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

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

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.

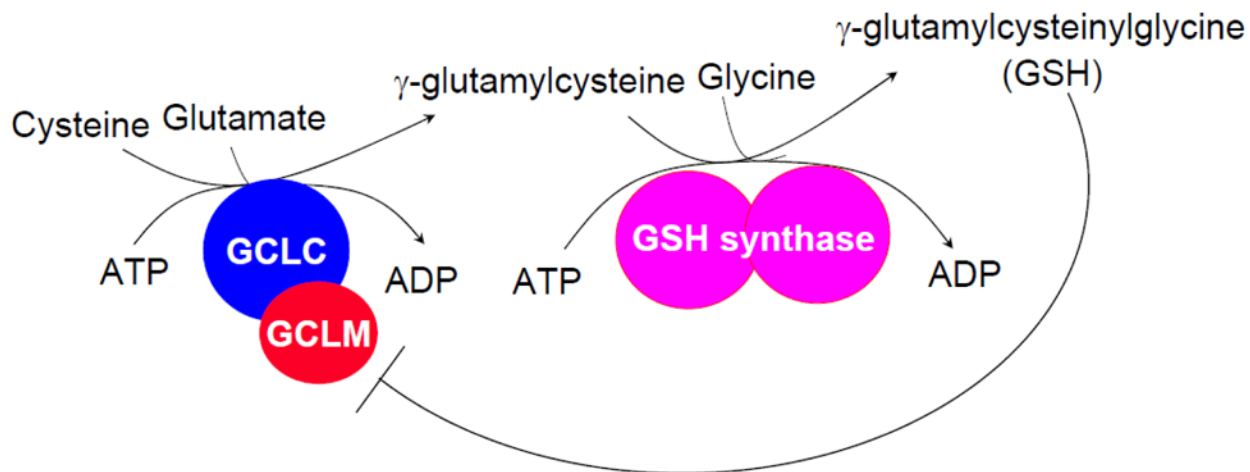- GSH synthesis is dysregulated in multiple human diseases.

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.
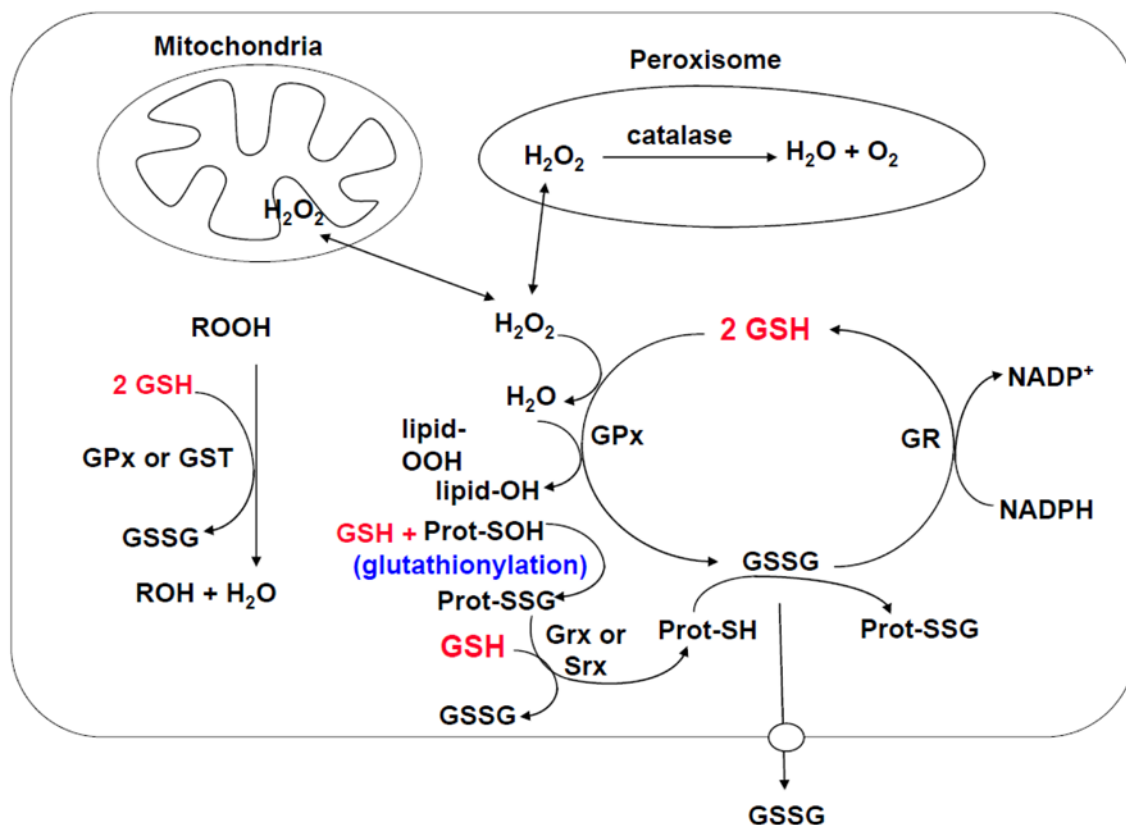
Fig. 2. Antioxidant function of GSH

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).
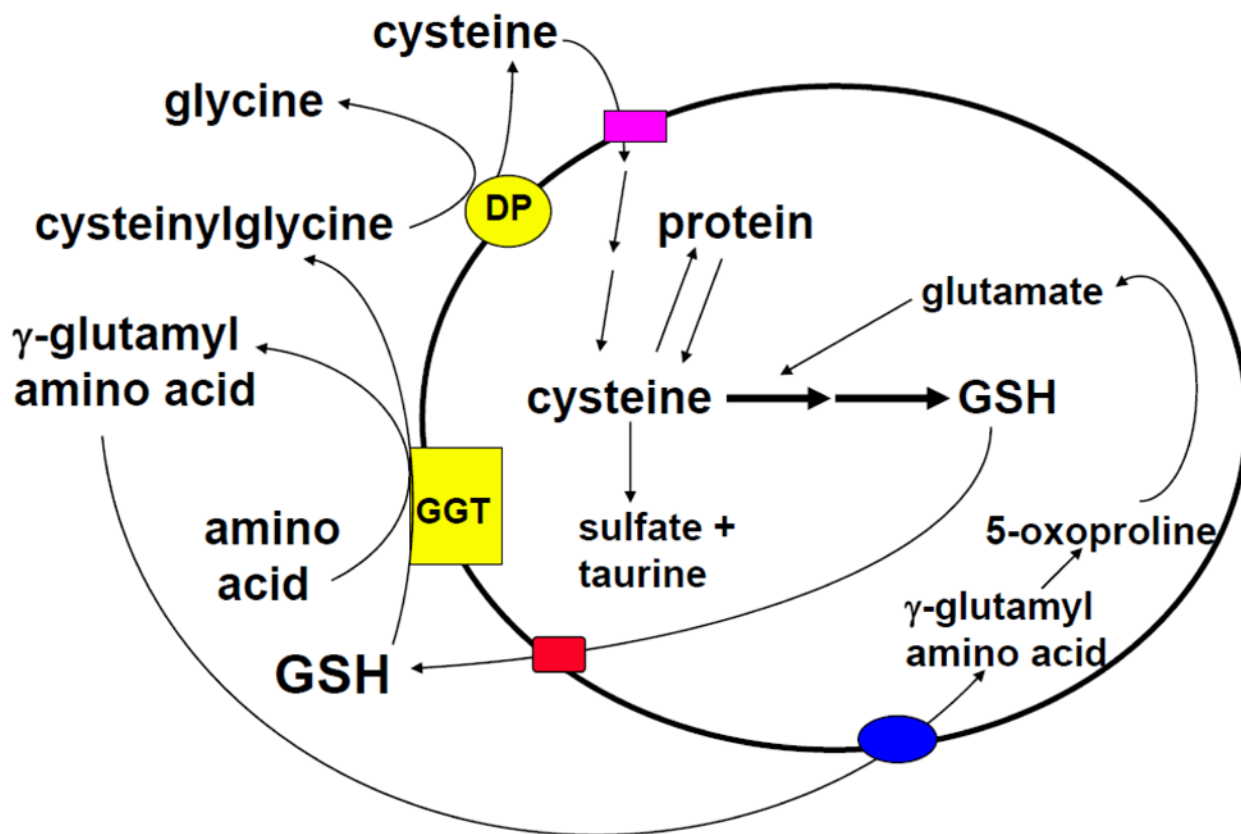
Fig. 3. GSH is a continuous source of cysteine via the γ-glutamyl cycle
GSH is transported out of the cell where the ecto-enzyme γ-glutamylpeptidase (GGT) transfers the γ-glutamyl moiety of GSH to an amino acid (the best acceptor being cystine), forming γ-glutamyl amino acid and cysteinylglycine. The 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.
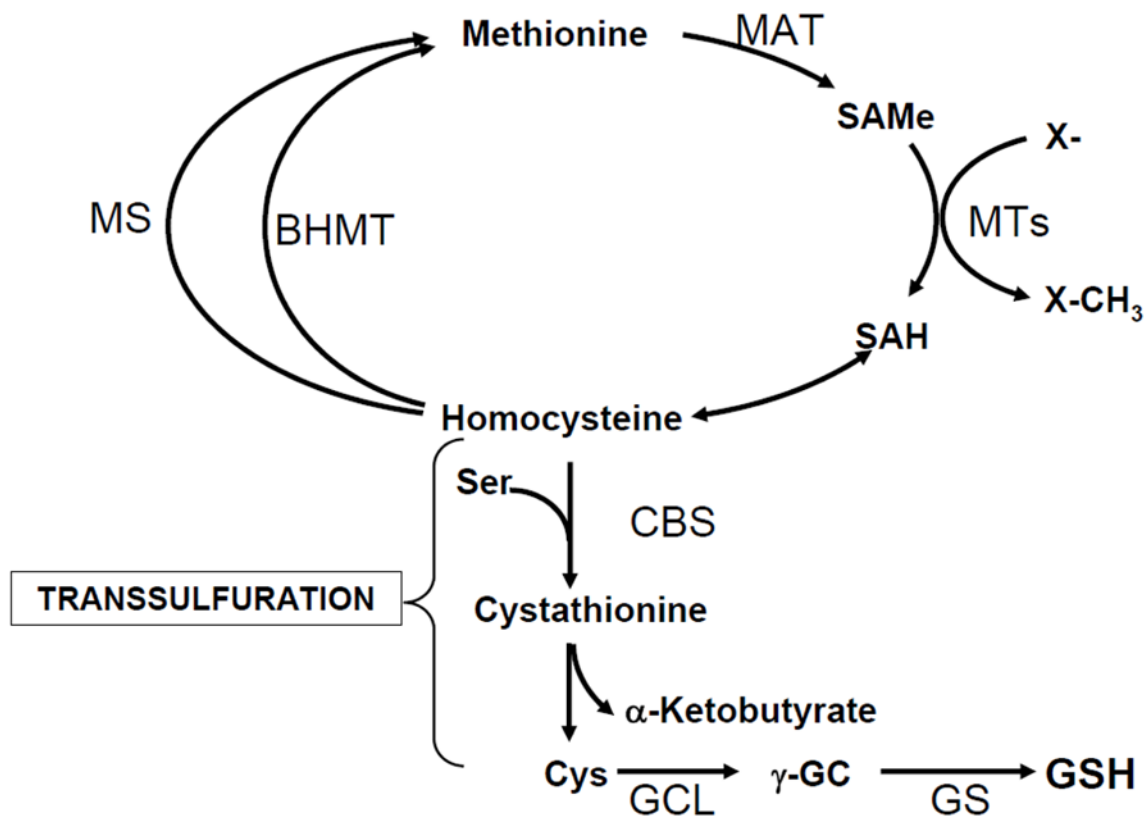
Fig. 4. Hepatic methionine metabolism
Liver plays a central role in methionine catabolism as up to half of the daily intake of methionine is catabolized to S-adenosylmethionine (SAMe) in the liver in a reaction catalyzed by methionine adenosyltransferase (MAT). SAMe is the principal biological methyl 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.