significant reductions in the level of an enzyme that utilizes GSH, GPx. These results suggest that GSH levels are lower in post-mortem prefrontal cortex from patients with SCZ, MDD, or BD, which can compromise the antioxidant capacity and make brain from these patients more vulnerable to oxidative damage.

GSH's antioxidant system is the primary endogenous means by which the brain defends against oxidative stress and includes GSH, GPx, and GR. We found significantly lower levels of GSH in psychiatric illness and supporting our findings, GSH levels are significantly reduced in MDD patient blood samples (Kodydkova *et al*. 2009) and reduced in erythrocytes (Altuntas *et al*. 2000), cerebrospinal fluid, and post-mortem brain from SCZ patients (Yao *et al*. 2006). GSH is a tri-peptide that contains the amino acids glutamate, cysteine, and glycine. In fact, both GSH (Agarwal & Shukla, 1999) and glutamate levels (Grant *et al*. 2009) can be elevated with pharmacological treatment by *N*-acetylcysteine (NAC). Translating these studies from the bench to the clinic; double-blinded, randomized, placebo-controlled trials have demonstrated that NAC improves the positive and negative symptoms of SCZ (Berk *et al*. 2008*b*) and the depressive symptoms of BD patients (Berk *et al*. 2008*a*), which

emphasizes the importance of maintaining cerebral GSH levels. To date, no trial has examined if GSH-promoting compounds have a beneficial effect for MDD symptoms.

The levels and activities of GCL, GR, GPx and GSTs affect GSH levels. The enzymes GCL and GSH synthetase, with GCL being rate limiting to the reaction, catalyse GSH synthesis. To probe if altered GSH levels were due to alterations to GSH synthesis, we measured the levels of the catalytic subunit for GCL in prefrontal cortex and did not find any differences between groups. Others have found that GCL alterations modulate GSH levels (Lavoie *et al*. 2009), this enzyme's gene is a potential susceptibility gene in SCZ (Tosic *et al*. 2006), and its expression is affected by mood-stabilizer treatment (Cui *et al*. 2007). Since GSH synthesis was not altered, we examined the enzymes that use GSH, GR and GPx, which function to remove hydrogen peroxide. Peripheral studies on psychiatric illnesses have reported increased activity of GR in MDD (Bilici *et al*. 2001; Kodydkova *et al*. 2009) but to our knowledge no changes have been observed in SCZ or BD. Studies on GPx in patients with psychiatric illness have been inconsistent. In SCZ, GPx has been reported elevated (Herken *et al*. 2001), decreased (Altuntas *et al*. 2000), and unchanged (Abdalla *et al*. 1986) in erythrocytes. Whereas in BD patients the activity of GPx is increased in serum (Andreazza *et al*. 2007), unchanged (Abdalla *et al*. 1986), and decreased (Ozcan *et al*. 2004) in erythrocytes. Last, in MDD, GPx activity is diminished in blood (Kodydkova *et al*. 2009) and elevated in erythrocytes (Bilici *et al*. 2001). Although the GSH antioxidant system has not been previously studied in post-mortem brain from individuals with MDD or BD, evidence demonstrates reductions to cerebral levels of GSH and GPx activities in patients with SCZ (Yao *et al*. 2006). Abnormal expression of antioxidant genes, largely associated with the GSH metabolic pathway, has been reported in BD (Benes *et al*. 2006; Sun *et al*. 2006). Our results in combination with data published by others suggests that the GSH antioxidant system is altered in brain from patients with psychiatric illness; however, conclusive evidence that GSH_R levels are due to specific modifications to GSH synthesis and detoxification enzyme levels, activities, or expression has not been demonstrated. Our data suggest that in the central nervous system of subjects with psychiatric illness, changes to GSH levels are not due to alterations to GSH synthesis, but in MDD and SCZ may be associated with decreased GPx enzyme levels. Interestingly it appears that the mechanism(s) responsible for reduced prefrontal cortex GSH levels in patients with BD is different than the mechanism(s) for individuals with MDD and SCZ.

The GSH antioxidant system has been studied in animal models of depression, such as chronic mild stress. Chronic mild stress reduces GSH levels (Eren *et al*. 2007*a*), and GPx activity in rat cerebral cortex (Eren *et al*. 2007*a*, *b*). These studies support our results of significantly decreased GPx levels in MDD compared to controls. In addition, chronic mild stress induces oxidative stress and lipid peroxidation (Eren *et al*. 2007*a*, *b*; Lucca *et al*. 2009*a*, *b*), protein carbonylation (Lucca *et al*. 2009*a*), superoxide (Lucca *et al*. 2009*b*), and nitric oxide (Eren *et al*. 2007*b*) levels in rat brain. Our observations that GSH_R and GPx levels are reduced in prefrontal cortex from MDD patients support these findings and emphasize the importance of maintaining GSH levels. Furthermore, our findings suggest that GSH-elevating compounds may protect against oxidative damage in these psychiatric illnesses.

While our results are of potential interest, there are a number of limitations. There exists variability between individuals for measurements of biological factors and lengthy post-mortem intervals for their collection and storage. These factors are matched between groups in the Stanley Consortium in order to minimize confounding variables but it may be that the measurement of a factor's levels may be vastly different from each person's true levels. Addition of GSH synthesis and recycling enzyme activity would provide a more detailed cerebral analysis of this antioxidant system in these patient groups; however, tissue amounts required for these assays constrained us from gathering these measurements. Unfortunately perfect animal models of psychiatric illness do not exist.

Our study demonstrates decreased GSH_R, GSSG, and GSH_T levels in post-mortem prefrontal brain from patients with MDD, BD, or SCZ. We observed significantly diminished levels of GPx in MDD and SCZ patient samples but not in BD patient samples.

Acknowledgements

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Statement of Interest

Dr Young is an occasional speaker for Eli Lilly and AstraZeneca.

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Glutathione: Pharmacological aspects and implications for clinical use in non-alcoholic fatty liver disease

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Glutathione is a tripeptide synthesized at cytosolic level, that exists in cells in a reduced form (thiol-reduced-GSH-) and in an oxidized form (disulfide-oxidized). The antioxidant function of GSH has led to speculation about its therapeutic role in numerous chronic diseases characterized by altered redox balance and reduced GSH levels, including, for instance, neurodegenerative disorders, cancer, and chronic liver diseases. Among these latter, non-alcoholic fatty liver disease (NAFLD), characterized by lipid accumulation in hepatocytes, in the absence of alcohol abuse or other steatogenic factors, is one of the most prevalent. The umbrella term NAFLD includes the pure liver fat accumulation, the so-called hepatic steatosis or non-alcoholic fatty liver, and the progressive form with inflammation, also known as non-alcoholic steatohepatitis, which is related to the increase in oxidative stress and reactive oxygen species, eventually leading to liver fibrosis. Although the pathogenetic role of oxidative stress in these diseases is well established, there is still limited evidence on the therapeutic role of GSH in such conditions. Hence, the aim of this review is to depict the current molecular and pharmacological knowledge on glutathione, focusing on the available studies related to its therapeutic activity in NAFLD.

KEYWORDS

chronic liver disorder, metabolic syndrome, non-alcoholic fatty liver disease, oxidative stress, oral glutathione

1. Introduction

Glutathione is a tripeptide found in many tissues at relatively high concentrations, namely 1–10 mM in cells, similarly to glucose, potassium, and cholesterol, with a critical role in several physiological processes, such as redox balance preservation, reduction of oxidative stress through detoxification from xenobiotic and endogenous compounds, and immune system modulation (1). The action of glutathione on oxidative stress has led to speculation on the possible therapeutic role of this molecule for several chronic diseases with altered redox balance.

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease worldwide, characterized by an excessive hepatic fat accumulation in the absence of alcoholic abuse, steatogenic medications or others concomitant liver diseases (2). NAFLD could be considered an hepatic manifestation of metabolic syndrome, with a strong association with obesity, type

2 diabetes, hypertension, and dyslipidaemia (3). Given the close association between NAFLD and metabolic dysfunction, a panel of experts proposed in 2020, not without some controversy, to rename this condition as metabolic-(dysfunction) associated fatty liver disease (4).

Although the pathogenic role of oxidative stress in NAFLD pathogenesis is well established (5), there are limited studies available in the literature investigating the potential effect of glutathione supplementation in this condition. The aim of this narrative review is to provide a broad overview on the pharmacologic aspects of glutathione, with a focus on current clinical data on its use for metabolic liver disease.

2. Glutathione: Pharmacological aspects

Glutathione is a tripeptide (γ-L-glutamyl-L-cysteinylglycine) consisting of glutamate, cysteine and glycine, with an atypical peptide bond between glutamate residue and cysteine, *via* the γ-carboxyl group. It exists in cells in two states: thiol-reduced (GSH) and disulfide-oxidized (GSSG). The reduced form (GSH) is the predominant one, accounting for more than 98% of total glutathione. Most of the GSH (80–85%) is stored in the cytosol, 10–15% in the mitochondria (with an equal concentration between matrix and cytosol, thus requiring specific transport systems) and a small part in the endoplasmic reticulum (ER) (1, 6, 7).

2.1. Thiol-reduced glutathione (GSH) synthesis

GSH is made available in cells through 3 processes, summarized in Figure 1.

The first process consists in the *de novo* synthesis of GSH from its component amino acids, through two ATP-consuming enzymatic reactions. It occurs exclusively in the cytosol, where glutamate cysteine ligase (GCL) and glutathione synthetase (GS) perform their function. The first step involves the binding of glutamic acid and cysteine, catalyzed by GCL, to form glutamylcysteine. This rate limiting step is controlled by the cellular availability of cysteine and GCL activity. This assumption is supported by the finding that only the overexpression of GCL, and not of GS, results in increased GSH levels (6). On the other hand, GSH exerts a negative feedback inhibition on GCL. In the second step, the homodimeric enzyme GS, a member of the ATP-grasp superfamily, rapidly catalyzes the binding of γ-glutamylcysteine with glycine, obtaining GSH (8, 9). GS, differently from GCL, is not feedback-inhibited by GSH and it is not associated with a regulatory subunit. Thus, GS activity appears to be mainly controlled by substrate availability.

The second pathway of GSH synthesis is that related to the recycling of cysteine. GSH is exclusively degraded at the extracellular level by cells expressing γ-glutamyltranspeptidase (GGT), such as those of the hepatobiliary tree and of other organs like the heart, kidney, lungs, pancreas, and seminal vesicles. GGT allows the degradation of GSH and the recycling of its constituent amino acids, such as glutamic acid and cysteine, to generate new GSH (so called γ-glutamyl cycle). The resulting cysteine is unstable at extracellular level, and it is rapidly autoxidizes to cystine. Nevertheless, cystine is taken up by some cells (i.e., endothelial cells) and, given the high reducing conditions, is intracellularly reduced to cysteine, employed for the synthesis of GSH (10, 11). This direct transport of extracellular cystine does not take place in hepatocytes, where the reduction of cystine to cysteine occurs mainly in the outer cell membrane as a consequence of GSH efflux (11). The extracellular L-cysteine/L-cystine redox balance, and thus the synthesis of GSH, is finely regulated by the intracellular conversion of L-cystine into L-cysteine (10), and its impairment is related to oxidative stress and other pathological disorders.

The last GSH synthetic process is the one depending on the conversion of the oxidized dimer GSSG to 2 reduced GSH molecules in cells by glutathione reductase (GR), an ubiquitous enzyme of the family of disulfide reductases, in the presence of NADPH and flavin adenine dinucleotide -FAD-. GR can perform its enzymatic activity in the cytoplasm, but also in the ER and within lysosomes, mitochondria and the nucleus. Since it participates in the synthesis of GSH, this enzyme plays a key role in the cellular redox homoeostasis (12).

The majority of plasmatic GSH originates from the liver, and for this reason an impairment in hepatic GSH synthesis has a systemic impact on redox balance and oxidative stress (1, 6, 7, 11).

2.2. The antioxidant role of GSH

GSH is implicated in several functions, including antioxidant defense with reduction of oxidative stress and maintenance of redox balance, metabolic detoxification from xenobiotics and exogenous compounds, cell cycle regulation, and immune system modulation, as well as fibrogenesis (1).

Its main role is to shield cellular macromolecules from endogenous and exogenous reactive oxygen species (ROS) and nitrogen ones. In particular, GSH catalytically detoxifies from hydroperoxides, peroxynitrite, and lipid peroxides and directly scavenges various oxidant molecules, like superoxide anion, hydroxyl radical, nitric oxide, and carbon radicals. Furthermore, GSH deals directly with heavy metals and persistent organic pollutants (POPs), direct causes of oxidative stress. POPs are mainly excreted through conjugation with GSH and this mechanism is extremely important for the health status, since exposure to POPs has been associated to diabetes, cardiovascular diseases and many other chronic diseases (1, 13).

ROS production can occur at several intracellular sites but, for most cells, takes place in the mitochondria and the mitochondrial electron transport chain is the main cellular process of ROS generation in physiological circumstances (14). For superoxide anion, the main ROS, a first line of defense is represented by the enzyme superoxide dismutase, localized in the mitochondrial matrix. This enzyme is able to convert the superoxide anion into hydrogen peroxide (H_2O_2). Once obtained, H_2O_2 can be degraded in mitochondria *via* the GSH redox system, employing glutathione peroxidases (Gpxs) and GRs, but also through peroxiredoxins (Prxs), a family of thiol-specific peroxidases.

Gpxs exist in multiple isoforms, with different cellular localization and different substrate specificity (15). Of these, Gpx1, localized mainly at the mitochondrial level (16), is the isoform most active in the liver (17). Gpxs are the main enzymes involved in scavenging ROS at high intracellular concentrations, protecting cells from oxidative stress-induced damage. At nanomolar concentrations of H_2O_2, Prxs seem to be more active, given their higher intracellular concentration and rate constant (18).

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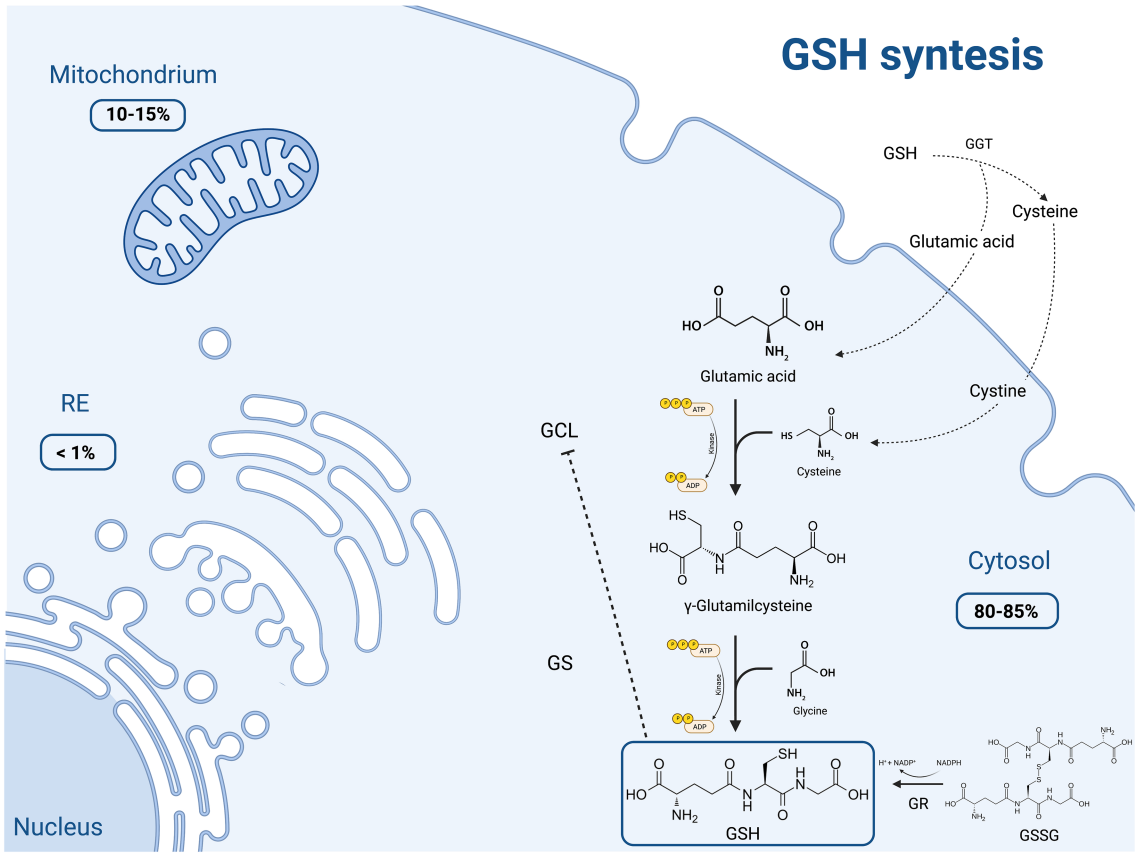

FIGURE 1
Schematic representation of the main molecular mechanisms of reduced glutathione (GSH) synthesis and the intracellular distribution of this molecule. GSH production occurs through three main pathways: (1) *de novo* synthesis *via* a 2-step process catalyzed by glutamate cysteine ligase (GCL) and glutathione synthetase (GS), which is primarily controlled by the cellular levels of cysteine. Moreover GCL activity is in part regulated by GSH feedback inhibition; (2) recycling of cysteine from conjugated glutathione *via* γ-glutamyltranspeptidase (GGT) in the γ-glutamyl cycle; (3) regeneration of the oxidized glutathione (GSSG) to GSH by glutathione reductase (GR). Most (80–85%) of the cellular GSH is in the cytosol; 10–15% is in the mitochondria and a small percentage is in the endoplasmic reticulum (ER). Created with "Biorender.com." ADP, adenosine diphosphate; ATP, adenosine triphosphate; ER, endoplasmic reticulum; GCL, glutamate cysteine ligase; GGT, γ-glutamyltranspeptidase; GR, glutathione reductase; GS, glutathione synthetase; GSH, thiol-reduced glutathione; GSSG, disulfide-oxidized glutathione; NADP+, oxidized nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; ---|, inhibition.

GSH, exploiting the redox-active thiol residue (-SH) of cysteine, exerts its antioxidant function mainly *via* Gpxs-mediated reactions, which result in peroxide buffering with simultaneous oxidation of GSH to GSSG (19). The obtained GSSG is potentially toxic and, under oxidative stress, its excessive accumulation can manifest its toxicity. First of all, GSSG may activate the SAPK/MAPK pathway, leading to cell apoptosis. In addition, GSSG retained in mitochondria during oxidative stress can lead to the S-gluthionylation of target proteins with mitochondrial dysfunction (20, 21). The S-glutathionylation is a process in which the interaction between GSSG and cysteinyl residues of proteins results in the formation of mixed disulfides (22). This physiological mechanism, which is useful for the post-translational modification of multiple proteins and for the regulation of signal and metabolic pathways, can turn harmful in case of oxidative stress, with the above-mentioned mitochondrial damage. To prevent the GSSG toxicity, this molecule is rapidly transformed in its reduced variant (GSH) by high intracellular levels of GRs, with the aim of maintaining an appropriate redox balance in the cell (1). Hence, the GSH/GSSG ratio represents a marker of oxidative stress.

Besides the neutralization of free radicals produced in phase 1 liver metabolism of chemical toxins, GSH also participates in the protection from the resulting electrophilic substrates through the intervention of glutathione-S-transferases (GSTs). GSTs are phase 2 enzymes, ubiquitously distributed in the cell. The ones locate in mitochondria have both GSH-transferase and peroxidase activity (23). They are therefore able, by exploiting the properties of GSH, to activate conjugation and peroxide reduction of dangerous products.

Also, GSH facilitates the transport and excretion of toxins, through the formation of S-conjugates of activated intermediates, which are water soluble and undergo renal excretion.

Finally, GSH is also a cofactor for several antioxidant enzymes. Among the antioxidant molecules of low molecular weight are vitamins E and C, obtained from the diet. In particular, vitamin E, after acting as an antioxidant by reducing lipid radicals, is restored to its reduced form by vitamin C. In turn, the oxidized vitamin C, thanks also to GSH, can revert to its reduced form (7). GSH therefore enables the recycling of vitamins C and E, again protecting the body from oxidative stress (1).

2.3. Depletion of GSH and therapeutic implications

The depletion of GSH levels has been demonstrated in aging and multiple chronic degenerative diseases, including neurodegenerative, cardiovascular, pulmonary, immune disorders and cancers (24, 25). There are cumulating data on reduced GSH levels and the consequent increased susceptibility to oxidative stress in many human diseases, contributing to the onset and worsening of these conditions.

For this reason, many studies have been conducted on the best methods to increase intracellular and intramitochondrial levels of GSH. A first approach to promote glutathione production might be the administration of specific precursors, cofactors or specific foods and nutrients that may increase or maintain optimal glutathione levels. Examples are cysteine supplements in the form of whey or *N*-acetylcysteine, antioxidant vitamins (B,C,E), alpha-lipoic acid, selenium or phytonutrients (i.e., Brassica vegetables and green tea) (25). However, data are scant and controversial, resulting in limited efforts to study the effect of nutritional interventions on GSH status. Further studies are needed to clarify optimal dose and delivery forms and one mandatory target should be the identification of sub-groups of individuals most likely to respond to particular supplements, nutrients or foods.

On the other hand, the obvious strategy to increase GSH levels is its direct administration. The main routes of administration of glutathione are oral, intramuscular, and intravenous. Intravenous GSH has a short half-life but has shown to be effective in several diseases. For example, the GSH intravenous administration in patients with Parkinson's disease determined significant improvements, which lasted for 2–4 months after the administration (26). Also oral administration, although with conflicting results, resulted in increased serum GSH levels with reduced oxidative stress and beneficial effects in several diseases (27, 28). Richie et al. recently found that oral GSH at either 250 or 1,000 mg/day was associated to significant increase in the body storage of GSH in non-smoking adults, in a dose-dependent manner (27). They also noticed a decrease in the markers of oxidative stress at 6 months, as shown by the improvement in the GSSG/GSH ratio. Furthermore, recent studies suggested that GSH oral administration in liposomal or sublingual forms may have a better bioavailability, with a favorable impact on systemic GSH levels (29, 30). For example, a novel GSH formulation bypassing the gastrointestinal digestion through an oral absorption, gave positive results in raising GSH blood concentration *in vitro* and *in vivo* (31). Moreover, this molecule showed a promising hepatoprotective function in a murine model of acute liver injury (32).

3. Non-alcoholic fatty liver disease: Pathogenesis and clinical features

NAFLD is the most common liver disease, characterized by an excessive hepatic fat accumulation in the absence of alcoholic abuse, steatogenic medications or others concomitant liver diseases (2). NAFLD could be considered an hepatic manifestation of metabolic syndrome, given the strong association with obesity, type 2 diabetes, hypertension and dyslipidaemia (3). This entity comprises NAFL and NASH, the latter being its progressive form and affecting about 10–20% of patients.

NAFLD appears to be more frequent in industrialized countries, its global prevalence is about 25% and varies across different geographical areas, being higher in Middle East and South America and lower in Africa (33). The overall prevalence of NASH is uncertain, as it relies on third-level referral centers with availability of liver biopsies, and is estimated between 1.5 and 6.45% (34).

According to the European Clinical Guidelines, NAFLD is defined either by the presence of steatosis in >5% of hepatocytes at the liver biopsy or by a proton density fat fraction >5,6% assessed by proton magnetic resonance spectroscopy or magnetic resonance (2). NASH is defined by evidence of hepatocyte injury (ballooning) and inflammation, with or without fibrosis, in a liver biopsy. 20% of patients with NASH develop cirrhosis and have a high risk of hepatocarcinoma. (35). The pathogenesis of NAFLD, schematically represented in Figure 2, is multifactorial and multiple mechanisms have been proposed to explain the process of excessive liver lipid accumulation, with the subsequent possible inflammation and fibrosis (36). According to the classic "two-hits" model, the increased insulin resistance, secondary to metabolic syndrome, determines an excessive lipid accumulation in healthy hepatocytes (first "hit"), mainly through an increased mobilization of free fatty acids from visceral adipose tissue to the liver. This process leads to NAFL, still reversible condition. The increased oxidative stress seems to be the second "hit" in the progression from NAFL to NASH: the increase in ROS and consequently in lipid peroxidation has been associated to hepatocellular damage, inflammation, and eventual fibrosis due to the activation of hepatic stellate cells. Moreover, ROS inhibit hepatocyte secretion of VLDL and promote hepatic insulin resistance, inducing liver fat accumulation and necro-inflammation. The oxidative stress also contributes to atherosclerosis, representing a possible link between NAFLD and metabolic syndrome (37). In addition, antioxidants that protect the liver from ROS damage and lipid peroxidation may be depleted: GSH, vitamin E, vitamin C and beta-carotene were found to be reduced in the NASH setting (38, 39). Closely related to oxidative stress is the so-called dicarbonyl stress, i.e., the accumulation of dicarbonyl metabolites leading to cell and tissue disfunction (40). Altered functioning of the glyoxalase enzyme system, with associated accumulation of glycation products, has been shown as another possible pathogenic mechanism in NAFLD (41, 42).

More recently, a "multiple hits" theory has been proposed, with the addition of other pathogenic factors in NAFLD development: low-grade chronic inflammation, genetic and epigenetic mutations, and intestinal microbiota. A condition of low-grade inflammation has been related to NAFLD, with an abnormal production of cytokines and adipokines, such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-1, leptin. In particular, lower levels of adiponectin and increased expression of TNF-α and its soluble receptor have been recently related to the development of NASH (43). A role for genetic has been demonstrated, with evidence of an increased risk of NAFL in patients with polymorphisms, like those associated to patatin-like phospholipase domain containing 3 (PNPLA3) rs738409, transmembrane 6 superfamily member 2 (TM6SF2) rs58542926 and membrane bound O-acyltransferase domain containing 7 (MBOAT7) rs641738 (44–47). Conversely, patients who carry variants in hydroxysteroid 17-beta dehydrogenase 13 (HSD17B13, rs72613567) and mitochondrial amidoxime-reducing component 1 (MARC1, rs2642438) are more protected than the general population (48, 49). Finally, the gut microbiome may

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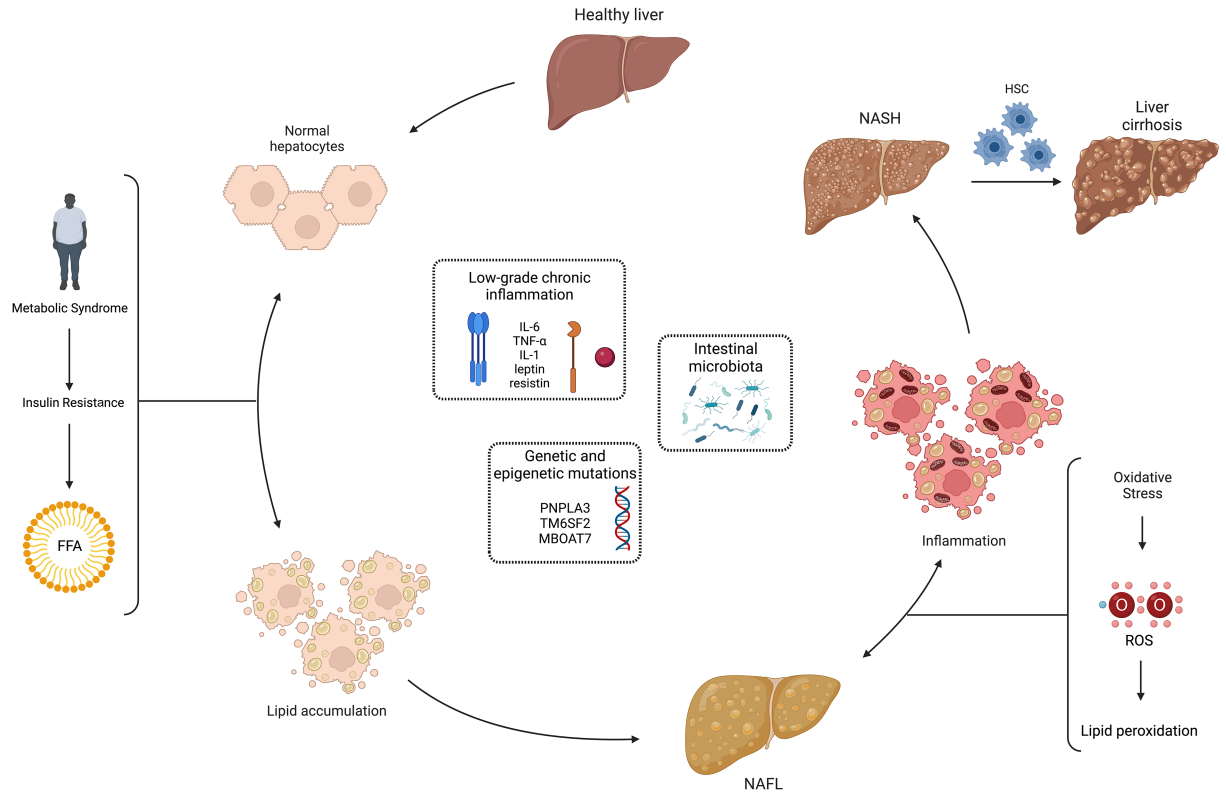

FIGURE 2
Non-alcoholic fatty liver disease (NAFLD) pathogenesis: "multiple hits" theory. The pathogenesis of NAFLD is multifactorial and multiple mechanisms have been proposed to explain the process of excessive liver lipid accumulation, with the subsequent possible inflammation and fibrosis. According to the classic "two-hits" model, the increased insulin resistance, secondary to metabolic syndrome, determines an excessive lipid accumulation in healthy hepatocytes (first "hit"), mainly through an increased mobilization of free fatty acids (FFA) from visceral adipose tissue to the liver. This process leads to the non-alcoholic fatty liver (NAFL), still reversible condition. The increased oxidative stress seems to be the second "hit" in the progression from NAFL to non-alcoholic steatohepatitis (NASH): the increase in reactive oxygen species (ROS) and consequently in lipid peroxidation has been associated to hepatocellular damage, inflammation and eventual fibrosis due to the activation of hepatic stellate cells (HSC). More recently, a "multiple hits" theory has been proposed, with the addition of other pathogenic factors in NAFLD development: low-grade chronic inflammation, genetic and epigenetic mutations, and intestinal microbiota. Created with "Biorender.com." FFA, free fatty acids; HSC, hepatic stellate cells; IL, interleukin; MBOAT7, membrane bound O-acyltransferase domain containing 7; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; PNPLA3, patatin-like phospholipase domain containing 3; ROS, reactive oxygen species; TM6SF2, transmembrane 6 superfamily member 2;TNF-α, tumor necrosis factor-alpha; ↔, reversible condition.

contribute to NAFLD pathogenesis: an increased intestinal permeability could lead to the entry of endotoxins in portal circulation and activation, through toll-like receptor 4, of Kupffer cells, with consequent inflammation (50–53).

Most patients with NAFLD are asymptomatic, and the diagnosis is mainly made incidentally on the basis of liver biochemistry or abdominal ultrasound abnormalities. Common symptoms include right upper quadrant pain and fatigue. The most common laboratory alterations are elevation of liver enzymes, with serum alanine aminotransferase higher than aspartate aminotransferase (ALT>AST) (54), although transaminase levels may be within limits in more than one third of cases, and hyperferritinemia (55), which has been demonstrated to be a marker of sever histologic damage and an independent predictor for liver fibrosis (56, 57). In a recent work by Corradini et al., variants of genes related to iron metabolism were shown to be associated with hyperferritinemia and more severe NAFLD (58).

Diagnosis is made by exclusion of alcohol abuse and other causes of liver disease (HBV-related hepatitis, HCV-related hepatitis, autoimmune liver diseases, polycystic ovary syndrome, drug-induced liver disease and congenital causes such as hereditary hemochromatosis, Wilson's disease, alpha-1 antitrypsin deficit). In association with the assessment of liver enzymes levels in serum, ultrasound (US) is the first line procedure to screen patients for NAFLD. Although US in a non-invasive and practical method, it has low sensitivity for mild levels of steatosis and cannot be used for the distinction between NAFLD and NASH, without a concomitant liver biopsy. Thus, vibration-controlled transient elastography (VCTE) or magnetic resonance elastography are used to identify early phases of the disease. If significant fibrosis is confirmed, patients should be referred to a specialist to perform liver biopsy and confirm the diagnosis histologically (59). In Chinese guidelines, high serum levels of CK-18 fragments (M30 and M65) have been proposed as a possible indicator to perform a liver biopsy (60).

Chronic inflammation is the driving force for the onset and progression of fibrosis in NASH (61). Liver fibrosis represents, together with the comorbidities of metabolic syndrome, a significant

TABLE 1 Main studies on the role of reduced glutathione (GSH) treatment in patients with metabolic liver disease.

Study	Country	Year	Type of the study	Study population	Treatment	Outcomes	Follow-up	Main findings	Ref
Dentico et al.	Italy	1995	50 pts 25 controls	NAFL No biopsies	1800 mg/day IV for 30 days (25 pts) 600 mg/day IM for 30 days (25 pts)	AST, ALT, GGT, bilirubin, MDA	4 month	↓AST, ALT and GGT ↓MDA No bilirubin improvement No adverse effects Efficacy high-dose treatment	77
Irie et al.	Japan	2016	15 pts	NAFL (n = 5) NASH (n = 10) Pre-treatment biopsies	300 mg/day PO for 3 months	ALT, GGT, 8-OHdG, IHC expression of GSH	3 month	↓ ALT, GGT and 8-OHdG in NASH (in NAFLD no statistical significance) GSH liver expression abundant (especially in NAFLD) Possible prevention of progression from NAFLD to NASH	78
Honda et al.	Japan	2017	29 pts	NAFL No biopsies	300 mg/day PO for 4 months	ALT, US (CAP)	4 month	↓ ALT CAP Improvement	79

ALT, alanine aminotransferase; AST, Aspartate aminotransferase; CAP, controlled attenuation parameter; GGT, gamma-glutamyl transferase; IHC, immunohistochemistry; IM, intramuscular; IV, intravenous; MDA, malondialdehyde; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; PO, per os; pts patients; US, ultrasound; 8-OHdG, 8-hydroxy-2-deoxyguanosine; ↓, reduction.

prognostic determinant in NAFLD. For this reason, a major goal in NAFLD management is the prevention of fibrosis and its detection in the earliest stages to avoid progression to cirrhosis. Liver biopsy is the diagnostic gold standard for fibrosis. However, it is an invasive technique with possible complications, therefore non-invasive tests (NITs) have been identified (62). According to the latest EASL Clinical Practice Guidelines, non-invasive scores, serum markers, liver stiffness and imaging methods should be used for ruling out rather than diagnosing advanced fibrosis in low-prevalence populations and should be preferentially employed in patients at risk of advanced liver fibrosis (63). Crucial NITs in NAFLD patients stratification are especially the fibrosis-4 (FIB-4) - an index that takes into account age, transaminases and platelet count- and the liver stiffness evaluation by VCTE. As concerns fibrosis evaluation through cross-sectional imaging techniques, especially magnetic resonance elastography, their use is limited at the moment to tertiary referral centers and for experimental studies, in light of their cost, the limited availability and the procedural length. Finally, it is worth mentioning the new glutamate-serine-glycine (GSG) index which, combining three amino acids involved in glutathione synthesis, provides a good assessment of NAFLD severity and allows the discrimination of liver fibrosis (64).

4. The therapeutic role of GSH in NAFLD

As already mentioned, oxidative stress is a pathophysiological hall-mark of metabolic liver disease (65–68). Under this condition, ROS overproduction appears to be associated with an impairment of intracellular GSH homeostasis, leading to a reduction in GSH levels and in its antioxidant and hepato-protective function (69, 70). Based on these assumptions, a role for GSH in the treatment of liver disease has been hypothesized for NAFLD (71).

While several clinical studies examined the favorable effect of reduced GSH short-term or long-term administration on alcohol-induced liver diseases (72–76), the available literature on the effect of GSH on NAFLD is limited -see Table 1-, and the studies at hand are to be considered pilots (77–79).

An early work, presented in 1995 by Dentico and colleagues, evaluated the effect of 30-day administration of high doses of intravenous or intramuscular GSH on liver cytolysis indexes in patients with chronic steatosic liver disease (77). No adverse effects were reported and a significant reduction in liver tests (specifically transaminases and gamma-glutamyltranspeptidase -GGT-), with many cases of bio humoral parameters normalization, was detected in

all treated patients, even several months after treatment interruption. In addition, confirming the efficacy of GSH treatment, a reduction in malondialdehyde, a marker of hepatic cell damage, was detected.

A subsequent study of Irie and colleagues in 2016 showed that the use of oral glutathione, at a daily dosage of 300 milligrams per day, may prevent NASH progression from NAFLD (78). A higher level of oxidative stress was detected in patients with NASH compared to NAFLD and a reduction in the levels of 8-hydroxy-2-deoxyguanosine (8-OHdG) and GGT, as markers of oxidative stress, was highlighted in NASH patients treated with GSH, with a consequent reduction of alanine transaminase (ALT). Also, they evaluated the immunohistochemical expression of GSH on pre-treatment biopsies, finding a stronger expression of GSH in NAFL than NASH. These results suggested a possible progression from NAFLD to NASH due to oxidative stress and demonstrated a potential therapeutic role for GSH in controlling the progression of liver damage.

The study by Honda et al., conducted in 2017, was an open-label, single-arm, multicentre pilot study that evaluated the therapeutic effect of oral glutathione administration (300 mg/day) in patients with NAFLD through the evolution of biochemical indices (ALT) and liver fat levels assessed by VCTE (79). ALT levels significantly decreased following treatment with GSH for 4 months, with a consequent decrease in liver fat levels non-invasively evaluated using elastography with controlled attenuation parameter.

These preliminary studies suggest a potential therapeutic effect of oral administration of GSH in NAFLD. However, the small sample-size, the short treatment period, the absence of control groups, the lack of liver biopsy evaluation after treatment are just some of the limitations of these studies. More studies are needed to elucidate the mechanism behind the effect of GSH and large-scale trials are necessary to confirm the therapeutic role for GSH. According to ClinicalTrials.gov, as of 5th December 2022, no phase III clinical trial on the use of GSH in NAFLD is currently ongoing or recruiting.

5. Conclusion

NAFLD is a liver disease characterized by a high prevalence in the general population. Although several drugs are under investigation, there are currently no approved drugs for NAFLD (80). The complex pathophysiology and heterogeneity of the disease raises the speculation that combined treatment will be required for many patients. Therefore, the need for new therapies able to cure and prevent the progression of this condition is increasingly urgent.

The pathogenetic role of oxidative stress in NALFD is well known and would explain the rationale for the use of GSH as a potential therapy. The studies currently available on the use of both oral and parenteral GSH are promising but represent only pilot studies for the time being. Indeed, these studies are burdened by several limitations, most importantly the small sample size and the lack of evaluation of the therapeutic effect of GSH by liver biopsy, which to date is the gold standard for the definition of steatosis and fibrosis levels. Further studies are needed to confirm the actual benefit of this molecule on metabolic liver diseases and define the best route of administration and the most appropriate dosage, allowing its use in clinical practice.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Glutathione Metabolism and Its Implications for Health[1]

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ABSTRACT Glutathione (γ-glutamyl-cysteinyl-glycine; GSH) is the most abundant low-molecular-weight thiol, and GSH/glutathione disulfide is the major redox couple in animal cells. The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes, γ-glutamylcysteine synthetase and GSH synthetase. Compelling evidence shows that GSH synthesis is regulated primarily by γ-glutamylcysteine synthetase activity, cysteine availability, and GSH feedback inhibition. Animal and human studies demonstrate that adequate protein nutrition is crucial for the maintenance of GSH homeostasis. In addition, enteral or parenteral cystine, methionine, *N*-acetylcysteine, and L-2-oxothiazolidine-4-carboxylate are effective precursors of cysteine for tissue GSH synthesis. Glutathione plays important roles in antioxidant defense, nutrient metabolism, and regulation of cellular events (including gene expression, DNA and protein synthesis, cell proliferation and apoptosis, signal transduction, cytokine production and immune response, and protein glutathionylation). Glutathione deficiency contributes to oxidative stress, which plays a key role in aging and the pathogenesis of many diseases (including kwashiorkor, seizure, Alzheimer's disease, Parkinson's disease, liver disease, cystic fibrosis, sickle cell anemia, HIV, AIDS, cancer, heart attack, stroke, and diabetes). New knowledge of the nutritional regulation of GSH metabolism is critical for the development of effective strategies to improve health and to treat these diseases. J. Nutr. 134: 489–492, 2004.

KEY WORDS: • amino acids • oxidative stress • cysteine • disease

The work with glutathione (γ-glutamyl-cysteinyl-glycine; GSH)[3] has greatly advanced biochemical and nutritional sciences over the past 125 y (1,2). Specifically, these studies have led to the free radical theory of human diseases and to the advancement of nutritional therapies to improve GSH status under various pathological conditions (2,3). Remarkably, the past decade witnessed the discovery of novel roles for GSH in signal transduction, gene expression, apoptosis, protein glutathionylation, and nitric oxide (NO) metabolism (2,4). Most recently, studies of in vivo GSH turnover in humans were initiated to provide much-needed information about quantitative aspects of GSH synthesis and catabolism in the whole body and specific cell types (e.g., erythrocytes) (3,5–7). This article reviews the recent developments in GSH metabolism and its implications for health and disease.

Abundance of GSH in Cells and Plasma. Glutathione is the predominant low-molecular-weight thiol (0.5–10 mmol/L) in animal cells. Most of the cellular GSH (85–90%) is present in the cytosol, with the remainder in many organelles (including the mitochondria, nuclear matrix, and peroxisomes) (8). With the exception of bile acid, which may contain up to 10 mmol/L GSH, extracellular concentrations of GSH are relatively low (e.g., 2–20 μmol/L in plasma) (4,9). Because of the cysteine residue, GSH is readily oxidized nonenzymatically to glutathione disulfide (GSSG) by electrophilic substances (e.g., free radicals and reactive oxygen/nitrogen species). The GSSG efflux from cells contributes to a net loss of intracellular GSH. Cellular GSH concentrations are reduced markedly in response to protein malnutrition, oxidative stress, and many pathological conditions (8,9). The GSH + 2GSSG concentration is usually denoted as total glutathione in cells, a significant amount of which (up to 15%) may be bound to protein (1). The [GSH]:[GSSG] ratio, which is often used as an indicator of the cellular redox state, is >10 under normal physiological conditions (9). GSH/GSSG is the major redox couple that determines the antioxidative capacity of cells, but its value can be affected by other redox couples, including $NADPH/NADP^+$ and $thioredoxin_{red}/thioredoxin_{ox}$ (4).

GSH Synthesis. The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes, γ-glutamylcysteine synthetase (GCS) and GSH synthetase (**Fig. 1**). This pathway occurs in virtually all cell types, with the liver being the major producer and exporter of GSH. In the GCS reaction, the γ-carboxyl group of glutamate reacts with the amino group of cysteine to form a peptidic γ-linkage, which protects GSH from hydrolysis by intracellular peptidases. Although γ-glutamyl-cysteine can be a substrate for γ-glutamylcyclotransferase, GSH synthesis is favored in animal cells because of the much higher affinity and activity of GSH synthetase (9).

Mammalian GCS is a heterodimer consisting of a catalytically active heavy subunit (73 kDa) and a light (regulatory) subunit (31 kDa) (8). The heavy subunit contains all substrate binding sites, whereas the light subunit modulates the affinity of the heavy subunit for substrates and inhibitors. The K_m values of mammalian GCS for glutamate and cysteine are 1.7 and 0.15 mmol/L, respectively, which are similar to the intracellular concentrations of glutamate (2–4 mmol/L) and cysteine (0.15–0.25 mmol/L) in rat liver (9). Mammalian GSH

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[3] Abbreviations used: GCS, γ-glutamylcysteine synthetase; GSH, glutathione; GSSG, glutathione disulfide.

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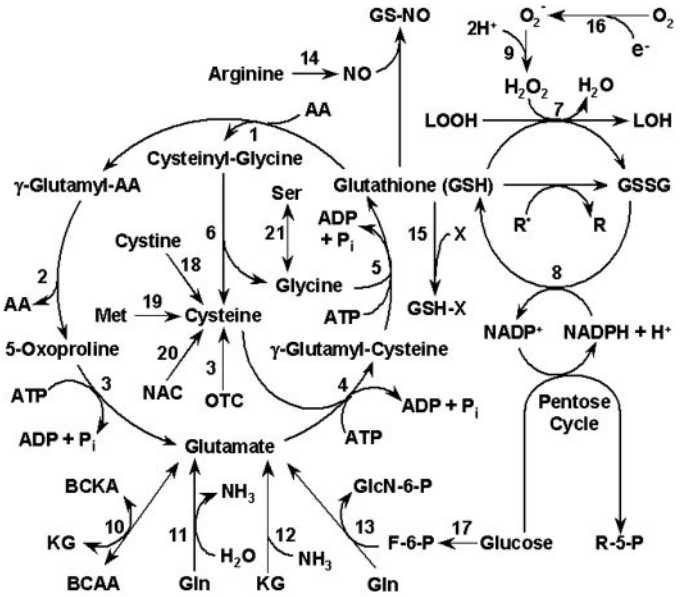

FIGURE 1 Glutathione synthesis and utilization in animals. Enzymes that catalyze the indicated reactions are: *1*) γ-glutamyl transpeptidase, *2*) γ-glutamyl cyclotransferase, *3*) 5-oxoprolinase, *4*) γ-glutamylcysteine synthetase, *5*) glutathione synthetase, *6*) dipeptidase, *7*) glutathione peroxidase, *8*) glutathione reductase, *9*) superoxide dismutase, *10*) BCAA transaminase (cytosolic and mitochondrial), *11*) glutaminase, *12*) glutamate dehydrogenase, *13*) glutamine:fructose-6-phosphate transaminase (cytosolic), *14*) nitric oxide synthase, *15*) glutathione *S*-transferase, *16*) NAD(P)H oxidase and mitochondrial respiratory complexes, *17*) glycolysis, *18*) glutathione-dependent thioldisulfide or thioltransferase or nonenzymatic reaction, *19*) transsulfuration pathway, *20*) deacylase, and *21*) serine hydroxymethyltransferase. Abbreviations: AA, amino acids; BCKA, branched-chain α-ketoacids; GlcN-6-P, glucosamine-6-phosphate; GS-NO, glutathione–nitric oxide adduct; KG, α-ketoglutarate; LOO•, lipid peroxyl radical; LOOH, lipid hydroperoxide; NAC, *N*-acetylcysteine; OTC, L-2-oxothiazolidine-4-carboxylate; R•, radicals; R, nonradicals; R-5-P, ribulose-5-phosphate; X, electrophilic xenobiotics.

synthetase is a homodimer (52 kDa/subunit) and is an allosteric enzyme with cooperative binding for γ-glutamyl substrate (10). The K_m values of mammalian GSH synthetase for ATP and glycine are ~0.04 and 0.9 mmol/L, respectively, which are lower than intracellular concentrations of ATP (2–4 mmol/L) and glycine (1.5–2 mmol/L) in rat liver. Both subunits of rat GCS and GSH synthetase have been cloned and sequenced (9), which facilitates the study of molecular regulation of GSH synthesis. γ-Glutamylcysteine synthetase is the rate-controlling enzyme in de novo synthesis of GSH (8).

Knowledge regarding in vivo GSH synthesis is limited, due in part to the complex compartmentalization of substrates and their metabolism at both the organ and subcellular levels. For example, the source of glutamate for GCS differs between the small intestine and kidney (e.g., diet vs. arterial blood). In addition, liver GSH synthesis occurs predominantly in perivenous hepatocytes and, to a lesser extent, in periportal cells (11). Thus, changes in plasma GSH levels may not necessarily reflect changes in GSH synthesis in specific cell types. However, recent studies involving stable isotopes (5–7) have expanded our understanding of GSH metabolism. In healthy adult humans, the endogenous disappearance rate (utilization rate) of GSH is 25 μmol/(kg · h) (6), which accounts for 65% of whole body cysteine flux [38.3 μmol/(kg · h)]. This finding supports the view that GSH acts as a major transport form of cysteine in the body. On the basis of dietary cysteine intake [9 μmol/(kg · h)] in healthy adult humans (6), it is estimated that most of the cysteine used for endogenous GSH synthesis is derived from intracellular protein degradation and/or endogenous synthesis. Interestingly, among extrahepatic cells, the erythrocyte has a relatively high turnover rate for GSH. For example, the whole-blood fractional synthesis rate of GSH in healthy adult subjects is 65%/d (6), which means that all the GSH is completely replaced in 1.5 d; this value is equivalent to 3 μmol/(kg · h). Thus, whole blood (mainly erythrocytes) may contribute up to 10% of whole-body GSH synthesis in humans (5,6).

Regulation of GSH Synthesis by GCS. Oxidant stress, nitrosative stress, inflammatory cytokines, cancer, cancer chemotherapy, ionizing radiation, heat shock, inhibition of GCS activity, GSH depletion, GSH conjugation, prostaglandin A_2, heavy metals, antioxidants, and insulin increase GCS transcription or activity in a variety of cells (2,8). In contrast, dietary protein deficiency, dexamethasone, erythropoietin, tumor growth factor β, hyperglycemia, and GCS phosphorylation decrease GCS transcription or activity. Nuclear factor κB mediates the upregulation of GCS expression in response to oxidant stress, inflammatory cytokines, and buthionine sulfoximine–induced GSH depletion (2,8). *S*-nitrosation of GCS protein by NO donors (e.g., *S*-nitroso-L-cysteine and *S*-nitroso-L-cysteinylglycine) reduces enzyme activity (8), suggesting a link between NO (a metabolite of L-arginine) and GSH metabolism. Indeed, an increase in NO production by inducible NO synthase causes GCS inhibition and GSH depletion in cytokine-activated macrophages and neurons (12). In this regard, glucosamine, taurine, n-3 PUFAs, phytoestrogens, polyphenols, carotenoids, and zinc, which inhibit the expression of inducible NO synthase and NO production (13), may prevent or attenuate GSH depletion in cells. Conversely, high-fat diet, saturated long-chain fatty acids, low-density lipoproteins, linoleic acid, and iron, which enhance the expression of inducible NO synthase and NO production (13), may exacerbate the loss of GSH from cells.

Regulation of GSH Synthesis by Amino Acids. Cysteine is an essential amino acid in premature and newborn infants and in subjects stressed by disease (14). As noted above, the intracellular pool of cysteine is relatively small, compared with the much larger and often metabolically active pool of GSH in cells (15). Recent studies provide convincing data to support the view that cysteine is generally the limiting amino acid for GSH synthesis in humans, as in rats, pigs, and chickens (6,14,15). Thus, factors (e.g., insulin and growth factors) that stimulate cysteine (cystine) uptake by cells generally increase intracellular GSH concentrations (8). In addition, increasing the supply of cysteine or its precursors (e.g., cystine, *N*-acetylcysteine, and L-2-oxothiazolidine-4-carboxylate) via oral or intravenous administration enhances GSH synthesis and prevents GSH deficiency in humans and animals under various nutritional and pathological conditions (including protein malnutrition, adult respiratory distress syndrome, HIV, and AIDS) (2). Because cysteine generated from methionine catabolism via the transsulfuration pathway (primarily in hepatocytes) serves as a substrate for GCS, dietary methionine can replace cysteine to support GSH synthesis in vivo.

Cysteine is readily oxidized to cystine in oxygenated extracellular solutions. Thus, the plasma concentration of cysteine is low (10–25 μmol/L), compared with that of cystine (50–150 μmol/L). Cysteine and cystine are transported by distinct membrane carriers, and cells typically transport one more

efficiently than the other (8). It is interesting that some cell types (e.g., hepatocytes) have little or no capacity for direct transport of extracellular cystine. However, GSH that effluxes from the liver can reduce cystine to cysteine on the outer cell membrane, and the resulting cysteine is taken up by hepatocytes. Other cell types (e.g., endothelial cells) can take up cystine and reduce it intracellularly to cysteine (Fig. 1); cellular reducing conditions normally favor the presence of cysteine in animal cells.

Extracellular and intracellularly generated glutamate can be used for GSH synthesis (16). Because dietary glutamate is almost completely utilized by the small intestine (16), plasma glutamate is derived primarily from its de novo synthesis and protein degradation. Phosphate-dependent glutaminase, glutamate dehydrogenase, pyrroline-5-carboxylate dehydrogenase, BCAA transaminase, and glutamine:fructose-6-phosphate transaminase may catalyze glutamate formation (Fig. 1), but the relative importance of these enzymes likely varies among cells and tissues. Interestingly, rat erythrocytes do not take up or release glutamate (17), and glutamine and/or BCAAs may be the precursors of glutamate in these cells (Fig. 1). Indeed, glutamine is an effective precursor of the glutamate for GSH synthesis in many cell types, including enterocytes, neural cells, liver cells, and lymphocytes (18). Thus, glutamine supplementation to total parenteral nutrition maintains tissue GSH levels and improves survival after reperfusion injury, ischemia, acetaminophen toxicity, chemotherapy, inflammatory stress, and bone marrow transplantation (19).

Glutamate plays a regulatory role in GSH synthesis through two mechanisms: *1*) the uptake of cystine, and *2*) the prevention of GSH inhibition of GCS. Glutamate and cystine share the system X_c^- amino acid transporter (8). When extracellular glutamate concentrations are high, as in patients with advanced cancer, HIV infection, and spinal cord or brain injury as well as in cell culture medium containing high levels of glutamate, cystine uptake is competitively inhibited by glutamate, resulting in reduced GSH synthesis (20). GSH is a nonallosteric feedback inhibitor of GCS, but the binding of GSH to the enzyme competes with glutamate (9). When intracellular glutamate concentrations are unusually high, as in canine erythrocytes, GSH synthesis is enhanced and its concentration is particularly high (9).

Glycine availability may be reduced in response to protein malnutrition, sepsis, and inflammatory stimuli (21,22). When hepatic glycine oxidation is enhanced in response to high levels of glucagon or diabetes (23), this amino acid may become a limiting factor for GSH synthesis. In vivo studies show that glycine availability limits erythrocyte GSH synthesis in burned patients (7) and in children recovering from severe malnutrition (21). It is important to note that dietary glycine supplementation enhances the hepatic GSH concentration in protein-deficient rats challenged with TNF-α (22).

The evidence indicates that the dietary amino acid balance has an important effect on protein nutrition and therefore on GSH homeostasis (8). In particular, the adequate provision of sulfur-containing amino acids as well as glutamate (glutamine or BCAAs) and glycine (or serine) is critical for the maximization of GSH synthesis. Thus, in the erythrocytes of children with edematous protein-energy malnutrition and piglets with protein deficiency, GSH synthesis is impaired, leading to GSH deficiency (3). An increase in urinary excretion of 5-oxoproline, an intermediate of the γ-glutamyl cycle (Fig. 1), is a useful indicator of reduced availability of cysteine and/or glycine for GSH synthesis in vivo (7,21)

Interorgan GSH Transport. Glutathione can be transported out of cells via a carrier-dependent facilitated mechanism (2). Plasma GSH originates primarily from the liver, but some of the dietary and intestinally derived GSH can enter the portal venous plasma (8). Glutathione molecules leave the liver either intact or as γ-Glu-$(Cys)_2$ owing to γ-glutamyl transpeptidase activity on the outer plasma membrane (Fig. 1). The extreme concentration gradient across the plasma membrane makes the transport of extracellular GSH or GSSG into cells thermodynamically unfavorable. However, γ-Glu-$(Cys)_2$ is readily taken up by extrahepatic cells for GSH synthesis. The kidney, lung, and intestine are major consumers of the liver-derived GSH (8). The interorgan metabolism of GSH functions to transport cysteine in a nontoxic form between tissues, and also helps to maintain intracellular GSH concentrations and redox state (8).

Roles of GSH. Glutathione participates in many cellular reactions. First, GSH effectively scavenges free radicals and other reactive oxygen species (e.g., hydroxyl radical, lipid peroxyl radical, peroxynitrite, and H_2O_2) directly, and indirectly through enzymatic reactions (24). In such reactions, GSH is oxidized to form GSSG, which is then reduced to GSH by the NADPH-dependent glutathione reductase (Fig. 1). In addition, glutathione peroxidase (a selenium-containing enzyme) catalyzes the GSH-dependent reduction of H_2O_2 and other peroxides (25).

Second, GSH reacts with various electrophiles, physiological metabolites (e.g., estrogen, melanins, prostaglandins, and leukotrienes), and xenobiotics (e.g., bromobenzene and acetaminophen) to form mercapturates (24). These reactions are initiated by glutathione-*S*-transferase (a family of Phase II detoxification enzymes).

Third, GSH conjugates with NO to form an *S*-nitrosoglutathione adduct, which is cleaved by the thioredoxin system to release GSH and NO (24). Recent evidence suggests that the targeting of endogenous NO is mediated by intracellular GSH (26). In addition, both NO and GSH are necessary for the hepatic action of insulin-sensitizing agents (27), indicating their critical role in regulating lipid, glucose, and amino acid utilization.

Fourth, GSH serves as a substrate for formaldehyde dehydrogenase, which converts formaldehyde and GSH to *S*-formyl-glutathione (2). The removal of formaldehyde (a carcinogen) is of physiological importance, because it is produced from the metabolism of methionine, choline, methanol (alcohol dehydrogenase), sarcosine (sarcosine oxidase), and xenobiotics (via the cytochrome P450–dependent monooxygenase system of the endoplasmic reticulum).

Fifth, GSH is required for the conversion of prostaglandin H_2 (a metabolite of arachidonic acid) into prostaglandins D_2 and E_2 by endoperoxide isomerase (8).

Sixth, GSH is involved in the glyoxalase system, which converts methylglyoxal to D-lactate, a pathway active in microorganisms. Finally, glutathionylation of proteins (e.g., thioredoxin, ubiquitin-conjugating enzyme, and cytochrome c oxidase) plays an important role in cell physiology (2).

Thus, GSH serves vital functions in animals (**Table 1**). Adequate GSH concentrations are necessary for the proliferation of cells, including lymphocytes and intestinal epithelial cells (28). Glutathione also plays an important role in spermatogenesis and sperm maturation (1). In addition, GSH is essential for the activation of T-lymphocytes and polymorphonuclear leukocytes as well as for cytokine production, and therefore for mounting successful immune responses when the host is immunologically challenged (2). Further, both in vitro and in vivo evidence show that GSH inhibits infection by the

TABLE 1

Roles of glutathione in animals

Antioxidant defense
Scavenging free radicals and other reactive species
Removing hydrogen and lipid peroxides
Preventing oxidation of biomolecules
Metabolism
Synthesis of leukotrienes and prostaglandins
Conversion of formaldehyde to formate
Production of D-lactate from methylglyoxal
Formation of mercapturates from electrophiles
Formation of glutathione-NO adduct
Storage and transport of cysteine
Regulation
Intracellular redox status
Signal transduction and gene expression
DNA and protein synthesis, and proteolysis
Cell proliferation and apoptosis
Cytokine production and immune response
Protein glutathionylation
Mitochondrial function and integrity

influenza virus (29). It is important to note that shifting the GSH/GSSG redox toward the oxidizing state activates several signaling pathways (including protein kinase B, protein phosphatases 1 and 2A, calcineurin, nuclear factor κB, c-Jun N-terminal kinase, apoptosis signal-regulated kinase 1, and mitogen-activated protein kinase), thereby reducing cell proliferation and increasing apoptosis (30). Thus, oxidative stress (a deleterious imbalance between the production and removal of reactive oxygen/nitrogen species) plays a key role in the pathogenesis of many diseases, including cancer, inflammation, kwashiorkor (predominantly protein deficiency), seizure, Alzheimer's disease, Parkinson's disease, sickle cell anemia, liver disease, cystic fibrosis, HIV, AIDS, infection, heart attack, stroke, and diabetes (2,31).

Concluding Remarks and Perspectives. GSH displays remarkable metabolic and regulatory versatility. GSH/GSSG is the most important redox couple and plays crucial roles in antioxidant defense, nutrient metabolism, and the regulation of pathways essential for whole body homeostasis. Glutathione deficiency contributes to oxidative stress, and, therefore, may play a key role in aging and the pathogenesis of many diseases. This presents an emerging challenge to nutritional research. Protein (or amino acid) deficiency remains a significant nutritional problem in the world, owing to inadequate nutritional supply, nausea and vomiting, premature birth, HIV, AIDS, cancer, cancer chemotherapy, alcoholism, burns, and chronic digestive diseases. Thus, new knowledge regarding the efficient utilization of dietary protein or the precursors for GSH synthesis and its nutritional status is critical for the development of effective therapeutic strategies to prevent and treat a wide array of human diseases, including cardiovascular complications, cancer, and severe acute respiratory syndrome.

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vertebrates. Given the essential role of SLC25A39 in erythropoiesis (*6*, *32*), this feature may have evolved to cope with more complex iron metabolism in vertebrates. Understanding how such closed-loop regulation functions in other physiological or pathological processes may provide important insights into the systemic role of metabolic compartmentalization.

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Glutathione Metabolism and Its Implications for Health[1]

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ABSTRACT Glutathione (γ-glutamyl-cysteinyl-glycine; GSH) is the most abundant low-molecular-weight thiol, and GSH/glutathione disulfide is the major redox couple in animal cells. The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes, γ-glutamylcysteine synthetase and GSH synthetase. Compelling evidence shows that GSH synthesis is regulated primarily by γ-glutamylcysteine synthetase activity, cysteine availability, and GSH feedback inhibition. Animal and human studies demonstrate that adequate protein nutrition is crucial for the maintenance of GSH homeostasis. In addition, enteral or parenteral cystine, methionine, *N*-acetylcysteine, and L-2-oxothiazolidine-4-carboxylate are effective precursors of cysteine for tissue GSH synthesis. Glutathione plays important roles in antioxidant defense, nutrient metabolism, and regulation of cellular events (including gene expression, DNA and protein synthesis, cell proliferation and apoptosis, signal transduction, cytokine production and immune response, and protein glutathionylation). Glutathione deficiency contributes to oxidative stress, which plays a key role in aging and the pathogenesis of many diseases (including kwashiorkor, seizure, Alzheimer's disease, Parkinson's disease, liver disease, cystic fibrosis, sickle cell anemia, HIV, AIDS, cancer, heart attack, stroke, and diabetes). New knowledge of the nutritional regulation of GSH metabolism is critical for the development of effective strategies to improve health and to treat these diseases. J. Nutr. 134: 489–492, 2004.

KEY WORDS: • amino acids • oxidative stress • cysteine • disease

The work with glutathione (γ-glutamyl-cysteinyl-glycine; GSH)[3] has greatly advanced biochemical and nutritional sciences over the past 125 y (1,2). Specifically, these studies have led to the free radical theory of human diseases and to the advancement of nutritional therapies to improve GSH status under various pathological conditions (2,3). Remarkably, the past decade witnessed the discovery of novel roles for GSH in signal transduction, gene expression, apoptosis, protein glutathionylation, and nitric oxide (NO) metabolism (2,4). Most recently, studies of in vivo GSH turnover in humans were initiated to provide much-needed information about quantitative aspects of GSH synthesis and catabolism in the whole body and specific cell types (e.g., erythrocytes) (3,5–7). This article reviews the recent developments in GSH metabolism and its implications for health and disease.

Abundance of GSH in Cells and Plasma. Glutathione is the predominant low-molecular-weight thiol (0.5–10 mmol/L) in animal cells. Most of the cellular GSH (85–90%) is present in the cytosol, with the remainder in many organelles (including the mitochondria, nuclear matrix, and peroxisomes) (8). With the exception of bile acid, which may contain up to 10 mmol/L GSH, extracellular concentrations of GSH are relatively low (e.g., 2–20 μmol/L in plasma) (4,9). Because of the cysteine residue, GSH is readily oxidized nonenzymatically to glutathione disulfide (GSSG) by electrophilic substances (e.g., free radicals and reactive oxygen/nitrogen species). The GSSG efflux from cells contributes to a net loss of intracellular GSH. Cellular GSH concentrations are reduced markedly in response to protein malnutrition, oxidative stress, and many pathological conditions (8,9). The GSH + 2GSSG concentration is usually denoted as total glutathione in cells, a significant amount of which (up to 15%) may be bound to protein (1). The [GSH]:[GSSG] ratio, which is often used as an indicator of the cellular redox state, is >10 under normal physiological conditions (9). GSH/GSSG is the major redox couple that determines the antioxidative capacity of cells, but its value can be affected by other redox couples, including $NADPH/NADP^+$ and $thioredoxin_{red}/thioredoxin_{ox}$ (4).

GSH Synthesis. The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes, γ-glutamylcysteine synthetase (GCS) and GSH synthetase (**Fig. 1**). This pathway occurs in virtually all cell types, with the liver being the major producer and exporter of GSH. In the GCS reaction, the γ-carboxyl group of glutamate reacts with the amino group of cysteine to form a peptidic γ-linkage, which protects GSH from hydrolysis by intracellular peptidases. Although γ-glutamyl-cysteine can be a substrate for γ-glutamylcyclotransferase, GSH synthesis is favored in animal cells because of the much higher affinity and activity of GSH synthetase (9).

Mammalian GCS is a heterodimer consisting of a catalytically active heavy subunit (73 kDa) and a light (regulatory) subunit (31 kDa) (8). The heavy subunit contains all substrate binding sites, whereas the light subunit modulates the affinity of the heavy subunit for substrates and inhibitors. The K_m values of mammalian GCS for glutamate and cysteine are 1.7 and 0.15 mmol/L, respectively, which are similar to the intracellular concentrations of glutamate (2–4 mmol/L) and cysteine (0.15–0.25 mmol/L) in rat liver (9). Mammalian GSH

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[3] Abbreviations used: GCS, γ-glutamylcysteine synthetase; GSH, glutathione; GSSG, glutathione disulfide.

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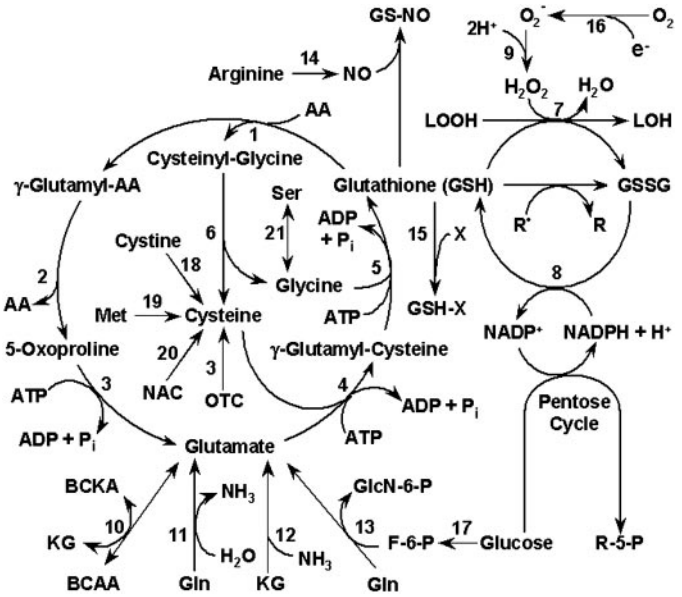

FIGURE 1 Glutathione synthesis and utilization in animals. Enzymes that catalyze the indicated reactions are: *1*) γ-glutamyl transpeptidase, *2*) γ-glutamyl cyclotransferase, *3*) 5-oxoprolinase, *4*) γ-glutamylcysteine synthetase, *5*) glutathione synthetase, *6*) dipeptidase, *7*) glutathione peroxidase, *8*) glutathione reductase, *9*) superoxide dismutase, *10*) BCAA transaminase (cytosolic and mitochondrial), *11*) glutaminase, *12*) glutamate dehydrogenase, *13*) glutamine:fructose-6-phosphate transaminase (cytosolic), *14*) nitric oxide synthase, *15*) glutathione *S*-transferase, *16*) NAD(P)H oxidase and mitochondrial respiratory complexes, *17*) glycolysis, *18*) glutathione-dependent thioldisulfide or thioltransferase or nonenzymatic reaction, *19*) transsulfuration pathway, *20*) deacylase, and *21*) serine hydroxymethyltransferase. Abbreviations: AA, amino acids; BCKA, branched-chain α-ketoacids; GlcN-6-P, glucosamine-6-phosphate; GS-NO, glutathione–nitric oxide adduct; KG, α-ketoglutarate; LOO•, lipid peroxyl radical; LOOH, lipid hydroperoxide; NAC, *N*-acetylcysteine; OTC, L-2-oxothiazolidine-4-carboxylate; R•, radicals; R, nonradicals; R-5-P, ribulose-5-phosphate; X, electrophilic xenobiotics.

synthetase is a homodimer (52 kDa/subunit) and is an allosteric enzyme with cooperative binding for γ-glutamyl substrate (10). The K_m values of mammalian GSH synthetase for ATP and glycine are ~0.04 and 0.9 mmol/L, respectively, which are lower than intracellular concentrations of ATP (2–4 mmol/L) and glycine (1.5–2 mmol/L) in rat liver. Both subunits of rat GCS and GSH synthetase have been cloned and sequenced (9), which facilitates the study of molecular regulation of GSH synthesis. γ-Glutamylcysteine synthetase is the rate-controlling enzyme in de novo synthesis of GSH (8).

Knowledge regarding in vivo GSH synthesis is limited, due in part to the complex compartmentalization of substrates and their metabolism at both the organ and subcellular levels. For example, the source of glutamate for GCS differs between the small intestine and kidney (e.g., diet vs. arterial blood). In addition, liver GSH synthesis occurs predominantly in perivenous hepatocytes and, to a lesser extent, in periportal cells (11). Thus, changes in plasma GSH levels may not necessarily reflect changes in GSH synthesis in specific cell types. However, recent studies involving stable isotopes (5–7) have expanded our understanding of GSH metabolism. In healthy adult humans, the endogenous disappearance rate (utilization rate) of GSH is 25 μmol/(kg · h) (6), which accounts for 65% of whole body cysteine flux [38.3 μmol/(kg · h)]. This finding supports the view that GSH acts as a major transport form of cysteine in the body. On the basis of dietary cysteine intake [9 μmol/(kg · h)] in healthy adult humans (6), it is estimated that most of the cysteine used for endogenous GSH synthesis is derived from intracellular protein degradation and/or endogenous synthesis. Interestingly, among extrahepatic cells, the erythrocyte has a relatively high turnover rate for GSH. For example, the whole-blood fractional synthesis rate of GSH in healthy adult subjects is 65%/d (6), which means that all the GSH is completely replaced in 1.5 d; this value is equivalent to 3 μmol/(kg · h). Thus, whole blood (mainly erythrocytes) may contribute up to 10% of whole-body GSH synthesis in humans (5,6).

Regulation of GSH Synthesis by GCS. Oxidant stress, nitrosative stress, inflammatory cytokines, cancer, cancer chemotherapy, ionizing radiation, heat shock, inhibition of GCS activity, GSH depletion, GSH conjugation, prostaglandin A_2, heavy metals, antioxidants, and insulin increase GCS transcription or activity in a variety of cells (2,8). In contrast, dietary protein deficiency, dexamethasone, erythropoietin, tumor growth factor β, hyperglycemia, and GCS phosphorylation decrease GCS transcription or activity. Nuclear factor κB mediates the upregulation of GCS expression in response to oxidant stress, inflammatory cytokines, and buthionine sulfoximine–induced GSH depletion (2,8). *S*-nitrosation of GCS protein by NO donors (e.g., *S*-nitroso-L-cysteine and *S*-nitroso-L-cysteinylglycine) reduces enzyme activity (8), suggesting a link between NO (a metabolite of L-arginine) and GSH metabolism. Indeed, an increase in NO production by inducible NO synthase causes GCS inhibition and GSH depletion in cytokine-activated macrophages and neurons (12). In this regard, glucosamine, taurine, n-3 PUFAs, phytoestrogens, polyphenols, carotenoids, and zinc, which inhibit the expression of inducible NO synthase and NO production (13), may prevent or attenuate GSH depletion in cells. Conversely, high-fat diet, saturated long-chain fatty acids, low-density lipoproteins, linoleic acid, and iron, which enhance the expression of inducible NO synthase and NO production (13), may exacerbate the loss of GSH from cells.

Regulation of GSH Synthesis by Amino Acids. Cysteine is an essential amino acid in premature and newborn infants and in subjects stressed by disease (14). As noted above, the intracellular pool of cysteine is relatively small, compared with the much larger and often metabolically active pool of GSH in cells (15). Recent studies provide convincing data to support the view that cysteine is generally the limiting amino acid for GSH synthesis in humans, as in rats, pigs, and chickens (6,14,15). Thus, factors (e.g., insulin and growth factors) that stimulate cysteine (cystine) uptake by cells generally increase intracellular GSH concentrations (8). In addition, increasing the supply of cysteine or its precursors (e.g., cystine, *N*-acetylcysteine, and L-2-oxothiazolidine-4-carboxylate) via oral or intravenous administration enhances GSH synthesis and prevents GSH deficiency in humans and animals under various nutritional and pathological conditions (including protein malnutrition, adult respiratory distress syndrome, HIV, and AIDS) (2). Because cysteine generated from methionine catabolism via the transsulfuration pathway (primarily in hepatocytes) serves as a substrate for GCS, dietary methionine can replace cysteine to support GSH synthesis in vivo.

Cysteine is readily oxidized to cystine in oxygenated extracellular solutions. Thus, the plasma concentration of cysteine is low (10–25 μmol/L), compared with that of cystine (50–150 μmol/L). Cysteine and cystine are transported by distinct membrane carriers, and cells typically transport one more

efficiently than the other (8). It is interesting that some cell types (e.g., hepatocytes) have little or no capacity for direct transport of extracellular cystine. However, GSH that effluxes from the liver can reduce cystine to cysteine on the outer cell membrane, and the resulting cysteine is taken up by hepatocytes. Other cell types (e.g., endothelial cells) can take up cystine and reduce it intracellularly to cysteine (Fig. 1); cellular reducing conditions normally favor the presence of cysteine in animal cells.

Extracellular and intracellularly generated glutamate can be used for GSH synthesis (16). Because dietary glutamate is almost completely utilized by the small intestine (16), plasma glutamate is derived primarily from its de novo synthesis and protein degradation. Phosphate-dependent glutaminase, glutamate dehydrogenase, pyrroline-5-carboxylate dehydrogenase, BCAA transaminase, and glutamine:fructose-6-phosphate transaminase may catalyze glutamate formation (Fig. 1), but the relative importance of these enzymes likely varies among cells and tissues. Interestingly, rat erythrocytes do not take up or release glutamate (17), and glutamine and/or BCAAs may be the precursors of glutamate in these cells (Fig. 1). Indeed, glutamine is an effective precursor of the glutamate for GSH synthesis in many cell types, including enterocytes, neural cells, liver cells, and lymphocytes (18). Thus, glutamine supplementation to total parenteral nutrition maintains tissue GSH levels and improves survival after reperfusion injury, ischemia, acetaminophen toxicity, chemotherapy, inflammatory stress, and bone marrow transplantation (19).

Glutamate plays a regulatory role in GSH synthesis through two mechanisms: *1*) the uptake of cystine, and *2*) the prevention of GSH inhibition of GCS. Glutamate and cystine share the system X_c^- amino acid transporter (8). When extracellular glutamate concentrations are high, as in patients with advanced cancer, HIV infection, and spinal cord or brain injury as well as in cell culture medium containing high levels of glutamate, cystine uptake is competitively inhibited by glutamate, resulting in reduced GSH synthesis (20). GSH is a nonallosteric feedback inhibitor of GCS, but the binding of GSH to the enzyme competes with glutamate (9). When intracellular glutamate concentrations are unusually high, as in canine erythrocytes, GSH synthesis is enhanced and its concentration is particularly high (9).

Glycine availability may be reduced in response to protein malnutrition, sepsis, and inflammatory stimuli (21,22). When hepatic glycine oxidation is enhanced in response to high levels of glucagon or diabetes (23), this amino acid may become a limiting factor for GSH synthesis. In vivo studies show that glycine availability limits erythrocyte GSH synthesis in burned patients (7) and in children recovering from severe malnutrition (21). It is important to note that dietary glycine supplementation enhances the hepatic GSH concentration in protein-deficient rats challenged with TNF-α (22).

The evidence indicates that the dietary amino acid balance has an important effect on protein nutrition and therefore on GSH homeostasis (8). In particular, the adequate provision of sulfur-containing amino acids as well as glutamate (glutamine or BCAAs) and glycine (or serine) is critical for the maximization of GSH synthesis. Thus, in the erythrocytes of children with edematous protein-energy malnutrition and piglets with protein deficiency, GSH synthesis is impaired, leading to GSH deficiency (3). An increase in urinary excretion of 5-oxoproline, an intermediate of the γ-glutamyl cycle (Fig. 1), is a useful indicator of reduced availability of cysteine and/or glycine for GSH synthesis in vivo (7,21)

Interorgan GSH Transport. Glutathione can be transported out of cells via a carrier-dependent facilitated mechanism (2). Plasma GSH originates primarily from the liver, but some of the dietary and intestinally derived GSH can enter the portal venous plasma (8). Glutathione molecules leave the liver either intact or as γ-Glu-$(Cys)_2$ owing to γ-glutamyl transpeptidase activity on the outer plasma membrane (Fig. 1). The extreme concentration gradient across the plasma membrane makes the transport of extracellular GSH or GSSG into cells thermodynamically unfavorable. However, γ-Glu-$(Cys)_2$ is readily taken up by extrahepatic cells for GSH synthesis. The kidney, lung, and intestine are major consumers of the liver-derived GSH (8). The interorgan metabolism of GSH functions to transport cysteine in a nontoxic form between tissues, and also helps to maintain intracellular GSH concentrations and redox state (8).

Roles of GSH. Glutathione participates in many cellular reactions. First, GSH effectively scavenges free radicals and other reactive oxygen species (e.g., hydroxyl radical, lipid peroxyl radical, peroxynitrite, and H_2O_2) directly, and indirectly through enzymatic reactions (24). In such reactions, GSH is oxidized to form GSSG, which is then reduced to GSH by the NADPH-dependent glutathione reductase (Fig. 1). In addition, glutathione peroxidase (a selenium-containing enzyme) catalyzes the GSH-dependent reduction of H_2O_2 and other peroxides (25).

Second, GSH reacts with various electrophiles, physiological metabolites (e.g., estrogen, melanins, prostaglandins, and leukotrienes), and xenobiotics (e.g., bromobenzene and acetaminophen) to form mercapturates (24). These reactions are initiated by glutathione-*S*-transferase (a family of Phase II detoxification enzymes).

Third, GSH conjugates with NO to form an *S*-nitrosoglutathione adduct, which is cleaved by the thioredoxin system to release GSH and NO (24). Recent evidence suggests that the targeting of endogenous NO is mediated by intracellular GSH (26). In addition, both NO and GSH are necessary for the hepatic action of insulin-sensitizing agents (27), indicating their critical role in regulating lipid, glucose, and amino acid utilization.

Fourth, GSH serves as a substrate for formaldehyde dehydrogenase, which converts formaldehyde and GSH to *S*-formyl-glutathione (2). The removal of formaldehyde (a carcinogen) is of physiological importance, because it is produced from the metabolism of methionine, choline, methanol (alcohol dehydrogenase), sarcosine (sarcosine oxidase), and xenobiotics (via the cytochrome P450–dependent monooxygenase system of the endoplasmic reticulum).

Fifth, GSH is required for the conversion of prostaglandin H_2 (a metabolite of arachidonic acid) into prostaglandins D_2 and E_2 by endoperoxide isomerase (8).

Sixth, GSH is involved in the glyoxalase system, which converts methylglyoxal to D-lactate, a pathway active in microorganisms. Finally, glutathionylation of proteins (e.g., thioredoxin, ubiquitin-conjugating enzyme, and cytochrome c oxidase) plays an important role in cell physiology (2).

Thus, GSH serves vital functions in animals (**Table 1**). Adequate GSH concentrations are necessary for the proliferation of cells, including lymphocytes and intestinal epithelial cells (28). Glutathione also plays an important role in spermatogenesis and sperm maturation (1). In addition, GSH is essential for the activation of T-lymphocytes and polymorphonuclear leukocytes as well as for cytokine production, and therefore for mounting successful immune responses when the host is immunologically challenged (2). Further, both in vitro and in vivo evidence show that GSH inhibits infection by the

TABLE 1

Roles of glutathione in animals

Antioxidant defense
Scavenging free radicals and other reactive species
Removing hydrogen and lipid peroxides
Preventing oxidation of biomolecules
Metabolism
Synthesis of leukotrienes and prostaglandins
Conversion of formaldehyde to formate
Production of D-lactate from methylglyoxal
Formation of mercapturates from electrophiles
Formation of glutathione-NO adduct
Storage and transport of cysteine
Regulation
Intracellular redox status
Signal transduction and gene expression
DNA and protein synthesis, and proteolysis
Cell proliferation and apoptosis
Cytokine production and immune response
Protein glutathionylation
Mitochondrial function and integrity

influenza virus (29). It is important to note that shifting the GSH/GSSG redox toward the oxidizing state activates several signaling pathways (including protein kinase B, protein phosphatases 1 and 2A, calcineurin, nuclear factor κB, c-Jun N-terminal kinase, apoptosis signal-regulated kinase 1, and mitogen-activated protein kinase), thereby reducing cell proliferation and increasing apoptosis (30). Thus, oxidative stress (a deleterious imbalance between the production and removal of reactive oxygen/nitrogen species) plays a key role in the pathogenesis of many diseases, including cancer, inflammation, kwashiorkor (predominantly protein deficiency), seizure, Alzheimer's disease, Parkinson's disease, sickle cell anemia, liver disease, cystic fibrosis, HIV, AIDS, infection, heart attack, stroke, and diabetes (2,31).

Concluding Remarks and Perspectives. GSH displays remarkable metabolic and regulatory versatility. GSH/GSSG is the most important redox couple and plays crucial roles in antioxidant defense, nutrient metabolism, and the regulation of pathways essential for whole body homeostasis. Glutathione deficiency contributes to oxidative stress, and, therefore, may play a key role in aging and the pathogenesis of many diseases. This presents an emerging challenge to nutritional research. Protein (or amino acid) deficiency remains a significant nutritional problem in the world, owing to inadequate nutritional supply, nausea and vomiting, premature birth, HIV, AIDS, cancer, cancer chemotherapy, alcoholism, burns, and chronic digestive diseases. Thus, new knowledge regarding the efficient utilization of dietary protein or the precursors for GSH synthesis and its nutritional status is critical for the development of effective therapeutic strategies to prevent and treat a wide array of human diseases, including cardiovascular complications, cancer, and severe acute respiratory syndrome.

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Review

How to Increase Cellular Glutathione

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Abstract: Glutathione (GSH) has special antioxidant properties due to its high intracellular concentration, ubiquity, and high reactivity towards electrophiles of the sulfhydryl group of its cysteine moiety. In most diseases where oxidative stress is thought to play a pathogenic role, GSH concentration is significantly reduced, making cells more susceptible to oxidative damage. Therefore, there is a growing interest in determining the best method(s) to increase cellular glutathione for both disease prevention and treatment. This review summarizes the major strategies for successfully increasing cellular GSH stores. These include GSH itself, its derivatives, NRf-2 activators, cysteine prodrugs, foods, and special diets. The possible mechanisms by which these molecules can act as GSH boosters, their related pharmacokinetic issues, and their advantages and disadvantages are discussed.

Keywords: glutathione; oxidative stress; glutathione boosters; cysteine prodrug; Nrf2 activators; N-acetyl cysteine

1. Introduction

Glutathione (GSH) is a tripeptide (γGlu-Cys-Gly) that is the major low molecular mass thiol in mammals. It is characterized by a gamma-peptide bond between the carboxyl group of the glutamate side chain and cysteine (Figure 1). This unusual amide bond protects the molecule from degradation by the action of cellular proteases. The carboxyl group of the cysteine residue is attached to glycine via a normal peptide bond. From a chemical point of view, this tripeptide has one positively charged amino group and two negatively charged carboxyl groups at a physiological pH. These functional groups make GSH an extremely hydrophilic molecule, capable of interacting with various macromolecules through electrostatic attraction, also through hydrogen bonds.

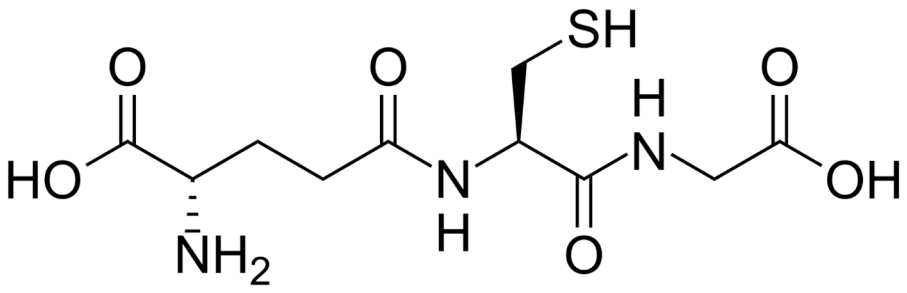

Figure 1. Chemical structure of glutathione.

GSH occurs in millimolar concentrations in cells, where it participates in detoxification reactions of both oxidants and electrophilic compounds. Therefore, the maintenance of physiological GSH levels is crucial to protect cells from harmful molecules [1].

It has been reported that various diseases with known or unknown etiology have decreased GSH levels. This phenomenon is thought to be due to the inability of cells to

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restore normal GSH levels or to increased production of reactive oxygen species (ROS), which can damage macromolecules fundamental to cell survival before being deactivated by antioxidant defenses. In this sense, it is evident that strategies aimed at restoring GSH levels could represent a new therapeutic option to prevent or alleviate the progression of diseases caused by oxidative stress. Numerous preclinical studies have shown that increasing GSH levels is possible, but the clinical implications of these results are still negligible [2]. In other words, currently, almost any disease seems to benefit from treatment with molecules capable of increasing GSH in vivo. This may depend on various factors, including the fact that several molecules are only able to increase GSH only under extreme conditions (i.e., very high concentrations) and in in vitro models but are unable to do the same in vivo.

The purpose of this review is to analyze what is reported in the literature in this field, focusing on the molecules and/or strategies that are more suitable to be used as GSH boosters in vivo. The molecular mechanisms involved (if known) will also be described.

2. Reactions of GSH

The most important chemical functionality of GSH is its cysteinyl moiety, which can act as a radical scavenger and reducing agent via its sulfhydryl group (SH) or participate in detoxification reactions through its addition to the electrophilic center of xenobiotics [1]. Although the pKa value of Cys-thiol GSH is relatively high (8.9–9.4) [3], indicating low reactivity in the intracellular environment, its central role in protecting cells from reactive oxygen/nitrogen species (RONS) is based on enzyme-catalyzed reactions, efficient recycling pathways, as well as the mass effect achieved by its high intracellular concentrations (2–10 mM). Among the important enzyme-mediated cellular processes that use GSH as a cofactor is the GSH-dependent reduction of hydrogen peroxide and other peroxides catalyzed by glutathione peroxidase (Figure 2). Hydrogen peroxide can also be destroyed by catalase through a dismutation reaction [4]. Cells, especially erythrocytes, are extremely rich in catalase (>300,000 U/mg protein), but the role of GSH peroxidase under physiological conditions is not insignificant, as shown by experiments with fibroblasts, which demonstrated that 80% to 90% of H_2O_2 is degraded by GSH peroxidase at H_2O_2 concentrations below 10 µM [5]. GSH forms *S*-conjugates with either xenobiotics (or their metabolites) or endogenous compounds. These reactions are usually catalyzed by a family of isoenzymes called glutathione *S*-transferases [6] (for a review). However, there are also some examples where the reaction occurs spontaneously. In the reaction with free radicals, GSH generates a sulfur-centered thiyl radical (reaction 1):

$$GSH + R^{\bullet} \rightarrow GS^{\bullet} + RH \quad (1)$$

$$2GS^{\bullet} \rightarrow GSSG \quad (2)$$

$$GS^{\bullet} + GSH \rightarrow GSSG^{\bullet} \quad (3)$$

$$GSSG^{\bullet} + O_2 \rightarrow O_2^{\bullet -} {}^{+}GSSG \quad (4)$$

The thiyl radical is rather stable and can usually react in the presence of oxygen either with another thiyl radical or with another GSH molecule to form a disulfide radical. This, in turn, generates a superoxide anion in the presence of oxygen (reactions 2–4). In both cases, glutathione disulfide (GSSG) is eventually formed [7].

The availability of GSH is ensured by its recycling and biosynthesis, which can be upregulated in situations of depletion due to oxidative/nitrosative stress or *S*-conjugation with electrophiles. In summary, intracellular GSH levels are influenced by several factors: (i) oxidative stress, (ii) conjugation with electrophiles, (iii) export from the cell, (iv) enzymatic reduction of GSSG, and (v) de novo synthesis. Reactions i–iii can be classified as depleting GSH reactions, whereas iv–v are considered as replenishing GSH reactions. The decrease in GSH may be due to reduced GSH production because of certain pathophysiological conditions as well as drug treatment. In addition, GSH levels may also be

determined by nutritional status, some hormones, psychogenic stress, and physical activity and may also exhibit diurnal variations [8]. Evidently, under oxidative stress, a significant percentage of GSH is converted to GSSG, and this also represents a situation in which GSH is depleted. In mammalian cells, glutathione is present mainly (>99%) in the reduced form (GSH), whereas GSSG normally constitutes < 1% of GSH. Hundreds of papers report GSSG concentrations much higher than 1% of GSH, likely due to artifacts in sample manipulation (i.e., oxidation) [9–11]. This is mainly due to acid precipitation of proteins, which is crucial for GSH determination. Under these conditions, a significant percentage of GSH tends to oxidize. This strongly affects the measured GSH values, which can be 1–2 orders of magnitude higher compared to the actual ones. This phenomenon occurs primarily in blood, where hemoglobin plays a central role in the formation of this artifact [12] (for a review).

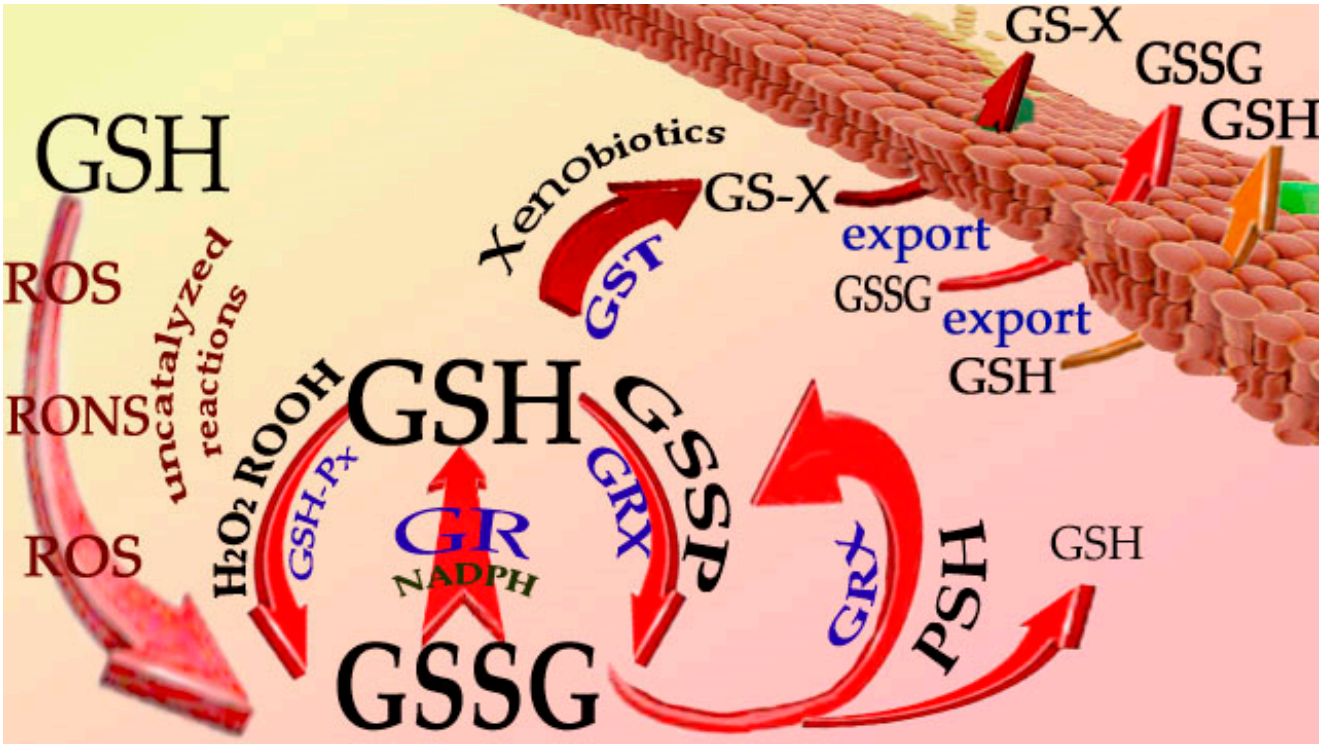

Figure 2. Schematic representations of GSH reactions. Within cells, GSH reacts with oxidants either spontaneously or catalyzed by glutathione peroxidase (GSH-Px). Glutathione disulfide (GSSG) is reduced back to GSH by glutathione reductase (GR). Trans-sulfuration reactions are catalyzed by glutaredoxin (GRX), whereas conjugation with xenobiotics requires catalysis by glutathione transferase (GST). Export of GSH conjugates (GS–X), GSSG and GSH, depends on the activity of multi-drug resistance proteins. ROS, reactive oxygen species; RONS, reactive oxygen/nitrogen species; H_2O_2, hydrogen peroxide; ROOH, organic peroxides; GSSP, S-glutathionylated proteins; PSH, protein SH groups.

A minimal percentage of GSH can also be found to be protein-bound (GSSP); this percentage, in parallel with GSSG, can be increased by pro-oxidant conditions. The formation of GSSP may play an important role in the allosteric regulation of protein function. This process, called protein *S*-glutathionylation, results in the inhibition of enzymes carrying—SH in the catalytic center, allosteric modifications by introducing a negative charge into the protein (Glu residue), and protection of protein—SH groups from further oxidation [13]. As the *S*-glutathionylation of proteins is mainly due to transglutathionylation reactions (5), whose equilibria are close to unity, and the cellular GSH/GSSG ratio seems to play a central role:

$$PSH + GSSG \leftrightarrow GSSP + GSH \quad (5)$$

Although these reactions may occur spontaneously, the nonenzymatic reaction in vivo appears to be negligible [14]. In general, cellular thiols other than GSH, such as cysteine (Cys), coenzyme A, and, more importantly, critical protein thiols, are maintained in a reduced state by this mechanism involving the two enzymes thioltransferase and glutaredoxin. Therefore, reaction (5) can be rewritten as follows:

$$RSH + GSSG \leftrightarrow RSSG + GSH \quad (6)$$

where RSH can be either a low molecular weight thiol or a protein thiol. Thioltransferase has been reported to have broad specificity ranging from asymmetric low molecular weight disulfides to mixed protein disulfides. It is unlikely that only those protein disulfide groups that are sterically inaccessible will react. It should be emphasized that thioltransferase can maintain several enzymes such as phosphofructokinase and pyruvate kinase in the reduced active state [15].

Normally, the GSH/GSSG ratio is finely regulated in the GSSG formed after the reaction of GSH with oxidants can be reduced back to GSH by glutathione reductase (GR) with NADPH donating reducing equivalents. Riboflavin is also required in this reaction because flavin dinucleotide (FAD) forms the prosthetic group of GR [16]; therefore, its deficiency may lead to an increase in GSSG.

Increasing the GSH/GSSG ratio appears to be critical for cell survival and for the regulation of the cell cycle [17], which is why the de novo synthesis of GSH, reduction of GSSG to GSH, and export of GSSG are considered protective [18].

GSH competes with other cellular redox-active biomolecules for the reduction of reactive oxygen species and thereby exerts its well-known protective function. However, GSH is also involved in several vital functions in animals: (i) it is essential in some phase IIs of xenobiotic metabolism; (ii) it may provide a reservoir for nitric-oxide-forming S-nitrosoglutathione [19], which is essential for the regulation of blood pressure; (iii) it is necessary for the conversion of prostaglandin H2 (a metabolite of arachidonic acid) into prostaglandins D2 and for leukotriene synthesis; (iv) there is evidence both in vitro and in vivo that GSH inhibits influenza virus infection [20]; (v) SARS-CoV2 infection impairs cellular glutathione metabolism and redox function, thus the replenishment of GSH during infection is thought to play a protective role against disease [21].

Last but not least, a decrease in GSH/GSSG activates several signaling pathways that reduce cell proliferation and induce apoptosis [22]. It follows that a decrease in GSH can impair normal physiological functions and cell survival [18] (for a review).

3. Synthesis and Catabolism of Glutathione

GSH is synthesized intracellularly by the sequential actions of γ-glutamylcysteine and GSH synthetases (γ-GCS and GSHS, respectively). The first step is of central importance: the activity of γ-GCS is subject to nonallosteric inhibition by GSH (Ki = 2.1 mM), and the availability of cysteine drives the kinetics of the whole reaction (Figure 3). Cysteine is mainly derived from the trans-sulfuration pathway (TSP) of methionine and/or the reduction of cysteine [23].

GSH is degraded extracellularly, where it is converted to cysteinylglycine (CysGly) and then to Cys at tissue sites rich in the ectoenzymes γ-glutamyltranspeptidase (γ-GT) and dipeptidases (mainly kidney and lung) by the sequential action of these two enzymes Figure 3) [24] These amino acids can be uploaded by cells and used for de novo GSH biosynthesis. GSH turnover is relatively rapid in most cells, with half-lives as short as 2–6 h [25], indicating high rates of both GSH synthesis and export. In addition to GSH, GSSG and GSH conjugates can also be exported when formed by cellular exposure to oxidants or electrophilic molecules. It is known that the export of glutathione conjugates from cells occurs primarily via proteins associated with multidrug resistance. Less is known about the export of GSH and GSSG, but some results suggest that the phenomenon is mediated by the same class of transporter proteins [26]. GSH appears to be released from

all tissues, particularly the liver (and to a lesser extent skeletal muscle and red blood cells), where cysteine derived from food or methionine via TSP is converted to tripeptide.

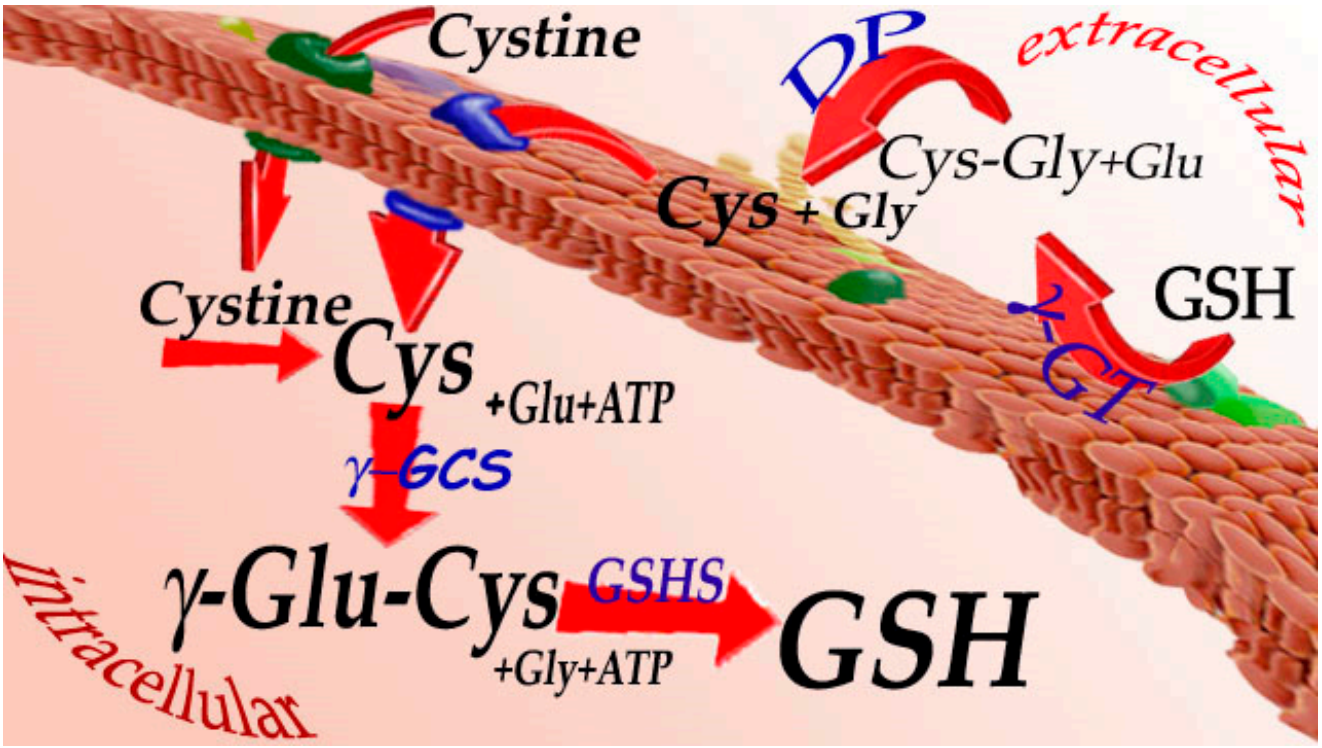

Figure 3. GSH synthesis and degradation. GSH is synthesized by a two-step enzymatic reaction involving γ-glutamylcysteine syntethase (γ-GCS) and GSH synthetases (GSHS) in sequence. γ-GCS catalyzes the formation of the dipeptide γ-glutamylcysteine (γ-Glu-Cys), and GSHS catalyzes the binding of glycine to γ-Glu-Cys to form GSH. This reaction occurs in the cytoplasm of each cell. Cysteine (Cys), which is the rate-limiting factor for this synthesis, can also be obtained by reduction of cystine once it enters the cells. GSH is enzymatically degraded to cysteinylglycine (Cys-Gly) by γ-glutamyltranspeptidase (γ-GT), which is located on the outer surface of plasma membranes. Cys-Gly can then be hydrolyzed into the individual amino acids by extracellular dipeptidases (DP). Glu, glutamic acid; Gly, glycine.

4. GSH Levels in Tissues

GSH is present in all organs, with the highest concentration found in the liver, where it ranges from 6 to 8 mM in both rats (Table 1) and mice [27]. However, GSH levels are also above 1 mM in all other organs (Table 1). Instead, low micromolar GSH concentrations occur in adipose tissue [28]. In cultured cells, GSH levels vary between 20 and 150 nmol/mg protein [29,30].

Unlike in the intracellular compartment, GSH is present in the extracellular fluids at low micromolar concentrations (Table 1), and, in humans, it is almost equimolar with GSSG [31]. In contrast to humans, rat plasma has higher levels of GSH, i.e., about 20 μM, which far exceeds GSSG (GSH/GSSG ratio = 6) [32]. Glutathione is present in very low amounts in the extracellular cortex, whereas its concentration is much higher in the epithelial mucosal fluid of healthy humans, where it is important in maintaining the fluidity of the mucus by reducing protein disulfide bridges [33].

Table 1. Levels of GSH measured in different tissues and cells.

Tissue	Species	Concentration	Reference
Kidney	Human	1564 ± 106 μM	[34]
Muscle	Human	2543 ± 267 μM	[35]
RBCs	Human	8470 ± 1750 nmol/g Hb	[36]
Neutrophils	Humans	13.2 ± 1.8 nmol/10^7 cells	[37]
Lymphocytes	Human	5.7 ± 0.35 nmol/10^7 cells	[38]
Platelets	Human	13.4 ± 2.64 nmol/10^9 platelets	[12]
Plasma	Human	3.1 ± 0.26 μM	[31]
ELF	Human	0.2–0.4 mM	[33]
Liver	Rat	8221 ± 558 μM	[39]
Kidney	Rat	2221 ± 302 μM	[39]
Lung	Rat	2314 ± 182 μM	[39]
Heart	Rat	1835 ± 244 μM	[39]
Spleen	Rat	2648 ± 55 μM	[39]
Brain	Rat	1801 ± 59 μM	[39]
Extracellular brain cortex	Rat	2.10 ± 1.78 μM	[40]
White adipose tissue	Mouse	~4.3 nmol/mg protein	[28]
Brown adipose tissue	Mouse	~2 nmol/mg protein	[28]

Abbreviations: RBCs, red blood cells, ELF, epithelial lining fluid, Hb, hemoglobin.

5. GSH and Disease

GSH plays a central role in cellular functions and viability, and its levels are very tightly regulated. Therefore, a significant decrease in its concentration can cause severe pathological manifestations. This is especially true for people suffering from genetic diseases caused by congenital defects in GSH metabolism. The relationship between oxidative stress, GSH depletion, and disease is much less clear. In this case, the decrease in GSH levels is due to its oxidation caused by the overproduction of RONS. Due to the physiological regulation of homeostatic GSH concentration, it is not easy to identify those pathological conditions in which oxidative stress may have this effect. In any case, much research has been performed on this topic, and some data suggest that this relationship does exist.

5.1. Inborn Alterations in GSH Metabolism

GSH levels are fine-tuned by the activity of several enzymes involved in both its synthesis and metabolism. Although these are rare events, there is evidence that deficiency of any of these enzymes can lead to a significant drop in GSH levels that is incompatible with life in most cases [41]. Thus, the deficiency of γ-GCS, the enzyme that catalyzes the first step of GSH synthesis, is a rare autosomal recessive disease characterized by hemolytic anemia and, in a smaller number of cases, neurological disorders. Similarly, deficiency of glutathione synthetase, which catalyzes the addition of glycine to glutamylcysteine to form GSH, also causes hemolytic anemia. Patients with more severe deficiency experience recurrent bacterial infections and neurodegenerative diseases. In both cases, GSH levels are far below physiological levels. Low activity of GSSG reductase results in low GSH levels and hemolytic anemia [41].

5.2. Ageing and Related Diseases

The free radical theory suggests that age-related loss of function is the result of cumulative damage by endogenous radicals released primarily by mitochondria. RONS trigger various effects, such as the induction of matrix metalloproteases, pro-inflammatory interleukins, and the activation of some specific signaling pathways responsible for organ-related senescence [42]. In particular, a close relationship has been suggested between aging, oxidative stress, and inflammation, a vicious cycle in which chronic oxidative stress and inflammation reinforce each other and, consequently, increase age-related morbidity. Age-related diseases such as diabetes, cardiovascular disease, chronic obstructive pul-

monary disease (COPD), cognitive function loss, macular degeneration, and sarcopenia are thought to have oxidative stress as a cause. Considering the protective properties of GSH against oxidative damage, the deficiency of GSH may be responsible for the onset and/or progression of these diseases.

5.2.1. Age-Related Ocular Diseases

A local decrease in GSH has been noted in age-related eye diseases such as cataract, where a direct correlation between GSH loss and lens browning has been observed [43]. Indeed, lens transparency requires that the thiol group of the structural proteins is in the reduced redox form, and the formation of high molecular weight agglomerates is attributed to the overproduction of disulfide bonds. Therefore, GSH is thought to be necessary to maintain the cysteine residues of proteins in the reduced form. GSH also plays an important homeostatic role in the retina as it is exposed to sunlight, leading to an increase in RONS formation. For example, a decrease in GSH was measured in the retinal pigment epithelium of mice after exposure to white fluorescent light (8000 lux) for 2 h. This observation has been linked to the development and progression of age-related macular degeneration in humans [44].

5.2.2. COPD

COPD is characterized by chronic progressive airway obstruction, with local inflammation playing an important role and tobacco smoke acting as a cofactor. Collectively, these etiologic factors may cause local oxidative injury and significant GSH depletion. Indeed, GSH content in alveolar cells has been shown in animal studies to be decreased after acute exposure to cigarette smoke [45]. GSH concentration is also decreased in COPD patients [46], which indirectly correlates with the severity of respiratory disease [47].

5.2.3. Diabetes Mellitus

Oxidative stress is involved in the pathogenesis of diabetes mellitus and its micro (e.g., retinopathy, neuropathy, and nephropathy) and macrovascular (essentially cardiovascular) complications. Several mechanisms may be responsible for this, such as the autoxidation of glucose, the increased formation of advanced glycation and lipoxidation end products, and the increase in polyol pathway flux, which, in turn, consumes NADPH [48]. As an additional mechanism, there is also some evidence that the activity of antioxidant enzymes can be modulated by glycation. This information has been obtained, for example, from in vitro experiments with lens cells exposed to glucose [49]. As hyperglycemia occurs in both type I and type II diabetes, oxidative stress is considered a pathogenic factor in both cases. Indeed, there are several clinical studies showing a significant decrease in blood GSH levels in type I and II diabetics [50,51]. Our research group has also found a significant increase in oxidized forms of GSH in the erythrocytes of patients with type I diabetes [52].

5.2.4. Cardiovascular Diseases

Inflammation plays an important role in the pathogenesis of atherosclerosis, which is triggered by endothelial injury, which, in turn, promotes compensatory responses and, in particular, the recruitment of inflammatory cells in the arterial wall. Therefore, atherosclerosis is characterized by a local increase in RONS and a decrease in antioxidants. Several studies, either in animal models or in humans, support the notion that the decrease in GSH can lead to cardiovascular events such as stroke [53] and cardiac transplantation [54]. Interestingly, Julius et al. demonstrated that elderly people affected by arthritis, diabetes, or heart disease had lower blood GSH levels than healthy age-matched individuals [55].

5.2.5. Neurodegenerative Diseases

GSH is central to brain function, as this organ consumes a high percentage of oxygen to produce ATP. Some RONS are formed during this mitochondrial process but are counteracted by intracellular antioxidants. Several neurodegenerative diseases are characterized by

mitochondrial degeneration associated with an increase in ROS production and oxidative damage. Evidence that GSH plays a neuroprotective role was confirmed by data from Sofic et al., who measured it in the substantia nigra of people with Parkinson's disease (PD) [56]. The fact that oxidative stress plays an important role in PD was also confirmed by the evidence that paraquat, a free radical generating herbicide, is able to induce the degeneration of dopaminergic neurons and PD in mice [57,58]. It has been reported that antioxidant defenses in the substantia nigra are relatively low compared with other regions of the central nervous system, due to low GSH levels, especially in the early stages of PD, when extravesicular dopamine and its degradation products may act as GSH-depleting agents. These neurons are protected by restoring normal GSH levels [59].

5.2.6. Other Age-Related Conditions

Unlike in younger people, a slight reduction in protein intake (less than 10% of calories) has been observed to increase the risk of death in people over 65 years of age [60]. No explanation for this observation has yet been found, but, among the various hypotheses, the possibility that some specific amino acids are essential for health, and, in particular, cysteine, the precursor of GSH, has also been considered. As GSH decreases with age, maintaining physiological GSH levels may protect against general health worsening due to tissue deterioration (e.g., muscle atrophy, osteoporosis, etc.).

5.3. Cystic Fibrosis

Cystic fibrosis is a genetic disease characterized by the chronic inflammation of the lungs leading to progressive damage to the airway epithelium, bronchiectasis, and chronic obstructive pulmonary disease. The etiology of the disease is due to a mutation of the transmembrane chloride channel CFTR, which is mainly expressed in the apical membrane of the epithelial cells of the lung. However, it is generally accepted that disease progression is favored by the local release of RONS from inflammatory cells. GSH is found in the epithelial lining fluid of the airways, where it protects the lungs from oxidative damage. There are several data indicating a decrease in GSH levels in lung epithelial fluid, plasma, and neutrophil granulocytes in the blood of patients with cystic fibrosis [61–63]. In addition, defective CFTR in lung epithelial cells has been shown to affect the transport of GSH within the cells, contributing to its decrease [64].

5.4. Other Diseases

The above are just a few examples of pathological conditions in which it is more likely that GSH depletion may play a role either as a primary cause or as a secondary contributor to disease progression. Indeed, oxidative stress and GSH decrease are associated with a wide variety of diseases. In recent decades, a wealth of research has emerged, demonstrating a link between reduced GSH concentration and the pathogenesis or progression of almost all known human pathological conditions [65] (for a review).

6. GSH Levels Regulation

There is mounting interest in identifying the best method(s) to increase cellular glutathione for both disease prevention and treatment. GSH concentrations in cells appear to be very finely tuned. Briefly, the substrates for de novo synthesis, impairment of GSH export, and activation of Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) leading to induction of γ-GCS and GSHS could be the main targets of treatments if the goal is to increase GSH concentration in cells (Table 2).

Table 2. In vivo treatments with different GSH enhancers.

Molecule	Treatment	Species	Mechanism	Reference
GSH	Liposomal	Human	Direct	[66]
GSH	Intranasal	Human	Direct	[67]
GSH	Endovenous	Human	Direct	[67]
GSH	Oral	Human	Direct	[67]
γ-Glu-Cys	Oral	Human	Enzymatic synthesis	[68]
NAC	Oral	Human	Cys delivery	[69]
OTC	Oral	Human	Cys indirect formation	[70]
Green tea	Oral	Human	GSH sparing? Up-regulation antioxidant enzymes?	[71]
DASH diet	Oral	Human	Unknown	[72]
GSH mono ester	Oral	Mouse	Enzymatic release	[73]
γ-Glu-Cys	Diet	Mouse	Enzymatic synthesis	[74]
NACET	Oral	Mouse	NAC and Cys delivery	[75]
S-ethyl cysteine	Oral	Mouse	Cys delivery	[76]
S-methyl cysteine	Oral	Mouse	Cys delivery	[76]
S-propyl cysteine	Oral	Mouse	Cys delivery	[76]
Fumaric acid	Oral	Mouse	Nrf2 inducer	[77]
Taurine	Oral	Mouse	Cys sparing	[78]
Silymarin	Oral	Rat	Antioxidant/Nrf2 inducer/cysteine sparing	[79]
NACET	Oral	Rat	NAC and Cys delivery	[80]
ADT	Oral	Rat	γ-GT inhibition? Nrf2 inducer?	[81]
Bardoxolone	Oral	Monkey	Nrf2 inducer	[82]

Abbreviations: GSH, glutathione, γ-Glu-Cys, γ-glutamylcysteine; Cys, cysteine; NAC, N-acetylcysteine; OTC, 2-oxothiazolidine-4-carboxylate, DASH diet, Dietary Approaches to Stop Hypertension; NACET, N-acetylcysteine ethyl ester; ADT, anethole dithiolthione.

6.1. GSH, GSH Esters, γ-Glutamylcysteine

The direct administration of GSH is probably the most evident method to increase GSH. However, orally administered GSH has low bioavailability because it is broken down in the gut into the amino acids of which it is composed. In addition, GSH is not taken up by cells to any significant extent. To circumvent these problems, Shina et al. investigated the efficacy of oral supplementation with liposomal GSH in humans. Although statistical power was limited due to the small sample size, this approach appeared promising [66]. Other routes of GSH administration, such as intravenous, intranasal, and novel sublingual formulations, have been investigated. However, these treatments appeared to be problematic or ineffective as GSH enhancers for a variety of reasons [67].

To improve bioavailability and cell entry, esters of GSH have been synthesized and studied. After the intraperitoneal administration of GSH ethyl ester (GSH-EE), an increase in GSH content was observed in various tissues, including cerebrospinal fluid and erythrocytes. The oral administration of the ester to mice also increased cellular GSH content in their kidneys and livers after it had been decreased by treatment of the animals with buthionine sulfoximine (an inhibitor of GSH synthesis) [73,83]. However, in experiments with endothelial cells (HUVEC), we did not find such efficacy in increasing GSH levels [84]. It was also found that GSH diesters are effectively transported into cells and cause an increase in intracellular GSH concentrations [85]. However, even if diesters are rapidly transported into and out of cells, they are rapidly cleaved into GSH monoesters, which are transported more slowly than GSH diesters. Preliminary studies by Levy et al. suggested that the GSH diester was about four times more effective than the GSH monoester in raising GSH levels in hamster livers [85].

γ-Glutamylcysteine (γ-Glu-Cys) is an immediate precursor of GSH, and only the catalysis of glutathione synthetase is required for the formation of glutathione. In a recent clinical study, γ-Glu-Cys was shown to successfully increase GSH levels in lymphocytes of healthy humans when administered orally (2 g) [68]. The results were promising, although the number of subjects participating in the study was small (thirteen); thus more data are needed to confirm this observation. It was also shown that exogenous γ-Glu-Cys could

easily cross the blood–brain barrier (BBB) and be taken up by many cell types. Liu et al. found that dietary supplementation with γ-Glu-Cys improved spatial memory in mice with Alzheimer's disease by increasing total GSH and reducing levels of oxidative stress and nerve cell apoptosis [74].

6.2. Nrf2

Nrf2 is a redox-sensitive transcription factor that binds to specific response elements in promoter sequences and drives the expression of numerous cytoprotective genes involved in the antioxidant response. Enzymes upregulated by Nrf2 include superoxide dismutase, enzymes involved in glutathione production (γ-GCS, GSHS), glutathione peroxidase, thioredoxin, the X_c^- system that facilitates cystine uptake, and many others with direct or indirect antioxidant function [86,87]. Nrf2 resides in the cytoplasm under basal conditions and has a short half-life, as it is ubiquinated by Kelch like-ECH-associated protein 1 (KEAP1) and cullin 3 [88]. After ubiquination, it is rapidly destroyed. Electrophilic molecules or oxidative stress oxidize the cysteinyl residues in Keap1, inhibiting the Keap1-Cul3 ubiquitination system [89]. Therefore, molecules that can activate Nrf2 have been the focus of interest due to their potential as GSH-enhancing drugs. Resveratrol, sulforaphane, lipoic acid, bardoxolone, curcumin, and dimethylfumaric acid have been shown to be Nrf2 activators [90–93]. The molecular mechanisms of action of these molecules, most of which are antioxidants (e.g., resveratrol) rather than oxidants, are unclear. It has been hypothesized that they may cause the indirect oxidation of a specific thiol residue in Keap1, leading to the stabilization of Nrf2 by inhibiting its proteasomal degradation and subsequent translocation to the nucleus [91,94,95]. Triterpendoids such as bardoxolone have been reported to induce cytoprotective genes through Keap1-Nrf2 antioxidant response element signaling and, in particular, increase cellular GSH levels [82]. Bardoxolone has been tested for the treatment of diseases in which oxidative stress is thought to play a key role, such as diabetic nephropathy. However, a phase 3 clinical trial evaluating bardoxolone for the treatment of chronic kidney disease was discontinued after patients were found to have a higher rate of heart-related adverse events [96]. Currently, the efficacy and safety of bardoxolone is being evaluated in the treatment of pulmonary hypertension [97].

Dimethyl fumarate has been approved by the US Food and Drug Administration as a treatment option for adults with relapsing–remitting multiple sclerosis [98]. Oral treatments with dimethyl fumarate appear to be able to increase GSH levels through the activation of Nrf2, leading to protection against oxidative stress [77]. As oxidative stress plays an important role in neurodegeneration in multiple sclerosis, increasing GSH levels seems to be a reasonable therapeutic option [99]. In 2015, a Cochrane systematic review found moderate-quality evidence of a reduction in the number of people with relapsing-remitting MS, who relapsed over a 2-year treatment period on dimethyl fumarate compared with placebo; low-quality evidence of a reduction in disability worsening; and a general need for higher-quality trials with longer follow-up [100].

6.3. Cysteine Pro-Drugs

As mentioned earlier, cysteine availability is a rate-limiting factor for GSH synthesis. Therefore, treatments that can increase cellular cysteine levels are considered an appropriate strategy to increase GSH. To this end, several cysteine prodrugs have been developed that are more suitable than cysteine itself, given its high chemical instability and tendency to oxidize to the highly insoluble cystine.

6.3.1. N-Acetylcysteine (NAC)

The best-studied cysteine pro-drug is NAC, which has been used in clinical practice for several decades both as an expectorant and as emergency therapy to treat acetaminophen intoxication. In addition to these specific applications, NAC is also being investigated as a potential therapeutic agent for all diseases in which oxidative stress and the resulting decrease in GSH play a role. The efficacy of NAC in increasing intracellular GSH levels is

due to its de-acetylation to cysteine within cells by various aminoacylases. The kinetics of this process were investigated in an in vitro study [101]. The results suggest that oral administration likely provides little cysteine for accelerated GSH synthesis, due to both poor uptake and minimal intracellular de-acetylation. However, an indirect mechanism could be the reaction of NAC with extracellular cysteine-containing disulfides (i.e., cystine, mixed disulfides with proteins, mixed disulfides with other low molecular weight thiols), allowing it to be released and enter cells [84]. NAC appears to function well as a GSH precursor in animal studies and in small clinical trials for a variety of indications, but, often, when studies were repeated, results were inconsistent [102] (for review). One reason for this could be its low bioavailability. In fact, it is about 10% after oral ingestion [80,103]. Numerous in vitro experiments (e.g., with bovine pulmonary artery endothelial cells, Chinese hamster ovary cells [104,105]) and in vivo data indicate the efficacy of NAC in increasing GSH in cells. An 8-week oral treatment with NAC (8 g/day in divided doses) was effective in restoring GSH levels in the whole blood and T cells of HIV-infected patients [69]. However, in another clinical trial, no change was observed in peripheral blood mononuclear cells (PBMC) of the healthy control group and only a moderate increase in the HIV-positive group [106]. The effect of the intravenous administration of NAC at various doses was compared between patients with known idiopathic pulmonary fibrosis and healthy subjects, and no difference in GSH levels was found in the healthy subjects group [107], supporting the concept that NAC has an effect only on replenishing GSH levels in tissues deficient in the tripeptide. Higher GSH levels were also found in the blood of autism patients after oral treatment with NAC (60 mg/kg/day in divided doses, 12 weeks). A similar effect was observed in the blood of patients with cystic fibrosis [63] and diabetes [108]. There are two aspects to consider when using NAC to replenish intracellular GSH levels: (a) Is NAC equally effective in increasing GSH levels in the specific organs damaged by a pathological condition? (b) Do the typical pharmacokinetics of NAC possibly require too high of a dosage to achieve a significant effect on GSH that patients are unlikely to take for an extended period of time? In experiments conducted in our laboratory with rats, 50 mg/kg NAC (*per os*) twice daily for a period of two weeks was ineffective for increasing GSH levels in most organs analyzed (including the brain) [80], but this was in contrast to other results [109]. Our data also shed light on another aspect that is not well studied: Is NAC able to increase GSH content in the central nervous system because the blood–brain barrier hinders the delivery of NAC to the central nervous system?

Supplementation of NAC and glycine (GlyNAC) has been proposed as an alternative strategy to NAC administration [110,111]. This idea stems from the observation that GSH concentration in the blood of the elderly is low compared to younger control subjects. The same authors found that two of the three amino acids that make up GSH, glycine, and cysteine were significantly lower compared to younger subjects and that this could be responsible for GSH deficiency. The simultaneous administration of glycine and NAC at a fixed dose (1.33 mol/kg/day and 0.81 mmol/kg/day, respectively) over a 24-week period to elderly subjects was able to correct GSH depletion in red blood cells and reduce some hematological markers of oxidative stress such as F2-isoprostanes. A summary of the observed effects of this supplement on age-related defects and the profile of tolerability and safety is provided in a recent report by Sekhar [112].

6.3.2. N-Acetylcysteine Ethyl Ester (NACET)

As an alternative to NAC and with the aim of improving its pharmacokinetic properties, we developed and studied NACET. This molecule was found to cross the plasma membrane much better than NAC with a higher hydrophobicity (logD = −5.4 for NAC and 0.85 for NACET) [80]. NACET was also characterized by a 10-fold bioavailability compared to NAC. Taken orally, it is rapidly absorbed by the GI tract and enters cells as a lipophilic substance. In the cell, it is de-esterified to the more hydrophilic NAC, which, in turn, is trapped in the cell and slowly converted to cysteine, acting as a cysteine pro-drug. After IV bolus administration to rats, the greatest amount of NACET was found in the cells of

various tissues, including the brain, mainly as NAC and Cys. Similarly, the chronic treatment of rats with equivalent doses of NAC or NACET showed that only NACET was able to significantly increase the GSH content of most tissues studied, including the brain [80]. Due to these properties, NACET is much more effective than NAC as a GSH enhancer. Comparative in vitro experiments in which HUVEC were treated with pharmacological concentrations of different GSH boosters (NAC, GSH monoethyl ester, oxothiazolidine-4-carboxylic acid) showed that only NACET was able to significantly influence intracellular GSH [84]. A similar effect was obtained with other cells, i.e., cells of the retinal pigment epithelium (ARPE-19 cells). In the same study, rats were orally administered 50 mg/kg NAC or NACET, and the GSH concentration in the eyes was measured. GSH levels were significantly increased by NACET treatment, with a peak 4 h after drug administration. In contrast to NACET, NAC had no significant effect on GSH concentration [75]. These data indicate that NACET is a promising alternative to other drugs for increasing intracellular GSH concentration, thanks to its best pharmacokinetic properties. It is desirable that NACET continues its preclinical characterization and hopefully can be tested in clinical trials in the future to confirm these positive properties.

6.3.3. N-Acetylcysteinamide (NACA)

An amide derivative of NAC, namely, N-acetylcysteinamide (NACA), was also developed by modifying the carboxyl group. This is another way to improve the lipophilicity of NAC and thus optimize its pharmacokinetics. NACA showed high bioavailability (about 60%), but it was assumed that it was metabolized to NAC in a first step [113]. NACA has been shown to be able to restore basal levels of GSH in erythrocytes or cultured cells treated with oxidants [114].

6.3.4. Thiazolidines

Another class of compounds that can increase intracellular cysteine is thiazolidines. The first observation of these molecules comes from the L-thiazolidine-4-carboxylic acid, which can be converted to N-formyl-cysteine by proline oxidase. Cysteine can then be generated by hydrolysis. The best studied molecule of this class is 2-oxothiazolidine-4-carboxylate (OTC), which, when tested in isolated hepatocytes, showed a low uptake rate and slow conversion to Cys once it entered the cells [115]. However, when administered by IV infusion to patients with acute respiratory distress syndrome (63 mg/kg every 8 h for 10 days), the replenishing effect on GSH in red blood cells was similar to that of NAC [116]. OTC (500 mg orally, every 8 h for 14 days) increased, circulating GSH levels in patients on peritoneal dialysis [70]. However, as indicated above, in HUVEC cells, treatment with OTC for 12–72 h did not significantly increase intracellular GSH [84].

6.3.5. Other Cysteine Derivatives

S-ethylcysteine, S-methylcysteine, and S-propylcysteine increased GSH levels in the striatum and reduced MPTP-induced oxidative stress, inflammatory damage, and loss of dopaminergic neurons in an animal model of Parkinson's disease. These compounds added to drinking water (1 g/L for 3 weeks) showed antioxidant and anti-inflammatory activities by acting as GSH precursors [76]. S-allylcysteine, as well as S-ethylcysteine, S-methylcysteine, and S-propylcysteine, increased GSH content in the kidneys and livers of orally treated Balb/c mice. In addition, these compounds had significant effects on antioxidant enzymes and spared alpha-tocopherol [117].

6.4. Taurine

Recently, taurine was shown to provide protection against oxidative stress by increasing glutathione stores [118,119]. Similarly, high-dose taurine treatment (400 mg/kg via oral gavage for 9 weeks) was able to increase GSH content in whole-brain homogenate of rats exposed to D-galactose [78]. The effects of taurine on GSH levels can be explained by the fact that cysteine is a precursor of both molecules (GSH and taurine); thus, sparing

cellular cysteine in the provision of taurine may lead to increased production of GSH. In this context, it is helpful to remember that the availability of cysteine determines the rate of cellular GSH production.

6.5. Silymarin

Silymarin is a mixture of flavonolignans extracted from the milk thistle *Silybum marianum*. It has been shown to prevent liver damage caused by various chemicals or toxins. This natural herbal medicine has been successfully used for centuries to treat liver diseases [120]. Silymarin acts as an antioxidant but also has a number of other biological properties, such as reducing inflammatory responses by inhibiting NF-κB signaling pathways [121] (for a review). The hepatoprotective role of silymarin against xenobiotic electrolytes such as carbon tetrachloride, ethanol, diethylnitrosamine, and cisplatin is well established [122,123]. Silymarin is a direct scavenger of many RONS, but it is also able to increase the levels of several antioxidant enzymes and of GSH [79]. It has also been shown to significantly increase Nrf2 protein levels in CON1 cells after 48 h of treatment [124]. The main mechanisms by which silymarin increases GSH levels are both the upregulation of Nrf2 and the increase in cysteine availability by increasing its synthesis and inhibiting its degradation to taurine [125]. In summary, silymarin spares the direct reaction of GSH with RONS, upregulates Nrf2, and increases cysteine levels; all of these actions combine to increase GSH levels in cells.

6.6. Food and Diet

There is growing evidence that diet may be an alternative to drugs to increase GSH levels. First of all, given the chemical structure of GSH, it is possible that its levels can be increased by ingesting dietary proteins that contain its precursors, particularly cysteine. In this context, bovine whey, a byproduct of cheese production that is particularly rich in cystine-containing proteins, is thought to play an important role. Moreover, bovine whey is not only rich in cysteine-containing proteins [126], but also contains significant amounts of the GSH precursor γ-glutamilcysteine. It can thus provide a good source of cysteine without the disadvantages of direct supplementation of cysteine or cystine, i.e., low solubility and toxicity. To date, several clinical studies have been conducted confirming the GSH-enhancing effect of whey protein isolates taken over several weeks by healthy individuals [127] or by individuals with various pathologies (e.g., nonalcoholic steatohepatitis, cancer) [128,129]. A pilot clinical study with Parkinson's' disease patients treated with a whey protein supplement showed that the treatment significantly increased plasma GSH in Parkinson's patients but not in the control group [130].

Immunocal® is an undenatured whey protein supplement designed to augment the available cellular GSH pool. Immunocal® has been shown to reduce oxidative stress by increasing lymphocyte GSH in both HIV-infected patients and cystic fibrosis patients [131].

Various nutrients (such as vitamins and minerals) have also been studied for their ability to increase GSH levels. For example, vitamin B6 could increase GSH levels because it is a cofactor for enzymes involved in the TSP, the metabolic pathway that converts homocysteine to cysteine. Vitamins C and E, as well as selenium, have also been tested for their antioxidant effects, which may be mediated by increasing GSH levels, but there is no clear clinical evidence to date [132]. Daily treatment with 500 mg of L-ascorbate for two weeks increased mean glutathione in red blood cells by nearly 50% compared to baseline in healthy, nonsmoking volunteers [133]. Ascorbic acid supplementation in a group of healthy ascorbic acid-deficient volunteers was associated with an 18% increase in lymphocyte GSH [134]. These experiments suggested that each change of 1 mole of ascorbate was accompanied by a change of about 0.5 mole of GSH. Presumably, ascorbate conserved glutathione by limiting its reaction with RONS. The lack of efficient biochemical systems to regenerate GSH when it was oxidized to species other than disulfides (e.g., sulfonic acids) may have led to its depletion. Ascorbic acid competes with GSH by acting as an electron donor, but it also forms semidehydroascorbyl radicals, which are relatively unreactive and

are efficiently reduced to ascorbate by specific NADPH-dependent reductases [135]. This may be the reason why high concentrations of vitamin C enhance GSH.

Some foods may contribute to the maintenance of physiological GSH levels. First, some fruits and vegetables contain significant amounts of GSH (e.g., asparagus, avocado, spinach) or its precursor cysteine (asparagus, red bell pepper, papaya) [136]. However, it is also clear from this study that there is a large heterogeneity between different food types and that the contribution of other aspects such as storage, preparation, and seasonal differences has not yet been elucidated. The effect of cooking was evaluated in a previous article and showed that this process could reduce GSH content. In the same study, it was also found that GSH may be present in foods as glutathione disulfide, which could be reduced back to GSH in cells by GR [137]. Therefore, this point is also worth considering.

Cruciferous vegetables are also of interest because they contain molecules capable of increasing GSH, such as sulforaphane and dithiolethiones. Since the 1980s, it has been observed that some dithiolthione compounds, such as anethole dithiolthione (ADT) and oltipraz, were able to increase GSH content in various organs of rats and mice and protect against the toxic effects of acetaminophen [138]. More recently, we have confirmed this GSH-replenishing effect, especially in different organs of rats such as the liver, kidneys and brain [81]. The molecular mechanisms of this effect remain unclear. We have observed a decrease in the activity of γ-GT in rat kidneys, which correlated with an increase in GSH in the same tissue, especially in the cells of the proximal tubule [139]. It could be speculated that the inhibition of γ-GT by ADT leads to a decreased degradation of GSH and induces the reuptake of intact GSH by the proximal tubule cells. There is also increasing evidence that ADT is able to stimulate the Nrf2 signaling pathway, but this point has not been fully elucidated [140]. The GSH-enhancing effect for this class of compounds was also demonstrated in a clinical study with oltipraz, which was able to increase lymphocyte glutathione in healthy humans [141]. To date, however, there is no direct evidence as to whether a cruciferous vegetable diet could affect physiological GSH levels. Other foods are also of interest because of their antioxidant properties, e.g., polyphenol-containing foods such as green tea and fruit juices, but there are few data on their effects on GSH levels [71].

Interestingly, there is some evidence that the adherence to specific dietary approaches such as the DASH diet (Dietary Approaches to Stop Hypertension) can successfully increase blood GSH levels. This was recently demonstrated in a meta-analysis that examined the effects of this type of diet on some oxidative stress biomarkers [72]. Among the parameters analyzed, GSH was one of those increased in the patients who followed this diet, which consisted mainly of fruits, vegetables, low-fat dairy products, whole grains, fish, poultry, and nuts [142]. However, considering that the participants of the clinical trials selected in this meta-analysis suffered from various diseases such as hypertension, obesity, and polycystic ovary syndrome, and the number of subjects studied was not very high (8 trials, 217 patients), further well-designed studies are needed to confirm this potential beneficial effect.

7. Conclusions and Future Perspectives

Undoubtedly, there is a great and growing interest in molecules that increase GSH levels due to their antioxidant and detoxifying effects. Nevertheless, some of the mechanisms involved remain enigmatic, and, more importantly, for many molecules, the available data are inconsistent. The most commonly used molecule NAC appears to perform this function in some experiments but fails in others or is effective only at very high doses. Other approaches using cysteine prodrugs similar to NAC are also promising but require further research. In particular, we have found that NACET is able to rapidly increase GSH in most tissues and cultured cells because of its unique pharmacokinetic properties (it is deesterified to NAC and deacetylated to Cys). Although NACET is an excellent cysteine precursor, we observed that high concentrations of NAC competitively inhibited γ-GCS, so that it may paradoxically exert a hormetic effect once converted to NAC [84]. The same aspect should be considered for other NAC donors.

Molecules that act via Nrf2 activation and, in turn, affect the expression of various antioxidant enzymes, including those required for GSH production, have been extensively studied in detail. However, the mechanism of Nrf2 activation by these molecules remains largely unexplained. These issues contribute to the high degree of uncertainty surrounding the best conditions for treating animals and/or humans (i.e., dose, duration of treatment, frequency of administration in long-term studies) to achieve the desired effect. Finally, it should be remembered that cells tightly regulate GSH levels, and, even if treatment causes a transient increase in GSH concentration, both the allosteric inhibition of γ-GCS by GSH and the increased GSH export cause cellular GSH levels to return to pretreatment levels. In this sense, little is known about the importance and regulation of GSH exports. This is another aspect that may account for the heterogeneity of the data obtained thus far. Indeed, the experimental results obtained may change depending on whether the treatments are performed with cells/animals having either normal or depleted GSH levels or with healthy or diseased humans with probably different GSH concentrations.

In addition, as a general concept, it also needs to be clarified whether there is sufficient evidence that increasing GSH levels is associated with improved prognosis or protection against disease. Increasing GSH levels is certainly possible, but there is little clinical evidence to support the impressive promises that theory and experimental research have made. In other words, it is not yet fully established whether increasing GSH levels is beneficial, as there are still no solid clinical trials to support this, and there are doubts about the efficacy of treatments in humans. It is not the aim of this review to investigate the reasons for the failures or uncertainties in this area, but we have limited ourselves to assessing which molecules might actually be able to increase GSH levels and by what mechanism, as some are likely to be much more efficient than others.

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Review

Cellular Compartmentalization, Glutathione Transport and Its Relevance in Some Pathologies

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Abstract: Reduced glutathione (GSH) is the most abundant non-protein endogenous thiol. It is a ubiquitous molecule produced in most organs, but its synthesis is predominantly in the liver, the tissue in charge of storing and distributing it. GSH is involved in the detoxification of free radicals, peroxides and xenobiotics (drugs, pollutants, carcinogens, etc.), protects biological membranes from lipid peroxidation, and is an important regulator of cell homeostasis, since it participates in signaling redox, regulation of the synthesis and degradation of proteins (S-glutathionylation), signal transduction, various apoptotic processes, gene expression, cell proliferation, DNA and RNA synthesis, etc. GSH transport is a vital step in cellular homeostasis supported by the liver through providing extrahepatic organs (such as the kidney, lung, intestine, and brain, among others) with the said antioxidant. The wide range of functions within the cell in which glutathione is involved shows that glutathione's role in cellular homeostasis goes beyond being a simple antioxidant agent; therefore, the importance of this tripeptide needs to be reassessed from a broader metabolic perspective.

Keywords: glutathione; S-glutathionylation; transporters; deficiency

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1. Introduction

Oxidative stress is one of the main causes of the development of different types of diseases, such as cancer [1,2], neurodegenerative pathologies [3,4], liver [5,6], cardiac [7,8], pulmonary [9,10] and renal diseases [11,12]. Therefore, strategies have been developed to reduce its effects, such as modifying the lifestyle of patients, that is, changes in diet and physical activity; abolishing any habit that generates oxidizing molecules (such as smoking or drinking alcohol) is also important. With such measures, it is sought to strengthen the antioxidant systems of the patient, for prevention of disease or to decrease its effects [13–16].

Regarding oxidative stress, the enzymatic systems that contribute the most to the generation of ROS include the proteins that are bound to the plasma membrane, such as the family of NADPH oxidases [17,18], the enzymatic systems that participate in the lipid metabolism within peroxisomes and the activity of various cytosolic enzymes such as cyclooxygenases. Although all these sources contribute to the increase in the oxidative state of the cell, the vast majority of cellular ROS (approximately 90%) originates from the mitochondria [19,20].

To counteract the effect of ROS, the cell has a series of antioxidant compounds. One of the most important antioxidant molecules in cellular systems is reduced glutathione (GSH).

This tripeptide (glutamate, cysteine and glycine) [21,22] is the most abundant non-protein thiol in cells, with concentration reaching up to 15 mM [20]. Most of this glutathione is in a reduced state (about 99%), the remaining 1% being oxidized glutathione (GSSG) [23,24]. The concentration of glutathione is regulated by different processes, such as its own synthesis, its re-oxidation, its use for the detoxification of diverse substances (such as alcohol and drugs), and its transport to the different intracellular and extracellular compartments. (Figure 1) [25,26]. Glutathione, through the multiple activities and functions in which it participates (neutralization of free radicals, donor of reducing equivalents, coenzyme, elimination of xenobiotics and other endogenous metabolites, etc.), is important for cellular homeostasis, since it is involved in the dynamic balance that the organism requires for its proper functioning and morphological integrity [27–29].

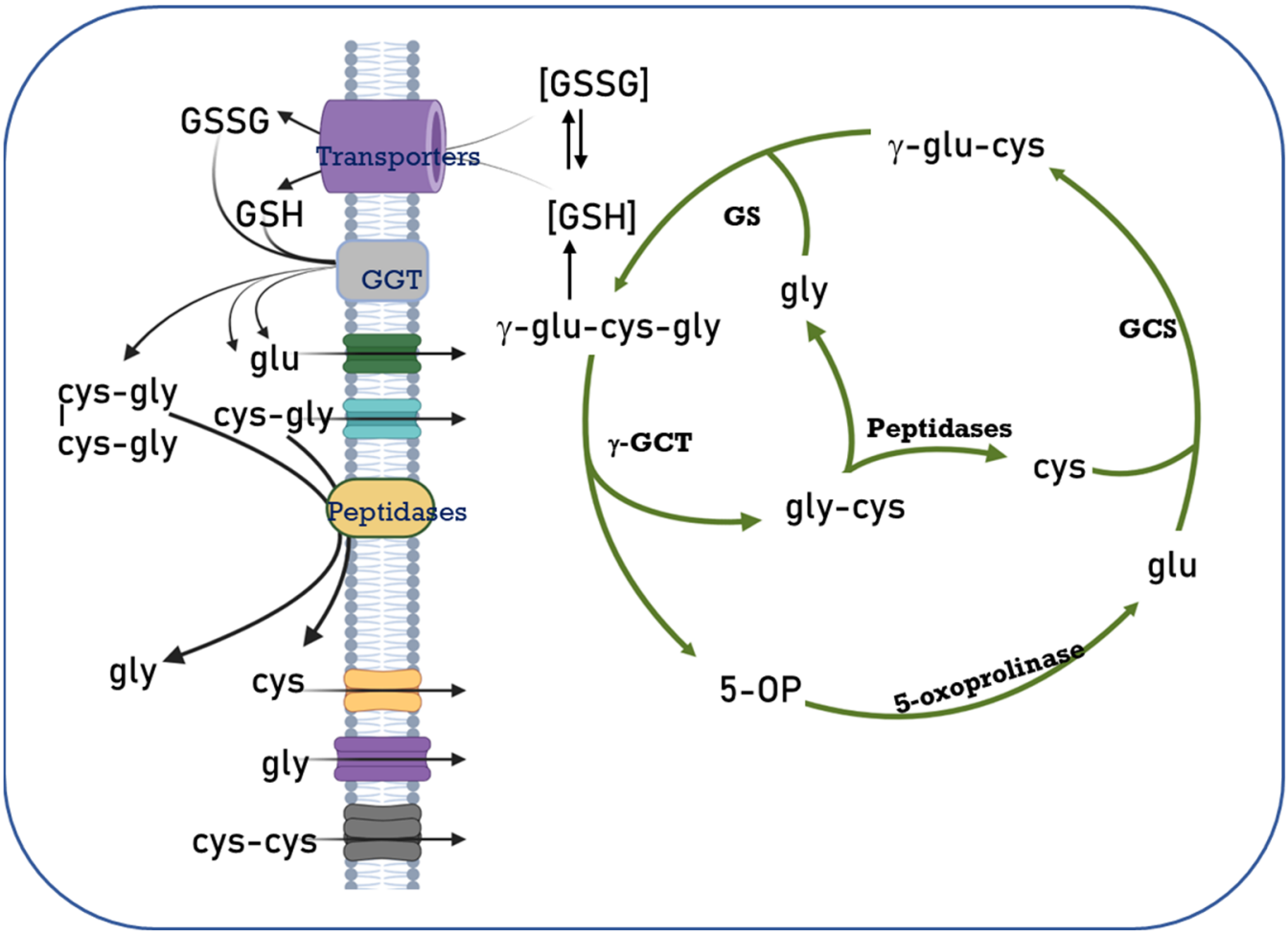

Figure 1. The Glutathione Cycle. Cellular glutathione homeostasis is directly related to its synthesis, degradation, transport, and the availability of the amino acids that make up the non-protein thiol. Different enzymes participate in this regulation process, such as γ-glutamylcysteine synthetase (GCS), glutathione synthetase (GS), 5-oxoprolinase, glutathione transporters, γ-glutamyl transpeptidases (GGT), membrane peptidases and amino acid transporters. 5-OP: 5 oxoproline. Modified from [30].

In this review, we describe the importance of glutathione, both inside and outside of the cell, its transport, its cellular compartmentalization and some associated deficiency diseases.

2. Glutathione Intracellular Compartmentalization

The conservation of hepatic glutathione levels is a dynamic process resulting from the balance between the synthesis rate, transport, use and removal of such thiols [26]. Its synthesis takes place only in the cellular cytosol since all the necessary enzymes for its

synthesis are found there [31]. Nevertheless, within the cell, glutathione is compartmentalized into different cell organelles and ratios. A concentration of 1–15 mM is found in the cell cytosol [20,29]. GSH is also present in the endoplasmic reticulum, nuclear matrix and peroxisomes, but at concentrations that need to be determined [27,32].

Mitochondria lack the enzymes needed for GSH biosynthesis, therefore the mitochondrial GSH pool must be imported from the cytoplasm [26]. This tripeptide is mainly found in mitochondria in its reduced form. It represents 10–15% of total cellular GSH, with a concentration range of 5–10 mM [31]. Glutathione cannot freely cross a lipid bilayer because it is negatively charged at physiological pH, so the outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) must be equipped with transporters or channels to facilitate the entry of GSH. The OMM is rich in porins that form aqueous channels through the lipid bilayer and allow diffusion between the intermembrane space (IMS) and the cytosol of molecules smaller than ~5 kDa, including glutathione [26]. Kojer demonstrated that glutathione pools in the IMS and the cytosol are linked by porins [33]. The inner membrane (IMM) is where, in mammalian cells, the dicarboxylate carrier (DIC) and the oxoglutarate carrier (OGC) were described to carry most of the GSH [34]. On the other hand, it has been reported that DIC and OGC together represent only 45–50% of the total glutathione uptake in hepatic mitochondria, so it has been proposed that the glyoxalase system contributes to mitochondrial GSH supply. This metabolic pathway is widespread in all biological systems and is involved in the cellular detoxification of α-ketoldehydes produced during glycolysis; it catalyzes the conversion of 2-oxaldehyde to 2-hydroxyacid, through the intermediate S-2-hydroxyacylglutathione. The glyoxalase system consists of two enzymes, glyoxalase I (Glo I) and glyoxalase II (Glo II) and GSH as a cofactor. In the cytosol, Glo I catalyzes the formation of S-D-Lactoylglutathione (SLG) from hemithioacetal (MeCOCH(OH)-SG) generated from methylglyoxal (MG) and GSH. The SLG can enter the mitochondria and through Glo II is hydrolyzed into D-lactate and GSH; this represents a complementary mechanism for the supply of GSH to the mitochondria [35].

The concentration of GSH present in the mitochondria is kept constant due to the transport of GSH from the cytosol, through two GSH transportation systems, one of high-affinity, stimulated by ATP, and one of low-affinity, stimulated by ATP and ADP [36]. In the case of endoplasmic reticulum, evidence suggests the presence of a transportation system that allows the selective passage of GSH onto GSSG [37]. In this organelle, GSH contributes to the reduction of protein-disulfide isomerase (PDI), responsible for catalyzing the formation of disulfide bonds in proteins [32,37]. The use of GSH to maintain oxidoreductases in their reduced form leads to a constant production of GSSG in the lumen of the endoplasmic reticulum. GSSG is transported to the cytosol with facilitation of diffusion through the Sec61 protein-conducting channel [38], where it is reduced by the enzyme glutathione reductase [31,37,38].

The mechanisms of nuclear glutathione transport and sequestration are under discussion [39]. Certainly, the synthesis of GSH does not take place in the nucleus because, like mitochondria, it lacks the enzymes required for GSH biosynthesis [26]. Bcl-2 proteins possess a BH-3 domain where GSH binds and since its presence seems to be correlated to the increase of the GSH pool in the nucleus, it is possible that Bcl-2 proteins are involved in GSH translocation into the nucleus through Bcl-2 associated athanogene pores (BAG) [39–41]. Diaz Vivancos et al. (2010) proposed a model for the glutathione cycle in the nucleus [27]. In this model, GSH is recruited and directed to the nucleus in the early G1 phase of cellular division; thus, GSH increases in the nucleus while cytosolic GSH is depleted. The altered cytosolic redox environment promotes the synthesis of new GSH, whereby the overall glutathione pool significantly increases; the nuclear envelope dissolves so that there is a rebalancing between cytosolic and nuclear GSH during G2 and M phase. During telophase, the nuclear membrane reassembles, the cell divides and the total GSH pool is allocated equally among the daughter cells (Figure 2) [27].

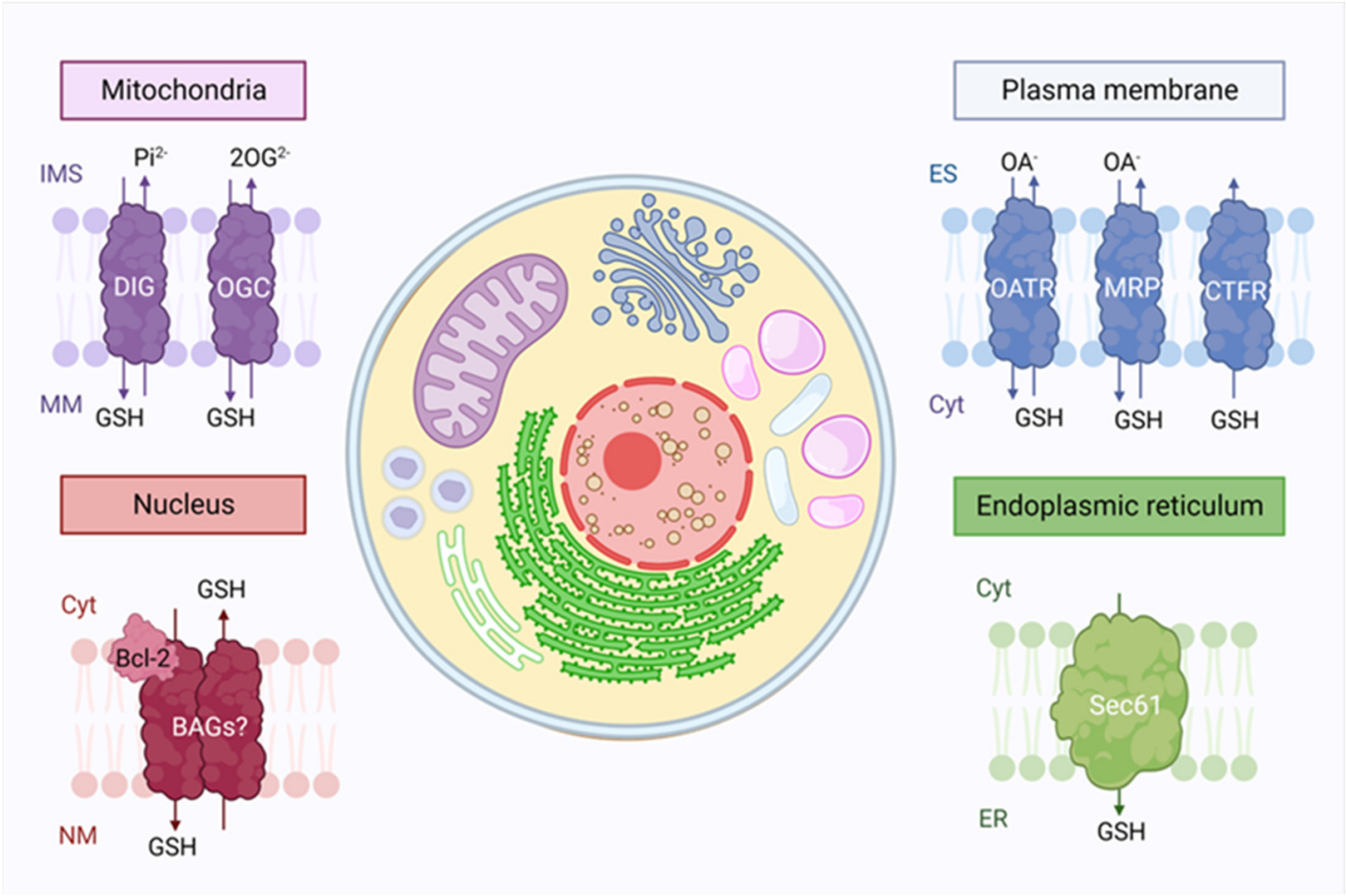

Figure 2. Glutathione intracellular compartmentalization. Glutathione synthesis takes place only in the cytosol (cyt), but it is distributed to many organelles due to the presence of transporters. In mitochondria, the outer membrane contains a large amount of porins, which allow glutathione transport, while dicarboxylate (DIG) and the oxoglutarate (OGC) transporters are present in the inner membrane. In the nucleus, Bcl-2 proteins are believed to be involved in the GSH translocation through Bcl2-associated athanogene pores (BAG). Glutathione is also found in the endoplasmic reticulum (ER), where its facilitated diffusion occurs through the Sec61 protein-conducting channel. Finally, the exchange between extracellular and intracellular glutathione in the plasma membrane occurs through the functioning of three families of transporters: the organic-anion-transporting polypeptide (OATR), the drug resistance-associated proteins (MRP) and cystic fibrosis transmembrane conductance regulator (CTRF). IMS: Intermembrane space, MM: Mitochondrial matrix, NM: Nuclear matrix, ES: Extracellular space.

The redox state of GSH/GSSG in plasma is controlled by multiple processes, including the synthesis of GSH from its constitutive amino acids, cyclic oxidation and reduction involving GSH peroxidase and GSSG reductase, protein S-glutathionylation, transport of GSH into plasma, and degradation of GSH and GSSG by γ-glutamyltranspeptidase [31,42].

GSH is present in all mammalian cells in a constant state of metabolic recirculation (synthesis, degradation, and irreversible loss of GSH). Its half-life is 4 days in human erythrocytes, 2 to 4 h in the cytosol of rat hepatic cells and 30 h in the mitochondrial lumen [43]. Many different conditions affect the intracellular GSH contents, some of them being the presence of heavy metals, high glucose concentrations, heat shock, exposure to reactive oxygen and nitrogen species including H_2O_2 and nitric oxide, ozone exposure, ionizing radiation, cigarette smoke [25,44–46]. Differences between GSH content in some mammalian cells are listed in Table 1.

Table 1. Glutathione distribution and homeostasis in different cell types.

Cell Type	GSH Cytosolic Concentration	GSH Homeostasis	References
Astrocytes	8–10 mM	Generate GSH conjugates exported from the cells by MRPs. Protect brain cells from ROS and xenobiotics	[47,48]
Neurons	0.2–2 mM	Lack of cystine transportation system, synthesis depends on cystine uptake via the cystine/glutamate exchange transporter	[49,50]
Hepatocytes	5–10 mM	Synthesis of GSH protects against oxidative stress, about 10% of total cytosolic GSH is transported to mitochondria	[51–54]
Erythrocytes	2.3–3 mM	Its levels are influenced by the environment. In addition, erythrocytes have the enzymatic machinery for the synthesis of GSH and the release of its derivates	[55–57]
Pneumocyte	400 μM in epithelial lining fluid	GSH protects lungs against oxidative damage. Type II pneumocytes contain more γ-glutamyl transferase than type I	[58–60]
Cardiomyocyte	2 mM	The insulin-signaling cascade regulates GSH concentration in ventricular myocytes by PI 3-kinase and MAP kinase pathways for controlling redox state	[61,62]

3. S-Glutathionylation

Redox regulation of cell function often involves the conversion of reactive thiols on specific cysteine residues from reduced to oxidized forms [63]. The main types of thiol modification that have been shown to play an important redox-dependent role include protein S-glutathionylation which is produced in the cell under physiological conditions and oxidative stress, both spontaneous and enzymatic [64,65]. Under S-glutathionylation, GSH may bind to cysteinyl residues in proteins by creating reversible disulfide bonds, depending on the cysteine position and redox potential [66,67].

This post-translational modification of the protein is primarily catalyzed by glutaredoxin (Grx), which leads to enhanced or suppressed activity; it can prevent protein degradation by proteolysis or sulfhydryl overoxidation, plays a key role in cellular signaling and participates in some pathological processes, including atherosclerosis, neurodegenerative disorders, cardiovascular diseases, and several types of cancer [65,68–71]. Reports suggest that protein S-glutathionylation and Grx1 carry out a wide range of antioxidant, anti-inflammatory, and anti-apoptotic functions in the body, participating in acute and chronic inflammatory responses [65,72,73].

The glutaredoxin catalytic mechanism depends on the GSH/GSSG ratio. Under an increase in GSH/GSSG, Grx can catalyze the deglutathionylation of proteins, but under conditions of decreased GSH/GSSG ratio, Grx can catalyze S-glutathionylation of proteins. It should also be noted that not only glutaredoxin can participate in these processes [64,65].

Protein S-glutathionylation regulates the structure and function of target proteins, including actin, Ras, integrins, transcription factors (NF-κB and AKT) [74], and metabolic enzymes (GAPDH, succinate dehydrogenase, and pyruvate kinase); therefore, it is required for cellular homeostasis [71,75].

4. Glutathione Transporters and Associated Diseases

There are studies that relate mitochondrial redox state and glutathione content with diseases and oxidative-induced cell death. Since GSH in mitochondria comes from the cytoplasmic reserve, the role of transporters becomes important [76]. The participation of three families of transporters in mammalian cells involved in the transportation and

movement of glutathione has been demonstrated: a family of drug resistance-associated proteins (MRP), cystic fibrosis transmembrane conductance regulator family (CFTR) and organic-anion-transporting polypeptide family (OATP) (Figure 3) [77,78].

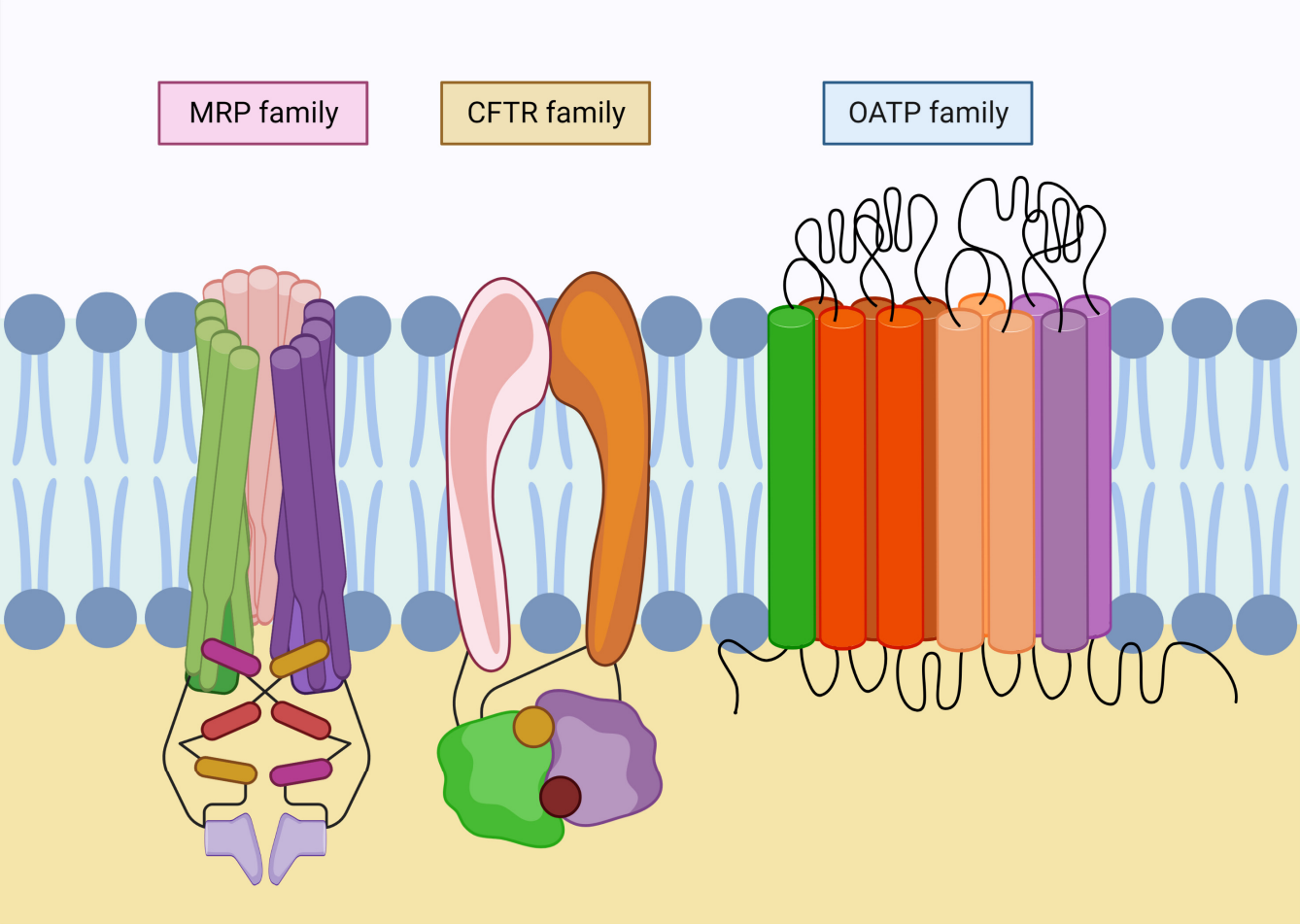

Figure 3. Families of glutathione transport in the plasma membrane. Glutathione transporters belong to the ABC superfamily, which requires ATP to carry out their functions. Multidrug resistance-associated proteins (MRP) are involved in GSH export and homeostasis. In addition, they conduct its derivates efflux. Moreover, the cystic fibrosis transmembrane conductance regulator family (CFTR) is involved in the export of GSH in the kidney and lungs. Finally, organic-anion-transporting polypeptide family (OATP) is widely expressed throughout the organs, acting bidirectionally and regulating the uptake of metabolites in the exchange for reduced glutathione.

4.1. MRP Family

Multidrug resistance-associated proteins (MRP/ABC) are involved in GSH export and homeostasis. MRP proteins not only regulate GSH efflux, but also transport oxidized glutathione derivatives such as glutathione disulfide (GSSG), S-nitrosoglutathione (GS-NO) and glutathione-metal complexes, as well as other glutathione S-conjugates [77,79]. MRP proteins belong to the C family of the ABC transporter superfamily, which requires ATP for transportation [80]. These transporters are responsible for the movement of a wide variety of xenobiotics, including drugs, lipids and metabolic products across plasma and intracellular membranes [81]. MRPs are located in the plasma membrane of mammalian cells, while in yeast and plants they are widely located in the vacuole [76]. Moreover, the MRP family of proteins is made up of 9 transporters (MRP1-MRP9), almost all of which accept glutathione S conjugates as substrates. One of the first studies that indicated that glutathione was transported by MRP proteins was with the use of a lung carcinoma cell

line that overexpresses the MRP1 protein; as a result it was found that it had lower levels of intracellular GSH and higher levels of extracellular GSH [82].

4.1.1. MRP1

The first human MRP identified as a GS-X and/or GSSG transporter was the ATP-binding cassette (ABC) protein ABCC1, first known as GSH conjugate pump and later identified as multidrug resistance protein MRP1 [83,84]. MRP1 is one of the most described transporters; knowledge of its molecular mechanisms and physiological functions related to GSH transport and GSH conjugates is the most advanced of all MRP-related proteins [79].

MRP1 carries a wide variety of anticancer drugs, including but not limited to vincristine, etoposide, anthracycline, and methotrexate (MTX). MRP1 has also been shown to transport other drugs used in the treatment of non-malignant diseases, such as opioids, antidepressants, statins, and antibiotics [85]. In addition to its role in the cellular extrusion of xenobiotics, MRP1 exports other physiologically important molecules. These include proinflammatory molecules (e.g., leukotriene C4), hormones (e.g., estrogens and prostaglandins), and antioxidants (e.g., oxidized and reduced glutathione) [86].

MRP1 is involved in inflammation, detoxification, and oxidative stress. A high level of MRP1 expression was associated with poor clinical outcomes in children with neuroblastoma [87]. Overexpression of MRP1, which represents the strength of cancer cells, can be targeted by substances such as verapamil, which specifically target this transporter and trigger lethal oxidative stress in the cancer cell. MRP1, when overexpressed, has been shown to regulate basal and apoptotic GSH release, suggesting that it plays a key role in these processes [87,88]. Recently, it was also found to act as a player in ferroptosis by regulating the abundance of intracellular glutathione. MRP1 is identified as a GSSG transporter. Inhibition of MRP activity has been shown to promote the accumulation of GSSG, which is cytotoxic to endothelial cell tumors. MRP inhibition could reduce drug resistance in cancer cells, and MRP acts as a potential target in cancer therapy [89].

4.1.2. MRP2

Meanwhile, the MRP2 transporter can also transport organic anions, including sulfate, glucuronide, and GSH conjugates. In addition, MRP2 is also responsible for the biliary elimination of certain endogenous conjugates, such as leukotriene-C4 (LTC4) and conjugated bilirubin [90].

Mutations in the MRP2 gene are associated with Dubin–Johnson syndrome, a condition due to the lack of hepatobiliary transport of organic anions without bile salts resulting in conjugated hyperbilirubinemia [81,82]. Studies have demonstrated that this transporter is one of the ABC pumps with the highest-level of expression in organs important for endo- and xenobiotic metabolism, such as the liver, kidneys, and intestine [81]. The MRP2 transporter is known to be present in some malignant human tumors, as demonstrated by immunostaining of hepatocellular, clear cell renal, colorectal, ovarian, leukemia, mesothelioma, lung, breast, bladder, and gastric cancer samples [83].

4.1.3. MRP3

MRP3 is a relatively poor transporter of GSH-conjugated organic anions compared to MRP1 and MRP2 [91]. In humans, MRP3 is primarily expressed in the adrenal glands, kidney, small intestine, colon, pancreas, and gallbladder, with a lower magnitude of expression in the lungs and spleen. MRP3 appears to play a compensatory role in the loss of MRP2 in the liver. Elevated levels of MRP3 expression have been detected in human hepatocellular carcinomas, primary ovarian cancer, and adult acute lymphoblastic leukemia. Furthermore, MRP3 overexpression was predicted to be a prognostic factor in childhood and adult acute lymphoblastic leukemia and adult acute myeloid leukemia [90].

4.1.4. MRP4

MRP4 is a widely versatile transporter that exhibits high substrate specificity composed of a wide variety of amphipathic anions, including steroid and eicosanoid conjugates, as well as cyclic nucleotides and nucleotide analogs. MRP4 has been shown to play a role in cyclic adenosine monophosphate (cAMP) homeostasis in vascular smooth cells and cardiac myocytes [92]. Other tasks have been proposed for MRP4 in platelets, considering that its location can shift from the granules to the plasma membrane when platelets are activated and under certain pathophysiological conditions [93]. These include the release of lipid mediators, as well as a role in aspirin resistance under certain conditions, such as in patients after coronary artery bypass graft surgery [94].

In addition to its localization in the plasma membrane, MRP4 was shown to be found in large amounts in the membrane of dense granules. An altered distribution of MRP4 was observed in platelets from a patient with Hermansky–Pudlak syndrome, in which MRP4 was only detected in the plasma membrane due to the lack of dense granules [95].

4.1.5. MRP5

MRP5 was identified as transporting cAMP, cyclic guanosine monophosphate (cGMP), and antiretroviral compound PMEA (9-(2-phosphonomethoxyethyl)adenine). It has also been shown to transport nucleotide/nucleoside analogues and GSH conjugates [96]. MRP5 expression was widely detected in human tissues such as liver, placenta, and cornea, and in carcinomas. MRP5 expression level was also associated with cisplatin exposure. Several in vitro studies suggested that MRP5 would transport several anticancer drugs, including MTX, purine and pyrimidine analogs [97].

4.1.6. MRP6

MRP6 is an organic anion transporter that is mainly distributed on the basolateral side of hepatocytes and on the proximal tubules of the kidney [98]. Although MRP6 is not involved in drug resistance, it may be a constitutive transporter in normal and abnormal hepatocytes [90]. MRP6 can also mediate the transport of glutathione conjugates, LTC4 and N-ethylmaleimide S-glutathione (NEM-GS) [99].

Mutations found in the MRP6 gene are associated with genetic abnormalities of the autosomal inherited connective tissue disorder called pseudoxanthoma elasticum (PXE), which is characterized by the presence of dystrophic elastic fibers in the skin, retina, and large blood vessels, causing the appearance of bags in the skin, loss of vision and calcification of blood vessels [100].

4.1.7. MRP7

MRP7 is a lipophilic anion transporter found primarily in the heart, liver, skeletal muscle, and kidney. MRP7 has a similar substrate range to MRP1-MRP4 and is involved in phase III (cell extrusion) of detoxification [101], but MRP7 does not engage in direct GSH transportation [102].

4.1.8. MRP8

MRP8 is an amphipathic anion transporter that is functional for the efflux of purine and pyrimidine nucleotide analogs including cAMP and cGMP, and may also transport GSH conjugates [103]. MRP8 is widely expressed in the human body, with the highest levels in the liver, brain, placenta, breasts, and testis [99]. Although there is a report showing a decrease in MRP8 level in breast cancer, a high level of MRP8 was reported in breast cancer and gastric cancer cell lines [96].

4.1.9. MRP9

MRP9 does not transport typical substrates such as drug conjugates and other substances as do other MRP members [104]. It is highly expressed in breast cancer, normal breast, and testis; however, its functions are still unknown [105]. There is a study showing

that in the joint absence of MRP5 and MRP9, some metabolites such as heme and some others are poorly transported or distributed, causing mitochondrial damage [106].

The available information for this family of transporters is summarized in Table 2.

Table 2. Family of MRP transporters and the molecules they transport.

Transporter	Endogenous Substrates	References
MRP1	GSH conjugates, cysteinyl leukotrienes, glucuronic acid conjugates, bilirubin, estradiol, sulfate conjugates, bile salts, sulfated steroids, GSH, GSSG	[82,107,108]
MRP2	GSH conjugates, cysteinyl leukotrienes, glucuronic acid conjugates, bilirubin, estradiol, sulfate conjugates, bile salts, sulfated steroids, GSH, GSSG	[82,107,109,110]
MRP3	GSH conjugates, cysteinyl leukotrienes, glucuronic acid conjugates, bilirubin, estradiol, sulfate conjugates, bile salts	[82,107,111]
MRP4	GSH conjugates, cysteinyl leukotrienes, glucuronic acid conjugates, estradiol, sulfate conjugates, sulfate conjugates, cyclic nucleotides, bile salts	[82,111–113]
MRP5	GSH conjugates, glucuronic acid conjugates, cyclic nucleotides, GSH	[82,111,114]
MRP6	GSH conjugates, cysteinyl leukotrienes	[82,115]
MRP7	GSH conjugates, cysteinyl leukotrienes, glucuronic acid conjugates, estradiol	[82,116]
MRP8	GSH conjugates, cysteinyl leukotrienes, glucuronic acid conjugates, estradiol, sulfate conjugates, cyclic nucleotides, GSH	[82,116]
MRP9	Unknown, but not drug conjugates or other organic anions	[117]

4.2. Family CFTR

CFTR proteins belong to the C family of the ABC transporter superfamily [80]. CFTR is best known as a chloride channel, but it has also been shown to facilitate GSH export in kidney cell lines and lung tissue [118].

The absence of functional CFTR disrupts epithelial water and ion homeostasis, leading to the accumulation of dehydrated mucus, recurrent bacterial infections, and ultimately organ failure and other life-threatening consequences [78]. Cystic fibrosis primarily affects the lungs, but also affects the pancreas, intestine, liver, kidneys, and sweat glands [119]. For example, a study in 16-HBE bronchial epithelial cells showed that CFTR gene expression is increased after 48 h of exposure to cigarette smoke, demonstrating that CFTR expression can be induced. It is possible that CFTR expression decreases as an initial response, but as exposure time increases, and as an adaptive antioxidant response, CFTR expression is induced [120]. However, in a previous study carried out in Calu-3 cells, it was shown that exposure to cigarette smoke causes a decrease in the synthesis of CFTR mRNA, which was reflected in the expression of the protein [121].

Another study demonstrated that CFTR deficiency occurs in the nasal respiratory epithelium of smokers [120]. One of the most likely causes of the decreased function would be the increase in heavy metals found in cigarette smoke, particularly cadmium, which has been shown to inactivate CFTR [122].

One report from 2005 indicates that human CFTR channels are reversibly inhibited by several reactive forms of glutathione (i.e., glutathione disulfide S-oxide [GS(O)SG], nitrosylated glutathione [GSNO] and GSSG), and glutathione treated with diamide, a strong thiol oxidizer. The underlying mechanism appears to involve the glutathionylation of cys-1344 near the signature sequence in the cytoplasmic nucleotide binding domains (NBDs); this region is predicted to participate in ATP-dependent channel opening [123,124]. Channels could be protected from inhibition by pretreating with *N*-ethylmaleimide (NEM)

a thiol alkylating agent, or by reducing agents such as dithiothreitol (DTT) or by the actions of GSH and glutaredoxin [124]. This finding is important because the CFTR channel is expressed in the lung and gut; these tissues are continuously exposed to thiol oxidants under a variety of inflammatory conditions, allowing the reactive glutathione species that are formed to have the potential for glutathionylation of these channels [125].

4.3. OATP Family

The family of organic-anion-transporting polypeptides (OATPs) consists of eleven human OATPs, which are classified into six different OATP1–6 subfamilies. OATP1 is mainly found in human hepatocytes [126]. However, its expression has been demonstrated in different tissues such as the blood-brain barrier (BBB), choroid plexus, lung, heart, intestine, kidney, placenta, and testis [127]. The three OATPs that are most abundantly expressed in the liver are OATP1B1, OATP1B3 and OATP2B1. These transporters act bidirectionally and regulate the uptake of amphipathic and anionic substances in exchange for reduced glutathione or bicarbonate [128].

The OATP family of transporters functions independently of ATP and sodium gradients, and instead, relies on the large GSH electrochemical gradient across the plasma membrane. Two members of the OATP family, OATP1 and OATP2, have been shown to mediate GSH export by exchanging GSH for solute uptake.

OATP1A1 has been found in cell types other than the proximal tubules, such as hepatocytes, which use the GSH electrochemical gradient to drive organic anion uptake [129].

There have been no studies showing that lack of OATP function causes disease. However, in several models of cholestatic liver diseases, such as endotoxin treatment, ethinylestradiol treatment and bile duct ligation, the expression of hepatocellular OATPs was down-regulated [130].

It is well known that malignant cell transformation alters the pattern of OATP expression in organs. In fact, the gonad specific OATP6A1 has been identified as a carcinogenic antigen in lung tumors and lung tumor cell lines [131]. Human Rotor syndrome is an inherited disorder associated with OATPs. It is an autosomal recessive disorder characterized by conjugated hyperbilirubinemia, coproporphyrinuria and almost no hepatic uptake of anionic diagnostic agents due to genetic variants in OATP1B1 and OATP1B3 [132].

5. Glutathione Deficiency Causes

The alteration of glutathione transport activity is related to the deficiency or low activity of the transporters mentioned in the previous section, which is reflected in an increase in intracellular glutathione concentrations. This condition is associated with some pathologies [87,88,119].

In humans, a decrease in GSH has been associated with different conditions, such as deficiency of the enzymes involved in glutathione synthesis [133]; in this case, individuals show a limited or generalized deficiency of GSH and an accumulation of 5-oxoproline (in blood and cerebrospinal fluid) leading to metabolic acidosis [43], mental retardation, neuropsychiatric dysfunction, spinocerebellar degeneration, peripheral neuropathy, myopathy, hepato-splenomegaly, hemolytic anemia, aminoaciduria, and severe neurological complications [134]. These individuals may also develop hypersensitivity to antibiotics and are more prone to influenza virus infections [43].

For the aforementioned reasons, the maintenance of high concentrations of GSH is vital for most types of cell, since it plays important roles in the control of biological process, including metabolic detoxification, protein folding, vitamin regeneration, mitochondrial health, immune defense against viruses, cellular proliferation regulation, apoptosis, and redox balance, among others. Control of GSH levels is a proposed strategy for health improvement and disease prevention [135,136].

Altered levels of GSH could be the result of defects in the enzymes involved in its metabolism [133] and its excretion through the plasma membrane [137]. An example is deficiency of CFRT, a protein involved in GSH transmembrane transportation, resulting in

a decrease in the GSH efflux, reducing its extracellular availability [138] and inducing an oxidative state, which ends in the apoptosis process [31,138,139].

Another cause is the deficiency of enzymes related to the reduction of GSSG to GSH. For example, in erythrocytes, a deficiency of glucose-6-phosphate dehydrogenase (G6PDH) generates a decrease in the concentration of NADPH, necessary for the regeneration of GSH. This contributes to a decrease in the intracellular content of GSH and therefore, less is exported to the outside of the cell [134]. Furthermore, stress-promoting exogenous agents, for example, smoking [140], acetaminophen consumption [141] bacterial and viral infections, alcoholism, excessive exercise, emotional stress, X-ray, or sun ultraviolet light exposure [133,141] could alter GSH levels due to the amount of ROS generated. Moreover, age also influences the loss of GSH, even in healthy individuals, yet antioxidant defenses decrease [133].

Many other pathologies result in a decrease of GSH levels (Figure 4). The reasons are diverse, but most coincide with defects in the synthesis or transportation of enzymes or a shortage of precursors. The organism uses different pathways to successfully increase the intracellular levels of glutathione and during exogenous regulation, the involved reactions are summarized in Figure 5 [142–147].

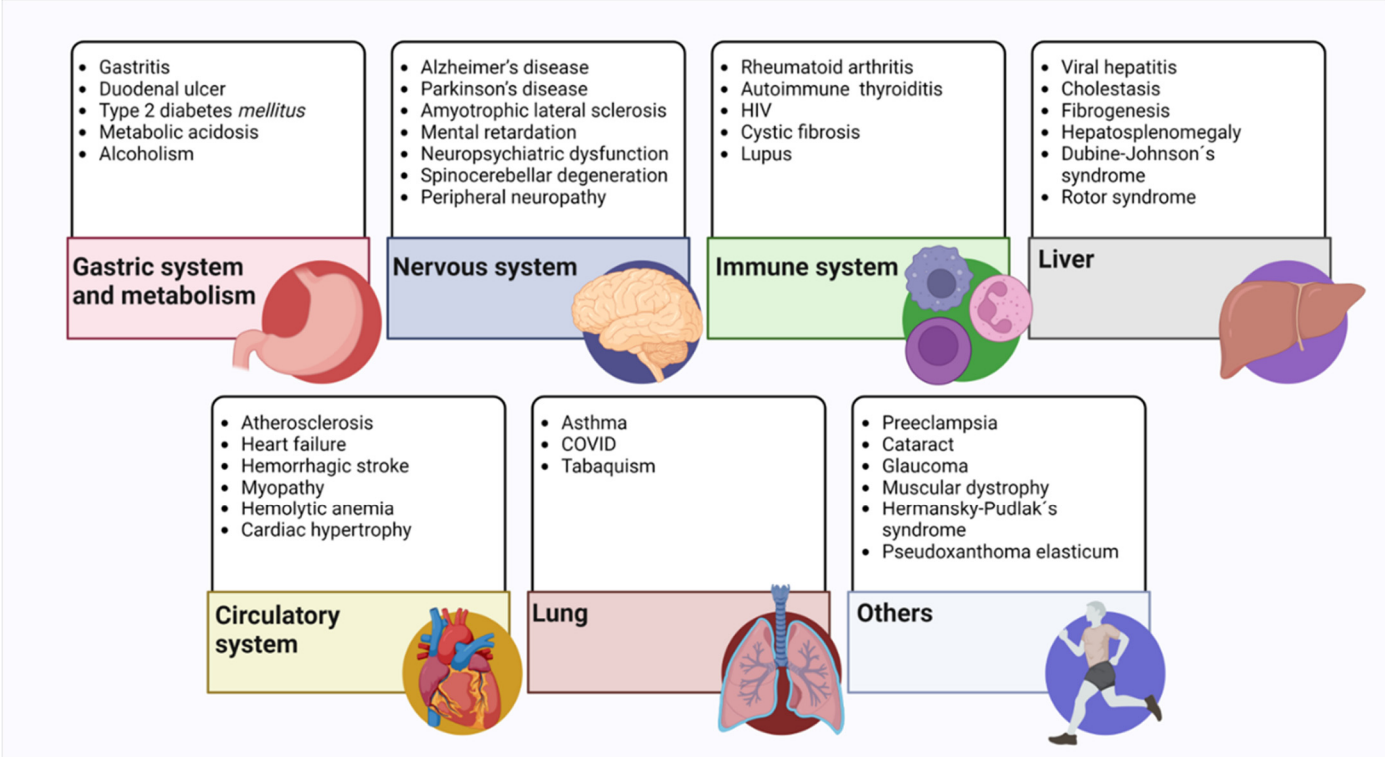

Figure 4. Pathologies related to the decrease in glutathione levels. These are mainly illnesses that cause a decrease in endogenous glutathione levels. All the pathologies mentioned in the text are classified according to the system or organ involved [81,82,87,95,96,98,119,130,132,134,148–152].

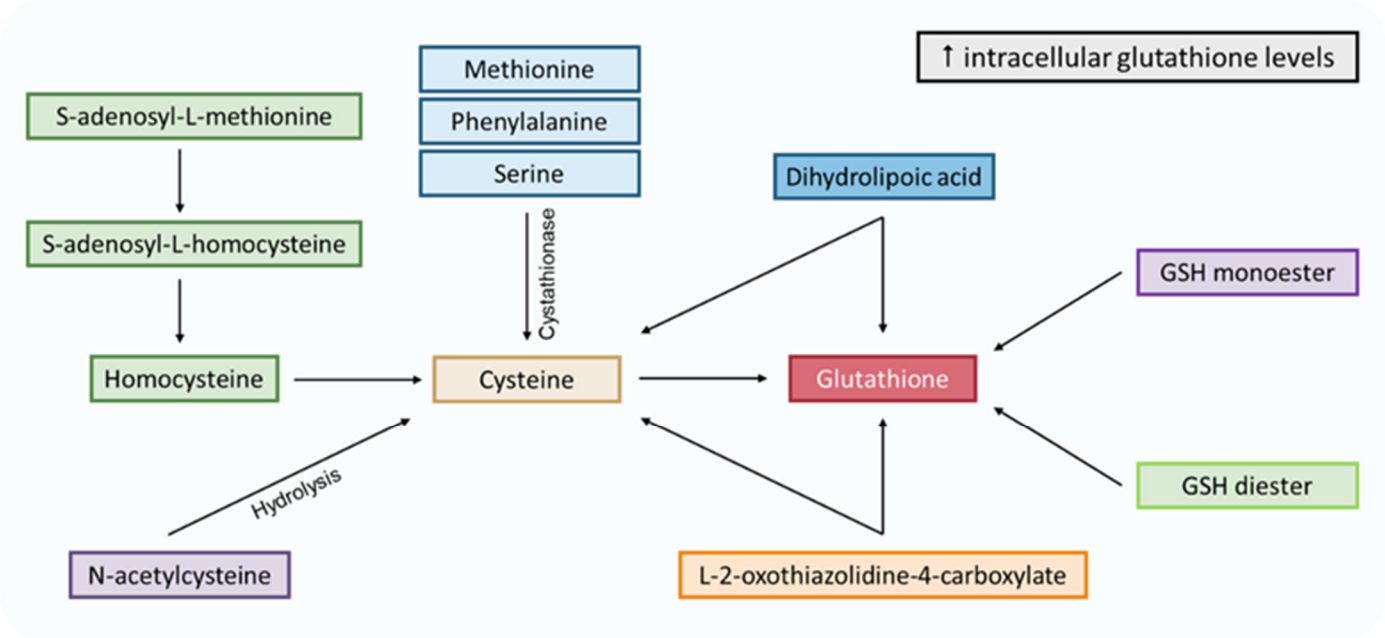

Figure 5. Pathways to increase intracellular levels of glutathione. Methionine, phenylalanine, serine and S-adenosyl-L-methionine are cysteine precursors through the transsulfuration pathway. N-acetylcysteine could be transformed into cysteine via hydrolysis. L-2-oxothiazolidine-4-carboxylate is an analog of 5-oxoproline (glutamate cycle form), which increases cysteine and glutathione intracellular levels. Dihydrolipoic acid comes from the α-lipoic acid reduction and can reduce glutathione and cysteine. The latter enters the γ-glutamyl cycle, where it stimulates glutathione synthesis. Finally, the GSH monoester and diester can be hydrolyzed to GSH.

6. Glutathione and Disease

Plasma glutathione GSH and GSSG levels vary, depending on the life span of a healthy individual (see Table 3). However, these concentrations can vary significantly when a disease generates and maintains oxidative stress for long periods, which would produce a decrease in the concentration of GSH and an increase in GSSG. Below, we describe some diseases that increase oxidative stress and thereby considerably affect the recovery time of patients.

Table 3. GSH, GSSG concentrations, GSH:GSSG ratio and EhGSH/GSSG at different stages of an individual's life.

Life Period	GSH	GSSG	GSH/GSSG	E_h GSH/GSSG	Refs.
Childhood	2.7 ± 0.17 mM	0.16 ± 0.02 mM	16.8	−200–220 mV	[153,154]
Maturity	2.8 ± 0.9 mM	0.14 ± 0.4 mM	20	−200–240 mV	[154,155]
Old age	2.2 ± 2.0 mM	0.15 ± 0.03 mM	14.7	−200–240 mV	[154,155]

6.1. Cardiovascular Disease (CVD)

An imbalance in redox homeostasis could cause cardiovascular complications. Development and progression of CVD have been characterized by changes in the concentration of GSH or its oxidation state [20]. There are some mechanisms involved in GSH diminution: increased oxidation by intracellular oxidizing agents, increased conjugation to molecules, and increased exit across the cell membrane [137].

Many animal studies have demonstrated the role of GSH in CVD. For example, heat shock proteins (HSPs) have shown protection against several stress stimuli in mammalian cells. Human heat shock protein 27 (Hsp27) and murine heat shock protein 25 (Hsp25) protect against H_2O_2 by increasing levels of reduced GSH in a G6PDH-dependent man-

ner [156]. Furthermore, degradation of nuclear factor erythroid 2–related factor 2 (Nrf2) has been found to contribute to the decreased expression of several antioxidant enzymes [157]. In addition, Nox4 facilitates cardiac-related adaptation to chronic stress by activating Nrf2, which increases concentrations of GSH and, consequently, increases the GSH/GSSG ratio [158]. In serum from atherosclerotic mice or mouse models with apolipoprotein E deficiency, GSH, transported in liposomes, reduced susceptibility to oxidation by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). GSH levels in peritoneal macrophages increased in these mice, but lipid peroxides and oxidized LDL levels decreased [159]. Another research study found that N-acetyl cysteine (NAC) might boost GSH levels and reduce liver and plasma cholesterol levels in mice fed a high-fat diet [160]. Moreover, in the process of atherogenesis, the ability of macrophages to synthesize glutathione is inversely related to the initiation and progression of atherosclerosis in apolipoprotein E deficient mice (Apo E-/-). Oxidative stress is an important factor in atherogenesis. Under oxidative stress, lipid peroxidation is observed in LDL and arterial wall cells, leading to the formation of atheromatous plaques. Macrophage GSH decreases the cellular oxidative state, the ability of macrophages to oxidize LDL, and the development of atherosclerotic lesions in Apo E-/- mice [161]. Again, glutathione peroxidase 1 (GPx-1) is a critical enzyme in the protection of vessels against atherogenesis. In the diabetic apolipoprotein E-deficient mouse model, decreased levels or lack of GPx-1 accelerate diabetes-associated atherosclerosis [162,163].

Furthermore, several human studies demonstrate that GSH has a positive effect on the cardiovascular system. According to several studies, patients with heart disease and diabetes have reduced plasma GSH levels. Additionally, patients with CVD have lower GSH levels than subjects without a CVD history [164]. Type 2 diabetes mellitus (T2DM) patients showed decreased levels of GSH, and of enzymes involved in GSH synthesis. In other studies, GSSG and transforming growth factor-beta (TGF-β) levels were higher in diabetic patients. In this case, an increased level of proinflammatory cytokines and a decreased expression of enzymes involved in GSH synthesis were observed [150]. The increased level of GSH in plasma leads to reduced values of systolic and diastolic pressure and a decreased incidence of diabetes [164]. In addition, the levels of GSH and GSSG were measured in mononuclear cells in hypertensive patients with or without different antihypertensive therapies. In hypertensive patients, the levels of GSH decreased while the levels of GSSG increased. Three months of antihypertensive treatment reduced oxidative stress and GSSG and increased the levels of GSH [165].

Plasma GSH, on the other hand, decreased by 21% and 40% in patients with asymptomatic and symptomatic CVD, respectively. These results indicate that decreases in the level of GSH are related to cardiac abnormalities in patients with CVD [166]. The blood test to measure the level of GSH should be used as a new biomarker to detect CVD in asymptomatic patients [166]. Additionally, increased oxidative stress could lead to myocardial infarction (MI) in cardiac procedures. The glutathione S-transferase (GST) polymorphism has been identified as a factor that could increase MI in cardiac surgery. Decreased GPx-1 activity increases risks of stroke and coronary heart disease. Thus, measuring erythrocyte GPx-1 levels might be used as a predictive value, and increasing the level of GPx-1 could have a beneficial effect on CVS. Furthermore, studies in patients with T2DM suggest that GPx-1 is an essential enzyme that plays a protective role in the development of endothelial dysfunction and atherosclerosis in diabetes [149]. The effect of antioxidants in patients with atherosclerosis has also been studied in humans. The results demonstrated that administrating NAC increased GSH levels and reduced endothelial adhesion molecule levels, potentially preventing vascular damage in diabetic patients. These results showed how glutathione has antioxidative and antiatherogenic properties and can lead to the remission of atherosclerosis [167].

Accordingly, treatment with GSH could reduce oxidative stress and prevent related diseases. However, the administration of GSH would not be the best solution because intestinal and hepatic gamma-glutamyl transferase (GGT) metabolizes GSH and decreases the level of administered GSH [168,169]. Therefore, the administration of pure GSH in the

form of an orobuccal fast-slow-release tablet on healthy volunteers has been evaluated. In this trial, it was observed that an increased level of GSH in the blood could result from GSH absorption through mouth mucosa [170]. Other researchers compared the level of GSH and other oxidative stress markers in the blood of subjects with metabolic syndrome after administration of different forms of GSH (oral and sublingual) and NAC [171].

6.2. Neurodegenerative Diseases

Neurodegenerative diseases, such as Parkinson's (PD), Alzheimer's (AD), amyotrophic lateral sclerosis (ALS), and Huntington's, share several common features in pathogenesis, such as (i) the accumulation of abnormally aggregated proteins (pathological inclusions), (ii) oxidative damage and (iii) mitochondrial dysfunction [172]. Each condition causes alterations in different pathways that enable oxidative damage to establish itself. Below is a brief description of these changes in some of the most common neurodegenerative diseases.

6.2.1. Alzheimer's Disease (AD)

AD is pathologically characterized by amyloid β (Aβ) deposition and neurofibrillary tangles in the brain, and loss of synaptic connections in specific areas of the brain [173]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a crucial redox-regulated gene in controlling ROS levels; as intranuclear Nrf2 is decreased in AD, this causes the accumulation of ROS, senescent organelles, and misfolded proteins [174]. The regulation of the expression of excitatory amino acid carrier 1 (EAAC1) is promoted by Nrf2. EACC1 is the regulatory mechanism of neuronal GSH production; therefore, by decreasing the expression of Nrf2, the expression of the EAAC1 protein is suppressed, which leads to a decrease in cysteine uptake and consequently to decreased brain GSH levels, and vulnerability to oxidative stress [174,175].

6.2.2. Parkinson's Disease (PD)

Neuronal loss in the substantia nigra (SN) is a neuropathological characteristic of PD, which leads to striatal dopaminergic insufficiency and an increase in the synthesis of α-synuclein in neuronal inclusions [176]. The α-synuclein binds to ubiquitin and forms cytoplasmic inclusions of proteins called Lewy bodies; α-synuclein could induce generation of abundant ROS and inflammatory factors, causing lipid peroxidation and death of neurons [177]. This causes a decrease in the level of GSH, which constitutes the main antioxidant defense of dopaminergic neurons [178]. As with AD, the Nrf2 is the main protein involved in the development of ROS-caused PD [177].

6.2.3. Amyotrophic Lateral Sclerosis (ALS)

ALS is a progressive, fatal neuromuscular disorder characterized by the degeneration of upper and lower motor neurons leading to somatic muscle dysfunction in the body [179]. In 90% of hereditary cases of ALS, patients have mutations in the superoxide dismutase 1 (SOD1) enzyme which converts $O_2\bullet-$ to H_2O_2 and O_2 to protect cells from ROS, and is directly associated with oxidative stress and inflammation [180]. Recent clinical studies showed that GSH levels in the brains of ALS patients were decreased compared to those of age-matched healthy volunteers [181], and the decrease of GSH levels was more prominent in the motor cortex than in the white matter of ALS patients [182]. These results suggest that the brains of patients with ALS have limited antioxidant capacity [180].

6.2.4. Huntington's Disease (HD)

This disease is characterized by an increase in the number of repeats of the cytosine, adenine, and guanine (CAG) triplet in the Huntington gene, located on the short arm of chromosome 4, which codes for a protein rich in glutamine residues known as huntingtin (HTT); therefore, this is considered a hereditary disease with an autosomal dominant pattern [183]. Some of the indicators of oxidative damage that have been observed in the striatum and cerebral cortex of patients with HD are an increase in the concentration of

malondialdehyde (MDA) and 4-hydroxynonenal (lipid oxidation products), increase in carbonylation and in protein nitration, as well as a decrease in GSH and an increase in the activity of glutathione peroxidase, catalase, and superoxide dismutase [184].

6.3. Diabetes Mellitus Type 2

Oxidative stress contributes to the pathogenesis of diabetes mellitus type 2 (DM2) by increasing insulin resistance or affecting insulin secretion [185]. Hyperglycemia increases free radical production and impairs the antioxidant defense system [186]. In patients with DM2, there is a decrease in antioxidant defenses, together with reduced levels of specific antioxidants such as ascorbic acid and vitamin E, and decreased activity of antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase [187]. In addition, antioxidant vitamins such as ascorbic acid and tocopherols have been reported to improve insulin sensitivity [188]. Patients with DM2 have a reduced level of GSH, high levels of GSSG and TGF-β, increased levels of proinflammatory cytokines, and decreased expression of enzymes involved in GSH synthesis. Oral GSH supplementation improves insulin sensitivity, reduces oxidative stress levels, and prevents GSH depletion in individuals with DM2. GSH supplementation also increases the levels of Th1-associated cytokines, IFN-γ, TNF-α, and IL-2, and decreases the levels of proinflammatory cytokines such as IL-6 and IL-10 in these individuals [189]. Reduced GSH concentration levels in DM2 patients' red blood cells, plasma, and monocytes are accompanied by decreased expressions of glutamate cysteine ligase (GCL), GSH synthetase (GS), and gamma-glutamyl transpeptidase (GGT), and a decreased substrate, since cysteine and glycine supplementation partially restore the GSH concentration in these patients [190]. Sodium tungstate is an alternative to reduce hyperglycemia in the treatment of diabetes. The reduction of hyperglycemia by sodium tungstate reduces lipid peroxidation and causes alterations in the antioxidant system in the salivary glands of diabetic rats induced by streptozotocin (STZ) increasing the GSH/GSSG ratio [191].

On the other hand, both in animal models and in patients, DM2 is frequently accompanied by islet fibrosis. Several in vivo and in vitro studies have shown that antioxidants can successfully inhibit pancreatic fibrosis. GSH can inhibit the activation and proliferation of pancreatic stellar cells (PSCs), thereby inhibiting pancreatic fibrosis and protecting islet β cells from damage [192].

6.4. Cancer

ROS are important in the processes of growth, proliferation, metastasis, and survival of tumor cells. These cells have higher levels of ROS and greater expression and activity of antioxidant systems than non-cancerous cells. Upregulation of NRF2 (nuclear factor, erythroid-derived 2-like factor 2) and elevated GSH levels have been observed in various tumors, including breast, ovarian, prostate, skin, lung, and pancreatic tumors [193]. NRF2 regulates the expression of several enzymes responsible for glutathione synthesis [194]. High ROS production in cancer cells requires high activity of cellular antioxidant systems, making cancer cells hypersensitive to agents that impair their antioxidant capacity. Therefore, the reduction in the production or availability of GSH may be important for tumor therapy [195]. Additionally, GSH acts as a detoxifying agent, so in cancer cells, this process can be used for the removal of chemotherapeutic drugs. Thus, GSH plays an important role in chemotherapy resistance, and its inhibition as part of combination therapies has been shown to be an important approach to improve the efficacy of chemotherapies [196]. On the other hand, because of the high concentration of GSH in many tumors and its high reactivity, GSH is used as an activator of prodrugs, such as romidepsin, which is used to treat cutaneous T-cell lymphoma and other peripheral T-cell lymphomas [197]. In addition, the development of ROS- and GSH-sensitive nanoparticle drug delivery systems has been proposed to deliver a highly toxic load more specifically and safely to cancer cells. The GSH-induced disintegration of nanoparticles was demonstrated as an example of this system, allowing the release of active platinum metabolites, which covalently bound to

the target DNA and induced apoptosis in cancer cells; however, this technology requires further investigation for optimization before clinical use [198]. Moreover, cancer cells that are resistant to radiation and chemotherapy have elevated GSH levels, probably due to GSH's ability to quench the ROS generated by these therapies [199].

Beyond its role in cancer cells, GSH synthesis has implications in the tumor surrounding microenvironment (TME) for non-malignant cells. For example, obesity is a risk factor for numerous malignancies and may promote tumorigenesis. Lowering GSH levels, either genetically or pharmacologically, prevents obesity induced by a high-fat diet. Therefore, GSH could promote lipid accumulation or storage and support a TME that favors tumor growth [200,201]. Another group of cells that have been implicated in GSH metabolism are immune cells. T cells show similar dependence on GSH as cancer cells due to ROS increase during periods of proliferation. Deletion of proteins important for GSH synthesis in T cells leads to an altered immune response, suggesting that GSH is required for antitumor immunity [202].

6.5. COVID-19

It has been reported that patients infected with COVID-19 disease have higher oxidative/nitrosative stress and a substantial decrease in vitamin D, as well as alterations in thiol levels, total antioxidant capacity, GSH and selenium. This appears to be a common pathway related to the high mortality from COVID-19 [152,203]. It was also found that in Covid patients, there was a decrease not only in GSH levels but also in vitamins such as A, C and E, as well as enzymes that combat oxidative stress such as glutathione peroxidase, superoxidodismutase and catalase [204].

GSH deficiencies have been found in people hospitalized with COVID-19, particularly in younger humans. This is a significant finding because younger humans are not expected to be GSH deficient [205]. Furthermore, it has been reported that even children with COVID-19 showed this GSH deficiency when compared to control values [206]. Finally, it is known that this deficiency depended on age and was more pronounced in older people. In addition, in patients with COVID-19, increased lipid peroxidation and damage due to oxidative stress were observed. Compared to control samples, significantly reduced levels of GSH were observed in postmortem cortical samples from COVID-19 patients. SARS-CoV-2 also induced oxidative stress-mediated changes in the testes and epididymis, as seen from COVID-19 postmortem autopsies compared with controls. GSH levels decreased with increasing severity of COVID-19 [207].

7. Conclusions

Glutathione plays an important role in antioxidant defense and in the regulation of the pathways necessary for cellular homeostasis, not only as a detoxifier of endogenous and exogenous compounds, but also through its participation in processes related to the modulation of the synthesis of DNA, gene expression, cell proliferation, apoptosis, S-glutathionylation of proteins, signal transduction, regulation of the immune system and metabolism of cellular compounds, among others.

Furthermore, glutathione is important for the proper functioning of the metabolism, considering its cellular distribution and transport. Glutathione transporters are particularly essential because they minimize fluctuations in its concentration, as well as regulating the redox state of glutathione in different cellular compartments, while their synthesis, degradation, and recycling functions act in a coordinated manner.

Finally, glutathione deficiency is known to contribute to oxidative stress, and has an important role in aging, as well as in the pathogenesis of different diseases, such as neurodegenerative diseases, liver and kidney disorders, cystic fibrosis, diabetes, and cardiovascular illness. In any case, it is desirable to maintain an optimal state (concentrations and redox state) of this cellular tripeptide. As described in this review article, different glutathione forms are present in the cell, all of which have specific and important functions. All this makes the study of this small tripeptide even more interesting. Although a wealth

of information exists about glutathione, more remains to be discovered about its role in cellular regulation. Therefore, the study of glutathione is an important and extensive field of research that demands further examination to develop new prevention strategies and even therapies for many age-related diseases.

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antioxidants

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Review

Neuroprotection against Aminochrome Neurotoxicity: Glutathione Transferase M2-2 and DT-Diaphorase

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Abstract: Glutathione is an important antioxidant that plays a crucial role in the cellular protection against oxidative stress and detoxification of electrophilic mutagens, and carcinogens. Glutathione transferases are enzymes catalyzing glutathione-dependent reactions that lead to inactivation and conjugation of toxic compounds, processes followed by subsequent excretion of the detoxified products. Degeneration and loss of neuromelanin-containing dopaminergic neurons in the nigrostriatal neurons generally involves oxidative stress, neuroinflammation, alpha-synuclein aggregation to neurotoxic oligomers, mitochondrial dysfunction, protein degradation dysfunction, and endoplasmic reticulum stress. However, it is still unclear what triggers these neurodegenerative processes. It has been reported that aminochrome may elicit all of these mechanisms and, interestingly, aminochrome is formed inside neuromelanin-containing dopaminergic neurons during neuromelanin synthesis. Aminochrome is a neurotoxic ortho-quinone formed in neuromelanin synthesis. However, it seems paradoxical that the neurotoxin aminochrome is generated during neuromelanin synthesis, even though healthy seniors have these neurons intact when they die. The explanation of this paradox is the existence of protective tools against aminochrome neurotoxicity composed of the enzymes DT-diaphorase, expressed in these neurons, and glutathione transferase M2-2, expressed in astrocytes. Recently, it has been reported that dopaminergic neurons can be protected by glutathione transferase M2-2 from astrocytes, which secrete exosomes containing the protective enzyme.

Keywords: glutathione; glutathione transferase; dopamine; Parkinson's disease; neuron; astrocytes; neuroprotection; aminochrome

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1. Glutathione as Antioxidant

Glutathione is an abundant tripeptide in essentially all aerobic organisms, and a pivotal component in the cellular protection against oxidative stress, electrophilic mutagens, and carcinogens [1]. The thiol group of the γ-L-glutamyl-L-cysteinyl-glycine (glutathione, GSH) can serve as a reductant, scavenge unpaired electrons, and conjugate various toxins and thereby enable their elimination. The biochemistry of glutathione has been extensively covered in a two-volume treatise [2] including a general review of its mechanism of action [3], and a recent review details recent advances with particular emphasis on redox homeostasis in the brain [4]. Glutathione is the most abundant low-molecular-mass thiols in the cell and is present in millimolar tissue concentrations, which reach 10 mM in liver [5]. Glutathione occurs predominantly in the reduced form (normally > 98%) owing to the activity of the ubiquitous NADPH-dependent glutathione reductase [6]. The enzyme shows specificity for glutathione disulfide (GSSG), even though modest activity is obtained

with the mixed glutathione-coenzyme A disulfide [7]. In general, disulfides (RSSR) and thiosulfate esters, such as S-sulfoglutathione, are reduced via thiol-disulfide interchange with glutathione (GSH), catalyzed by thioltransferase (also called glutaredoxin 1) [8].

$$RSSR + 2\ GSH \rightarrow 2\ RSH + GSSG$$

Thus, by coupling to glutathione reductase, efficient reduction of cystine and other disulfides formed by oxidative processes is afforded by glutathione and thioltransferase [9]. Similarly, disulfides in proteins formed under oxidative conditions can be reduced by the same device, as well as by thioredoxin coupled to thioredoxin reductase [10].

Primary products of oxygen metabolism, which include reactive oxygen species (ROS) such as singlet oxygen, superoxide, hydroxyl radical, and hydrogen peroxide are also detoxified by glutathione. Furthermore, a diverse range of toxic secondary products derived from reactions of ROS with cell constituents are inactivated by reactions catalyzed by glutathione transferases and selenium-dependent glutathione peroxidases [11,12]. The harmful products include alkenals, epoxides, quinones, and organic peroxides, and it has been suggested that their toxicity has contributed to the evolutionary trajectories of the glutathione-dependent enzymes.

The biosynthesis of glutathione from its constituent amino acids, glutamic acid, cysteine, and glycine, proceeds via γ-glutamylcysteine catalyzed by γ-glutamylcysteine ligase followed by addition of the C-terminal glycine catalyzed by glutathione synthetase [1]. The first reaction can be rate-limited by either the availability of cysteine or by the activity of the γ-glutamylcysteine ligase, which is negatively feedback regulated by glutathione. Liver is a major site of glutathione biosynthesis, and micromolar concentrations of the thiol circulate in blood plasma, but the blood–brain barrier prevents uptake into the brain. Thus, the precursors have to be taken up by membrane-bound transport proteins for glutathione biosynthesis in the brain tissues. Glutathione plays a pivotal protective role in the nervous system since the brain is subject to oxidative stress and ROS are produced in mitochondria. In addition to the ROS listed above, nitric oxide and superoxide can combine to form peroxynitrite, which can reach farther than other ROS and cause more widespread tissue damage. Both the primary and the secondary products of oxidative stress can be inactivated by glutathione-dependent reactions, and thereby counteract degenerative processes in the brain.

A role of glutathione in the cellular protection against Parkinson's disease was suggested several decades ago based on the deficiency in substantia nigra [13], but it remains unclear whether disruption of the proper glutathione status is a causative effect or a result of the disease [13]. A decrease in the cellular glutathione concentration in the brain of Parkinson's disease patients, in particular in the substantia nigra, is well established. On this basis clinical trials involving intravenous [14] or intranasal [15] glutathione administration to patients have been made in order to supplement the endogenous glutathione.

Precursors of glutathione biosynthesis, such as *N*-acetyl-L-cysteine, which have been demonstrated to increase glutathione concentrations following intravenous administration, have also been considered for clinical applications [8,16]. More recently a combination of *N*-acetylcysteine and a regenerative secretome preparation of mesenchymal stem cells has been suggested [17].

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) regulates the coordinated biosynthesis of glutathione and various proteins enabling antioxidant and protective functions. In particular, γ-glutamylcysteine ligase catalyzing the rate-limiting step in glutathione synthesis, several glutathione transferases (GSTs), as well as DT-diaphorase are upregulated by activators of Nrf2 [18]. Experiments suggests that triggering the higher antioxidant potential in astrocytes causes release of glutathione, thereby supporting the less capable neurons in mixed populations [6,19].

Furthermore, the excitatory amino acid carrier 1, the glutamate/cysteine transporter selectively present in neurons, plays a central role in the regulation of neuronal GSH

production, and it has been suggested as a new target, in addition to Nrf2, in therapeutic strategies for neurodegenerative diseases [4,20].

2. Glutathione Transferase

GSTs are enzymes catalyzing glutathione-dependent reactions that lead to inactivation and conjugation of toxic compounds, processes followed by subsequent excretion of the detoxified products. GSTs occur abundantly in multiple forms and the "GSTome" [21] encompasses both soluble and membrane-bound proteins. The homologous soluble GSTs, which occur in several classes [22], are prominent components of the cellular defense against toxicants. Discovered as enzymes detoxifying xenobiotics, we later found that the natural substrates are primarily products of lipid peroxidation and other noxious compounds derived from endogenous cellular components [11,23,24]. Recent findings relevant to Parkinson's disease show that α-synuclein oligomers formed with the dopamine-derived aminochrome-glutathione conjugate are not neurotoxic [25]. We have also demonstrated that GSTs can be secreted from cells in culture and taken up in catalytically functional form by other cells [26], suggesting extracellular trafficking of GSTs in tissues.

Crystal structures of the soluble GSTs [27] show that the enzymes are composed of two protein subunits, each carrying a binding site for glutathione (the G-site), as well as a binding site for the electrophilic second substrate (the H-site). The subunits are identical, or in some cases nonidentical but homologous, subunits from the same GST class [28]. The mu class encompasses a cluster of five homologous genes *GSTM1-GSTM5* on human chromosome 1, where *GSTM2*, assigned to cytogenetic band 1p13.3, encodes the homodimeric GST M2-2(Figure 1).

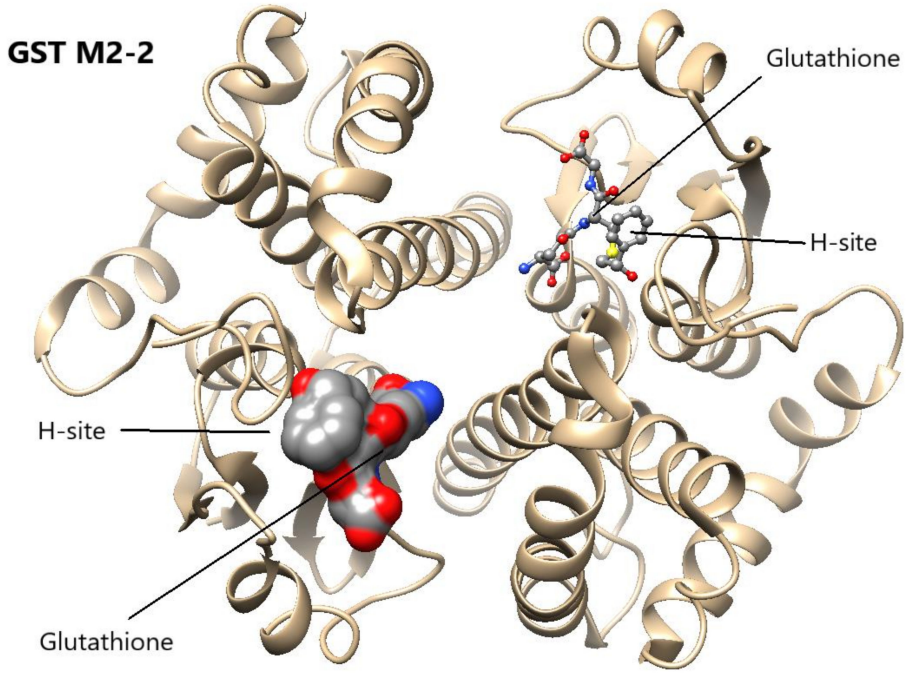

Figure 1. Human GST M2-2 showing location of the G-site and the H-site in each subunit. A structure of the enzyme in complex with aminochrome or its glutathione conjugate is not available, but the location of glutathione (G-site) and the H-site can be identified by the bound glutathione conjugate of S-styrene 7,8-oxide (shown left in space-filling and right in ball-and-stick representations). The figure is based on the crystal structure (PDB ID: 2C4J) and rendered in UCSF Chimera [29].

The enzyme GST M2-2 was first purified from human skeletal muscle under the name GST-4 [30] and was later also found at high levels in brain, testis, and heart, but not detectable in liver [31]. The catalytic activity of GST M2-2 with the toxic ortho-quinones derived from catecholamines is several orders higher than those of all other human GSTs

suggesting a designated protective role of GST M2-2 against oxidative stress caused by redox cycling of the orthoquinone substrates [32]. The highest specific activity has been observed with aminochrome, but dopachrome and noradrenochrome are also highly active substrates [24]. Notably, GST M2-2 is inducible by treatment with aminochrome, as demonstrated in astrocytoma U373MG cells, and GST M2-2 released from the cells protect dopaminergic neurons from aminochrome [26].

3. Parkinson's Disease Neurodegeneration

Parkinson's disease is the second most prevalent neurodegenerative disease, which affects neuromelanin-containing dopaminergic neurons, generating motor symptoms such as rest tremors, bradykinesia, rigidity, and postural instability [22]. However, the existence of non-motor symptoms such as hyposmia, depression, sleep disorders, constipation, anxiety, cognitive decline, orthostatic hypotension, and visual disturbances appear several years before the appearance of motor symptoms [33–36]. The motor symptoms appear when 60–70% of neuromelanin-containing dopaminergic neurons have been lost. The degenerative process and the progression of the disease is extremely slow and takes years to progress from non-motor to motor symptoms, suggesting that environmental or exogenous neurotoxins are not involved in the degenerative process of idiopathic Parkinson's disease. This idea is supported by the contrasting fact that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) developed severe Parkinsonism in just 3 days in drug addicts who used synthetic illegal drugs contaminated with this neurotoxin [37]. In addition, Parkinsonism has been observed in young workers occupationally exposed to manganese in mining or welding [38], copper mining [39], and paraquat in agriculture [40].

Therefore, these findings suggest that in the idiopathic form of the disease, the degenerative process of neuromelanin-containing dopaminergic neurons is not induced by exogenous neurotoxins or prion-like propagative process, as evidenced by the extremely slow degeneration over multiple years [35,40,41]. It seems plausible that the extreme slowness of the degenerative process in idiopathic Parkinson's disease depends on degeneration focused on a single neuromelanin-containing dopaminergic neuron that ends with its death. This degenerative event is focalized and does not trigger the degeneration of neighboring neuromelanin-containing dopaminergic neurons. At another time, a new and independent neurotoxic event occurs that ends with the loss of a new neuromelanin-containing dopaminergic neuron. These neurotoxic events accumulate over time and after years of the focalized degenerative process motor symptoms finally appear when at least 60–70% of the neuromelanin-containing dopaminergic neurons have been lost. According to this model of neurodegeneration focused on a single neuron, the degenerative process must be triggered by an endogenous neurotoxin that is generated within the neuromelanin-containing dopaminergic neurons.

The identity of the neurotoxin that triggers the degenerative process of the neuromelanin-containing dopaminergic neurons of the nigrostriatal system is unclear. However, there is general agreement in the scientific community that this degenerative process involves oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, neuroinflammation, dysfunction of protein degradation of the lysosomal and proteasomal systems, and aggregation of alpha-synuclein to neurotoxic oligomers [33,42–48]. However, what triggers these neurotoxic mechanisms in idiopathic Parkinson's disease is still unknown.

The discovery that mutations in some genes that code for proteins, such as *SNCA*, *PRKN*, *PINK1*, *UCHL1*, *LRRK2*, *ATP13A2*, *GBA*, *VPS35*, *DJ-1/PARK7*, *PLA2G6*, *SYNJ1*, *DNAJC6*, and *FBXO7*, associated with a familial form of Parkinson's disease has been an important input in the basic research of this disease by revealing the association of specified proteins to the disease [49–51]. For example, mutations of the alpha-synuclein gene generate the formation of neurotoxic oligomers that induce oxidative stress, synaptic dysfunction, autophagy impairment, mitochondrial dysfunction, endoplasmic reticulum stress [52]. However, these mutations do not explain the role of these proteins in idiopathic Parkinson's disease where the patients do not have these mutations.

The question is why neuromelanin-containing dopaminergic neurons degenerate and what initiates the process. The possible endogenous neurotoxins triggering the degenerative process in Parkinson's disease include neurotoxic alpha-synuclein oligomers, DOPAL, and aminochrome generated during the oxidation of dopamine to neuromelanin [53].

3.1. Alpha-Synuclein Oligomers

Alpha-synuclein aggregates in two different ways: (i) to form alpha-synuclein fibrils that are not considered neurotoxic but seem to be involved in prion-like propagation of these aggregates [35]. Alpha-synuclein fibrils are one of the major components of Lewy bodies, which is a hallmark of Parkinson's disease. Lewy bodies are also composed of a large number of proteins from mitochondria, autophagy and proteasome systems, and gene products associated to familial forms of Parkinson disease such as PINK-1, LRRK2, DJ1, and [54–56]. The role of Lewy bodies in Parkinson's disease pathogenesis is controversial since it has been proposed to play key role in the propagation of the disease from one to another region [35]; on the other side, the formation of Lewy bodies is not required to induce two familial forms of Parkinson's disease, because patients with LRRK2 and parkin mutation do not develop Lewy bodies [57,58]. Lewy bodies have been found to be present in postmortem material of Parkinson's disease patients, where the melanin-containing dopaminergic neurons involved in the motor symptoms were lost long before. When a neuron dies the microglia phagocyte and remove all cell components of the degenerated neuron, and therefore, the postmortem material includes the tissue that survives the degenerative process [59]. It has been proposed that Lewy bodies play a neuroprotective role in Parkinson's disease [54] by preventing the formation of neurotoxic alpha-synuclein oligomers. (ii) Alpha-synuclein is found membrane bound or in soluble state that can also aggregate to pre-fibrillar transitional species called oligomers. Alpha-synuclein structure modification plays an important role in its aggregation to oligomers. Point mutations in human alpha-synuclein DNA sequence change alpha-synuclein folding, inducing the formation of neurotoxic oligomer associated a familial form of Parkinson's disease. However, these points mutations cannot explain alpha-synuclein aggregation to neurotoxic oligomers in idiopathic Parkinson's disease. Another way to disrupt normal alpha-synuclein folding is the formation of adducts with some molecules, such as aminochrome that induce the formation of neurotoxic oligomers [60]. However, in vitro experiments showed that the formation of alpha-synuclein oligomers is not restricted to soluble monomer aggregation because it has been reported that alpha-synuclein fibrils ends are able to release oligomers. Short fibrils release more oligomers than long fibrils and, therefore, are more toxic [61].

3.2. 3,4-Dihydroxyphenylacetaldehyde (DOPAL)

Excess of cytosolic dopamine in dopaminergic neurons is degraded by the enzyme monoamine oxidase by catalyzing the oxidative deamination of dopamine. The product of this reaction, DOPAL, is converted to 3,4-dihydroxyphenylacetic acid, a reaction catalyzed by the enzyme aldehyde dehydrogenase-1. A study performed with postmortem brain tissue from Parkinson's disease patients revealed that the aldehyde dehydrogenase-1 protein level was decreased in these samples in comparison with control human brains [62]. The decrease in aldehyde dehydrogenase-1 expression will result in accumulation of DOPAL that can be oxidized to ortho-semiquinone and later to ortho-quinone species. DOPAL has been reported to be neurotoxic by promoting oxidative stress and stimulating alpha-synuclein aggregation to oligomers [63–65]. However, the decrease of aldehyde dehydrogenase-1 in postmortem Parkinson's disease material is not a direct consequence of degeneration of neuromelanin-containing dopaminergic neurons in the nigrostriatal system, because the decrease in the expression of aldehyde dehydrogenase-1 expression was measured in the neurons that had survived the degenerative process. The neuromelanin-containing dopaminergic neurons lost during years of neurodegeneration of the nigrostriatal system would have been removed by microglia long before the level of expression of aldehyde dehydrogenase was measured [66,67].

3.3. Aminochrome: The Neuromelanin Precursor

A possible explanation for the neurotoxic effect of aminochrome is that neuromelanin synthesis requires the formation of neurotoxic ortho-quinones [68,69]. Neuromelanin is synthesized in dopaminergic neurons when the catechol structure of dopamine is oxidized by dioxygen, metals, and enzymes, generating three ortho-quinones: dopamine ortho-quinone, which is stable only at pH lower than 2.0 and, therefore, immediately cyclizes to aminochrome. Aminochrome is a more stable molecule and is the most extensively investigated of the ortho-quinones. Finally, aminochrome can be further oxidized to 5,6-indolequinone, which can polymerize to generate neuromelanin (Figure 2).

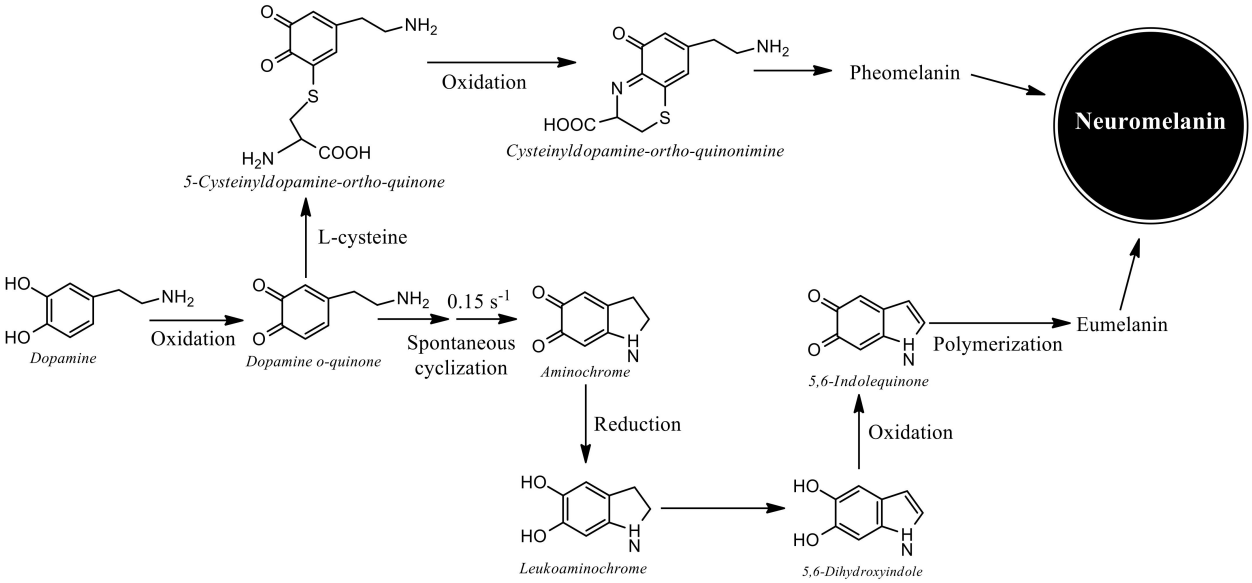

Figure 2. Synthesis of melanin. The synthesis of neuromelanin requires the oxidation of the catechol group of dopamine, where three ortho-quinones are generated in a sequential manner (dopamine ortho-quinone, aminochrome, and 5,6-indolequinone). Dopamine ortho-quinone is the first intermediate formed when the hydroxy groups of dopamine are oxidized. This ortho-quinone is stable at a pH lower than 2.0, which implies that at physiological pH in the cytosol it cyclizes in two steps forming aminochrome at a rate of 0.15 s^{-1}. Aminochrome is the ortho-quinone most stable quinone that finally, after 40 min via oxidation, rearranges its structure generating 5,6-indolequinone at rate of 0.06 min^{-1}, which is the direct precursor of neuromelanin and finally polymerizes to neuromelanin [48,68].

Human neuromelanin-containing dopaminergic neurons in the nigrostriatal system play a crucial role in movement control and the loss of these neurons in substantia nigra pars compacta of more than 60% induces motor symptoms in Parkinson's disease. Dopaminergic neurons located in substantia nigra pars compacta in the midbrain send projections to the dorsal striatum in the forebrain. Interestingly, neuromelanin accumulates with the age in the cell body of dopaminergic neurons of substantia nigra pars compacta due to these neurons have lower expression of VMAT-2 in comparison with dopaminergic neurons of mesolimbic system located in ventral tegmental area [70]. VMAT-2 mediated transport of dopamine into monoaminergic vesicles is essential to prevent dopamine oxidation to neuromelanin. Dopamine within monoaminergic vesicles is completely stable due the slight low pH environment that prevents dopamine oxidation to neuromelanin.

Human neuromelanin is composed by pheomelanin and eumelanin in a ratio of 1:3. These different melanin structures arise from the existence of two different pathways of melanin synthesis. The eumelanin synthesis involves dopamine oxidation to dopamine orthoquinone → aminochrome → 5,6-indolequinone → eumelanin, while pheomelanin synthesis encompasses dopamine oxidation to dopamine ortho-quinone, which, in the presence of L-cysteine, generates 5-cysteinyldopamine. 5-Cysteinyldopamine is oxidized to 5-cysteinyldopamine ortho-quinone. The cysteinyl group is oxidized to

cysteinyldopamine ortho-quinonimine, which finally polymerizes to pheomelanin. Glutathione conjugation of dopamine ortho-quinone forms 5-glutathionyldopamine that is degraded to 5-cysteinyldopamine, which polymerizes to pheomelanin or is excreted [71]. 5-Cysteinyldopamine is a stable final product that can be detected in serum or urine of melanoma patients [72] (Figure 2).

The most neurotoxic of these ortho-quinone is aminochrome, which induces (i) oxidative stress by being reduced with a single electron to leukoaminochrome *o*-semiquinone radical [73]; (ii) the formation of neurotoxic oligomers of alpha-synuclein [60]; (iii) mitochondrial damage including membrane impairment and inhibition of complex I that ultimately leads to mitochondrial dysfunction [73–77]; (iv) neuroinflammation [78,79]; (v) stress of the endoplasmic reticulum [80]; and (vi) impairment of protein degradation of both lysosomal and proteasomal systems [81,82]. Aminochrome is not a stable molecule that can be secreted from dopaminergic neurons to neighboring neurons, generating a propagative neurotoxic effect, since aminochrome immediately after its formation can be reduced by flavoenzymes or form adducts with proteins such as mitochondrial complex I, alpha-synuclein, actin, alpha- and beta-tubulin in cytoskeleton, and lysosomal vacuolar-type H + -ATPase, among other proteins [60,74,83,84]. Therefore, aminochrome cannot induce a propagative neurotoxic effect, but rather induces a neurotoxic effect focused on a single dopaminergic neuron.

The synthesis of neuromelanin in dopaminergic neurons is a harmless and normal process because neuromelanin formation increases with age in the substantia nigra, and healthy older adults at death have these neurons intact loaded with this dark pigment. This observation seems paradoxical, since neuromelanin synthesis in dopaminergic neurons generates the neurotoxic aminochrome, while neuromelanin synthesis generally is a harmless process. The explanation for this apparent contradiction is that there are neuroprotective mechanisms that prevent the neurotoxic effects of aminochrome in healthy individuals, who have their neuromelanin-containing dopaminergic neurons intact in the nigrostriatal system. There are two neuroprotective enzymes that prevent aminochrome-dependent neurotoxicity, DT-diaphorase and GST M2-2.

3.4. DT-Diaphorase

DT-diaphorase (NQO1; NAD(P)H: quinone oxidoreductase; EC.1.6.99.2) is the unique flavoenzyme that reduces quinones to hydroquinones by transfer of two electrons from NADH or NADPH [85]. Other flavoenzymes catalyze one-electron reduction of quinones to generate semiquinone radicals that in general are very reactive with oxygen. DT-diaphorase catalyzes the two-electron reduction of aminochrome to leukoaminochrome by using NADH or NADPH as electron donors. Leukoaminochrome can slowly autoxidize in the presence of dioxygen generating superoxide, but the presence of superoxide dismutase inhibits the auto-oxidation of leukoaminochrome, and glutathione peroxidase removes H_2O_2 formed in this autoxidation. DT-diaphorase is expressed in different organs, and in the brain, the enzyme is expressed in substantia nigra, striatum, hypothalamus, hippocampus, frontal cortex, ventral tegmental area, and cerebellum. Interestingly, DT-diaphorase is responsible for 97% of the total quinone reductase activity in the substantia nigra [86].

DT-diaphorase occurs in both dopaminergic neurons and astrocytes, but glutathione transferase M2-2 is expressed only in astrocytes. The oxidative pathway of dopamine oxidation to neuromelanin (dopamine → dopamine ortho-quinone → aminochrome → 5,6-indolequinone → neuromelanin) exists in dopaminergic neurons of substantia nigra where the expression of VMAT-2 is not enough high to complete prevent dopamine oxidation in the cytosol. Dopamine inside monoaminergic vesicles is completely unreactive because the protons of hydroxyl groups are firmly bound. Dopamine transport into monoaminergic vesicles mediated by VMAT-2 is coupled to an ATPase that pumps protons, decreasing the pH of the vesicles. The level of VMAT-2 expression in dopaminergic neurons of the mesolimbic system is much higher than in nigral neurons, preventing neuromelanin formation. Astrocytes express both dopamine transporter and nonspecific transporter that

take up dopamine released under neurotransmission, which can be oxidized to dopamine ortho-quinone and aminochrome. However, glutathione transferase M2-2 catalyzes glutathione conjugation of both dopamine ortho-quinone and aminochrome, preventing the formation of 5,6-indolequinone, which is the direct precursor of neuromelanin. Astrocytes do not have dopamine synthesis and the level of dopamine in astrocytes cytosol depends on the competition between dopamine re-uptake mediated by dopamine transporter into dopaminergic neurons and dopamine uptake into astrocytes of dopamine released during neurotransmission [85].

DT-diaphorase competes with other flavoenzymes that reduce aminochrome in a one-electron process to leukoaminochrome *o*-semiquinone radical, which is extremely reactive with oxygen, as evidenced by experiments performed with electron spin resonance [87]. One-electron reduction of aminochrome induces the generation of redox cycling between aminochrome and leukoaminochrome *o*-semiquinone radical by reducing dioxygen to superoxide until dioxygen and NADH are depleted. This redox cycle is a potent inducer of oxidative stress and depletion of NADH that inhibits mitochondrial electron transport and ATP formation. The silencing of DT-diaphorase expression by using a siRNA targeting this gene induces aminochrome neurotoxicity, supporting the notion of a protective role of this enzyme [88]. Thus, two-electron reduction of aminochrome catalyzed by DT-diaphorase prevents oxidative stress and mitochondrial electron transport chain inhibition caused by NADH depletion [73]. Another neurotoxic mechanism caused by aminochrome involves its ability to induce the formation of alpha-synuclein neurotoxic oligomers, which have been proposed to induce mitochondrial dysfunction, autophagy dysregulation, oxidative stress, and endoplasmic reticulum stress [52,60]. DT-diaphorase prevents the formation of neurotoxic alpha-synuclein oligomers [60]. DT-diaphorase also thwarts aminochrome-induced proteasomal dysfunction [89] as well as aminochrome-induced lysosomal dysfunction that it is essential to perform autophagy-dependent protein degradation [83]. Aminochrome-induced aggregation of alpha- and beta-tubulin prevents microtubules assembly and stability. DT-diaphorase also prevents aminochrome-induced cytoskeleton disruption by inhibiting actin and alpha- and beta-tubulin aggregation, which dramatically affects neurons morphology due to a phenomenon called cell shrinkage [84]. Microtubules play an important role in neuron cytoskeleton structure, as well as an essential role in axonal transport of proteins and neurotransmitter vesicles that is strongly decreased in animals treated with aminochrome [90] (Figure 3). Furthermore, microtubules are involved in the fusion between lysosomes and autophagosomes that it is crucial for autophagy-dependent protein degradation, and DT-diaphorase prevents tubulin aggregation caused by aminochrome [82].

3.5. Glutathione Transferase M2-2

Glutathione is an important antioxidant in neurons, but also plays an important protective role in Parkinson's disease by participating in aminochrome conjugation catalyzed by glutathione transferase [85]. Human class Mu glutathione transferases catalyze aminochrome glutathione conjugation where GST M2-2 is 193- and 1000-times more active than GST M1-1 and GST M3-3 enzymes, respectively. Human glutathione transferase M2-2 is expressed in astrocytes but not in neurons and aminochrome induces an increase in the expression of this enzyme [26]. GST M2-2 catalyzes aminochrome conjugation to 4-S-glutathionyl-5,6-dihydroxyindoline that is resistant to biological oxidants such as dioxygen, superoxide and hydrogen peroxide, suggesting that this is a final product that it is not able to contribute to neurotoxic oxidation and reduction reactions [32].

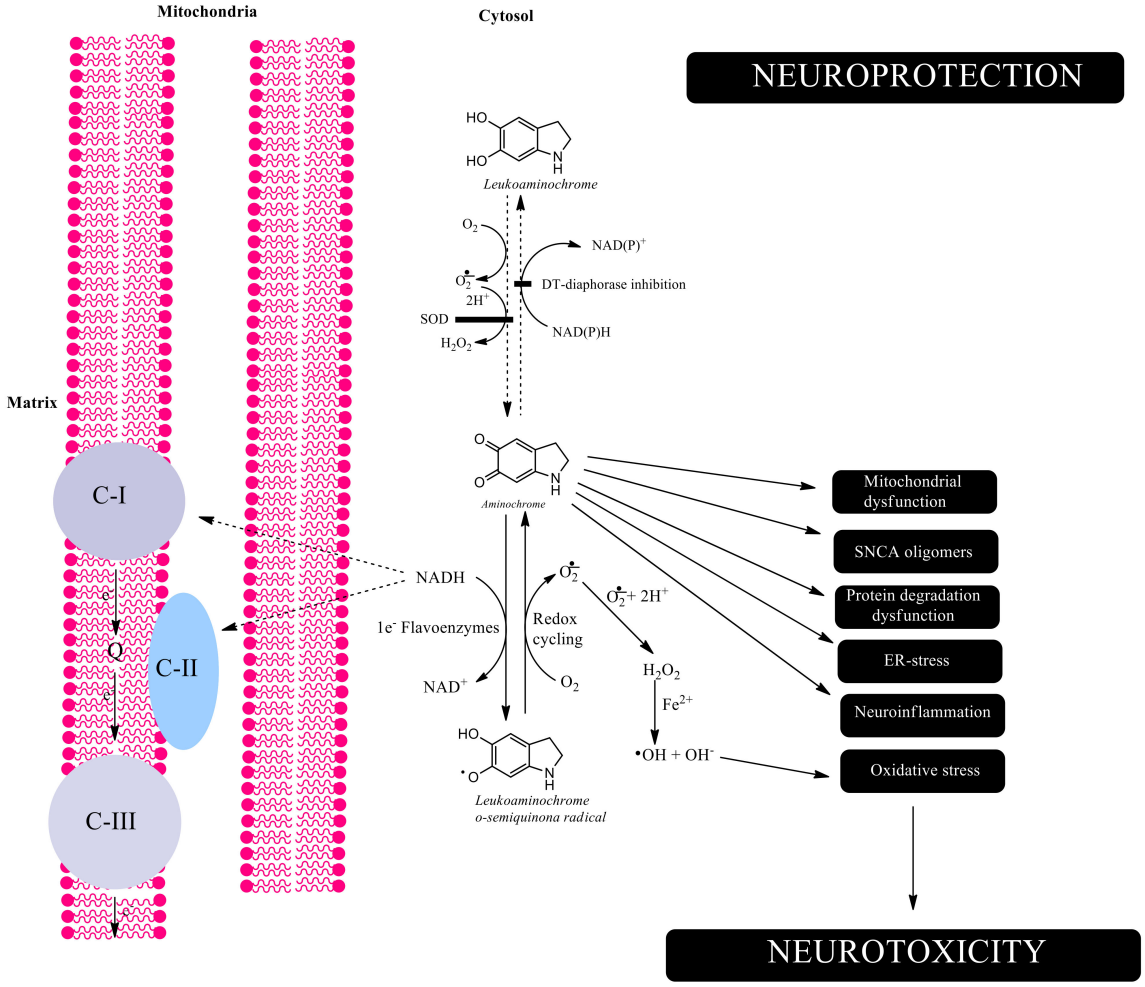

Figure 3. Aminochrome-induced neurotoxicity. DT-diaphorase catalyzes the reduction of aminochrome with two electrons to leukoaminochrome, preventing the neurotoxic effects of aminochrome. Leukoaminochrome can autoxidize very slowly in the presence of dioxygen, generating superoxide. However, the autoxidation of leukoaminochrome is significantly faster in the presence of superoxide and the presence of superoxide dismutase in the cytosol inhibits the autoxidation, since it removes the superoxide molecules, which are the true motor of the autoxidation of leukoaminochrome. Inhibition of DT-diaphorase allows aminochrome to be neurotoxic in two different ways: (i) aminochrome can be reduced with one-electron donors to leukoaminochrome *o*-semiquinone, which is extremely reactive with dioxygen generating superoxide. Reduction of aminochrome with a single electron generates a redox cyclization between aminochrome and leukoaminochrome o-semiquinone radical, which depletes dioxygen and NADH. NADH depletion affects the activity of the mitochondrial respiratory chain and ultimately ATP production. Superoxide spontaneously or in the presence of superoxide dismutase is converted to hydrogen peroxide, which in the presence of reduced iron (Fe^{2+}) generates hydroxyl radicals. The hydroxyl radical is a powerful reactive oxygen species that induces oxidative stress; and (ii) aminochrome forms adducts with different proteins such as alpha-synuclein, complex-I in the mitochondrial respiratory chain, and other cell components. Aminochrome induces mitochondrial dysfunction, formation of neurotoxic alpha-synuclein oligomers, dysfunction of both lysosomal and proteasomal protein degradation systems, endoplasmic reticulum stress and neuroinflammation.

GST M2-2 also catalyzes glutathione conjugation of dopamine *o*-quinone to 5-glutathionyl dopamine [91]. In general, all glutathione conjugates are degraded by the enzyme γ-glutamyl transpeptidase and dipeptidase. 5-Glutathionyldopamine is degraded to 5-cysteinyldopamine which has been found in human neuromelanin of substantia nigra, cerebrospinal fluid and

other dopamine-rich regions, such as putamen, globus pallidus and caudate nucleus [92–94]. These data suggest that 5-cysteinyldopamine is a final product, supporting the notion that GST M2-2 is a neuroprotective enzyme against aminochrome neurotoxicity [81] (Figure 4).

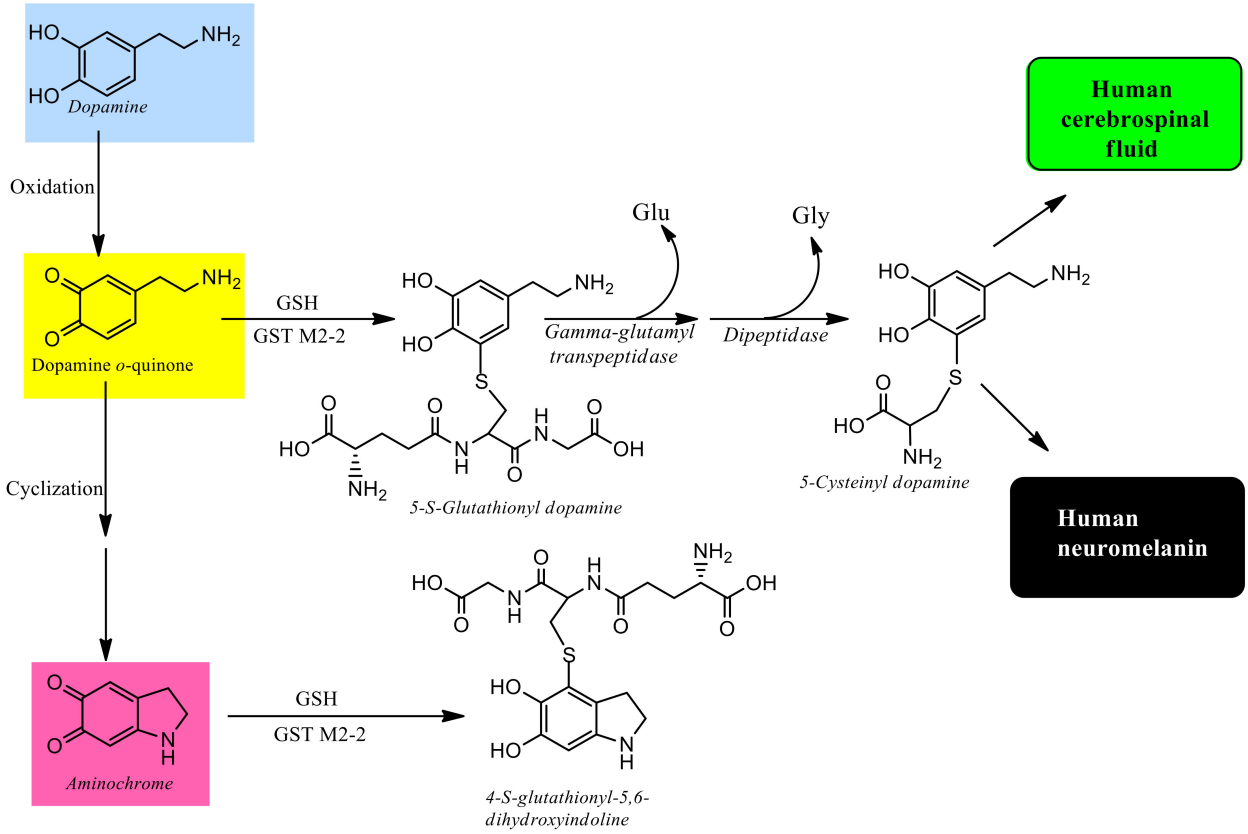

Figure 4. Aminochrome conjugation with glutathione. Glutathione transferase M2-2 catalyzes glutathione conjugation of dopamine *o*-quinone to 5-S-glutathionyl dopamine, which degrades to 5-cysteinyl dopamine. 5-cysteinyl dopamine has been found in neuromelanin and human spinal brain fluid, suggesting that it is a final product. Glutathione transferase M2-2 also catalyzes the conjugation of aminochrome with glutathione to 4-S-glutathionyl-5,6-dihydroxyindoline, which is resistant to biological oxidizing agents such as dioxygen, superoxide, and hydrogen peroxide. The aminochrome conjugation prevents neurotoxic effects and appears to be a major neuroprotective mechanism.

4. Astrocytes Protects Dopaminergic Neurons

There is an interrelation between neurons and astrocytes, where astrocytes secrete glutathione and cysteine that neurons take up to maintain stable levels of thiols to prevent oxidative stress generated by the loss of electrons from the mitochondrial transport chain. However, this transfer of glutathione and cysteine is only in one direction, from astrocytes to neurons [95]. The interrelation between neurons and astrocytes is not restricted to exchange of glutathione and cysteine. Astrocytes play an important role in neuronal survival and function by supporting neuron energy demand by providing lactate, a precursor of glucose synthesis in the gluconeogenesis pathway, by generating astrocyte-neuron lactate shuttle [96]. Neurons are completely dependent on ATP availability for essential functions, such as neurotransmission or axonal transport, and glucose is the main source of energy production under normal conditions in the brain. Astrocytes also play an important role by taking up lipid droplets generated by neurons. Astrocytes detoxify neurotoxic fatty acids created in hyperactive neurons that transport these fatty acids into astrocytes lipid droplets by using apolipoprotein E-positive lipid particles. Astrocytes' mitochondrial β-oxidation oxidizes these neuronal fatty acids and induces detoxifying enzyme expression [97]. Another interrelation between astrocytes and neurons is glutamate-glutamine metabolism.

An important part of release glutamate by glutaminergic neurons is take up by astrocytes due to neurons have lower capacity to take up glutamate. Astrocytes convert most of the glutamate to glutamine that neurons take up [98].

A new interrelation between neurons and astrocytes, in order to protect dopaminergic neurons against aminochrome neurotoxicity, has been reported [25,26,85,99,100]. DT-diaphorase and GST M2-2 play key neuroprotective roles against aminochrome neurotoxicity in neuromelanin-containing dopaminergic neurons in the nigrostriatal system, explaining why neuromelanin synthesis is a normal and harmless pathway. DT-diaphorase is expressed in dopaminergic neurons and astrocytes, while GST M2-2 is expressed only in human astrocytes. Astrocytes surrounding dopaminergic neurons can take up dopamine released during neurotransmission through the dopamine transporter and other non-specific transporters such as organic cation transporter-3 and plasma membrane transporter. Dopamine within astrocytes can be oxidized, thereby generating aminochrome that induces an increase in the expression of GST M2-2. This enzyme conjugates aminochrome with glutathione within astrocytes, but, in addition, astrocytes secrete GST M2-2 through exosomes [85]. GST M2-2 loaded exosomes penetrate dopaminergic neurons to prevent aminochrome neurotoxicity in collaboration with DT-diaphorase (Figure 5). Significantly, a study of senescence processes demonstrated that small extracellular vesicles from young human donors contained GST M2-2 which could ameliorate aging of old fibroblasts seemingly mimicking the protective effect of astrocytes on neurons.

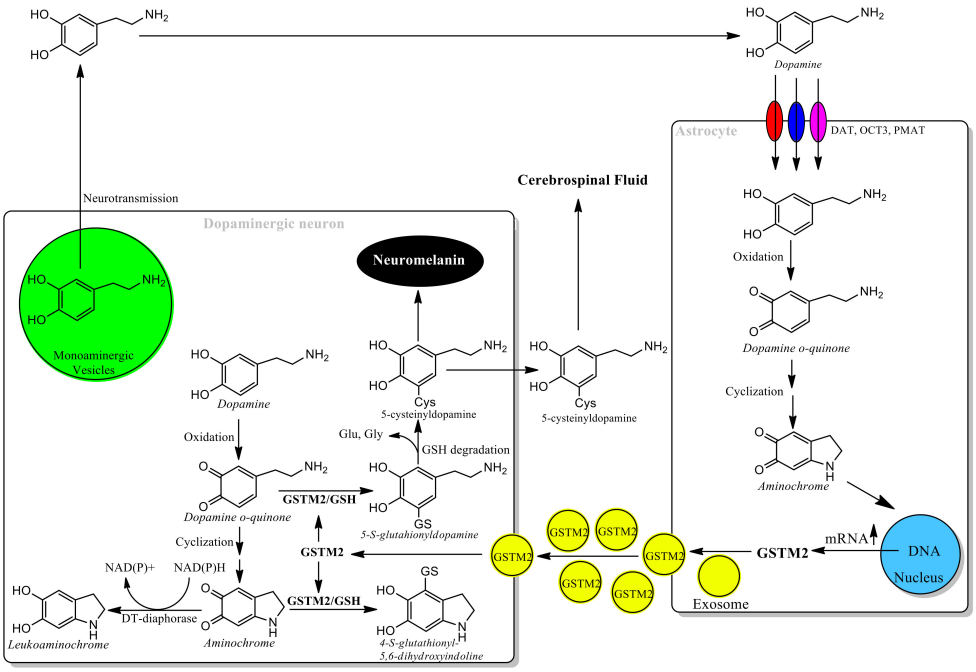

Figure 5. Astrocytes protects dopaminergic neurons against aminochrome neurotoxicity. A new mechanism of neuroprotection of dopaminergic neurons against the neurotoxic effects of aminochrome mediated by astrocytes has been reported. Dopaminergic neurons express DT-diaphorase to prevent aminochrome neurotoxic effects. Dopamine neurons release dopamine normally during the neurotransmission process, which astrocytes are also able to take up through transporters that are expressed in astrocytes such as dopamine transporter (DAT), organic cation transporter-3 (OCT3), and plasma membrane monoamine transporter (PMAT). Dopamine within astrocytes can be oxidized to form aminochrome, which increases the expression of glutathione transferase M2-2 (GSTM2). GST M2-2 prevents the neurotoxic effects of aminochrome within astrocytes by conjugating it with glutathione, but GST M2-2 is also exported through exosomes that are released from astrocytes into dopaminergic neurons. Exosomes loaded with GST M2-2 penetrate dopaminergic neurons, releasing this enzyme into the cytosol of these neurons, where together with DT-diaphorase they prevent the neurotoxic effects of aminochrome.

5. Conclusions

The protective mechanism against aminochrome neurotoxicity, where astrocytes collaborate with dopaminergic neurons, plays a crucial role in preventing neurotoxic effects during neuromelanin synthesis where aminochrome is generated. This protective system composed, of DT-diaphorase and GST M2-2, explains why neuromelanin synthesis is a harmless and normal chemical pathway, and why neuromelanin-containing dopaminergic neurons of the nigrostriatal system are intact in healthy seniors.

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antioxidants

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Review

Is There a Glutathione Centered Redox Dysregulation Subtype of Schizophrenia?

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Abstract: Schizophrenia continues to be an illness with poor outcome. Most mechanistic changes occur many years before the first episode of schizophrenia; these are not reversible after the illness onset. A developmental mechanism that is still modifiable in adult life may center on intracortical glutathione (GSH). A large body of pre-clinical data has suggested the possibility of notable GSH-deficit in a subgroup of patients with schizophrenia. Nevertheless, studies of intracortical GSH are not conclusive in this regard. In this review, we highlight the recent ultra-high field magnetic resonance spectroscopic studies linking GSH to critical outcome measures across various stages of schizophrenia. We discuss the methodological steps required to conclusively establish or refute the persistence of *GSH-deficit* subtype and clarify the role of the central antioxidant system in disrupting the brain structure and connectivity in the early stages of schizophrenia. We propose in-vivo GSH quantification for patient selection in forthcoming antioxidant trials in psychosis. This review offers directions for a promising non-dopaminergic early intervention approach in schizophrenia.

Keywords: glutathione; glutamate; psychosis; schizophrenia; redox; antioxidant; oxidative stress; myelin; spectroscopy

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1. Introduction

Schizophrenia is one of the most devastating of adolescent onset illnesses. Despite the advances in pharmacological, psychological, and social aspects of care in the last 50 years, only a small sub-group achieves combined clinical and functional recovery (~13%) [1,2]. Over several decades, barely any improvement has occurred in life expectancy and the years of potential life lost [3]. All the currently available antipsychotics focus on dopamine, but the symptom relief they provide does not translate to functional recovery in most cases. Remarkably, around 10% of patients do not develop further episodes after the first [4]; but in those who experience persistent illness or recurrences, antipsychotics do not reverse the cognitive deficits and negative symptoms that contribute to most of the functional disability [5,6]. Currently there are no truly 'disease-modifying' interventions available [7] though many promising leads have emerged in recent times.

While a complete mechanistic account of the schizophrenia or the 'group of schizophrenias', as Bleuler surmised 110 years ago [8], is still lacking, three key neurobiological

substrates of the disabling illness trajectory have emerged in recent times. (1) glutamatergic dysfunction in early stages [9,10] related to excitation–inhibition imbalance resulting from prefrontal parvalbumin interneuron deficits in the cortical microcircuit [11,12] (2) dysconnectivity of large-scale brain networks (especially involving the dorsal anterior cingulate cortex [ACC] and insula) in prodromal stages before the first-episode [13–16], related to persistent symptom burden [17–19] and cognitive deficits [20–22], and (3) microstructural changes in the grey [23,24] and white matter [25–27], predating the illness but becoming more prominent during the first-episode [24], possibly reflecting the loss of dendritic spines [28,29] and myelination deficits [30,31]. Despite these mechanistic insights no accessible therapeutic targets have emerged yet. This is because most changes occur in early developmental periods that precede the illness onset by many years. By the time symptoms first appear, most neural disruptions are already well established and often irreversible. Therefore, there is an urgent need to identify pathways of poor outcome in schizophrenia that remain 'modifiable' *after* the symptom onset.

2. Glutathione in Schizophrenia

One of the pathways of poor outcome that has its roots in early life but continues to remain modifiable in later stages of illness may be the antioxidant pathway. Destructive free radicals that damage brain tissue are by-products of oxidative metabolism but are effectively scavenged by antioxidants. Glutathione [GSH] is the cardinal antioxidant in brain cells. In preclinical models of schizophrenia, early GSH deficit contributes to dysfunction of prefrontal parvalbumin interneurons [32,33], increased susceptibility of excitotoxic pyramidal cell damage especially in the presence of a hyperdopaminergic state [34,35], reduced dendritic spines [36], reduced stability of axonal projections [37], and facilitates oligodendrocyte cell death disrupting myelin formation [38–41]. Crucially, agents that improve GSH levels ameliorate the effects of oxidative stress in various preclinical models of schizophrenia (ketamine [42], GluN2A [43] or neonatal ventral hippocampal lesions [44,45], perinatal infection [46], stimulant exposure [46], and maternal immune activation [47]). This large body of evidence has led to the claim that various mechanistic strands underlying schizophrenia converge on the "hub of oxidative stress" indexed by GSH [48,49]. Preclinical models with early GSH deficit (i.e., from postnatal day 0 onwards) display various schizophrenia-like features in adult life, including a sensitivity to dopamine excess [50], and prefrontal hypomyelination [51]. Nevertheless, chronic peri-adolescent treatment with the glutathione precursor N-acetylcysteine (postnatal days 5–90) restores antioxidant-related and myelin-related mRNA expression improving cognitive flexibility in later life [51]. Thus, despite the likely developmental origins of the GSH-deficit, a phenotype 'rescue' is possible in preclinical models with interventions in later life. Such 'rescue' effects on myelination have also been reported in patients with early psychosis taking GSH precursors as supplements [52], offering GSH-deficit as a potentially modifiable pathway of poor outcomes in schizophrenia. In this context, early identification of individuals with GSH-deficit from clinical samples of patients with schizophrenia assumes paramount importance.

3. Intracortical GSH in Schizophrenia: MRS Studies

Peripheral antioxidant markers are reduced in patients with schizophrenia [53,54]. Genetic [55,56] and cell biology studies [57–59] indicate that the ability to produce GSH in the face of oxidative stress is likely to be reduced in the patients, at least a subset of them. More direct demonstration of low GSH levels comes from in vivo Magnetic Resonance Spectroscopic (MRS) studies and cerebrospinal fluid measurements or post-mortem quantification of glutathione. Early in vivo studies reported 27–52% GSH reduction [60–62] in established schizophrenia. For many subsequent MRS GSH studies, the anterior cingulate cortex (ACC) has been the chosen region of interest given its relevance to schizophrenia as well as the technical advantage (uniform field homogeneity, higher signal-to-noise, narrow spectral peak width, low probability of susceptibility artefacts) offered by a midline MRS

voxel placement [63]. A recent synthesis of cross-sectional MRS studies demonstrated a small but significant GSH reduction (effect size = 0.26) in the ACC region in schizophrenia [64]. Interestingly, of the 12 studies that were included in this meta-analysis, only two reported significant GSH differences between patients and HCs [60,65], contributing to a modest reduction in GSH among patients. This small but significant GSH reduction in schizophrenia has been reported by two other meta-analyses, one restricted to ultra-high field 7T MRS studies (effect size = 0.21) [66] and the other including all central measures of GSH MRS, CSF and post-mortem samples (effect size = 0.26) [67].

Since 2018, several MRS studies on ACC GSH in schizophrenia have been published [68–74] (Table 1). While the largest study to date supports Das and colleagues' report of GSH reduction in early stages of schizophrenia [74], smaller studies have found no differences in patients when compared to healthy controls. Interestingly, samples of acutely symptomatic, untreated first-episode patients report higher levels of ACC GSH than healthy controls [72,73] which is not seen in other post-acute samples beyond 1–2 years of illness that are likely to include more treatment-resistant subjects [68,71,74].

These observations present a more nuanced picture of intracortical GSH aberrations than what can be expected from preclinical studies or from the large effect of size reduction of peripheral GSH measures in schizophrenia.

Table 1. Studies of intracortical GSH published since Das et al., 2018.

Study	No. Patients/ Controls	Females/ Males Patients	No. of Females/Males Controls	Age of Patients (Years) Mean (SD)	Age of Controls (Years) Mean (SD)	Clinical Features	Duration of Illness (Years) Mean (SD)
Coughlin et al., 2021	46/50 (16/10 *)	12/34	16/34	34.17 (11.8)	32.06 (11.28)	Chronic, stable phase of schizophrenia; 13% no APD. ACC GSH patients = HC	12.36 (11.45)
Dempster et al., 2020 [a]	26/27	5/21	10/17	24.04 (5.4)	21.48 (3.57)	Acute, untreated psychosis; dACC GSH patients = HC; Higher GSH in patients with faster response to APD.	0.54 (1.25)
Godlewska et al., 2021	17/18 (14/18 *)	0/17	0/18	25.6 (1.1)	27.1 (0.8)	Stabilized first-episode; diagnostic information N/A; 12% no APD; ACC GSH patients = HC	2.54 (0.28)
Iwata et al., 2021 [b]	21/26	5/16	7/19	46.3 (12.7)	40.8 (13.2)	First line treatment responders; dACC GSH patients = HC	20.0 (12.2)
Iwata et al., 2021 [b]	27/26	8/19	7/19	40.5 (11.2)	40.8 (13.2)	TRS—Clozapine responders; dACC GSH patients = HC	16.4 (9.7)
Iwata et al., 2021 [b]	24/26	5/19	7/19	44.8 (13.2)	40.8 (13.2)	TRS—Clozapine non-responders; dACC GSH patients = HC	23.5 (13.2)
Limongi et al., 2021 [a]	19/20	7/12	9/11	21.7 (3.3)	21.3 (3.7)	Acute, untreated psychosis; 60% no APD; dACC GSH patients > HC	1.1 (1.8)
Pan et al., 2021 [a,b]	16/25	3/13	11/14	21.81 (3.17)	22.12 (3.54)	Acute psychosis with high disorganization; >65% schizophrenia. dACC GSH patients > HC	0.98 (1.13)
Pan et al., 2021 [a,b]	24/25	6/18	11/14	23.71 (5.43)	22.12 (3.54)	Acute psychosis with low disorganization; >80% schizophrenia. dACC GSH patients = HC	0.91 (1.7)

Table 1. *Cont.*

Study	No. Patients/ Controls	Females/ Males Patients	No. of Females/Males Controls	Age of Patients (Years) Mean (SD)	Age of Controls (Years) Mean (SD)	Clinical Features	Duration of Illness (Years) Mean (SD)
Wang et al., 2019	81/91 (74/88 *)	24/57	49/42	22.3 [4.4]	23.3 (3.9)	Stabilized first-episode; <65% of sample had schizophrenia. dACC GSH HC > patients	1.27 (0.8)

* Final sample with available MRS glutathione (GSH) data; all demographic information refers to the original larger sample. [a] overlapping samples; [b] same healthy control samples, distinct patient samples, reported in the same manuscript. APD: Antipsychotic Drugs, dACC: Dorsal Anterior Cingulate Cortex. HC: Healthy control subjects, NA: Data not available; SD: Standard Deviation; TRS: Treatment Resistant Schizophrenia.

The heterogeneity observed in MRS studies of ACC GSH in schizophrenia highlights two distinct possibilities. First, there are likely to be at least two subgroups of patients, one with notable GSH deficit, and the other with near-normal or supra-normal levels of GSH compared to healthy subjects. Both these subgroups are most likely to be apparent among the untreated first episode patients with more florid positive symptoms but less cognitive deficits and more favourable treatment response profile than more established cases. One of the earliest reports linking increased intracortical GSH with favourable treatment outcomes came from the study of Wood and colleagues [75]. They reported a 22% increase in medial temporal GSH levels in first episode psychosis; in a sub-sample from this study [76], treatment related increase in GSH was associated with a gain in global functioning scores. ACC GSH levels were not examined in their sample. We observed that in untreated first episode patients, an elevated ACC GSH at the time of presentation occurred in those with more florid disorganization [73] but most of these subjects responded briskly to the regular antipsychotic treatments over the next 6 months [69]. Help seeking subjects with subthreshold psychotic symptoms display better social and occupational functioning when ACC GSH levels are higher [77]. In the meta-analysis of Das and colleagues we noted a small but significant increase in ACC GSH in bipolar disorder, a phenotype that is often associated with better functional outcomes than schizophrenia [64]. Samples with more established schizophrenia with residual symptoms and impaired functioning are likely to have an over-representation of the subgroup with GSH deficit. Based on the reported effect-sizes of the observed GSH deficit across chronic and partially treatment-resistant samples [64,67], it is likely that only a subgroup of patients have a notable GSH-deficit. Nonetheless, this subgroup of patients with GSH-deficit are likely to be treatment-resistant; a recent 7-Tesla MRS study in first-episode psychosis reports a large effect-size (Cohen's d = 0.83) reduction in ACC GSH in 32 treatment-resistant patients compared to 106 non-treatment-resistant patients [78].

The second possibility is that in a patient with schizophrenia, GSH levels may vary with the stage of illness. Acute, untreated symptomatic state may relate to higher levels of GSH than stable clinical states of schizophrenia. Increase in GSH level may mark a compensatory response to acute oxidative stress that may not be sustained as the illness progresses to a more persistent stage. Longitudinal studies of intracortical GSH are limited; most patient cohorts with repeated MRS acquisitions to date did not measure GSH [79–83]. To our knowledge, only two studies report on longitudinal GSH measurements in psychosis to date. In a sample of 21 subjects with first episode schizophrenia scanned at baseline with <3 days of lifetime antipsychotic exposure and followed after 6 months of antipsychotic treatment, GSH levels were highly stable (Mean (SD) at baseline =1.71 (0.36), at 6-months = 1.75 (0.23)) [84]. The same stability in GSH levels was also seen in the 10 demographically healthy controls scanned at two time points (Mean(SD) at baseline =1.64 (0.25), at 6-months = 1.63 (0.32)) [84]. In a larger sample of 38 patients with first episode psychosis (onset within 2 years) and 48 healthy controls followed up over 4 years [85], GSH levels was found to have a near zero change in all five studied brain regions (ACC, thalamus, DLPFC, centrum semiovale, and orbitofrontal cortex) over time, strongly arguing for a 'trait-like' stability of GSH levels compared to other metabolites. These observations do

not rule out within-individual differences over time, and the possibility of an early excess in untreated state; but provide a strong support for the presence of a distinct subgroup with GSH-deficit that is over-represented in more established phase of schizophrenia, in association with reduced treatment responsiveness and poor functional outcomes.

4. Factors Contributing to the Putative Intracortical GSH Deficit

In the next two sections we focus on factors contributing to the putative intracortical GSH-deficit and the pathophysiological consequences of this deficit.

Preclinical studies discussed earlier insinuate the possibility of a constitutional defect in GSH synthesis in schizophrenia. Defective production of GSH due to reduced expression of the GSH synthesizing enzyme Glycine Cysteine Ligase (GCL), has been demonstrated in patient-derived cell culture studies [57]. The high-risk variant gene encoding GCL's catalytic subunit (GCLC) with eight or nine GAG repeats as opposed to seven was seen in 36% of patients, but only 3% of healthy controls in some samples [57]. Later studies failed to replicate an association between high-risk GCLC variant and lower GSH levels in schizophrenia [68,71,86]. Thus, the high risk variants do not consistently indicate low intracortical GSH [62]. In the same vein, low GSH levels in post-mortem brain tissue of patients with schizophrenia are observed despite normal levels of GCL and GSH peroxidase-like protein [86]. Furthermore, large-scale genome-wide association studies (GWASs) to date have not observed aberrations in the GSH synthesis pathway in schizophrenia (reviewed by Ermakov et al. [87]). Thus, a constitutional defect in GSH synthesis, if present, may be limited to a small number of patients. In most others, intracortical GSH levels are likely influenced by indirect factors (e.g., transcriptional regulators or epigenetic factors regulating gene expression [87,88]) affecting redox status.

Environmental factors, in particular lifestyle factors, also determine intracortical GSH. In fact a number of lifestyle factors in patients with mental illnesses may transiently affect GSH levels [89,90]. For example, in youth with mood disorders [91], apparent intracortical GSH-deficits are mostly explained by lifestyle factors (alcohol or smoking) [89,92,93]. In depression, this early-life reduction in GSH levels [94,95] appears to normalise later (15.7% increase, 1 year after first-episode depression [96]), likely explaining the higher than expected levels in later life [97]. At present, the relative influence of lifestyle factors on intracortical GSH in schizophrenia is unknown.

While several studies have examined the influence of lifestyle factors on peripheral antioxidant markers [98,99], intracortical GSH levels do not always reflect the peripheral antioxidant status in schizophrenia. For example, while the levels of scavenging antioxidant enzymes such as glutathione peroxidase levels correlate positively with ACC GSH in healthy subjects, such an effect is inconsistent, and absent in patients [55,100]. Peripheral antioxidant deficit in schizophrenia is of several magnitudes larger than the central GSH reduction reported so far (effect sizes 1.02 vs. 0.26 [67]). Further, localised brain tissue changes, such as an increase in free water concentration in grey matter (likely an effect of neuroinflammation), can affect the intracortical GSH in schizophrenia [101].

Taken together, intracortical GSH-deficit may represent a 'failure mode' of the redox system occurring in some patients with schizophrenia, with multifactorial pathways (genetic and lifestyle-related) converging to result in a relative GSH-deficit state over the course of this illness.

5. Consequences of the Putative Intracortical GSH Deficit

Irrespective of its origins, the presence of a pervasive inability to counter oxidative stress with increased intracortical GSH is likely to produce multiple downstream consequences in schizophrenia. Preclinical studies reviewed above indicate that a GSH-deficit is likely to facilitate excitotoxic damage [34,35] affecting dendritic spines [36] and axonal stability [37] and disrupting myelin formation [38–41]. Myelin deficits in schizophrenia (termed *dys*myelination [102,103]) are likely multifactorial [104,105]; oxidative stress is hypothesized to preferentially affect prefrontal myelin-generating precursor cells [106–108], affecting cortical

microcircuits in the early phase of psychosis [49]. In first episode schizophrenia, GSH levels correlate with white matter integrity [39]; its relationship with myelin content and microstructure of the grey matter is still unknown. Most of the recorded grey matter changes in schizophrenia occurs in the immediate post-onset phase [24,109,110], coinciding with the critical period of intracortical myelination [111–113]. Intracortical myelin mostly insulates parvalbumin containing interneurons [114] that are highly susceptible to developmental factors influencing redox balance [115] as well as GSH deficit and associated glutamate-mediated excitotoxicity [30,116]. In a subsample of patients reported by Pan and colleagues [73], we obtained quantitative intracortical myelin measurement and noted several regions where patients had lower intracortical myelin in the presence of higher glutamate, only when GSH levels were lower than the median (bilateral dorsolateral prefrontal cortex, right superior temporal, and left precentral gyrus and right subgenual ACC). Thus, higher ACC glutamate related to lower prefrontal intracortical myelin, only when ACC GSH levels were also lower; this indicates a gatekeeping role for GSH in glutamate-related *dys*myelination (Figure 1). Relating GSH-deficit at the onset to subsequent intracortical myelin changes will provide compelling proof for the downstream effects of antioxidant aberrations on the illness trajectory.

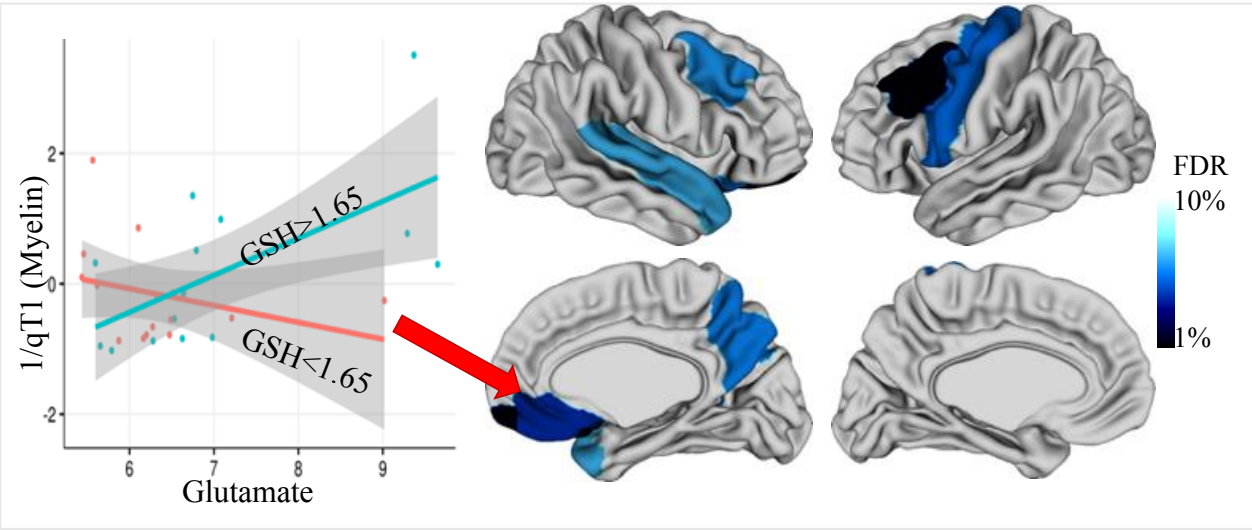

Figure 1. Permissive role of GSH on glutamate-related demyelination. Intracortical myelin (qT1) was mapped at the mid-cortical surface (50% depth between the white matter on pial surfaces) using the CIVET pipeline (https://github.com/aces/CIVET (accessed on 24 October 2021)) with T1-weighted images. Median qT1 values were gathered over cortical regions defined by the Desikan–Killiany Atlas (DKT40). In First Episode Psychosis (n = 30), glutamate relates to reduced prefrontal, orbitofrontal, and superior temporal intracortical myelin (1/qT1) only when GSH levels are also lower (red slope) in the dorsal ACC [MRS voxel not shown]—results were significant in 8 cortical regions after FDR correction (10%). Scatterplot shows the right lateral orbitofrontal cortex [contiguous with the medial surface that is shown on display], indicating significant glutamate-by-GSH interaction effect (t_{24} = 5.59, $p = 9.52 \times 10^{-6}$) on qT1, accounting for age and sex as covariates in multiple linear regression. Mean age 23.13 (SD 5.30), with 24/6 male/female subjects.

In terms of clinical and functional consequences of low intracortical GSH, we observed a predictive relationship between low GSH and delayed response to antipsychotics. For every 10% move towards the lower end of GSH levels, patients had seven additional days of non-response to antipsychotics [69]. Lack of early response is a critical indicator of long-term poor outcomes in schizophrenia [117–119]. Significant deficits in ACC GSH observed in patients with early stage of treatment resistance is highly suggestive of a poor outcome trajectory [78]. Correlational studies also relate lower GSH to higher residual symptom burden [65], negative symptoms [120], and cognitive deficits [74] in established cases of schizophrenia.

In summary, patients with intracortical GSH deficit are likely to display structural and functional features indicative of poor outcome trajectories in schizophrenia. Nevertheless, due to the lack of temporal information required to separate causes vs. consequences, it is not clear if low ACC GSH plays a causal role in persistent poor outcome trajectory across the course of schizophrenia [67]. Longitudinal follow-up studies linking baseline GSH measurement to later long-term functional outcome are required in this regard.

6. Glutathione—Glutamate Relationship

In healthy physiological states, ACC GSH and glutamate levels are tightly correlated [65,68,69]. This may be related to the co-dependency of intracellular GSH and glutamate synthetic processes, with GSH acting as a reservoir for glutamate [121,122]. If GSH synthesis is reduced by blocking glutamate to GSH conversion, this increases cytosolic glutamate levels and synaptic excitatory potential. On the other hand, if GSH to glutamate conversion is blocked, this reduces glutamate concentration and synaptic excitation. Thus, intracortical GSH levels influence the prevailing glutamatergic tone, and vice versa. This raises the question of whether the subgroup of patients with higher-than-normal GSH levels in early stages of psychosis have a concurrent increase in glutamate levels.

Among the untreated first episode patients, we indeed noted higher glutamate levels in those with higher GSH levels, though this relationship was weaker than in healthy subjects [69]. Several observations indicate opposing effects of GSH and glutamate on the course of schizophrenia. Higher intracortical glutamate levels relate to reduced social function [69,123] and poorer treatment response [81] in the early stages and reduced cortical grey matter [83,124,125]. On the other hand, higher GSH levels relate to faster treatment response [69], better functioning [77], and preserved cortical grey matter [101] (See also [126,127] for association with peripheral GSH). We recently observed an opposing influence of glutamate and GSH on the intrinsic connectivity within the dACC node of the Salience Network in first episode psychosis [72]. Thus, GSH and glutamate may covary in their levels but have contrasting effects on the course of schizophrenia.

In schizophrenia, although the exact nature of glutamate dysfunction in schizophrenia is yet to be clarified, meta- and mega-analyses of MRS data demonstrate a glutamatergic deficit state, at least in the prefrontal cortex [66,128,129]. Emerging observations implicate antipsychotics in progressive glutamate reduction in schizophrenia [128] but evidence in this regard is still inconclusive (see [84,129]). Interestingly, lower GSH levels co-occur with reduced glutamate in the chronic stage [65,68], especially in ultra-treatment-resistant patients [71]. Thus, a GSH-deficit phenotype may be characterised by concurrent reduction in glutamate.

To understand the concurrent glutamate and GSH reduction in established schizophrenia, several explanatory models can be invoked. First, this concurrent reduction may reflect a persistent reduction in neural activity. In a healthy physiological state, sustained neural activity inevitably increases mitochondrial oxidative stress; as a result, increase in GSH may occur to reduce free radical burden. This activity-dependent GSH increase has been observed in short time scales (over a few minutes) using functional MRS in several [130–132] but not all [133–135] studies to date. While some increase in free radical production is inevitable in physiological states [136], there is no convincing evidence that this is sufficient to increase GSH levels, in the absence of a specific disruption in mitochondrial activity [137]. Instead, the task-dependent increase in GSH in physiological states may reflect an increase in conversion of the excess synaptic glutamate released during sustained neural activity; this diversion to GSH-synthesis can in turn reduce the availability of the precursor glutamate and reduce further excitation in a homeostatic manner. The presence of a negative correlation between BOLD signal and task-related GSH increase [130] in healthy subjects further supports this view. In this context, sustained reduction of intracortical GSH in a subgroup of patients may reflect a pervasively low neural activity of a given brain region. Thus, in chronic stages of schizophrenia when sustained effortful activity is diminished, low GSH levels can be expected accompanying a glutamatergic deficit state.

Another equally viable explanation for concurrent GSH and glutamate deficit in schizophrenia focusses on the putative primacy of the glutamatergic deficit [138]. A reduction in glutamate transport in the mitochondria may disrupt mitochondrial function and cause an increase of free radical production [139] and the consequent adaptive increase in GSH consumption. Finally, a third factor such as altered resting-state cerebral blood flow may lead to mitochondrial dysfunction, leading to both an increase in free radical species followed by lowered GSH and concomitant glutamatergic deficit. Recent evidence supports the notion of impaired mitochondrial function (especially, mitophagy, the elimination of defective mitochondria) in schizophrenia [140], though its relationship with GSH and glutamate in schizophrenia requires further investigation.

In summary, the fate of GSH and glutamate are highly intertwined throughout the longitudinal trajectory schizophrenia. Delineating the putative functional relevance of intracortical GSH deficit requires concomitantly measuring glutamate to clarify its relationship, as well as tracking the functional outcomes and cumulative treatment exposure [67] in schizophrenia.

7. Treatment-Engagement and Stratification Markers (GSH) for Antioxidant Trials

An exciting clinical utility of prospectively identifying patients with GSH-deficit is the therapeutic possibility of correcting it. A number of compounds with the potential to correct the effects of GSH deficit are in the pipeline (Table 2) [44,76,141–171]. Of these, N-acetylcysteine has been shown to improve cognition and negative symptoms (6 RCTs) [172], though the effect size is modest. Antioxidant therapies are more likely to benefit patients with a central antioxidant-deficit. Reliable characterisation of the GSH-deficit phenotype is critical in this regard. Furthermore, while the antioxidant pipeline is promising [44,76,141–171], reversing cognitive/negative symptoms requires longer trials that are substantially difficult to complete. We need reliable markers of biological efficacy that indicate engagement of the mechanistic target; this will help overcome several obstacles in clinical translation (e.g., targeted in vivo assay, dose finding, estimating trial duration, understanding placebo response). While MRS ACC GSH measurement provides a marker for treatment-engagement [173], given its variation with the illness phase, it is unlikely to become a standalone aid in patient selection for long-term trials. More accessible behavioural readouts (delayed response to antipsychotics or poor social or occupational functioning) may help in patient selection.

Table 2. Potential therapeutic agents that can alter intracortical GSH.

Drugs that Activate Nrf2-Mediated GSH Regulation	Drugs that Increase or Stabilise GSH Levels via Other Mechanisms
Sulforaphane (NCT02880462; NCT02810964; NCT01716858; NCT04521868)	N-acetylcysteine [172,174] (NCT02505477, NCT03149107)
Curcumin [154,155] (NCT02104752, NCT02298985)	Direct liposomal GSH [157] (NCT01967667)
Resveratrol [158,159]	Ebselen [44,146] (NCT03013400)
Quercetin [160,161] (NCT04063124),	Ethyl eicosopentanoic acid [76,145]
Genistein [162,163] (NCT01982578)	Glucose-dependent insulionotropic polypeptide [143]
Andrographolide [164]	Alpha-lipoic acid [144] (NCT03788759)
CXA-10 [165]	L-arginine [142] (NCT04054973)
Bardoxolone [171]	S-adenosylmethionine [149,150]
Omaveloxolone [166] (NCT02255435),	Sarcosine [167,168]
Sulforadex (SFX-01) [175] (NCT02614742)	Serine [147,148] (NCT04140773, NCT03711500)
Dimethylfumarate [152,153]—now used for MS	Telmisartan [141] (NCT03868839)
Luteolin [169,170]	Trehalose [156] (NCT02800161)

NCT numbers indicate selected ongoing or recently completed clinical trials in neuropsychiatric disorders. Nrf2 = Nuclear factor-erythroid factor 2-related factor 2.

In terms of neuroimaging-based stratification markers for long-term clinical trials, two promising approaches need further study. One is the use of measures reflecting the likely downstream effects of the GSH-deficit. Given the critical importance of restoring glutamate homeostasis by manipulating GSH levels, indices of glutamatergic dysfunction may prove to be useful indirect markers of the need for antioxidant trials. Using dynamic causal modelling (DCM) of resting state functional MRI, Limongi and colleagues linked higher ACC glutamate in untreated schizophrenia to a model of cortical disinhibition in the ACC-insula network [18]. Glutamate-related disinhibition in this network predicted computational parameters of cognitive dysfunction as well as social withdrawal [18], while GSH levels had a robust relationship with the state of excitation–inhibition imbalance in this assay, with higher GSH predicting reduced disinhibition, an opposite effect from glutamate. An antioxidant that reliably reduces such markers of glutamate-related cortical disinhibition (likely by increasing intracortical GSH) is likely to be 'hitting the target' relevant to schizophrenia.

Another potential stratification marker is the estimation of early 'response' in MRS GSH to antioxidants as an indicator of the likelihood of success of long-term treatment. In essence, this is similar to the use of early symptom reduction as a longer-term prognostic indicator. Several antioxidants act by supplying the precursor for GSH synthesis, taking a longer time to increase intracortical GSH. For example, NAC increases intracortical GSH by 23% in schizophrenia only after 24 weeks but not immediately after administration (single dose effect = 1.3% increase [176]). In contrast, some antioxidants such as sulforaphane have a more rapid and stable effect on GSH levels [177,178] by activating the Nrf2 gene, the most dominant regulator of antioxidant transcription pathways. Sulforaphane is 80% bioavailable, reaches peak plasma levels 1 h after oral ingestion, with first-order kinetics [$t_{1/2}$ ~2.5 h, 60% renal excretion at 8 h [179], full washout recorded in 3–5 days [180]] and has already been shown to increase intracortical GSH (using ultra-high field 7T MRS) in healthy volunteers (24% in 7 days) [181]. It is one of the antioxidants whose pharmacokinetics have been well studied [182], and whose safety profile has been established in clinical trials [183–185] with four ongoing trials in schizophrenia, making it a suitable drug to evaluate markers of biological efficacy. A notable sulforaphane-induced increase in MRS GSH level may predict a relative deficit state at the baseline, and thus a superior long-term functional response in patients.

8. Challenges and Opportunities

In summary, intracortical GSH is not abnormal in all patients who initially present with psychosis; but in a latent subgroup of patients with particularly adverse outcomes of schizophrenia, a pervasive intracortical GSH-deficit may result from a confluence of risk factors. A question of great translational importance is whether we can identify the subgroup of patients who will develop GSH-deficit at the onset of illness. The studies reviewed above suggest that this subgroup cannot be identified simply on the basis of genotyping or peripheral antioxidant measurements alone [67]. We suggest the following approaches for a reliable characterization of a pervasive intracortical GSH-deficit subtype:

1. The putative consequences of GSH-deficit in schizophrenia likely involve aberrant functional connectivity within key brain networks (e.g., the Salience Network for dorsal ACC GSH deficit), myelination as well as grey matter microstructure. Longitudinal multimodal imaging, preferably starting from untreated states, and experimental 'perturb-and-measure' approaches with pharmacological agents such as sulforaphane or NAC will provide the required temporal information to characterize a causal role for intracortical GSH on these features. This is essential to establish the biological construct validity of the GSH-deficit phenotype in vivo.
2. Attrition of the inception cohort is an important challenge in longitudinal studies of early-stage psychosis. Multi-site involvement is likely to be of critical importance to overcome this issue.

3. Subgroup identification based on continuous biological measures is a statistical challenge; a single cut-off value for clinical decisions may not readily emerge. To mitigate this, in addition to the use of growth mixture and clustering models, normative estimates of MRS GSH values and classification approaches to inform cutoff optimization may be required.
4. Several potential confounders/mediators of intracortical GSH (lifestyle variables, genetic variants, antipsychotic/antidepressant exposure, duration of illness, and substance use) require careful quantification to establish a relationship with outcomes of interest.
5. Isolated measures of intracortical GSH do not provide the context in which the observed reduction occurs; concurrent static or dynamic measurement of glutamate will provide the relevant information to study putative mechanistic changes. As of now, 7T-MR spectroscopy (MRS), with its attendant improvisation in signal detection hardware, pulse sequences, and spectral modelling, is positioned as the only human in vivo technique that can confidently isolate glutamate from other molecules and concurrently estimate glutathione resonance. Among the MRS studies specifically optimized for GSH detection, 7T studies [65,186] report higher effect size GSH reduction in schizophrenia compared to 3T [55,187].

Current methods for measuring human brain intracortical GSH levels via MRS in conjunction with other critically relevant metabolites of the cortical microcircuit such as glutamate and GABA currently have quantification precision in the range of 20–40%. The use of MRS methods optimized for one of these key metabolites improves the quantification precision below 10% for one metabolite at the detriment of others (see [188] for a detailed review). Some recent developments to study redox status in vivo utilizing hyperpolarized ^{13}C N-acetyl cysteine [189,190] or thiol-water proton exchange saturation transfer are promising to extend our insights into the glutathione system. Ultimately, developing MRS simultaneously optimized to provide quantification precision better than 10% for GSH, glutamate as well as GABA with greater spatial precision will open opportunities to explore mechanistic interventions targeting the schizophrenia redox-dysregulation subtype.

On the translational front, characterizing a redox-dysregulation subtype of schizophrenia using GSH-centered imaging holds significant promise for early intervention. Unlike data-driven subtyping approaches that are currently prevalent in the field, the redox-dysregulation or GSH-deficit subtype is based on the prediction of varying psychopharmacological outcomes i.e., superior response to antioxidants/poor response to antipsychotics. This may open the possibility of a stratified approach to pharmacological intervention in one subgroup and may reduce the iatrogenic burden of blanket trials across an entire diagnostic group over long time periods. Identifying reliable peripheral proxies for intracortical GSH will move this quest even closer to our clinics [191,192].

9. Conclusions

The GSH-deficit hypothesis offers a clinically actionable prognostic model in schizophrenia with a well-defined therapeutic utility. Longitudinal multimodal imaging studies combined with experimental 'perturb-and-measure' approaches can help delineate a putative redox-dysregulated subtype and establish its mechanistic primacy in the long-term trajectory of schizophrenia. This approach, if successful, will be a decisive step towards non-dopaminergic early intervention in schizophrenia.

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antioxidants

Review

Glutathione in the Nervous System as a Potential Therapeutic Target to Control the Development and Progression of Amyotrophic Lateral Sclerosis

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Abstract: Amyotrophic lateral sclerosis (ALS) is a rare neurological disorder that affects the motor neurons responsible for regulating muscle movement. However, the molecular pathogenic mechanisms of ALS remain poorly understood. A deficiency in the antioxidant tripeptide glutathione (GSH) in the nervous system appears to be involved in several neurodegenerative diseases characterized by the loss of neuronal cells. Impaired antioxidant defense systems, and the accumulation of oxidative damage due to increased dysfunction in GSH homeostasis are known to be involved in the development and progression of ALS. Aberrant GSH metabolism and redox status following oxidative damage are also associated with various cellular organelles, including the mitochondria and nucleus, and are crucial factors in neuronal toxicity induced by ALS. In this review, we provide an overview of the implications of imbalanced GSH homeostasis and its molecular characteristics in various experimental models of ALS.

Keywords: glutathione; oxidative stress; amyotrophic lateral sclerosis; neurogenerative disease

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1. Introduction

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a progressive neurodegenerative disease characterized by the selective loss of motor neurons in the spinal cord and motor cortex [1,2]. Muscle weakness and degradation at 2–5 years after the onset of symptoms can result in fatal muscle dystrophy, paralysis, and death [3,4]. Most ALS cases (90–95%) occur sporadically with no clearly related risk factors, while approximately 5–10% of ALS cases are considered to be hereditary, attributed to various mutations in specific genes [1]. Pathogenic mutations in genes encoding *Cu/Zn-superoxide dismutase 1* (*SOD1*), *chromosome 9 open reading frame 72* (*C9orf72*), *optineurin* (*OPTN*), *p97/valosin-containing protein* (*VCP*), *TAR DNA-binding protein* (*TDP-43*), *fused in sarcoma* (*FUS*), *Ewing sarcoma breakpoint region 1* (*EWSR1*), and *TATA box-binding protein-associated factor 15* (*TAF15*), have been linked to the sporadic and familial forms of ALS [5–9]. There are only two FDA-approved drugs for ALS, and both extend patient lifespan by a few months [10,11]. Therefore, there is an urgent demand for the development of therapeutics for ALS.

SOD1, the first discovered ALS-linked gene, codes for an important enzyme in the defense mechanism against oxidative stress. Mutations in the *SOD1* gene account for approximately 20% of familial ALS cases [12]. Previous studies have suggested that mutant SOD1 forms insoluble aggregates in the mitochondria and induces mitochondrial defects that lead to cell death [13]. Recently, dominant mutations in specific genes coding for different RNA-binding proteins with prion-like domains, including *TDP-43*, *FUS*, *EWSR1*, and *TAF15*, have been found in sporadic and familial ALS cases [14–17]. Several studies have discovered mutations and functions of RNA-binding proteins in the pathogenesis of ALS. The expression of wild-type or mutant human TDP-43 in mice has been found to result in the degeneration of motor neurons [18]. There is evidence of ALS onset as a

result of the presence of cytoplasmic inclusions in TDP-43 mutants in familial and sporadic ALS patients [3]. Furthermore, FUS is involved in RNA metabolism, including RNA processing, splicing, transport, and translation, by regulating cellular localization and degradation [9,15]. FUS is also localized in the nucleus and has been identified as a component of cytoplasmic inclusions in patients with familial ALS [19]. ALS-linked mutations in FUS lead to the mislocalization of FUS to the cytoplasm from the nucleus [20,21]. A recent study found a missense mutation in the *EWSR1* gene in patients with ALS [17]. Similar to other FET proteins, the mutant EWSR1 protein is mislocalized in the cytoplasm of the spinal cord [22]. The overexpression of EWSR1, namely EWSR1^{G511A} and EWSR1^{P552L}, in neurons leads to neuronal dysfunction in *Drosophila* [17]. In addition, mutations in the *TAF15* gene have been implicated in the pathogenesis of familial and sporadic ALS, and the TAF15 protein also forms prion-like aggregates in the cytoplasm of spinal cord neurons of rats, leading to increased neuronal toxicity [16,23,24]. The property of cytoplasmic aggregate formation in several ALS-linked proteins has been suggested to play a critical role in the development and progression of ALS. Therefore, the clearance and degradation of cytoplasmic aggregates in motor neurons could be a therapeutic strategy for ALS. However, despite efforts by several research groups, the pathogenic mechanisms underlying neurodegeneration through gene mutations and the screening of potential therapeutics remain poorly understood.

Oxidative stress arises from an imbalance between the production of reactive oxygen species (ROS) and the antioxidant system, which removes oxidative damage in cells and neurons. Therefore, maintaining this balance is critical in the pathogenesis of neurodegenerative diseases, including ALS. Numerous pathological studies have found evidence of increased oxidative stress in ALS. Previous studies have revealed increased oxidative damage to proteins in the tissue of ALS patients postmortem. Increased levels of protein carbonyl were observed in the spinal cord and motor cortex of patients with sporadic ALS [25]. Furthermore, several biomarkers for antioxidant defense and ROS damage are altered in the peripheral tissues and cerebrospinal fluid of patients with ALS [26,27]. The activity and expression of SOD, catalase, glutathione reductase (GR), and glutathione transferase (GST) are also reduced in the cerebrospinal fluid or peripheral blood mononuclear cells of patients with familial or sporadic ALS [28–30]. The reduced form of glutathione (GSH), a tripeptide, is well-known to non-enzymatically react with ROS to scavenge free radicals. A reduction in the GSH/oxidized glutathione (GSSG) ratio and GSH levels in cerebrospinal fluid has also been observed in patients with ALS [31]. Thus, the antioxidant defense mechanism against oxidative stress, which includes reactive species scavengers, is an important system in ALS, and the dysregulation of GSH homeostasis is believed to contribute to the development and progression of ALS. Although uncertainties remain regarding the mechanisms underlying the association between GSH and the dysfunction of neuronal cells and the specific functions of GSH that are critical for inducing neuronal toxicity, such as ROS production and protein aggregation in ALS pathogenesis, dysfunctional GSH metabolism, GSH-related enzymatic systems, such as GR and GST, and an imbalanced redox status are increasingly postulated to be crucial to the development and progression of ALS.

In this review, we provide an update on recent advances in our understanding of the relationship between GSH and the progression of ALS and an overview of the pathophysiological role of GSH deficiency in the brain. Specifically, we focus on evidence of aberrant GSH metabolism in various ALS models and summarize experimental studies supporting GSH redox imbalance as the cause of ALS both in vitro and in vivo. To our knowledge, this review is the first to focus on the functions of GSH in ALS conditions and its role as a potential therapeutic agent in ALS pathogenesis.

2. The Roles of GSH as an Antioxidant in the Nervous System

GSH is found at different levels in all cells. It accounts for approximately 95% of the total non-protein thiol groups in cells and is ubiquitously distributed in the body. High

levels of GSH have been found in the nervous system [32]. Many studies have suggested that an intensive metabolic exchange of GSH occurs between astrocytes and neurons, whose interactions appear to be critical for neuronal GSH homeostasis and the protection of neurons in the brain against ROS and oxidative damage.

GSH Synthesis and Cellular Distribution in Neurons

GSH is a tripeptide composed of glutamate, cysteine, and glycine. It is the most abundant thiol molecule found in tissues, including the brain, with a concentration of approximately 1–10 mM in the latter compared to approximately 2–3 mM in neurons, which is higher than that in the blood or cerebrospinal fluid (approximately 4 μM) [33–36]. GSH is synthesized in the cytosol by consecutive ATP-dependent reactions catalyzed by two enzymes, namely γ-glutamate-cysteine ligase (γ-GCL) and GSH synthetase (GS) [37,38]. γ-GCL mediates the first step of GSH synthesis, an ATP-dependent enzymatic process, with glutamate and cysteine to form the dipeptide, γ-glutamyl cysteine. γ-GCL is composed of a catalytic subunit (GCLC) and a modulatory subunit (GCLM), and is a rate-limiting enzyme in GSH synthesis [39]. Conditional *GCLC* knockout mice in whole neuronal cells displayed GSH depletion and neuronal cell death [40]. *GCLM* knockout mice exhibited reduced GSH levels and abnormal behavior phenotypes [41]. GS in the last step of GSH synthesis mediates the formation of GSH, which combines with glycine to form γ-glutamyl cysteine [42].

GSH is oxidized to glutathione disulfide (GSSG) by the reaction of glutathione peroxidase (GPx) coupled with the reduction of hydrogen oxide or hydroperoxides. GSH is regenerated from GSSG by GR, which uses NADPH as an electron donor [43]. Therefore, the reaction catalyzed by GPx and GR mediates GSH recycling. GSH is consumed by the extracellular release of GSH from cells via the generation of GSH-conjugates in the cytosol or oxidation to form GSSG. These processes induce a reduction in intracellular GSH. Thus, to maintain the intracellular levels of GSH, the synthesis, and recycling of GSH and the inhibition of GSH release must be induced. The depletion or redox imbalance of GSH (decreased GSH/GSSG ratio) has been reported to be involved in various neuronal dysfunction and neurodegenerative diseases, including autism, Alzheimer's disease, Parkinson's disease, and Huntington's disease [44–47].

Although GSH is synthesized exclusively in the cytosol, it is present in the most important cellular organelles, namely the mitochondria, endoplasmic reticulum (ER), peroxisome, and nucleus [48]. These results suggest that GSH has specific functions in different cell compartments. Mitochondrial GSH represents 10–15% of the cellular GSH [49]. As the mitochondria lack the enzymes involved in de novo GSH synthesis, the maintenance of mitochondrial GSH levels depends on its uptake from the cytosol via carrier-mediated transporter systems. Many studies have shown that mitochondrial protection systems against free radicals and ROS, such as GSH, are critical for protecting neuronal cells from oxidative stress in the mitochondria. Moreover, mitochondrial GSH is important for protecting the organelles from ROS generated via the oxidative phosphorylation system. Muyderman et al. found that the selective depletion of mitochondrial GSH in astrocytes significantly increased hydrogen peroxide-induced apoptotic cell death and provided evidence for the crucial role played by mitochondrial GSH in preserving cell viability [50]. Wüllner et al. investigated the effects of acute GSH depletion and reduced mitochondrial GSH in relation to mitochondrial dysfunction in the cerebellar granule neurons of rats [51]. They found that the depletion of neuronal mitochondrial GSH led to a significant increase in ROS production and cell death in the nervous system. Furthermore, Wilkins et al. investigated the mechanism of mitochondrial GSH transport in the brain [52]. They showed that the dysfunction of mitochondrial GSH transporters, such as dicarboxylate and 2-oxoglutarate carriers, could result in an increased susceptibility of neurons to oxidative stress. These results demonstrate that the maintenance of mitochondrial GSH via sustained mitochondrial GSH transport is critical for protecting neurons from oxidative stress. Feng et al. also showed a reduction in mitochondrial GSH in the brain and spinal cord of *GCLC*-

deficient mice [40]. Therefore, reduced mitochondrial GSH levels might be associated with mitochondrial defects in the nervous system.

The cytosol in cells is maintained in a reduced state to stabilize the free thiol groups. However, the ER environment is more oxidized than the cytosol to promote disulfide bond formation [48,53]. The redox environment in the ER influences the activity of various enzymes, including protein disulfide isomerase (PDI), which is responsible for the formation of disulfide bonds [53]. Previous studies have reported that the maintenance of redox status by regulating the GSH:GSSG ratio in the ER is considerably more oxidized than that in the cytosol [54]. Measuring the GSH levels in the ER revealed that the GSH:GSSG ratio in the ER is between 1:1 and 3:1 [55]. Furthermore, several studies have suggested the role of ER GSH in the formation of protein disulfide bonds; GSH is suggested to act as a net reductant in the ER by maintaining ER oxidoreductases, including PDI and endoplasmic reticulum oxidation 1 (ERO1) in a reduced state or by directly reducing non-native disulfide bonds in folding proteins [56–58]. Tsunoda et al. revealed that the selective depletion of ER GSH by expressing a cytosolic GSH-degrading enzyme, ChaC1, in the ER did not alter protein folding or ER stress response [59]. Although this study suggests the existence of an alternative electron donor that maintains the redox status in the ER, this result does not exclude the importance of the role of GSH in protein folding in the ER. However, direct evidence for the role of ER GSH in neuronal cells has yet to be found. However, as misfolded and aggregated proteins in the ER are one of the causes of neurodegenerative diseases, there is a need to determine the precise role of GSH in the ER of neurons.

GSH is present in the peroxisome—it is transported from the cytosol to the peroxisome by diffusion across the peroxisomal membrane [60,61]. Furthermore, GSSG is thought to be exported to the cytosol through a peroxisomal glutathione transporter, Opt2, and is subsequently reduced to GSH by cytosolic GR in an NADPH-dependent manner [62]. Catalase, peroxidase, and GSH are major components of the peroxisomal antioxidant system. GSH peroxidase (GPx) in the peroxisome requires GSH as a cellular reductant to reduce hydrogen peroxide to water [63].

GSH also plays a critical role in the nucleus. Previous studies have shown that the distribution of GSH to the nucleus is a critical factor in cell proliferation [64]. In plants, GSH depletion blocks the transition from G1 to S phase in the cell cycle of the root [65]. Moreover, GSH recruitment into the nucleus can regulate chromatin structure and condensation, which controls gene expression [66]. Therefore, the recruitment and translocation of GSH from the cytosol to the nucleus during the cell cycle has a great influence on cellular redox homeostasis and gene expression. Jeong et al. investigated GSH levels in living mammalian cells using a fluorescent real-time thiol tracer (FreSHtracer) [67]. They found that GSH levels were markedly higher in the nucleus than in the cytosol, and GSH was required for the maintenance of stem cell functions. Miller et al. also investigated the precise localization of GSH in the mouse central nervous system and found that GSH is synthesized in neurons and diffuses into the nucleus to protect DNA from oxidative stress [68]. However, the molecular functions of GSH in the nucleus of neurons and the mechanisms of GSH transport to the nucleus in neurons are not yet clearly understood and remain a topic of debate.

3. Evidence for the Dysfunction of GSH Metabolism in ALS

3.1. GSH Redox Imbalance in Cellular Models of ALS: In Vitro Studies

Extensive research has been conducted to investigate GSH metabolism and redox imbalance in experimental cellular models of ALS. As a result, an aberrant GSH redox balance, including GSH depletion in the nervous system, has been identified as a pathogenic mechanism of ALS in various in vitro models (Table 1). Lee et al. found that the levels of GSH and relative enzymes were decreased in familial ALS models [69]. They also examined the levels of GSH and GSSG in NT-2 cells (human teratocarcinoma cells) and SK-N-MC cells (human neuroblastoma cells) expressing human wild-type SOD1 or SOD1 mutants, including G37R and G85R, which are associated with fALS [69]. The GSH levels were found

to be significantly reduced in NT-2 cells expressing SOD1^{G37R} or SOD1^{G85R}. Furthermore, The GSH levels were significantly decreased in SK-N-MC cells expressing SOD1^{G37R} or SOD1^{G85R}. However, the levels of GSSG in both mutants of the SOD1-expressing cells were increased. The activity of Gpx and protein carbonylation, a marker of protein damage induced by oxidative stress, was increased in cells transfected with the SOD1 mutants [69]. Rizzardini et al. showed that GSH depletion induced by ethacrynic acid impairs mitochondrial functions, including ROS production and membrane potential, in NSC-34 mouse motor neurons [70]. To investigate the effects of GSH depletion in motor neurons, NSC-34 cells were treated with ethacrynic acid, a GSH-depleting agent that directly conjugates GSH. The decreased GSH levels led to increased ROS, loss of mitochondrial membrane potential, and apoptosis. Furthermore, these researchers developed a cellular model of fALS by transfecting NSC-34 cells with human wild-type SOD1 or mutant SOD1^{G94A}. SOD1^{G94A}-expressing cells are more sensitive to mitochondrial dysfunction, including decreased mitochondrial membrane potential induced by ethacrynic acid, a GSH-depleting agent [71]. These results indicate that GSH depletion may be a suitable indicator in studies of the pathogenic mechanisms of oxidative stress-induced toxicity in motor neurons, suggesting that oxidative stress coupled with mitochondrial damage induces the development of disease onset similar to ALS.

Chi et al. investigated the effect of cellular GSH alterations on the cell death of motor neurons [72]. Their results showed that the treatment of NSC-34 cells with ethacrynic acid or L-buthionine sulfoximine significantly reduced GSH production and was accompanied by increased ROS generation. GSH depletion by ethacrynic acid enhanced the expression of the oxidative stress markers AP-1, c-Jun, c-Fos, and HO-1. Moreover, GSH depletion promoted apoptotic cell death by increasing cytochrome c release and capase-3 activation in NSC-34 cells [72]. Collectively, these results strongly suggest that decreased cellular GSH production and availability leads to increased intracellular oxidative stress and apoptosis, and promotes oxidative stress propagation and motor neuronal degeneration. Muyderman et al. also investigated the role of mitochondrial GSH in NSC-34 cells with human SOD1^{G93A}. Interestingly, cells stably expressing SOD1^{G93A} showed significantly decreased mitochondrial GSH levels [73]. Furthermore, the treatment of SOD1^{G93A}-expressing cells with ethacrynic acid resulted in increased apoptotic cell death and decreased the mitochondrial GSH pool. Such finding suggests that SOD1^{G93A} regulates mitochondrial oxidative stress by inducing the selective loss of the GSH pool in mitochondria.

Glutamate, an excitatory neurotransmitter in the mammalian nervous system, is a substrate for glutamate cysteine ligase together with cysteine in an ATP-dependent reaction [74]. This is the rate-limiting step in the synthesis of GSH, the main antioxidant in the central nervous system [74]. Abnormalities in both glutamate and glutamine levels were identified in the nervous tissues of a mouse model of fALS [75]. As a result, Cantoni's group investigated the relationship between GSH synthesis and glutamine metabolism in a cellular model of fALS [76]. They found that both GSH and glutamate levels were significantly decreased in SOD1^{G93A}-expressing NSC-34 cells cultured with the standard concentrations of glucose and glutamine, as well as alterations in the metabolic pathways involving glutamine/glutamate [76]. These results indicate that the decrease in GSH caused by SOD1^{G93A} expression is due to mitochondrial dysfunction associated with the reduction of the flux of glucose-derived pyruvate.

GSH production is regulated by the Nrf2 signaling pathway. This signaling pathway is impaired in various SOD1 models of fALS [77], and may therefore play a critical role in ALS pathogenesis. Moujalled et al. studied the GSH content in primary astrocytes from TDP-43^{Q331K}-expressing mice and found a significant impairment in total GSH induction in response to sodium arsenite treatment in the astrocytes of TDP-43^{Q331K}-expressing mice [78]. This result suggested that the mutant form of TDP-43 impairs the production of GSH by regulating the Nrf2 antioxidant signaling pathway. Moreover, Muyderman's group showed that GSH is depleted, resulting in increased ROS levels in TDP-43^{A315T}-expressing NSC-34 cells [79]. The depletion of GSH in TDP-43 ^{A315T}-expressing cells

or the loss of TDP-43 function resulted in increased intracellular ROS production, cell death, and cytosolic mislocalization of TDP-43, whereas protection against mutant TDP-43-mediated cytotoxicity was restored by increasing intracellular GSH levels by treatment with GSH monoethyl ester [79]. Collectively, these results suggest that oxidative stress is a critical factor in TDP-43-associated ALS pathogenesis and may result from the loss of GSH production. Therefore, novel therapeutics that restore GSH content in motor neurons may be beneficial in the prevention of TDP-induced neurotoxicity.

3.2. GSH Redox Imbalance in Animal Models of ALS: In Vivo Studies

Several in vitro studies have implicated GSH redox imbalance as a critical mediator of increased ROS generation and apoptosis in motor neurons and astrocytes. Mutations in ALS-causing genes have been found to lead to GSH depletion and neuronal toxicity. Therefore, there is a need to determine the precise role of GSH depletion and novel regulators of ALS pathogenesis. In particular, the novel regulatory mechanisms of GSH redox imbalance, including GSH depletion, dysfunction of GSH metabolism, and GSH transport, associated with ROS production in in vivo ALS models, need to be explored. Consistent with in vitro cellular studies, in vivo studies using various ALS animal models have provided evidence that the expression of ALS-causing genes activates various pathways and regulators that lead to a GSH redox imbalance. Astrocytes may play a critical role in the survival of motor neurons in ALS (Figure 1) [80]. According to previous studies, increased levels of GSH caused by activated astrocytes and released GSH from astrocytes improve the antioxidant status of co-cultured neurons [81–84]. Vargas et al. showed that GSH production in spinal cord astrocytes from $SOD1^{G93A}$-expressing rats can prevent motor neuron apoptosis induced by nitric oxide [85]. Further, these researchers found that the neurotoxic effects of $SOD1^{G93A}$ expression in astrocytes can be counteracted by increased GSH levels induced by the activation of the Nrf2 signaling pathway [85]. They also revealed that Nrf2 activation in astrocytes protects against neuronal toxicity in $SOD1^{G93A}$ transgenic mice [86]. Nrf2 overexpression in astrocytes increased the survival rate and delayed neuromuscular denervation in $SOD1^{G93A}$ transgenic mice. Furthermore, an increased GSH content was observed in the spinal cord and cerebellum tissues of $SOD1^{G93A}$-coexpressing mice with Nrf2 [86]. Such a finding indicates that the activation of the Nrf2 signaling pathway can restore motor neuronal toxicity by increasing GSH synthesis and release in astrocytes (Figure 1).

Both Liu's and Linseman's groups studied the levels of GSH and GSSG in whole blood and the spinal cord lumbar region of $SOD1^{G93A}$-expressing mice during disease progression [72,87]. As a result, decreased extracellular GSH levels were observed in the whole blood of $SOD1^{G93A}$-expressing mice. Moreover, intracellular GSH levels in motor neurons were found to be significantly reduced during disease progression in the mice [87]. However, the GSSG levels were significantly increased in $SOD1^{G93A}$-expressing mice [72]. Therefore, reduced GSH may contribute to motor neuron cell death by inducing the nuclear translocation of apoptosis-inducing factor (AIF) in $SOD1^{G93A}$ transgenic mice. Vargas et al. examined the effect of decreased GSH in ALS models with the knockout of a modifier subunit of glutamate-cysteine ligase (GCLM) [88]. Decreased GSH was found to reduce survival and neurons in the ventral horn and spinal cord tissues of $SOD1^{G93A}$-expressing mice with *GCLM* knockout. Moreover, increased ROS production and mitochondrial dysfunction were also observed in $SOD1^{G93A}$-expressing mice with GCLM knockout [88]. The researchers also investigated the effects of *GCLM* loss in $SOD1^{WT}$-expressing mice [89]. A reduction of approximately 70% in the total GSH content was observed in the brain cortex, brainstem, and spinal cord in the *GCLM* knockout of $SOD1^{WT}$-expressing mice. Interestingly, the loss of *GCLM* resulted in a decreased survival, increased motor neuron loss, and accelerated muscle denervation in $SOD1^{WT}$-expressing mice [89]. A reduction in GSH synthesis caused by the loss of *GCLM* may contribute to disease development and progression by modulating mitochondrial function in $SOD1^{WT}$ and $SOD1^{G93A}$-induced mouse models of ALS.

The cystine/glutamate antiporter (system X_C-) is an important factor for GSH synthesis, tasked with transporting cystine into the cell to facilitate the release of glutamate into the extracellular space [90,91]. Cytoplasmic cystine is reduced to cysteine and is used in GSH synthesis in the brain [92,93]. Albano et al. revealed increased cystine uptake in the spinal cord of $SOD1^{G93A}$ transgenic mice [94]. Therefore, it is possible that enhanced system X_C- activity could be a protective mechanism for oxidative damage in motor neurons by maintaining intracellular GSH levels.

Treatment with urate has been found to suppress oxidative stress-induced toxicity in vitro [95]. Moreover, Serum uric acid levels were found to be reduced in patients with ALS [96,97]. Thus, decreased urate levels may be associated with the development and progression of neurodegenerative diseases, including ALS. Zhang et al. showed that treatment with urate increased the levels of GSH in the $SOD1^{G85R}$-expressing *Drosophila* model of fALS by upregulating the Akt signaling pathway and catalytic subunit of glutamate-cysteine ligase (GCLC) [98]. This finding indicates that urate plays a neuroprotective role in motor neuronal damage by activating GSH synthesis.

Glucocorticoids are cholesterol-derived steroid hormones secreted exclusively by the adrenal gland and are critical regulators of homeostasis under basal and stress conditions [99,100]. Glucocorticoids are involved in excessive ROS production, which is associated with various neurodegenerative diseases, including TDP-43-induced proteinopathies [101,102]. Caccamo et al. found that dexamethasone, a synthetic glucocorticoid, increased the susceptibility to TDP-43-induced neurotoxicity in a mouse model [103]. Dexamethasone treatment was also found to exacerbate memory deficits and impair autophagy activation in the C-terminal fragment of TDP-43 and TDP-25-expressing mice. By examining the alteration of cellular redox status following dexamethasone treatment, the researchers found that the GSH/GSSG ratio was significantly decreased in dexamethasone-treated TDP-25-expressing mice [103]. The data indicate that there is a correlation between the decrease in the GSH/GSSG ratio and TDP-25-induced neurotoxicity in the brains after treatment with dexamethasone. Finally, accumulating evidence suggests that further research on the dysregulation of GSH metabolism and GSH-related enzymes induced by neurotoxic conditions could facilitate the development of a potential therapeutic strategy for ALS.

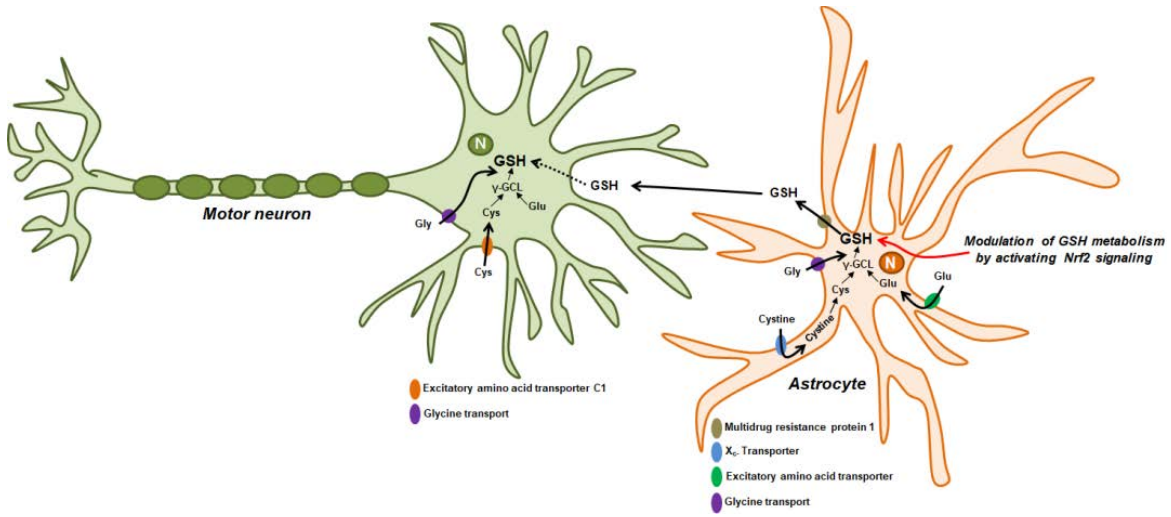

Figure 1. Astrocyte-mediated regulation of GSH biosynthesis and transport to motor neurons. The biosynthesis and release of GSH are associated with the Nrf2 signaling pathway in astrocytes [83]. Astrocytes take up cysteine, glutamate, and glycine through various transporters; thereafter, GSH is released from astrocytes via multidrug resistance protein 1 and transported to the motor neurons [104]. Cysteine and glutamate uptake are mediated by the X_{c-} transporter and excitatory amino acid transporter in astrocytes [91,105]. Glycine is transported by astrocytic glycine transport type 1 [106]. Intracellular GSH is synthetized from these amino acids in astrocyte [107]. Extracellular GSH may be taken up by motor neurons directly or indirectly [108]. Motor neurons also take up three amino acids for GSH synthesis. Increased GSH synthesis in astrocytes may exert a protective effect against motor neuronal toxicity under oxidative stress and in ALS.

Table 1. Alteration of GSH content in various experimental models of ALS.

Cell Lines and Animals	Experimental ALS Models	Phenotypes Associated with Oxidative Stress	GSH Status	Reference
NT-2 SK-N-MC	Human $SOD1^{WT}$ Human $SOD1^{G37R}$ Human $SOD1^{G85R}$	Increased protein carbonyl Increased 8-OHG Increased lipid peroxidation	Decrease GSH Increased GSSG	[69]
NSC-34	Ethacrynic acid treatment	Decreased mitochondrial membrane potential Increased ROS generation Increased apoptosis	Decreased GSH	[70]
NSC-34	Human $SOD1^{WT}$ Human $SOD1^{G93A}$	Decreased cell viability Increased ROS generation Decreased mitochondrial membrane potential Increased mitochondrial toxicity	-	[71]
NSC-34	Ethacrynic acid treatment	Increased ROS generation Increased oxidative response gene expression Increased apoptosis	Decreased GSH	[72]
NSC-34	Human $SOD1^{G93A}$	Decreased cell proliferation Decreased cell viability Increased apoptosis	Decreased mitochondrial GSH	[73]
NSC-34	Human $SOD1^{WT}$ Human $SOD1^{G93A}$	Increased mitochondrial dysfunction	Decreased GSH	[76]
NSC-34	Human $TDP\text{-}43^{M337V}$	Increased gene expression of Nrf2 signaling pathway	Decreased GSH	[78]
NSC-34	Human $TDP\text{-}43^{WT}$ Human $TDP\text{-}43^{A315T}$	Increased ROS generation Decreased cell viability Increased cell death	Decreased GSH	[79]
NSC-34	Human $SOD1^{G93A}$	Restored cell viability by treating urate	Increased GSH by treating urate	[98]
Rat astrocytes	Human $SOD1^{G93A}$	Restored cell survival by activating Nrf2	Increased GSH by activating Nrf2	[85]
Mouse motor neurons	Human $SOD1^{G93A}$	Increased apoptosis	Decrease GSH Increased GSSG	[72]
Mouse astrocytes	Human $SOD1^{G93A}$	Extended survival by expressing Nrf2 Delayed muscle denervation by expressing Nrf2	Increased GSH secretion by expressing Nrf2	[86]
Mouse astrocytes	Human $SOD1^{G93A}$ Human $SOD1^{H46R/H48Q}$	Decreased cell survival by *GCLM* knockout Increased motor neuron loss by *GCLM* knockout Increased oxidative stress by *GCLM* knockout Decreased complex IV activity by *GCLM* knockout	Decrease GSH by *GCLM* knockout	[88]
Mouse	Human $SOD1^{G93A}$	Increased cystine uptake by cystine/glutamate antiporter	-	[94]
Fly	Human $SOD1^{WT}$ Human $SOD1^{G85R}$	Extended survival by treatment with urate Improved motor defect by treatment with urate Enhanced antioxidant enzyme activity by treatment with urate Decreased ROS level by treatment with urate	-	[98]
Mouse	Human TDP-25	Increased memory deficit by treatment with dexamethasone	Decreased GSH/GSSG ratio by treatment with dexamethasone	[103]
Mouse	Human $SOD1^{G93A}$	Decreased survival	Decreased GSH in whole blood and spinal cord	[87]
Mouse	Human $SOD1^{WT}$	Decreased cell survival by *GCLM* knockout Increased motor neuron loss by *GCLM* knockout	Decrease GSH by *GCLM* knockout	[89]

3.3. GSH Redox Imbalance in ALS Patients

Previous studies have identified binding sites for GSH in the synaptic membranes of the brain and spinal cord [109,110]. In 1993, Lanius et al. examined GSH binding in the spinal cord of sALS patients using a radioactively labeled GSH, [^{35}S]-GSH, and found that [^{35}S]-GSH binding in the spinal cord was increased in patients with sALS [111]. Similarly, Babu et al. observed an imbalance in the antioxidant system in the erythrocytes of patients with sALS [28]. In fact, they found that GSH levels were significantly decreased in the erythrocytes of patients with sALS. Intracellular GSH recycling is catalyzed by GR using NADPH as a reducing agent to convert GSSG to two molecules of GSH [112]. GR activity was also found to be reduced in sALS patients compared to that in control patients [28]. Another group found that erythrocyte GPx activity was significantly impaired in patients with sALS, which remained low during disease progression [29]. GPx catalyzes the detoxification of hydrogen peroxide by utilizing GSH as a major source of protection against oxidative damage in the nervous system [113]. Thus, it can be hypothesized that an antioxidant imbalance, including the depletion of GSH and GSH-related enzymes, could be a contributing factor to the development and progression of ALS.

Weiduschat et al. investigated the in vivo levels of GSH in the motor cortex region of patients with ALS using the J-edited spin-echo difference magnetic resonance spectroscopy (MRS) technique [114]. Consistent with this study, other groups have reported a decrease in GSH levels in the motor cortex and corticospinal tract in patients with ALS compared to healthy controls [115,116]. The GSH levels in the corticospinal tract were more strongly correlated with disease progression than those in the motor cortex. Decreased GSH levels in the motor cortex of patients with ALS are likely to be a manifestation of clinical changes and pathogenic processes specific to ALS. Yang et al. also performed a Mendelian randomization analysis to identify ALS-associated metabolites, and detected 18 metabolites, including γ-glutamyl amino acids, that may exert causal effects on the development and progression of ALS [117]. The γ-glutamyl cycle is responsible for GSH biosynthesis [118]. Therefore, oxidative damage caused by the dysfunction of GSH metabolism may be a critical risk factor associated with the pathogenesis of ALS.

4. Clinical Trials in ALS

In 1998, Schiffer's group studied the effect of GSH treatment on the rate of progression of ALS in patients and found that GSH treatment did not have a significant effect on ALS progression [119]. Some cysteine-containing molecules, such as N-acetylcysteine (NAC) and procysteine, are used to increase GSH levels. Brown's group studied the pharmacokinetic properties of procysteine, a cysteine prodrug that increases intracellular GSH levels in patients with ALS [120]. However, the subcutaneous infusion of NAC did not reduce disease progression in patients with ALS [121]. Of note, the GSH levels in the cerebrospinal fluid of patients with ALS are markedly reduced with aging. Although the oral administration of cysteine-containing molecules did not have statistically beneficial effects on ALS patients, cysteine-containing molecules may be a valuable therapeutic option given its ability to increase GSH content in the cerebrospinal fluid.

5. Conclusions

GSH exerts important functions in the central nervous system as an antioxidant, enzyme cofactor, redox buffer, and neuromodulator. GSH deficiency and the dysfunction of GSH metabolism are common to several neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease. Although accumulating evidence suggests that aberrant GSH homeostasis is linked to the development and progression of ALS, the precise role and mechanism of redox imbalance in neuronal cells in this disease remains to be determined. In this review, we first summarized the experimental evidence supporting the role of GSH in the development and progression of ALS. Many studies on ALS have focused on the decreased GSH content and imbalanced GSH redox status in the nervous system, which result in ALS progression. Furthermore, several GSH-related enzymes that

target oxidative stress and regulate GSH homeostasis have also been studied. The mechanism of ALS progression may involve decreased GSH, increased GSSG, and an impaired GSH-associated antioxidant system, suggesting that an imbalance in redox status beyond its physiological limit may be detrimental to motor neuronal functions and survival that regulate disease initiation and conditions. Although several studies provide indirect or direct evidence that the dysregulation of GSH homeostasis and metabolism in the nervous system are associated with the pathogenesis of ALS, it remains unclear whether reduced GSH is a causative factor in ALS or whether various mutations in ALS-causing genes are responsible for the impaired GSH redox status observed in ALS. In addition, the molecular mechanisms and pathways related to the synthesis, transport, and degradation of GSH in neurons and astrocytes are complex, and our understanding of the imbalanced GSH redox status in the brain of ALS patients remains incomplete. Therefore, future studies should aim to intensively investigate whether treatments that increase the GSH/GSSG ratio in motor neurons and astrocytes by activating GSH biosynthesis have clinical efficacy in the treatment of ALS. In addition, there is a need to study and develop effective pharmacological agents that may enhance GSH functions to reduce oxidative stress and damage cellular organelles, including the mitochondria, in motor neurons.

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Review Article

Glutathione Homeostasis and Functions: Potential Targets for Medical Interventions

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Glutathione (GSH) is a tripeptide, which has many biological roles including protection against reactive oxygen and nitrogen species. The primary goal of this paper is to characterize the principal mechanisms of the protective role of GSH against reactive species and electrophiles. The ancillary goals are to provide up-to-date knowledge of GSH biosynthesis, hydrolysis, and utilization; intracellular compartmentalization and interorgan transfer; elimination of endogenously produced toxicants; involvement in metal homeostasis; glutathione-related enzymes and their regulation; glutathionylation of sulfhydryls. Individual sections are devoted to the relationships between GSH homeostasis and pathologies as well as to developed research tools and pharmacological approaches to manipulating GSH levels. Special attention is paid to compounds mainly of a natural origin (phytochemicals) which affect GSH-related processes. The paper provides starting points for development of novel tools and provides a hypothesis for investigation of the physiology and biochemistry of glutathione with a focus on human and animal health.

1. Introduction

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) with multiple functions in living organisms [1–4]. As a carrier of an active thiol group in the form of a cysteine residue, it acts as an antioxidant either directly by interacting with reactive oxygen/nitrogen species (ROS and RNS, resp.) and electrophiles or by operating as a cofactor for various enzymes [5–8]. Glutathione is moderately stable in the intracellular milieus because intracellular peptidases can cleave peptide bonds formed by the α-carboxyl groups of amino acids, but typically not the γ-carboxyl groups.

The reduced and oxidized forms of glutathione (GSH and GSSG) act in concert with other redox-active compounds (e.g., NAD(P)H) to regulate and maintain cellular redox status [9]. The former is quantitatively described by the redox potential, calculated according to the Nernst equation. In most cells and tissues, the estimated redox potential for the GSH/GSSG couple ranges from −260 mV to −150 mV (cited after [10]).

GSH is synthesized in a two-step process catalyzed by L-glutamate: L-cysteine γ-ligase, (γGLCL, EC 6.3.2.2) (also called γ-glutamyl-L-cysteine ligase or γ-glutamylcysteine synthase), and glutathione synthase (GLS, EC 6.3.2.3). GSH is consumed in many ways, such as by oxidation, conjugation, and hydrolysis [11]. GSH can be directly oxidized by ROS and RNS or indirectly during GSH-dependent peroxidase-catalyzed reactions. Conjugation with endogenous and exogenous electrophiles consumes a substantial portion of cellular GSH. In addition, cells may lose GSH due to export of its reduced, oxidized or conjugated forms. Extracellularly, GSH can be hydrolyzed by γ-L-glutamyl transpeptidase (GGT, EC 2.3.2.2) transferring the γ-glutamyl functional group to water during hydrolysis to form free glutamate [12]. The enzyme may also transfer the γ-glutamyl moiety of GSH to amino acids and peptides. Frequently, products of GSH hydrolysis are taken up by cells either as individual amino acids, or as dipeptides. The intra- and extracellular GSH levels are determined by the balance between its production, consumption, and transportation. Due to important physiological functions of GSH, these processes are tightly regulated. The activities of the enzymes involved in GSH metabolism are controlled

at transcriptional, translational, and posttranslational levels [3, 11].

Since GSH participates not only in antioxidant defense systems, but also in many metabolic processes, its role cannot be overestimated. Therefore, it is not surprising that the GSH system has attracted the attention of pharmacologists as a possible target for medical interventions. The main efforts in this field have been applied to decreasing or increasing GSH levels in organisms. General strategies involve specific inhibition of γGLCL, a key enzyme of GSH biosynthesis, and depletion of cellular reserves by externally added electrophiles (usually for research purposes). The use of buthionine sulfoximine (BSO) is probably the most popular approach to depleting GSH. BSO was first synthesised as the D,L-form [13, 14] and later as the L-BSO enantiomer [15]. Usually a mixture of D- and L-BSO is used in experiments [16–18]. GSH levels may be enhanced by supplementation with precursors, mainly cysteine in the form of different esters. However, during the the last decade a new approach for the regulation of GSH-utilizing enzymes has emerged. It is evident that many of these are induced at the transcriptional level by mild oxidative stress, which involves binding of the Nrf2 transcription factor to the antioxidant response element (ARE) (also called the electrophile response element; EpRE) in the promoter region of genes encoding certain enzymes, particularly γGLCL and glutathione *S*-transferases [19–22].

Glutathione has several additional functions in cells. For example, it is (i) a reserve form of cysteine, (ii) stores and transports nitric oxide, (iii) participates in the metabolism of estrogens, leukotrienes, and prostaglandins, the reduction of ribonucleotides to deoxyribonucleotides, the maturation of iron-sulfur clusters of diverse proteins, (iv) involved in the operation of certain transcription factors (particularly those involved in redox signalling), and (v) the detoxification of many endogenous compounds and xenobiotics [11].

The present review will focus on the molecular mechanisms of operation of the GSH system, with special attention to regulatory pathways controlling the expression of the enzymes involved. Information on GSH biosynthesis, hydrolysis and utilization, intracellular compartmentalization, and interorgan transfer will be highlighted. Special sections will deal with GSH functions, such as antioxidant properties and relationship to specific enzymes. On the basis of these mechanisms, some potential approaches for medical interventions will also be evaluated.

2. Glutathione Biosynthesis, Hydrolysis, Excretion, and Utilization

Intracellular GSH concentrations usually range from 0.5 to 10 mM, whereas extracellular values in animals are one to three orders of magnitude lower [2, 11]. GSH is commonly the most abundant low molecular mass thiol in animal and plant cells. Most microorganisms also possess GSH in high concentrations, but there are some species and viable mutants lacking GSH [23–25].

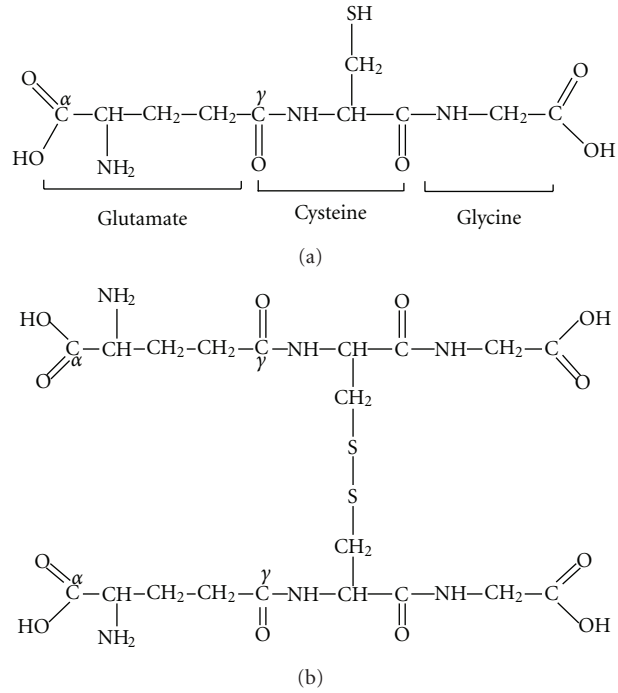

Figure 1: Glutathione is a tripeptide: L-γ-glutamyl-L-cysteinyl-glycine. In its reduced form (a) the N-terminal glutamate and cysteine are linked by the γ-carboxyl group of glutamate, preventing cleavage by common cellular peptidases and restricting cleavage to γ-glutamyltranspeptidase. The cysteine residue is the key functional component of glutathione, providing a reactive thiol group that plays an essential role in its functions. Furthermore, cysteine residues form the intermolecular dipeptide bond in the oxidized glutathione molecule (b).

Figure 1 shows the chemical structure of reduced and oxidised glutathione forms. GSH is formed from glutamate, cysteine, and glycine (Figure 1(a)), but it possesses an unusual peptide bond. The N-terminal glutamate and cysteine residues are linked by the γ-carboxyl group of glutamate, rather than the common linkage in proteins of an α-carboxyl peptide bond. This specific peptide bond prevents GSH from being hydrolyzed by most peptidases that cleave at the α-carboxyl peptide bond of N-terminal amino acids. This configuration also restricts the cleavage of GSH by GGT localized on the external surface of certain cell types. As a result, GSH is relatively stable in the cell and is cleaved by GGT only at external sides on the membranes of certain cells. In addition, the presence of the C-terminal glycine residue in the GSH molecule protects it against cleavage by intracellular γ-glutamyl cyclotransferase. The major oxidized form of glutathione (i.e., glutathione disulfide, GSSG) consists of two residues of GSH that have been oxidized in such a fashion as to be connected by an intermolecular disulfide bond (Figure 1(b)).

The steady-state level of cellular GSH is provided by the balance between production and consumption, as well as by extrusion from the cell as reduced, oxidized, or bound forms (summarized in Figure 2). GSH is produced in two steps. In the first step, the enzyme γGLCL forms a peptide bond

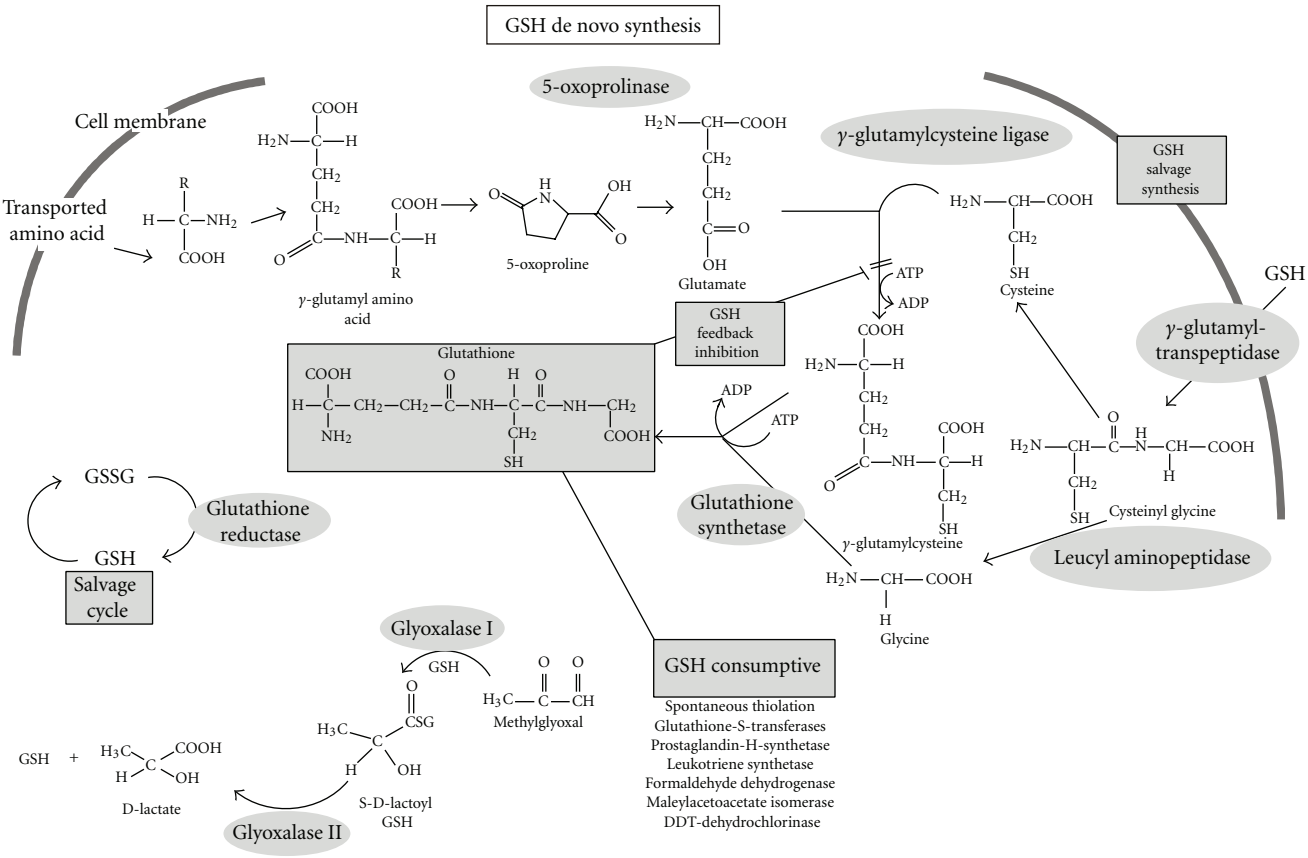

Figure 2: Glutathione homeostasis involves both intra- and extracellular mechanisms. Glutathione is synthesized in both *de novo* and salvage synthesis pathways. *De novo* synthesis requires the three amino acids and energy in the form of ATP. Glutamate may be provided in part from the conversion of a γ-glutamyl amino acid to 5-oxoproline, which is then converted to glutamate. Two ATP molecules are used for the biosynthesis of one GSH molecule. Salvage synthesis involves either reduction of GSSG or uses precursors formed from the hydrolysis of GSH or its conjugates by γ-L-glutamyl transpeptidase at the external surface of the plasma membrane that are transported back into the cell as amino acids or dipeptides. GSH is consumed in various processes. In addition to detoxification of reactive species and electrophiles such as methylglyoxal, GSH is involved in protein glutathionylation and several other processes, such as the biosynthesis of leukotrienes and prostaglandins, and reduction of ribonucleotides. Modified from [27].

between the γ-carboxyl of glutamate and the amino group of cysteine using energy provided by the hydrolysis of ATP:

$$\gamma\text{-L-Glutamate} + \text{L-cysteine} + \text{ATP} \longrightarrow \gamma\text{-L-glutamyl-L-cysteine} + \text{ADP} + \text{P}_\text{i} \quad (1)$$

In the next step, the dipeptide is combined with glycine by glutathione synthetase (GLS), again driven by the hydrolysis of ATP:

$$\gamma\text{-L-Glutamyl-L-cysteine} + \text{glycine} + \text{ATP} \longrightarrow \text{GSH} + \text{ADP} + \text{P}_\text{i} \quad (2)$$

It should be noted that, in some cases, the provision of ATP for GSH synthesis can be a limiting factor for GSH metabolism [26]. The first step, catalyzed by γGLCL, is the rate-limiting step for overall GSH biosynthesis process. The enzyme is inhibited by GSH, the end product of the pathway, indicating that its biosynthesis is regulated via a negative feedback control mechanism.

GSH may be oxidized directly by oxidants such as hydroxyl radical ($HO^\bullet$) [28, 29] or peroxynitrite ($ONOO^-$) [30, 31]. Direct oxidation leads to the production of thiyl radicals [32], the fusion of which results in GSSG formation (Figure 2). GSH is extensively used as a cosubstrate by glutathione peroxidases (GPx, EC 1.11.1.9) reducing hydrogen peroxide (H_2O_2) or organic peroxides (generally abbreviated as ROOH or LOOH in the case of lipid peroxides) with the production of GSSG, water, or alcohols. Figure 3 shows the dismutation of H_2O_2 by catalase.

How do catalases and GPxs cooperate in H_2O_2 catabolism? Firstly, they are mainly localized in different cellular compartments—GPxs are cytosolic residents, whereas catalases are found mainly in peroxisomes. Secondly, the affinity of GPx for H_2O_2 is one to two orders of magnitude higher than that of catalase. So, one may conclude that the two enzymes operate in concert, complementing each other. GSSG produced from the consumption of GSH can be either restored again by the action of glutathione reductase (GR, EC 1.6.4.2) (reaction (3)), or excreted from the cell.

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \longrightarrow 2\text{GSH} + \text{NADP}^+ \quad (3)$$

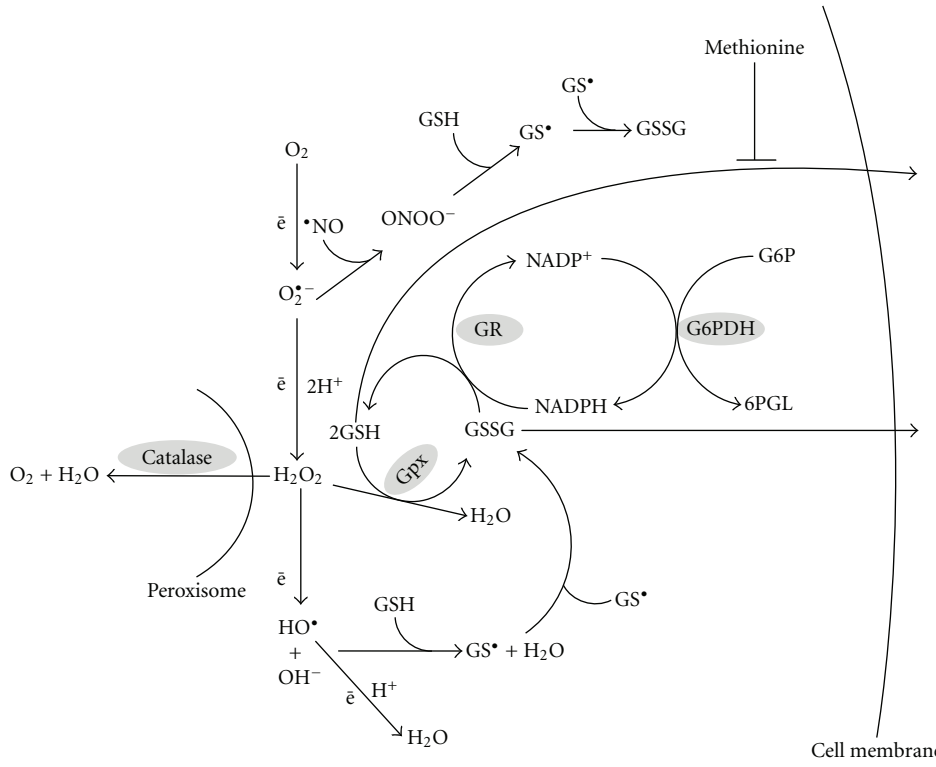

Figure 3: Involvement of glutathione in elimination of reactive oxygen and nitrogen species. Hydroxyl radical and nitric oxide (after oxidation to the NO^+ form) or peroxynitrite may interact directly with GSH leading to GSSG formation. Hydrogen peroxide may be removed by catalase or by glutathione peroxidase (GPx). The latter requires GSH to reduce peroxide.

Glutathione excretion from cells is inhibited by methionine [33]. Three forms of glutathione, namely, GSH, GSSG, and GSH-conjugates, can be excreted into extracellular spaces. There the conjugates are mainly hydrolysed to different components and reabsorbed. However, cysteine residues usually remain conjugated to xenobiotics and are released by organisms in feces. Most glutathione *S*-conjugates are metabolized to the corresponding *N*-acetyl cysteine *S*-conjugates (mercapturic acids) and released in the urine and bile [34]. Glutamate and glycine residues are usually recovered, but the cysteine residues remain conjugated and are lost. Both GSH and GSSG are substrates for the extracellular membrane-bound enzyme GGT:

$$\text{GSH} + \text{amino acid} \longrightarrow \gamma\text{-glutamyl-amino acid} + \text{L-cysteinyl-glycine} \quad (4)$$

$$\text{GSH} + \text{H}_2\text{O} \longrightarrow \text{L-glutamate} + \text{L-cysteinyl-glycine} \quad (5)$$

γ-L-Glutamyl transpeptidase cleaves only the γ-peptide linkage. The enzyme can transfer the γ-glutamyl group of GSH, GSSG, or GSH-conjugates onto amino acid acceptors to form γ-glutamyl peptides and cysteinyl-glycine (reaction (4)), or to water thereby hydrolyzing GSH and related compounds to glutamate and cysteinylglycine (reaction (5)). Cysteinylglycine can be further hydrolyzed by a dipeptidase to cysteine and glycine. The products, namely, amino acids and γ-glutamyl amino acids, may be transported back into cells and used for GSH resynthesis or other needs. This provides the basis for recycling of excreted GSH and GSSG (salvage cycle) by the cell of origin or by other cells [34]. Upregulation of this process provides an additional mechanism for GSH maintenance in the cell.

3. Intracellular Compartmentalization and Interorgan Transfer

Although GSH is synthesized in the cytosol, it is distributed to different intracellular organelles where it is used in organelle-specific functions related to its role in the regulation of cellular redox status. In addition to the cytosolic pool, GSH functions in somewhat independent pools in the endoplasmic reticulum (ER), nucleus, and mitochondria. In most of these compartments GSH is typically found in a highly reduced state, but in the ER a substantial portion is oxidised and the ratio [GSH]/[GSSG] may be as high as 3 : 1, whereas in the cytoplasm the oxidized form is usually on the order of about 1% of the total or less [35, 36]. In the ER, GSSG is the main source of oxidizing power that supports the efficient production of the functional conformation of nascent polypeptides by the formation of the required intramolecular disulfide bonds between cysteine residues. In the nucleus, GSH maintains the appropriate redox status of the sulfhydryl groups in proteins involved

in nucleic acid biosynthesis and DNA repair in addition to standard antioxidant functions. In this compartment, it is also used in the reduction of ribonucleotides to produce deoxyribonucleotides by ribonucleotide reductase [37].

About 10–15% of cellular GSH is located in mitochondria. Since mitochondria have a very small volume, the local GSH concentration in these organelles is usually higher than that in the cytosol. Of the various subcellular compartments, most attention has been paid to the mitochondrial GSH pool (mGSH) because of the close relationship between mGSH and cell survival that has been demonstrated in many cases. This topic is covered in an excellent recent review of Mari et al. [38] and readers are directed to this review for extensive details. Here, I will mention just a few important aspects of the mGSH system. As mentioned above, GSH is synthesized only in the cytosol and is transported into intracellular organelles. It easily crosses the outer mitochondrial membrane through porin channels but, being an anion, cannot diffuse across inner mitochondrial membrane into the matrix. At least two systems are believed to be involved in GSH import into the mitochondria across the inner membrane. GSH transport into the matrix must overcome an unfavourable electrochemical gradient [39–44]. This is provided by two mitochondrial membrane carriers [45, 46] that exchange GSH for dicarboxylates and 2-oxoglutarate (α-ketoglutarate). These two antiport carriers provide electroneutral exchange of selected anions across the inner mitochondrial membrane with no charge transfer. The role of these two mitochondrial GSH carriers was also evidenced by a reconstitution of recombinant mitochondrial dicarboxylate carriers into proteoliposomes [45]. However, it should be noted that during GSH import the mitochondria lose important intermediates of the Krebs cycle so that anaplerotic mechanisms may be needed to replenish these. It should also be noted that GSSG cannot leave the mitochondria and therefore needs to be regenerated in the matrix by GR using NADPH (reaction (3)).

In addition to its "classic" functions, GSH plays organelle-specific roles in the mitochondria and a few of them will be mentioned here. Due to the pivotal role of mitochondria in programmed cell death (apoptosis) as well as extensive ROS involvement in this process, and adding the fact that mitochondria produce over 90% of cellular ROS, the role of GSH in cell protection cannot be overestimated. GSH may either directly bind some ROS species or serve as a source of reductive power for certain antioxidant systems. The inner mitochondrial membrane is particularly rich in cardiolipin, whereas it is virtually absent from other membranes and only the outer mitochondrial membrane contains minor amounts of this phospholipid. When mGSH levels are compromised, cardiolipin is one of the important targets of oxidative damage. Due to its unique chemical structure among phospholipids, cardiolipin confers stability and fluidity to the mitochondrial membrane. In addition, cytochrome *c* is normally bound to the inner mitochondrial membrane via its association with cardiolipin. By protecting cardiolipin from oxidative damage, GSH prevents changes in the physicochemical properties of the mitochondrial inner membrane that lead to membrane destabilization and the dissociation of cytochrome *c*. ROS also induce an increase in permeability of the internal mitochondrial membrane for calcium. Enhanced ROS and calcium levels, acting in concert, may trigger the cell death machinery via apoptosis or necrosis. Hence, mitochondrial GSH clearly has an important role in preventing apoptosis triggered by cytochrome *c* release from the inner membrane.

Not surprisingly, therefore, a decrease in mGSH levels is closely associated with certain pathologies in both humans and animals. This relationship has been described for hypoxia/reperfusion injury [47, 48], certain liver diseases such as alcoholic steatohepatitis [49, 50], nonalcoholic steatohepatitis [51, 52], and liver cirrhosis [53, 54], neurological diseases such as Alzheimer and Parkinson diseases, diabetes mellitus and associated complications [55–57]. Many of the abovementioned pathologies are included in the group of so-called age-related diseases and, therefore, it is not easy to differentiate aging as a normal physiological process and age-related or age-induced pathologies. Harman [58] proposed the oxidative stress theory of aging, which he later modified to the mitochondrial theory of aging [59]. This theory suggested that oxidative damage to organisms is connected with the progressive accumulation of oxidized/modified products of ROS attack that ultimately determine the lifespan of organisms. Insofar as they are cornerstones of the oxidative stress and/or mitochondrial theories of aging, ROS and mitochondrial function are intimately regulated by GSH and the [GSSG]/[GSH] ratio, thereby linking these theories of aging to mitochondrial GSH levels. Other pathologies, such as several diseases of the lungs (e.g., chronic pulmonary disease, acute respiratory distress syndrome, neonatal lung damage, and asthma) and of the immune system are also associated with a compromised mitochondrial GSH system [60–62]. Finally, mGSH involvement in combating the toxicity of different xenobiotics, particularly drugs such as cisplatin, is clearly evident [63–65].

One more important point related to mGSH should also be mentioned here. The correct analysis of the mitochondrial GSH pool is an experimentally complicated issue. To study this, cells are typically disrupted in order to isolate mitochondria and this can substantially affect not only redox status, but also total GSH content. Hence, there is a need to introduce new techniques for the proper evaluation of the operation of the mitochondrial GSH system. Some interesting ideas on this topic can be found in recent studies by Winther and colleagues [66, 67].

Another important topic is GSH distribution between different organs of animals. Glutathione can be transported across the plasma membrane, which is the first step of a complicated interorgan transfer network [4, 13]. Liver is the main source of GSH exported into the blood [68–71]. The export of GSH and its conjugates from liver cells occurs via transporters referred to as organic anion-transporting polypeptides (OATPs), which are generally believed to carry out electroneutral exchange, in which the cellular uptake of organic anions is coupled to the efflux of anions such as HCO_3^-, GSH, GSSG, and/or glutathione *S*-conjugates

[72, 73]. Both GSH and GSSG are circulated and are used to supply other organs, particularly kidney. The production in liver and export from it are related to GSH functions, and at least two principles may be implicated. The first one involves epithelial cells that contact with the exterior, such as intestine and lungs. The primary GSH function here is directed to detoxification of injurious external agents to prevent damage to the organism. There is a large body of data indicating that this is an important role of GSH in normal intestinal function. The lungs are exposed to high oxygen levels and also to inhaled toxins. Alveolar macrophages provide an additional ROS source in this tissue. Hence, there are multiple reasons for maintaining adequate GSH levels in lungs. The second principle is related to high intensity oxygen-based metabolism and detoxification of certain compounds by internal organs. Liver and kidney are probably the best representatives of this group. The portal vein brings blood from the intestine to the liver and, if not detoxified in the intestine, xenobiotics must be neutralized by hepatocytes [52, 74–76]. In addition, the liver is an important biosynthetic organ where ROS are produced in substantial amounts as side products of energy production in the mitochondrial electron transport chain or as the result of biosyntheses involving diverse oxygenases. Kidney also requires a highly efficient GSH system to perform its functions [13, 77, 78]. The problems with extracellular GSH investigation and intertissue transfer are to a large extent based on inadequate methodology. Since the concentrations of extracellular GSH are more than an order of magnitude lower than intracellular levels, correct redox ratios are often difficult to determine.

4. Glutathione Functions

The chemical structure of GSH determines its potential functions and its broad distribution among all living organisms reflects its important biological role. GSH has been found in all mammalian cells. Probably most importantly, GSH is responsible for protection against ROS and RNS, and detoxification of endogenous and exogenous toxins of an electrophilic nature. Other functions include (i) maintaining the essential thiol status of proteins and other molecules; (ii) storage of cysteine reserves both in the cell and for interorgan transfer; (iii) involvement in the metabolism of estrogens, leukotrienes, and prostaglandins; (iv) participation in the reduction of ribonucleotides to deoxyribonucleotides; (v) participation in the maturation of iron-sulfur clusters in proteins; (vi) copper and iron transfer; (vii) signal transduction from the environment to cellular transcription machinery. The above-listed GSH functions and a few others will be covered in this section.

4.1. Elimination of Reactive Oxygen and Nitrogen Species. GSH is an important antioxidant, directly reacting with ROS, RNS, and other reactive species, particularly $HO^{\bullet}$, HOCl, $RO^{\bullet}$, $RO_2^{\bullet}$, $^1O_2$, and $ONOO^-$, often resulting in the formation of thiyl radicals ($GS^{\bullet}$) (Figure 3). GSH is also involved as an antioxidant in the detoxification of products from ROS-promoted oxidation of lipids such as malonic dialdehyde and 4-hydroxy-2-nonenal [79, 80], and probably many other products of ROS interaction with cellular components [11, 19, 81, 82]. The thiyl radicals formed from these reactions can also combine with different molecules, as well as with other thiyl radicals leading to the formation of oxidized glutathione (glutathione disulfide, GSSG) in the latter instance. GSSG is also produced in reactions catalyzed by GPx (reaction (6)) and glutaredoxins (reaction (7)):

$$\mathrm{ROOH} + 2\mathrm{GSH} \longrightarrow \mathrm{ROH} + \mathrm{GSSG} + \mathrm{H_2O} \tag{6}$$

$$\text{Oxidized glutaredoxin} + 2\mathrm{GSH} \longrightarrow \text{reduced glutaredoxin} + \mathrm{GSSG} \tag{7}$$

GSSG may be either excreted from the cell, or reduced by GR at the expense of NADPH (reaction (3)). Most of the reductive power for this reaction is provided by the pentose phosphate shunt-two molecules of NADPH are produced per molecule of glucose-6-P that cycle through the pathway. The first and limiting step is catalyzed by glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49):

$$\text{Glucose-6-phosphate} + \mathrm{NADP^+} \longrightarrow \text{6-phosphoglucolactone} + \mathrm{NADPH} + \mathrm{H^+} \tag{8}$$

The second molecule of NADPH is provided by the next pentose phosphate shunt reaction, catalyzed by 6-phosphogluconate dehydrogenase (6-PGDH). These two enzymes are not the only cellular NADPH producers. NADPH is also formed by NADP-dependent isocitrate dehydrogenase, malic enzyme, and some others, but it is widely believed that most cellular NADPH is generated by the pentose phosphate pathway.

As mentioned above, the glutathione couple GSH/GSSG is a critically important redox player and together with other redox active couples, including NAD(P)/NAD(P)H, $FAD/FADH_2$, regulates and maintains cellular redox status. The estimated *in vivo* redox potential for the GSH/GSSG couple ranges from −260 mV to −150 mV depending on the conditions (cited after [10]).

Under normal conditions, when a cell is not stressed, the processes that generate ROS are well counterbalanced by antioxidant systems. In this respect, GSH is often considered to be a key player of the defense system. However, under various circumstances the steady-state ROS level increases leading to oxidative damage to the cell, called "oxidative stress," the term first defined by Sies [83] "*Oxidative stress*" "*came to denote a disturbance in the prooxidant-antioxidant balance in favor of the former*." The definition was later expanded to "*An imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage, is termed "oxidative stress*"" to emphasize the damage to certain cellular components [84]. Owing to extensive studies on oxidative stress and the discovery of many intricacies related to this phenomenon over the two last decades, the

definition could be modified to "*Oxidative stress is a situation where the steady-state ROS concentration is transiently or chronically enhanced, disturbing cellular metabolism and its regulation and damaging cellular constituents*" [81]. This definition underlines the dynamic nature of the processes of ROS generation and elimination, damage to cellular core and regulatory pathways, and potential negative consequences of enhanced ROS levels either acutely or chronically. If cells are not capable of coping with the intensity of oxidative stress, this can culminate in their death via necrosis or apoptosis.

The dynamics of ROS-related processes are shown in Figure 4. Under control conditions, steady-state ROS levels fluctuate over a certain range [81, 82, 85]. However, ROS levels can exceed this range due to an increase in ROS production either as a result of internal physiological changes or external induction. If the cellular antioxidant potential is high enough, acutely increased ROS levels can be quickly reduced again back to the initial (control) range. But if the existing antioxidant potential is not capable of eliminating extra ROS, the cell can increase its antioxidant defenses, but it will require some time to respond, and this will also consume energy and important biomolecules (e.g., amino acids). Upregulation of the antioxidant potential may result in the restoration of ROS levels back into the initial range, or due to a prolonged increase in ROS levels the cell may enter a state of "chronic oxidative stress" (Figure 4). In many cases, acute oxidative stress has no serious consequences for organisms, but the chronic state may lead to or accompany certain pathologies. Oxidative stress is well-documented to occur, for example, in cardiovascular and neurodegenerative diseases, diabetes mellitus, cancer, and aging [9, 12, 47, 51, 86–89]. Under some circumstances, ROS levels do not return to the initial range and the system may be stabilized at new, higher ROS level referred to as "quasistationary" that occurs in various pathological states [81]. Interestingly, the opposite situation of decreased ROS levels can occur in some instances and is sometimes called "reductive stress." However, there has been very little investigation of this situation and, therefore, it will not be further discussed here.

The above short excursion into oxidative stress theory underscores not only the importance of GSH for ROS combating in unstressed conditions, but also the augmented role that GSH must play during oxidative stress. Enhanced ROS levels may require not only enhanced GSH action to maintain redox status, but also enhanced energy and material consumption to replace consumed GSH and/or transport it to the places where it is needed.

As mentioned above, GSH may be involved in detoxification of RNS [6]. For example, nitric oxide ($^{\bullet}$NO) was initially thought to interact directly with GSH to produce *S*-nitrosoglutathione (GSNO). However, further investigation demonstrated $^{\bullet}$NO must first be converted to NO^{+} (nitrosonium ion) in an iron- or copper-catalyzed reaction before reacting with GSH to form GSNO [90, 91]. It should be noted that GSNO and other nitrosothiols can be used for storage and transportation of $^{\bullet}$NO because as unstable compounds they can be decomposed easily to generate $^{\bullet}$NO and GSSG.

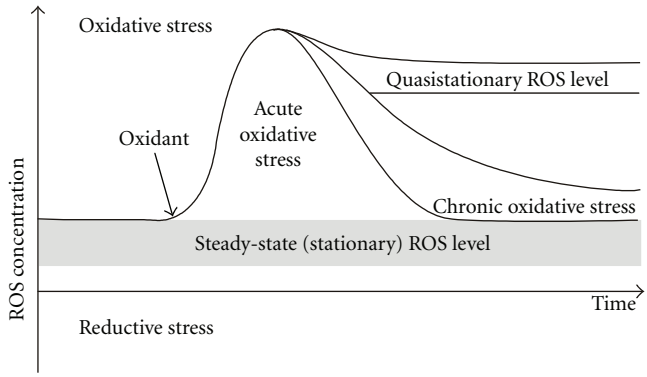

Figure 4: The dynamics of reactive oxygen species in biological systems. Steady-state levels of reactive oxygen species fluctuate over a certain range under normal conditions. However, under stress ROS levels may increase or decrease beyond the normal range resulting in acute or chronic oxidative or reductive stress. Under some conditions, ROS levels may not return to their initial range and stabilize at a new quasistationary level.

4.2. Elimination of Endogenously Produced Toxicants. The role of GSH in detoxification of the end products of lipid peroxidation such as malonedialdehyde and 4-hydroxy-2-nonenal was mentioned above. Many other toxic metabolites are produced as side-products of the normal cellular metabolism. For example, methylglyoxal (2-oxopropanal) is one of these and it can be generated both enzymatically and nonenzymatically [92, 93]. Glycolysis appears to be the main source of methylglyoxal where it is produced from triose phosphates, particularly due to spontaneous decomposition of glyceraldehyde-3-phosphate [94, 95]. Methylglyoxal toxicity is based on its capacity to interact with any molecule containing free amino groups such as amino acids, nucleotide bases of nucleic acids, and cysteine residues in proteins [96–99]. Methylglyoxal and other α-dicarbonyls, in turn, may be involved in ROS generation. Glutathione acts as a cofactor in the system of methylglyoxal elimination which consists of two enzymes called glyoxalases [92, 100, 101]. The first enzyme in this pathway, glyoxalase I (Glo I, EC 4.4.1.5), catalyses the isomerization of hemiacetal adducts, which are formed in a spontaneous reaction between a glutathione and aldehydes such as methylglyoxal:

$$\text{glutathione} + \text{methylglyoxal} \longleftrightarrow \text{hemithioacetal adduct} \longleftrightarrow \text{(R)-S-lactoylglutathione} \quad (9)$$

The second enzyme, glyoxalase II (Glo II, EC 3.1.2.6), catalyzes the hydrolysis of the product of the above reaction:

$$\text{(R)-}S\text{-Lactoyl-GSH} + H_2O \longrightarrow \text{D(-)lactic acid} + \text{GSH} \quad (10)$$

This pathway is the main route for methylglyoxal catabolism in yeasts [3, 95, 102, 103] and mammals [104–107].

GSH also may be involved in the detoxification of endogenously produced formaldehyde. For example, some

yeasts produce formaldehyde as part of methanol catabolism [108–111]. The reaction is catalyzed by formaldehyde dehydrogenase (FaDH, EC 1.1.1.1) which uses GSH as a cosubstrate:

$$\text{Formaldehyde} + \text{GSH} + \text{NAD}^+ \longrightarrow \textit{S}\text{-formylglutathione} + \text{NADH} + \text{H}^+ \quad (11)$$

Formaldehyde also may be produced from the catabolism of certain amino acids and, therefore, reaction (11) may be important for its detoxification in animals and plants [112, 113]. Interestingly, formaldehyde dehydrogenase also catalyzes the decomposition of *S*-nitroso-glutathione and it is not limited to yeasts [24], but also found in plants and animals [114–117].

4.3. Metal Homeostasis. GSH can interact with certain metal ions. It contains six potential coordination sites for metal ion binding such as cysteinyl sulfhydryl, glutamyl amino, glycyl, and glutamyl carboxyl groups, and two peptide bonds. Among these, the sulfhydryl group possesses the highest affinity for metal cations, particularly cadmium, copper, zinc, silver, mercury, arsenic, and lead [118]. The interaction of a metal ion with the GSH sulfhydryl group can be stabilized by coordination with other potential binding sites. The most stable complexes are formed by divalent cations in a 1 : 2 ratio with GSH. The complexes form spontaneously because they are thermodynamically favored and the resulting mercaptides are relatively stable. Several metabolic functions for these metal-GSH complexes have been proposed: (i) they can help in the mobilization and transfer of cations between ligands; (ii) they can serve to transport metal ions across membranes; (iii) they serve as a source of cysteine, playing a central role in metal homeostasis; (iv) they serve as a cofactor for redox reactions yielding metal compounds with different speciation or biochemical forms [118]. The remainder of this section focuses only on GSH involvement in the metabolism of chromium, copper, and iron ions.

GSH is involved in Cr^{6+} reduction in many organisms (reviewed in [82, 119]). GSH-dependent reduction of Cr^{6+} results in the formation of Cr^{3+}, effectively converting the ion from an anionic form ($Cr_2O_7^{2-}$ or CrO_4^{2-}) to a cationic form [82, 120]. Cr^{6+} in its anion form (associated with oxygen) is readily transported into cells via nonspecific anion carriers, but Cr^{3+} as a cation is not so bioavailable and is believed to be less toxic due to its interaction with many cellular ligands [121]. Therefore, Cr^{6+} reduction to Cr^{3+} can be characterized as a way to decrease chromium toxicity [122]. Although Cr^{6+} can be reduced nonenzymatically, studies suggest that in cells GSH and GSH-dependent enzymes, either alone or in concert with ascorbic acid and cysteine, play an important role in these processes [119, 123–126]. For example, the inhibition of GR by carmustine prevented Cr^{6+} reduction in isolated rat hepatocytes [127]. Nontoxic biological effects of chromium are also associated with GSH-related transformation of Cr^{6+}. Although it is not clear how this occurs, these effects are related to the ability of chromium to affect carbohydrate metabolism potentiating the effects of insulin [128, 129]. It is worth noting that although Cr^{3+} is thought to be a regulator of carbohydrate metabolism, the capacity of biological systems to reduce Cr^{6+} with the participation of the GSH system may be used to deliver chromium into biological systems.

GSH plays a more specific and well-documented role in the metabolism of copper and iron. GSH is believed to be responsible for the mobilization and delivery of copper ions for the biosynthesis of copper-containing proteins [118]. In this case, GSH is involved in (i) reduction of Cu^{2+} to Cu^+, (ii) mobilization of copper ions from stores, and (iii) delivery of copper ions during the formation of "mature" proteins. For the last function, Cu^{2+} must be reduced to Cu^+ before it can be incorporated into apoproteins, and GSH provides the reducing power [130]. Interestingly, GSH is not only the carrier for Cu^+, but is also involved in copper mobilization from metallothioneins in a reversible manner. The Cu(I)-GSH complex is used for copper incorporation into Cu,Zn-superoxide dismutase (Cu,Zn-SOD) from bovine erythrocytes [131] lobster apohemocyanin [132], and blood plasma albumin [133].

The role of GSH in iron metabolism is not as well studied. However, by analogy with copper, GSH may be involved in iron reduction, transportation, mobilization from different stores, and incorporation into certain target molecules. GSH involvement in iron metabolism in the yeast *S. cerevisiae,* has been investigated in details [134]. GSH was not required for iron adsorption, delivery to mitochondria, maintenance of mitochondrial Fe,S-proteins, or for their maturation. However, the maturation of extramitochondrial Fe,S-proteins required GSH. Although the precise role of GSH in this process is not clear, GSH involvement in facilitated transport of components of Fe,S-clusters was suggested [134].

5. Glutathione Peroxidases and Transferases and Their Regulation

These enzymes play very specific roles in cellular metabolism that should be specially highlighted. As mentioned above, GPx catalyzes the GSH-dependent reduction of many peroxides (reaction (6)). GPx enzymes are particularly involved in the removal of LOOH, thereby terminating lipid peroxidation chain reactions and protecting biological membranes. Four isoenzymes of GPx have been identified in mammalian tissues [135, 136]. The active site of these enzymes contains a selenocysteine residue which is responsible for the catalytic activity. Mammalian isoenzymes GPx-1, GPx-2, and GPx-3 reduce H_2O_2 and peroxides of free fatty acids, whereas GPx-4 reduces peroxides of phospholipids and cholesterol [137].

Certain glutathione *S*-transferases (GST, EC 2.5.1.18) catalyze GSH conjugation with electrophiles, but some also catalyze the reduction of lipid peroxides and as a consequence they are also called selenium-independent peroxidases [138]. These GSTs do not possess a selenocysteine residue in their active site. GSTs are an enzyme superfamily responsible for biotransformation of electrophilic compounds. In this way GSTs protect organisms against

genotoxic and carcinogenic compounds of both exogenous (xenobiotics) and endogenous origin. Mammalian GSTs are organized in multiple classes designed by Greek letters. Major classes include Alpha, Mu, Pi, abbreviated in Roman capitals as A, M, P. [139]. Traditionally, GST activity is measured with 1-chloro-2, 4-dinitrobenzene (CDNB), cumene hydroperoxide, or *tert*-butyl hydroperoxide as the substrates. Due to selenium-independent GPx activity, α-class GSTs can efficiently reduce peroxides of free fatty acids and phospholipids, as well as cholesterol hydroperoxides [140]. It is worth noting that α-class GSTs can reduce peroxides of membrane phospholipids without requiring phospholipase A_2-mediated release of the peroxidized fatty acids from the membrane phospholipids [141, 142]. The role of α-GST in peroxide metabolism is highlighted in excellent reviews of Awasthi and colleagues [140, 143].

By regulating the level of certain electrophiles, GSTs and GSH may indirectly affect regulatory pathways controlled by these compounds. For example, 4-hydroxynonenal (4-HNE) is a well-known product of lipid peroxidation, which has a key role in stress-mediated signalling. Its steady-state intracellular level is determined by the balance between production due to lipid peroxidation and elimination via various pathways. One of the subgroups of the anionic α-class of GSTs can utilize 4-HNE as a preferred substrate, conjugating it to GSH with high efficiency [140]. The enzyme shows a much higher affinity toward 4-HNE than to most xenobiotics suggesting its critical role in the regulation of cellular 4-HNE levels. The adduct formed, GS-HNE, is exported from cells in an ATP-dependent manner by a primary transport system similar to the system that extrudes other GSH conjugates [144, 145].

However, GSTs may not only play positive roles in cell protection against xenobiotics. In certain cases, they can be responsible for the need to increase the doses of specific drugs. For example, in many solid tumors enhanced resistance to drugs is associated with the increased activity of GSTs that detoxify xenobiotics [27, 146]. GST was identified as a prominent protein in many cases and is overexpressed in many cancers resistant to several drugs. These GSTs have been proven to be a viable target for prodrug activation with at least one candidate in late-stage clinical development [146].

The activities of GPxs and GSTs, like other antioxidant enzymes, are regulated in many ways. Most attention has been paid to their upregulation via specific regulatory pathways involving ROS or electrophiles at certain stages. Many reviews extensively describe these pathways [147–152], and here we will describe just a few of them where GSH is known to be an active participant. OxyR-related regulatory protein was described in bacteria about 20 years ago (reviewed in [153–158]). Subsequently, the YAP1/GPx3-regulated system was found to be responsible for augmentation of antioxidant potential in yeast [85, 154, 157, 158]. Finally, in animals the operation of ROS-based regulatory cascades, involving GSH and GSH-related enzymes, has been identified. In this context, the Nrf2/Keap1 system of animals is often considered to be the most important and finely controlled pathway that regulates the activities of antioxidant and phase II detoxification enzymes via interaction with antioxidant response elements (ARE) in regulatory regions of many of the genes that encode antioxidant enzymes [21, 159–168] (the same gene region is also known as the electrophile response element (EpRE) to designate its involvement in the cellular response to electrophiles). In animals, the activities of many phase II detoxifying enzymes, including GSTs and GPxs, are also upregulated via the Nrf2/Keap1 system. The dilemma of the simultaneous regulation of GSTs and antioxidant enzymes was solved when the mechanism by which the Nrf2/Keap1 system operation was uncovered (Figure 5). Under normal (nonstressed) conditions Nrf2 protein interacts with Keap1 in the cytosol and is quickly ubiquitinated followed by the proteasomal degradation. However, when ROS levels rise, Keap1 is oxidized and becomes incapable of binding Nrf2. This results in its migration (possibly related to phosphorylation by certain protein kinases) into the nucleus. In the nucleus, Nrf2 binds to the ARE (EpRE) DNA element of target genes together with a small Maf protein and perhaps with other proteins. The complex stimulates the expression of target genes, including those encoding GSTs and antioxidant enzymes. Clearly, enhanced expression of antioxidants and phase II detoxification enzymes is an important factor in increasing cellular resistance to xenobiotics. In addition to GSTs, a key enzyme of GSH-biosynthesis, γGLCL, is also among the targets of the Nrf2/Keap1 regulatory pathway. Because of its involvement in the regulation of diverse physiological processes, and especially those related to GSH, the Nrf2/Keap1 system has gained attention not only at the basic biological level, but also from a pharmacological viewpoint.

Detoxification of xenobiotics in animals is usually, but not always, provided by a specific system consisting of so-called phase I, phase II, and phase III enzymes. Phase I enzymes are represented by hydroxylases such as endoplasmic reticulum members of the cytochrome P450 family, which introduce oxygen onto molecules of hydrophobic xenobiotics and endogenous compounds, transforming them in more hydrophilic forms. Phase II detoxification enzymes catalyze conjugation reactions that add glutathione, amino acids, sulphate, glucuronic, acetyl, or methyl residues to activated xenobiotics. Plasma membrane antiporters represent phase III detoxification; these energy-dependent pumps export conjugates from the cell, thereby decreasing their intracellular concentration. Although this system of nomenclature for the detoxification of xenobiotics can be useful, the classification may not always hold for detoxification reactions involving GSH. For example, many electrophilic xenobiotics can react directly with GSH without the prior need for activation by phase I enzymes [34].

6. Glutathionylation of Cellular Sulfhydryls

An increase in cellular levels of mixed disulfides formed between GSH and protein thiols, a process called glutathionylation, was demonstrated to be caused by oxidative stress about three decades ago [169–171]. Since that time many studies of the role of glutathionylation have been carried out.

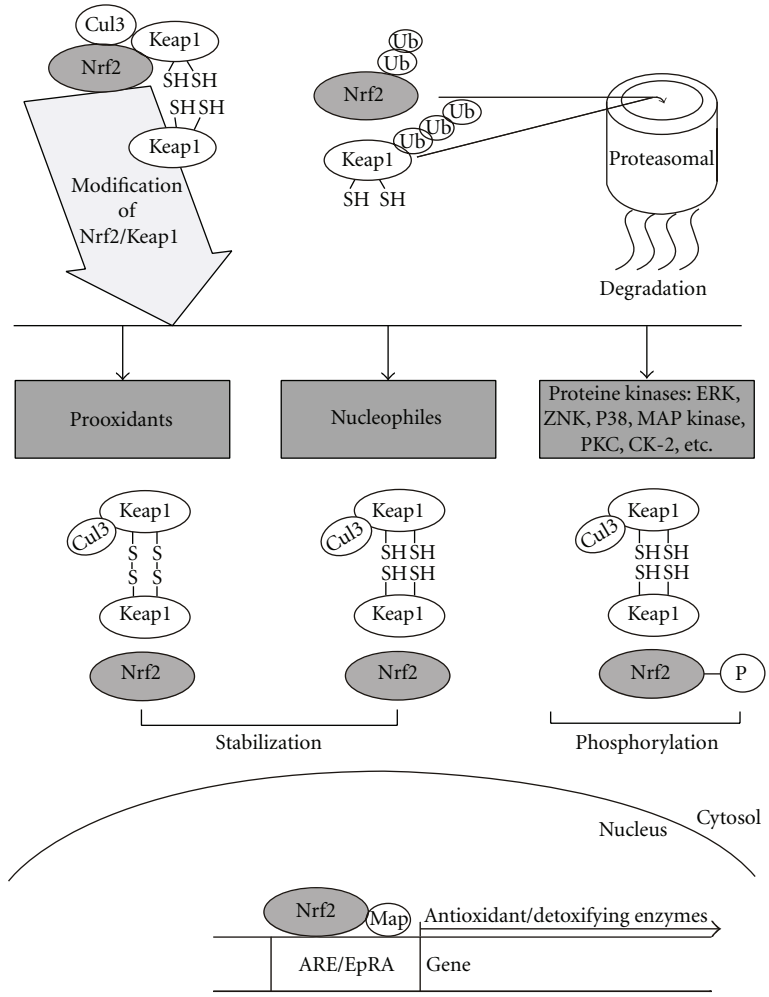

FIGURE 5: Operation of the Nrf2/Keap1 system during response to oxidative stress in animals. Under nonstressed conditions the transcription factor Nrf2 binds to the Keap1 homodimer. The resulting protein complex can then further complex with Cullin 3 leading to ubiquitination of Nrf2 followed by proteasomal degradation. Following an oxidative insult or electrophilic attack, Keap1 cannot bind Nrf2 which allows Nrf2 to diffuse into the nucleus and, in concert with small Maf proteins (sMaf), Map and others, Nrf2 binds to the ARE/EpRE elements of regulatory regions in genes encoding antioxidant or phase 2 detoxification enzymes. Nrf2 migration into the nucleus is promoted by at least three different mechanisms: oxidation of Keap thiol groups to form disulfides, modification of Keap1 cysteine residues by electrophiles, or phosphorylation of Nrf2 by protein kinases that, in turn, may be activated by oxidants.

Work from the laboratory of Sies and others implicated the process in the regulation of the activity of specific enzymes and certain regulatory pathways [6, 172–176]. From this, glutathionylation was recognised as one of the physiologically relevant mechanisms of posttranslational modification of certain proteins. Exposure of cysteine residues of proteins to ROS leads to their oxidation with the consequent formation of stable sulfenic, sulfinic, or sulfonic acid derivatives and unstable transient forms (Figure 6). Sulfenic acid may be returned to the original cysteine form by several reductases ([6, 177] and cited therein) whereas sulfinic acid can be reduced only by the specific action of sulfiredoxin [178–181]. It is believed that sulfonic acid cannot be reduced in living organisms. Cysteine oxidation to sulfenic acid may be used for ROS sensing and in this case it plays a positive role in cell adaptation. However, more frequently the oxidation may inhibit certain proteins if the oxidized cysteine residues are important for protein function. Therefore, in addition to direct reduction of sulfenic acid to cysteine, living organisms possess other ways of dealing with this moiety (Figure 6). Sulfenic acid residues may interact with reduced glutathione forming mixed disulfides [182, 183]. This issue is not so straightforward, because formation of this dithiol can be implicated in the regulation of some metabolic pathways. Many proteins are subject to glutathionylation and some of them lose biological activity as the result, whereas others may be activated [182]. In human T lymphocytes, Fratelli and colleagues [184] found that cell exposure to oxidants (diamide and H_2O_2) enhanced glutathionylation of certain proteins. These included cytoskeletal proteins (vimentin, myosin, tropomyosin, cofilin, profilin, and actin), metabolic enzymes (enolase, aldolase, 6-phosphoglucolactonase, adenylate kinase, ubiquitin-conjugating enzyme, phosphoglycerate kinase, triose phosphate

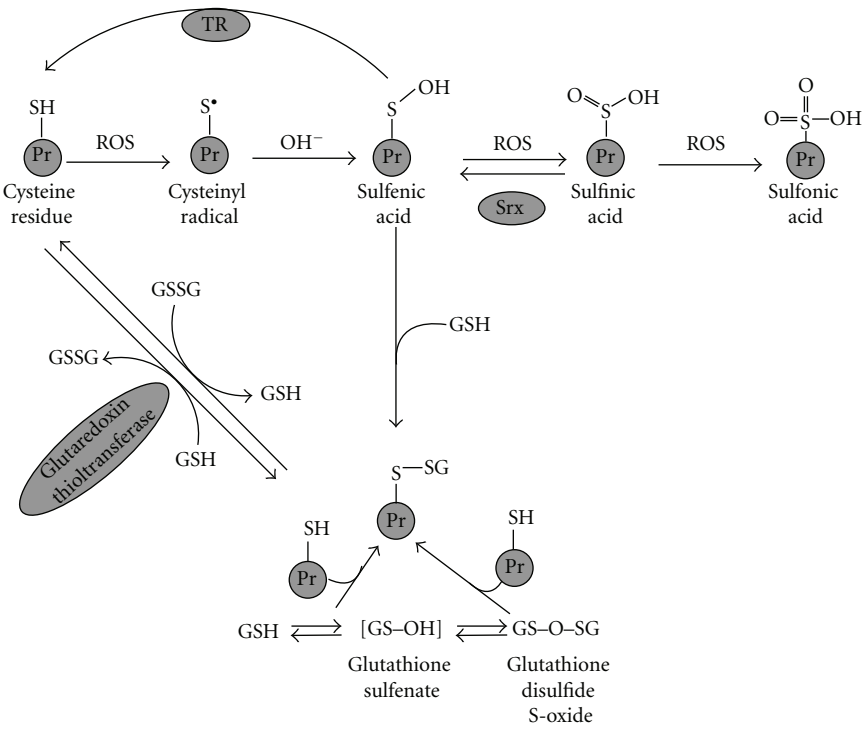

Figure 6: Oxidation of protein cysteine residues to sulfenic, sulfinic, or sulfonic derivatives and formation of glutathionylated proteins. In biological systems, sulfenic and sulfinic derivatives may be reduced by thioredoxin (TR) and sulfiredoxin (Srx), respectively, whereas sulfonic moieties cannot be reduced. Glutathionylated proteins are formed by direct interaction of GSH with sulfenic acid derivatives, exchange between cysteine residues and GSSG, or interaction with oxidized glutathione forms.

isomerase and pyrophosphatase), redox enzymes (peroxiredoxin 1, protein disulfide isomerase, and cytochrome c oxidase), cyclophilin, stress proteins (HSP70 and HSP60), nucleophosmin, transgelin, galectin, and fatty acid binding protein. *S*-Glutathionylation is thought to be one of the mechanisms preventing ROS-induced irreversible protein inactivation under oxidative stress insults. During recovery, GSH residues can be removed from the glutathionylated proteins resulting in restoration of their functional activity.

Figure 6 shows the known pathways of glutathionylation/deglutathionylation and oxidation of cysteine residues in cellular processes. The routes leading to the formation of mixed disulfides are interactions between: (i) sulfenic acid derivatives and GSH, (ii) GSSG and protein cysteine residues, (iii) protein cysteine residues and glutathione sulfenate, and, finally (iv) protein cysteine residues and glutathione disulfide S-oxide. Connected to the protein via a disulfide bond, GSH can be removed via a thiol-disulfide exchange reaction. GSH is used by glutaredoxin releasing GSSG. It is now clear that glutathionylation as a posttranslational modification of proteins can be involved in the regulation of the activity of diverse proteins.

In addition to formation of mixed thiols with proteins, GSH may also form mixed disulfides with low molecular mass thiols. In many cases, the biological relevance is uncertain, but in the case of coenzyme A the formation of the mixed disulfide may be biologically important. For example, CoASSG was found to inhibit GR [185], phosphofructokinase [186], and fatty acid synthase [187], whereas fructose-1,6-bisphosphatase was activated by CoASSG [188]. A very potent vasoconstrictory effect of CoASSG has also been described [189].

GSTs also play regulatory roles in many cellular processes in ways that are not usually directly related to their catalytic activity. Frequently their direct interaction with certain regulatory enzymes/proteins has been shown to be involved in cellular responses to oxidative stress, regulation of proliferation, differentiation, and apoptosis. Most information on these mechanisms is associated with the pi-type GSTs (GSTπ). For example, GSTπ inhibits c-Jun aminoterminal kinase (JNK) [183]. JNK phosphorylation activates c-Jun and triggers activation of multiple downstream effectors related to proapoptotic signalling and certain cytotoxicities but its sequestration in a complex with GSTπ blocks these events. Under oxidative or nitrosative stresses the above complex dissociates, and GSTπ undergoes glutathionylation with subsequent oligomerization. The GSTπ isoenzyme is believed to be the main isoenzyme involved in this effect, although other soluble isoforms of GST may also be involved in this type of regulation [183].

The glutathionylation process is thought to be responsible for the anticancer effect of PABA/NO [O^2-{2,4-dinitro-5-[4-(*N*-methylamino)benzoyloxy]phenyl}1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate] [190]. Overexpression of GSTπ in solid tumors is linked to the development of resistance to a number of anticancer agents. PABA/NO is catalytically activated by GSTπ releasing $^{\bullet}$NO that elicits antitumor activity both *in vitro* and *in vivo*

[191]. Locally produced •NO extensively modifies specific target proteins, particularly protein disulfide isomerase (PDI). Nitrosylation or glutathionylation of PDI leads to enzyme inactivation, activation of the unfolded protein response (UPR), and cancer cell death. It has been suggested that •NO itself may not be directly responsible for the toxicity of PABA/NO, but rather that peroxynitrite, which is much more reactive, provides the effect. Peroxynitrite is a product of the interaction between nitric oxide and superoxide anion radical and is known to be a powerful nitrosating agent [190].

7. Regulation of Transcription of GSH-Related Genes

Being an important antioxidant either directly, or via GSH-related enzymes, GSH is a key component in the regulation of redox homeostasis. It is well known that changes in GSH levels or deregulation of the redox status are caused or at least are associated with diverse pathologies and aging. The most thoroughly investigated cases include cardiovascular and neurodegenerative diseases, cancer, AIDS, cystic fibrosis, liver disorders, diabetes mellitus, and associated complications. Regulation of the activities of GSH-related enzymes is often considered as a way to prevent or ameliorate the disease. Several cellular signalling systems are known to be involved. However, the mostly efficient approaches are related to the possibility of manipulating GSH biosynthesis and phase II detoxification enzymes. In the former case, attention is focused on the first key enzyme of GSH synthesis, γGLCL, and in the latter case on GSTs. These enzymes are mainly regulated at the expression level and some of the mechanisms involved have been deciphered. Although it is known that the promoter regions of the genes encoding γGLCL and GSTs possess binding sites for such transcriptional regulators as NF-κB, AP-1, AP-2, SP-1, and others [192–194], most attention has been concentrated on the Nrf2/Keap1 system [160, 195]. This is connected, at least partially, to its high sensitivity to effectors relative to other regulatory systems [81]. The Nrf2/Keap1 system is responsive to many challenges, particularly to oxidants and electrophiles. As mentioned above, Nrf2 operates in concert with an adaptor protein, Keap1, a cytoplasmic resident. In nonstressed cells the binding of Nrf2 to Keap1 promotes ubiquitination of Nrf2 followed by proteasomal degradation. This system is tightly regulated in cells (Figure 5). Enhanced levels of oxidants or electrophiles, as well as activation of various protein kinases disrupt the Nrf2/Keap1 association resulting in Nrf2 stabilization and migration into the nucleus. Therein Nrf2 binds to the ARE/EpRE in the promoter region of target genes and in concert with small proteins of the Maf family stimulates their transcription. In a series of elegant studies several mechanisms that direct Nrf2 into the nucleus have been described (reviewed in [81]): (i) oxidation of specific cysteine residues of Keap1 resulting in its inability to bind Nrf2, (ii) interaction of nucleophilic molecules with cysteine residue(s) of Keap1 leading to the formation of adducts that prevent binding to Nrf2, (iii) phosphorylation of Nrf2 by different protein kinases, and (iv) ubiquitination of Keap1 followed by proteasomal hydrolysis (Figure 5).

Deciphering the mechanisms of operation of the Nrf2/Keap1 system helped to explain various previously puzzling data on chemoprevention in several disease states. Chemoprevention has attracted much attention as one of the most practical and realistic strategies for decreasing the global burden of diseases related to xenobiotics and certain oxidants. A mechanistic approach has gained acceptance recently because it not only provides the rationale to reveal potential mechanisms, but it also predicts and identifies potentially effective chemicals. A broad spectrum of substances have been reported that exhibit chemopreventive potential, and it is noticeable that many of these substances were identified in plants, particularly those that are medicinal and/or edible. Numerous phytochemicals derived from fruits, vegetables, grains, spices, and herbs are capable of affecting certain diseases related to disrupted GSH homeostasis. Extensive reviews on chemopreventive phytochemicals have been published. Thus, there is no need for in depth coverage of this field, and interested readers are directed instead to several excellent recent reviews [21, 22, 86, 166, 195–198]. In the present review, discussion will be limited to well-studied phytochemicals that operate by affecting the Nrf2/Keap1 system. These have been exceptionally well discussed by Surh and colleagues [21] and are summarized in Table 1.

Sulforaphane [1-isothiocyanato-(4*R*,*S*)(methylsulfinyl) butane] is an isothiocyanate found in broccoli and other cruciferous plants. It is a known inducer of genes encoding phase II defense and antioxidant enzymes including GPx, GST, and γGLCL [196, 211]. Sulforaphane appears to modulate upstream MAP kinases, but reliably demonstrated effects are associated with Nrf2 activation via the direct modification of Keap1 cysteine residue(s) [199]. As an electrophile, sulforaphane directly interacts with protein thiols forming thionoacyl adducts. In addition, sulforaphane induces structural changes in Keap1 leading to its polyubiquitination and proteasomal degradation [200].

Curcumin (diferuloylmethane) is derived from the rhizomes of turmeric (*Curcuma longo*). It stimulates the expression of antioxidant and phase II detoxification enzyme genes in several experimental models [212–214]. Curcumin-induced expression is also mediated via Nrf2 activation in a ROS-related manner. ROS activate PKC and P38 MAP kinase which then have downstream effects by phosphorylation of Nrf2 [201, 215].

Epigallocatechin gallate (EGCG) is a major active catechin of green tea that exerts antioxidant, anti-inflammatory and chemopreventive properties [86, 216, 217]. It stimulates Akt, ERK1/2 and P38 MAP kinase leading to Nrf2 phosphorylation and its import into the nucleus [202, 218].

Several allyl sulfides, namely, diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) are major components of garlic that are capable of inducing phase II detoxification enzymes in a Nrf2-dependent manner [203, 204, 219]. DAS transiently increases ROS concentrations

Table 1: Phytochemicals that are known to activate the Nrf2/Keap1 signalling pathway in human and animal systems with identified mechanisms.

Phytochemical	Keap1		Nrf2		References
	Oxidation	Alkylation	Ubiquitination	Phosphorylation	
Sulforaphane	−	+	?	?	[199, 200]
Curcumin	+	−	−	?	[201]
Epigallocatechin gallate				+	[202]
Allyl sulfides	?			+	[203, 204]
Resveratrol				+	[205]
Capsaicin				+	[206]
(10)-Shogaol		+			[207]
Lycopene					[208]
Carnosol				+	[209]
Xanthohumol		+			[210]

stimulating, ERK and P38 MAP kinase which phosphorylate Nrf2 [203, 220].

Resveratrol (trans-3,5,4′-trihydroxystilbene) is a polyphenol found in grapes, bilberry, blueberry, other berries, and other plant species. It exerts antioxidant, anti-inflammatory, antiaging, and chemopreventive activities affecting cellular signalling [205, 221, 222]. These activities are mediated, at least partially, by Nrf2 phosphorylation.

Pungent vanilloids such as capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide), a major pungent of hot chili pepper (*Capsicum annuum*) [206, 223], and (10)-shogaol from ginger (*Zingiber officinale*) also activate phase II detoxification enzyme expression in a Nrf2-dependent manner [207]. The former acts in a ROS-dependent manner via PI3/Akt mediated Nrf2 phosphorylation, whereas the latter acts via electrophilic alkylation of Keap1.

Lycopene, a natural carotenoid found in tomato and tomato products also exerts chemopreventive activity in an Nrf2-dependent manner [208, 224]. However, there is no available information on the mechanisms involved. It should be noted, that absorption of lycopene by the intestine is much more efficient from processed tomatoes than from fresh tomatoes due to a higher bioavailability in the processed products [225–227].

Carnosol, an orthophenolic diterpene found in rosemary (*Rosmarinus officinalis*), also enhances the expression of phase II detoxification enzyme genes in an Nrf2-related manner [228]. Upregulation of ERK, P38 MAP kinase, and JNK pathways was found to be responsible for the effects, which potentially show the involvement of Nrf2 phosphorylation [228]. Cinnamaldehyde from dried bark of *Cinnamomum cassia* also induced phase II enzyme expression via Nrf2 translocation into the nucleus [209, 229, 230]. Xanthohumol, a sesquiterpene from hop (*Humulus lupulus*) also shows chemopreventive activity, inducing antioxidant and phase II detoxification enzymes [210]. Its action was linked with Nrf2 activation resulting from the alkylation of Keap1. Hence, a great variety of diverse agents of natural origin have been found that activate the Nrf2 signalling pathway, but it is likely that many more remain to be discovered.

Many diverse studies on the involvement of Nrf2 and associated components were discussed above. However, in our opinion, the authors have not always provided clear evidence of direct or mediated Nrf2 involvement in the upregulation in certain systems. Although Nrf2 involvement could be expected logically, other signalling pathways should also be investigated. This is especially true when dealing with natural extracts instead of pure compounds because even a minor component in the extract may affect the system via an unidentified pathway(s) and imitate Nrf2 involvement. Unfortunately, in many cases the data presented do not provide definitive evidence to support the involvement of Nrf2.

The chemopreventive efficacy of various phytochemicals that has been demonstrated in cell models frequently cannot be extrapolated to whole organisms due to low bioavailability. Only a very small portion of consumed phytochemicals is absorbed in the gastrointestinal tract, usually much less than 1% [231, 232]. In addition, there are often potentially negative effects on organisms due to supposedly useful phytochemicals. They often activate the expression of genes encoding phase I detoxification enzymes such as cytochrome P450. This can create problems because many xenobiotics may be activated by oxidation mediated by these oxygenases and thereby express their toxic potential. In this case, the transcriptional activation of genes encoding these oxygenases would be considered a negative side effect of phytochemical treatment. In some cases, these compounds may simultaneously activate the expression of phase I and phase II enzyme genes, in which case the final result would be unpredictable in many circumstances. Simultaneous induction of the expression of genes encoding phase II detoxification and antioxidant enzymes may take place with so-called phase III detoxification enzymes which are membrane pumps providing active extrusion of GSH conjugates of electrophiles that are formed either spontaneously or enzymatically in GST-catalyzed reactions. A final important issue must be emphasized when analyzing effects

due to phytochemicals. Phase II and phase III detoxification enzymes may be responsible for catabolizing certain drugs (such as drugs used to treat cancer) via conjugation with GSH and extrusion from cells. This could lead to the need to increase doses of some drugs to make them effective or could even result in resistance to the drugs.

The mechanism of induction of phase II enzyme expression by plant polyphenols has been elucidated by Zoete and colleagues [233]. They investigated the ability of these compounds and their synthetic analogs to induce the activity of NADP(H) quinone reductase (NQ01), a prototypic phase II detoxification enzyme. By using quantum-mechanical methods the authors calculated the tendency of these compounds to release electrons by the energy of the highest occupied molecular orbital (E_{HOMO}). They found that the smaller the absolute E_{HOMO} of an agent (i.e., the lower its reduction potential), the stronger its electron donor property was and the greater its inducer potency. That allowed inducers to be ranked and led to predictions of the efficiency of inducers based on their reduction potential [233]. However, it should be noted that the experiments were carried out in cell culture, which does not take into account factors such as the absorption and transportation of polyphenols when they are administered to the whole organism. However, the approach may give some clues for the prediction of the biological effects of polyphenols in regulating the activity of antioxidant and phase II and III detoxification enzymes.

8. Relationship between GSH Homeostasis and Pathologies

Elevated ROS levels as well as the presence of different xenobiotics are well-known factors in various pathologies and aging, but in some cases these relationships are not straightforward. Many details of GSH involvement in these processes including regulation of GSH-related enzymes were discussed above. Therefore, the current section will provide a general summary as well as highlight some potentially useful therapeutic avenues.

Figure 7 shows general routes of enhanced ROS levels and/or the presence of xenobiotics associated with various pathologies. Elevated ROS levels are a key finding in many diseases [234] including cardiovascular and neurodegenerative diseases, cancer, diabetes mellitus, and aging [88, 89, 197, 235–237]. ROS concentration may be enhanced for many reasons of both an internal or external nature, such as inflammation or exposure to xenobiotics. GSH can interact directly with ROS to reduce their levels and in this manner delay the development of pathologies. The potential of various phytochemicals to disrupt this link between ROS elevation and increased pathology may be related to the inherent antioxidant activity possessed by various plant components. However, potentially more potent protective effects of phytochemicals may arise from indirect effects. Since this review is focused on GSH, the ways in which GSH participates in these processes must be highlighted. They include (1) activation of GSH biosynthesis via supplementation of substrates and energy, (2) increased enzymatic potential to produce GSH and reduce GSSG, (3) increased activities of detoxification enzymes that use GSH, and (4) activation of routes for extrusion of GSSG and glutathione *S*-conjugates from cells. It is clear from this list that there are several good targets for pharmacological interventions in pathologies in which oxidative stress may be a contributing factor.

The uptake of xenobiotics and their interaction with biomolecules in living organisms depend on various factors such as their chemical and physical properties, type of organism, and its physiological state. Here, we will not focus on specific aspects, but rather will provide the general principles of xenobiotic metabolism leading to pathologies, GSH involvement and potential protective effects of certain phytochemicals. Some xenobiotics can be directly autoxidized leading to ROS production and the potential pathological consequences were described above. However, most xenobiotics are not autoxidized directly and contribute to pathology only after transformation via different mechanisms. Many xenobiotics are oxidized by various endogenous oxygenases with the production of ROS at this stage. The biotransformed xenobiotics that result may also have enhanced potential to induce pathology via direct interaction with cellular constituents due to their electrophilic nature. Biotransformed xenobiotics may also undergo autoxidation with concomitant ROS generation. In order to prevent this scenario, cells utilize phase II detoxification enzymes. GSH plays a prominent role in this process, either directly conjugating with xenobiotics or participating as a substrate in enzymatically catalyzed conjugation reactions. Finally, conjugates are excreted from the cell by the phase III detoxification system of plasma membrane active transporters. However, cellular GSH is not lost to a great extent; most is reclaimed via GSH salvage processes (Figure 2). This means that extracellular transpeptidases cleave the conjugates releasing different GSH components which may be reabsorbed by cells and reused for tripeptide resynthesis. Overall, then, GSH may prevent the development of pathology related to electrophiles either by directly interacting with them or in an enzyme-catalyzed manner. Some phytochemicals also directly interact with electrophiles, but their action may also be realized through activation of GSH synthesis/resynthesis and reduction. Activation of phase II and III detoxification enzymes is thought to be the main route for xenobiotic detoxification and excretion from the organism. Activation of the transcription of genes encoding enzymes that combat xenobiotics is one of the main pharmacological strategies for treating xenobiotic-induced diseases. As described above, the Nrf2/Keap1 system, in concert with other signal transduction systems, regulates the expression of genes encoding many of the enzymes involved in phase I, II, and phase III xenobiotic detoxification. Some phytochemicals may stimulate phase I detoxification enzymes and also increase cellular potential for detoxification of drugs, which may cause either a decrease in sensitivity to the drug or even complete resistance. This emphasizes the need for a clear understanding of the

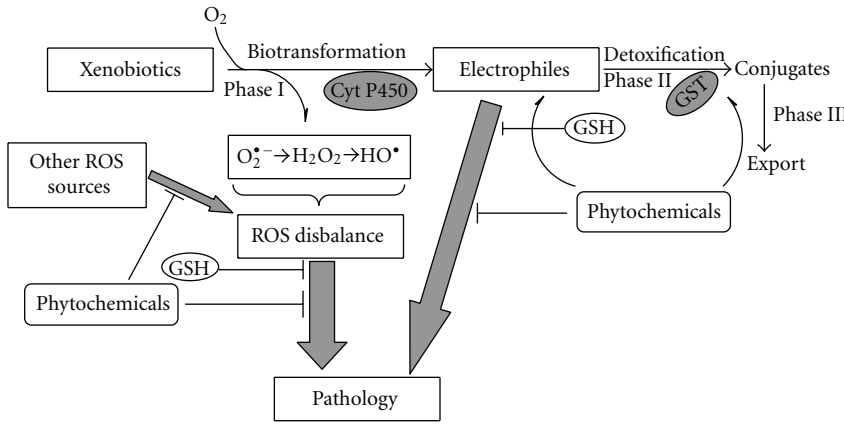

Figure 7: Involvement of glutathione in the detoxification of xenobiotics and reactive oxygen species, its relationship with pathological development and the potential role of different phytochemicals. Glutathione is responsible for helping to maintain redox balance by directly or indirectly interacting with ROS, and is also involved in detoxification of electrophiles either via direct interactions or via enzyme-catalysed conjugation. Certain phytochemicals may affect GSH action on ROS and electrophiles either by directly interacting with ROS and electrophiles, or by upregulating defensive enzymes.

molecular mechanisms of both drug and phytochemical action for the development of new medical strategies.

9. Research Tools and Pharmacological Approaches to Manipulate Glutathione Levels

The role of GSH in the function of living organisms is clearly reflected by a phrase coined by Sies [4]—the term "inevitable GSH." The great importance of GSH has been revealed in multiple experiments either by depletion or repletion of cellular GSH reserves.

Cellular GSH reserves can be depleted in at least three different ways—by increasing GSH oxidation, by inhibition of biosynthesis, or by inactivation of the genes encoding the enzymes of GSH synthesis. Experimentally, the cellular GSH pool can be reduced by treatment with different oxidants such as hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide [238, 239], or diamide [240, 241]. In 1979, a specific inhibitor of γGLCL was synthesized—buthionine sulfoximine (BSO) [13, 14], that when introduced into cells depletes GSH reserves [16, 17, 242]. These approaches have helped researchers investigate the function of cellular GSH. Since the use of oxidants to deplete GSH pools in the treatment of different pathologies usually causes many side effects, BSO was soon tested not only for basic research purposes, but also for clinical investigations in cancer research. For example, local BSO application to certain skin cancers may sensitize them to irradiation [243], drug [244], and photodynamic [245] treatments.

Frequently used tools in GSH research and therapy are interventions that increase GSH levels [246]. This is usually achieved by supplementation with GSH monoesters and diesters [247–249], GSH precursors such *N*-acetyl cysteine (NAC); [250–252] or α-mercaptopropionylglycine [87, 253]. Importantly, cysteine is not usually utilized as a precursor presumably due to its toxicity at high concentrations [254]. On the other hand, cysteine in protein-bound form, particularly as a component of whey, has some potential to increase GSH levels [255–258]. The above compounds are used as precursors for GSH biosynthesis, both experimentally and in some therapies; for example, NAC is broadly used in therapies that combat HIV [259–261] and other infections [262–264]. Although used less frequently than NAC, cysteine precursor in the form of prodrug, 2-oxothiazolidine-4-carboxylate (OTC), is also used to enhance cellular GSH level [247]. Experimentally, the overexpression of certain genes involved in GSH production also may enhance its level.

At least one important factor needs to be taken into account when treatments are used to elevate GSH. It is well known that many "classic" antioxidants can, under certain conditions, become prooxidants. These include low molecular mass antioxidants such as ascorbic acid [265, 266], epigallocatechin-3-gallate [267], α-tocopherol [268], and retinol [265], as well as antioxidant enzymes such as superoxide dismutase [269, 270]. Although information on possible prooxidant properties of GSH is very limited [271–273], its potential prooxidant effects cannot be ignored. Virtually all compounds known as antioxidants possess prooxidant properties [274]; these are two sides of the same coin. The relationship between pro- and antioxidant properties depends on the nature of the compound and specific conditions.

10. Conclusions and Perspectives: Glutathione—Two Faced Janus Pharmacological Target

GSH has a very complicated pattern of involvement in diverse biological processes. Consequently, any experimental and clinical intervention should be undertaken with precaution due to the complicated, interrelated, and tightly regulated networking of living processes. In many cases, any modification of one parameter may result in unpredictable

responses from diverse processes. For example, at first glance, an increased GSH level through supplementation of its esters may augment defense mechanisms of not only normal cells, but also of cancer cells, especially considering that cancer cells may be rather aggressive in sequestering resources. This can result in a need to enhance the doses of anticancer drugs.

The same ideology can be applied to upregulation of detoxification and antioxidant enzymes. They are frequently regulated at the transcriptional level via enhanced Nrf2 binding to ARE/EpRE DNA elements. However, in many cases, phase II and III detoxification enzymes are also responsible for the detoxification of anticancer drugs and their extrusion from the cell. In addition, some inducers of these enzymes affect phase I detoxification enzymes, which frequently may transform procarcinogens to actual carcinogens via metabolic activation by hydroxylases such as cytochrome P450.

However, taking into account the potential undesirable effects of pharmacological interventions, there is a need to investigate them carefully and many different models may be used for that purpose. Based on available information, some specific molecules with expected properties can be synthesized and tested. Several important notes should be provided in this case. Many potential effectors can exist in several forms and chemical synthesis may lead to the production of, for example, mixtures of different racemates or diastereoisomers, some of which may be pharmaceutically effective, but others of which may cause deleterious effects such as what occurred with thalidomide. One of its racemates was teratogenic [275]. The second important consideration in the chemical synthesis of putative drugs is related to the production of intermediates and side products, which needs special attention and investigation.

Another important factor should be reiterated here. Innumerable studies have shown that GSH is an antioxidant. However, virtually any antioxidant can, under certain conditions, act as a prooxidant [274]. For example, in studies with yeast we found that superoxide dismutase may act either as an anti- or prooxidant depending on its expressed activity [269, 270]. Under certain conditions GSH also can be a prooxidant [276]. Therefore, precaution should be paid to interventions that enhance GSH levels.

Because of the above caveats, modern pharmacology research has refocused on natural products, mainly of plant origin, although bacteria, fungi, and animal sources cannot be ignored. The ideal situation is when these components are possessed by edible vegetables, fruits, herbs, and spices or products formed during their processing. Excellent examples of these include sulforaphane from cruciferous plants [196, 211], epigallocatechin gallate from green tea [86, 216, 217], curcumin from turmeric [201, 212–215], allyl sulfides from garlic [203, 204, 219, 220], anthocyanins and resveratrol from different berries and grapes [196, 205, 221, 222], and carnosol from rosemary [228]. These and other examples demonstrate the great potential for discovery of natural compounds that can be used as pharmaceuticals that may affect GSH homeostasis.

Careful selection of experimental models is very important. Cell cultures are extremely useful for the identification of potential drugs. They allow rapid testing of diverse potential compounds at low cost. This approach is especially helpful for revealing molecular mechanisms of investigated processes. In some cases, simpler cellular models such as bacteria and unicellular yeasts can also be used as models, but in many cases their pathways of xenobiotic catabolism are very different from those of mammals thereby limiting their use. However, all isolated cell systems have at least two serious limitations. The first is that isolated cells are not under systemic control by the whole organism, lacking factors such as the regulatory effects of endocrine and nervous systems, which may substantially modify cellular responses. The second is that chemicals or mixtures for testing are applied directly to cells, which avoids complicated whole organism processes such as absorpton, transportation, transformation, and excretion. These processes can lead to large differences in the responses of isolated cells versus cells in intact organisms, emphasizing the fact that both basic and applied studies must ultimately rely on the use of whole animal models.

Animal models also have some limitations, both technical and ethical. The second is beyond the scope of this review, and, therefore, we will focus only on the first item. First, animal experimental models are much more expensive and require many more resources than cellular models. Certainly, mammalian models are the most valuable because these animals are closest to the human condition. However, much information may be gained from simple animal models that may be ultimately applied to mammals. The fruit fly, *Drosophila melanogaster*, is one of the most popular and tractable animal models. Although it is an invertebrate, it is easy to care for, thousands of different strains exist, and it is possible to manipulate its genome. As a result several experimental models of human pathologies have been developed in *D. melanogaster*, making it a very useful biomedical tool. Many biological processes and their regulation are highly conserved in eukaryotes, particularly from yeasts through insects and to vertebrates. For example, the Nrf2/Keap1 system has recently been described in *D. melanogaster* [277] and fish [278]. Warm-blooded mammals, such as rats, mice, and primates are also extremely useful subjects, but ethical issues often substantially limit the use of mammalian models. As a result, cellular models are often preferred to animal models. Certainly, clinical trials in human populations are the final step before introduction of certain drugs.

One more aspect which is frequently ignored should be highlighted here. This is the problem of accurate measurement of the levels of different glutathione forms, particularly reduced (GSH) and oxidized (GSSG) forms, their ratio (an index of redox potential), and mixed thioethers need further experimental development. This is very important because these parameters are used to characterize the development of oxidative or nitrosative stresses under some circumstances, particularly in certain pathologies [9, 10, 27, 146]. When dealing with cell cultures or unicellular organisms it is practically impossible to isolate cells from the cultivation media and fix GSH level quickly. Other problems exist when studying multicellular organisms. One is the need

for very rapid dissection and freezing of target tissues because the redox state in cells can change very rapidly. Another is the fact that many organs consist of multiple cell types which can possess different glutathione levels and forms. In other words, global analysis of the whole tissue may give incorrect assessments of glutathione status in different cell types. Finally, there is an issue of the intracellular distribution of glutathione and its metabolites. Disintegration of the cell to isolate subcellular components may result not only in glutathione redistribution, but alter the redox ratio of reduced to oxidized forms, that is, redox potential. New approaches, particularly to resolve *in vivo* glutathione quantification, are needed to solve these and related problems.

Therefore, a scheme for investigation of potential chemicals, pharmaceuticals, or phytochemicals that target GSH homeostasis may be proposed. At the first stage of investigation, cell cultures and unicellular organisms can be used to identify potential candidate compounds and potential effectors and, if possible, to identify mechanisms involved. Selected compounds would then be evaluated at the whole organism level. Studies with *D. melanogaster* are easy and cheap to perform, and existing or specially produced fly lines with deleted regulatory/effector systems may be tested to provide further clues as to the biological action and side-effects of the candidate compound. Zebrafish (*Danio rerio*) also can be used as an alternative genetically tractable model organism, the genome of which has been sequenced, and many tools for molecular interventions in this organism have been developed. Indeed, there are reliable data on the possibility of manipulating the Nrf2/Keap1 system in these fish [279]. If successful in these organisms, candidate compounds of interest may then be studied in mammalian models. The development of molecular biological tools and production of lines with deleted genes or chimeric lines may also provide some additional information. Research with genetically transformed mice would provide the most useful information, but they are expensive and time consuming to work with. So, the final strategy would depend on many circumstances and rely on the facilities available, particular interests, skills and experience of reserchers.

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Potential use of glutathione as a treatment for Parkinson's disease

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Abstract. The aim of the present study was to assess the efficacy and safety of glutathione (GSH) for the treatment of Parkinson's disease (PD). The PubMed, Cochrane Library, OvidSP, Web of Science, China Science and Technology Journal Database, Chinese National Knowledge Infrastructure and China Wanfang Standards Database databases were systematically searched from the inception dates to October 1st, 2019, using the key words'glutathione' or 'GSH' and 'Parkinson' or 'Parkinson's disease' or 'PD'. The quality of the included articles was assessed using the bias risk assessment tool of the Cochrane systematic evaluator manual (version 5.1.0). Pooled analysis of the relevant data was performed using RevMan 5.3 software and subgroup analysis was performed to determine the impact of the dosage (300 vs. 600 mg) on the outcome measures. A total of seven randomized controlled trials involving 450 participants were included in the meta-analysis. The results of the present study indicated a statistically significant difference between the GSH and control groups, in terms of the Unified Parkinson's Disease Rating Scale (UPDRS) III [standard mean difference (SMD), -0.48; 95% CI, -(0.88-0.08); P=0.02] and GSH peroxidase (SMD, 1.88; 95% CI, 0.52-3.24; P=0.007). However, the differences in the UPDRS I (SMD, -0.04; 95% CI, -0.25-0.16; P=0.70) and UPDRS II (SMD, 0.03; 95% CI, -0.17-0.24; P=0.77) score and in side effects were not statistically significant between the groups. Subgroup analyses revealed that the dosage (300 vs. 600 mg) was an influencing factor for UPDRS III. The present study demonstrated that GSH may mildly improve motor scores in PD, but not at the expense of increased adverse events.

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Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease associated with aging, which is characterized by the selective loss of nigrostriatal dopaminergic neurons (1-3). PD is the second most common neurodegenerative disease in the world with a prevalence that is estimated to reach between 8.7 and 9.3 million by 2030 (4). To date, the pathophysiology of PD remains to be fully elucidated, though studies indicate that oxidative stress may be one of the mechanisms contributing to PD (5). There is currently no cure for PD; thus, further research into the development of novel treatment strategies is critical (6). Increasing evidence has demonstrated that oxidative stress has an important role in the events contributing to the degeneration of dopaminergic neurons (7), and that redox reactions are a possible source of oxidative stress in nigral dopaminergic neurons (8). Glutathione (GSH) is a ubiquitous thiol tripeptide that protects against oxidative stress-induced damage by neutralizing reactive oxygen species (5). GSH deficiency has been identified as an early event in the progression of PD (9). Therefore, supplementing GSH may effectively improve the symptoms of PD. In recent years, a number of clinical trials have sought to investigate the effects of GSH treatment for PD (10-12). Regrettably, the sample size of these studies was small and the clinical evidence is insufficient (10-12). To the best of our knowledge, no previous meta-analyses have assessed the efficacy and safety of GSH in patients with PD. Hence, in the present study, a meta-analysis was performed with the aim of providing medical evidence-based support for GSH treatment in these patients.

Materials and methods

Search strategy. To identify eligible studies, a primary search was conducted using electronic databases (PubMed, Cochrane Library, OvidSP, Web of Science, China Science and Technology Journal Database, Chinese National Knowledge Infrastructure and China Wanfang Standards Database) from the inception dates to October 1st, 2019, using the keywords 'glutathione' or 'GSH' and 'Parkinson' or 'Parkinson's disease' or 'PD'. Specific retrieval strategies were adjusted according to different databases. The procedure was concluded by: i) The perusal of the reference sections of all relevant studies; ii) a manual search for GSH in key journals and abstracts from the major annual meetings in the field of PD; and iii) contact with experts to request unpublished data. The primary search

Table I. Characteristics of the included trials and participants.

First author (year)	Design	Follow-up	Age (years) GSH/Control	Participants (males/females) GSH/Control	Intervention Route, dose, frequency	Outcomes	(Refs.)
Hauser (2009)	RCT	4 w	62.6+7.9/ 65.9+12.6	(5/5)/(6/4)	Intravenous push, 1400 mg, Qd	A; B; C; E	(10)
Mischley (2017)	RCT	3 m	60.9+11/ 60.9+11	11; 14/14	Intranasal administration, 300 mg or 600 mg, Qd	A; B; C	(11)
Mischley (2015)	RCT	3 m	-	10; 10/10	Intranasal administration, 300 mg or 600 mg, Qd	A; B; C; E	(12)
Bao (2018)	RCT	4 m	64.6+8.2/ 65.1+9.6	(56/44)/(55/45)	Intravenous drip, 600 mg, Bid	A; B; C; D	(14)
Bao (2003)	RCT	6 w	61.41+9.68/ 58.87+7.94	(14/16)/(16/14)	Intravenous drip, 600 mg, Bid	D	(15)
Hu (2019)	RCT	21 d	66.8+6.9/ 70.7+7	(17/15)/(18/13)	Intravenous drip, 1200-1400 mg, Qd	A; B; C; D; E	(16)
Zhang (2005)	RCT	4 m	56+4.5/ 57+4.9	(12/7)/(11/8)	Intravenous drip, 600 mg, Bid	D	(17)

GSH, reduced glutathione; RCT, randomized controlled trial; Bid, bis in die; Qd, quaque die; w, weeks; m, months; d, days; UPDRS, Unified Parkinson's Disease Rating Scale; A, UPDRS I; B, UPDRS II; C, UPDRS III; D, glutathione peroxidase; E, adverse events; m, months; w, weeks.

was completed by independent investigators (HLW and JZ) and any discrepancies were resolved by consultation with an investigator (YZC) not involved in the initial procedure.

Inclusion criteria. The inclusion criteria for the present study were as follows: i) Participants were clinically diagnosed with PD; ii) GSH was administered as an intervention treatment; iii) patients treated with GSH were directly compared with a non-GSH or placebo group; iv) outcomes were determined using the Unified Parkinson's Disease Rating Scale (UPDRS) and/or GSH peroxidase (GSH-Px) and/or related adverse events (AEs); and v) the study was a published randomized controlled trial (RCT).

Exclusion criteria. Articles fulfilling the following criteria were excluded from the present study: i) Randomized trials without a placebo or control group; ii) studies lacking original data; and iii) abstracts, conference papers, letters or comments.

Quality assessment. The risk of bias in the included studies was assessed by two independent reviewers (WHL and JZ) using the Cochrane Handbook for Systematic Reviews of Interventions (13). Bias was evaluated in the following seven domains: i) Random sequence generation; ii) allocation concealment; iii) blinding of participants and personnel; iv) blinding of outcome assessment; v) incomplete outcome data; vi) selective outcome reporting; and vii) other bias, of which random sequence generation, blinding of participants and personnel, and blinding of outcomes assessment were of most interest. Any disagreements were resolved by discussion among all of the reviewers. The risk of bias in each domain was rated as low, unclear or high, according to methods used to ensure the minimization of each form of bias. Using the following methods, individual studies were categorized as having low, high or unclear risk of bias: i) Low risk of bias (plausible bias unlikely to markedly alter the findings) if the risk of bias was low in all domains; ii) unclear risk of bias (plausible bias that raises certain doubt about the results) if the risk of bias was unclear in one or more domains; or iii) high risk of bias (plausible bias that seriously weakens confidence in the results) if a high risk of bias was present in one or more domains. Any disagreements were resolved through a discussion within the entire review team.

Data extraction. Data were extracted by two independent reviewers (WHL and YPL) using a predefined data extraction method. Disagreements were resolved by discussion or consensus with a third independent author (CYZ). The extracted data included the first author, study characteristics (i.e. year, duration and design), participant characteristics (i.e. age, sample size and systemic therapy) and outcomes (UPDRS/GSH-Px/related AEs). For studies with insufficient information, the reviewers contacted the corresponding authors where possible to acquire the data.

Statistical analysis. When conditions permitted, the study was divided into three arms based on the administered dose of GSH used to obtain the two-arm data (300 mg/d groups vs. control groups, and 600 mg/d groups vs. control groups). Dichotomous data were analyzed using the risk ratios (RRs) with 95% confidence intervals (CIs). When the result unit, measurement method or measurement time was inconsistent, continuous outcome measurements were analyzed using standard mean differences (SMDs) with 95% CIs; 95% CIs

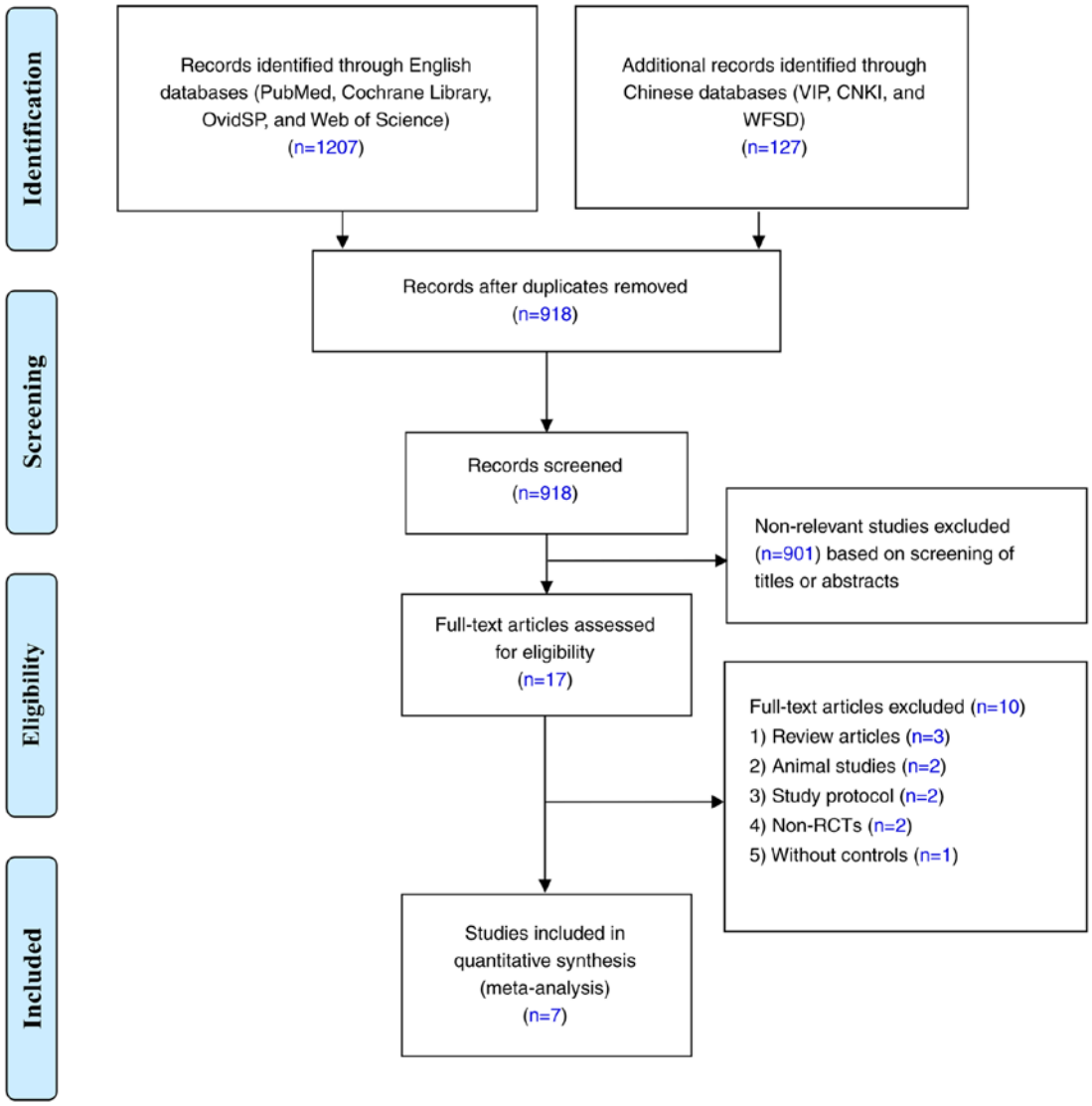

Figure 1. Literature search and screening process. RCT, randomized controlled trial; VIP, China Science and Technology Journal Database; CNKI, Chinese National Knowledge Infrastructure; WFSD, China Wanfang Standards Database.

were calculated using the inverse variance (IV) statistical method. I-square (I^2) statistics and the Q test were performed to assess the impact of study heterogeneity on the results of the meta-analysis. According to the Cochrane review guidelines (13), if severe heterogeneity was present at $P<0.1$ or $I^2>50\%$, the random-effects model was chosen; otherwise, the fixed-effects model was used. Subgroup analyses were performed according to GSH dose.

Results

Search results. According to the aforementioned retrieval strategy, a total of 1,334 related articles were initially retrieved and 918 studies were retained after 416 papers with duplicate data were excluded. Of the identified articles, 901 that did not meet the inclusion criteria were excluded after reading the title and abstract. Of the remaining 17 studies (which were evaluated for applicability by reading the full text), a further 10 were omitted per the exclusion criteria, leaving a total of 7 included studies (10-12,14-17). A flow diagram of the screening process is depicted in Fig. 1. The age distribution of the patients within these studies was 43-84 years and the included studies were published between 2003 and 2019. The studies primarily reported on the outcomes of UPDRS, GSH-Px and related AEs. The specific basic characteristics of the included studies are listed in Table I.

Quality assessment. The quality of the included RCTs was assessed according to the Cochrane Handbook (Figs. 2 and 3) (13). In the category random sequence generation, the seven studies had a low risk. There were two articles with sufficient allocation concealment, while the allocation and concealment schemes of the other five articles were not clear. Furthermore, performance bias of three studies were low-risk and four articles were unclear. There were two papers with low detection bias, while another five articles were rated as unclear with regard to this bias. In terms of incomplete data, seven articles were all rated as having low risk, and the risk of selective reporting was low in five articles and was high in two

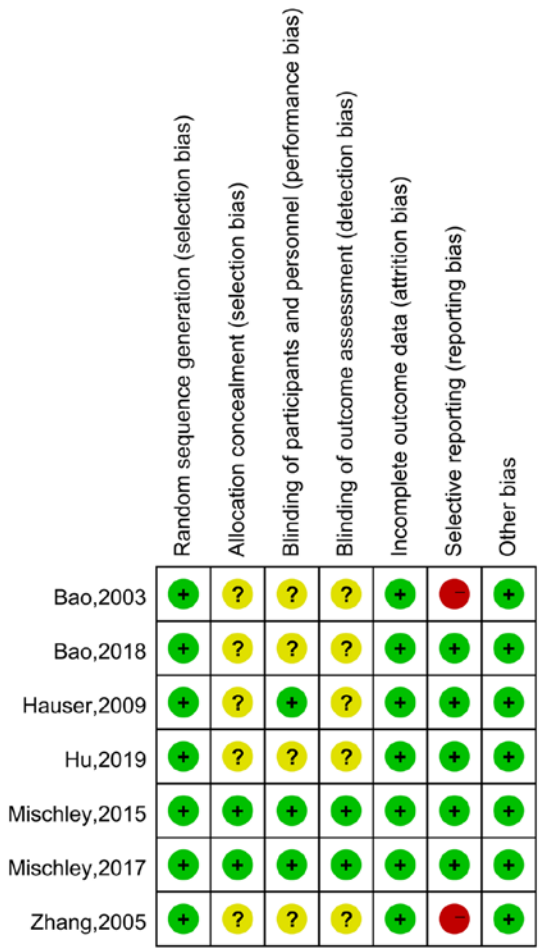

Figure 2. Risk of bias assessment; authors' judgments regarding each risk of bias item for each included study (summary).

studies. There were seven studies with a low risk of other bias. In conclusion, the overall quality of the seven included studies was moderate.

Pooled results

UPDRS. There were five studies reporting data on UPDRS I, II and III (10-12,14,16). Due to differences in data type (end value and end value minus baseline value), the SMD was applied to determine differences in the UPDRS I, II and III scores between the GSH and control groups. The heterogeneity test did not reveal any differences between studies reporting data regarding the UPDRS I (χ^2=3.51, I^2=0%, P=0.74); thus, the fixed-effects model was used (Fig. 4A). In addition, heterogeneity between the studies that reported data on UPDRS II was low (χ^2=7.51, I^2=20%, P=0.28) and thus, the fixed-effects model was applied once again (Fig. 4B). However, the heterogeneity test indicated moderate differences between studies reporting data on UPDRS III (χ^2=14.95, I^2=60%, P=0.02), and therefore, the random-effects model was used (Fig. 4C). The pooled SMD was -0.04 (95% CI=-0.25-0.16, P=0.70) for UPDRS I, 0.03 (95% CI=-0.17-0.24, P=0.77) for UPDRS II and -0.48 [95% CI=-(0.88-0.08), P=0.02] for UPDRS III. These pooled results demonstrated that, compared with the control groups, GSH may slightly improve the motor scores of patients with PD.

GSH-Px. In total, four studies (14-17) presented GSH-Px data for the GSH and control groups. The SMD was used to estimate differences in GSH-Px between the two groups. The results of the pooled SMD are presented in Fig. 5. There was significant heterogeneity among the studies (χ^2=72.36, I^2=96%, P<0.00001) and thus, the random-effects model was used. The pooled SMD was 1.88 (95% CI=0.52-3.24, P=0.007), indicating that compared with the control groups, serum GSH-Px levels were significantly higher in the GSH groups.

AEs. There were three studies reporting on the incidence of gastrointestinal reactions (10,12,16); two papers containing data on dizziness or headache (10,16); two articles reporting on involuntary movement (10,16); two papers on labored breathing (10,12); two articles presenting strep throat-associated data (10,12); and two studies reported on insomnia (10,16) in the GSH and control groups (Table II). The heterogeneity test revealed no differences between the studies (separately, I^2=0, 0, 0, 41, 41 and 0%, respectively); thus, the fixed-effects model was applied. Separately, the pooled RRs were 0.78 (95% CI=0.28-2.14, P=0.62), 0.99 (95% CI=0.28-3.49, P=0.99), 0.33 (95% CI=0.44-2.99, P=0.32), 1.59 (95% CI=0.29-8.59, P=0.19), 1.59 (95% CI=0.29-8.59, P=0.59) and 1.64 (95% CI=0.23-11.74, P=0.62). These pooled results of AEs demonstrate that the use of GSH appears to be safe.

Subgroup analysis: Influence of GSH dosage on UPDRS III. In total, two studies (11,12) reported data of UPDRS III with the use of GSH (300 mg/d) for PD and two papers (11,12) included data on the use of GSH (600 mg/d) (Fig. 6). The heterogeneity test indicated minimal differences between these studies (individually, χ^2=1.06, I^2 =6%, P=0.30; and χ^2=2.87, I^2=30%, P=0.24, respectively), and therefore, the fixed-effects model was used. The pooled SMDs were -0.67 [95% CI=-(1.30-0.04), P=0.04] and -0.16 (95% CI=-0.41-0.09, P=0.21), respectively, suggesting that the dose (300 vs. 600 mg) was an influencing factor for UPDRS III. Therefore, it was conservatively hypothesized that in patients with PD, a 300-mg dose of GSH may be more effective than a 600-mg/d dose.

Discussion

To the best of our knowledge, the present study was the first meta-analysis to evaluate the efficacy and safety of GSH for the treatment of PD. The study provided medical evidence-based support for the effectiveness and safety of GSH. The results of the meta-analysis were as follows: i) GSH does not have the potential to improve mentality, behavior, mood or the ability to perform daily activities, but has the ability to slightly improve motor function in patients with PD; ii) compared with the control groups, serum GSH-Px levels were significantly higher in the GSH groups, though there was notable heterogeneity between the studies (I^2=96%); iii) GSH appears to be safe and, compared with the control groups, does not increase the rate of AEs; and iv) the dose of GSH (300 vs. 600 mg/d) may be one of the factors influencing motor function in patients with PD.

GSH (an antioxidant) is a tripeptide formed by the dehydration condensation of cysteine, glycine and glutamic

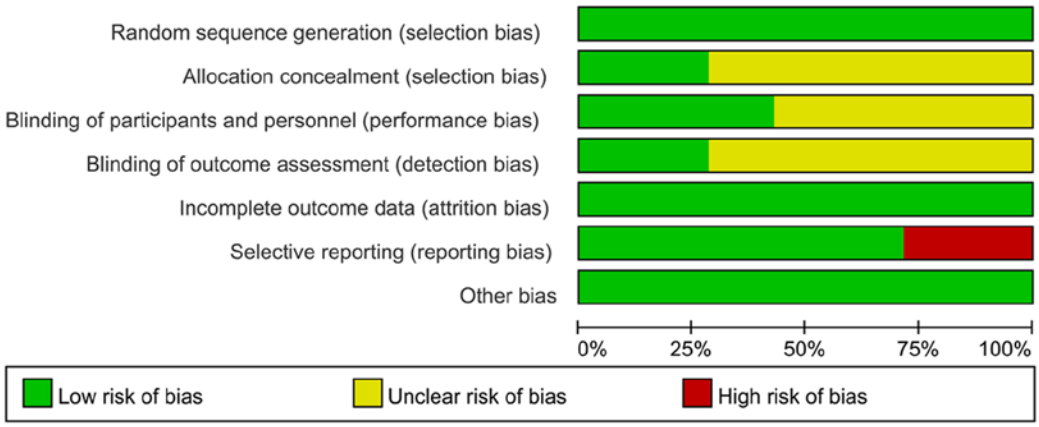

Figure 3. Risk of bias assessment, authors' judgments regarding each risk of bias item for each included study (graph).

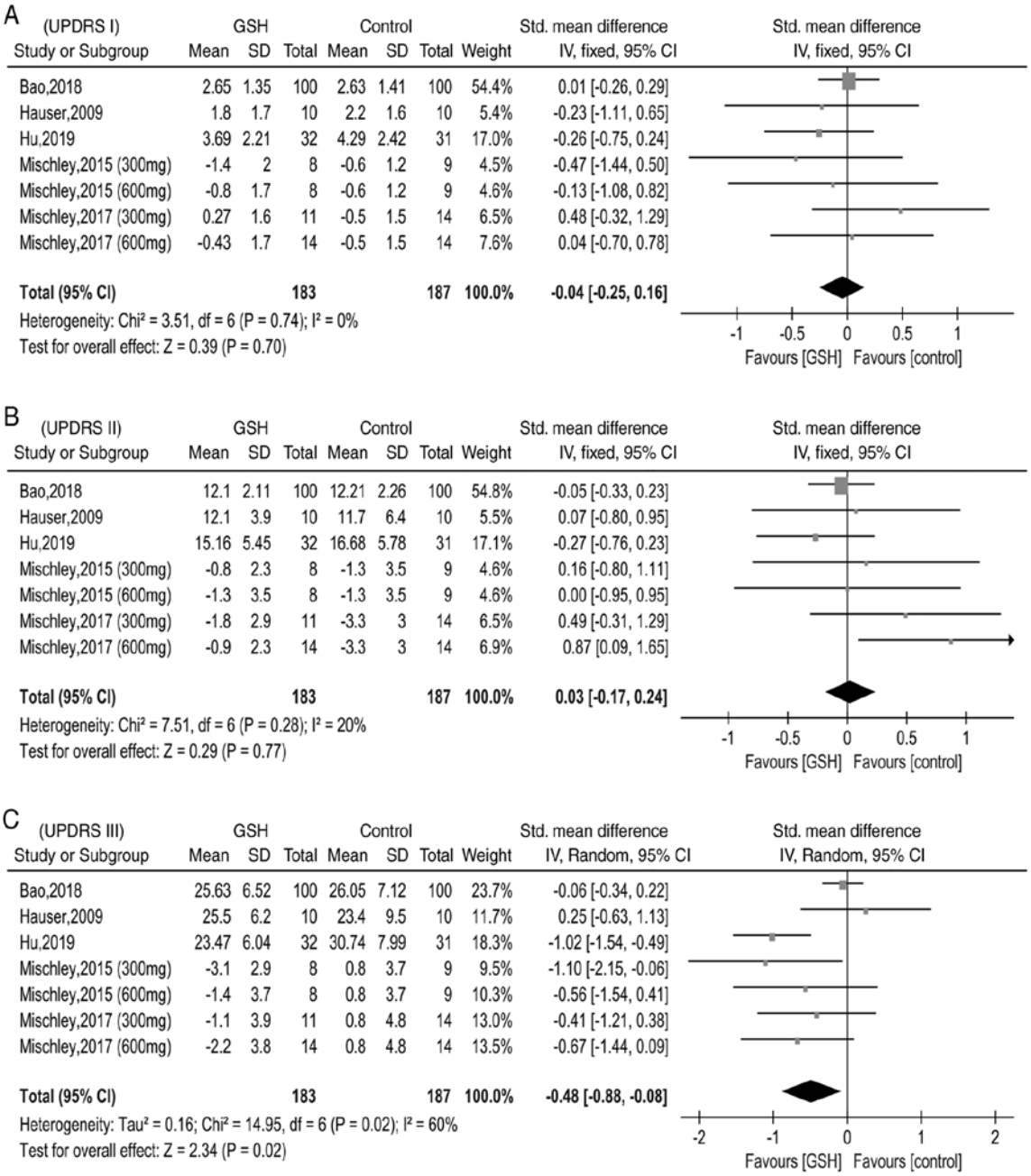

Figure 4. Meta-analysis of UPDRS between the GSH and control groups. (A) UPDRS I; (B) UPDRS II; (C) UPDRS III. UPDRS, Unified Parkinson's Disease Rating Scale; IV, inverse variance; df, degrees of freedom; SD, std. deviation; std., standard; GSH, glutathione.

Table II. Meta-analysis of adverse effects compared between the GSH and control group.

	Pooled results					
Adverse effect	RR	95%CI	P-value	I^2 (%)	P-value	n-value
Gastrointestinal reaction	0.78	[0.28, 2.14]	0.62	0	0.49	7
Dizziness or headache	0.99	[0.28, 3.49]	0.99	0	0.43	4
Involuntary movement	0.33	[0.44, 2.99]	0.32	0	0.99	4
Labored breathing	1.59	[0.29, 8.59]	0.19	41	0.19	5
Strep throat	1.59	[0.29, 8.59]	0.59	41	0.19	5
Insomnia	1.64	[0.23, 11.74]	0.62	0	0.59	4

N, number of study arms in the forest plot; RR, risk ratio.

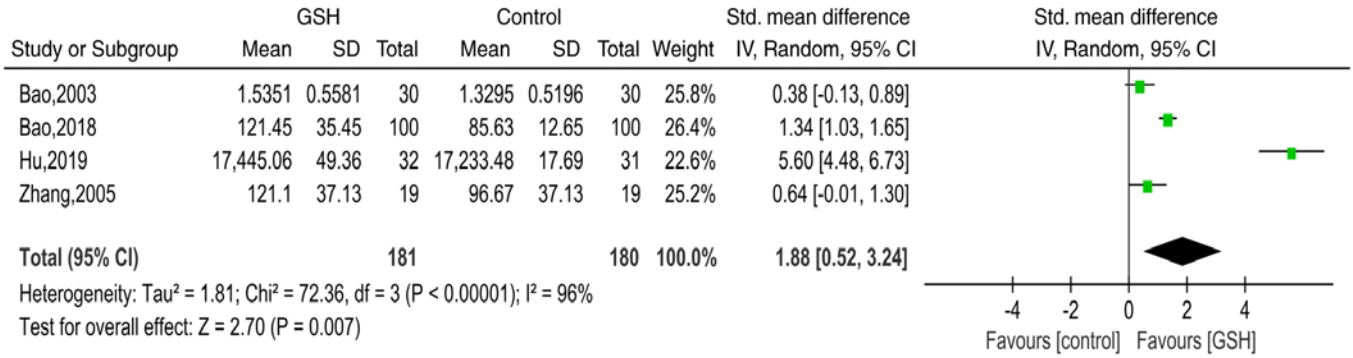

Figure 5. Meta-analysis of relevant studies assessing serum GSH-Px in Parkinson's disease. IV, inverse variance; df, degrees of freedom; SD, std. deviation; std., standard; GSH-Px, glutathione peroxidase.

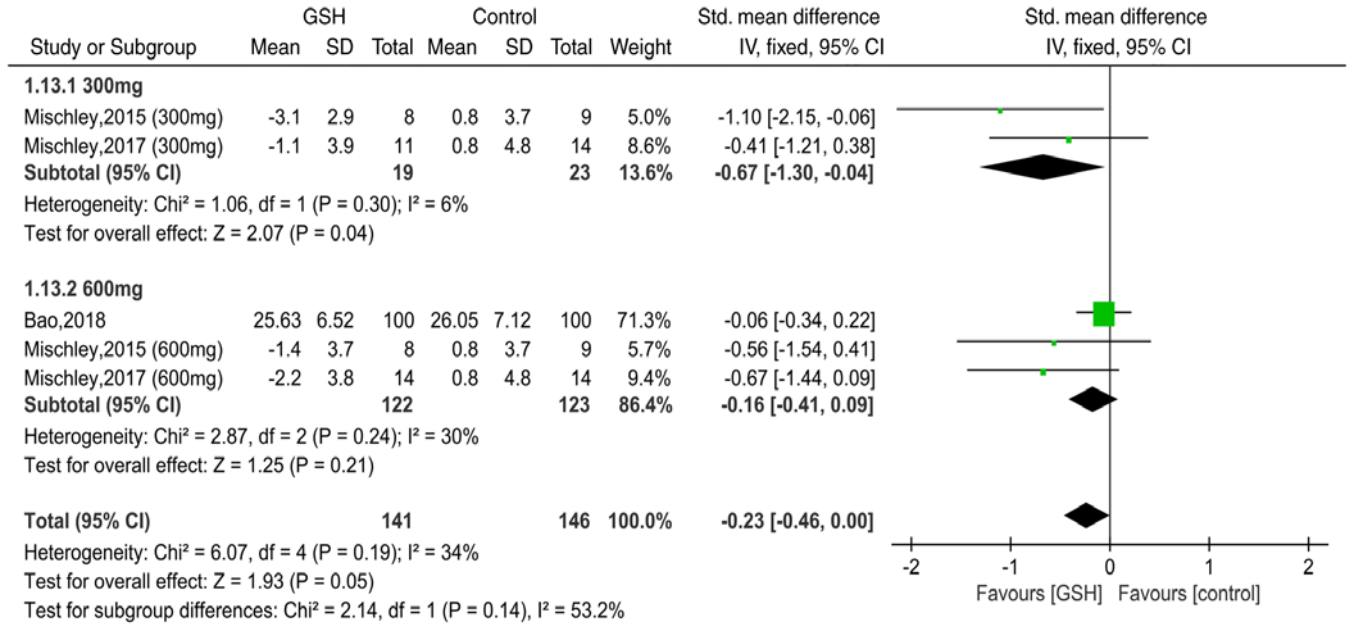

Figure 6. Subgroup analyses were performed in terms of the dose. IV, inverse variance; df, degrees of freedom; SD, std. deviation; std., standard; GSH, glutathione.

acid (11,18). The tripeptide participates in redox reactions, which reduce damage to nerve cells caused by oxygen free radicals (11). Although most individuals synthesize sufficient GSH to maintain a redox balance, this is not the case in patients with PD or other neurodegenerative diseases, which has been demonstrated to be associated with GSH consumption (11).

Several studies have indicated that significant GSH depletion (30-50%) is associated with an increased proportion of oxidized GSH in post-mortem PD substantia nigra tissues (19-21). Furthermore, a clinical study by Mischley *et al* (22) demonstrated that the whole-blood GSH concentration is negatively correlated with the clinical severity of PD. Furthermore, *in vitro* experiments have suggested that increased depletion of GSH results in selective impairment of mitochondrial complex I activity (23). To a certain extent, GSH replacement may provide symptomatic benefits to patients with PD by preventing mitochondrial dysfunction and thus reducing the impairment of dopaminergic function (10). In light of this, a series of clinical studies have been performed. In RCTs by Hauser *et al* (10), 21 subjects were randomly assigned to the GSH (n=11) and control (n=10) groups, which demonstrated that GSH is safe for use in patients with PD. However, there is currently no evidence to suggest that GSH is able to effectively improve the symptoms of PD, which may be the result of the study sample being too small (10). In addition, Mischley *et al* (11,12) also performed RCT studies, though the sample sizes of these studies were also small. In the present study, pooling data from Chinese and English studies revealed that GSH may mildly improve motor function in patients with PD. The results of several animal and clinical trials support these findings (24-26). However, although the present study provides preliminary medical evidence-based data on clinical studies, the effectiveness and safety of GSH supplementation requires further clarification.

Pooling the results of previous studies suggested that GSH-Px is positively associated with GSH levels. GSH non-enzymatically reacts with toxic free radicals and also acts as an electron donor in the reduction of peroxides catalyzed by GSH-Px (27). The resultant oxidized GSH is then being processed by GSH reductase and thus, GSH is recycled (27). GSH-Px has a major role in the recycling of GSH, which is supported by the fact that GSH-Px-knockout mice challenged with toxins (such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) exhibited greater dopamine depletion compared with age-matched control mice (28). Although the underlying mechanisms remain elusive, elevated GSH-Px concentrations appeared to be beneficial in alleviating the AEs of PD treatment.

In addition, several studies (10,12,16) have reported data surrounding gastrointestinal reactions, dizziness or headache, involuntary movement, labored breathing, strep throat and/or insomnia. The pooled results of these studies revealed that the therapeutic dose of GSH is safe. Further patient studies also indicated that when GSH was repeatedly administered at doses of up to 5 g per day, both orally or intravenously, no toxicity was observed (29,30).

In the present study, a subgroup analysis was used to identify the source of heterogeneity surrounding UPDRS III and to perform in-depth data mining. Subgroup analysis suggested that 300 mg/day GSH was more effective than 600 mg/d. However, it is worth noting that 300 mg/day can not yet be confirmed as the optimal dose, because it is not known whether there are other possible optimal doses (the present study only compared the difference between 600 and 300 mg/day). In addition, due to just a few studies with a relatively low population size reporting these data, particularly for 300 mg/day, only two studies published by the same group with only 19 patients treated with GSH in total were included. Therefore, it was conservatively hypothesized that the administration of 300 mg/d or other doses of GSH warrants further investigation in future studies. Such research should be actively pursued in the future; animal experiments provide good evidence that GSH is not only safe but also potentially effective, though findings in humans require further clarification. The dose differences may be a cause of heterogeneity among studies. Furthermore, other confounding factors may also be a source of heterogeneity (such as ethnicity, sex, age, conventional treatment protocol, route of administration, course of disease and degree of disease severity).

The present meta-analysis has several limitations: i) Only seven articles comprising 450 patients were included and the quality of these articles was variable; ii) only studies published in the English and Chinese languages were included, which may have resulted in potential language bias; iii) due to data limitations, subgroup analysis by ethnicity, sex, age, course of disease and disease severity ere not performed; and iv) the pooled results warrant further clarification.

In conclusion, despite the limitations of the present study (which may have influenced these results), it was concluded that GSH may slightly improve the motor scores of patients with PD, though not at the expense of increased AEs. Furthermore, the GSH dosage may influence the efficacy. However, these conclusions warrant further investigation in the future.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HLW, JZ, YPL, LD and YZC contributed to the interpretation of the data and writing of the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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REDOX EXPERIMENTAL MEDICINE

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REVIEW

Glutathione in cancer progression and chemoresistance: an update

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Abstract

Glutathione (GSH) is one of the most important components of the cellular antioxidant system, and it is able to exert several pleiotropic functions influencing cell growth, proliferation, adaptation and death. It has been demonstrated that changes in GSH levels underlie the pathogenesis of many human diseases, including cancer. In detail, although on one hand GSH homeostasis plays a protective role from the onset of cancer, on the other, it is involved in cancer progression and in therapy resistance. In this review, after a brief report on the physiological role of GSH, we have focused the attention on its role in cancer and refractoriness to anticancer therapy giving an update on the preclinical and clinical studies relied on the compounds targeting GSH system. Based on these considerations, a deeper knowledge of GSH-dependent network can be crucial to identify new strategies for preventing and/or curing cancer.

Keywords

- glutathione
- oxidative stress
- cancer
- chemoresistance

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Introduction

Glutathione (GSH) is mainly present in the cytosol, and its distribution between the intracellular compartments is crucial to regulate redox homeostasis, gene expression, cell signaling and proliferation (Sies 1999). The increase in GSH content is associated with early cell proliferation and is crucial to stimulate the shift from G0 to G1 phase of the cell cycle (Lu & Ge 1992). Moreover, it has been reported that GSH sequestration in the nucleus correlates with a reduced transcription of genes encoding for stress and defense proteins and is involved in the modulation of DNA synthesis (Diaz Vivancos *et al.* 2010). It is also likely that the presence of GSH and GSH-related enzymes in the nucleus (Soboll *et al.* 1995) can contribute to maintain nuclear proteins, such as histones and other chromatin-related proteins, in a reduced state in order to guarantee chromatin stability and cell cycle progression.

In this context, the glutathionylation of histone H3, which increases cell proliferation, has been shown to increase the susceptibility of human breast cancer cells to doxorubicin treatment (de Luca *et al.* 2011).

With regard to cancer development and treatment, a different role of GSH has been described in the literature. In fact, on one hand GSH is involved in the detoxification and elimination of carcinogens (Forman *et al.* 2009), and on the other hand the elevated levels of GSH and of other antioxidants detected in different cancers (i.e. melanoma, hepatocarcinoma, bone marrow, breast, colon, pancreatic and lung cancer) can contribute to neoplastic progression and support the acquisition of drug resistance (Gamcsik *et al.* 2012, Traverso *et al.* 2013).

In fact, the anticancer strategy is often based on the induction of oxidative stress through the administration of pro-oxidant chemotherapeutic drugs or ionizing radiation (Pelicano *et al.* 2004, Holley *et al.* 2014). Unfortunately, these approaches, after an initial success, lead to the onset of chemoresistance due to the increase

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of antioxidant defense (Kim *et al.* 2019, Domenicotti & Marengo 2022).

Then, the antioxidant response in cancer is triggered by the increased production of reactive oxygen species (ROS) as a result of mitochondrial dysfunctions, metabolic alterations or long-term anticancer therapy (Sabharwal & Schumacker 2014) (Fig. 1). It has been demonstrated that GSH and other antioxidants maintain ROS at physiological levels stimulating cell survival and proliferation through the activation of redox signaling pathways (e.g. protein kinase B (Akt), mitogen-activated protein kinase (MAPK) and protein kinase C (PKC)) and suppressing cell death induced by supraphysiological ROS concentrations (Dickinson & Chang 2011, Marengo *et al.* 2016).

Biosynthesis and physiological role of GSH

GSH is a tripeptide, consisting of glutamic acid, cysteine and glycine. The majority of GSH is represented by the reduced form (GSH), while the oxidized form (glutathione disulfide, GSSG) is found to be less than 1% of the total GSH (Lu 2013). Notably, the GSH/GSSG ratio has been reported to be decreased under some physiological conditions as observed in newborns (full-term and preterm) (Frosali *et al.* 2004) and following anaerobic exercise (Wiecek *et al.* 2015).

GSH is present at millimolar concentrations mainly in the cytosol, reaching over 70% of total intracellular content. The remaining intracellular GSH (30%) is distributed in the mitochondria (Marí *et al.* 2009), in the nucleus (García-Giménez *et al.* 2013) and in the endoplasmic reticulum

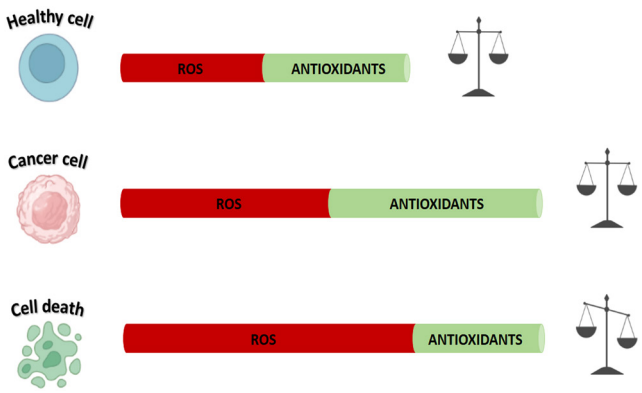

Figure 1
Redox balance in healthy and cancer cells. Healthy and cancer cells maintain redox homeostasis by balancing ROS production with adequate levels of antioxidants. Cancer cells produce greater amounts of ROS, due to their high metabolic rate, and consequently have an increased production of antioxidants. In both cells, when redox balance is not maintained, a condition of oxidative stress occurs, leading to cell death.

where GSSG is the predominant form (Hwang *et al.* 1992). The distribution between different intracellular compartments is important to better regulate cell needs and functions. In fact, GSH can act as a substrate for GSH peroxidases (GPXs) and GSH-S-transferases (GSTs) (Forman *et al.* 2009), it takes part in iron and sulfur metabolism (Liochev 1996) and it is involved in maintaining the reduced form of several dehydrogenases, ATPases (Huang & Philbert 1995) and thioredoxin which is required for the activity of ribonucleotide reductase, a critical enzyme in DNA synthesis (Holmgren 1981).

GSH synthesis is strongly influenced by the availability of cysteine and by the activity of glutamate cysteine ligase (GCL), an enzyme consisting of a modifier subunit (GCLM) and a catalytic subunit, which, in the presence of ATP and Mg^{2+}, forms γ-glutamylcysteine starting from cysteine and glutamate (Lu 2013). Subsequently, the synthesis is finished through the activity of GSH synthetase (GS) which adds glycine to the dipeptide (Forman *et al.* 2009, Lu 2013). Moreover, the maintenance of intracellular GSH and its aminoacids as well as *de novo* synthesis is guaranteed by the 'GSH or γ-glutamyl cycle' (Fig. 2) (Orlowski & Meister 1970). In fact, in this pathway, GSH is degraded into cysteinyl glycine and 5-oxoproline by the enzyme γ-glutamyltransferase (GGT) which is followed by

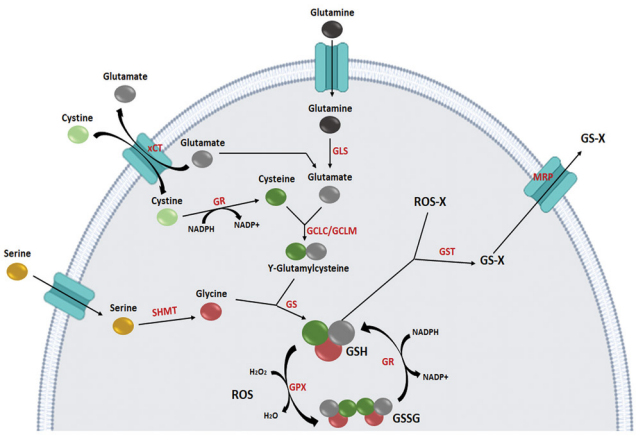

Figure 2
GSH cycle. GSH is synthesized within the cytosol from its precursor aminoacids cysteine, glutamate and glycine through two consecutive reactions controlled by the catalytic and modifier subunits of GSH cysteine ligase (GCLC and GCLM) and by GSH synthase (GS). Cystine is imported through xCT, a cystine/glutamate transporter, and converted to cysteine by GSH reductase (GR); glutamate and glycine can be obtained from their precursors, glutamine and serine, through a reaction catalyzed by glutaminase (GLS) and serine hydroxymethyltransferase (SHMT). GSH can be conjugated with other molecules (GS-X) through the activity of GSH-S-transferase (GST) and then exported via multidrug resistance protein (MRP), or it can detoxify ROS by means the activation of glutathione peroxidase (GPX). If GSH is oxidized to GSSG, it can be regenerated via GR and nicotinamide adenine dinucleotide phosphate (NADPH).

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the release of cysteine, glycine and glutamate. Indeed, GSH is the main source of cysteine, controlling and maintaining the pool and allowing its storage in the reduced form, and in the free form it is rapidly oxidized to cystine (Lu 2013, Traverso *et al.* 2013).

GSH plays a key role not only in maintaining intracellular oxidative balance, acting as an antioxidant, but also in driving metabolism (Meister 1995, Sies 1999) and detoxification mechanisms (Lu 2013). In fact, GSH is able to neutralize free radicals and ROS, and it is also crucial for the activity of GPXs, by acting as a substrate (Forman *et al.* 2009). Upon reaction with oxidizing compounds, GSH is converted to GSSG that, being potentially toxic to cells, is extruded or converted to GSH through the action of GSH reductase (GR), whose coenzyme is nicotinamide adenine dinucleotide phosphate (NADPH) (Couto *et al.* 2016). Moreover, GSH prevents lipid peroxidation of membranes through the regeneration of alpha-tocopherol and protects from ferroptosis, an iron-dependent form of cell death characterized by intracellular lipid hydroperoxide accumulation (Ursini & Maiorino 2020).

In addition, GSH can form conjugates with xenobiotic compounds, either directly or via GSTs which catalyze the conjugation of the sulfhydric residue of GSH with the electrophilic residue of xenobiotic compounds inactivating their toxic potential and favoring their export out of the cell (Strange *et al.* 2001). According to substrate specificity and amino acid sequences, GST isoenzymes are classified as alpha (A), pi (P), mu (M), sigma (S), theta (T) and zeta (Z), which are located into the cytosol, and kappa (K) and omega (O) which are detected respectively into mitochondria and associated to membranes (Hayes *et al.* 2005). In addition to the transferase function, GSTs have been shown to form protein–protein interactions with members of the MAPKs involved in cell survival and death signaling. For instance, GSTP1 inhibits the activity of c-Jun N-terminal kinase *in vivo*, blocking apoptosis and favoring cellular transformation (Chatterjee & Gupta 2018).

Notably, GSH is also involved in (i) the transport and metabolism of copper and iron, (ii) in the homeostasis of nitric oxide, (iii) in the metabolism of estrogens, leukotrienes, prostaglandins and nucleotides, (iv) in DNA repair and (v) in the modulation of cell death (Sies 1999, Lu 2013).

Therefore, GSH has several pleiotropic functions that are mainly due to its ability to maintain intracellular proteins (e.g. metabolic enzymes, transcription factors and antioxidant molecules) in a reduced state, supporting and guaranteeing cell metabolism and survival (Aquilano *et al.* 2014) (Fig. 3).

Role of GSH in cancer

The pathogenesis of several human diseases including cancer is characterized by alterations of intracellular redox homeostasis (Traverso *et al.* 2013, Nitti *et al.* 2022).

As reported in the introduction, ROS production is enhanced in cancer cells (Weinberg *et al.* 2010) that trigger an adaptive response by increasing GSH levels and activating GSH-dependent enzymes (Estrela *et al.* 2006, Gamcsik *et al.* 2012). Also, many anticancer therapies (anthracyclines, alkylating agents, platinum coordination complexes and camptothecins) act by increasing ROS production, and cancer cells defend their survival keeping low ROS thresholds and abolishing senescence or cell death (O'Brien & Tew 1996). However, it has not yet been fully clarified whether the increase in the antioxidant defense, and in particular of GSH content, is the adaptive response triggered by cancer cells as a consequence of the altered redox balance or it is an innate property of cancer cells which makes them 'ready in advance' to counteract any possible increase of ROS generation.

However, an important role is played by the nuclear factor erythroid 2-related factor 2 (NRF2) that is involved in the regulation of several antioxidant genes and, in particular, of those coding for the enzymes involved in GSH synthesis, such as GCL, GR, GPX and GST, which have been found to be overexpressed in several cancers (e.g. pancreatic, lung, breast, ovarian, skin and prostate cancers) (Gorrini *et al.* 2013, Rojo de la Vega *et al.* 2018). Moreover, it has been reported that NRF2 can modulate GSH by promoting the expression of solute carrier family

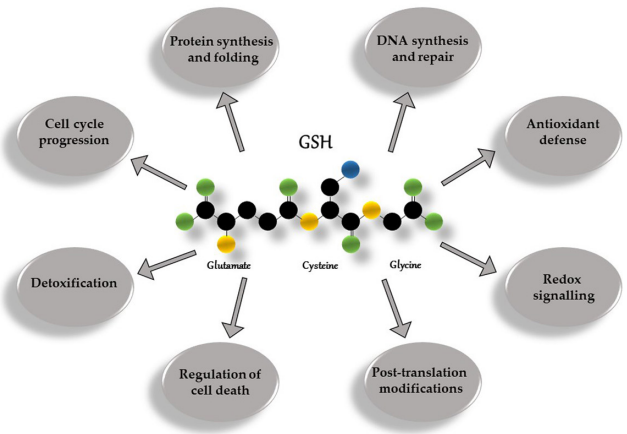

Figure 3
The biological functions of GSH. GSH plays pleiotropic functions including antioxidant defense, redox signaling, cell cycle progression, xenobiotic detoxification, regulation of cell death, post-translation modifications, protein synthesis and folding, DNA synthesis and repair.

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7 member 11 (*SLC7A11*) encoding xCT (Sasaki *et al.* 2002), a cysteine/glutamate antiporter, which is overexpressed in many cancers (Jiang *et al.* 2015) and in chemoresistant ones (Monteleone *et al.* 2021).

Interestingly, it has been reported that the expression of GCLM and *SLC7A11* genes are regulated by hypoxia-inducing factor with an upregulation of GSH biosynthesis in hypoxic cancers (Cuperlovic-Culf *et al.* 2016).

As above reported, cells can resynthesize GSH from GSSG via GR activity which requires the presence of NADPH as a substrate (Lu 2013). With this regard, high GSH/GSSG ratios in cancer cells can be due to the activation of pentose phosphate pathway (PPP), leading to NADPH production indispensable for GSH reduction (Li *et al.* 2014, Zhang *et al.* 2016).

PPP activity in cancer cells can be upregulated by the activation of oncogenes (i.e. Ras, mTORC1 and Nrf2) or the inactivation of oncosuppressors (p53) and is increased in response to ionizing radiation or chemotherapy (Patra & Hay 2014). This metabolic response plays a crucial role in helping glycolytic cancer cells to synthesize nucleic acids, coenzymes, ATP and fatty acids and to fight oxidative stress via GSH reduction.

Although cancer cells prefer aerobic glycolysis (Warburg effect), in order to satisfy their energy demands, under pro-oxidant conditions their metabolism can shift toward oxidative phosphorylation (OXPHOS), which is a dynamic and reversible process strongly involved in cancer progression and therapy resistance (Nitti *et al.* 2022). In this connection, our recent study demonstrated that the inhibition of PKCα, by favoring the switch from OXPHOS to aerobic glycolysis, is able to reduce GSH levels and stimulate ferroptosis (see the next paragraph) of chemoresistant neuroblastoma stem cells (Monteleone *et al.* 2021).

GSH and cancer cell death

GSH plays a crucial role in the induction of apoptosis, autophagy, necroptosis and ferroptosis, and consequently modulation of GSH levels may have important therapeutic implications.

In detail, it has been recognized that the reduction in the GSH/GSSG ratio and the resulting increase in ROS production lead to mitochondrial damage and induce apoptosis (Franco & Cidlowski 2009). However, since GSH loss precedes mitochondrial injury followed by cytochrome c release and caspase activation, the stimulation of GSH synthesis could be a strategy of cancer cells to escape apoptosis.

GSH is also involved in necroptosis, a form of necrosis regulated by receptor interacting protein kinases 1 (RIPK1) and 3 (RIPK3), and characterized by translucent cytoplasm, organelle swelling, increased cell volume and disruption of the plasma membrane (Gong *et al.* 2019). In this regard, it has been reported that dimethyl fumarate and artesunate are used for inducing necroptosis in colon cancer cells and in renal carcinoma cells, respectively (Xie *et al.* 2015, Chauhan *et al.* 2017). Conversely, the use of necrostatin-1, an inhibitor of RIPK1, prevents cell death by preserving them from GSH depletion (Xu *et al.* 2007).

The drastic reduction in GSH levels is also accompanied by ferroptosis, an iron-dependent cell death that can be induced by GPX4 inhibition resulting in lipid peroxidation and ROS accumulation (Ursini & Maiorino 2020). In this regard, it has been shown that RSL3 induces ferroptosis by inactivating GPX4 active site and, consequently, leading to lipid peroxidation and ROS accumulation (Sui *et al.* 2018). Another ferroptosis inducer is erastin, an xCT inhibitor that acts by inducing GSH depletion (Dixon *et al.* 2014). On the contrary, compounds able to inhibit lipid peroxidation, such as liproxstatin-1 and ferrostatin-1, counteract ferroptotic death (Zilka *et al.* 2017).

However, it has been demonstrated that GSH depletion due to xCT inhibition can also lead to autophagy which represents a mechanism of tumor suppression (Jin & White 2008) even though more studies are needed to clarify the relationship between GSH and autophagy.

GSH and chemoresistance

Chemoresistance is a complex and frequent consequence of cancer treatment, and it is responsible for a poor prognosis for cancer patients. There are several mechanisms underlying cancer cell resistance to chemotherapeutic drugs: (i) drug inactivation, (ii) induction of efflux transporters, (iii) inhibition of apoptosis, (iv) deregulation of cell cycle and checkpoints, (v) enhanced DNA repair and (vi) genetic and epigenetic alterations of cellular oxidative metabolism (Zheng 2017). Indeed, as reported earlier, the increase in GSH levels and the activation of GSH-related enzymes can support tumor growth and counteract the efficacy of therapy, contributing to promote the onset of chemoresistance (Kim *et al.* 2019). In this context, our study demonstrated that chronic treatment with etoposide, a drug which exerts its cytotoxic effect by stimulating ROS overproduction, leads to a selection of multidrug resistant neuroblastoma cells displaying higher levels of GSH with

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respect to parental cells (Colla *et al.* 2016). In addition, the drugs targeting xCT, by limiting the influx of cysteine and GSH biosynthesis, were able to sensitize neuroblastoma stem cells to etoposide counteracting chemoresistance (Monteleone *et al.* 2021).

Notably, the overexpression of GGT in ovarian, colon, liver, prostate, sarcoma, melanoma and breast cancer has been associated with a poor prognosis and resistance to treatment with alkylating agents (e.g. cisplatin and oxaliplatin), which are metabolized and inactivated by GGT (Pompella *et al.* 2006).

Another GSH-dependent enzyme involved in chemoresistance is GST which is highly expressed in many cancers that become chemoresistant (Singh & Reindl 2021). In fact, as reported earlier, GST catalyses the conjugation of GSH with drugs (e.g. cisplatin) and facilitates drug efflux through multidrug resistance proteins (MRPs), which are membrane transporters whose enhanced expression can be associated with the acquisition of chemoresistance (Lautier *et al.* 1996). Also, the overexpression of GR, a key enzyme involved in the GSH cycle, contributes to the development of chemoresistance as observed in temozolomide-resistant glioblastoma cells (Zhu *et al.* 2018).

Furthermore, the overexpression of the enzymes involved in GSH synthesis such as GCL and GS can contribute to chemoresistance by enhancing GSH levels (Backos *et al.* 2012).

An eminent role is played by GPX4, an enzyme involved in the regulation of ferroptosis and that has been found overexpressed in drug-resistant tumors with poor prognosis (Liu *et al.* 2021).

Based on these findings, GSH and GSH-dependent antioxidant pathways are under investigation as potential targets of innovative therapeutic strategies aimed at counteracting cancer progression and therapy resistance.

GSH-based therapies

Therefore, it is clearly evident that GSH is related to cancer progression and therapy resistance, although many aspects need to be investigated. However, several strategies aimed at targeting GSH system have been proposed, and many of them are currently included in clinical trials (Table 1).

The most used strategy is represented by GSH depletion. In fact, since many anticancer therapies act by stimulating ROS overproduction, their failure is due to scavenging effects of GSH and of GSH-dependent antioxidant system. Therefore, the combination of GSH-depleting agents with a pro-oxidant therapy can be effective to enhance therapeutic sensitivity. In addition, the depletion of GSH, which is involved in drug metabolism, could lead to an increase in the bioavailability of chemotherapeutic drugs, enhancing their efficacy and allowing the clinical use of a lower drug dosage with a decrease in side effects.

The main strategies that can be exploited to induce GSH depletion are the following: (i) to inhibit GSH biosynthesis; (ii) to block precursor amino acids of GSH; (iii) to promote GSH efflux or (iv) to consume intracellular GSH reservoir (Fig. 4).

GSH biosynthesis inhibitors

In order to inhibit GSH biosynthesis, it is necessary to target the enzymes involved in its synthesis and regeneration. Indeed, GCL is critical for GSH synthesis, and one of its inhibitors mainly used is L-buthionine sulfoximine (BSO, Table 1), which has been shown to induce cancer cell death and to increase chemotherapeutic drug sensitivity of neuroblastoma cells (Domenicotti *et al.* 2003, Marengo *et al.* 2008, Marengo *et al.* 2011, Monteleone *et al.* 2021). However, although these results are encouraging, the clinical use of BSO is restricted due to its short half-life and the non-selective effect of GSH depletion in both healthy and cancer cells. In addition, a further limitation is the difficulty to distinguish and produce the active stereoisomer that might be tumor selective (Sandor *et al.* 1995, Lewis-Wambi *et al.* 2009, Wu & Batist 2013, Hamilton *et al.* 2007).

In order to overcome this limitation, interesting studies have been carried out using polymeric nanoparticles loaded with BSO and other anticancer agents (Cruz *et al.* 2020).

Moreover, also GS could be a therapeutic target. In fact, Wang and coauthors have realized a novel L-cysteine-based poly(disulfideamide) polymer encapsulated with UNC0638 (a histone methyltransferase G9a inhibitor) which is able to inhibit GSH synthesis and to eliminate its intracellular pool (Wang *et al.* 2020). The nanodrug, in comparison with UNC0638 *per se*, had an improved anticancer activity on pancreatic ductal adenocarcinoma and, more importantly, a greater tolerability and absence of toxic effects (Wang *et al.* 2020).

Notably, another possibility to induce GSH depletion is to counteract its regeneration by inhibiting the enzymes involved in the reduction reaction of GSSG. In this context, Xia *et al.* have recently investigated the effects of Stattic, a STAT3 inhibitor, able to inhibit GR and to induce ROS-mediated death of cervical cancer cells (Xia *et al.* 2021).

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Table 1 Drugs targeting GSH system and currently included in clinical trials.

Drug	Cancer type	Status	Identifier
Blocking aminoacid precursors			
Sulfasalazine			
	Breast cancer	Recruiting	NCT03847311
	Glioblastoma	Recruiting	NCT04205357
Sorafenib			
	Prostate cancer	Completed	NCT00090545
	Hepatocellular cancer	Completed	NCT00813293
	Breast cancer	Completed	NCT00101400
	Ovarian cancer	Completed	NCT00436215
	Lung cancer	Completed	NCT00609804
	Solid tumors	Completed	NCT00572078
Artemisinin			
	Breast cancer	Completed	NCT00764036
	Cervical neoplasia	Recruiting	NCT04098744
	Colorectal cancer	Recruiting	NCT03093129
	Ovarian cancer	Recruiting	NCT04805333
Luteolin			
	Tongue carcinoma	Unknown	NCT03288298
Synthesis inhibitors			
BSO			
	Neuroblastoma	Completed	NCT00005835
	Neuroblatoma	Completed	NCT00002730
B-lapachone (ARQ 501)			
	Advanced solid tumors	Completed	NCT00524524
	Solid tumors	Completed	NCT00099190
	Solid tumors	Completed	NCT01502800
	Head-and-neck cancer	Completed	NCT00358930
	Pancreatic cancer	Completed	NCT00102700
	Pancreatic cancer	Stopped	NCT02514031
Efflux promoters			
Verapamil			
	Brain cancer	Completed	NCT00706810
	Gastric cancer	Completed	n.a.
	Lymphoma	Active	NCT03013933
	Colorectal cancer	Completed	n.a.
Resveratrol			
	Colon cancer	Completed	NCT00256334
	Colon cancer	Completed	NCT00433576
	Colorectal cancer	Completed	NCT00920803
	Colorectal cancer	Completed	n.a.
	Cancer prevention	Completed	NCT00098969
	Multiple myeloma	Completed	NCT00920556
Apigenin			
	Colorectal cancer	Unknown	NCT00609310
Quercetin			
	Prostate cancer	Unknown	NCT01538316
	Prostate cancer	Completed	NCT01912820
	Prostate cancer	Completed	NCT03493997
	Childhood cancer	Recruiting	NCT04733534
	Oral cancer	Recruiting	NCT05456022
	Colon cancer	Completed	NCT00003365
	Colorectal cancer	Completed	NCT02195232
	Kidney cancer	Unknown	NCT02446795
	Squamous cell carcinoma	Recruiting	NCT03476330
	Lymphoma	Unknown	NCT00455416
Sulforaphane			
	Lung cancer	Active	NCT03232138
	Prostate cancer	Completed	NCT01228084
	Breast cancer	Recruiting	NCT03934905

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Table 1 (*continued*)

Drug	Cancer type	Status	Identifier
	Melanoma	Completed	NCT01568996
Romidepsin			
	Lung cancer	Completed	NCT01302808
	Solid tumors	Completed	NCT00379639
	Solid tumors	Completed	NCT01537744
	Solid tumors	Completed	NCT00019318
	Prostate cancer	Completed	NCT00106418
	Colorectal cancer	Completed	NCT00077337
	Colorectal cancer	Completed	NCT02512172
	Thyroid cancer	Completed	NCT00098813
	Leukemia	Completed	NCT00042822
Reserve consumption			
Disulfiram			
	Breast cancer	Recruiting	NCT04265274
	Breast cancer	Recruiting	NCT03323346
	Prostate cancer	Completed	NCT01118741
	Solid tumors	Completed	NCT00742911
	Multiple myeloma	Recruiting	NCT04521335
	Glioblastoma	Completed	NCT02678975

Also, 2-acetylamino-3-(4-(2-acetylamino-2-carboxye thylsulfanylcarbonylamino) phenyl carbamoylsulfanyl) propionic acid has been identified as a GR inhibitor that is able to induce cell cycle arrest of human esophageal cancer cells through a generation of oxidative stress (Li *et al.* 2017).

An additional approach to reduce GSH availability is the inhibition of GGT that, as above reported, is upregulated in many cancers (Pompella *et al.* 2006). Glutamate analogues, such as acivicin, azaserine and boronate derivatives, which have been identified as GGT inhibitors have shown a marked toxicity in patients (Joyce-Brady & Hiratake 2011). Notably, the nanoparticle-encapsulated compound OU749 revealed a greater efficacy than the GGT inhibitor alone, and it has been tested on cisplatin-resistant human non-small cell lung cancer cells (Wang *et al.* 2021).

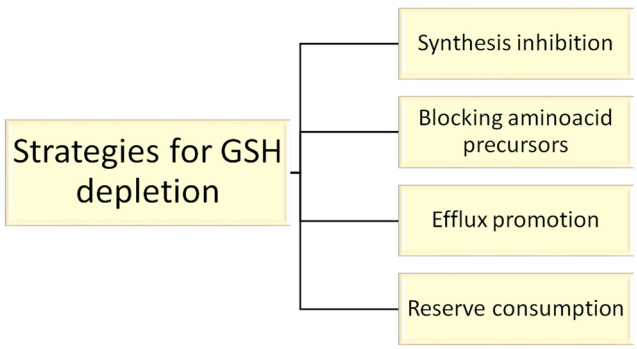

Figure 4
Strategies aimed at inducing GSH depletion.

GSH precursor amino acid uptake inhibitors

Another possibility to limit GSH synthesis is to reduce the availability of cysteine by inhibiting xCT transporter. In this context, erastin and its analogues and sulfasalazine (Table 1) have been found to be effective in depleting GSH by reducing cysteine influx. Consequently, erastin has been found to induce ferroptosis and to counteract the progression of several cancers (Zhao *et al.* 2020). Furthermore, erastin combined with docetaxel reduced the onset of chemoresistance in ovarian cancer (Zhou *et al.* 2019), while an encapsulated form was effective in breast cancer (Yu *et al.* 2019). Notably, two erastin analogues, imidazole ketone erastin and piperazine erastin, due to improvement of solubility, potency and stability, induced ferroptosis in mouse models of fibrosarcoma, lymphoma and hepatocellular carcinoma (Zhang *et al.* 2019). Also, sorafenib acts by inhibiting xCT, and its administration alone or in combination with other drugs has been shown to activate ferroptosis in hepatocellular carcinoma and in kidney cancer (Table 1) (Lachaier *et al.* 2014). Among xCT inhibitors, sulfasalazine, initially identified as an immunosuppressant for chronic inflammatory diseases, has been recognized as ferroptosis inducer in breast cancer, lymphoma, bladder cancer and colon cancer treated in combination with cisplatin (Table 1) (Liu *et al.* 2017). Moreover, it has been recently demonstrated that C2-4, a PKCα inhibitor, analogously to sulfasalazine, sensitizes etoposide-resistant neuroblastoma stem cells by inducing ferroptosis (Monteleone *et al.* 2021).

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Other compounds, such as capsazepine, pseudolaric acid B, artemisinin (Table 1) and its derivatives and metaderin, are able to inhibit xCT (Zhang *et al.* 2021), and among them, artesunate and dihydroartesiminin have shown anticancer properties by inducing ferroptotic death (Zhang *et al.* 2021).

Since xCT overexpression may be due to the increased activity of Nrf2, the compounds targeting this transcription factor have been proposed to treat cancer and/or to counteract the onset of chemoresistance. In this regard, natural compounds such as brusatol, halofuginone luteolin, chrysin and emetine showed anticancer properties (Table 1) (Xiang *et al.* 2018, Panda *et al.* 2022) and a chemosensitizer action (Panieri & Saso 2019).

Notably, the glucocorticoid clobetasol propionate (Choi *et al.* 2017) and antitubercular drugs such as isoniazid and ethionamide, by interfering with Nrf2 nuclear translocation, were able to enhance therapy sensitivity in lung cancer and leukemia cells, respectively (Verma *et al.* 2015, Peng *et al.* 2016).

Also trigonelline, by inhibiting Nrf2, has been demonstrated to increase the sensitivity of lung cancer cells to etoposide and cisplatin (Fouzder *et al.* 2021). Analogously, Zhou *et al.* reported that digoxin, a drug used to treat heart failure, sensitizes chemoresistant pancreatic cancer cells to gemcitabine by inhibiting Nrf2-dependent pathways (Zhou *et al.* 2019). In addition, luteolin, a natural flavonoid, and its derivative apigenin were found to sensitize resistant colorectal cancer and hepatocarcinoma cells to doxorubicin via Nrf2 inhibition (Chian *et al.* 2014, Gao *et al.* 2017).

With regard to synthetic Nrf2 inhibitors, (i) Im3829 has been shown to sensitize lung cancer patients to radiotherapy (Lee *et al.* 2012); (ii) AEM1 sensitized A549 lung cancer cells to various anticancer agents (Bollong *et al.* 2015); (iii) compound f4 counteracted A549 and leukemia cell growth and proliferation (Zhang *et al.* 2014, Zhang *et al.* 2017); (iv) compound ML385 sensitized lung cancer cells to carboplatin (Singh *et al.* 2016) and (v) Stattic, above cited as GR inhibitor, was able to sensitize colon cancer cells to 5-fluorouracil (Tajmohammadi *et al.* 2019).

GSH efflux promoters

Furthermore, another strategy to induce GSH loss is to promote its efflux from cells (Table 1). In this context, the modulation of MRP1, the main transporter of free and drug-conjugated GSH, may lead to GSH depletion circumventing chemoresistance in cancer cells (Lorendeau *et al.* 2017). In addition, verapamil and derivatives, used in the treatment of cardiovascular disease, and flavonoids such as resveratrol, apigenin, quercetin and aminothienopyrimidine derivatives, have been demonstrated to inhibit MRP1 in preclinical studies (Lorendeau *et al.* 2017). Several of these compounds have been also tested in clinical trials which are still ongoing (Table 1). However, the majority of clinical studies (phase 1) carried out are focused to analyze the toxicity of the drug, and only a little number of them (phase 2) are aimed at analyzing the efficacy of the treatment with results not yet available. Recently, a novel MRP1 inhibitor, YAN, has been identified, and a promising effect was observed in the treatment of multidrug-resistant lung cancer cells (Gao *et al.* 2021).

GSH-consuming drugs

Interestingly, the binding of GSH to anticancer drugs leading to a consumption of intracellular GSH stores is determinant to induce cancer chemoresistance. Therefore, isothiocyanates, such as β-phenylethyl isothiocyanate and sulforaphane, having affinity for GSH binding, can have a promising antitumor activity (Table 1). In detail, it has been found that sulforaphane conjugated with polymer nanoparticles reduces drug toxicity and is effective in counteracting breast cancer cell survival (Xu *et al.* 2019). Aldehydes or α,β-unsaturated ketones as quinone methide, oridonin and cinnamaldehyde are able to form conjugates with GSH and showed anticancer properties (Luo *et al.* 2018). In particular, romidepsin binds to GSH and has been proposed for the treatment of cutaneous T-cell lymphoma and urothelial carcinoma (Table 1) (Pattarawat *et al.* 2020).

Notably, intracellular GSH levels can be depleted by exposure to pro-oxidant compounds, leading to GSSG formation. They are commonly encapsulated in nanocarriers to avoid systemic toxicity and to obtain a major selectivity. In fact, several metal-based nanomaterial approaches, such as manganese dioxide, metal-organic frameworks based on copper, iron or platinum or complexes of several metals, have shown a marked antitumor activity (Wang *et al.* 2019). In addition, it has been demonstrated that disulfiram is able to convert GSH to GSSG and is effective in the combined treatment of metastatic melanoma (Meraz-Torres *et al.* 2020).

Conclusions

Cancer cells, due to their increased metabolic rate, produce high levels of ROS that are balanced by the presence of

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efficient antioxidants and in particular of GSH-related system. The maintenance of the redox homeostasis guarantees cancer cell survival and proliferation and drives the adaptation to therapy-induced stress. In fact, several traditional anticancer drugs exert their cytotoxic action by stimulating ROS production, and although the initial treatment is able to kill cancer cells, the long-term treatment leads to a selection of more malignant cells equipped with antioxidant defense and characterized by drug refractoriness. Therefore, a strategy able of overcoming such 'adaptive tolerance threshold' could help to fight chemoresistant cancer cells. In this context, GSH depletion has been proposed as a strategy to counteract cancer progression and therapy resistance, via the inhibition of key enzymes or precursors of GSH synthesis, consumption of its intracellular stores and promotion of its efflux.

These GSH-depleting compounds, used alone or in combination with traditional therapies, not only potentiate the pro-oxidant effect of chemotherapeutic drugs but also enhance their biodisponibility as a consequence of the decrease in GSH conjugation and drug elimination. In addition, since GSH is fundamental for GPX4 activity, it is possible to induce ferroptosis of cancer cells by directly targeting GPX4 (Ursini & Maiorino 2020, Wei *et al.* 2020).

Interestingly, the most innovative strategies are focused to load the anticancer drugs or chemosensitizers on nanoparticles whose administration can limit toxic side effects and favor a more selective release of the compounds.

Furthermore, considering that oxidative state can be highly variable in each tumor and each phase of cancer progression, monitoring GSH levels in patients before and during therapy could be crucial to early identify refractory patients and to direct therapy toward a personalized approach.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributor statement

GEV, CD and BM conceived the study. GEV, BT, NT and BM wrote the original draft. CD reviewed the final version. All authors have read and agreed to the published version of the manuscript.

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Review

The Role of Glutathione in Protecting against the Severe Inflammatory Response Triggered by COVID-19

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Abstract: The novel COVID-19 pandemic is affecting the world's population differently: mostly in the presence of conditions such as aging, diabetes and hypertension the virus triggers a lethal cytokine storm and patients die from acute respiratory distress syndrome, whereas in many cases the disease has a mild or even asymptomatic progression. A common denominator in all conditions associated with COVID-19 appears to be the impaired redox homeostasis responsible for reactive oxygen species (ROS) accumulation; therefore, levels of glutathione (GSH), the key anti-oxidant guardian in all tissues, could be critical in extinguishing the exacerbated inflammation that triggers organ failure in COVID-19. The present review provides a biochemical investigation of the mechanisms leading to deadly inflammation in severe COVID-19, counterbalanced by GSH. The pathways competing for GSH are described to illustrate the events concurring to cause a depletion of endogenous GSH stocks. Drawing on evidence from literature that demonstrates the reduced levels of GSH in the main conditions clinically associated with severe disease, we highlight the relevance of restoring GSH levels in the attempt to protect the most vulnerable subjects from severe symptoms of COVID-19. Finally, we discuss the current data about the feasibility of increasing GSH levels, which could be used to prevent and subdue the disease.

Keywords: SARS-CoV-2; angiotensin-converting enzyme (ACE); angiotensin-converting enzyme 2 (ACE2); glutathione; inflammation; ROS; *N*-acetylcysteine; glycine; chloroquine; paracetamol

1. Introduction

Lung inflammation is the main cause of life-threatening respiratory disorders at the severe stage of SARS-CoV-2 infection, characterized by the so-called "cytokine release syndrome (CRS)".

The key to fighting this harmful inflammatory response resides in: (i) addressing the mechanism of the virus penetration into the cell, mediated by binding to and inactivation of the ACE2 protein; (ii) contrasting the exacerbation of the inflammatory response. The standard pharmacological approach would suggest either the use of an antiviral drug with the aim of blocking viral replication or the exploitation of drugs previously validated as inhibitors of some inflammatory pathway in other chronic diseases. Unfortunately, these drugs are ineffective in healing the most severe cases of SARS-CoV-2, and additionally, they have several side effects. The baffling aspect of this disease is the great heterogeneity of response among patients, ranging from severe symptoms to asymptomatic progression. Understanding the protective mechanisms and the reasons of their failure could provide a breakthrough in the quest for a cure.

The inflammatory response can be traced back to the pathway of viral entry through its receptor ACE2. Angiotensin-converting enzyme 2 (ACE2) is a protease that, with its companion

the angiotensin-converting enzyme ACE, takes part in the renin-angiotensin system (RAS). They are localized at the cell surface and compete for the same substrates, angiotensin I and II. ACE2 counters the activity of ACE by reducing the amount of angiotensin-II (ANGII) and increasing ang (1-7) peptide. The downstream effects of the two enzymes are opposite: ACE activity leads to vasoconstriction, oxidative stress, inflammation and apoptosis, whereas ACE2 causes vasodilatation, angiogenesis and anti-inflammatory, anti-oxidative and anti-apoptotic effects [1]. The oxidative stress generated by ACE activity is due to the effects of its product, ANGII, which increases the production of reactive oxygen species (ROS) through the activation of NADPH oxidase and the generation of peroxynitrite anions. In contrast, the ang (1-7) peptide synthesized by ACE2 activity leads to a downregulation of pro-oxidant pathways, which prevents or attenuates the cellular damage induced by oxidative stress.

Each person has a different balance between ACE and ACE2 and can be more prone to inflammation if ACE prevails. When this happens, and additionally infection by SARS-CoV-2 downregulates ACE2 abundance on cell surfaces, as suggested by evidence from related coronaviruses [2], the result is the toxic overaccumulation of ANGII, exacerbated inflammation and, finally, acute respiratory distress syndrome and fulminant myocarditis (Figure 1). A different balance of ACE/ACE2 can explain the heterogeneous responses to infection caused by the same virus. The link between the dysregulation of the RAS cascade and the likelihood or severity of SARS-CoV-2 infection has been discussed in some recent works [3,4], and it is a matter of importance when the effects of the RAS inhibitors are debated [5].

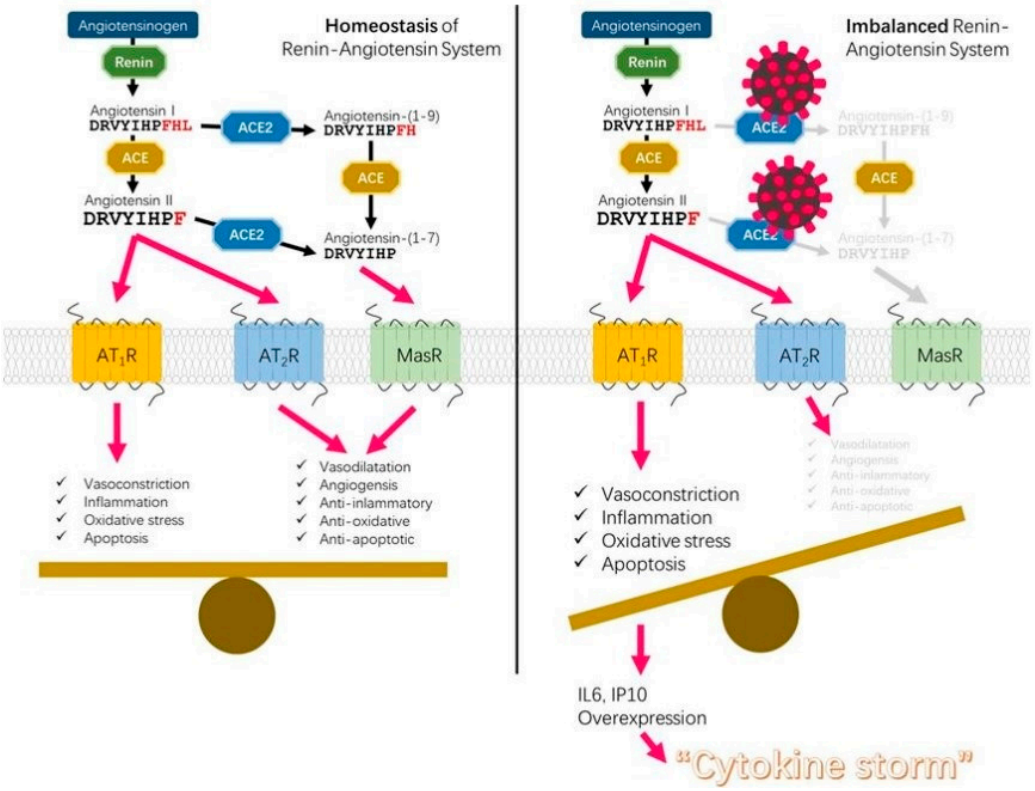

Figure 1. A comprehensive scheme of the interactions between the molecules involved in the renin-angiotensin system (RAS) from [3], copyright 2020 by Gang Niu. Reprinted with permission.

Reducing the oxidative stress secondary to the imbalance between ACE and ACE2 could be the best approach for the prevention and treatment of COVID-19. Oxidative stress constitutes a failure of anti-oxidation defense systems to keep ROS and reactive nitrogen species in check. ROS are signaling molecules that induce the release of pro-inflammatory cytokines [6], and the dysregulation of this response plays an essential role in the development of inflammation [7].

Glutathione (GSH) has the function of "master antioxidant" in all tissues; the high concentration of the reduced form (millimolar) highlights its central role in the control of many processes such as detoxification, protein folding, antiviral defense and immune response [8].

The aim of this review is to provide a brief overview of the protective action of GSH against the exacerbated inflammation triggered by COVID-19 upon ACE/ACE2 imbalance. Furthermore, we discuss the evidence of the low levels of GSH found in the conditions associated with the severe outcome of the disease, with the intension of looking into the relevance of restoring GSH levels in the attempt to protect the most vulnerable subjects from COVID-19.

2. Cellular GSH Homeostasis

Glutathione, a tripeptide composed of glutamate, cysteine and glycine, is an antioxidant molecule ubiquitous in most living organisms. Intracellular GSH balance is maintained by de novo synthesis, regeneration from the oxidized form, GSSG, and extracellular GSH uptake. In transporting epithelial cells, such as enterocytes, γ-glutamyl transferase (γ-GT) and dipeptidase (DP), the hydrolysis of extracellular GSH is catalyzed to its constituent amino acids, glutamate, cysteine and glycine. The three amino acids are adsorbed by transporters. Additionally, intestinal epithelial cells can import intact GSH from the lumen via specific plasma membrane transporters.

Cytosolic synthesis of GSH takes place in two ATP-dependent reactions catalyzed by glutamate-cysteine ligase (GCL) and glutathione synthase (GS). The activity of these enzymes is regulated by many pathways, suggesting that the synthesis of GSH must respond to a multitude of environmental conditions. TGFβ1 is one of the major repressors of GCL expression leading to lower levels of GSH [9]; because the cytokine activity is induced by ROS and leads to fibrosis [10,11], this implies that in a pro-fibrogenic environment, the depletion of GSH occurs through multiple mechanisms, either by oxidation or by decreased synthesis [12]. In addition, hypoxia represents an inhibitory signal for GSH synthesis, since it decreases the activity of the two key biosynthetic enzymes GCL and GS [13]. On the other hand, vitamin D induces the expression of GCL and glutathione reductase genes [14], thus increasing the levels of GSH. The GLC gene is induced also by exposure to reactive oxygen species and nitric oxide species [15].

Glutathione exists in reduced (GSH) and oxidized (GSSG) states. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to other unstable molecules, such as ROS. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form glutathione disulfide (GSSG). Such a reaction is possible due to the relatively high concentration of glutathione in cells (up to 5 mM in the liver). The regeneration of GSH from GSSG is catalyzed by glutathione reductase (GR) in the GSH redox cycle. GSSG reduction occurs at the expense of NADPH, produced by the pentose phosphate pathway (PPP) from glucose oxidation. The intracellular GSH pool, present in millimolar concentrations, is involved in various GSH-dependent reactions. Compartmentalization of GSH within the mitochondria, nucleus or endoplasmic reticulum creates distinct and independently regulated subcellular redox pools.

Glutathione plays a cytoprotective role through several mechanisms. As a reducing agent, it is the main cellular antioxidant agent in the reduction of hydrogen peroxide (H_2O_2) and lipid hydroperoxides (LOOH) catalyzed by glutathione peroxidases (GPXs). In the reactions of protection against ROS, the role of glutathione is illustrated by the reduction of peroxides:

$$2\,GSH + R_2O_2 \rightarrow GSSG + 2\,ROH$$

R = H or alkyl group, and by the neutralization of free radicals:

$$GSH + R \rightarrow 0.5\,GSSG + RH$$

GSH takes part also in the reduction of protein disulfides (PrSSG) catalyzed by glutaredoxins (GRXs). Moreover, it is employed in the preservation of protein-dithiols by the protein disulfide reductase (glutathione) (EC 1.8.4.2), an enzyme that catalyzes the chemical reaction:

$$2 \text{ glutathione} + \text{protein disulfide} \leftrightarrow \text{glutathione disulfide} + \text{protein-dithiol}.$$

This enzyme acts on different substrates, such as insulin, which is inactivated by cleavage of the disulfide bridge, or on protein disulfide, in which the reduction to protein-dithiol restores the catalytic activity, protecting the cell from oxidant stress.

Another important function of glutathione is mediated by its conjugation to several substrates. In the detoxifying defense systems, GSH participates in conjugation reactions catalyzed by glutathione-S-transferases (GSTs). Many heavy metals such as mercury and lead are eliminated as GSH conjugates to prevent their irreversible binding to SH groups of many enzymes, including many membrane ATPase. Subacute exposure to lead results in GSH pool depletion and accumulation of lipid peroxidation products and modifies the activity of the glutathione-related enzymes, such as GR, GST and glucose-6-phosphate dehydrogenase [16]. Many lipophilic xenobiotics are conjugated to GSH to facilitate their excretion or further metabolism. Conjugate compounds are exported from the cell by GSH transporters, which requires ATP for active pumping [17]. In particular, the multidrug resistance-associated proteins (MRP/ABCC) appear to mediate GSH export and homeostasis. The MRP proteins mediate not only GSH efflux, but they also export oxidized glutathione derivatives (e.g., glutathione disulfide (GSSG), S-nitrosoglutathione (GS-NO) and glutathione-metal complexes), as well as other glutathione S-conjugates [18]. Moreover, GSH can be conjugated to proteins in a process called protein S-glutathionylation (PSSG). PSSG protects protein cysteines from being overoxidized to sulfinic and sulfonic species that are not readily regenerated. S-glutathionylation also changes the protein structure and function.

Due to the strong cytoprotective effects of GSH, alterations in GSH homeostasis have been associated with neurodegenerative diseases, AIDS, liver and heart disease, aging, diabetes mellitus and cancer (reviewed in [19–21]).

3. The Protective Actions of GSH

Glutathione can prevent damage to important cellular components caused by ROS and their derivatives, such as free radicals, peroxides, lipid peroxides, or by organic pollutants and heavy metals. In addition, due to the peculiar reactivity of the –SH group, GSH is involved in several chemical reactions, from disulfide bridge reduction to conjugation to endogenous molecules and xenobiotics. As the pool of the available GSH molecules is fixed, any unexpected increase of its utilization leads to a decrease of the free molecules and impairment of the competing pathways. For example, when GSH is conjugated, it is stolen from the enzymes using the molecule as a cofactor or substrate. This observation is relevant to explain why a deficiency of GSH can occur and can affect pathways involved in severe viral symptoms. Among the many functions of GSH, some are worthy of mention in relation to their impact on the exacerbated inflammation taking place in COVID-19 and in relation to the symptoms developed in the disease.

1. GSH protects cells by neutralizing (i.e., reducing) ROS, which are key signaling molecules that play an important role in the progression of inflammatory disorders. The relationship between ROS production and proinflammatory cytokine activation is well established [22]. An enhanced ROS generation by polymorphonuclear neutrophils at the site of inflammation causes endothelial dysfunction and tissue injury [23].
2. The conjugation of GSH to xenobiotics is particularly abundant; glutathione S-transferase enzymes catalyze GSH conjugation to lipophilic xenobiotics, facilitating the excretion or further metabolism of many drugs. The conjugation process is illustrated by the metabolism of *N*-acetyl-*p*-benzoquinone imine (NAPQI). NAPQI is a reactive metabolite formed by the action of

cytochrome P450 on paracetamol (acetaminophen). Glutathione conjugates to NAPQI and the resulting product is excreted [24].

3. Many enzymes use GSH as a cofactor or substrate. For example reactions requiring GSH as a key cofactor are catalyzed by the prostaglandin H synthase, the rate-limiting enzyme in the production of prostaglandins and thromboxane [25], which are essential regulators of vascular function. Moreover, the enzyme leukotriene C(4) synthase conjugates LTA(4) with GSH to form the leukotriene LTC(4), the parent compound of the cysteinyl leukotrienes [26]; these molecules are potent mediators of airway narrowing.
4. GSH is used to synthesize S-nitrosoglutathione (GSNO), an endogenous S-nitrosothiol that plays a critical role in nitric oxide (NO) signaling and is a source of bioavailable NO. The generation of GSNO can serve as a stable NO pool which can properly transduce NO signaling [27]. NO produced by nitric oxide synthase eNOS and nNOS, in the presence of GSH, can effectively modulate vessels and neuronal functions, regulating the blood flow according to the local calcium influx.

As a consequence of the competition between these and many other GSH-consuming pathways, on one hand, the raging inflammation and oxidative stress triggered by the viral infection steals GSH from core functions such as NO-dependent vasodilatation; on the other hand, when other biochemical pathways are consuming GSH, the patient is not protected from an inflammation that can prove fatal.

4. The Beneficial Effects of GSH on the Inflammation Driven by the Imbalance of ACE/ACE2

The harmful increase of ANGII can depend on many factors. First, it can be due to the increased renin activity, which is not affected by GSH. Instead, ACE expression and activity are modulated by glutathione; in fact, the oxidized form GSSG shows an activating effect on ACE activity, whereas the reduced GSH provides an inhibitory effect [28]. Finally, the boosted ANGII production can be due to decreased ACE2 expression and activity; this is the case with coronavirus infection, which recognizes ACE2 as its extracellular binding site [29]. Compared to SARS-COV-1, SARS-CoV-2 has about 4-fold higher affinity for ACE2 [30]. Infection of cells by SARS viruses that bind ACE2 results in two effects: inhibition of ACE2 activity and decrease of ACE2 expression in infected cells [29,31,32]. The increased ANGII, through binding to AT1R, activates NADPH oxidases that transfer an electron from NADPH to O_2 generating several radical species, which can be scavenged by GSH. ROS-mediated oxidation can, in turn, alter gene expression through the induction of signaling cascades or the interaction with transcription factors [33]. Among these factors, a prominent role is played by NF-kB, whose role in inflammation in severe acute respiratory syndrome (SARS) has been demonstrated in both SARS-CoV-infected cultured cells and mice [34]. Drugs that inhibit NF-κB activation lead to a reduction in inflammation and lung pathology. NF-kB is involved in inflammation through multiple mechanisms. In vitro, the viral nucleocapsid (N) protein activates interleukin-6 (IL-6) expression through NF-kB binding at the promoter region of the gene [35]. High levels of IL-6 in the acute stage associated with lung lesions were found in SARS patients [36]. By reducing ROS production, GSH inhibits NF-kB activation and consequently keeps the cytokine storm under control. The effects of GSH are outlined in Figure 2.

The oxidant/antioxidant imbalance is not peculiar to SARS, but it is shared by all inflammatory lung diseases in which the activation of redox-sensitive transcription factors such as NF-kB is reduced by GSH [37]. In an animal model of oxidative stress, NF-κB binding activity was inversely related to liver glutathione and was further suppressed by oral administration of green tea extract [38].

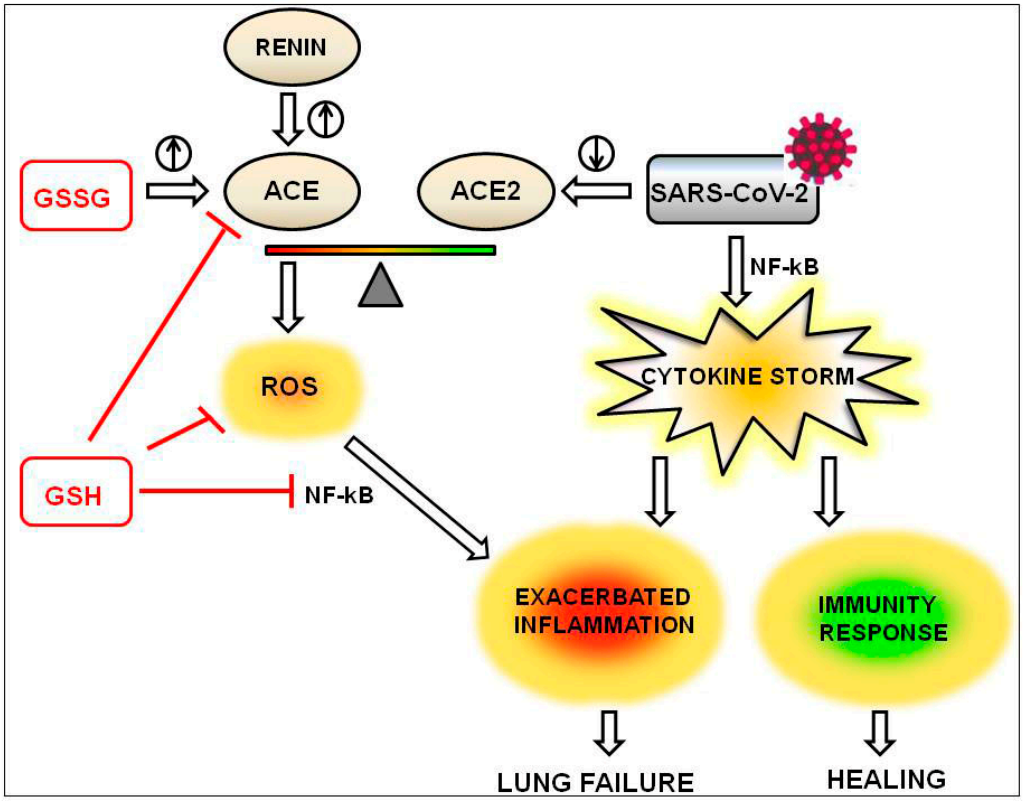

Figure 2. The anti-inflammatory effects of reduced glutathione (GSH) are exerted through the inhibition of ACE activity, decrease of reactive oxygen species (ROS) production and reduction of NF-kB activation (red lines). The balance ACE/ACE2 is shifted toward ACE by the oxidized form of glutathione (GSSG) and by renin (the circled arrows pointing upwards indicate the induction of ACE) and by viral infection (the circled arrow pointing downwards indicates the downregulation of ACE2).

5. Conditions Associated with Low GSH

GSH plays a central role in the pathophysiology of human diseases (reviewed in [39]). GSH imbalance is observed in a wide range of pathological conditions including lung infections, HIV, diabetes, cancer and age-related diseases [20,40]. If we consider the conditions clinically associated with severe COVID-19 disease [41–43], we find evidence of a perturbed GSH replenishment.

1. Age. Age is a major risk factor for both morbidity and mortality in COVID-19 patients [44,45]. In laboratory animals, the age-related changes in GSH content have been measured in different tissues. Old mice had lower GSH than young mice in several organs. The sharp decrease in the lung is highly remarkable because the sum of aging and air pollution can lead to very low GSH [46]. Similarly to the data obtained in animals, several studies have reported that also in human subjects the concentration of GSH declines with aging [47–52].

2. Sex. The severity of COVID-19 is strictly correlated to gender. While men and women have the same prevalence of infection, men with COVID-19 are more at risk for worse outcomes and death, independent of age. In the public data set, the number of men who have died from COVID-19 is 2.4 times that of women (70.3 vs. 29.7%, $p = 0.016$) [53]. Several studies report decreased GSH levels in men compared to women. Research investigating the effect of sex hormones on free radicals and lipid peroxides production found that the erythrocyte glutathione level was lower in healthy men than in healthy women and concluded that the decrease in GSH concentration was not the result of reduced production but probably the result of more rapid utilization against increased oxidative stress triggered by testosterone [54]. Another study reported that neonatal tissues show a gender-dependent modulation of their glutathione metabolism; in fact, in response to oxidative stress, endothelial cells as well as cells derived from tracheal aspirate of baby girls had a greater activity of

glutathione reductase compared to tissues derived from baby boys [55]. A study aimed at determining the influence of gender and antioxidant supplementation on exercise-induced oxidative stress reported that, before supplementation, women had higher reduced glutathione and total glutathione compared with men [56].

From data reported in the literature, the difference between sexes in GSH content can be related to hormones or can be ascribed to significant sex-specific differences in drug-metabolizing enzymes.

Estrogens can downregulate the production of ROS triggered by the imbalance of the ACE/ACE2 system; therefore, women have a lower risk of GSH depletion. Indeed, ACE2 expression is modulated by sex hormones. Estrogen modulates the local renin angiotensin system via downregulation of ACE and simultaneous upregulation of ACE2, AT2R and ang (1-7) receptor expression levels [57]. Estradiol directly activates ACE2 expression in different tissues, including differentiated airway epithelial cells [57–59]. Moreover serum ACE is significantly lower in female children compared to males after the age 12 [60]. 17beta-estradiol but not other sex hormones such as progesterone and testosterone increases the content of GSH-dependent protein disulfide reductase and protects endothelial cells from oxidative stress [61]. Estradiol production in the peripheral tissues is dependent on the local availability of the precursor DHEA, produced from DHEA sulfate (DHEAS) by steroid-sulfatase. Steroid-sulfatase becomes the limiting enzyme for the synthesis of estrogens in the elderly; whereas the serum DHEAS in young males is much higher than in females, after menopause/andropause, it is very similar. The overexpression of steroid-sulfatase in elderly women can lead to an estrogen production higher than in men even after menopause.

Cortisol also seems to affect glutathione homeostasis. In fact, a study analyzing the administration of anti-inflammatory glucocorticoids in cases of acute respiratory distress syndrome (ARDS) showed that hydrocortisone administration was followed by glutathione depletion and lower glutathione reductase activity in alveolar epithelial type II cells, thus failing to show beneficial effects [62]. Based on these observations, it is possible to infer a role of cortisol in the gender difference of GSH level, as in elderly women, urinary free cortisol excretion is lower than in the elderly men [63].

As for the different sex-related activities of metabolizing enzymes, the specific activity of the catabolic enzyme gamma-glutamyltranspeptidase in female mice was 73% of that in male mice, suggesting that the faster glutathione turnover in males could account for the higher susceptibility to oxidative injury [64]. A time-dependent reduction of hepatic and renal cortical glutathione was observed in both male and female mice following a dose of acetaminophen, but the depletion in male mice was significantly greater than that in the females [65]. This means that even if in basal conditions the GSH levels are similar, after challenges with drugs or pollutants requiring the GSH-dependent detoxification system, the decrease of GSH is more evident in males than in females.

On a large sample of adults from North-Western Italy, vitamin D levels were found significantly higher in women than in men, more elevated in summer than in winter and higher in individuals aged less than 64 years compared to those older than 65 years [66]. As vitamin D induces the expression of GCL and GR genes [14], thus increasing the levels of GSH, these small but significant differences must also be taken into account as preventive factors of severe COVID-19.

3. Diabetes. The correlation between low GSH and diabetes is well established. Diabetes has been widely associated with oxidative damage, increased GSSG/GSH ratio and decreased GSH content in different tissues [67,68]. Decreased GSH is, in most cases, associated with increased activity of NF-kB [69]. A study by Samiec and colleagues showed that the levels of total glutathione and its reduced form were lower in plasma of older subjects and even lower in diabetic patients [70]. In diabetes, the role of GSH as an antioxidant molecule is as critical as its contribution in maintaining the levels of GSNO, the main donor of NO, as discussed above. Insulin resistance is negatively related to the activity of endothelial NO synthase (eNOS), thus creating a link between metabolic and cardiovascular diseases. Lower NO production induces both insulin resistance and hypertension [71]. The increase of insulin sensitivity in muscle, adipocytes and liver depends more on GSNO than on pure NO [72,73].

4. Hypertension. Diabetes and hypertension seem to be the most frequent comorbidities in dying patients [74]. Hypertension may depend on multiple factors; among them it is relevant to cite the activation of the renin-angiotensin system mainly by renin overexpression secondary to low vitamin D, the decreased activity of eNOS and the decreased levels of GSH. As already described for diabetes, the combination low GSH/low NO leads to a higher calcium influx into the vessel wall and in smooth muscle with vasoconstriction, as demonstrated also in animal models [75]. Since in hypertensive patients the low levels of GSH are often accompanied by a decrease of companion enzymes (catalase, GSH-peroxidase and GST) and increased lipid peroxides, the prevailing hypothesis is that the GSH decrease is due to a burst of ROS production, secondary to an inflammatory process [76]. It has been demonstrated that GSH depletion induces chronic oxidative stress and causes hypertension in normal rats. This is accompanied by inactivation and sequestration of NO by ROS, leading to diminished NO bioavailability [77].

5. Obesity. Together with the most common comorbidities, hypertension (56.6%) and diabetes (33.8%), obesity was also found to be closely associated with COVID-19 (41.7%) in patients hospitalized in a US health care system [78]. Many studies suggest that in obese subjects oxidative stress and chronic inflammation are important underlying factors leading to development of many pathologies such as diabetes and cardiovascular diseases. Several studies have reported that in obese patients oxidative stress is associated with diminished glutathione levels [79,80] and decreased GSH/GSSG ratio [81]. In addition, nutritional stress caused by a high fat high carbohydrate diet promotes oxidative stress, as evident by increased lipid peroxidation products, a diminished antioxidant system and decreased glutathione levels [82,83].

6. Pharmacotherapy. Glutathione S-transferase (GST) enzymes catalyze the conjugation of GSH to lipophilic xenobiotics, which include most of the drugs. Acetaminophen (paracetamol) is the best-known drug affecting GSH levels by this mechanism (more than 600 entries in PubMed on this interaction). Many other drugs decrease GSH levels because they induce oxidative stress, for example doxorubicin (adriamycin), antimalarial drugs, chloroquine (CQ), etoposide, opiates, ethanol [84] and antidepressants [85]. The use of CQ deserves special caution because of its chemical properties. In addition to its prooxidant activity leading to GSH depletion [86], chloroquine accumulates into lysosomes leading to their alkalinization and to the impaired uptake of many nutrients from the blood, including transferrin-bound iron. Iron deficiency in the nerve reduces cytochrome C synthesis, respiratory chain activity and ATP synthesis. This toxicity is untreatable and can progress to blindness [87].

Based on these observations, we conclude that many seemingly marginal health problems can concur in creating an individual fragility in the antioxidant defenses, which could be aggravated by the viral infection and could lead to the severe outcome of COVID-19.

6. Treatment with GSH and Thiols

Anti-oxidant therapies exert beneficial effects on many diseases characterized by inflammation consequent to impaired redox homeostasis [88–90]. In the context of inflammatory diseases, systemic oxidative stress is detected as decreased total free thiol levels (free sulfhydryl groups of cysteine in proteins such as albumin as well as low-molecular-weight free thiols, for example cysteine, glutathione, homocysteine and related species). A recent study has concluded that low molecular mass systemic thiols might play a role in the inflammatory and oxidative stress pathways involved in both chronic obstructive pulmonary disease (COPD) and cardiovascular disease [91]. The levels of systemic free thiols can be influenced by nutritional or therapeutic intervention [92]. For these reasons, many clinical trials have evaluated the efficacy of *N*-acetylcysteine (NAC) administration, and many are still ongoing (714 studies, 349 completed), as well as the effects of GSH supplementation (162 studies, 100 completed) (https://clinicaltrials.gov/).

NAC is both a thiol with antioxidant properties and one of the substrates in GSH biosynthesis. The protective effects of the administration of antioxidant NAC on a murine asthma model was

demonstrated in two studies. Treatment with NAC was able to attenuate the diminished ratio GSH/GSSG in animals [93], and the repletion of glutathione pool by NAC counteracted allergen induced airway reactivity/inflammation and restored oxidant-antioxidant balance [94].

In humans, NAC and GSH supplementation has demonstrated its efficacy in several pathologies, and the most interesting results have been obtained in cardiovascular, pulmonary and viral diseases.

NAC has been found effective in acute myocardial infarction (AMI) [95]; indeed, oral NAC supplementation reduced the levels of some inflammatory markers in AMI patients receiving fibrinolytic therapy, and it was judged a therapeutic option for the successful management of these patients.

A study investigating the acute anti-hypertensive effect of anti-oxidant agents in hypertensive subjects and diabetic patients found that the anti-oxidant GSH showed a significant hypotensive effect probably due to the control exerted over the nitric oxide-free radical interaction [96].

In several chronic pulmonary diseases, the correlation between oxidative stress and pathogenesis has been described. The levels of GSH were reported to be markedly decreased in lung fluids and plasma of patients with idiopathic pulmonary fibrosis (IPF). Numerous studies have demonstrated that the administration of various antioxidants is protective against the development of fibrosis in this pathology; among them, some trials aimed at testing the efficacy of GSH precursor, NAC, reported its efficacy in augmenting pulmonary GSH levels and alleviating oxidative stress, although with different results (reviewed in [97]). Many studies found beneficial effects of NAC in COPD patients (reviewed in [98]), although the efficacy of NAC in reducing disease severity was ascribed to NACs mucolytic activity rather than its function as GSH precursor [97]. Alterations in GSH levels and in some GSH-dependent enzymes have also been reported in asthma [97].

The levels of oxidative stress are also critical in the immune response to viruses. During viral infections, an intracellular GSH depletion is mediated by multiple mechanisms and is crucial for viral replication [99]. Although several in vitro and in vivo studies demonstrate that the administration of GSH inhibits viral replication, to date very few clinical trials support the pharmacological use of NAC in respiratory viral infection in vivo (reviewed in [100]). GSH treatment is a promising approach, but high doses of GSH are necessary to achieve a therapeutic efficacy, due to its poor transport into the cells and tissues. GSH delivery could be improved by some derivatives with hydrophobic chains of different length or by I-152, which is a conjugate of NAC and s-acetyl-β-mercaptoethylamine (MEA), which is able to release NAC and MEA and increase GSH content [100].

GSH has been used locally in the treatment of emphysema, where experimental pieces of evidence demonstrated that the oxidative downregulation of the activity of α-1-proteinase inhibitor was curtailed by glutathione, and the authors of the study suggest that this treatment can be considered an option for acute respiratory crises due to COPD [101]. Previous clinical trials of nebulized GSH have demonstrated the bioavailability and safety of up to 600 mg twice daily [101]. As an increased ROS production in COVID-19 is the currently prevailing hypothesis, this approach could be suitable in this case as well.

GSH is one of the more represented molecules in our body: its concentration is 2–5 mM, comparable to very abundant molecular species such as glucose in the blood (5 mM) and intracellular ATP (5–10 mM). If we assume 40 kg of tissue with an average of 2.5 mM GSH (about 750 mg/L), it means that there is 30 g of GSH in the whole body. With a half-life of 48 h, its life is around 10 days; this means that tissue loses 3 g of GSH a day; therefore, the dietary support for full replacement is approximately 1.5 g glutamate, 0.75 g glycine and 1.20 g cysteine (1.63 g if assumed as *N*-acetyl-L-cysteine, NAC) a day. Body GSH concentration may be increased with oral intake of either GSH, or proteins enriched in the amino acid constituents of GSH, or the supplementation of the two limiting amino acids cysteine and glycine, as the body availability of glutamate is usually not limiting. Oral administration of GSH is more expensive than supplements with cysteine and glycine, and its systemic bioavailability may be poor due to degradation in the gut; therefore, its suitability for use on a large population could be limited. Interestingly, a case report study has shown that the repeated use of both 2000 mg of oral administration and intravenous injection of glutathione was effective in relieving the severe

respiratory symptoms of COVID-19, showing for the first time the efficacy of this antioxidant therapy for COVID-19 [102].

Dietary supplementation with the glutathione precursors cysteine and glycine, in proper conditions, fully restores glutathione synthesis and concentrations. Some observations support this practical and effective approach to decrease oxidative stress in aging and diseases associated with low GSH concentration [103]. It was demonstrated that elderly subjects had markedly lower red blood cell concentrations of GSH (53%) compared with younger controls. After oral treatment of 14 days with 0.81 mmol cysteine (132 mg NAC)·kg^{-1}·d^{-1} and 1.33 mmol glycine (100 mg)·kg^{-1}·d^{-1}, the elderly reached the GSH concentration of the younger controls [103].

Five clinical trials are at the moment evaluating the effects of supplementation with *N*-acetylcysteine plus glycine (https://clinicaltrials.gov/).

NAC has been largely used in the past as a treatment for bronchitis. A meta-analysis evaluating 13 studies and a total of 4155 people with COPD concluded that the standard dose of 1200 milligrams of *N*-acetylcysteine per day reduces the incidence and severity of flares (known as exacerbations) compared to a placebo [104]. Considering NAC as one of the substrates in GSH biosynthesis, the stoichiometric dose of glycine should be about 1000 mg (exactly 938 mg). This amount roughly corresponds to the daily turnover in healthy people and seems a reasonable dosage free of side effects, either for a preventive or therapeutic approach.

7. Conclusions

The outlined overview describes how SARS-CoV-2 can unbalance a high activity of the renin-angiotensin system in the lung via ACE2 downregulation, followed by free radical mediated inflammation, and unveils the protective role of GSH; this biochemical approach to COVID-19 disease opens novel avenues for further investigation aimed at understanding the involved molecular mechanisms.

Several pieces of evidence reported in our biochemical analysis suggest that low levels of GSH could be one of the major causes of the excessive inflammatory response linked to severe COVID-19 symptoms and indicate that increasing body GSH could reduce the number of symptomatic patients. Future clinical studies investigating the levels of GSH in COVID-19 patients may be the starting point to explore this possibility.

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Abbreviations

GSH	glutathione
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
ACE	angiotensin-converting enzyme
RAS	renin-angiotensin system
ROS	reactive oxygen species
GST	glutathione S-transferase
GPX	glutathione peroxidase
NAPQI	*N*-acetyl-*p*-benzoquinone imine
LT	leukotriene
GSNO	S-nitrosoglutathione

NO	nitric oxide
NOS	nitric oxide synthase
ANGII	angiotensin II
AT1R	angiotensin II receptor type 1
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
IL-6	interleukin-6
CQ	chloroquine
PEDV	porcine epidemic diarrhea virus
NAC	*N*-acetyl-L-cysteine
COPD	chronic obstructive pulmonary disease

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RESEARCH ARTICLE Open Access

Efficacy of glutathione for the treatment of nonalcoholic fatty liver disease: an open-label, single-arm, multicenter, pilot study

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Abstract

Background: Glutathione plays crucial roles in the detoxification and antioxidant systems of cells and has been used to treat acute poisoning and chronic liver diseases by intravenous injection. This is a first study examining the therapeutic effects of oral administration of glutathione in patients with nonalcoholic fatty liver disease (NAFLD).

Methods: The study was an open label, single arm, multicenter, pilot trial. Thirty-four NAFLD patients diagnosed using ultrasonography were prospectively evaluated. All patients first underwent intervention to improve their lifestyle habits (diet and exercise) for 3 months, followed by treatment with glutathione (300 mg/day) for 4 months. We evaluated their clinical parameters before and after glutathione treatment. We also quantified liver fat and fibrosis using vibration-controlled transient elastography. The primary outcome of the study was the change in alanine aminotransferase (ALT) levels.

Results: Twenty-nine patients finished the protocol. ALT levels significantly decreased following treatment with glutathione for 4 months. In addition, triglycerides, non-esterified fatty acids, and ferritin levels also decreased with glutathione treatment. Following dichotomization of ALT responders based on a median 12.9% decrease from baseline, we found that ALT responders were younger in age and did not have severe diabetes compared with ALT non-responders. The controlled attenuation parameter also decreased in ALT responders.

Conclusions: This pilot study demonstrates the potential therapeutic effects of oral administration of glutathione in practical dose for patients with NAFLD. Large-scale clinical trials are needed to verify its efficacy.

Trial registration: UMIN000011118 (date of registration: July 4, 2013).

Keywords: Nonalcoholic fatty liver disease, Glutathione, Controlled attenuation parameter

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Background

Nonalcoholic fatty liver disease (NAFLD) is an important cause of chronic liver injury worldwide [1, 2]. The spectrum of NAFLD ranges from nonalcoholic fatty liver to nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma [3]. NAFLD is associated with metabolic syndromes and the incidence of NAFLD has increased over time [4, 5]. First-line treatment for NAFLD is lifestyle modification to achieve weight reduction, particularly through diet and exercise [6]. However, weight reduction is very difficult to accomplish and maintain. Effective therapy for NAFLD has not yet been established.

Glutathione, γ-L-glutamyl-L-cysteinyl-glycine, is a tripeptide present in every cell of the human body [7]. Although its functions are complex and remain the subject of current research, glutathione is thought to play crucial roles in the detoxification and antioxidant systems in cells. Because a reduction of glutathione levels in cells has been found to increase the risks for diseases and poisoning, direct intravenous injection of glutathione has been used to treat patients with chronic liver diseases and poisoning [8, 9].

Glutathione is synthesized in cells from glutamic acid, cysteine, and glycine. Cysteine and glycine are generated from methionine and serine, respectively, and glutamic acid is synthesized from α-ketoglutarate, a metabolite of glucose. These amino acids are generally supplied from food. It has been reported that oral administration of glutathione did not change the levels of glutathione and glutathione disulfide in the deproteinized fraction of blood [10], and it has been suggested that orally administered glutathione is degraded into constituting amino acids and does not exert specific activity beyond the amino acid source. However, it has been reported that glutathione can pass through the mono layer of Caco-2 cells without degradation [11]. In addition, Park et al. reported an increase in the protein-bound form of glutathione in human blood after oral administration, while glutathione in the deproteinized fraction did not change [12]. These studies suggest that orally administered glutathione is absorbed into the blood and might have effects on the redox status in the human body. Such findings have encouraged us to examine the therapeutic effects of oral administration of glutathione on NAFLD.

The objective of the current study was to demonstrate the therapeutic potential of oral administration of glutathione in an open-label, single-arm, multicenter, pilot study prior to subsequent large-scale clinical trials. In this study, we compared clinical parameters before and after treatment with glutathione. We also evaluated controlled attenuation parameter (CAP) and liver stiffness measurement (LSM), as determined by vibration-controlled transient elastography (VCTE).

Methods

Patients and study design

The study protocol was conducted in accordance with the guidelines contained within the Declaration of Helsinki and was approved by the ethics committees of Yokohama City University and Kyoto Prefectural University of Medicine. Written informed consent was obtained from all participants before entry into the study. The trial is registered with the University Hospital Medical Information Network (UMIN) Clinical Trials Registry (UMIN000011118).

Patient enrollment began in January 2014 and ended when the target sample size was reached in September 2014. Follow-up of participants ended in December 2014. We prospectively evaluated 34 NAFLD patients with liver dysfunction. NAFLD was diagnosed based on ultrasonography. All 34 patients provided a detailed medical history and underwent a physical examination. Patients were excluded if they had infectious hepatitis (hepatitis B or C or Epstein–Barr virus infection), autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, hemochromatosis, α1-antitrypsin deficiency, Wilson's disease, drug-induced hepatitis, alcoholic hepatitis, or excessive alcohol consumption (present or past consumption of >20 g alcohol/day). No NAFLD patient had clinical evidence of hepatic decompensation, such as hepatic encephalopathy, ascites, variceal bleeding, or a serum bilirubin level greater than twice the upper limit of normal. All patients were started on a standard diet (30 kcal/kg/day, consisting of 50–60% carbohydrate, 20–30% fat, and 15–20% protein) and received exercise counseling beginning 3 months before glutathione treatment. Exercise consisted of 5–6 metabolic equivalents for 30 min daily. Patients taking medication for lifestyle-related comorbid diseases, such as hypertension, dyslipidemia, and diabetes, were included; however, no change in medication or dose was allowed.

Because serum alanine aminotransferase (ALT) levels have been reported to predict the histological course of NASH and because strict control of ALT is required to prevent the progression of NASH [13], the primary outcome of this study was a change in ALT levels.

Anthropometric and laboratory evaluations

Patient weight and height were measured using a calibrated scale after patients removed their shoes and any heavy clothing. Venous blood samples were obtained after patients had fasted overnight (12 h). Platelet counts and concentrations of fasting blood sugar (FBS), hemoglobin A1c (HbA1c), immunoreactive insulin (IRI), high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, non-esterified fatty acids (NEFA), aspartate aminotransferase (AST), ALT, γ-glutamyl transpeptidase, ferritin, and type

IV collagen 7 were measured using standard laboratory techniques before and after glutathione treatment. Patients with FBS ≥126 mg/dL, HbA1c ≥6.5%, and/or currently using antidiabetic medication were defined as having diabetes according to the criteria of the Japan Diabetes Society [14].

Glutathione in the deproteinized fraction and protein-bound fraction of plasma were determined using the method described by Park et al. [12]. Briefly, 100 μL of plasma was mixed with three parts ethanol. The supernatant was used as the deproteinized fraction. The precipitate was extracted using 100 μL of 5% trichloroacetic containing 2% 2-mercaptoethanol. The supernatant was used as the protein-bound fraction. Glutathione in these fractions were alkalized and derivatized with 6-aminoquinolyl-N hydroxy succinimidyl carbamate as described previously. The derivatives were resolved and detected using liquid chromatography/electron spray ionization/tandem mass spectrometry in multi-reaction monitoring mode.

Vibration-controlled transient elastography

VCTE was performed using an M-probe device (Fibroscan; EchoSens, Paris, France). Details of the technique and the examination procedure for LSM have been described previously [15, 16]. CAP was measured using VCTE to stage steatosis. The technique is a proprietary algorithm based on the ultrasonic attenuation coefficient of the shear wave of VCTE, an estimate of the total ultrasonic attenuation at 3.5 MHz. CAP uses the same radiofrequency data as LSM and is only appraised if the acquisition is valid. It is expressed in decibels per meter. Measurements were obtained from the right lobe of the liver through the intercostal spaces, with a patient lying in the dorsal decubitus position and the right arm in maximal abduction. Only VCTE measurements based on at least 10 valid shots and success rates ≥60% were considered reliable and were used for statistical analysis.

Statistical analysis

Data are expressed as mean ± standard deviation, unless indicated otherwise. The sample size was determined by reference to a previous report [17]. We estimated that with this sample size, the study would have 80% power to detect an absolute difference in the rate of improvement in ALT of 30 percentage points, with a two-sided type 1 error of 0.05. All statistical analyses were performed using JMP ver. 11.2.0 software (SAS Institute, Cary, NC, USA). Univariate comparisons between patient groups were analyzed using the Student's *t*-test or the Mann–Whitney's U-test, as appropriate. A p-value <0.05 was considered statistically significant.

Results

Biochemical response after 4 months of glutathione treatment

The study flowchart is shown in Fig. 1. Of the 34 patients enrolled, two withdrew before the start of treatment. The remaining 32 were treated with L-glutathione (300 mg/day; KOHJIN Life Sciences, Tokyo, Japan, US FDA GRAS #GRN000293) for 4 months by oral administration. Twenty-nine patients (14 men, 15 women, mean age 56.0 ± 13.3 years) finished the study protocol.

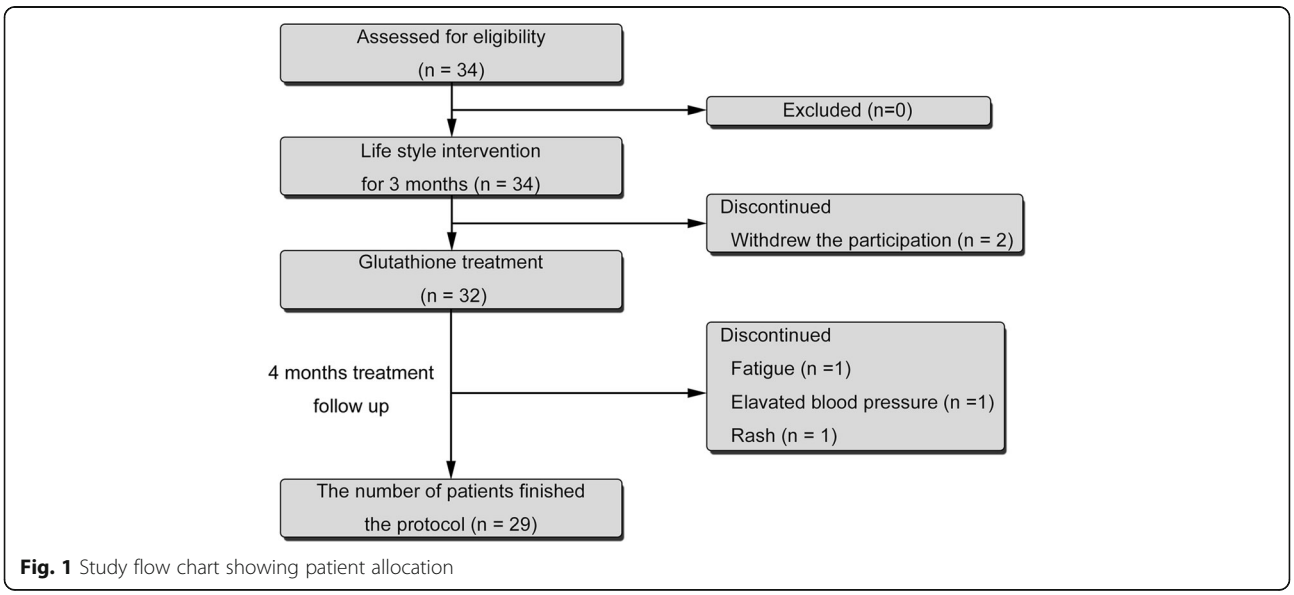

Fig. 1 Study flow chart showing patient allocation

Table 1 Characteristics of patients before and after glutathione treatment (n = 29)

	Before treatment	After treatment	*P*-value
Age (year)	56.0 ± 13.3		-
Male/female (n)	14/15		-
Dyslipidemia (%)	24 (82.8)		-
Statin (%)	12 (41.4)		-
Diabetes (%)	14 (48.3)		-
BMI (kg/m^2)	26.5 ± 3.9	26.5 ± 3.9	0.32
FBS (mg/dL)	118.4 ± 34.3	120.0 ± 27.8	0.24
IRI (μU/mL)	23.1 ± 29.8	23.4 ± 33.8	0.38
HbA_{1c} (%)	6.37 ± 1.18	6.46 ± 1.23	0.016
HDL cholesterol (mg/dL)	55.2 ± 16.3	55.0 ± 15.4	0.32
LDL cholesterol (mg/dL)	114.0 ± 28.8	111.3 ± 28.0	0.08
Triglycerides (mg/dL)	195.2 ± 135.9	163.6 ± 121.9	0.007
NEFA (μEq/L)	651.2 ± 242.5	533.5 ± 209.7	0.013
AST (IU/L)	46.7 ± 17.2	47.6 ± 21.2	0.39
ALT (IU/L)	68.9 ± 36.1	58.1 ± 33.5	0.014
GGT (IU/L)	70.4 ± 46.5	66.6 ± 47.5	0.29
Ferritin (ng/mL)	219.8 ± 150.8	194.4 ± 139.2	0.015
Platelet count (×10^4 /μL)	20.8 ± 5.7	20.9 ± 5.3	0.30
Type IV collagen 7 s	5.08 ± 1.95	4.84 ± 1.34	0.40
Glutathione in protein fraction (μM)	1.42 ± 0.87	0.93 ± 0.63	0.010
Glutathione in deproteinized fraction (μM)	0.025 ± 0.040	0.019 ± 0.024	0.27
CAP (db/m)	295.7 ± 44.9	285.4 ± 48.8	0.07
LSM (kPa)	9.94 ± 4.93	9.24 ± 4.48	0.16

Data are expressed as mean ± standard deviation. *BMI* body mass index, *FBS* fasting blood sugar, *IRI* immunoreactive insulin, *HBA1c*, hemoglobin A1c, *HDL cholesterol* high-density lipoprotein cholesterol, *LDL cholesterol* low-density lipoprotein cholesterol, *NEFA* non-esterified fatty acid, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* γ-glutamyl transpeptidase, *CAP* controlled attenuation parameter, *LSM* liver stiffness measurement

Three patients dropped out, one each owing to fatigue, elevated blood pressure, and rash.

The clinical and laboratory characteristics of the study participants are shown in Table 1. Twenty-four patients (82.8%) had dyslipidemia and 12 (41.4%) were taking statins. Fourteen patients (48.3%) had diabetes.

After 4 months of glutathione treatment, ALT levels decreased significantly. Glutathione treatment decreased the concentrations of triglycerides, NEFA, and ferritin. HbA1c levels increased after glutathione treatment. Unexpectedly, glutathione in the plasma protein fraction decreased significantly after glutathione treatment. There was no significant difference in glutathione levels in the deproteinized fraction. Although glutathione treatment did not significantly affect CAP and LSM values, both tended to decrease.

Glutathione treatment improved CAP values in ALT responders

The median decrease in ALT level from baseline was 12.9%. The 29 patients were divided into ALT responders (n = 15), defined as those with an ALT reduction ≥12.9%, and ALT non-responders (n = 14), defined as those with an ALT reduction <12.9%, and the factors associated with responses to glutathione were evaluated (Table 2). ALT responders were significantly younger than ALT non-responders (50.7 ± 12.1 years vs. 61.7 ± 12.4 years, p = 0.011). Body mass index (BMI) did not differ between ALT responders and non-responders (26.5 ± 4.1 kg/m^2 vs. 26.6 ± 3.8 kg/m^2, p = 0.47). Although the percentages of ALT responders and non-responders with dyslipidemia did not differ (80.0% vs. 85.7%, p = 0.68), HDL cholesterol and LDL cholesterol levels were higher in ALT responders. Rates of statin use tended to be lower in ALT responders than in non-responders (26.7% vs. 57.1%, p = 0.10). Rates of diabetes also tended to be lower (33.3% vs. 64.3%, p = 0.10) and HbA1c levels were significantly lower in ALT responders compared with non-responders. There were no significant differences in glutathione levels in the plasma protein and deproteinized fractions between ALT responders and non-responders before glutathione treatment.

The characteristics of ALT responders and non-responders before and after glutathione treatment are

Table 2 Characteristics of ALT responders and non-responders

	ALT responders (n = 15)	ALT non-responders (n = 14)	P-value
Age (year)	50.7 ± 12.1	61.7 ± 12.4	0.011
Male/female (n)	9 (6)	5 (9)	0.191
Dyslipidemia (%)	12 (80.0)	12 (85.7)	0.68
Statin (%)	4 (26.7)	8 (57.1)	0.10
Diabetes (%)	5 (33.3)	9 (64.3)	0.10
BMI (kg/m^2)	26.5 ± 4.1	26.6 ± 3.8	0.47
FBS (mg/dL)	115.7 ± 37.9	121.4 ± 31.2	0.33
IRI (μU/mL)	23.5 ± 22.7	22.6 ± 38.0	0.47
HbA_{1c} (%)	5.94 ± 1.03	6.9 ± 1.2	0.019
HDL cholesterol (mg/dL)	60.3 ± 18.6	49.7 ± 11.7	0.04
LDL cholesterol (mg/dL)	124.5 ± 33.1	102.9 ± 18.6	0.021
Triglycerides (mg/dL)	202.4 ± 164.1	187.6 ± 103.3	0.39
NEFA (μEq/L)	720.2 ± 285.0	563.3 ± 143.0	0.055
AST (IU/L)	46.3 ± 20.2	47.1 ± 14.1	0.45
ALT (IU/L)	77.1 ± 38.6	60.1 ± 32.2	0.104
GGT (IU/L)	81.9 ± 58.0	58.0 ± 26.6	0.085
Ferritin (ng/mL)	260.2 ± 164.5	176.6 ± 126.1	0.07
Platelet count ($\times 10^4$ /μL)	20.3 ± 4.6	21.3 ± 6.8	0.32
Type IV collagen 7 s	4.61 ± 1.13	5.59 ± 2.51	0.09
Glutathione in protein fraction (μM)	1.53 ± 0.92	1.27 ± 0.83	0.230
Glutathione in deprotenized fraction (μM)	0.017 ± 0.020	0.036 ± 0.057	0.116
CAP (db/m)	300.3 ± 41.1	290.4 ± 50.2	0.29
LSM (kPa)	8.71 ± 4.63	11.36 ± 5.05	0.080

Data are expressed as mean ± standard deviation

Abbreviations: BMI body mass index, *FBS* fasting blood sugar, *IRI* immunoreactive insulin, *HBA1c* hemoglobin A1c, *HDL cholesterol* high density lipoprotein cholesterol, *LDL cholesterol* low density lipoprotein cholesterol, *NEFA* non-esterified fatty acid, *AST* asparate aminotransferase, *ALT* alanine aminotransferase, *GGT* γ-glutamyl transpeptidase, *CAP* controlled attenuation parameter, *LSM* liver stiffness measurement

shown in Table 3. Glutathione treatment decreased ALT levels in ALT responders (Fig. 2a) but increased AST and ALT levels in ALT non-responders (Fig. 2b). In ALT responders, glutathione treatment decreased NEFA, ferritin, and HDL cholesterol levels but increased HbA1c levels. In ALT non-responders, glutathione treatment reduced triglyceride levels but increased FBS levels. Glutathione treatment significantly decreased glutathione in the plasma protein-boud fraction in ALT responders; there was no change in ALT non-responders. Surprisingly, CAP values were significantly reduced in ALT responders; there were no differences in ALT non-responders.

Discussion

Glutathione has a long history for the treatment of chronic liver disease by intravenous injection. This study demonstrates a therapeutic effect of glutathione by oral administration in patients with NAFLD. The primary outcome of this study was a change in ALT levels. The 29 patients who were treated with oral administration of glutathione (300 mg/day) for 4 months showed a reduction in ALT levels as well as reductions in triglycerides, NEFA, and ferritin levels. The findings of the current study suggest the beneficial effects of glutathione by oral administration for NAFLD patients. It is thought that glutathione is degraded into amino acids during digestion and absorption processes. Orally administered glutathione is suggested to serve as a source of amino acids in the synthesis of endogenous glutathione. Supplementation of large doses of glycine and serine, precursors of glutathione, can attenuate NAFLD in human and animal models [18, 19]. In the current study, the dose of glutathione was 300 mg/day. The amount of cysteine potentially released from 300 mg of glutathione is less than 120 mg, the amount that can be obtained from 10 to 20 g of meat or 100 mL of milk. It is, therefore, very unlikely that the current dose of orally administered glutathione attenuates the pathogenesis of NAFLD via an amino acid source for glutathione synthesis.

It is reported that the level of the protein-bound form of glutathione increases 1–2 h after ingestion of glutathione, which suggests that orally administered glutathione is

Table 3 Characteristics of ALT responders and non-responders before and after glutathione treatment

	ALT responders (n = 15)			ALT non-responders (n = 14)		
	Before treatment	After treatment	P-value	Before treatment	After treatment	P-value
Age (year)	50.7 ± 12.1		-	61.7 ± 12.4		-
Male/female (n)	9 (6)		-	5 (9)		-
Dyslipidemia (%)	12 (80.0)		-	12 (85.7)		-
Statin (%)	4 (26.7)		-	8 (57.1)		-
Diabetes (%)	5 (33.3)		-	9 (64.3)		-
BMI (kg/m^2)	26.5 ± 4.1	26.5 ± 4.0	0.23	26.6 ± 3.8	26.4 ± 3.9	0.45
FBS (mg/dL)	115.7 ± 37.9	113.0 ± 23.0	0.38	121.4 ± 31.2	128.2 ± 31.4	0.004
IRI (μU/mL)	23.5 ± 22.7	17.9 ± 14.3	0.14	22.6 ± 38.0	30.5 ± 31.4	0.11
HbA_{1c} (%)	5.94 ± 1.03	6.08 ± 1.10	0.017	6.9 ± 1.2	6.89 ± 1.28	0.14
HDL cholesterol (mg/dL)	60.3 ± 18.6	57.4 ± 16.7	0.001	49.7 ± 11.7	52.2 ± 13.8	0.08
LDL cholesterol (mg/dL)	124.5 ± 33.1	117.1 ± 34.8	0.06	102.9 ± 18.6	104.1 ± 14.5	0.41
Triglycerides (mg/dL)	202.4 ± 164.1	178.8 ± 157.6	0.15	187.6 ± 103.3	146.2 ± 62.1	0.003
NEFA (μEq/L)	720.2 ± 285.0	576.0 ± 230.1	0.032	563.3 ± 143.0	473.9 ± 170.7	0.13
AST (IU/L)	46.3 ± 20.2	40.0 ± 20.7	0.11	47.1 ± 14.1	55.8 ± 19.3	0.003
ALT (IU/L)	77.1 ± 38.6	47.9 ± 28.1	<0.0001	60.1 ± 32.2	68.9 ± 36.3	0.005
GGT (IU/L)	81.9 ± 58.0	64.8 ± 48.4	0.07	58.0 ± 26.6	68.5 ± 48.3	0.06
Ferritin (ng/mL)	260.2 ± 164.5	217.7 ± 162.3	0.015	176.6 ± 126.1	169.5 ± 109.9	0.28
Platelet count (×10^4 /μL)	20.3 ± 4.6	20.2 ± 4.7	0.45	21.3 ± 6.8	21.8 ± 6.0	0.25
Type IV collagen 7 s	4.61 ± 1.13	4.39 ± 1.06	0.10	5.59 ± 2.51	5.42 ± 1.49	0.13
Glutathione in protein fraction (μM)	1.53 ± 0.92	0.88 ± 0.53	0.004	1.27 ± 0.83	1.00 ± 0.77	0.24
Glutathione in deprotenized fraction (μM)	0.017 ± 0.020	0.019 ± 0.029	0.60	0.036 ± 0.057	0.018 ± 0.017	0.19
CAP (db/m)	300.3 ± 41.1	285.1 ± 53.2	0.049	290.4 ± 50.2	285.8 ± 44.9	0.31
LSM (kPa)	8.71 ± 4.63	7.91 ± 4.22	0.19	11.36 ± 5.05	10.9 ± 4.38	0.32

Data are expressed as mean ± standard deviation

Abbreviations: BMI body mass index, *FBS* fasting blood sugar, *IRI* immunoreactive insulin, *HBA1c* hemoglobin A1c, *HDL cholesterol* high density lipoprotein cholesterol, *LDL cholesterol* low density lipoprotein cholesterol, *NEFA* non-esterified fatty acid, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* γ-glutamyl transpeptidase, *CAP* controlled attenuation parameter, *LSM* liver stiffness measurement

absorbed into the blood [12]. This protein-bound glutathione may be deposited in the liver, attenuating hepatitis.

The levels of protein-bound glutathione were reported to return to baseline levels after an overnight fast [12]. In the current study, we found that the baseline level of the protein-bound form of glutathione significantly decreased after an overnight fast following 4 months of glutathione administration, especially in ALT responders. The levels of protein-bound glutathione in patients in the current study were considerably higher than those of healthy

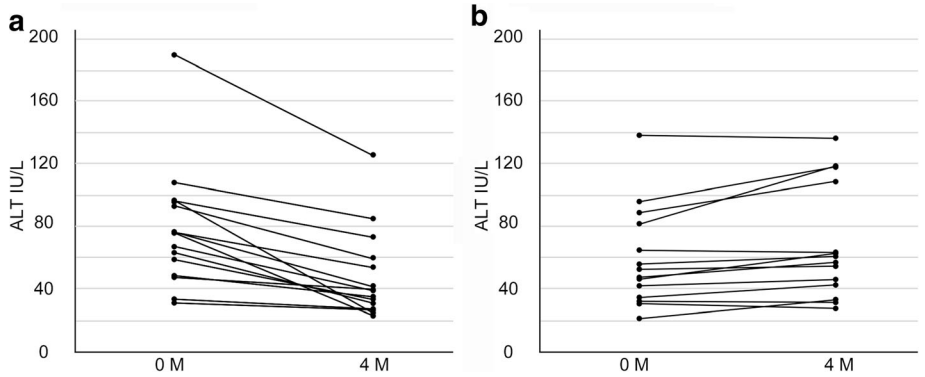

Fig. 2 Alanine aminotransferase (ALT) levels before and after treatment with glutathione in **a** ALT responders and **b** ALT non-responders

volunteers in previous studies [12] estimated using the same method. Glutathione treatment also decreased protein-bound glutathione to normal baseline levels. These findings suggest that oral administration of glutathione may increase the incorporation of protein-bound glutathione into the liver or decrease the pathological excretion of glutathione from the liver.

NAFLD is a complex disease. Its pathogenesis is thought to involve various factors, including insulin resistance, lipotoxicity, gut/nutrient-derived signals, adipocytokines, oxidative stress, and genetic factors. Dyslipidemia has been reported in 20–80% of patients with NAFLD [20]. Our previously study revealed that orally administrated glutathione accelerates fatty acid utilization by upregulating levels of the protein peroxisome proliferator-activated receptor-γ coactivator-1α and mitochondrial DNA with reduced plasma NEFA levels [21]. The current study also revealed that 24 (82.8%) of our patients had dyslipidemia, and glutathione treatment reduced triglyceride and NEFA levels significantly.

Increases in ferritin and body iron stores have been detected frequently in NAFLD patients [22, 23]. Ferritin and iron can promote the development of NAFLD through oxidative stress [24]. Results from the PIVENS trial showed that oral administration of the anti-oxidant vitamin E improved liver dysfunction and the pathological conditions of NASH [17]. However, long-term treatment with vitamin E has been associated with increases in all-cause mortality and the risk for prostate cancer [25–27], suggesting the need to evaluate the efficacy and safety of this agent. In the current study, glutathione treatment significantly decreased ferritin levels, but the mechanism behind the decrease remains unclear. Glutathione is thought to ameliorate hyperferritinemia and oxidative stress, and to have therapeutic effects in patients with NAFLD.

Liver fat was non-invasively assessed using VCTE with CAP. A meta-analysis found that CAP has good sensitivity and specificity for detecting liver fat [28]. CAP values in our study tended to decrease in all patients and significantly decreased in ALT responders following 4 months of glutathione treatment. Although the relationship between histologic improvement of hepatic steatosis and the reduction of CAP values has not yet been determined, glutathione may reduce hepatic steatosis.

We also investigated the patient factors associated with the therapeutic effects of glutathione. We found that HDL cholesterol and LDL cholesterol levels were higher and HbA1c levels lower in ALT responders than in non-responders. Although the percentage of patients using statins did not differ significantly between the two groups, the percentage tended to be lower in ALT responders than in ALT non-responders. While it can be nothing more than speculation because of the small sample size, patients who showed therapeutic effects following glutathione treatment appeared to be younger and did not have diabetes or had mild diabetes.

Three patients withdrew from the study because of fatigue, elevated blood pressure, and a rash. In ALT responders, HbA1c levels increased and HDL cholesterol levels decreased after glutathione treatment. A study of 6522 patients found that 24 (0.4%) had experienced adverse reactions, the most frequent being anorexia, nausea, vomiting, and rash [29]. Although administration of glutathione may have been associated with a rash in one patient in the current study, the causal associations between glutathione and other adverse effects are unclear.

This study had some limitations. First, our study was a single-arm study without a control group. Second, the study was limited by the small sample size and the short treatment period (4 months). Third, as the pathological conditions of the patients were not evaluated by liver biopsy, incorporation of orally administered glutathione in the liver was not confirmed. Fourth, a number of patients withdrew from the study but no causal association can be determined.

Conclusions

Treatment with glutathione significantly improved ALT levels. In addition, CAP values were significantly reduced in ALT responders. Our pilot study suggests that oral administration of glutathione supports hepatic metabolism and improves NAFLD. To elucidate the mechanism behind the beneficial effects of glutathione, further studies that examine the incorporation of orally administrated glutathione into the liver and the effects on the host redox system using stable isotope-labeled glutathione and animal models are required. Large-scale clinical trials are necessary to confirm the therapeutic effects of glutathione.

Abbreviations

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; CAP: Controlled attenuation parameter; FBS: Fasting blood sugar; HbA1c: hemoglobin A1c; HDL: High-density lipoprotein; IRI: Immunoreactive insulin; LDL: Low-density lipoprotein; LSM: Liver stiffness measurement; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; NEFA: Non-esterified fatty acid; UMIN: University Hospital Medical Information Network; VCTE: Vibration-controlled transient elastography

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Availability of data and materials
The data generated and analyzed during this study are available from the corresponding author on reasonable request.

Authors' contributions
Designed and coordinated the study: TK, YH, YS, and AN. Performed the experiments: TK, YH, YS, TK, TK, YO, WT, KI, KF, MY, YS, ST, SS, MO, SO, YE, YI, WA, KS, and AN. Performed the statistical analyses: TK, YH, and AN. Collected the data and critically reviewed the manuscript: TK, YH, YS, KK, MT, TY, WA, KS, and AN. Wrote the manuscript: TK, YH, YS, WA, KS, and AN. All authors approved the final version of the manuscript.

Ethics approval and consent to participate
The study protocol was conducted according to the guidelines contained within the Declaration of Helsinki and was approved by the ethics committees of Yokohama City University and Kyoto Prefectural University. Written informed consent was obtained from all participants before entry into the study.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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The Therapeutic Potential of Glutathione Supplement: A Review of Clinical Trials

Abdelrahim Hunaiti

***Abstract*—Glutathione (GSH) is a tripeptide (γ-glutamyl cysteinyl glycine) involved in a variety of biological processes indispensable to sustain life and the most abundant free radical scavenger synthesized endogenously in humans. There are adverse health consequences from glutathione deficiency. The present mini–review aims to provide an extensive overview to glutathione supplement therapeutic effects in human subjects.**

***Index Terms*—Glutathione; Therapeutic Supplementation; Free radical scavenger.**

I. Introduction

Glutathione (GSH) is an antioxidant present in plants, animals, fungi, and some bacteria and the most important antioxidant found in mammalian cells. structurally glutathione is a tripeptide consisting of glutamic acid attached via its side chain to the N-terminus of cysteinylglycine to synthesis GSH, (γ-L-glutamyl-L-cysteinylglycine). Due to the sulfhydryl group of cysteine it can exists in either reduced (GSH) or oxidized (GSSG) form. Because of its regulatory functions against oxidative stress and maintaining thiol homeostasis, 90% of glutathione under normal circumstances are in a reduced form (GSH) while in abnormal condition of oxidative stress, glutathione concentration in oxidized form (GSSG) is more abundant [1,2]. The GSH: GSSG ratio is the primary determinant of the cellular redox state and the processes of glutathione synthesis, transport, utilization, and metabolism are tightly controlled to maintain intracellular glutathione homeostasis and redox balance. An elevation in the GSSG content and decreases in GSH content are indicative of an increases production of free radicals and ultimately lead to systemic oxidative stress and the risk of acquiring serious and life-threatening diseases [2]. Many studies indicate that disturbances in cellular glutathione homeostasis and redox status are implicated in the etiology and/or progression of a number of acute and chronic human diseases, including neurodegenerative diseases , cancer, cystic fibrosis, cardiovascular, inflammatory, immune, metabolic, and aging diseases [3,4,5,6]. Supplementation with the glutathione precursor's cysteine and glycine fully restores glutathione synthesis and lowers levels of oxidative stress and oxidant damages [7, 8]. However, supplementing with glutathione precursors relies upon the body's ability to synthesize glutathione from these precursors. Supplementing GSH for people at risk for glutathione redox balance has limited due to rapid breakdown during oral ingestion. Allen J, and Bradley RD [9] studied the effects of oral glutathione supplementation on systemic oxidative stress biomarkers in forty adult volunteers without acute or chronic disease participated in their study. They reported no significant changes in biomarkers of oxidative stress, including GSH status, GSSG, and ratio GSH: GSSG in the human volunteers. While Richie JP et al [10] in their 6-month randomized, double-blinded, placebo-controlled trial of supplementation oral GSH (250 or 1,000 mg/day) showed, for the first time, that daily consumption of GSH supplements was effective at increasing body stores of glutathione. Similarly Sinha, R. et al [11] showed that Oral supplementation with liposomal glutathione elevates body stores of glutathione and markers of immune function. Schmitt, B et al. [12] in their study demonstrated the significant superiority of a new sublingual form of GSH over the oral form, both in terms of bioavailability and positive effects on oxidative stress. Recent study in our lab highlighted the Clinical uses of bilingual glutathione supplementation as a novel treatment for cystine stone [13] our results showed that patients who took glutathione over 3 months had no new stone formation and the size of the already presented stones was still the same or reduced, while those patients who took placebo for the same period developed new stones and the size of already presented stones increased. Overwhelming evidence shows that maintaining glutathione levels may help with the symptoms of neurodegenerative diseases. Brain glutathione level was suggested as a biomarker for mild cognitive impairment and Alzheimer's disease and elevated glutathione as a therapeutic strategy in Alzheimer's disease and Cognitive Improvement with Glutathione Supplement [14,15 ,16,17,18]. Their findings appear to support glutathione as a potential therapeutic agent. Kern JK et al [19] found that oral glutathione supplements or injections might reduce some effects of Autism spectrum disorders (ASD) Al-Omari et al [20] reported that oxidative stress played a crucial role in the wide spread of ASD in Jordan in combination with, the C677T polymorphism of MTHFR gene in the etiology of the disorder and that Oral sublingual -glutathione given to autistic children improved their deterioration of GSH homeostasis and oxidative stress as well as some of their behavioral symptoms such as cooperation, imitation, play ,leisure and social interaction.

II. Glutathione and its Anti-aging and Skin Whiting Effects

Another major effect of Glutathione imbalance in the body is premature aging.

The functional application and therapeutic potential of

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glutathione in the treatment of aging-associated diseases and the antimelanogenic properties of glutathione are well documented in the literature [21, 22] .It is well known that Glutathione deficiency in elderly humans occurs because of a marked reduction in glutathione synthesis and dietary supplementation with the glutathione precursors cysteine and glycine fully restores glutathione synthesis and concentrations and lowers levels of oxidative stress and oxidant damages. The antimelanogenic properties of glutathione is due to its ability to scavenge ultraviolet radiation induced reactive oxygen species generated in epidermal cells and blocking the induction of tyrosinase the enzyme essential for the skin pigment melanin by these peroxides [22] these findings has led to its promotion as a skin-lightening agent. GSSG was preferred over GSH, as GSH is unstable in aqueous solutions. GSSG eventually generates GSH after cutaneous absorption [23, 24].

III. Conclusion

Glutathione is generally a safe ingredient for use and is regarded as food or health supplements in most countries, while it is considered a pharmaceutical agent in some others. Could be used both as a protective and therapeutic agent against several diseases. Despite the therapeutic benefits of glutathione, the major challenge is attributed to its poor bioavailability due to rapid breakdown during oral ingestion the bioavailability can be enhanced through several innovative ways. We feel that this product has the potential to do a great deal of good for a large number of people

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Nebulized Glutathione Induces Bronchoconstriction in Patients with Mild Asthma

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To assess the effects on bronchial responsiveness of nebulized glutathione (GSH), one of the most efficient scavengers of oxidant substances in the airways, we studied eight patients with mild asthma (FEV_1, 88 ± 11% predicted [SD]) in a randomized, double-blind, cross-over, placebo-controlled fashion. Bronchial challenge was measured using both FEV_1 and total pulmonary resistance (Rrs) by the forced oscillation technique. Patients received nebulized GSH (600 mg with 4 ml of 0.9% sodium chloride) or placebo (identical saline solution) over a period of 25 min, 1 wk apart. Placebo provoked subclinical mild bronchoconstriction (changes from baseline: FEV_1, −1%; Rrs, +17%); by contrast, GSH caused major airway narrowing (changes from baseline: FEV_1, −19%; Rrs, +61%) and induced cough (four patients) or breathlessness (three patients). Differences between placebo and GSH after challenge were also noticeable in both FEV_1 ($p = 0.03$) and Rrs ($p = 0.02$). Neither osmolarity (660 mosm · kg^{-1}) nor pH (3.0) of the GSH solution accounted for these effects. Nebulized salbutamol (5.0 mg) given before the GSH challenge blocked GSH-induced bronchoconstriction. Furthermore, GSH-induced FEV_1 falls were inversely correlated with metabisulfite bronchoprovocation (provocative dose [PD_{20}], 1.49 ± 1.83 μmol) but not with methacholine challenge. The detrimental effects of nebulized GSH on the airway bronchial tone in patients with mild asthma strongly suggests bronchoconstriction provoked by sulfite formation. Marrades RM, Roca J, Barberà JA, de Jover L, MacNee W, Rodriguez-Roisin R. Nebulized glutathione induces bronchoconstriction in patients with mild asthma.

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An imbalance between oxidants and antioxidants may affect patients with obstructive airway disease, such as bronchial asthma and chronic obstructive pulmonary disease (COPD). There are data suggesting that increased production of reactive oxygen species (ROS), or oxidants, by inflammatory cells contributes to the pathogenesis of asthma (1). Glutathione (L-γ-glutamyl-L-cysteinyl-glycine, or GSH), a sulfhydryl-containing tripeptide produced by most mammalian cells, is an efficient scavenger of ROS. Glutathione is present in the epithelial lining fluid (ELF) of the normal lower respiratory tract (2), where it is thought to play a major role in preventing or minimizing the adverse consequences of ROS. Under appropriate conditions, an increase in one or more antioxidants might offset increases in oxidant production. Both systemic and ELF deficiency of GSH have been demonstrated in patients with idiopathic pulmonary fibrosis (IPF) (3) and cystic fibrosis (4), and in individuals who are HIV-seropositive but symptom-free (5). GSH has been nebulized safely in patients with IPF to augment GSH levels on the respiratory epithelial surface, thus enhancing antioxidant defense and controlling fibroblast proliferation (6). Likewise, in HIV-seropositive individuals, GSH has been shown efficacious, apparently by improving local host defenses (7).

Decreased peripheral blood GSH peroxidase activity has been documented in patients with asthma, thereby suggesting reduced antioxidant defenses and increased susceptibility to ROS (8, 9). Similarly, nocturnal asthma has been related to an imbalance between oxidants and antioxidants in the ELF (10). Smith and co-workers (11) found increased amounts of GSH in bronchoalveolar lavage fluid in patients with stable asthma amounts that were inversely correlated with bronchial hyperresponsiveness. Recently, Vachier and colleagues (12) demonstrated an enhancement of ROS formation by polymorphonuclear neutrophils and monocytes in stable asthmatic patients. Thus, the potential benefits of GSH inhalation in patients with unstable asthma or during an acute severe exacerbation, to reinforce the antioxidant defenses, could enhance the efficacy of both bronchodilators and anti-inflammatory agents. However, many substances given by nebulization may affect the underlying bronchomotor tone, particularly in asthma. The major aim of this study was to assess the effects and the safety of nebulized GSH in patients with mild asthma.

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METHODS

Patients

We studied eight patients, five males and three females (age, 29 ± 7 [SD] yr), all nonsmokers who had mild asthma (FEV_1, 88 ± 11% predicted FEV_1/FVC, 77 ± 5% predicted [13]). The diagnosis of asthma was based on a history of recurrent episodes of wheezing, chest tightness, and reversible airway obstruction previously documented by a physician. No attempt was made to differentiate between atopic and nonatopic patients. Their asthma was clinically mild with PD_{20} (provocative dose of methacoline producing a 20% fall in FEV_1) values ranging from 0.07 to 0.78 μmol (0.32 ± 0.31 μmol), compared to normal values > 1.9 μmol. All patients controlled asthma symptoms by inhaling short-acting β-adrenergic agonists given as needed. In addition, two patients were taking budesonide; one, theophylline; and another, long-acting β-adrenergic agonist. These medications remained unchanged throughout the period of study. Short-acting β-adrenergics were withdrawn for 6 h before the study, while the rest of the medication was withheld for 12 h. No subject had a chronic illness other than asthma and none had had a chest infection or a major exposure to allergen within the previous 6 wk. All subjects volunteered for the study and gave informed consent in writing to participate. The protocol was approved by the Ethical Committee of Hospital Clínic, Universitat de Barcelona.

Glutathione Preparation

Glutathione solution (600 mg · $vial^{-1}$) was kindly provided by R. G. Crystal, M.D. (New York, NY), and reconstituted with 4 ml of 0.9% NaCl. We used this dosage based on previous studies (6) that showed an increased antioxidant effect on the airway fluid lining. Four milliliters of this solution were placed in the reservoir of a pneumatic nebulizer (Ultravent; Mallinckrodt, St. Louis, MO) that generates aerosol droplets appropriate for alveolar deposition. The size of aerosol droplets, determined by laser particle-size analysis, indicated a mass median aerodynamic diameter of 2.8 μm with a geometric SD of 1.3 μm. The nebulizer was driven at 40 psi (1 psi = 6.9 kPa) with compressed air to generate 10 L · min^{-1} of aerosol. Using a one-way valve, noseclip and mouthpiece in series, the system was closed; that is, all gas, including aerosolized GSH, was either inspired or expired through a filter to collect all expired drug. Using this system, aerosolization of 600 mg GSH to spontaneously breathing individuals with the nostrils occluded required 25 min. The pH and osmolarity of the GSH solution were 3.0 and 660 mosm · kg^{-1}, respectively. Osmolarity was measured with an osmometer (Advanced Micro-Osmometer model 3MO-Plus; Advanced Instruments, Inc., Norwood, MA). Using this delivery system, Holroyd and co-workers reported (7) that the percentage of reduced GSH in the preparation was above 97% and remained unchanged following aerosolization.

Design

All studies were designed in a randomized, double-blind, cross-over placebo-controlled manner. Airway response was measured by assessing the resistance of the respiratory system (Rrs) using the forced oscillation technique applied at the mouth at a frequency range of 6–10 Hz (14) and by forced spirometry (Datospir 92; Sibelmed, Barcelona, Spain). Three sequential studies were designed (a) to examine the effects of GSH on airway caliber, (b) to evaluate the influence of osmolarity and pH of GSH solution, and (c) to analyze the role of pretreatment with salbutamol in influencing GSH challenge. The subjects inhaled the solutions at approximately the same time of the day, 1 wk apart, to avoid circadian interference. Bronchodilators were administered, if required, after the challenge. Each study lasted approximately 3 mo and the time elapsed between each study was at least 2 mo.

In the first challenge, patients inhaled a GSH solution or placebo (0.9% NaCl; osmolarity, 309 mosm · kg^{-1}; pH, 6.0) to evaluate the effects of GSH on airway tone. Measurements of Rrs were done repetitively over a period of 20 min before and 25 min after completion of the challenge. Similarly, FEV_1 was measured before and 5 min after completion of GSH or placebo aerosolization. The final prechallenge Rrs value was the average of five individual measurements done at 4-min intervals. Because no differences were found in Rrs value after challenge throughout the period of study, the final value corresponded to the average of seven measurements done at approximately 4-min intervals. An identical procedure was used for the two other studies.

In the second challenge, a similar protocol was developed to evaluate the effects of osmolarity and pH of GSH solution. In this case, placebo solution was made by adding HCl to 2% NaCl to obtain a pH of 3.0. This solution had similar osmolarity (648 mosm · kg^{-1}) and identical pH (3.0) to that of the GSH solution.

In the third challenge, patients received salbutamol (5.0 mg) or placebo (0.9% NaCl) from a pneumatic nebulizer (Oximask; Proclinics, La Llagosta, Spain) over 20 min before the GSH challenge. After salbutamol or placebo administration, FEV_1 and Rrs (two measurements) were assessed.

Finally, because of the possibility of sulfite generation during GSH nebulization due to the sulfhydryl group of cysteine, one of the aminoacids of GSH, we carried out an additional challenge, namely a dose-response study to inhaled metabisulfite. Metabisulfite challenge is one of the most widely used approaches to measure the airway's response to inhaled sulfites. We used the method described by Nichol and colleagues (15). Thus, the provocative dose of metabisulfite required to cause a 20% fall in FEV_1 (PD_{20}) was established.

Data Analysis

Results are expressed as mean ± SD. Changes in FEV_1 and Rrs were analyzed using a two-way repeated measures analysis of variance (ANOVA). In the third challenge, *post hoc* comparisons were performed using paired *t* tests. The PD_{20} values of methacholine and metabisulfite challenges were log transformed for statistical correlations. Pearson's correlations were used when appropriate to assess relationships between variables. Statistical significance was set at $p < 0.05$.

RESULTS

Measurements performed at baseline of each challenge (means for 7 challenges: FEV_1, 3.45 ± 0.7 L, 92 ± 10% predicted; Rrs, 3.72 ± 1.16 cm H_2O · L^{-1} · s) were within normal limits (FEV_1) (13) or slightly increased (Rrs) (14) and failed to reach significant differences among them (FEV_1, $p = 0.07$; Rrs, $p = 0.41$).

Comparison between Glutathione and Placebo

All patients completed the study (Figure 1). However, in one patient (baseline FEV_1, 2.68 L, 90% predicted; FEV_1/FVC, 75% predicted), GSH provoked marked bronchoconstriction. The FEV_1 decreased by −1.91 L (−69% from baseline), Rrs increased by 5.06 cm H_2O · L^{-1} · s (+154% from baseline), and the patient had severe wheezing and breathlessness. The study was interrupted and nebulized salbutamol (5.0 mg) was immediately administered, producing rapid clinical improvement. This patient was excluded from the rest of studies. In the remaining seven patients, GSH induced cough in four and breathlessness in two. Inhaled salbutamol (400 μg) was given to these two patients at the end of the measurements with immediate full recovery. No symptoms were reported after the nebulization of placebo. The administration of placebo did not induce any variation in FEV_1 (change from baseline, −0.02 ± 0.18 L [−1%]) and caused a marginal increase in Rrs (change from baseline, +0.61 ± 0.37 cm H_2O · L^{-1} · s [+17%]). By contrast with placebo, GSH induced marked bronchoconstriction, as shown by a decrease in FEV_1 of −0.61 ± 0.72 L (−19% from baseline, $p = 0.03$) and an increase in Rrs of 2.16 ± 1.62 cm H_2O · L^{-1} · s (+61% from baseline, $p = 0.02$). For both measures, $n = 8$.

Comparision between Glutathione and Hypertonic Acid Solution

Glutathione provoked cough in four patients and breathlessness in two, who recovered completely with inhaled salbutamol; by contrast, the hypertonic acid solution (placebo) in-

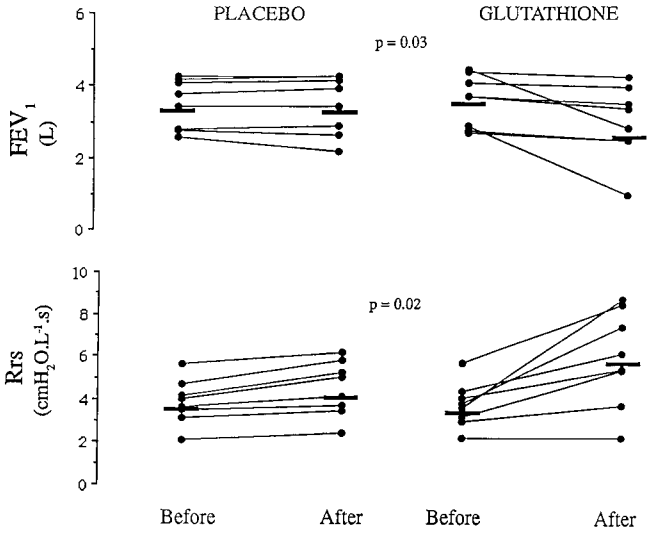

Figure 1. Individual responses of FEV_1 and Rrs to placebo or glutathione. Bars indicate mean values.

duced cough only in one patient. Compared with placebo, GSH challenge decreased FEV_1 by -0.40 ± 0.50 L (-11% from baseline, $p = 0.04$) and increased Rrs by $+1.95 \pm 1.25$ cm $H_2O \cdot L^{-1} \cdot s$ ($+51\%$ from baseline, $p = 0.02$), as shown in Figure 2. The nebulization of placebo altered both FEV_1 (change from baseline, $+0.13 \pm 0.09$ L [$+4\%$]) and Rrs (change from baseline, $+0.49 \pm 0.71$ cm $H_2O \cdot L^{-1} \cdot s$ [$+15\%$]), but less strikingly. Notably, GSH solution induced a similar degree of airway narrowing in this study and in the former one (Rrs, by $+51\%$ and $+48\%$, respectively; FEV_1, by -11% in both; $n = 7$, after excluding the patient who showed extreme bronchoconstriction in the first challenge).

Effect of Salbutamol on Glutathione Challenge

As expected, salbutamol inhalation produced mild bronchodilation (Figure 3). Compared with baseline, FEV_1 increased by $+0.29 \pm 0.23$ L ($+9\%$, $p = 0.02$), while Rrs decreased by -1.04 ± 0.79 cm $H_2O \cdot L^{-1} \cdot s$ (-25%, $p = 0.01$). By contrast, the nebulization of placebo produced no change in FEV_1 ($+0.09 \pm 0.16$ L [$+3\%$]), whereas Rrs decreased moderately -0.53 ± 0.51 cm $H_2O \cdot L^{-1} \cdot s$ [-12%], $p = 0.03$). The challenge with GSH after pretreatment with salbutamol still induced cough in two patients. However, compared with measurements performed after salbutamol, no significant change in FEV_1 ($p = 0.07$) nor in Rrs ($p = 0.21$) were shown after GSH challenge. By contrast, after pretreatment with saline placebo, GSH produced cough in three patients and mild breathlessness in one, inducing a significant increase in Rrs (change from placebo, $+0.89 \pm 0.78$ cm $H_2O \cdot L^{-1} \cdot s$ [$+24\%$]), $p = 0.02$), yet FEV_1 remained essentially stable (change from placebo, -0.07 ± 0.19 L [-2%]).

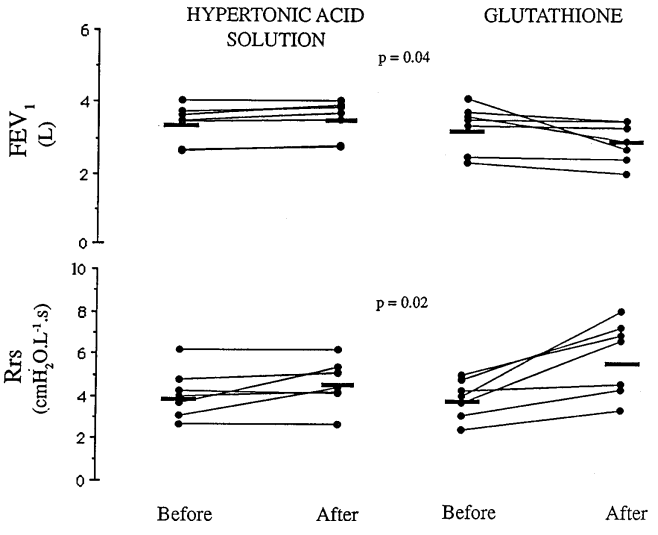

Figure 2. Individual responses of FEV_1 and Rrs to hypertonic acid solution or glutathione. Bars indicate mean values.

Metabisulfite Challenge

We performed this study in six patients, who showed a dose-dependent decrease in FEV_1 with inhaled doubling concentrations of metabisulfite with minor side effects (cough in four). The mean PD_{20} was 1.49 ± 1.83 μmol (range, 0.20–5.12 μmol), similar to other values shown previously (15, 16). A significant negative correlation was observed between airway hyperresponsiveness to metabisulfite and the GSH airway responses shown in the first two challenges (Figure 4) such that the greater the bronchoconstriction to inhaled GSH, the more intense the responsiveness to metabisulfite. By contrast, there was no correlation with methacholine bronchoprovocation.

DISCUSSION

The most novel finding of this study is that nebulized GSH at a dose of 600 mg had a bronchoconstrictor effect in this subset of patients with clinically stable mild asthma. This effect was repeatedly observed in the first three studies and was not related to osmolarity or pH of the GSH solution. Nebulized salbutamol prevented these deleterious functional effects.

It is notable that the GSH-induced airway changes in the third challenge were of lesser magnitude than in the other two, possibly because airway tone was conditioned by the previous nebulization with placebo, although changes in the degree of bronchial hyperresponsiveness by itself through the whole study also could play a role.

Glutathione has been administered safely at the same dose in patients with IPF and HIV-seropositive individuals, without adverse events. The former disorder is characterized by alveolar inflammation, increased release of oxidants, and decreased concentrations of the antioxidant GSH in respiratory ELF. Borok and colleagues (6) demonstrated that exogenous nebulized GSH in patients with IPF provoked an increase in total ELF GSH and oxidized GSH, with a decrease in spontaneous superoxide anion release by alveolar macrophages. Equally important, GSH is a metabolite or cofactor in several normal immune processes, so that a deficiency of reduced GSH can cause dysfunction of both lymphocytes and natural killer cells. As in patients with IPF, both systemic and ELF deficiency of GSH have been shown in HIV-seropositive patients. Because the lung is the most common site of infection in patients who progress to AIDS, it seems appropriate to suggest that increases of GSH levels in the ELF of these patients could improve local host defenses. Holroyd and colleagues (7) showed that nebulized GSH increased total GSH levels that remained within the normal range for at least 3 hr after treatment in HIV-seropositive patients. To our knowledge, however, GSH has never been administered to patients with bronchial asthma.

Several studies have evaluated the effects of acidity and/or high osmolarity of aerosol solutions in asthma and found that both cause cough and bronchoconstriction (17). Our results show that neither osmolarity nor pH account for the measured bronchoconstriction. Despite the fact that GSH solution is hypertonic

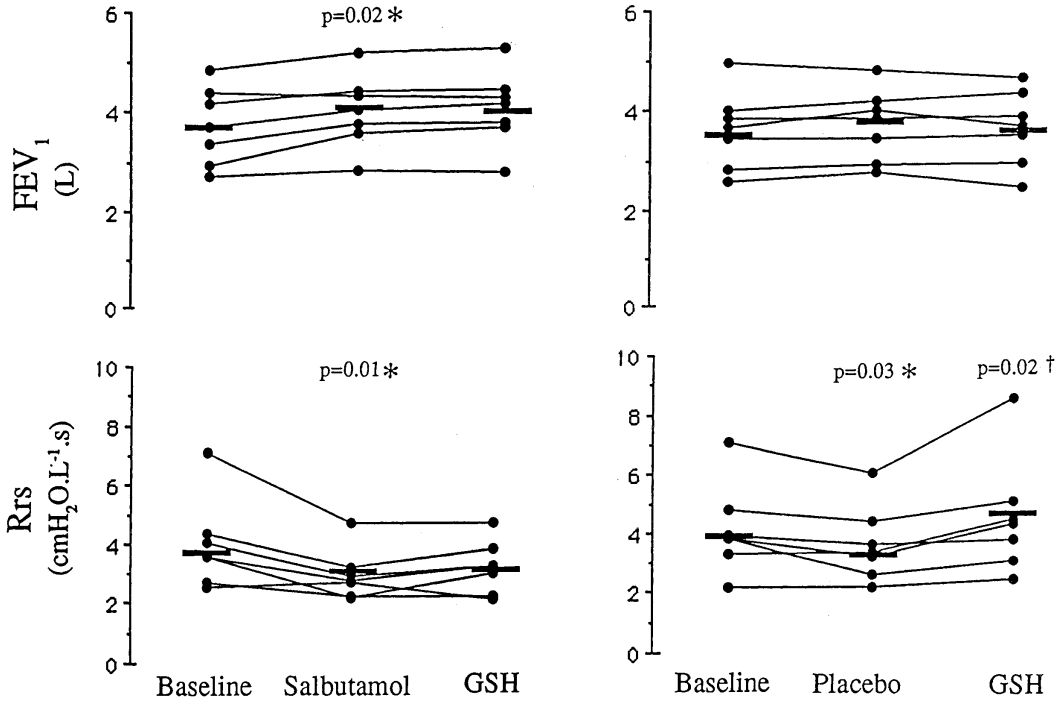

Figure 3. Individual responses of FEV_1 and Rrs to salbutamol or placebo and glutathione (GSH). *Bars* indicate mean values. *Comparing baseline with salbutamol or placebo. †Comparing GSH with placebo.

(660 mosm · kg^{-1}) and acidic (pH 3.0), the low volume of the solution given and the slow rate of administration (0.16 ml · min^{-1}) probably minimized the impact of these two factors. Anderson and Smith (17) recommended rates of aerosol delivery between 1.0 and 3.0 ml · min^{-1} and solutions with osmolarity up to 875 mosm · kg^{-1} for osmotic challenges in the assessment of bronchial hyperresponsiveness. The size of aerosol droplets generated by the pneumatic nebulizer used (2.8 μmol) was appropriate for alveolar deposition, with only 25% of the solution expected to be deposited in the tracheobronchial tree.

To explain the bronchoconstrictor effect of nebulized GSH in our patients, we suggest two potential hypotheses. The most salient could be a result of the fact that GSH is a highly hydrophilic substance containing cysteine, an aminoacid with a sulfhydryl group. When either sulfur species dissolves in aqueous solutions, a pH-dependent equilibrium is established among

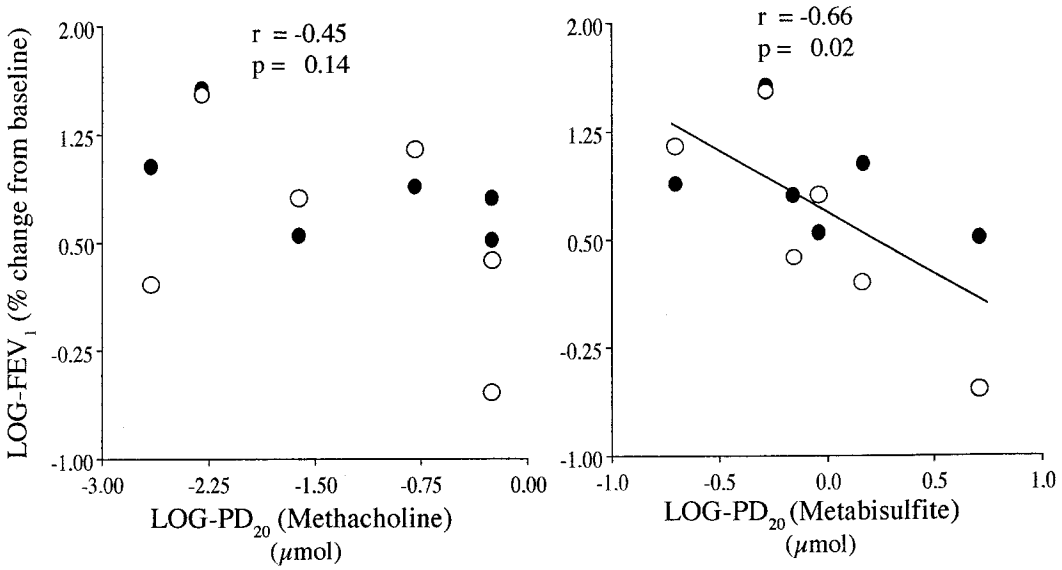

Figure 4. Individual plots of FEV_1 after GSH challenge (n = 6) against methacholine (*left panel*) and metabisulfite (*right panel*). Each patient is represented twice (*closed circles* = first challenge; *open circles* = second challenge). The close negative correlation shown between the responses to GSH and those to metabisulfite contrasts with the poor relationship shown after methacholine.

different sulfites (sulfur dioxide [SO_2], metabisulfite [$SO_5^=$], bisulfite [SO_3^-], sulfite [$SO_3^=$]) (18). These sulfites produce the characteristic "rotten eggs" smell of GSH solution, and their acute bronchoconstrictor effects are well established. In healthy subjects, however, this effect was significant only after inhalation of concentrations in excess of 5 parts per million, or ppm (19). Several studies have shown that patients with asthma are exquisitely sensitive to the bronchomotor effects of sulfites (concentrations below 1 ppm). The precise mechanism of SO_2-induced bronchoconstriction remains elusive, and both cholinergic and noncholinergic mechanisms have been implicated (20, 21). Recently, a sensory nerve activation with tachykinins also has been invoked (22). It has been suggested that when sulfites are inhaled from a mouthpiece, as in our study, their bronchoconstrictor effects could increase (23). Although we did not measure the levels of sulfites during GSH nebulization, it is likely that they could be implicated in the induced bronchoconstriction. The finding that all the patients tested showed a significant bronchoconstrictive response to metabisulfite challenge, correlated inversely with the threshold of responsiveness to GSH, lends further support to the mechanism of bronchoconstriction induced by sulfite formation. The lack of adverse reaction to GSH nebulization in either IPF patients or HIV-seropositive individuals could be related to the different bronchoconstrictor sensitivity to sulfites of these populations.

A complementary explanation of GSH-induced bronchoconstriction may be that GSH is a versatile molecule that plays a key role in multiple metabolic pathways, one of them being the airway inflammatory response. Glutathione is involved in the metabolism, through conjugation, of leukotriene A_4, which results in the formation of leukotriene C_4 (LTC_4), which can be converted to leukotriene D_4 (LTD_4) (24, 25). Both LTC_4 and LTD_4 are well-known potent bronchoconstrictors with proinflammatory effects, such as increasing vascular permeability and microvascular leakage, and both have been invoked in the pathogenesis of bronchial asthma (26). The exogenous administration of GSH could provoke an imbalance in the components of ELF in asymptomatic asthmatic patients, thereby triggering an acute inflammatory reaction similar to that shown during an asthma attack.

The results of the third challenge could support, in part, these two contentions. On the one hand, β-adrenergic agonist agents can prevent sulfite-induced bronchoconstriction (19); on the other, we recently demonstrated an antiedema effect of salbutamol after platelet-activating factor (PAF) challenge in both healthy (27, 28) and asthmatic individuals (29), possibly by preferentially preventing airway microvascular leakage.

In conclusion, we have shown that nebulized GSH is deleterious to patients with stable mild asthma because it induces clinical symptoms, essentially cough, breathlessness, and marked functional bronchoconstriction that was prevented with nebulized salbutamol. Inhalation of sulfites that come from GSH solution could be involved in these effects. Our results should be considered if future therapeutic strategies with antioxidant preparations for patients with asthma or other chronic obstructive disorders are planned.

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ORIGINAL RESEARCH

Glutathione and its antiaging and antimelanogenic effects

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Background: Previous studies showed that supplementation of reduced form of glutathione (GSH, 500 mg/d) has a skin-lightening efficacy in humans. This study was designed to evaluate the influences of both GSH and oxidized form (GSSG), at doses lower than 500 mg/d, on improving skin properties.

Patients and methods: A randomized, double-blind, placebo-controlled, parallel, three-arm study was conducted. Healthy female subjects were equally randomized into three groups and took GSH (250 mg/d), GSSG (250 mg/d), or placebo orally for 12 weeks. At each visit at baseline and for 12 weeks, skin features including melanin index, wrinkles, and other relevant biophysical properties were measured. Blood samples were collected for safety monitoring.

Results: In generalized estimating equation analyses, melanin index and ultraviolet spots of all sites including face and arm when given GSH and GSSG tended to be lower than placebo. At some sites evaluated, subjects who received GSH showed a significant reduction in wrinkles compared with those taking placebo. A tendency toward increased skin elasticity was observed in GSH and GSSG compared with placebo. There were no serious adverse effects throughout the study.

Conclusion: We showed that oral glutathione, 250 mg/d, in both reduced and oxidized forms effectively influences skin properties. Overall, glutathione in both forms are well tolerated.

Keywords: glutathione, melanin, pigment, aging, wrinkle, whitening

Introduction

The quest for means to alter skin color is endless. Caucasians seek ways to tan their skin, while many darker skin-type individuals are always in search of whitening or lightening agents.

Numerous topical agents available for melasma treatment are also used to lighten the skin color. However, as many people would prefer their skin to be thoroughly fairer, oral or even intravenous agents are administered to obtain these results. One of the widely used, systemic agents is glutathione, a thiol compound and one of the regulators of melanogenic pathway in the human system.

Glutathione is an antioxidant present in almost every cell in the body, playing a role in the detoxification of drugs and xenobiotics.[1] Furthermore, reduced glutathione (GSH) acts as a hydrogen donor in the detoxification of hydrogen peroxide.[2] As a dietary supplement, GSH possesses various systemic effects such as improvement of liver abnormalities,[3,4] improvement of diabetic complication,[5] protection from viral infection,[6] and antitumor activity.[7,8] It is even used to treat autism.[9]

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In vitro experiments have demonstrated that glutathione is related to melanogenesis.[10–13] Its antimelanogenic properties result from a variety of mechanisms including stimulation of pheomelanin synthesis rather than darker eumelanin, its antioxidant effects,[14] and interference with intracellular trafficking of melanogenic enzymes.[15] Glutathione also possesses certain antiaging properties.[16]

Glutathione is generally a safe ingredient for use as a dietary supplement. An oral acute toxicity study of GSH in mice found that the lethal dose 50 (LD50) was more than 5 g/kg, indicating that glutathione is nontoxic. In many clinical trials, no serious adverse reactions have been observed.[9,17–19] On the contrary, it can even reverse the toxic effects following excessive intake of other amino acids.[20]

In the human body, glutathione exists in two forms, reduced and oxidized (GSSG), which can be readily converted to each other. However, it is not clear whether the two forms are physiologically similar, especially when melanogenesis is concerned. Moreover, efficacy and long-term safety of either form have not been examined systematically.

Glutathione is regarded as food or health supplements in several countries including the Philippines, Malaysia, Taiwan, and Thailand, while it is considered a pharmaceutical agent in Korea, Japan, and People's Republic of China. Our group previously reported that oral GSH administration (500 mg/d) resulted in lightening of skin color, when given for 4 weeks.[21] The main objective of this study was to find out whether glutathione, in the reduced and oxidized forms, maintains its skin-lightening efficacy when given at a dose of 250 mg/d for 12 weeks, a dosage allowed by the Thai and Taiwanese Food and Drug Administrations.

Patients and methods

Study design

The study protocol was conducted in accordance with the Declaration of Helsinki, in compliance with the International Conference on Harmonization - Good Clinical Practice and reviewed and approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University. Clinical study was conducted at Chula Clinical Research Center, Faculty of Medicine, Chulalongkorn University. A randomized, double-blind, placebo-controlled, parallel, three-arm study was applied. The subjects were equally block-randomized into three groups: GSSG (250 mg/d), GSH (250 mg/d), or placebo. Each subject received their assigned capsules in identical packages at weeks 0, 4, and 8, with 30 capsules per visit (two extra capsules per package). The subjects were informed to take the study capsules once before bedtime. Subjects returned for evaluations at weeks 4, 8, and 12.

Subjects

Sixty healthy volunteers, aged between 20 and 50 years, were eligible for this study. They were residents of Bangkok and recruited through the dermatology clinic at King Chulalongkorn Memorial Hospital. Only female volunteers were enrolled to reduce gender variability and also because females are by far the majority of individuals who seek skin-whitening agents. All volunteers were found to be healthy based on their medical history and physical and clinical laboratory examination including serology, hematology, and biochemistry tests. All volunteers had to abstain from other medications, supplementary vitamins, and alcohol intake for 2 weeks prior to enrollment and throughout the study. The methods and conditions of the study were clearly explained to all volunteers. Signed written informed consent was obtained from each volunteer before screening processes for this study. However, each subject had the right to withdraw their consent at any time.

Study medications

Daily doses of GSSG (AquaGluta™; 250 mg/d), GSH (Setria®; 250 mg/d), or dibasic calcium phosphate as placebo in identical capsules and packages were provided by Kyowa Hakko Bio Co., Ltd (Tokyo, Japan). The total weight of the three capsules for each group was the same.

Objective evaluation of skin properties

At each visit, subjects rested in a room controlled at a temperature of 21 ± 3°C for 20 minutes before assessments. Prior to the evaluation, they washed their face with soap and water, pat-dried with paper towel, and waited 5–10 minutes for air drying.

Melanin index

For objective evaluation of skin color, melanin index as determined by Mexameter (Courage-Khazaka Electronic, Koln, Germany) was used as the primary outcome. All measurements were done in triplicate at six sites to represent the skin of the sun-exposed and sun-protected areas as follows:

1. Sun-exposed areas
   - Face: left and right; 2.5 cm caudally from the lateral canthi.
   - Extensor surfaces of the forearms, left, and right; 7 cm above the ulnar styloid processes.
2. Sun-protected areas
   - Upper, inner arms, left, and right; 10 cm from the axillary vault.

VISIA™ CR system

Standardized digital photographs were taken by the VISIA™ CR system (Canfield Scientific, Fairfield, NJ, USA), a software which was also used to quantitatively evaluate ultraviolet (UV) spots, pores, and evenness on the left and right sides of the face.

Transepidermal water loss (TEWL)

TEWL was measured using Tewameter® TM300 (Courage+Khazaka Electronic GmbH, Köln, Germany). All measurements were done in triplicate at sites designated for melanin index to represent the skin of the sun-exposed and sun-protected areas.

Water contents (Corneometer)

Water contents were measured by Corneometer® CM825 (Courage+Khazaka) in triplicate at sites as mentioned earlier.

Elasticity

Elasticity was measured by Cutometer MPA580® (Courage+Khazaka) in triplicate at the sites mentioned previously.

Wrinkle

Wrinkle formation was objectively measured by Visioscan® (Courage+Khazaka).

Subjective evaluation of skin properties

For global evaluation, at each visit, subjects were asked to grade the overall response using a 4-point rating scale: 4 = very satisfactory, 3 = moderately satisfactory, 2 = minimally satisfactory, and 1 = not satisfactory. In the questionnaires, the following features/items were addressed: skin lightening, facial skin evenness, pigmented spots lightening, pore size improvement, skin smoothness, wrinkle reduction – crow's feet, wrinkle reduction – nasolabial folds, wrinkle reduction – forehead, general skin condition, fatigability, sleep (e.g., quality and length)

Safety

To evaluate the safety of subjects, vital signs were determined at each visit. Also, at all visits, a blood aliquot of 7 mL was drawn for complete blood cell counts, chemistry, and lactate dehydrogenase.

Statistical analysis

Paired *t*-test was used to compare baseline values with those of the final visit. The generalized estimating equation (GEE) was performed to investigate how the efficacies of GSSG, GSH and placebo varied over time before and after treatment. Subjective evaluations of the two glutathione preparations and placebo were compared by analysis of covariance (ANCOVA), with the baseline values as covariates. Statistically significant level was defined as *P*-value <0.05 (two-tailed). Analyses were performed using STATA software version 11.0 (StataCorp, College Station, TX, USA).

Results

Sixty volunteers were enrolled in the study. Three volunteers had to terminate, two due to elevation in liver function tests and one due to unanticipated start of oral contraceptive pills. Fifty-seven volunteers were included for final analysis. The majority of subjects had skin phototype IV (96.4%). Subjects' demographic data are summarized in Table 1.

A total of 18 subjects received GSSG, 20 received GSH, and 19 received placebo. Mean baseline measurements (Mexameter, VISIA, Tewameter, Corneometer, Cutometer, Visioscan) in the three groups at six sites were not significantly different (ANCOVA, $P > 0.05$).

The GEE was performed to investigate how the efficacies of GSSG, GSH, and placebo varied over time during treatment (Table 2). As our subjects were recruited from a broad age range, we also decided to categorize the age groups into those younger and older than 40 years.

Table 1 Baseline demographics

Characteristics	Number (%) of patients
Age distribution (year)	
20–30	17 (29.8%)
31–40	20 (35.1%)
41–50	20 (35.1%)
Age (year)	
Mean ± SD	36.1 ± 8.1
Median	36
Min/max	21/48
Hair color	
Black	48 (84.2%)
Brown	9 (15.8%)
Skin phototype	
III	1 (1.8%)
IV	55 (96.4%)
V	1 (1.8%)
Homogeneity of facial skin color	
Homogenous	26 (45.6%)
Not homogenous	31 (54.4%)
Treatment	
GSSG	18 (31.6%)
GSH	20 (35.1%)
Placebo	19 (33.3%)

Abbreviations: SD, standard deviation; GSSG, oxidized glutathione; GSH, reduced glutathione.

Table 2 GEE analysis

Variables	Placebo vs. GSSG		Placebo vs. GSH	
	Mean difference	*P*-value	Mean difference	*P*-value
Mexameter				
Sun-exposed face, right	18.4241	N.S.	23.7472	N.S.
Sun-exposed face, left	18.9250	N.S.	18.8436	N.S.
Sun-exposed arm, right	15.7356	N.S.	37.0054	0.072
Sun-exposed arm, left	8.4700	N.S.	31.9076	0.091
Sun-protected arm, right	9.4779	N.S.	18.8066	N.S.
Sun-protected arm, left	7.4993	N.S.	15.1403	N.S.
Mexameter (age >40 years)				
Sun-exposed face, right	−10.6149	N.S.	−18.8602	N.S.
Sun-exposed face, left	−9.4868	N.S.	−23.2070	N.S.
Sun-exposed arm, right	−38.3149	N.S.	60.4821	0.031*
Sun-exposed arm, left	−43.4550	N.S.	53.7161	0.057
Sun-protected arm, right	−18.9622	N.S.	31.2671	0.078
Sun-protected arm, left	−25.8103	N.S.	17.7876	N.S.
VISIA				
Sun-exposed face, right	23.1060	N.S.	5.2296	N.S.
Sun-exposed face, left	21.1206	N.S.	7.9414	N.S.
VISIA (age >40 years)				
Sun-exposed face, right	−0.8889	N.S.	−6.8889	N.S.
Sun-exposed face, left	−18.5556	N.S.	−10.0198	N.S.
Tewameter				
Sun-exposed face, right	0.2515	N.S.	−0.3893	N.S.
Sun-exposed face, left	−0.0641	N.S.	−0.0011	N.S.
Sun-exposed arm, right	−0.5992	N.S.	0.0108	N.S.
Sun-exposed arm, left	−0.4294	N.S.	−0.1032	N.S.
Sun-protected arm, right	−0.6957	N.S.	−0.3497	N.S.
Sun-protected arm, left	−0.1941	N.S.	0.1534	N.S.
Tewameter (age >40 years)				
Sun-exposed face, right	−0.8032	N.S.	−0.5919	N.S.
Sun-exposed face, left	−1.4715	N.S.	−0.3575	N.S.
Sun-exposed arm, right	−0.2515	N.S.	−0.0403	N.S.
Sun-exposed arm, left	−0.0561	N.S.	−0.4224	N.S.
Sun-protected arm, right	−0.1284	N.S.	−0.3981	N.S.
Sun-protected arm, left	0.5856	N.S.	−0.0566	N.S.
Corneometer				
Sun-exposed face, right	0.3321	N.S.	3.6350	N.S.
Sun-exposed face, left	−0.2904	N.S.	2.4277	N.S.
Sun-exposed arm, right	3.0026	N.S.	3.9812	N.S.
Sun-exposed arm, left	2.8878	N.S.	3.0428	N.S.
Sun-protected arm, right	1.2492	N.S.	2.1739	N.S.
Sun-protected arm, left	1.3142	N.S.	3.8116	0.031*
Corneometer (age >40 years)				
Sun-exposed face, right	5.4455	N.S.	9.9433	0.048*
Sun-exposed face, left	4.6751	N.S.	7.9991	0.090
Sun-exposed arm, right	6.0388	N.S.	1.5504	N.S.
Sun-exposed arm, left	5.6142	N.S.	0.4824	N.S.
Sun-protected arm, right	3.5240	N.S.	0.1463	N.S.
Sun-protected arm, left	5.4652	0.052	0.1840	N.S.
Cutometer				
Sun-exposed face, right	−0.0372	0.082	0.0036	N.S.
Sun-exposed face, left	−0.0283	N.S.	−0.0116	N.S.
Sun-exposed arm, right	−0.0031	N.S.	−0.0088	N.S.
Sun-exposed arm, left	0.0001	N.S.	−0.0029	N.S.
Sun-protected arm, right	−0.0006	N.S.	−0.0052	N.S.
Sun-protected arm, left	0.0007	N.S.	−0.0010	N.S.
Cutometer (age >40 years)				
Sun-exposed face, right	−0.0395	N.S.	0.0089	N.S.
Sun-exposed face, left	0.0288	N.S.	−0.0052	N.S.
Sun-exposed arm, right	−0.0060	N.S.	−0.0008	N.S.
Sun-exposed arm, left	−0.0095	N.S.	−0.0096	N.S.
Sun-protected arm, right	−0.0097	N.S.	−0.0105	N.S.
Sun-protected arm, left	−0.0086	N.S.	−0.0029	N.S.
VISIO				
Sun-exposed face, right	−0.0542	N.S.	0.4993	N.S.
Sun-exposed face, left	0.0601	N.S.	1.1137	N.S.
Sun-exposed arm, right	0.5420	N.S.	0.7263	N.S.
Sun-exposed arm, left	0.7505	N.S.	0.8105	N.S.
Sun-protected arm, right	0.6234	N.S.	0.2277	N.S.
Sun-protected arm, left	0.7887	N.S.	2.3124	0.006*

Table 2 (*Continued*)

Variables	Placebo vs. GSSG		Placebo vs. GSH	
	Mean difference	*P*-value	Mean difference	*P*-value
VISIO (age >40 years)				
Sun-exposed face, right	−0.8735	N.S.	0.5056	N.S.
Sun-exposed face, left	0.1897	N.S.	1.3635	N.S.
Sun-exposed arm, right	−0.1998	N.S.	1.3337	N.S.
Sun-exposed arm, left	0.4910	N.S.	2.1052	0.043*
Sun-protected arm, right	1.6827	N.S.	0.8387	N.S.
Sun-protected arm, left	0.2899	N.S.	2.5568	0.066

Note: *$P<0.05$.
Abbreviations: GEE, generalized estimating equation; N.S., not significant; GSSG, oxidized glutathione; GSH, reduced glutathione.

Melanin index

In all subjects with an age range between 20 and 50 years, GEE model showed that melanin index and UV spots of all sites including face and arm from GSSG and GSH groups tended to be lower than placebo group (Table 2) but were not statistically significant ($P > 0.05$). There were no significant differences between GSSG and GSH groups. The subgroup analysis of middle-aged individuals showed that the melanin index of sun-exposed right forearm of subjects aged >40 years who received GSH (N = 7) was significantly lower than the index of those who received placebo (N = 10, $P = 0.031$). Melanin index measured at the sun-exposed left forearm from those receiving GSH was also lower than those receiving placebo. However, this did not reach statistically significant level ($P = 0.057$).

TEWL and water content

TEWL measurement of sun-exposed right forearm of the GSH group was significantly lower than that of GSSG group ($P = 0.044$). However, the water contents of sun-protected left arm of those who received GSH were lower than that of those who received placebo ($P = 0.031$). This was also true for subjects aged >40 years, for the sun-exposed right face ($P = 0.048$).

Wrinkles

Visioscan measurements of sun-protected left arm of subjects in the GSH group were significantly lower than those of the placebo group ($P = 0.006$). This was also true for those aged >40 years when measurements were taken at the sun-exposed left forearm ($P = 0.043$). A similar trend was seen for GSH vs. placebo for the sun-protected left arm, in advanced age group ($P = 0.066$).

Elasticity

Although statistically significant differences could not be demonstrated, GSSG and GSH supplementation tended to increase skin elasticity. Especially, the elasticity of sun-exposed right face of those who received GSSG was notably higher than the elasticity of those who received placebo ($P = 0.082$).

Subjects were asked to fill the questionnaires to subjectively evaluate skin properties at each visit. Satisfaction was scored as rating scale. There were no statistically significant differences in any of the ratings among the three groups ($P > 0.05$, ANCOVA).

Compliance

Compliance was not an issue in the present study. All subjects took the capsules as directed and assessed by the protocol throughout the study.

Adverse events

Adverse reactions included pruritus, macular erythema, transient minute red spots on the skin, and tiredness. In the treatment groups combined (GSSG, GSH), these occurred in five patients (13.15%), which included three with pruritus (7.89%), one with erythema (2.63%), three with red spots (7.89%), and one with tiredness (2.63%). In the placebo group, there were two incidents of pruritus (10.52%), one erythema (5.26%), and three red spots (15.79%) (Table 3). No serious adverse events took place. The two incidents of transaminitis were temporary and the liver function tests returned to normal within a few weeks. Detailed blood parameters are described in Table 4.

Discussion

Our group has previously demonstrated that oral glutathione, 500 mg/d, can reduce skin pigmentation after 4 weeks'

Table 3 Adverse events

Treatment	n	AE (%)	Pruritus (%)	Erythema (%)	Red spot (%)	Tiredness (%)
GSSG	18	2 (11.11)	1 (5.56)	0	2 (11.11)	0
GSH	20	3 (15.0)	2 (10.0)	1 (5.0)	1 (5.0)	1 (5.0)
Placebo	19	4 (21.05)	2 (10.53)	1 (5.26)	3 (15.79)	0
Total	57	9 (15.79)	5 (8.77)	2 (3.51)	6 (10.53)	1 (1.75)

Abbreviations: AE, adverse event; GSSG, oxidized glutathione; GSH, reduced glutathione.

Table 4 Blood parameters

Laboratory parameters		Baseline	EOT
WBC	Missing – n (%)	0	0
	Mean ± standard error	6.5 ± 1.5	6.4 ± 1.5
RBC	Missing – n (%)	0	0
	Mean ± standard error	5.0 ± 1.4	4.6 ± 0.4
Hb	Missing – n (%)	0	0
	Mean ± standard error	13.3 ± 2.2	12.6 ± 1.2
Platelets	Missing – n (%)	0	0
	Mean ± standard error	283.8 ± 66.2	280.4 ± 66.7
AST	Missing – n (%)	0	0
	Mean ± standard error	17.7 ± 7.5	18.1 ± 7.6
ALT	Missing – n (%)	0	0
	Mean ± standard error	16.6 ± 11.6	16.3 ± 11.1
GGT	Missing – n (%)	0	0
	Mean ± standard error	19.5 ± 11.0	21.9 ± 14.2
TP	Missing – n (%)	0	0
	Mean ± standard error	1.3 ± 0.4	1.4± 0.4
TC	Missing – n (%)	0	0
	Mean ± standard error	206.0 ± 35.2	204.1 ± 32.2
TG	Missing – n (%)	0	0
	Mean ± standard error	88.8 ± 41.5	87.7 ± 40.5
LDH	Missing – n (%)	0	0
	Mean ± standard error	154.0 ± 26.3	158.3 ± 23.2
BUN	Missing – n (%)	0	0
	Mean ± standard error	11.1 ± 2.7	10.5 ± 2.8
Cr	Missing – n (%)	0	0
	Mean ± standard error	0.8 ± 0.1	0.8 ± 0.1

Abbreviations: EOT, end of treatment; WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; AST, aspartate transaminase; ALT, alanine transaminase; GGT, gamma-glutamyl transferase; TP, total protein; TC, total cholesterol; TG, triglyceride; LDH, lactate dehydrogenase; BUN, blood urea nitrogen; Cr, creatinine.

administration in young, otherwise-healthy medical students.[21] Watanabe et al also demonstrated that topically applied GSSG can significantly reduce melanin indices.[22] Recently, Handog et al investigated the use of intraoral lozenge containing 500 mg of glutathione in an open-label study and demonstrated significant skin lightening after 8 weeks of administration.[23]

It is well established that glutathione can be transported across the intestinal epithelium after oral supplementation,[9,19] yet the fate of orally administered GSH is to be resolved as it is readily oxidized within the human body. On the contrary, its oxidized counterpart is much more stable. Until very recently, most studies were not able to detect blood or plasma glutathione, despite large doses of oral intake.[17] However, Park et al demonstrated that although no glutathione could be measured in the whole plasma compartment, GSH could be detected in the protein-bound fraction of the human blood between 60 and 120 minutes after oral intake.[24]

In this study, we have demonstrated that both GSSG and GSH exerted their effects on melanin indices, which reached statistically significant levels at specific site and higher age group. This is in agreement, yet to some degree dissimilar, to our earlier study, the explanations for which are several-fold. First, the dose of glutathione used in this study is half of that used in the prior study. This is to comply with the daily dosage of L-glutathione allowed in some countries including Thailand. Second, the subjects recruited in this study are all females and of more advanced age. Interestingly, with subgroup analysis, the changes in subjects aged more than 40 were even more pronounced than when the whole group was analyzed. Being affected with more photodamage can definitely affect the final outcomes measured, especially when pigment is concerned.

Our results also showed that GSH was significantly superior to placebo in its ability to improve wrinkles, at least at some anatomic locations. This is an extremely interesting and novel finding as cutaneous aging is a significant problem faced by the majority of people of any age. As the world's populations are rapidly heading toward an aging society and human life spans are increasing, this problem will certainly be of greater magnitude in the foreseeable future. Having to apply topical antiwrinkle preparation to the entire skin is both costly and in many circumstances impractical for the elderly, especially when compared with popping a pill.

Also of importance are the findings that both forms of glutathione showed trends in increased skin elasticity at various sites, both sun-exposed and sun-protected skin. These findings have never been reported before and deserve further investigations in larger populations.

Because glutathione has regulatory properties on melanogenesis and antioxidants in general are protective against aging process, the "dual" antimelanogenic and antiaging properties demonstrated in Watanabe's and our studies are not surprising. In fact, the link between melanization and aging has been studied in animal models.[25]

The strengths of our study are, first, objective and well-standardized measurements. Second, a randomized, double-blind study that analyzes the effect of glutathione in both reduced and oxidized forms in comparison with placebo has never been conducted. Limitations are that our subjects are all female, Asian, and of certain age range.

Overall, glutathione in both forms are well tolerated. No major adverse events took place during the study period. Increases in transaminases occurred in two subjects highlighting the fact that blood chemistry should be performed even when individuals are taking over-the-counter supplements. Nonetheless, these adverse events were transient and the blood parameters promptly returned to their normal values upon cessation of consumption.

Conclusion

In summary, we have shown that oral glutathione, 250 mg/d, in both reduced and oxidized forms have various beneficial effects on skin properties and is possibly an antiaging agent, at least in middle-aged female subjects. Further studies in larger and more diverse populations are warranted.

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Disclosure

The authors report no conflicts of interest in this work.

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Pharmacokinetics of Glutathione and Its Metabolites in Normal Subjects

To determine the loading and maintenance dosage of glutathione (GSH) for patients suffering from reactive oxygen species (ROS) injury such as acute paraquat intoxication, a kinetic study of reduced GSH was performed in synchrony with that of cysteine (Cys), cystine (Cys2), and methionine (Met). Human subject's porticipitation was voluntary. The effective dose of Cys, Cys2, and Met against ROS in fibroblast cells generated by paraquat was assessed using laser scanning confocal microscopy. Both Cys and Met suppressed ROS in a dose-dependent manner at concentrations of 1-1,000 μM; the concentration required to suppress ROS by 50% was 10 μM for Cys and 50 μM for Met. Using metabolite kinetics with the assumption that Cys and Met are the metabolites of GSH, expected concentrations of Cys and Met of above 20 and 50 μM were estimated when GSH was administered at 50 mg/kg body weights every 205.4 min for Cys and 427.4 min for Met.

Key Words : *Cysteine; Cystine; Glutathione; Methionine; Paraquat; Reactive Oxygen Species; Pharmacokinetics*

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INTRODUCTION

Accidental ingestion of paraquat is frequently fatal within a few days due to multiple-organ failure mediated by reactive oxygen species (ROS) (1). Over the past 30 yr, several methods for modifying the toxicity of paraquat have been examined: a) prevention of absorption by the gastrointestinal tract (2, 3), b) removal from the bloodstream (1, 4), c) prevention of accumulation in the lungs (5, 6), d) scavenging oxygen free radicals (7), and e) prevention of lung fibrosis (8, 9). Unfortunately, most of these methods have not proven effective, with the outcome already determined by the degree of exposure to paraquat.

Several sulfur-containing compounds have been examined as antioxidants in paraquat-induced lung injury due to their inherent antioxidant properties and an early observation that depletion of reduced glutathione (GSH) enhanced paraquat toxicity (10). Even though some studies have shown that alveolar type II cells can supplement endogenous synthesis of GSH with the uptake of exogenous GSH (11, 12), the antioxidant effectiveness of exogenously administered GSH is hindered by its instability when crossing cell membranes and its rapid hydrolysis in the circulation (13-15).

In circulation, GSH is degraded rapidly by gamma-glutamyltranspeptidase, an enzyme found on the extracellular surfaces of cells, yielding glutamate (Glu), cysteine (Cys), and glycine (Gly) (16). In some cells, degradation of GSH at the cell surface directly provides the cells with Cys required for GSH synthesis (17). Although Cys is a critical amino acid for the synthesis of GSH, it is sufficiently reactive in circulation for large amounts of Cys to be oxidized immediately to cystine (Cys2).

Recently we found that extracellular methionine (Met) is as strong an antioxidant as Cys against the intracellular ROS produced by paraquat. In man, this essential sulfur-containing amino acid is metabolized in the trans-sulfuration pathway (18). It is successively converted to S-adenosyl-methionine, S-adenosyl-homocysteine, and homocysteine. Homocysteine lies at a branch point from which sulfur metabolism can be controlled, either it can be remethylated to Met or converted to Cys via cystathione. The remethylation of homocysteine is catalyzed by betaine-homocysteine methyltransferase and 5-methyltetrahydro-folate-homocysteine methyltransferase. In this setting, Cys2 has been shown to increase the activity of betaine-homocysteine methyltransferase (19-21). In this regard, one of the potential functions of extracellular GSH is a source of both Cys and Met in the overall sulfur-containing amino acid balance. Aebi et al. (22) reported

that Cys in plasma increased after the GSH infusion, but the plasma concentration of Cys plus Cys2 decreases.

Taking together the antioxidant effect of both Cys and Met, and the metabolic interrelationships between Met, Cys, and Cys2, we hypothesized that it would be more accurate to estimate the antioxidant effect of GSH based on the kinetics of each sulfur-containing amino acid. In order to prove this hypothesis, we observed the change of sulfur-containing amino acids concentration in blood, synchronized with the changes in GSH after the intravenous administration of GSH.

MATERIALS AND METHODS

All of the reagents were obtained from Sigma (St. Louis, MO, U.S.A.) except where otherwise stated. This experimental study was approved by the Investigational Review Board at Soonchunhyang University Cheonan Hospital (Cheonan, Korea), and all human subjects provided written informed consent, and their participation was voluntary. Subjects ate a regular diet without alcohol and did not have any kinds of drugs including vitamins, for more than 3 days before the study.

Cell culture

Swiss 3T3 fibroblasts, obtained from American Type Culture Collection (ATCC CCL 92), were maintained at 37˚C in Dulbecco's modified Eagle's medium supplemented with 25 mM HEPES (pH 7.4), 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin. For experiments, cells were cultured on round coverslips in 12-well plates and then stabilized for 30 min with Dulbecco's modified Eagle's medium supplemented with 5 mg/mL apotransferrin, 1 mg/mL bovine serum albumin, 25 mM HEPES (pH 7.4), 2 mM glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin (serum-free medium).

Measurement of intracellular ROS

The amount of intracellular ROS was measured as described in Koo et al. (23). Cultured cells on round coverslips were stabilized in serum-free medium without phenol red for at least 30 min, and then stimulated with paraquat for varying durations.

ROS generation in cells was assessed using the probe 2,7-dichlorofluorescein (DCF) (Molecular Probes, Eugene, OR, U.S.A.). Some of the cells were treated with various concentrations of antioxidants (GSH, Cys, Cys2, and Met) for 30 min prior to imaging. For the last 5 min of stimulation, the membrane-permeable diacetate form of the DCF: was added to the perfusate at a final concentration of 5 μM. Esterases within the cell cleave the acetate groups on DCF-diacetate, thus trapping the reduced probe intracellularly. Intensity values (confocal laser scanning microscope, LSM 510, Carl Zeiss, Germany) are reported relative to initial values after subtracting the background. In preliminary experiments, paraquat at 50-500 μM produced ROS in Swiss 3T3 fibroblasts in a dose-dependent manner at 30-60 min. The cells detached within 30 min when the paraquat concentration was over 600 μM, or when the incubation time was over 60 min at lower concentrations. Therefore, we selected the optimal condition for our experiments to be 40 min of incubation with 500 μM paraquat.

Blood sampling for GSH and amino acids in volunteers

GSH (50 mg per kg of body weight, L-glutathione, reduced; Dong-A Pharmaceutical, Seoul, Korea) was infused into an antecubital vein over 10 min. Blood samples started from 6 o'clock in the morning after 12 hr overnight fasting through an indwelling intravenous catheter placed on other side of cubital vein, just before GSH administration (for basal level at time zero) and at 10, 20, 30, 60, 90, 120 and 240 min after GSH administration in seven male volunteers (medical students, aged 22 or 23 yr; body weight 65.5 ± 4.5 kg). During the sampling, only water drinking was permitted. No side effect has observed after glutathione infusion.

Basal blood cell count, urinalysis and blood chemistry including BUN, creatinine, liver function test, and fasting blood sugar were normal in the all subjects. They ate a regular diet without alcohol and did not take any drugs including vitamins for at least 3 days before the study.

Serum GSH measurement

Samples were prepared and derivatized for HPLC analysis using procedures (with slight modifications) as described previously (24, 25). Briefly, 0.1 mL of serum was mixed with 0.1 mL of 25 mM dithiothreitol and 0.05 mL of 0.1 M Tris (pH 8.5) for the measurement of total GSH. The GSH-*o*-phthalaldehyde adducts were separated on a 4.6×250 mm Luna C18 column (5 μm, Phenomenex, Torrance, CA, U.S.A.) using two Waters 510 pumps, a 717 autosampler, and a 474 fluorescence detector (Milford, MA, U.S.A.), and fluorescence was detected at 420 nm with excitation at 340 nm. The amount of oxidized glutathione (GSSG) was obtained by subtracting the amount of GSH from the amount of total GSH.

Serum amino acid measurement

Glu, Cys, Cys2, Met, and Gly were analyzed using the Pico-Tag method (Waters, Milford, MA, U.S.A.) after the serum was dried in a sample tube under vacuum (Korea Basic Science Institute, Daejeon, Korea) (26). Free-amino-acid samples were derived using derivatizing solution (ethanol:distilled H_2O:triethylamine:phenylisothiocyanate at 7:1:1:1, v/v/v/v) for 15 min. The derivatized free amino acids were

applied to a 30-cm Pico-Tag free-amino-acid-analysis column (3.9×300 mm) equilibrated with buffer A equipped with a Waters HPLC system (510 HPLC pump, 717 automatic sampler, 996 photodiode array detector, and Millennium 32 chromatography manager) and eluted with a linear gradient composed of buffer B (0%, 14%, 20%, 46%, and 100%) at a flow rate of 1 mL/min at 46°C. The absorbance at 254 nm was measured. Buffer A was 140 mM sodium acetate (6% acetonitrile), and buffer B was 60% acetonitrile.

Statistical analysis

Results are expressed as mean±SD unless stated otherwise. Intracellular ROS was measured in about 30 cells randomly selected from three separate experiments, and DCF fluorescence intensities of treated cells were compared with those of unstimulated control cells. Analysis of variance was used to detect differences in ROS between groups, and statistical significance was defined as a probability value of $p<0.05$.

The pharmacokinetics of GSH, GSSG, and total GSH were characterized by the peak plasma concentration (C_{max}), elimination half time ($t_{1/2}$), and the area under the plasma concentration-time curve (AUC) for the first 60 min (BA Calc 2002, KFDA, Ver 1.1.1). The elimination rate constant (k_e) was determined by linear-regression analysis of the log-linear part of the concentration-time curve. The value of $t_{1/2}$ was calculated as $t_{1/2}=\ln(2/k)$. The AUC was calculated by the log-linear trapezoidal rule from 0 to 60 min after subtraction of the basal concentration. In order to avoid the influence of endogenous GSH, two concentrations (obtained at 120 and 240 min) were not included in calculating pharmacokinetic parameters because of the values less than basal level. The loading dose was calculated as the desired plasma level×volume of distribution at steady state, and the infusion rate as the desired plasma level×clearance (volume/unit time).

RESULTS

Effect of GSH, Cys, Cys2, and Met on the intensity of ROS in Swiss 3T3 fibroblasts

Our preliminary study found that Swiss 3T3 fibroblasts had a good reproducibility in both the production and the suppression of ROS, each done by paraquat and by antioxidants respectively. GSH at concentrations of 1-10 mM suppressed ROS in a dose-dependent manner, with 50% suppression was done by 5 mM GSH (Fig. 1A). Each of Cys,

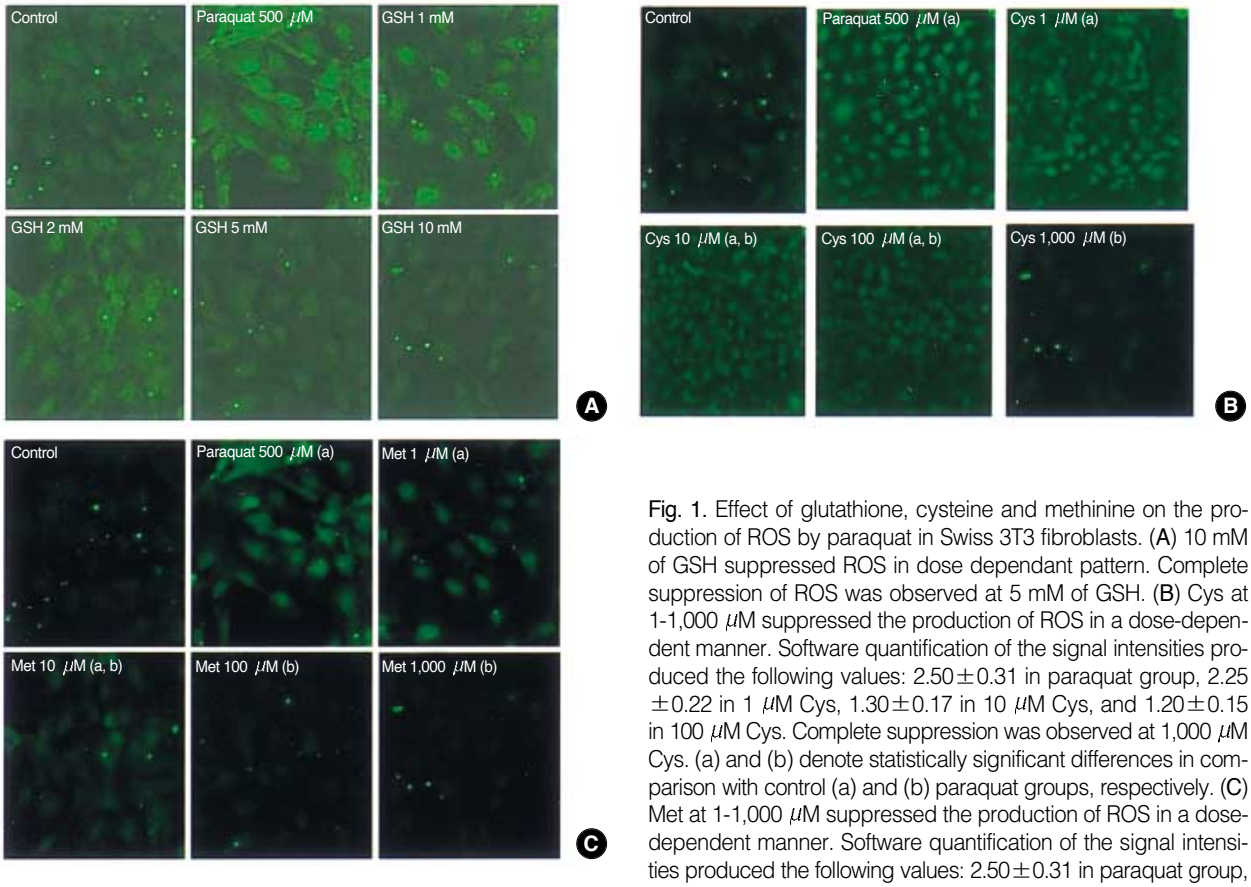

Fig. 1. Effect of glutathione, cysteine and methinine on the production of ROS by paraquat in Swiss 3T3 fibroblasts. (A) 10 mM of GSH suppressed ROS in dose dependant pattern. Complete suppression of ROS was observed at 5 mM of GSH. (B) Cys at 1-1,000 μM suppressed the production of ROS in a dose-dependent manner. Software quantification of the signal intensities produced the following values: 2.50±0.31 in paraquat group, 2.25±0.22 in 1 μM Cys, 1.30±0.17 in 10 μM Cys, and 1.20±0.15 in 100 μM Cys. Complete suppression was observed at 1,000 μM Cys. (a) and (b) denote statistically significant differences in comparison with control (a) and (b) paraquat groups, respectively. (C) Met at 1-1,000 μM suppressed the production of ROS in a dose-dependent manner. Software quantification of the signal intensities produced the following values: 2.50±0.31 in paraquat group, 2.40±0.30 in 1 μM Met, 2.05±0.25 in 10 μM Met, 1.19±0.15 in 100 μM Met, and 1.05±0.12 in 1,000 μM Met. (a) and (b) denote statistically significant differences in comparison with control (a) and paraquat (b) groups, respectively.

Table 1. Changes of amino acids and GSH during the observation period, in mean (SD) concentration (in μM) after intravenous administration of GSH (50 mg per kg of body weight) in 7 volunteers

Time (min)	0	10	20	30	60	120	240
GSH	4.3	154.4	11.1	5.3	4.7	4.0	5.7
	(2.5)	(70.0)	(7.3)	(2.9)	(2.6)	(2.3)	(2.7)
GSSG	9.7	1061.5	144.4	62.3	18.6	11.8	12.0
	(6.8)	(481.2)	(60.0)	(26.8)	(5.3)	(5.3)	(7.4)
Total GSH	14.0	1219.8	155.4	67.6	23.3	15.8	16.8
	(12.1)	(628.4)	(53.7)	(29.4)	(9.8)	(7.0)	(8.1)
Glu	47.4	268.0	191.3	121.0	38.4	53.3	31.9
	(14.3)	(78.8)	(19.6)	(30.7)	(16.5)	(19.4)	(11.4)
Cys	11.4	264.7	64.6	42.2	26.7	15.0	10.6
	(2.5)	(58.5)	(10.1)	(9.3)	(3.9)	(3.5)	(3.0)
Cys2	57.0	68.8	81.5	103.6	95.6	88.0	71.1
	(8.4)	(10.5)	(8.4)	(11.1)	(11.6)	(13.0)	(11.8)
Met	25.4	362.2	377.7	198.0	103.4	87.8	64.1
	(2.7)	(40.5)	(64.1)	(36.6)	(14.7)	(22.6)	(18.6)
Gly	220	477	455	314	271	193	216
	(23)	(101)	(112)	(93)	(81)	(80)	(41)

Cys2, and Met was observed to suppress ROS at various concentrations in dose-dependent manner: GSH at 1-10 mM, both Cys and Met at 1-1,000 μM, and Cys2 at 40-400 μM as shown in Fig. 1. 50% of the suppression was done by 5 mM GSH (Fig. 1A), by 10 μM Cys, 50 μM Met (Fig. 1B, C), and by 400 μM of Cys2 (data are not presented in figure).

Pharmacokinetics of GSH, Cys, Cys2, and Met

The basal and peak plasma concentrations of GSH, GSSG, and total GSH following the infusion of GSH (50 mg per kg of body weight) are listed in Table 1. The elimination rate constants, the elimination half-life, the systemic clearance, and the apparent volumes of GSH, GSSG, and total GSH following the infusion of GSH are listed in Table 2. It is postulated that at the lowest effective plasma GSH concentration is 1 mM. Assuming this as the target concentration, the optimal dose of GSH can be determined from the pharmacokinetic parameters as follows. The loading dose (1.69 g/kg) was calculated by multiplying the volume of distribution administered by the target concentration. The optimal infusion rate was calculated by multiplying the target concentration by the clearance rate, and was 5.70 g/hr/kg. Because GSH has a very short half-life (of 10 min), it is difficult to maintain it at a therapeutic concentration.

Changes in the metabolites of GSH were presented in Table 1. Glu and Cys reached their peak concentrations at 10 min, and Met at 20 min. However, Cys2 increased gradually, peaked at 30 min, and then slowly decreased. The 10- and 20-min concentrations of Glu, Cys, and Met were significantly higher than their baseline values, but no significant change was observed in Cys2. The repeated ANOVA revealed statistically significant nonlinear relations between concentration and time for all four amino acids. The pharmacokinetic profile of Cys after subtracting the baseline concentration was summarized in Table 2. Using metabolite kinetics whilst assuming that Cys is a metabolite of GSH, and that AUC $(Cys)_{single\ i.v.}$ is 4108.4 μM·min after administering GSH at 50 mg per kg of body weight, the expected Cys concentration is over 20 μM when this dose is given every 205.4 min. In the same way, when it comes to Met, the concentration is over 50 μM when the same dose is given every 427.4 min.

Table 2. Pharmacokinetic parameters of GSH, GSSG and total GSH following high-dosage intravenous administration of GSH (50 mg per kg of body weight) in 7 volunteers

	C_{basal} (μM)	C_{max} (μM)	AUC (μM min)	K (min^{-1})	$t_{1/2}$ (min)	C1 (mL min^{-1} kg^{-1})	Vd (1 kg^{-1})
GSH							
Mean	4.3	150	1242.8	0.07	10.9	309.2	5.528
SD	5.5	234.6	1905.2	0.026	3.3	360.9	6.935
GSSG							
Mean	9.7	1055.8	11148.1	0.066	10.8	10.6	0.164
SD	6.8	480.1	3705	0.012	2	3.7	0.061
Total glutathione							
Mean	14	1205.8	12427	0.066	10.9	9.8	0.152
SD	12.1	621.7	4618.3	0.014	2.2	3.8	0.058

DISCUSSION

The intracellular ROS produced by paraquat was suppressed by extracellular GSH at concentrations of 1-10 mM (Fig. 1A). This concentration range is three orders of magnitude higher than normal extracellular levels because the GSH concentration in circulation has known to be μM range. After intravenous administration, in agreement with a previous report (25), most of the GSH was oxidized to GSSG and disappeared immediately from circulation with a half-life of about 10 min. Pharmacokinetic investigations revealed that GSH at a loading dose of 1.69 g/kg and a maintenance dosage of 5.70 g/hr/kg are needed to reach 1 mM GSH which is a minimum requirement of extracellular concentration to suppress significantly the intracellular ROS. Considering that it is impossible to administer such a large amount of GSH in practical point of view, the intravenous administration of GSH would be an invalid treatment modality if GSH does not metabolized furthermore to other substances carrying antioxidant capacity.

In our study, five amino acids were measured over time: Glu, Cys, and Gly as metabolites of GSH, and Cys2 and Met as sulfur-containing compounds in plasma. Glu, Gly, and Cys reached peak concentrations at 10 min, Met peaked at 20 min, and Cys2 at 30 min. This finding suggests that GSH administered intravenously degraded immediately into the three amino acids. The intravenous infusion of GSH at 50 mg per kg of body weight increased the concentration of Cys

to over 20 μM, which suppressed intracellular ROS by approximately 50% for about 60 min. Using metabolite kinetics with the assumption that Cys is a metabolite of GSH, Cys concentration over 20 μM would be achieved when GSH were administered at 50 mg/kg body weights every 205.4 min. In the same way, the Met concentration of 50 μM, which suppresses intracellular ROS by about 50%, would be achieved if GSH were administered at 50 mg per kg of body weights every 427.4 min.

Occasionally, the result of in vitro study used to be challenged when it is going to be extended to that of in vivo, because of the uncertainty of whether the results from in vitro experiment is also true in vivo. In that sense, our study raises fundamental questions to be answered. First of all, there may be difference in the intensity of ROS formation by paraquat and/or in ROS suppression by antioxidant, between in vitro cell line and cells in physiologic state. This is one of the subjects frequently argued in vitro study, which has to be overcome by a careful interpretation of other adjunct experiments. In this regard, our results that derived from the in vitro experiments and being extended to the in vivo experiments should be understood as relative ones rather than absolute ones, even the data are presented in numbers. The other problem is that in vitro and in vivo have the quite different metabolism system of GSH and amino acids.

The purpose of our current study was to find out how we could determine the appropriate dose of GSH for the patients with critical ROS injury such as acute paraquat intoxication. Our study just observed the epiphenomenon of sulfa-containing amino acid without intensive check-up on each amino acid metabolism respectively. During the initial 30 min after administration, the Cys2 level increased slowly and thereafter it decreased. The Met concentrations were higher than basal levels during the first 30 min and decreased as the Cys2 decreased (Fig. 1C). This implies that the increase in Cys2 stimulated Met synthesis. As we mentioned above the current study is not designed to determine the metabolic interrelationship among sulfur-containing amino acids. However, keeping in mind that methylation of homocysteine is an essential pathway to the formation of Met, it seems likely that GSH metabolites influence the enzymes involved in Met synthesis.

The remethylation of homocysteine is catalyzed by betaine-homocysteine methyltransferase and 5-methyltetrahydrofolate-homocysteine methyltransferase. In this setting, Cys2 is known to increase the activity of betaine-homocysteine methyltransferase-this is the so-called Cys2-sparing effect of Met, which is readily observed when Cys2 is added to a low-Met diet in animal models (20). Therefore, it seem reasonable that practical guidelines for the dosage of GSH should be derived from pharmacokinetic studies of sulfur-containing amino acids synchronized with the changes in GSH after the intravenous administration of GSH.

In conclusion, not being conclusive, our results provide us relevant clinical information. GSH is a useful antioxidant for the patients suffering from acute, critical injury mediated by ROS, and the recommended dose appears to be determined more reasonably when the metabolite of GSH is encountered.

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Glutathione: Overview of its protective roles, measurement, and biosynthesis

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Abstract

This review is the introduction to a special issue concerning, glutathione (GSH), the most abundant low molecular weight thiol compound synthesized in cells. GSH plays critical roles in protecting cells from oxidative damage and the toxicity of xenobiotic electrophiles, and maintaining redox homeostasis. Here, the functions and GSH and the sources of oxidants and electrophiles, the elimination of oxidants by reduction and electrophiles by conjugation with GSH are briefly described. Methods of assessing GSH status in the cells are also described. GSH synthesis and its regulation are addressed along with therapeutic approaches for manipulating GSH content that have been proposed. The purpose here is to provide a brief overview of some of the important aspects of glutathione metabolism as part of this special issue that will provide a more comprehensive review of the state of knowledge regarding this essential molecule.

Keywords

Glutathione; Glutamate cysteine ligase; Hydroperoxide; Xenobiotic; Methods

1. Introduction

The tripeptide, γ-L-glutamyl-L-cysteinyl-glycine known as glutathione (GSH) (Fig. 1), is the most important low molecular weight antioxidant synthesized in cells. It is synthesized by the sequential addition of cysteine to glutamate followed by the addition of glycine. The sulfhydryl group (−SH) of the cysteine is involved in reduction and conjugation reactions that are usually considered as the most important functions of GSH. These reactions provide the means for removal of peroxides and many xenobiotic compounds; however, GSH is also involved in regulation of the cell cycle (Meister 1992).

2. Sources of oxidants

GSH plays a major role in removal of many reactive species. But, before addressing those aspects, it is important to understand from where these reactive species come and their pathological consequences that GSH helps avoid. Quinones are a class of redox cycling molecules that includes some drugs and xenobiotic compounds. Redox cycling in this context refers to the ability to cycle between oxidized and reduced forms and in the process, produce reactive oxygen species, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2). In this reaction (Fig. 2), the quinone is reduced by one electron transport reaction to produce a semiquinone, which is a free radical that can react with oxygen to produce O_2^-.

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There are other places in the cells where reactive oxygen species can be generated. In phagocytes, a major part of the mechanism of killing microorganisms involves production of reactive oxygen species (Forman and Thomas, 1986). The first enzyme involved is NADPH oxidase (NOX) that produces O_2^-. That enzyme is now known to be a member of a class of enzymes found in almost all cells (Vignais 2002). Once O_2^- is made, it can be dismuted into H_2O_2 both by a relatively fast non-enzymatic reaction and by very fast reaction catalyzed by one of the superoxide dismutases (SOD). Some phagocytes have the capacity to secrete enzymes called myeleoperoxidases that can catalyze a reaction of H_2O_2 and halides (chloride or bromide) to produce hypochlorous acid (HOCl) or hypobromous acid (HOBr) (Bakkenist et al., 1980). These hypohalous acids kill bacteria but can also damage normal tissue and thereby contribute to an inflammatory reaction.

The H_2O_2 formed can also be potentially hazardous if there are reduced metals present in the cells. H_2O_2 can react with ferrous iron (Fe^{2+}) and produce the hydroxyl radical (OH). This radical has capability to oxidize Teflon or fluorine and any organic molecule at near diffusion limited rates. In other words, OH can react with any molecule next to where it is produced. O_2^- can reduce ferric iron (Fe^{3+}) to Fe^{2+}, which suggests that it can play two roles in producing OH; however, reduction of Fe^{3+}can also occur with other reductants such as ascorbic acid (vitamin C).

One of the dangers of producing OH is when it is produced near a membrane. Lipids can be oxidized by OH and start a free radical chain reaction that will damage the membrane. In the initiation of lipid peroxidation by OH, the reaction with a reduced molecule of the lipid produces a lipid radical (L) and water. The L can react with oxygen to produce hydroperoxide radical (LOO), which then reacts with another lipid molecule, generating a lipid peroxide (LOOH) and another lipid radical L that can continue a chain reaction. One of the dangers from lipid peroxidation besides membrane damage is the production of byproducts such as 4-hydroxy-2-nonenal (HNE). Arachidonic acid is a polyunsaturated fatty acid found in membranes of all cells. When it becomes oxidized, it can break down yielding a large variety of compounds including a, β-unsaturated aldehydes (Poli et al., 1987) These are toxic compounds because they can react with proteins in the cells, particularly at cysteine, lysine or histidine by either Michael addition to the carbon–carbon double bond or by Schiff base formation at the carbon–oxygen double bond (Esterbauer et al., 1991; Eckl, 2003; Schaur, 2003). These reactions can inactivate the function of proteins. For example, reaction with an active site cysteine can destroy the activity of an enzyme.

The final component of oxidative damage considered here is peroxynitrite ($ONOO^-$). This ion is made in a reaction between nitrogen oxide (NO) and O_2^-. These two free radicals react at the fastest rate of any reaction known to occur in biology and is the only reaction that is faster than the dismutation reaction of O_2^- via superoxide dismutases. In its basic form, $ONOO^-$ does not react with organic molecule, it breaks down to form nitrite (NO_2^-) and nitrate (NO_3^-) But when peroxynitrite is protonated it becomes the highly reactive, peroxynitrous acid (ONOOH) that has the reactivity of nitrogen dioxide (NO_2), a very toxic free radical component in smog and cigarette smoke, and OH.

3. Protective functions of glutathione

3.1. Reduction

GSH is found in the cytosol of cells where it is in the range of 1–10 mM (Meister 1988). In most cells the GSH concentration is about 1–2 mM, while in hepatocytes, which export GSH, the concentration can reach about 10 mM. So why do we need GSH outside of the cells? In plasma GSH is in the micromolar range; however, in some extracellular spaces such as the lining fluid of the lung, a thin layer of fluid covering the air spaces where gas exchange occurs,

there is high concentration of GSH that is secreted by epithelial cells (Sutherland et al., 1985; Cantin et al., 1987). In people who smoke or inhale particles or other oxidants, there is potential inflammation that involves invasion of neutrophils from the blood through the endothelial and epithelial cells into the air spaces. As these neutrophils squeeze between the cells, they release HOCl, which can react with GSH secreted from the epithelial cells that normally protects the epithelial cells (Venglarik et al., 2003).

In cystic fibrosis patients, who secrete lower GSH than normal individuals into the lining fluid covering their alveoli, and in smokers, who have exposed their lungs to many oxidants including nitrogen dioxide and H_2O_2, there is both chronic inflammation and lower than normal GSH (Roum et al., 1993). In that case, HOCl can oxidize proteins in the lining fluid or on the surface of the epithelial cells. It can also react with lipid to produce even more dangerous compounds than are produced by lipid peroxidation itself (Pullar et al., 2000). Fig. 3 shows how GSH reacts with HOCl and removes it (Winterbourn and Brennan, 1997). While many studies of GSH in inflammation have been done of the lungs, these reactions can occur in any organ.

Secretion of GSH to the air space in cystic fibrosis is depressed because of a mutation of a protein called cystic fibrosis transport receptor (CFTR) (Roum et al., 1993). The CFT1 cell line, which is derived from a cystic fibrosis patient, has lower GSH secretion to the apical (air space) side. If the wild type CFTR is transfected into the cells, the rate of GSH secretion is increased to the level seen in normal cells (Gao et al., 1999). The generation of HOCl in the surface fluid covering normal epithelial cells to mimic the action of stimulated neutrophils can decrease in the electrical resistance of that epithelial cell layer; however, the presence of GSH at a concentration similar to normal lining fluid protects against the loss of electrical resistance (Venglarik et al., 2003). Similar events occur during inflammation and are exaggerated in cystic fibrosis patients. There is some evidence that other lung diseases, such as idiopathic pulmonary fibrosis, also have a lower GSH concentration (Cantin et al., 1989). Further studies on the potential contribution of GSH deficiency to these pathologies are needed. Understanding the transport of GSH across the plasma membrane is an important issue that is essential to treatment of diseases involving oxidative stress (see reviews by Ballatori et al., 2008 and by Yuan and Kaplowitz, 2008 in this issue).

Compared to the extracellular environment, what happens inside of the cells is quite different. Glutathione plays major roles in the different cellular compartments. In mitochondria it plays a key role in regulating apoptosis versus necrosis (see review by Yuan and Kaplowitz 2008 in this issue). In the nucleus, GSH is a key regulator of cellular division (see review by Pallardó et al., 2008 in this issue.) While lungs are clearly adversely affected by lowered intracellular and extracellular GSH, the majority of studies on the pathologies involving GSH transport and metabolism have been done in liver. Reviews of the involvement of altered intracellular GSH in lung diseases (Biswas and Rahman, 2008), liver diseases (Yuan and Kaplowitz, 2008) and viral diseases (Fraternale et al., 2008) can be found in this issue.

Most of the GSH in antioxidant defense in cells is utilized by three members of glutathione peroxidase (GPx) family (Brigelius-Flohe, 1999) and by one of the peroxiredoxins (Prdx 6). These enzymes catalyze the reduction of H_2O_2 by GSH into H_2O and GSSG. Prdx 6 also requires GSH S transferase Pi in order to be active (Ralat et al., 2006). Phospholipid hydroperoxide glutathione peroxidase (PHGPx or GPx IV) can reduce lipid peroxides to lipid alcohols (Imai and Nakagawa, 2003). GSSG is potentially toxic to the cells but cells normally contain high glutathione reductase activity, which maintain most of the GSH in the reduced form. Some GSSG is also secreted from cells. During oxidative stress, GSSG could react by disulfide exchange with a protein thiol to produce a protein mixed disulfide (PSSG), which can further exchange with another protein thiol to a protein disulfide (Huang and Huang,

2002). These reactions are actually quite slow unless catalyzed by an enzyme such as protein disulfide isomerase (PDI), an important enzyme that is particularly abundant in the endoplasm reticulum where protein folding occurs. In fact, the cysternae of the endoplasm reticulum is the only part of the cell with a relatively high ratio of GSSG/GSH. In the cytosol formation of PSSG is transient except during oxidative stress.

Formation of PSSG with some enzymes may play a role in signal transduction although the exact mechanism of their formation is uncertain. So how might PSSG form during normal metabolism in the cells? While protein disulfide exchange with a thiol can be catalyzed by PDI, some proteins contain a microenvironment in which thiolate ($-S^-$), which is far more reactive than is a thiol in both reaction with H_2O_2 or disulfide exchange, is formed. This requires that the microenvironment be composed in part by basic amino acids in proximity to the cysteine to allow dissociation of the thiol, which normally has a pK_a of around 8.3. GSH peroxidase catalyzes the production of GSSG, which could be potentially exchanged with a thiolate to form mixed disulfide. But in the cytosol, even during oxidative stress, the ratio of GSH/GSSG remains very high, which makes that exchange reaction unfavorable. The enzyme PDI can enhance the rate of that reaction but, like any catalyst, cannot change the equilibrium. Instead, it has been proposed that during physiological signaling when the H_2O_2 is used as the second messenger, some of protein thiolates could potentially react and form sulfenic acid (PSOH) (Fig. 4); however, for most thiolates including that formed by glutathione, the rate of the non-enzymatic reaction is too slow to account for the inactivation of the enzymes (Forman, 2007). We do know that in the active site of peroxiredoxins, where the reaction of H_2O_2 with a thiolate can occur up to six orders of magnitude faster than with glutathione in its thiolate form, the reaction can occur. Regardless, once formed, a protein sulfenate would rapidly react with GSH to produce the mixed disulfide, and this could be the mechanism through which PSSG formed for some proteins in the cytosol during oxidative stress when H_2O_2 is high enough to overcome a slow rate constant.

3.2. Conjugation

The elimination of many xenobiotic compounds can be accomplished through conjugation with GSH followed by secretion of the adduct from the cell (Boyland and Chasseaud 1969). Although the quinone, menadione, can react with GSH to form an adduct non-enzymatically, an enzymatic catalyzed Michael addition by a glutathione-S transferase (GST) is much faster. The glutathione adduct can then be secreted from cells through a membrane transporter such as the multidrug resistant proteins. The product of the addition of GSH can also rearrange into a quinol that are usually considered to be less toxic than the quinone (see above).

GSH is also used in the elimination of electrophiles such as HNE. Almost all these reactions are catalyzed by GSTs, and there is a specific one in human cells that can cause the conjugation of GSH to HNE at about 100 times faster rate than the non-enzymatic reaction. The conjugate, which is a Michael adduct (because the reaction is a Michael addition), can rearrange to form a cyclic hemiacetal (Fig. 5) (Alary et al., 2003). Both of the compounds however, can be excreted from the cells. This is the major route of elimination of HNE and other electrophiles that conjugate with GSH.

3.3. Interaction with other non-enzymatic antioxidants

While GSH is the most important small molecular weight antioxidant produced in the cells, there are other small molecular antioxidants obtained from the diet such as vitamins E (α-tocopherol) and C (ascorbic acid). Vitamin E can reduce lipid hydroxyl radicals and lipid peroxides that are produced from polyunsaturated fatty acids. The oxidized vitamin E is then reduced by vitamin C in a non-enzymatic but rapid reaction. The oxidized vitamin C can then be restored to the reduced form by enzymatic reactions, one of which uses GSH as substrate.

4. Measurement of glutathione

One of the important issues in determining the mechanisms of both oxidative stress and redox signaling is the measurement of the different forms of thiols in cells. The predominant forms are the reduced form of GSH and GSSG. Nitrosoglutathione (GSNO) and protein nitrosothiols (PSNO) are also formed in cells and play a role in NO signaling independent of the cyclic GMP pathway. Cysteine is a precursor amino acid of GSH and cystine is the disulfide form of cysteine. Protein thiols exist as cysteine, mixed disulfides between cysteine and GSH or other thiols, and disulfides between two protein cysteines that may be in the same or different protein molecules. It is important to recognize that an increase in the oxidized forms of these thiols in the cytosol will be transient even during oxidative stress. Therefore it can be very difficult to measure thiol oxidation, particularly that occurring in signal transduction.

GSH reacts with dithionitrobenzoic acid (DTNB) (Akerboom and Sies, 1981) and by reducing GSSG total GSH (GSH + GSSG) can be measured. DTNB reacts with GSH to produce a conjugate and TNB anion that can be detected by fluorescence or absorbance (Fig. 6a). To measure total GSH, a recycling assay is used in which GSH reacts with the conjugate producing GSSG and another molecule of TNB, which can be increases fluorescence or absorbance (Fig. 6b). The enzyme glutathione reductase then reduces the GSSG releasing the GSH that can react with another molecule of DTNB. Therefore, instead of a single determination of how much DTNB reacts with GSH, the rate of TNB production is measured, as that is proportional to the initial amount of GSH. To measure GSSG however, one must first modify the GSH present at the beginning so it is removed from the recycling assay. Modification of GSH is done with N-ethylmaleimide (NEM) or vinylpyridine. To measure protein mixed disulfides, the GSH can be released from the protein mixed disulfide with sodium borohydride ($NaBH_4$), and the GSH is then measured in the recycling assay.

A more commonly used procedure for measuring GSH and GSSG now is high performance liquid chromatography (HPLC) (Fariss and Reed, 1987). In this assay, thiol compounds are first modified by the addition of iodoacetate (Fig. 6c). The amino groups on the compound then are modified by 1-fluoro-2, 4-dinitrobenzene. This then allows separation of many compounds that can be identified by their movement on HPLC.

On method that has been developed to measure nitrosoglutathione involves the production of GSH from it followed by reaction with orthophthaldehyde (OPT) to produce a fluorescent compound (Fig. 6d) (Tsikas et al., 1999) while another method uses a biotinylated fluorescent label in a method called the biotin-switch (Gladwin et al., 2006). First however, as with the measurement of GSSG above, it is necessary to first remove any GSH in the original sample with methyl methanethiosulfonate before reducing GSNO to release GSH. Various reagents have been proposed as best for differentially reducing GSNO as well as PSNO especially as the presence of GSSG or protein mixed disulfides can also yield GSH upon reduction (Gladwin et al., 2006). After reaction with OPT the products are separated by HPLC with a fluorescence detector. There are other methods for measuring GSNO such as using ^{15}N labeling (Kluge et al., 1997), but this is not commonly used and requires mass spectrometry.

5. Glutathione synthesis

The first step in de novo GSH synthesis involves the combination of cysteine with glutamate to produce γ-glutamylcysteine. This reaction is catalyzed by the enzyme glutamate cysteine ligase (GCL), which is also called γ-glutamylcysteine synthetase (Fig. 7). This enzyme requires coupled ATP hydrolysis to form an amide bond between the γ-carboxyl group of glutamate and the amino group of cysteine (Huang et al., 1993). The next step involves the enzyme glutathione synthetase, responsible for adding glycine to the dipeptide to produce GSH (γ-glutamylcysteinylglycine) and also requires coupled ATP hydrolysis (Meister, 1974).

GSH can be transported out of cells. This mechanism is physiologically important as hepatocytes supply GSH found in the plasma, which is used as a source of cysteine for GSH synthesis in other cells (Anderson et al., 1980). In fact, GSH in the plasma is maintained at very low concentration because of the metabolism of GSH by many other cells (Sies and Graf, 1985; Hirota et al., 1986). This process requires two enzymes commonly found on the surfaces of cells. The enzyme γ-glutamyl transpeptidase transfers a glutamate to other amino acids releasing cysteinylglycine, which in turn can be broken down by a dipeptidase to produce cysteine and glycine (Kozak and Tate 1982; Hirota et al., 1986). Cysteine and glycine as well as γ-glutamyl amino acids are moved into cells by specific amino acid transporters and used for GSH biosynthesis (Meister, 1991).

5.1. Regulation of glutamate cysteine ligase activity

GCL is regulated at both the level of its enzymatic activity and the expression of its two subunits. One subunit is the relatively heavy (− 73 kDa) subunit, which has competent but low catalytic activity for production of γ-glutamylcysteine. The catalytic subunit, designated as GCLC, can be feedback inhibited by GSH (Huang et al., 1993). The lower molecular weight (− 28 kDa) subunit regulates the activity of the enzyme by reducing the inhibition by GSH (Huang et al., 1993; Choi et al., 2000) and with purified enzyme has been shown to also decrease the K_M for glutamate (Huang et al., 1993). This subunit, which is designated as GCLM for its modulatory activity can affect the steady state level of GSH found in cells when GCLM/GCLC expression is altered (Richman and Meister, 1975; Choi et al., 2000; Krzywanski et al., 2004). Thus, increased expression of GCLC will tend to elevate GSH while increasing GCLM/GCLC will further increase GSH. An example of when lowering GCLM/GCLC causes decreased GSH is the expression of the HIV-Tat protein, which suppresses GCLM expression (Choi et al., 2000). Finally, the kinetics of GCL seems to be regulated by phosphorylation of both subunits as well (Sun et al., 1996). The functional roles of the two GCL subunits are reviewed in this issue by Franklin et al. (2008)).

5.2. Regulation of glutamate cysteine ligase expression

The expression of GCL is also regulated at many levels. Oxidant species and electrophiles are able to increase the transcription of both the modulatory and catalytic subunits (Shi et al., 1994; Rahman et al., 1996; Tian et al., 1997) (also see review by Lu, 2008 in this issue). This occurs by the activation of signal transduction pathways involved in the control of transcription of GCLC and GCLM genes but also there is some evidence of mRNA stabilization by oxidants and electrophiles (Liu et al., 1998).

It has been known for almost twenty years that sublethal concentrations of electrophiles could increase GSH production (Ogino et al., 1989; Darley-Usmar et al., 1991); however, it was unclear whether the increase was on the kinetic or the transcriptional level or even whether GSSG reduction was increased. Using redox cycling quinones to increase production of hydrogen peroxide and by measuring transcription by nuclear run-on analysis, it was then shown that a sustained increase the amount of GSH in cells could be achieved by increasing the transcription of GCLC (Shi et al., 1994; Shi et al., 1994). Subsequently many labs showed that a variety of other agents, able to generate an oxidative stress throughH_2O_2 generation, increasing concentrations of electrophiles or nitric oxide could also induce GCLC or GCLM subunits or both (Rahman et al., 1996; Tian et al., 1997; Galloway and McLellan, 1998; Liu et al., 1998; Moellering et al., 1999; Wild and Mulcahy, 1999).

The GCLC and GCLM promoter sequences were described first from humans and then they were determined in rodents (Gipp et al., 1992; Gipp et al., 1995; Hudson and Kavanagh, 2000; Yang et al., 2001). The human and rodent promoters have some similar cis elements and appear to be regulated somewhat differently than the human genes (Iles and Liu 2005) (see

review by Lu, 2008 in this issue). For the human GCL genes, the promoter enhancer regions of the two genes contain several elements able to respond to oxidants and electrophiles (Gipp et al., 1992; Gipp et al., 1995; Yang et al., 2001; Dickinson et al., 2002). One of the important oxidant responsive cis elements (transcription factor binding sites) regulating GCL genes is the AP-1 binding site also called the TRE element. TRE binds members of the Jun and Fos family of transcription factors (Ofir et al., 1990; Binetruy et al., 1991). Another important element in human GCL gene promoters that responds to electrophiles in cells and increases gene expression is the EpRE or electrophile response element (Rushmore et al., 1991; Jaiswal, 1994; Vasiliou et al., 1995). EpRE elements are also present in both human GCLC and GCLM promoters (Gipp et al., 1992; Gipp et al., 1995). Initially EpRE was called the antioxidant response element (ARE) because the first compound, shown to activate ARE was a so-called antioxidant that was subsequently shown to generate H_2O_2 through redox cycling (Pinkus et al., 1996). The EpRE elements bind proteins members of the Nrf family, Jun family and small Maf family (Venugopal and Jaiswal, 1998;Kong et al., 2001; Moran et al., 2002; Itoh et al., 2004). One of the transcription factors established as able to bind EpRE is Nrf2, which located in the cytosol through the inhibitory interaction with Keap1 in resting cells. Upon stimulation, Nrf2 is translocated into the nucleus after dissociation from Keap1 (Itoh et al., 1999).

While the redox and electrophilic response cis elements have been identified, less has been done to identify the signaling mechanisms that activate the transcription factors that bind to those elements. We will describe here briefly what is understood regarding the signaling by HNE. Darley–Usmar and coworkers have shown that HNE directly modifies Keap1, which allows Nrf2 to avoid degradation and migrate to the nucleus where it can bind to EpRE elements in the promoters of the human GCLC and GCLM genes (Levonen et al., 2004). But, this cannot be the whole story as there are actually multiple EpRE elements in the promoters and not all of them are involved in regulating transcription (Dickinson et al., 2004). While Nrf2 is critical, EpRE binding also involves a partner protein. For the EpRE element that regulates transcription of GCLC in human bronchial epithelial cells that partner has not yet been firmly identified.

More is understood about the TRE element. Interestingly, the TRE element in the human GCLC promoter appears to bind c-Jun dimers preferentially (Rahman et al., 1999). For HNE induction, the activation of the critical AP-1 binding elements in both human GCL genes can is achieved through the Jun N-terminal kinase (JNK) pathway (Dickinson et al., 2002). JNK phosphorylates c-Jun, which translocates into the nucleus, and binds to the TRE element. Inhibition of JNK completely eliminates GCLC and GCLM gene expression in response to HNE in human bronchial epithelial cells while inhibition of the ERK or $p38^{MAPK}$ pathways had no effect. Recently, the activation of the JNK pathway by HNE has been shown to occur upstream at the protein tyrosine phosphatase SHP-1that is inhibited by HNE, which also appears to accelerate the degradation of the enzyme (Rinna and Forman, 2008).

6. Glutathione therapeutics

As an increase in GSH appears to be a ubiquitous response to oxidants and electrophiles and some diseases appear to be exacerbated by decreasing GSH, increasing GSH by using delivery of permeable esters (Levy et al., 1993) or increasing the availability of cysteine using the non-toxic precursor N-acetylcysteine (Thor et al., 1979) have been proposed. Increasing GSH through synthesis would also seem to be useful therapeutically but as oxidants and most electrophiles would not seem appropriate, natural compounds such as curcumin, a principal ingredient of curry powder (Dickinson et al., 2003), and sulforaphane, a potent Phase II gene-inducing compound in broccoli, (Brooks et al., 2001) have been proposed but none of these natural has actually become a major therapeutic agent.

On the other hand, compounds that decrease GSH and increase the susceptibility of tumors to chemotherapy or radiation have been used. GCL can be inhibited by a buthionine sulphoximine quite specifically making it a useful tool in studying GSH metabolism, and useful in cancer chemotherapy (Martensson et al., 1989; Anderson et al., 1997; Gartenhaus et al., 2002). An inhibitor of γ-glutamyl transpeptidase (GGT), acivicin (AT-125) (Griffith and Meister, 1980) was tried in chemotherapy before it was known to inhibit GGT; however, acivicin also inhibits enzymes in purine and pyrimidine biosynthesis, which may be its actual mode of action (Poster et al., 1981; Elliott and Weber, 1985). Thus, there is still much to be done in understanding how GSH synthesis and metabolism may be manipulated to therapeutic advantage. Further information about the use of GSH and related compounds in therapy for a variety of diseases including viral infection, cystic fibrosis and cancer, can be found in the reviews by Biswas and Rahman (2008)) and by Fraternale et al. (2008) in this issue.

Abbreviations

GSH, glutathione
−SH, sulfhydryl group
O_2^-, superoxide
H_2O_2, hydrogen peroxide
NOX, NADPH oxidase
SOD, superoxide dismutase
HOCl, hypochlorous acid
HOBr, hypobromous acid
Fe^{2+}, ferrous iron
OH, hydroxyl radical
Fe^{3+}, ferric iron
L, lipid radical
LOO, hydroperoxide radical
LOOH, lipid peroxide
HNE, 4-hydroxy-2-nonenal
$ONOO^-$, peroxynitrite
NO, nitrogen oxide
NO_2^-, nitrite
NO_3^-, nitrate
ONOOH, peroxynitrous acid
NO_2, nitrogen dioxide
CFTR, cystic fibrosis transport receptor
GPx, glutathione peroxidase
PHGPx GPx IV, phospholipid hydroperoxide glutathione peroxidase
GSSG, glutathione disulfide
Prdx, peroxiredoxin
PSSG, protein mixed disulfide
PDI, protein disulfide isomerase
$-S^-$, thiolate
PSOH, sulfenic acid
GST, glutathione-S transferase
GSNO, nitrosoglutathione
DTNB, dithionitrobenzoic acid
NEM, N-ethylmaleimide
$NaBH_4$, sodium borohydride
OPT, orthophthaldehyde
GCL, glutamate cysteine ligase

ARE, antioxidant response element
JNK, Jun N-terminal kinase
GGT, γ-glutamyl transpeptidase.

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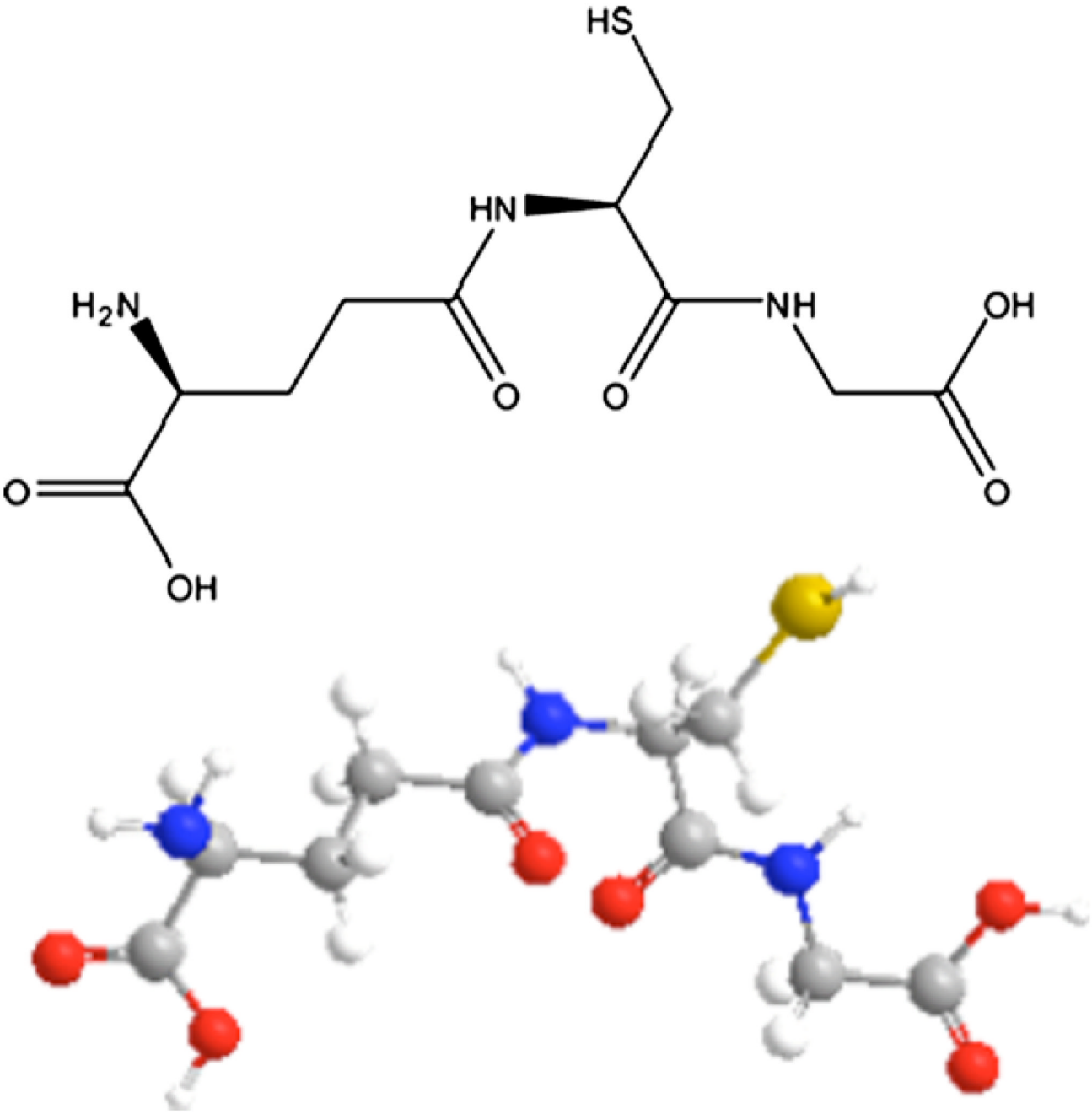


Fig. 1.
Glutathione structure. A stereochemical and ball and stick figure showing γ-glutamyl-cysteinyl-glycine are shown.

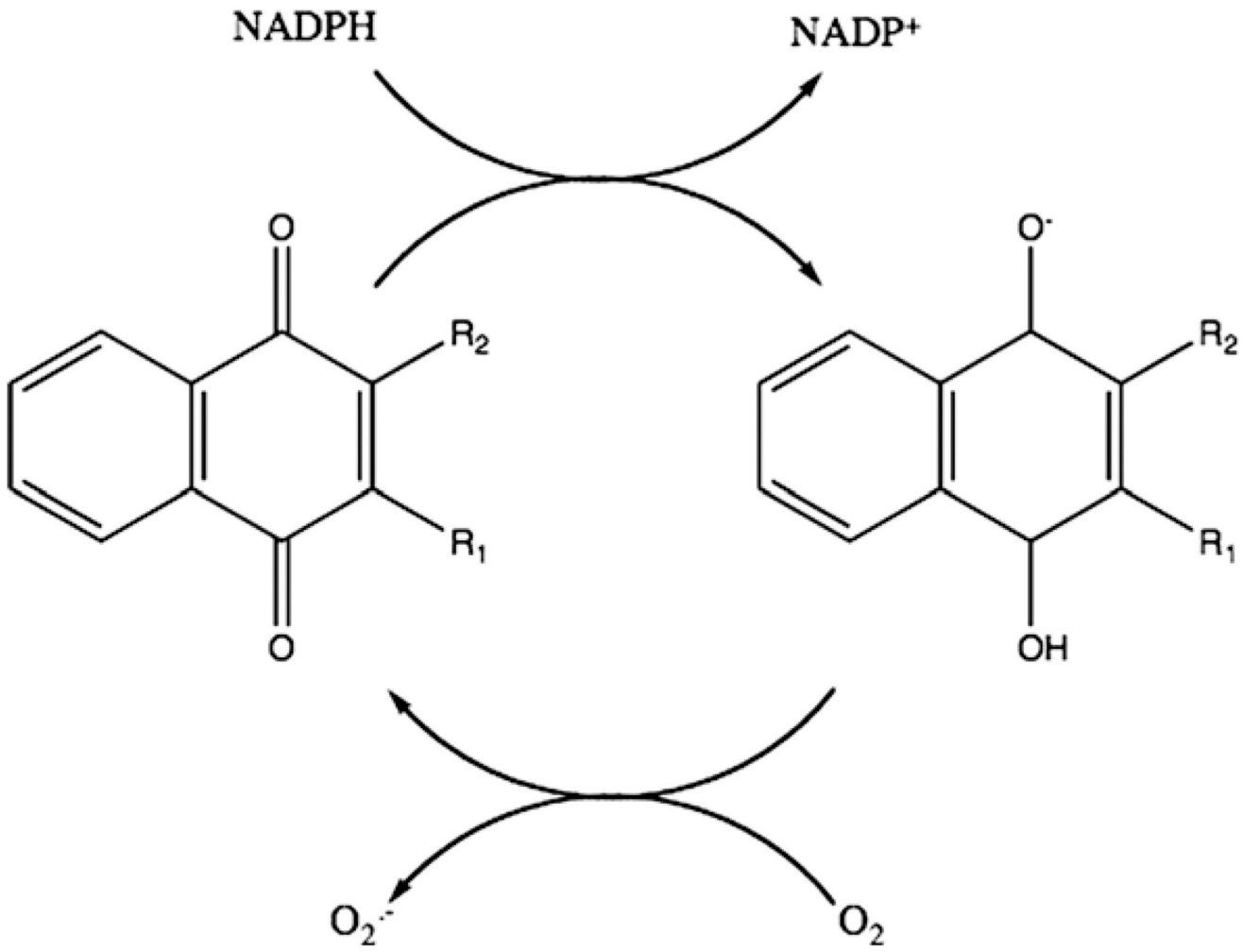


Fig. 2.

Redox cycling of 1,4-naphthoquinones. A naphthoquinone with two variable groups (R) can be reduced by NADPH (or NADH, which is not shown) enzymatically to the semiquinone radical and then will react with oxygen to generate superoxide and restore the naphthoquinone.

$$GSH + HOCl \rightarrow GSCl + H_2O$$

$$GSCl + GSH \rightarrow GSSG + HCl$$

$$GSCl + 2HOCl \rightarrow GSO_2Cl + 2HCl$$

$$GSO_2Cl \rightarrow GSO_2NH\,(\text{formed with an amino group of GSH itself}) + HCl$$

$$GSO_2Cl + GSH \rightarrow GSO_2SG + HCl$$

Fig. 3.
Reactions of glutathione with hypochlorous acid. GSH and HOCl can react to produce several different products.

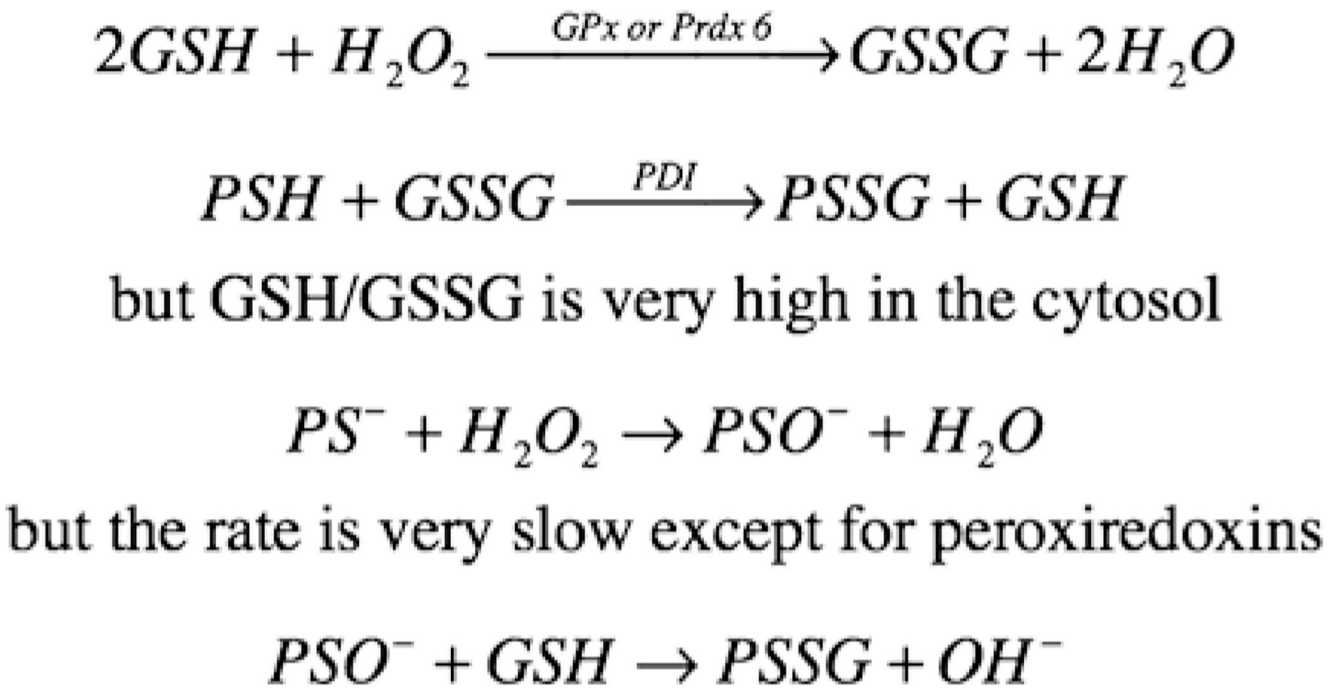


Fig. 4.

Formation of protein mixed disulfide. Both glutathione peroxidases and peroxiredoxin 6 can catalyze the oxidation of glutathione by hydrogen peroxide to glutathione disulfide and water. GSSG can then undergo an exchange reaction with protein sulfhydryl to form PSSG, which is usually catalyzed by a protein disulfide isomerase. An alternative mechanism is the oxidation of a protein thiolate to a sulfenic acid, which then will react with GSH to form PSSG and water.

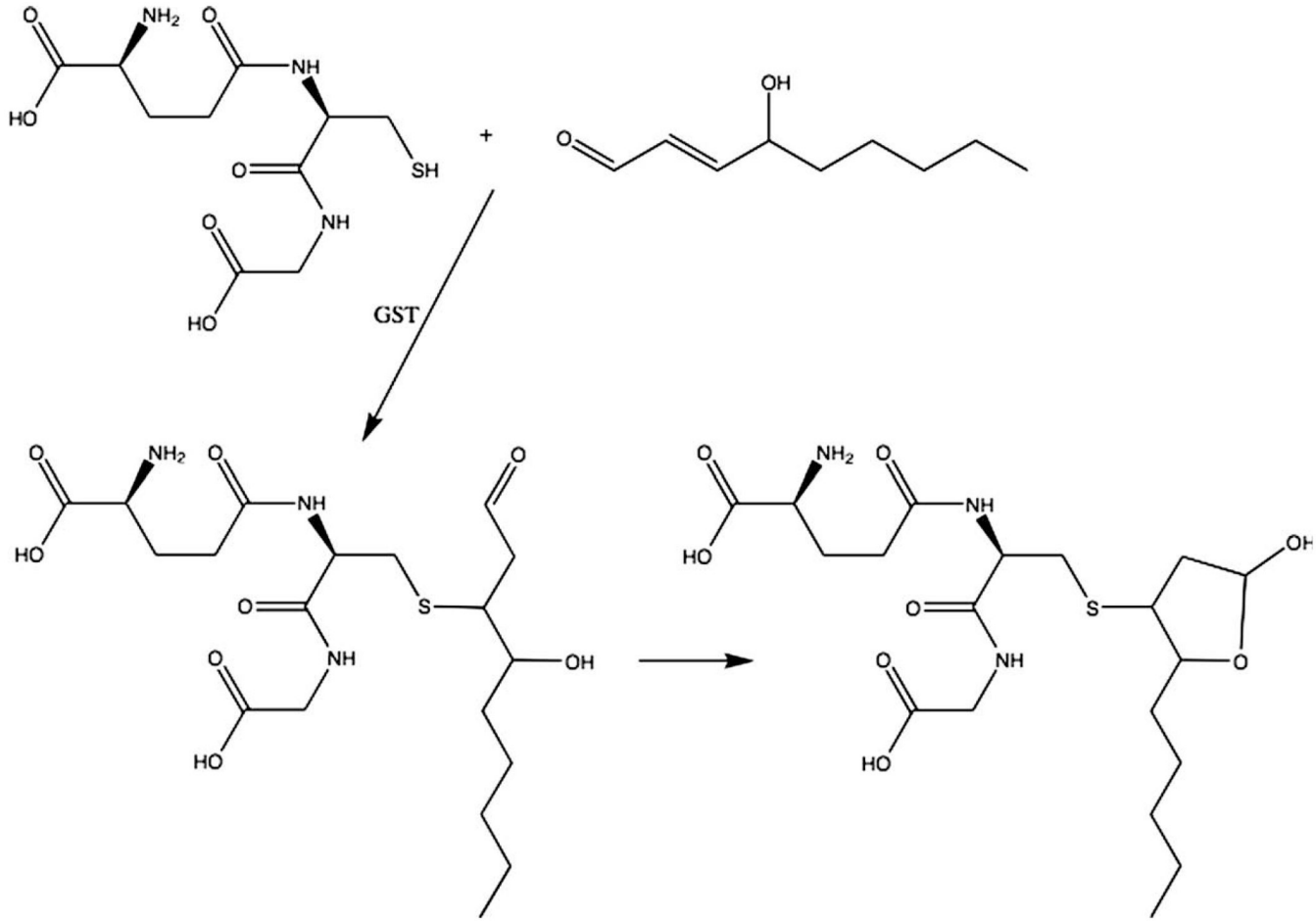
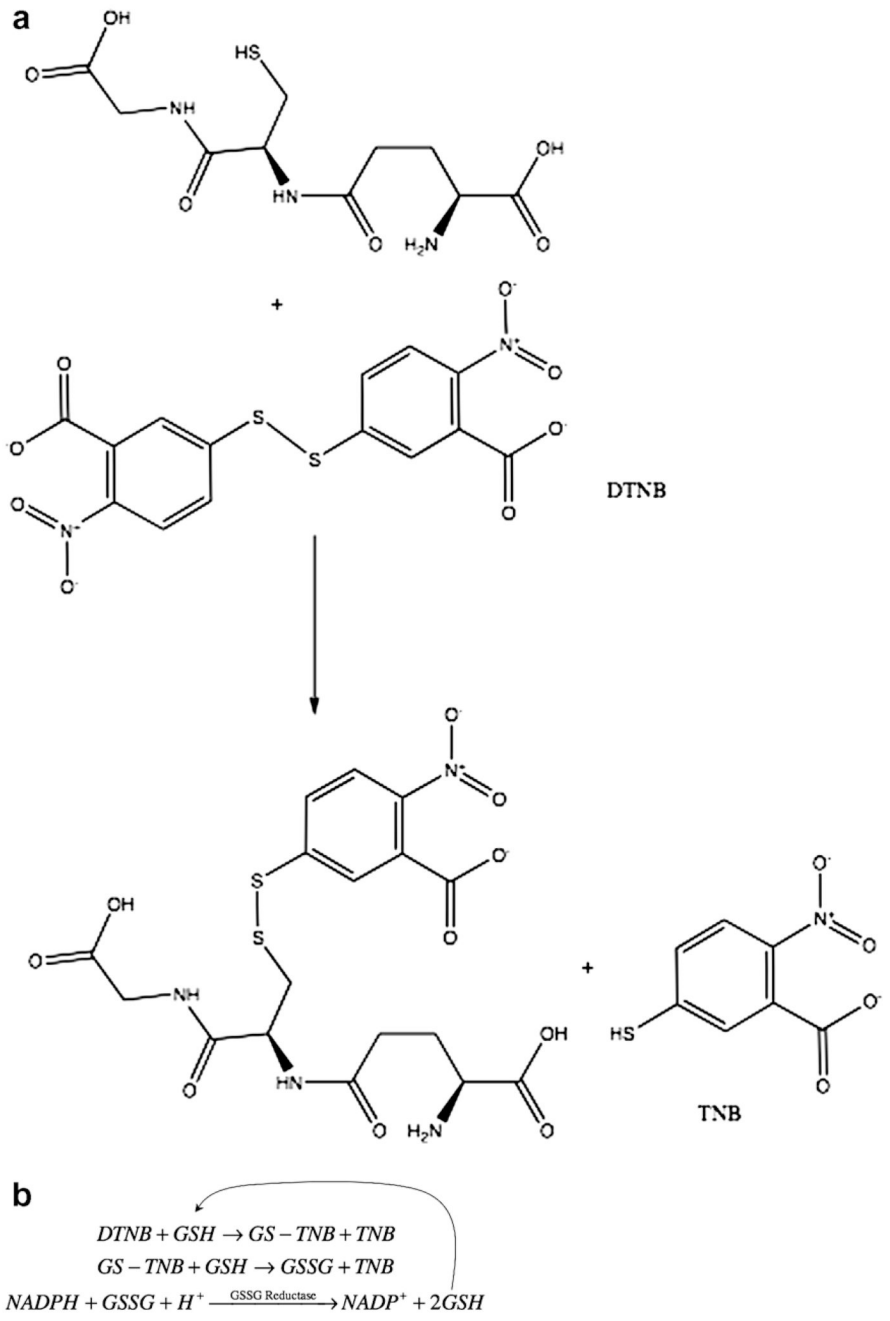


Fig. 5.
Glutathione conjugations with 4-hydroxynonenal. Glutathione S-transferases catalyze the conjugation of GSH with HNE. This is a Michael addition that can slowly occur non-enzymatically.



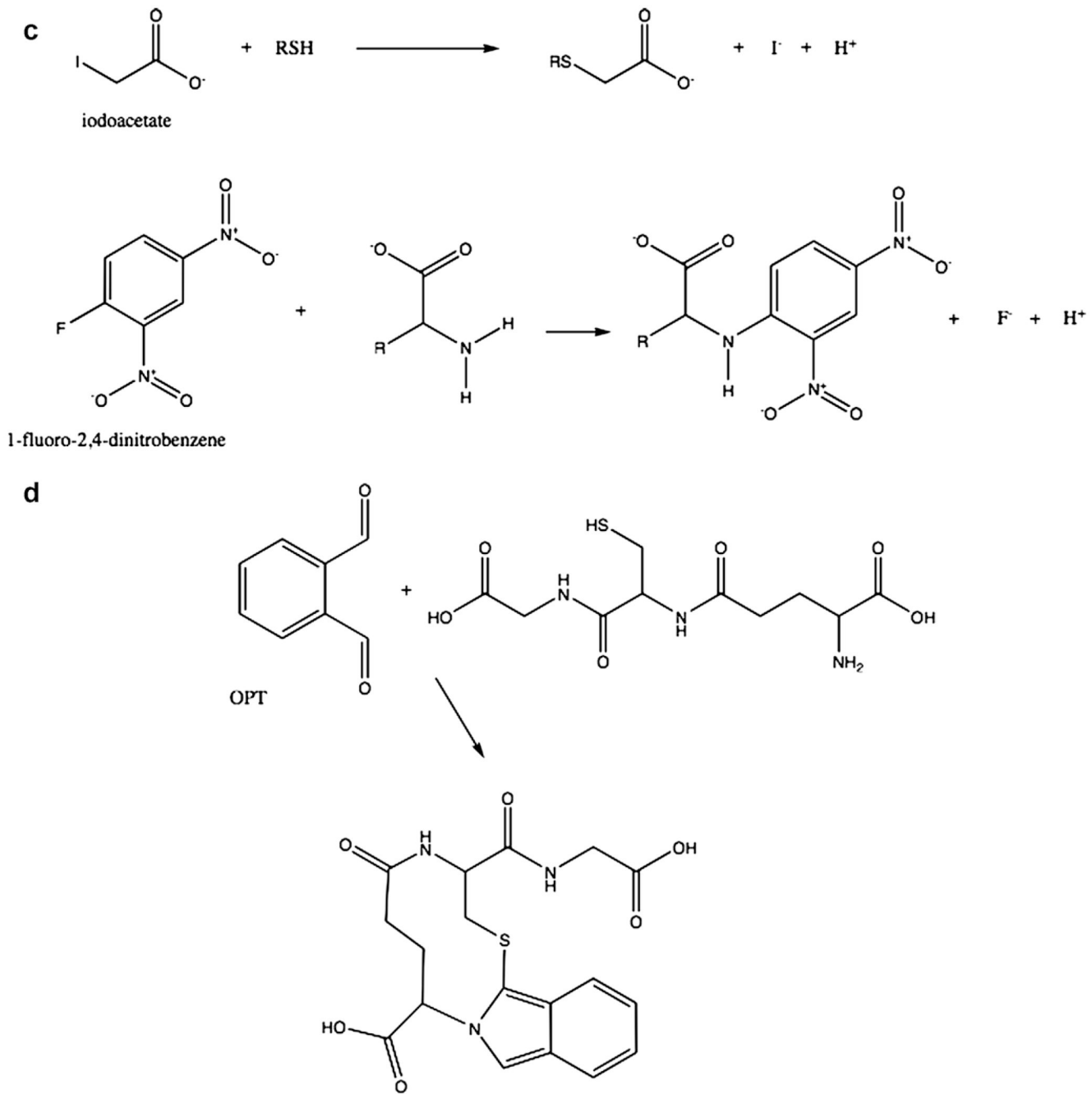


Fig. 6.

Measurements of thiols. (a) Reaction of GSH with DTNB produces an adduct and TNB, which is measured spectrofluorometrically or spectrophometrically; (b) total glutathione can be determined by recycling of GSSG produced in the reaction in (a) and measuring the rate of TNB; (c) Glutathione and related compounds are first derivatized with iodoacetate followed by a second derivatization with 1-fluoro-2,4-dinitrophenol. The second products are then separated by HPLC and measured spectrofluorometrically; (d) Reaction of glutathione with orthophthaldehyde (OPT) yields a product that can be measured spectrofluorometrically.

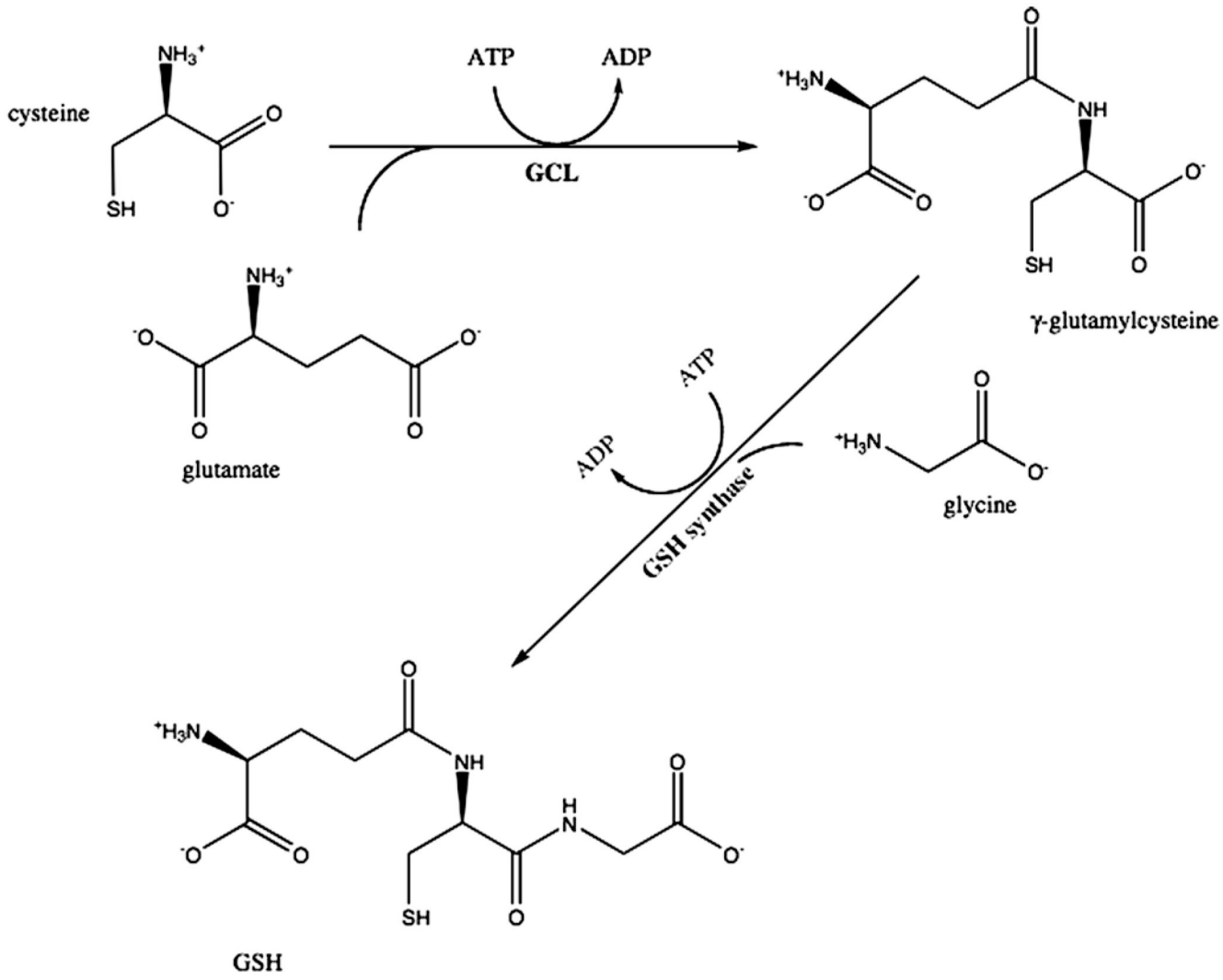


Fig. 7.
Glutathione synthesis. The sequential ATP dependent formation of amide bonds between cysteine and the γ-carboxyl group of glutamate and then between glycine and cysteine are shown.