

Glutathionylation: a regulatory role of glutathione in physiological processes

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Glutathione (γ -glutamyl-cysteinyl-glycine) is an intracellular thiol molecule and a potent antioxidant that participates in the toxic metabolism phase II biotransformation of xenobiotics. It can bind to a variety of proteins in a process known as glutathionylation. Protein glutathionylation is now recognised as one of important posttranslational regulatory mechanisms in cell and tissue physiology. Direct and indirect regulatory roles in physiological processes include glutathionylation of major transcriptional factors, eicosanoids, cytokines, and nitric oxide (NO). This review looks into these regulatory mechanisms through examples of glutathione regulation in apoptosis, vascularisation, metabolic processes, mitochondrial integrity, immune system, and neural physiology. The focus is on the physiological roles of glutathione beyond biotransformational metabolism.

KEY WORDS: *epigenetics; GSH; GSSG; immunity; transcriptional factors*

From the toxicological point of view, γ -glutamylcysteinylglycine or glutathione is an important molecule in toxin metabolism and biotransformation of xenobiotics, as it converts several classes of compounds through conjugation. Conjugated electrophiles include various complex natural molecules, environmental chemicals, and drugs (1). Phase II conjugations are well known to toxicology (2-5). Glutathione participates in the defence against reactive oxygen species (ROS) and has a role as an antioxidant, as it metabolises oxygen radicals and peroxides and establishes intracellular redox balance (6).

It binds to other endogenous molecules forming, for example, the haemoglobin-glutathione complex, which serves as one of the markers of oxidative stress in human blood (7). With the advancement of detection methods in recent years (such as redox proteomic techniques and others), we have also learned about a vast number of interactions between glutathione and cellular proteins (8, 9).

We usually note the glutathione imbalance under various physiological conditions, and literature keeps demonstrating its regulatory roles in organisms (10-13).

The aim of this review is to summarise current knowledge about direct and indirect mechanisms of redox regulation and glutathionylation, which could serve as a guide to glutathione functions beside the ones usually described and evaluated by toxicologists.

PHYSIOLOGY AND TRANSCRIPTIONAL REGULATION OF GLUTATHIONE METABOLISM

Glutathione is a low-molecular-weight tripeptide present in microorganisms, plants, and mammals. It consists of cysteine (Cys), glutamic acid, and glycine and can be free or bound to proteins. In eukaryotic cells it can be a powerful enzyme cofactor. Its active site is the thiol (-SH) group of the Cys residue at which conjugation reactions take place. Most often, glutathione appears in its reduced form (GSH), but it can also appear in its oxidised disulphide (GSSG) form. The normal ratio between GSH and GSSG (GSH/GSSG) in the cytoplasm and mitochondria is >10 to 1 and in human serum it is 6-10 to 1.

Its concentrations in the cells are usually in millimolar ranges (7). It is the most abundant of all cellular thiols, which contain more than 90 % of the total nonprotein sulphur in tissues. About 90 % of GSH is located in the cytosol, while the remaining 10 % is located in the mitochondria, endoplasmic reticulum, and even in the nucleus (7, 14-17). In the endoplasmic reticulum it regulates internal proteins (18). It can shift from one cellular compartment to the other. For example, it shifts from the cytosol to the mitochondrial matrix thanks to mitochondrial transporter proteins (9, 19).

GSH is synthesised by all organs, liver in particular. GSH in the liver has a rapid turnover, with half-life of approximately 2-3 h. (7, 20-24). High concentrations are also found in the bile acid, up to 10 mmol L⁻¹. Its concentrations can be high in various blood cells and other

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tissues (7, 20), even in the vitreous of the eye (13). GSH levels adapt to physiological needs; in the oocyte, for instance, they keep around 7 mmol L⁻¹ but drop rapidly to nearly 0 mmol L⁻¹ after fertilisation and stay that low until the 3rd day of the blastocyst phase. After day 7, in the gastrulation phase, GSH levels rise with cell division to about 2 mmol L⁻¹ (25).

Hansen and Harris (25) provide a comprehensive comparison of glutathione tissue concentrations between species that can serve as biomedical models. It is a valuable reference for the comparison with experimental data obtained in various biomedical and toxicological studies utilising *in vivo* models.

REGULATION OF PROTEIN ACTIVITY BY REDOX BALANCE AND GLUTATHIONYLATION

Redox status and radical species [such as ROS and reactive nitrogen species (RNS)] posttranslationally modulate metabolic pathways and signal transduction in cell necrosis, apoptosis, and proliferation. They govern signal proteins in inflammatory and toxic reactions and regulate the binding of transcriptional factors to cognate DNA (23, 26-30). Brigelius-Flohe and Flohe (8) have published a most comprehensive review of the principles of redox and ROS regulation of proteins and cellular processes.

Glutathione participates in these regulatory mechanisms, too. It regulates protein activity in two ways. The first by indirectly changing protein charge and redox state (without binding directly to a protein) and therefore modulating protein action. The second is by directly binding to proteins and forming disulphide bonds (P-SSG). This second process is known as glutathionylation.

Glutathionylation

Glutathionylation is a thiol modification of proteins that complement other posttranslational regulatory mechanisms such as phosphorylation, acetylation, methylation, ubiquitination, glucuronidation, and palmitoylation in the cell (8, 9). In some cases, a protein can be phosphorylated and glutathionylated on different amino acid residues at the same time. Combinations of these posttranslational modifications modulate and fine tune protein activity. In the cell, glutathione reversibly binds to various structural, signalling, regulatory, receptor, or channel proteins and transcriptional factors involved in maintaining cell and tissue homeostasis or in pathophysiological processes (9).

The extent of glutathionylation greatly depends on the GSH/GSSG ratio in the cell. Usually, the higher the GSSG content (and GSH/GSSG ratio of 1:1 towards 1:10), the stronger the glutathione binding (8, 9, 16, 19). Proteins interact with GSH by forming P-SSG bonds on Cys residues of both the protein and GSH. Other cellular thiols

complement glutathionylation. Proteins bind other thiols beside glutathione by forming disulphide (P-SS) bonds or even thiol disulphide bonds between proteins (P-SS-P) as part of regulatory activities.

Glutathionylation and deglutathionylation are highly regulated biochemical processes. Glutathionylation is usually preceded by several biochemical intermediary changes on the protein. Sometimes it is preceded by other thiol-protein interactions (P-SS or P-SS-P), whose aim is to activate reaction. Similarly, glutathionylation is sometimes preceded by redox changes in target proteins (change of protein charges) initiated by ROS or redox imbalance, activation of RNS, or nitrosoglutathione (GSNO) (8, 9, 31, 32). Sometimes interactions between proteins and thiols can also create sulphenic (SOH), sulphinic (SO₂H), and sulphonic (SO₃H) or *S*-nitrosothiol (P-SNO) formations that damage proteins (similar to ROS) and lead to cell death. Repair of proteins damaged in this way is regulated by the thioredoxin (TRX, EC 1.8.4.8) enzymes.

Enzymes in glutathione reactions and glutathionylation

The overall glutathione metabolism and shuttling is governed by an array of enzymes in the cell. In the Phase II conjugation reactions glutathione *S*-transferase (GST, EC 2.5.1.18) catalyses reactions between reactive electrophiles and GSH to form xenobiotic conjugates (GS-X). Being an antioxidant, GSH metabolises intracellular oxygen radicals and peroxides, principally through enzymes such as glutathione peroxidase (GPx, EC 1.11.1.9, forms 1-8 in human) and peroxiredoxins (PRDXs, EC 1.11.1.15, 1-7 in humans), which, in turn, restore cellular redox balance. Enzymes that reversibly convert GSH and GSSG are GPx and glutathione reductase (approved name glutathione-disulphide reductase, GR, EC 1.8.1.7) (20, 33-37). Glutathione *S*-transferase P (GSTP or GSTP π , EC 2.5.1.18) catalyses *S*-glutathionylation of proteins, and glutaredoxin-1 (GLRX1, EC 1.20.4.1) catalyses deglutathionylation of proteins (5, 9, 19, 38). During glutathionylation, GSTP binds GSH and lowers the dissociation constant (pKa) of the thiols, creating a thiolate anion (GS⁻) as the active site for protein binding. Other GSH-dependent enzymes involved in glutathionylation are glutathione *S*-transferase omega 1 (GSTO1) (39), GR, GPx, PRDX, GST, membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) superfamily of enzymes, and sometimes even glyoxalase I (or lactoylglutathione lyase, GloI, EC 4.4.1.5) and glyoxalase II (or hydroxyacylglutathione hydrolase, GloII, EC 3.1.2.6). Each of these enzymes can participate in protein glutathionylation. Glutathionylation of proteins is also regulated by thioredoxins (mentioned above as enzymes that repair thiol-damaged proteins), since they have the ability to remove glutathione from proteins (40-42).

Summing up, ROS, intracellular thiols, and glutathionylation play a key role in regulating physiological and cellular processes. They do it by activating or deactivating transcription factors and signalling proteins or by increasing their turnover, expression, translocation, or binding to DNA.

Table 1 lists a number of glutathionylated transcription factors and signalling proteins that are involved in normal cell function. Some of them are described in detail in the text.

MOLECULAR INTERACTIONS WITH TRANSCRIPTION FACTORS

Glutathione synthesis is regulated by glutathionylation

Glutathione regulates its own cycle (Figure 1) by modifying the nuclear factor (erythroid-derived 2)-like 2 (Nrf2, NFE2L2, p45-related factor 2). This transcription factor protects the cell from oxidative stress by regulating several hundreds of antioxidant genes (16). Nrf2 also controls the transcription of important enzymes in the GSH

Table 1 *Glutathionylated proteins and signalling molecules involved in the regulation of cellular processes*

CELL PROCESSES AND REGULATION		
<i>Function</i>	<i>Proteins shown to be glutathionylated</i>	<i>References</i>
DNA and gene regulation, protein synthesis, and proteolysis	H3 (histone 3); 20s α (proteasome subunit); HSP90β (heat shock protein 90); HSP70 (heat shock protein 70); HSP60 (heat shock protein 60); sirtuin 1 : NAD-dependent acetylase 1	9, 16, 69, 157, 206, 207
Cell signalling channel proteins	14-3-3γS100 (S100 Calcium binding protein A1); SERCA (sarco/endoplasmic reticulum Ca ²⁺ -ATPase); RyR1 and 2 (Ryanodine receptor type1); CFTR (cystic fibrosis transmembrane conductance regulator); NaH⁺ transporter ; NaK-ATPase	9, 16
Transduction and regulatory proteins	MKP-1 (mitogen activated protein kinase1) on Cys ²⁵⁸ glutathionylation diminishes phosphorylation; MAPK c(X)5-Rmotif ; AMPK (5' AMP-activated protein kinase) glutathionylated during low GSH levels; PTEN (phosphatase and tensin homolog) glutathionylation inhibits PTENs phosphorylation of PIP3 (phosphoinositol 3 phosphate) lowering cellular phosphoinositol-biphosphate; PP2A (protein phosphatase 2); Src on Cys ²⁷⁷ ; PKCα (protein kinase C alpha) and PKCδ glutathionylation on Cys ⁴⁹⁹ inhibits enzyme activity decrease of cell growth and proliferation; c-jun GSNO activated can be glutathionylated during low GSSG concentrations on Cys ²⁶⁹ decreases DNA binding and inhibit proliferation; MEKK1 (Mitogen-activated protein kinase) Cys ¹²³⁸ glutathionylation inhibits activity and cell proliferation while increase apoptosis; PTP1B (protein tyrosine phosphatase 1β) glutathionylation on Cys ²¹⁵ activate proliferation; P50 glutathionylation on Cys ⁶² inhibits proliferation; caspase 1 ; caspase 8 ; procaspase 3 glutathionylation inhibits apoptosis, deglutathionylation promotes apoptosis; Ras possibly increase in proliferation while glutathionylation on Cys ¹¹⁸ ; Fas : glutathionylation on Cys ²⁹⁴ increases apoptosis; PKA (protein kinase A); Ask1 , RIP1 ; RIP 3 ; DAPDH , SSH1L (cofilin-phosphatase slingshot-1L); PKM (pyruvate kinase muscle isoenzyme).	8, 9, 16, 19, 26, 82, 86, 88, 99, 171
Intracellular redox status regulation	TRX (thioredoxin) glutathionylation on Cys ⁷² abolishes the enzyme ability to catalyse disulfide reductase activity; Grx (glutaredoxin) by deglutathionating proteins Grx binds GSH to itself, glutathionylated on Cys ²² ; GSTπ and ω bind glutathione on Cys ⁴⁴ and Cys ¹⁰¹ while transferring it on other proteins; GPRX peroxiredoxines.	8, 26, 53, 158
NO and GSH-NO signalling	eNOS (endothelial NO synthetase)	16, 164, 168, 175, 177, 179
Leukotrienes and prostaglandins	mPGES	108, 102, 130, 131;134;135
Transcriptional factors and gene expression	NFκB ; Nrf2 ; AP-1 ; IκB through IKK glutathionylation on Cys ¹⁷⁹ inhibits proliferation; HIF-1 ; STAT3 glutathionylated on Cys ^{418,428,468} inhibits proliferation.	16, 29, 61, 62, 208, 217

pathways and of the cystine/glutamate exchange transporter protein, which maintains intracellular GSH levels through cystine intake. Normally, the intake of extracellular GSH by the cell is low and occurs through GSH transporters (17, 43-45). Cystine serves as a substrate for the cystine-glutamate antiporter. This transport system, which is highly specific for cystine and glutamate, increases the concentration of cystine inside the cell. In this system, the anionic form of cystine is transported in exchange for glutamate. Cystine is quickly reduced to cysteine (46). Therefore, beside the anti-inflammatory function (discussed in sections below), Nrf2 confers greater redox potential and resistance to oxidative stress (47, 48).

Oxidative stress is a major activation signal for Nrf2 to dissociate from the complex it forms with its inhibitor, the Kelch-like ECH-associated protein 1 (Keap1) (16) (Figure 1). Keap1 subunit is glutathionylated, and GSH regulates its inhibiting activity. Following the dissociation, the Nrf2 subunit translocates to the cell nucleus and binds to the antioxidant-response element (ARE) in the promoter of target genes coding for antioxidant enzymes such as glutathione peroxidase-2 (GPX2), GST, haeme-oxygenase-1 (HO-1, EC 1.14.99.3), nicotinamide adenine dinucleotide phosphate oxidase (NADPH) : quinone reductase (EC 1.6.5.5), and aldo-keto reductase (49-51).

There where the Nrf2 pathway is impaired, such cells have low GSH concentrations and are highly prone to oxidative damage. Nrf2 signalling is also regulated by other posttranslational, transcriptional, translational, and epigenetic mechanisms, other signal proteins (e.g. p62, p21, and IQ motif-containing GTPase-activating protein) (45), and even hormones such as aldosterone (52). Another important regulator of the Nrf2/Keap1 complex is the thioreductase system, more specifically the selenoprotein thioredoxin reductase 1 (TRXR1, EC 1.8.1.9), a highly reactive enzyme.

Thus, beside glutathionylation, TRX pathways have a vital role in thiol and disulphide regulation of protein activation in the cells (40, 53). The GSH system and TRX are two distinct pathways with complementary actions (54). Glutathionylation regulating Keap1 works as a positive feedback loop. For example, the GSTP enzyme is regulated at the transcriptional level by Nrf2. When the cell is under oxidative stress, GSTP stimulates glutathionylation of Keap1 with GSH. This, in turn, activates Nrf2 and consequently increases the expression of GSTP (55). With a few exceptions, this is the general scheme of regulatory loop in most cells.

In addition, Nrf2 directly induces the transcription of glutamate cysteine ligase (GCL, EC 6.3.2.2) and GSH synthase (GSS, EC 6.3.2.3), two ATP-dependent enzymes responsible for *de novo* GSH synthesis in the cytoplasm (51). GCL limits the rate of GSH biosynthesis. It is a holoenzyme consisting of a glutamate cysteine ligase catalytic (GCLC) subunit and a regulatory glutamate cysteine ligase modifier (GCLM) subunit (56). Redox

imbalance and accumulation of oxygen radicals trigger GCLC and GCLM expression, which results in elevated GCL activity (Figure 1) and, consequently, higher GSH levels (16, 24, 57).

How glutathionylation regulates the NF- κ B and AP-1 signalling pathways

Beside Nrf2, GCL transcription is also regulated by other redox-sensitive transcription factors, such as the nuclear factor kappa B (NF- κ B, a homo- and heterodimer of subunit proteins p50, p52, p65, Rel-B, and C-Rel) or activator protein 1 (AP-1). These two transcriptional factors, therefore, affect cellular GSH levels as well (Figure 1). NF- κ B stimulates GCLC and GCLM transcription, the first by binding to the *gclc* promoter and the second indirectly, by activating AP-1, which, in turn, activates the *gclm* promoter (16, 58-60).

The effects of GSH on NF- κ B are distinctly different between the cytoplasm and the nucleus (Figure 1). In the cytoplasm, NF- κ B forms a complex with an inactive, non-DNA-binding κ B inhibitor (I κ B) which cannot enter the nucleus (Figure 1). The key step in NF- κ B activation is the detachment of I κ B from the heterodimer as a consequence of its ubiquitination and phosphorylation through the action of I κ B-kinase (EC 2.7.11.10) and protein kinases (such as protein kinase C; PKC; EC 2.7.11.13). When the dissociated I κ B is ubiquitinated and degraded in the proteasome (Table 1), activated NF- κ B moves to the nucleus and binds to DNA response elements in the enhancer and promoter regions of genes (16, 28, 61-67). Glutathionylation occurs at the NF- κ B p65 and p50 subunits and at the I κ B kinase (66-68) and S20 subunit of proteasome that degrades the dissociated I κ B subunit (69). In the cytoplasm, NF- κ B activity is either enhanced by GSH depletion or inhibited by exposure to a reactive chemical through Cys modification by covalent binding (29, 70). Experiments show that oxidants such as diamide, which oxidise GSH, lead to the accumulation of I κ B glutathionylated on Cys¹⁷⁹, which promotes NF- κ B release (70). In contrast, high thiol levels and GSH might inactivate NF- κ B by scavenging the oxidants needed to activate the enzymes involved in NF- κ B activation. Moreover, high GSH levels block protein kinase folding and consequently the phosphorylation of the I κ B/NF- κ B complex needed for the activated NF- κ B to reach the nucleus (7, 16, 61).

In the nucleus, GSH has the opposite function from the one in the cytoplasm (Figure 1). When NF- κ B is translocated to the nucleus, GSH increases the binding of NF- κ B to DNA. To do that, NF- κ B must be in its reduced form (71). Beside GSH, many other gene products activate NF- κ B through the feedback loop, such as interleukins (IL-1 β , IL-6, IL-8) and tumour necrosis factor alpha (TNF- α) (72), which participate in the pathogenesis of several inflammatory/immunomediated diseases. Glutathionylation regulates response in many cell types, including B and T

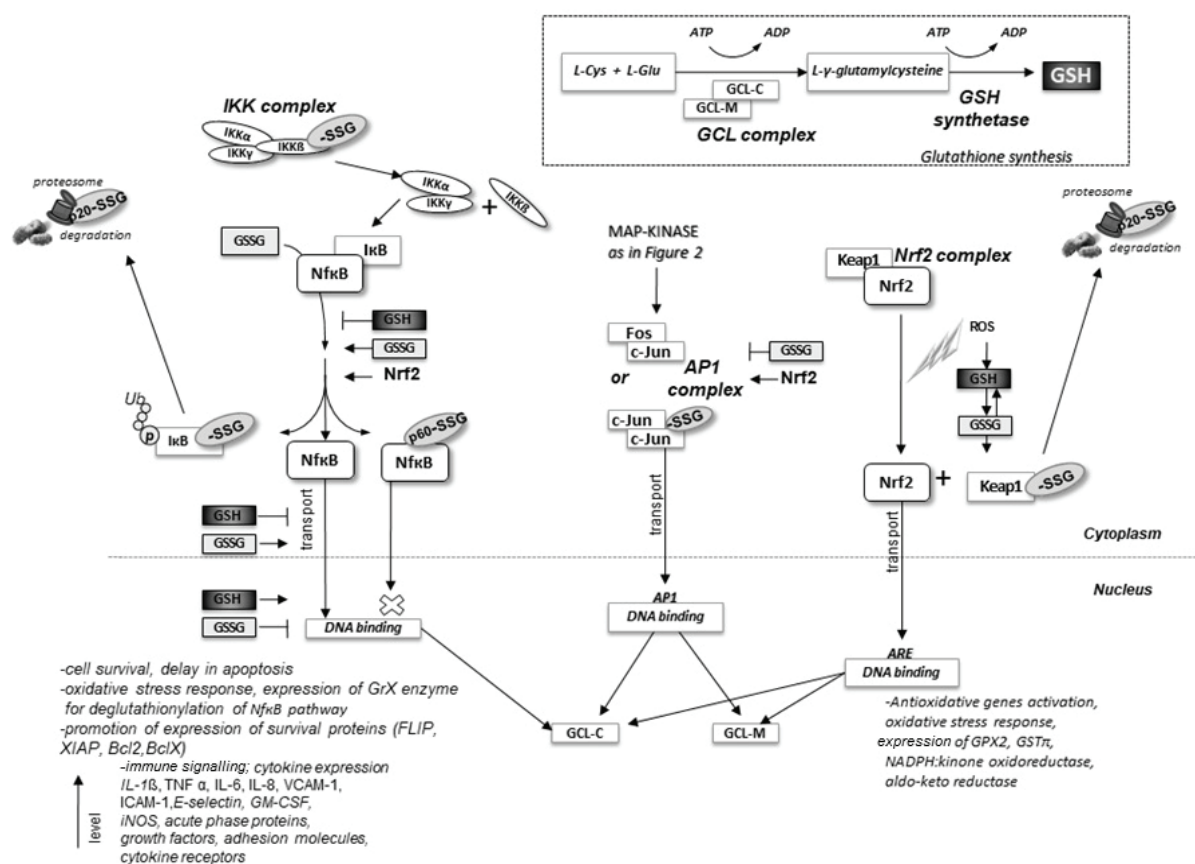


Figure 1 Biosynthesis of glutathione is regulated by Nrf2 through Nf-κB and AP1 signalling pathways

ROS – reactive oxygen species; GSH – reduced glutathione; GSSG – oxidised glutathione, glutathione disulphide; GST – glutathione transferase; -SSG – glutathione conjugate through disulphide bridges; Nrf2 – nuclear factor (erythroid-derived 2)-like 2 (NFE2L2); NF-κB – nuclear factor κB; IKK – IκB kinase; IKKα – α subunit of IκB kinase; IKKβ – β subunit of IκB kinase; IKKγ – γ subunit of IκB kinase; IκB – inhibitor of κB; L-Cys – L-cysteine; L-Glu – L-glutamate; GCL – glutamate-cysteine ligase; IL – interleukin; VCAM1 – vascular cell adhesion molecule 1; ICAM1 – intercellular adhesion molecule 1; Keap1 – Kelch-like ECH-associated protein 1; MAP kinase – mitogen-activated protein kinase (MAPK); AP-1 – activator protein 1; Fos – fos subunit of AP-1; c-Jun – c-Jun subunit of AP-1; GCLC – glutamate-cysteine ligase catalytic subunit; GCLM – glutamate-cysteine ligase modifier subunit; CFLAR – CASP8 and FADD-like apoptosis regulator; GRX – glutaredoxin; XIAP – X-linked inhibitor of apoptosis; BCL2 – apoptosis regulator Bcl-2; TNF-α – tumour necrosis factor alpha; SELE – selectin E; GM-CSF – granulocyte-macrophage colony-stimulating factor; iNOS – inducible nitric oxide synthase; GPx2 – glutathione peroxidase 2; GSTπ – glutathione transferase pi

lymphocytes, macrophages, and monocytes (62). For example, NF-κB activated in microglia and macrophages by cytokines, bacterial endotoxins, or other stimuli promotes the expression of several pro-inflammatory proteins, including TNF-α, cyclooxygenase 2 (COX-2), and inducible nitric oxide synthase (iNOS) (73).

Even in erythrocytes, NF-κB and major components of the canonical NF-κB signalling pathway interfere with the antioxidative system. Challenged by its inhibitors, compounds such as Bay 11-7082 and parthenolide, GSH levels drop, and so does erythrocyte survival (74). The examples of responses of NF-κB to GSH *in vivo* are numerous and specific (9, 19, 35, 64).

AP-1 is a transcription factor regulating physiological processes, cell growth and proliferation (including tumorigenesis), differentiation, apoptosis, and organogenesis (75-77). It is a complex composed of gene products (Figure

1) from the Jun and Fos proto-oncogene families (67, 71, 75). They form homodimeric (Jun/Jun) or heterodimeric (Jun/Fos) complexes that bind to DNA (71, 75). AP-1 transduces physiological signals from molecules such as cytokines, growth factors, and neurotransmitters to the nucleus (64). Environmental stimuli activate extracellular signal-regulated kinases (ERKs), which, in turn, activate AP-1, including its c-Fos and c-Jun family members (78). The AP-1 Jun/Fos complex binds to the tetradecanoylphorbol-13-acetate TPA-responsive element (TRE)/AP-1 on DNA (8, 75, 76). A “leucine zipper” domain between the Jun and Fos dimers of AP-1 is crucial for binding to TRE. Fos and Jun preserve cysteine residues in the basic motif, which, if oxidised, interferes with the binding of AP-1 to DNA (27). AP-1 gene transcription is activated by the phosphorylation of c-Jun on Ser⁶³ and Ser⁷³ through the activity Jun N-terminal kinase (JNK, also known as mitogen-activated

protein kinase 8, MAPK8) (64). However, glutathionylation can inhibit JNK (16, 78, 79).

In contrast, ROS can induce the synthesis, activation, and the translocation of AP-1 (8, 63, 67, 71, 77). Studies *in vitro* have shown that drops in GSH and rises in ROS levels can lead to the oxidation of c-Jun thiols, which promotes c-Jun redox-regulated AP-1 DNA binding activity, whereas an increase in GSSG inhibits c-Jun transcription (8, 16, 19, 80). The c-Jun subunit responds to both oxidative and nitrosative stress. Thus, a decrease in the GSH to GSSG ratio or even an increase in *S*-nitrosoglutathione (GSNO) can promote glutathionylation on c-Jun Cys²⁶⁹. The c-Jun subunit can also be activated by the formation of disulphide bridges between Cys³²⁰ residues. This is an example of how both oxidation mechanisms and disulphide formation on sites specific for S-glutathionylation on Cys residues regulate the DNA-binding of c-Jun (19, 66, 81-83). Glutathionylation of c-Jun promotes DNA binding of AP-1. Unlike other proteins undergoing glutathionylation, c-Jun is a protein that can be glutathionylated under relatively moderate GSSG concentrations (19). Binding of AP-1 can also be enhanced by TRX in cooperation with a nuclear protein known as redox factor 1 (Ref-1), as it promotes Cys transition from reduced to oxidised forms (27). AP-1 to DNA binding is inhibited by GSSG and GSNO in many cell types (19). Some researchers, however, report that AP-1 to DNA binding is stimulated if intracellular GSH is lowered with DL-buthionine-(*S,R*)-sulphoximine (BSO) or if the GSH to GSSG ratio is raised with diamide, like in HepG2 cells. This may partly be due to the redistribution of GSH in the nucleus in addition to the presence of Ref-1 and TRX (65, 66).

In addition to regulating many genes, AP-1 to DNA binding also upregulates the expression of GCL and consequently GSH levels (Figure 1). One example is the increased GSH in the bronchoalveolar lavage fluid (BALF) of chronic cigarette smokers (84), as oxidation probably activates AP-1. However, one hour after acute smoking up to two cigarettes, GSH depletes quickly because of inadequate adaptive increase in antioxidant levels in the epithelial lining fluid of chronic cigarette smokers, insufficient to protect the lung epithelium even when exposure is so short (84).

Glutathione interaction with apoptosis signal proteins

Evidence collected in recent years shows that a balance between GSH and GSSG is important for apoptosis regulation. Changes in the GSH/GSSG ratios can induce apoptosis through several intracellular signalling pathways (Table 1), but mainly through glutathionylation of specific proteins, as illustrated in Figure 2 (marked as disulphide conjugates, -SSG) (6, 19, 85, 86). They include NF- κ B, IKK, AP-1, JNK, mitogen-activated protein kinase 5 (MAP3K5, EC 2.7.11.25), caspases (Cas, EC 3.4.22.55-3.4.22.64), phosphatidylinositol-3,4,5-trisphosphate

3-phosphatase (PTEN, EC 3.1.3.67), protein kinase C (PKC, EC 2.7.11.13), and the death-inducing signalling complex (DISC). In DISC, glutathionylated is ASK (Figure 2), which determines whether the signal from the extracellular cell surface death receptor (Fas) will proceed to caspase-3 (Cas-3, EC 3.4.22.61) in the cytoplasm. Figure 2 also shows the mitochondrial activity and the GSH-dependent BCL2-associated X protein (BAX), which regulates apoptosis activation. Several experimental studies describe BAX as an early step in H₂O₂-induced apoptosis (26, 85). In them, cells were treated with the tert-butyl hydroperoxide (TBH), which causes redox imbalance in the mitochondria and generation of ROS. Mitochondria lose integrity, release cytochrome c, and activate Cas-9 and Cas-3. Ultimately, Cas-3 glutathionylation will determine whether DNA fragmentation factor subunit alpha (DEFA) will cause DNA fragmentation and apoptotic cell death (87, 88). Hansen and Harris (25) showed that cell death is mediated by an increase in cellular GSSG rather than by a decline in GSH. However, during differentiation and embryonic development, necrosis and apoptosis are characterised by GSH decline and oxidising conditions, while during proliferation they are characterised by reducing conditions and higher GSSG.

Glutathione, angiogenesis, and vascularisation

Hypoxia-induced factor-1 alpha (HIF-1 α) is a transcription factor (among those listed in Table 1) that interacts with GSH specifically under ischemic and hypoxic conditions (89). Watanabe et al. (89) have demonstrated that higher ROS levels in ischemic tissues lead to glutathionylation of the Cys⁵²⁰ residue of HIF-1 α . In experimental *in vitro* studies, HIF-1 α was glutathionylated by adding cell-permeable GSSG-ethyl ester or 2-acetyl-amino-3-[4-(2-acetyl-amino-2-carboxyethylsulphonylthiocarbonylamino)phenylthiocarbonylsulphonyl] propionic acid (2-AAPA), both of which inhibit GSR. GSR inhibition decreases GSH and increases GSSG, promoting glutathionylation on the Cys⁵²⁰ residue of HIF. This glutathionylation on Cys⁵²⁰ can be reversed by activating GLRX1, which then removes GSH from HIF-1 α . In another experiment, GLRX1 ablation caused accumulation of stabilised, glutathionylated HIF-1 α in C2C12 cells. Stabilised HIF-1 α promoted revascularisation by inducing higher vascular endothelial growth factor A (VEGF-A) production. In *in vivo* experiments, this recovered blood flow and improved ischaemic revascularisation of the muscles after femoral artery ligation (89).

Hughes et al. (39) also found that HIF-1 α is an important factor in macrophage function. Madamanchi and Runge (83) confirmed that, in addition to glutathionylation, the GSH/GSSG balance affects vascularisation indirectly (which can lead to pathological changes of blood vessels) through other complex redox signalling interactions (83). Future experiments will try to answer how can stabilised

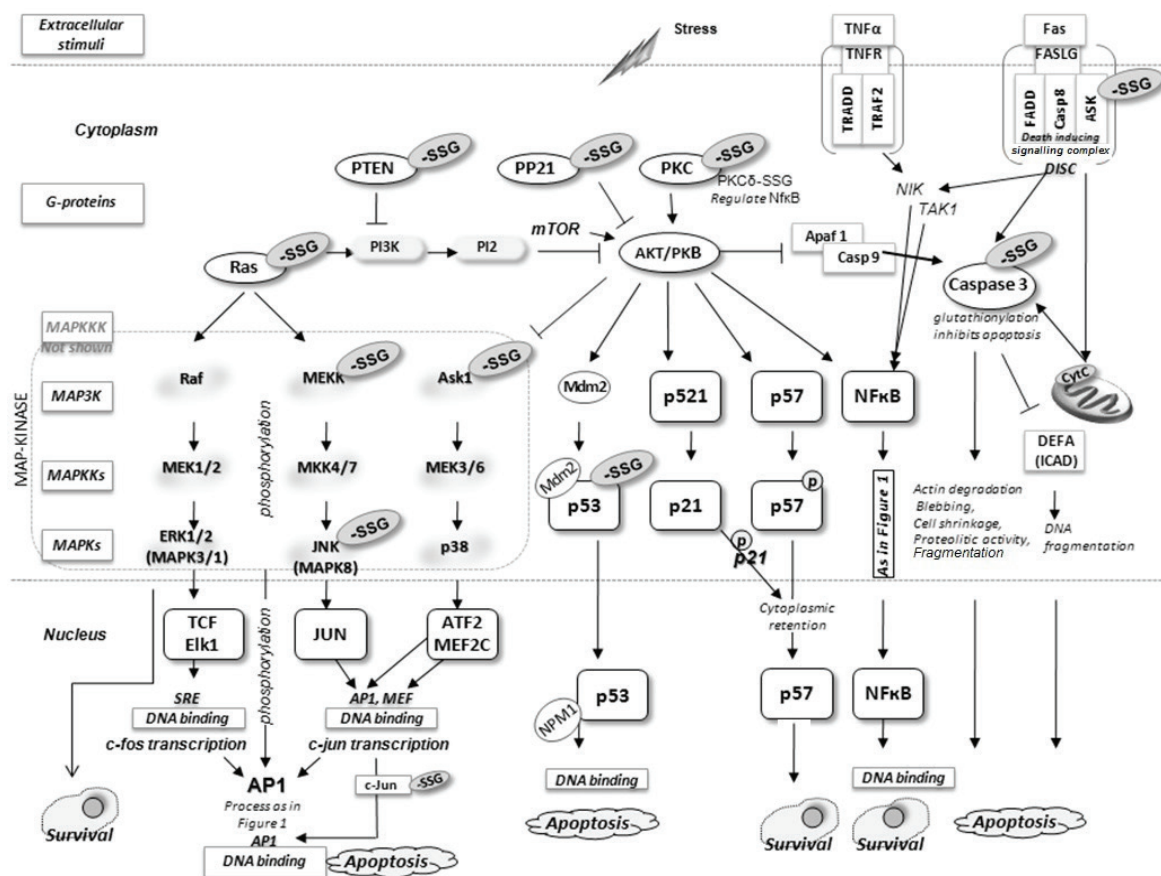


Figure 2 Glutathionylation of signalling proteins and transcription factors involved in pathways regulating cell death or survival -SSG – glutathione conjugate through disulphide bridges; Ras – a small GTP-binding protein GTP-ase); Raf kinase – proto-oncogene serine/threonine-protein kinase; PTEN – phosphatase and tensin homologue; PI3K – phosphoinositol 3 kinase; MAPK – mitogen-activated protein kinase; MAPKK – mitogen-activated protein kinase kinase; MEK1/2 – mitogen-activated protein kinase 1 or 2 (MAP2K1/2); ERK 1/2 – extracellular signal-regulated kinases 1 and 2 (MAPK 3/1); MEKK – mitogen-activated protein kinase kinase (MAP3K); MKK – mitogen-activated protein kinase kinase (MAP2K); Ask1 – apoptosis signal-regulating kinase 1 or mitogen-activated protein kinase kinase kinase 5 (MAP3K5); MEKK 3/6 – mitogen-activated protein kinase 3 or 6 (MAP3K3/6); TCF Elk1 – ternary complex factor (TCF) ETS domain-containing protein Elk-1; SRE – serum response element of DNA; AP-1 – activator protein 1; PI3K – phosphatidylinositol-4,5-bisphosphate 3-kinase; JNK – janus kinase c-Jun N-terminal kinase or mitogen-activated protein kinase 8 (MAPK8); JUN – c-Jun gene; p53 – tumour suppressor gene p53; ATF2 – activating transcription factor 2; MEF2c – myocyte-specific enhancer factor 2c; AKT/PKB – AKT1-RAC-alpha serine/threonine-protein kinase or protein kinase B (PKB); MDM2 – mouse double minute 2 oncoprotein; TNFα – tumour necrosis factor alpha; TNFR – tumour necrosis factor receptor superfamily 1A and 1B (TNFRSF1A/1B); TRADD – tumour necrosis factor receptor type 1-associated DEATH domain protein; TRAF – TNF receptor-associated factor 2; mTOR – mechanistic target of rapamycin; APAF1 – apoptotic peptidase activating factor 1; Casp 9 – caspase 9; Casp 3 – caspase 3; FAS – Fas cell surface death receptor; FasR – FAS receptor or apoptosis antigen 1 (APO-1 or APT); FADD – Fas-associated protein with death domain synonym MORT1; FASLG – Fas ligand; DEFA – DNA fragmentation factor subunit alpha (also known as ICAD); NPM1 – nucleophosmin 1

(glutathionylated) HIF-1α proteins affect tumour vascularisation and help treatment.

p53

Under oxidative stress, the activity of p53 is governed by S-glutathionylation (Figure 2). This is when GSH-adducts inhibit p53 on the Cys residues in the proximal DNA-binding domain (90). In stress, most posttranslational modifications on p53 enhance its transcriptional activity, which, in turn, triggers the cell cycle checkpoints. S-glutathionylation on Cys¹⁴¹ of p53 interferes with p53 dimerisation, which inhibits p53 to DNA binding and interferes with its main tumour suppressing function but

also with its inhibition of angiogenesis, its role in apoptosis, and genomic stability.

In its anti-cancer role, p53 activates DNA repair proteins and arrests cell growth by holding the cell cycle at the G1/S phase for as long as necessary for the DNA repair proteins to fix the damage. Only then will the cell cycle continue (83, 90).

GSH interaction is associated with yet another important protein, nucleophosmin (NPM1) (Figure 2). NPM1 is a histone chaperone (Table 1), important in the transport and synthesis of ribosome and nuclear acid stability. Under normal conditions, NPM1 is bound to RNA and partly to DNA. Cellular stress triggers its dissociation from nucleolar

nucleic acids and translocation from the nucleus to the nucleolus, which activates p53 to DNA binding and p53-dependent apoptosis. Using live-cell imaging and redox biosensors, Yang et al. (91) have demonstrated that NPM1 undergoes glutathionylation on Cys²⁷⁵, which triggers its dissociation from nucleolar nucleic acids. In contrast, its mutant protein C275S (cys/ser substitution) could not be glutathionylated and remained bound to DNA under nucleolar stress. Among several anti-proliferative compounds interacting with NPM1, the authors identified avrainvillamide, which binds specifically to NPM1 at Cys²⁷⁵ and disperses NPM1 into the nucleoplasm. The application of actinomycin D, which binds to glutathione at Cys²⁷⁵, causes a similar effect on NPM1. A recent clinical application of actinomycin D caused substantial remission in patients with acute myeloid leukaemia. This GSH involvement and manipulation with its active binding site on NPM in nucleolar stress sensing may help to develop therapeutic strategies (91).

Another protein involved in GSH metabolism and DNA control by p53 is the MDM2 oncoprotein, a major negative regulator of the p53 tumour suppressor protein. Riscal et al. (92) showed that MDM2 can regulate amino acid metabolism and redox homeostasis of serine/glycine metabolism and, therefore, GSH recycling in the cell.

Glutathione interactions with signalling and regulatory proteins in metabolic processes

Important evidence is emerging of GSH regulatory roles in various metabolic states (Table 2), although such research is just emerging in contrast to the research of glutathionylation in the immune system, cell growth, survival, apoptosis, or tumorigenesis.

Adipocytes are major energy depots, and peroxisome proliferator-activated receptor γ (PPAR γ) is the major transcription factor controlling the growth and differentiation of adipocytes. This transcription factor is regulated by two signalling molecules: 15-deoxyprostaglandin J2 (15d-PGJ2) and 4-hydroxynonenal (4-HNE), which is a product of lipid peroxidation. Both are conjugated with GSH, which lowers PPAR γ activity (93, 94). Additionally, obesity-induced insulin resistance is linked to 4-HNE as one of the most abundant lipid aldehydes in adipose tissue. In adipocyte oxidative stress, it is metabolised by glutathione S-transferase A4 (GSTA4) into pro-inflammatory glutathionyl-HNE (GS-HNE) and glutathionyl-1,4-dihydroxynonenal (GS-DHN). According to a recent discovery by Frohnert et al. (95), this is how adipocyte dysfunction results in tissue inflammation and insulin resistance.

In mitochondria, peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α), MAPK, and sirtuin (for example sirtuin 1, Table 1, possibly others too) are regulated by glutathionylation or ROS. Glutathionylation regulates complex I, which increases mitochondrial superoxide anion (O₂⁻) production. Complex II proteins are continuously

glutathionylated. Other mitochondrial proteins, uncoupling proteins (UCP 2 and 3), ATP synthetase, and succinyl-CoA-synthetase are also glutathionylated (16). GSH also binds to coenzyme A : glutathione-disulphide oxidoreductase (EC 1.8.4.3), an important enzyme in the metabolism of amino acids (7, 39, 94), and to dehydroascorbate reductase [GDOR, also known as glutathione dehydrogenase (ascorbate), EC 1.8.5.1], which reduces dehydroascorbate to ascorbate (7). Table 2 lists other mitochondrial proteins and effects related to glutathionylation, such as mitochondrial fusion.

It is believed that physical inactivity disrupts metabolic and redox homeostasis and eventually leads to ROS-associated diseases (7). There are clear connections between metabolic states and GSH. For example, physical ability in GSH-deficient rats is known to be reduced in half (54). Most of the research on this topic assumes that oxygen is consumed during physical exercise, that GSH is oxidised, and that GSSG tissue levels increase. This GSSG build up, however, upregulates glutathionylation. Exercise enhances GSH levels by inducing GSH synthesis and also by increasing GSH regeneration from GSSG. In other words, high oxygen consumption during physical exercise restores GSH through transcriptional mechanisms explained earlier in the text as well as through the TRX pathways. Animal experiments with exercise suggest that the GSH and TRX pathways are opposite pathways, as TRX-1 activity negatively correlates with GSH (54).

Exercise is also related to angiogenesis, which is regulated by HIF-1 α . Increased ROS production through exercise is believed to affect DNA methylation and posttranslational modifications of histone residues, which, in turn, create conditions for heritable epigenetic adaptive chromosomal conditions. Physiological response to exercise is elevated ROS and biochemical imbalance in the muscle tissue, which make muscles contract (54). Creatine kinase M isoenzyme (CK-M, EC 2.7.3.2), which regenerates ATP in muscles, has been shown to be glutathionylated at Cys²⁸³. However, this regulation was investigated only *in vitro* (16, 18). During muscle contraction, glutathionylation promotes ryanodine receptor (RyR) 1 and 2 activity, which controls the release of Ca²⁺ into muscle cells. Glutathionylation occurs on Cys⁶⁷⁴ of the sarcoplasmic reticulum Ca²⁺-ATPase (SERCA, EC 3.6.3.8), but GSH has also been shown to regulate other endoplasmic reticulum proteins beside SERCA, such as immunoglobulin heavy chain-binding protein (BiP), protein disulphide isomerase (PDI), calnexin, calreticulin, and endoplasmic (16, 18).

In metabolic syndrome-related cardiovascular diseases, reduction of plasma and vascular GSH results in increased oxidative stress, which promotes atherosclerosis (96). TNF- α activates NF- κ B, which, in turn, upregulates the expression of adhesion molecules on the surface of endothelial cells and vascular smooth muscle cells of blood vessels. By inhibiting mitochondrial electron transport chain activity, TNF- α promotes interaction of electrons with O₂

Table 2 *Glutathionylated proteins involved in the regulation of physiological systems*

PHYSIOLOGICAL SYSTEMS		
<i>Function</i>	<i>Proteins shown to be glutathionylated</i>	<i>References</i>
Mitochondrial ROS production and ROS scavenging	MMC I: mammalian mitochondrial complex I glutathionylated under various conditions) deactivates electron transfer and increases ROS production; MMC II: mammalian mitochondrial complex II glutathionylated increases activity and decreases ROS production	9, 16
Mitochondrial function and integrity	ANT: adenine nucleotide translocase on Cys ⁵⁶ ; mPTP: mitochondrial transport pore protein opens upon glutathionylation; OGC: oxoglutarate carrier; DIC: dicarboxylate carrier - GSH transport through mitochondrial membrane in liver and kidney; TTC: tricarboxylate carrier - GSH transport through mitochondrial membrane in brain; MFN2: Mitofusin-2 and GSSG binding stimulates mitochondrial fusion	22, 216
Mitochondrial Biochemistry, bioenergetics	ATP5A1: ATP synthetase α -subunit isoform 1 on Cys ²⁴⁴ and Cys ²⁹⁴ in conditions of high glucose; UCP 2: uncoupling protein 2	9
Energy metabolism and glycolysis	GAPDH: glyceraldehyde 3-phosphatase dehydrogenase; ATP5A1: ATP synthetase α -subunit isoform 1; carbonic anhydrase; haemoglobin; CK: creatine kinase; IDH: isocitrate dehydrogenase; αKGDH: α -ketoglutarate dehydrogenase; PDH: pyruvate dehydrogenase e2 subunit; aconitase; storage and transport of cysteines	2, 9, 16, 19, 67, 80, 211, 212, 213
Cell adhesion, architecture and movement, muscles	β-actin on Cys ³⁷⁴ ; β-tubulin; cofilin in monocyte chemotaxis; anexin A2; myosine; troponin I	9, 16, 214, 215
Neural system	Nrf2/Keap	55
	Amyloid	213
	Na,K-ATPase	185-195
	Parkinson's disease; Huntington's disease; Alzheimer's disease	
	MGST1: microsomal glutathione S-transferase 1; CPLX2: complexin-2;	
Immune system	PBK: lymphokine-activated killer T-cell-originated protein kinase; CPSF6: cleavage and poly adenylation specificity factor subunit 6, PRDX5: mitochondrial peroxiredoxin-5; RPLP1: 60S acidic ribosomal protein P1; PRDX3: mitochondrial thioredoxin-dependent peroxide reductase; DSTN: actin-depolymerizing factor; PCBP1: poly(rC)-binding protein 1; EIF5A: eukaryotic translation initiation factor 5A; AKR1C1: aldo-ketoreductase family 1 member C1; AKR1C3: aldo-ketoreductase family 1 member C3; MAL: MyD88 adaptor-like Cys ⁹¹ glutathionylation critical protein in innate immunity; NLRP3 /IL-1β inflammasome pathway	65, 66, 139, 189, 209, 210
	Monocytes; macrophages; T-lymphocyte function	9, 16, 19, 149, 150
	Cytokine production and immune response	7, 65, 66, 139, 142, 147

and increases the production of superoxide anion (O₂⁻) (65, 97) and hydrogen peroxide (79, 98), which intensify the pathological processes resulting in the activation of inflammatory cells in the adipose tissue, endothelial dysfunction, and atherogenesis in vascular tissues (72, 99). Glutathionylation, therefore, plays a complex regulatory role in obesity, metabolic syndrome-related cardiovascular diseases, and diet-induced dyslipidaemia and cholesterolaemia (83, 100).

The list of transcription factors and regulatory proteins (101) involving glutathionylation will expand in the future. Table 1 summarises other cellular regulatory proteins that have not been described in more detail in this text, because this review will focus on other signalling molecules such as eicosanoids, cytokines, and nitric oxide (NO) and their relation to glutathione.

GLUTATHIONE AND EICOSANOIDS

MAPEG superfamily enzymes and their relation to GSH

The superfamily of large, membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) share structural evolutionary relationship with the cytosolic GST. This superfamily includes enzymes crucial in leukotriene (LT) and prostaglandin (PG) physiology and synthesis, namely leukotriene synthases (LTCS), prostaglandin synthases (PGES), 5-lipoxygenase activating protein (FLAP), and microsomal glutathione S-transferases (MGST 1-3) (102).

Their structural similarity to GST makes glutathione an important cofactor in their activity and in the physiology of eicosanoids. The MAPEG enzymes participate in inflammatory processes and, to a certain extent, in biotransformation and detoxification. However, evidence of their glutathionylation is scarce, because of methodological challenges, since they are membrane-integrated proteins and therefore slightly more complex to isolate and investigate, but new data are being gathered as research methods advance (102).

Leukotrienes (LTs)

Leukotrienes are fatty acid signalling molecules first found in leukocytes (hence their name). In the synthesis of some of them GSH plays a major role (103). LTs are generally recognised as potent inflammatory factors, particularly activated during inflammation. Their role is to attract certain white blood cells, but also to enhance the action of PGs during reproduction by binding to G-protein-coupled receptors on target cells (104-107). Leukotriene B4 (LTB4), leukotriene C4 (LTC4), and their metabolites leukotriene D4 (LTD4) and E4 (LTE4) stimulate endothelial cell-leukocyte adhesion and chemokine excretion and act as potent neutrophil chemoattractants. They are mainly involved in vascular leakage and epithelial barrier functions (108-110). Cysteinyll LTs (LTC4, LTD4, and LTE4) are crucial mediators of inflammation and inflammatory diseases of the respiratory and cardiovascular systems, such as bronchial asthma (111), allergic reactions (104, 112), bronchoconstriction, and neutrophil extravasation (108).

LTs are synthesised through multistep enzymatic pathways (Figure 3). Their biosynthesis begins with cytosolic phospholipase A2 (EC 3.1.1.4) mediating the release of arachidonic acid from the cell membrane and converting it to unstable epoxide intermediate, leukotriene

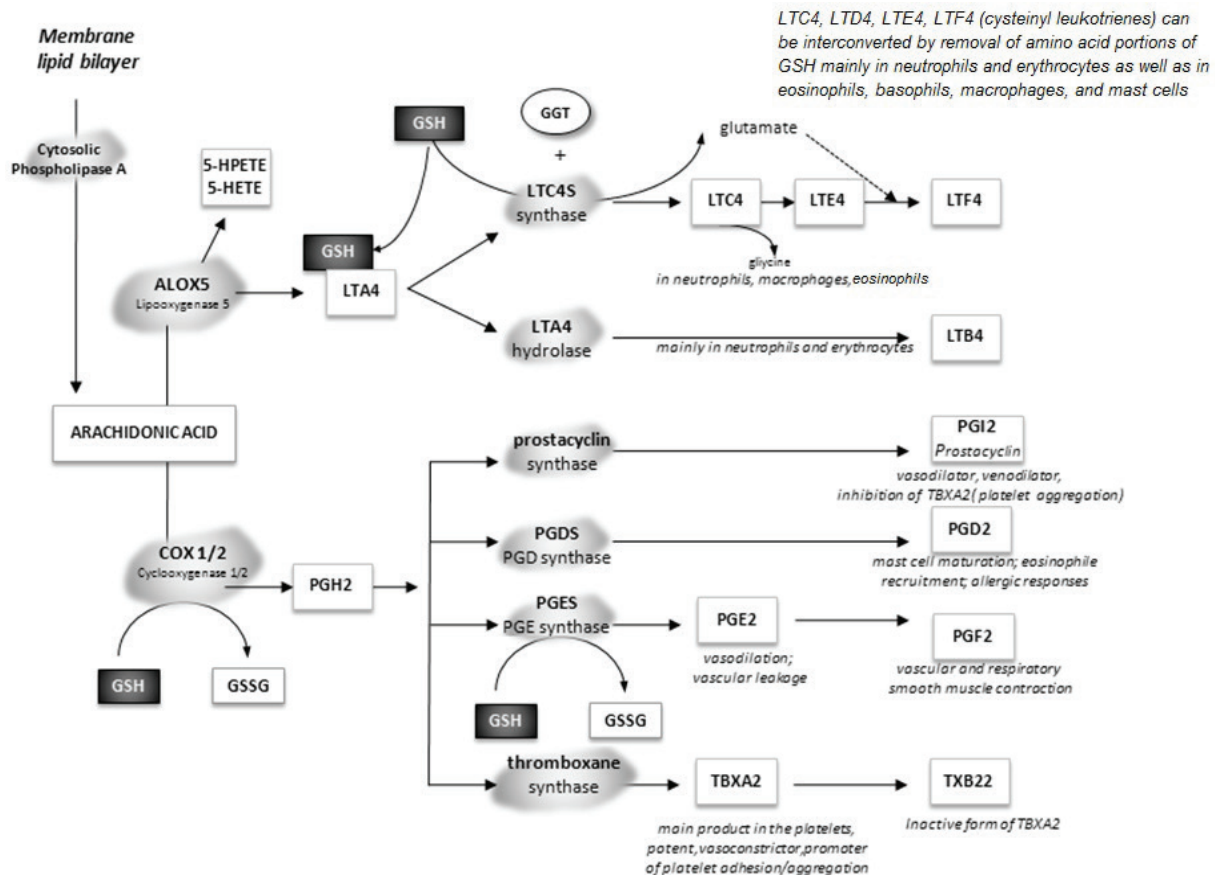


Figure 3 The role of glutathionylation in the synthesis of eicosanoid molecules

5-HPETE – hydroperoxyeicosatetraenoic acid or arachidonic acid 5-hydroperoxide; 5-HETE – 5(S)-hydroxyicosatetraenoic acid; ALOX5 (LOX5) – arachidonate 5-lipoxygenase; COX1/2 – cyclooxygenase 1 or 2, also known as PTGS1/2 – prostaglandin-endoperoxide synthase 1 or 2; LTA4 – leukotriene A4; LTC4 – leukotriene C4; LTE4 – leukotriene E4; LTF4 – leukotriene F4; LTB4 – leukotriene B4; PGI2 – prostaglandin I2; PGD2 – prostaglandin D2; PGF2 – prostaglandin F2; TBXA2 – thromboxan A2

A4 (LTA4). This intermediate can be transformed to LTB4 by hydrolysis or to LTC4 by conjugation with glutathione. Therefore, LT levels correlate with GSH levels (104, 108, 109, 110). The conjugation of LTA4 with reduced GSSG mediates cysteinyl LT synthesis (111-115) through glutathione S-transferase leukotriene C4 synthase (LTC4S, EC 4.4.1.20) from the MAPEG superfamily to form an intracellular parent compound, the pro-inflammatory LTC4 by adding GSH at C6 of LTA4 (113, 116). Synthesised and excreted LTC4 is highly involved in signalling by blood cells and degraded extracellularly by the liver, where it is secreted and metabolised into the biliary system. Bioconversion of LTC4 is the result of the interaction with γ -glutamyltransferase (GGT, EC 2.3.2.2), which is also the enzyme known to eliminate and degrade glutathione in the kidney, but here it has another, complementary role in inflammation signalling with LTC4 synthase (LTC4S) (116).

Leukotriene C4 synthase

LTC4S (EC 4.4.1.2) is a 17-kDa integral perinuclear homodimer membrane enzyme. It shows GST activity, strictly specific for LTA4 as a substrate (104, 117-119). LTC4S is similar to other enzymes important for GSH metabolism (covered at the beginning of the text), having the same precursors as the enzymes participating in GSH metabolism (120). It is the only enzyme in haematopoietic cells that utilise GSH and LTA4 to produce LTC4. LTC4S differs from conventional GST by its lack of homology, inability to conjugate GSH to xenobiotics, selectivity for LTA4, differential susceptibility to inhibitors, and inability to bind specific microsomal GST antibodies (118, 120-124, 144).

Human LTC4S cDNA encodes a protein of 150 amino acids (104, 117). The LTC4S monomer has four transmembrane α -helices and forms a symmetric trimer as a unit with functional domains across each interface. The structure of the enzyme is complex and has the active site that forms two "U" loop conformations (111, 119) on the cytoplasmic side of the membrane. The first hydrophilic loop of LTC4S binds LTA4 at amino acid residue Tyr⁹³, and the second hydrophilic loop is essential for GSH conjugation where GSH positions its thiol group on arginine (Arg) on the membrane-enzyme interface (125). At the LTA4 active site, Arg¹⁰⁴ activates GSH to produce a thiolate anion that attacks carbon C6 of LTA4. This creates a thioether bond that results in LTA4 glutathionylation (119). The expression of LTC4 synthase is limited to cells of the bone-marrow lineage. In other tissues, LTC4 synthesis is possible with microsomal GSH S transferase-II (MGST-II) when LTC4 synthase is not expressed, such as in the liver and endothelial cells (123). In such tissues LTC4 can be formed through the transcellular metabolism of LTA4, where GSH conjugates to LTA4 to form LTC4 through the MGST2 and MGST3 enzymes (104).

A recently synthesised nanomolar inhibitor of LTC4S glutathionylation, the so called TK04, will be a useful tool in future pharmacological research of GSH/LTC4S interactions and modulation of inflammatory signalling through glutathionylation in blood cells (110).

Prostaglandins

Figure 3 illustrates the involvement of glutathione in PG metabolism. PGs are biologically active derivatives of arachidonic acid and other polyunsaturated fatty acids. Prostaglandin E2 (PGE2) is expressed in inflammation, vasodilation, and vascular leakage; prostaglandin D2 (PGD2) in mast cell maturation, eosinophil recruitment, and allergic responses; prostaglandin F2 (PGF2) in vascular and respiratory smooth muscle contraction; and prostaglandin I2 (PGI2) in platelet inhibition (103, 108, 126).

The biosynthesis of PG is initiated by the release of arachidonic acid from membrane phospholipids, similar to LT metabolism described above (108, 126). It involves oxygenation and cyclisation to an unstable endoperoxide intermediate, prostaglandin G2 (PGG2). Subsequent reduction of C5 hydroperoxide moiety of PGG2 yields prostaglandin H2 (PGH2). This reaction is catalysed by cyclooxygenases [COX, aka prostaglandin-endoperoxide synthase (PTGS), EC 1.14.99.1], which come in two forms: the constitutive type COX-1 and the induced type COX-2. Both are indirectly controlled by GSH (103, 108). COX-2 is produced in fibroblasts, macrophages, and epithelial cells in response to external chemicals or IL-1 β and TNF α , which indirectly control PGE2 production.

Prostaglandin E synthase (PGES, EC 5.3.99.3) also needs reduced GSH to convert the endoperoxide group of PGH2 into the 9-keto-11-hydroxy moiety of PGE2. If GSH is not available, there is no PGE2, as demonstrated with A549 cells, whose microsomal fractions containing enzymes for PG synthesis were incubated without GSH (102, 108).

Prostaglandin E2 synthases

There are three types of PGES (EC 5.3.99.3): two microsomal (mPGES1 and mPGES2) and one cytosolic (cPGES aka PGES3). These enzymes have different origin, different physiological properties, different cell localisation, regulation, and catalytic mechanisms, yet they are all involved in the synthesis of the same PG (127, 128). Earlier it was believed that mPGES2 was glutathione-independent, in contrast to the rest of the PGESs, but recent research has confirmed that mPGES2 binding activity is possible only in the presence of glutathione (101).

As for mPGES1, it comes from the GSH-dependent MAPEG superfamily and is functionally linked to COX-1 in basal PGE2 and COX-2 in delayed PGE2 synthesis (129-133). It is highly expressed in the brain, heart, muscle, kidney, and liver (103). Although mPGES1 is closely related

to MGST1 (38 % homology), it is unable to catalyse the conversion of PGH₂ to PGE₂, and the requirement for GSH is the only functional similarity between the two (131). The enzyme is homotrimeric in structure, and the trimer contains four helix bundles which traverse the microsomal membrane (131-135). It catalyses PGE₂ in inflammation and pain, participates in cancer cell proliferation and tumour growth, and even participates in detoxification. Inhibiting its activity could significantly reduce hypertension, thrombosis, and myocardial damage compared to the inhibition of COX-2. Pharmaceuticals could target its active site for glutathione binding (136-138).

The third prostaglandin synthase, the cPGES is a GSH-dependent enzyme, expressed constitutively in a wide variety of tissues. It is linked to COX-1 and associated with the heat shock protein 90 (Hsp90) in immediate PG production (129, 130).

An important prostaglandin production regulator is the transcription factor Nrf2 (described earlier). It induces the transcription of peroxiredoxins 1 and 6 (PRDX1 and PRDX6) and of lipocalin-type prostaglandin D synthase (L-PGDS, EC 5.3.99.2). PRDX1 and PRDX6 protect cells against oxidative stress, but when produced in excessive amounts (thanks to Nrf2), they boost the synthesis of PGE₂ and PGD₂ (127). Again, the whole pathway is a loop, since PRDX1 binds Toll-like receptor 4, which reversibly induces NF-κB activation, and its accumulation stimulates COX2 and mPGES1 expression. The end result is more PGs, as described above.

Furthermore, activated MAPKs, p38, and ERK, which are regulated by GSH (Figure 2), additionally phosphorylate PRDX6, activating the synthesis of PGD₂. PGD₂ and its metabolite 1515d-PGJ₂ reactivate Nrf2 in a positive feedback loop for further synthesis.

GSH AND REGULATORY AND SIGNALLING MOLECULES IN THE IMMUNE SYSTEM

Apart from eicosanoids, inflammation is regulated by cytokines (interleukins for signalling between leukocytes, chemokines for inducing cell chemotaxis, and interferons for antiviral effects), whose release varies with the type of cell (127).

The sources of ROS and oxidative stress on the immune system can either be internal, i.e. the immune cells (macrophages, neutrophils, and eosinophils) and immune system components (cytokines) or external, i.e. a variety of pathological and toxic states that affect the immune cells. Cells of the immune system contain high levels of polyunsaturated fatty acids and are highly vulnerable to the damaging effects of free radicals. The resulting lipid peroxides act as oxidative stress generators (2). Regardless of the source, the intracellular redox state has a key function in regulating inflammatory response (139-141). ROS

activates multiple stress kinase pathways and redox-sensitive transcription factors, including the aforementioned NF-κB and AP-1. ROS also modulates cytokine production, peptide hormones, and immunoregulators (92, 142). Shifts in the GSH/GSSG balance (GSH decrease) during inflammation regulate cytokine production and immune response by activating different types of cells. However, if tissue GSH levels stay low for a long time, immune response may fail (61, 67, 142).

Several *in vitro* and *in vivo* studies have shown that GSH and its precursors downregulate cytokine synthesis, activation, and downstream processes. In lung cells they do it by inhibiting NF-κB activation and promoting the formation of a NF-κB/disulphide complex or by directly inhibiting DNA binding (23, 39, 142-145). Stimulation with lipopolysaccharides (LPS) increases the glutathionylation of IKKβ. Also, experimental silencing RNA (siRNA)-mediated knock-down of GSTP decreases the levels of the IKKβ-SG complex and promotes NF-κB nuclear translocation, transcriptional activity, and pro-inflammatory cytokine production in response to LPS. LPS-induced NF-κB transcriptional activity and pro-inflammatory cytokine production can also be induced pharmacologically by TLK117, an isotype-selective inhibitor of GSTP (145). This interference is considered a promising target pathway for drug development and strategies to reduce oxidative stress-induced inflammation (146).

On the other hand, cytokines, which themselves are mediators of oxidative stress, disturb the redox equilibrium and affect GSH/GSSG shuttling and recycling (142). Both IKKα and β of the NF-κB have been found in the nucleus, but only IKKα is believed to be involved in cytoplasm-nuclear shuttling in response to cytokine stimulation. Cytokines TNFα, IL-1, and IL-6 can also cause oxidative stress, which, in turn, triggers the activation of NF-κB (68).

Leukocytes are particularly sensitive to redox changes and changes in GSH levels (147-150). The immune response starts with the activation of monocytes, macrophages, and antigen-presenting cells (APC). Complex regulations of elevated ROS, thiol modifications, disturbances of the redox-sensitive signalling pathways, and protein glutathionylation lead to macrophage polarisation, foam cell formation, monocyte transmigration, and macrophage cell death (9).

Peroxiredoxin 2 (PRDX2) is an antioxidant enzyme activated by redox-dependent inflammatory mediators that promotes glutathionylation and controls cytokine-induced peroxide levels in macrophages. As it possesses inflammatory signal properties, PRDX2 stimulates macrophages to produce and excrete TNFα (151). PRDX2 and some other glutathionylated proteins are excreted *in vitro* in LPS-stimulated macrophages. TRXs are induced as well. PRDX2 and TRX from macrophages change the redox status of cell surface receptors and induce inflammatory response. This mechanism is being investigated as a pharmacological target for chronic inflammatory diseases (151).

In macrophages and other APCs, GSH is necessary for antigen unfolding (processing for presentation). As it forms disulphide bonds with the antigen, its levels determine which type of cytokines and other regulatory mediators will be secreted, which, in turn, will determine which type of T cell will be activated (2, 7, 39, 88, 152). In other words, the cellular response of the immune system will depend on GSH levels in macrophages and APCs (140).

GSH status will also determine a number of T cell functions. A decrease in or complete lack of GSH in APCs induces cytokine types that inhibit cytotoxic T lymphocytes (CD8+) and affect the proliferation of CD16+ NK cells. At the same time, cytokines will promote CD4+ cell activity (7, 88, 153). GSH can stimulate cytokines that induce CD4+ T lymphocytes apoptosis (7). How essential GSH is for T lymphocyte proliferation has been demonstrated by several studies (2, 152, 154), in which intracellular GSH was elevated or depleted with buthionine sulfoximine (BSO). At low GSH levels, human T lymphocytes did not proliferate after treatment with mitogenic lectins. Increased GSH levels (with 2-oxothiazolidine-4-carboxylate and 2-mercaptoethanol) enhanced mitogenic response by increasing IL-1 and IL-2 production and lymphocyte proliferation or by decreasing the production of the inflammatory mediators PGE and LTB4 (2, 7, 67, 152, 156, 157).

Several immune functions relevant to HIV-1 infection depend on adequate intracellular GSH levels, such as the activation of natural killer cells, T-lymphocyte-mediated cell death, and lymphocyte activation by mitogens (158). During HIV-1 infection, oxidised GSH in CD4+ lymphocytes increases significantly and disturbs their redox status (158). It has been demonstrated that TNF- α promotes HIV transcription via activation of the transcription factor NF- κ B. There were even attempts to improve the immunological status by administering *N*-acetylcysteine to HIV patients to raise their GSH levels. The maintenance of the thiol/oxidant balance, therefore, appears to be crucial for the protection against the toxic effects of TNF- α or other inflammatory cytokines (61, 67).

However, despite the general opinion that interleukins are not directly glutathionylated, some integrins are, such as the leukocyte very late antigen-4 (VLA-4, $\alpha^4\beta^1$), which binds to the vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressing cell adhesion molecule-1 (MadCAM-1). This binding is essential for the homing and recruitment of leukocytes and mobilisation of progenitor cells. *In vitro* glutathionylation of this integrin decreased its binding to VCAM-1 and decreased cell rolling on VCAM-1-coated flow chambers (144). The intercellular adhesion molecule 1 (ICAM-1), also known as cluster of differentiation 54 (CD54), is transcriptionally regulated by NF- κ B and thus indirectly controlled by glutathionylation. It is a primary inflammatory marker that promotes tight adherence of leukocyte integrins to endothelial cells, which is an essential step in leukocyte extravasation. In human

pulmonary aortic endothelial cells, ICAM-1 glutathionylation is additionally augmented by TNF- α (159-161), which promotes the expression of ICAM-1 on the cell surface and monocyte adhesion. GRx-mediated de-glutathionylation of ICAM-1 has the opposite effect. Therapeutic application of glutathionylation of ICAM could affect its binding to leukocyte integrins (144).

NITRIC OXIDE (NO), GSNO FORMATION, AND THE ROLES OF GSNO

NO is a pleiotropic physiological regulator of diverse functions in the cardiovascular, neuromuscular, neurological, genitourinary, gastrointestinal, and renal tissue (99, 162). Intracellular GSH determines the sensitivity of certain cell types to NO and its derivatives (163). One such derivative of NO and superoxide ($O_2^{\cdot-}$) is the reactive nitrogen species (RNS), a family of radical molecules catalysed by nitric oxide synthase 2 (NOS2, EC 1.14.13.39) and NADPH oxidase, respectively. With ROS, it constitutes the free radical species in the organism (ROS/RNS) (164).

Cell NO is produced by catalytic conversion of arginine to citrulline by specific NOSs, which take three isoforms: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS), each encoded by its own, highly distinct gene (77). The endothelial form is glutathionylated through GLRX1 to generate mesenteric nitric oxide (165).

NO is also produced in large amounts by cytokine-activated macrophages and monocytes via iNOS, which makes NO an effective tumour-killing cytotoxic agent (77). Figures 4 and 5 illustrate the interactions between different NO adducts and GSH during oxidative stress. As a free radical, NO contains one unpaired electron, which makes it unstable compared to many other chemical species (although it is mildly reactive compared to ROS) (164).

NO is a mild oxidant and reductant (165). Low and high concentrations of NO have different roles. At low concentrations it behaves as a signalling molecule in many physiological processes, such as modulation of the synaptic and non-synaptic communication between neurons in the brain tissue. At high concentrations it can cause neural cell damage, inflammation, and death, which may lead to neurodegeneration and memory loss (151). High levels of NO produced by inflammatory cells can damage DNA, RNA, lipids, and proteins, causing amplified mutations and changes in enzyme and protein function, such as those seen in carcinogenesis.

RNS are involved in numerous, potentially carcinogenic events; they may trigger lipid peroxidation, induce transversion mutations and DNA strand breaks, disturb the mitochondrial respiratory chain, and affect protein phosphorylation by nitrosylating tyrosine residues such as p53 (77, 166-168). However, beside its radical and damaging effects, *S*-nitrosylation (a covalent attachment of a nitrogen monoxide group to the thiol side chain of

cysteine) is another redox-based physiological regulation and posttranslational modification of the signalling proteins, just like glutathionylation.

Increased ROS concentrations reduce the amount of bioactive NO, because it reacts with superoxide anion, which results in chemical inactivation and the formation of a highly reactive and toxic peroxynitrite (ONOO⁻) with a half-life of less than one second (166). Susceptibility to NO and ONOO⁻ depends on factors such as intracellular glutathione and cellular stress resistance signalling pathways. For example, NO enhances the expression of GCS, a rate-limiting GSH synthesis enzyme described earlier in the text. The availability of GSH in situations of oxidative and nitrosative stress is ensured by GSH recycling and biosynthetic pathways (82). Increased GSH concentration then counteracts RNS cytotoxicity (169).

NO in more reactive RNS such as N₂O₃ can also react with sulphhydryls to form a nitrosothiol (RSNO) through S-nitrosylation. Such reactions in GSH also form S-nitrosoglutathione (GSNO) (168). It has been shown that GSH is readily nitrosylated *in vivo* to form GSNO, which is produced both in and outside the cell (162, 169-173). The formation of GSNO can also occur through a transnitrosation reaction between thiol and nitrosothiol, which is a reaction between a nitrosylated protein and GSH (163).

Peroxynitrite reacts with the sulphhydryl compounds in the cell due to high intracellular free thiol concentrations (162, 171). However, the chemistry involved will depend on a number of factors, including pH and GSH concentrations. At typical physiological GSH concentrations and pH, the reaction between ONOO⁻ and GSH will predominantly be a two-electron oxidation process leading to GSSG formation. The first step is sulphenic acid (RS-OH), which is then reduced to disulphide with another GSH, which can be recycled by glutathione reductase (162, 165). Another possibility is one-electron oxidation involving peroxyntrous acid and/or its derivatives. It produces a thiyl radical (GS[•]) that will start an oxygen-dependent chain reaction forming a peroxy radical that will deplete intracellular GSH (162).

However, most research has focused on GSNO. It modulates cellular processes and signalling through S-nitrosylation and/or S-glutathionylation (162-164, 171, 174). These processes modify Cys thiols in animal cell proteins (174), and any defects in these modifications can cause a disease, such as heart or Alzheimer's disease (163, 164, 172). In physiological conditions, GSNO and other S-nitrosothiols are present in blood and brain. In adult rat brain tissue GSNO ranges between 6 and 8 μmol L⁻¹, which is ~0.3 to 0.7 % of the tissue GSH level (172).

Whenever the GSH/GSNO physiological balance is disturbed, such as in GSH depletion, GSNO will be cleaved (Figure 4). Excess GSNO, in turn, is removed through the activity of S-nitrosoglutathione reductase (GSNOR, aka alcohol reductase 5, EC 1.2.1.46). The balance appears to determine whether NO will be cytotoxic or cytoprotective (169). Regulation of protein nitrosation is not well

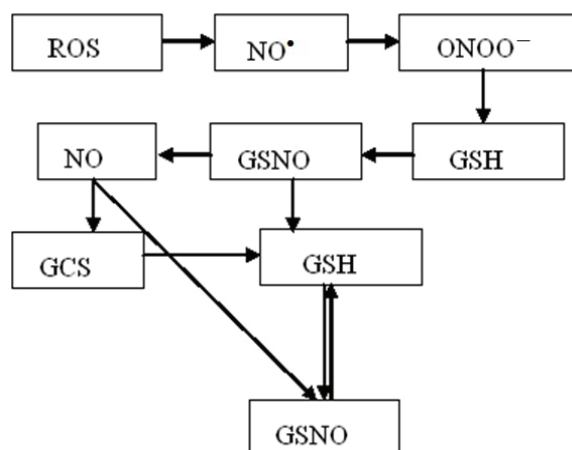


Figure 4 Balance between GSH and GSNO levels during oxidative stress

GSH – reduced glutathione; ROS – reactive oxygen species, NO – nitric oxide; NO[•] – nitrosonium ion; ONOO⁻ – peroxynitrite; GCS – γ -glutamylcysteine synthase; GSNO – S-nitrosoglutathione

understood yet, as NO-dependent events proceed without discernible changes in GSNOR expression (175). GSNO acts as an antioxidant by modifying the redox status, e.g. by increasing GSH or decreasing peroxynitrite levels. It is more stable and approximately 100 times more potent than the classic antioxidant GSH (163, 172, 176). It can also transport NO in plasma, which prolongs its half-life (168). If cell metabolism needs it, GSNO can serve as a donor of both NO and GSH (169). It also inhibits platelet activation, reduces embolisation in humans (172), and protects neurons (168, 173, 177) by inhibiting neuron apoptosis and Cas-3 activity. After injuries, it counters inflammation mainly by downregulating NF- κ B, adhesion molecules, cytokines, and inducible NOS (iNOS) (172, 173, 177). In macrophages, thymocytes, lymphocytes, and endothelial cells NO and GSNO can induce apoptosis through various signalling pathways, especially through S-nitrosylation/denitrosylation as a reversible redox switch that inhibits inflammatory events (172, 178). In *in vivo* experiments, LPS-challenged rats showed a drop in serum NO, and significant rise in creatinine and BUN, which were successfully countered with GSNO treatment (179). The therapeutic potential of GSNO is yet to be established with the application of new GSNO nanocarriers (180, 181) or administration methods suitable for oral applications as target drugs (182).

Glutathionylation in brain and neural tissues

In recent years, research has focused on glutathionylation and its relation with neurodegenerative diseases and neurophysiology (Table 2), particularly on the major transcription factors and regulatory molecules such as histones (186) in the brain (183-186). Carvalho et al. (55) investigated GSTP glutathionylation of Nrf2 in Parkinson's disease. Speaking of which, Johnson et al. (187) established that GLTRX deficiency exacerbates the neurodegenerative processes.

In brain cancers, posttranslational glutathionylation on the Cys¹⁴⁵ of O⁶-methylguanine-DNA methyltransferase (MGMT) has promising therapeutic effects (188). MGMT is highly expressed in brain cancer, where it repairs the mutagenic, carcinogenic, and cytotoxic O⁶-alkylguanine adducts, including those generated by anticancer alkylating agents used in cancer treatment (188).

In Huntington's disease, Cys^{176/178} glutathionylation on the Ca²⁺-permeable transient receptor potential cation channel (TRPC) is increased in the striatum in both transgenic mice and patients. Glutathione-activated TRPC5-like currents elevate cytosolic Ca²⁺, activate calmodulin-dependent protein kinases and the calpain-caspase pathways, and eventually induce striatal neuronal cell death. This occurs at the same time as the downregulation of the TRPC1 expression, which only adds to the neuronal damage in the striatum cells in Huntington's disease (189).

In Alzheimer's disease, glutathionylation is described as posttranslational control in various cellular processes (190-192). It regulates a key enzyme for ionic homeostasis in cell transduction, Na⁺, K⁺-ATPase (193, 194).

Future research will provide more evidence of glutathionylation and redox signalling as important control mechanisms in brain physiology (195).

PROMISING NEW METHODS AND CONCLUSION

Bioinformatics and various methods developed to detect glutathionylation enable prediction of hundreds of new glutathionylated sites within cells (9, 196-199). One such indirect method is the biotin switch assay, which labels oxidation states to identify reversible modifications, S-glutathionylation, S-nitrosylation, and conjugated sulphenic acids on proteins. The method is based on the reaction of free thiols with an alkylating agent maleimide. The site is first alkylated, then maleimide removed in a desalting column, and the free thiol can be labelled with a biotin-conjugated alkylating agent such as biotin-HPDP. Such biotin-HPDP labelled (biotinylated) proteins are purified with streptavidin-agarose beads and can be detected with Western blotting and avidin-based detection protocols.

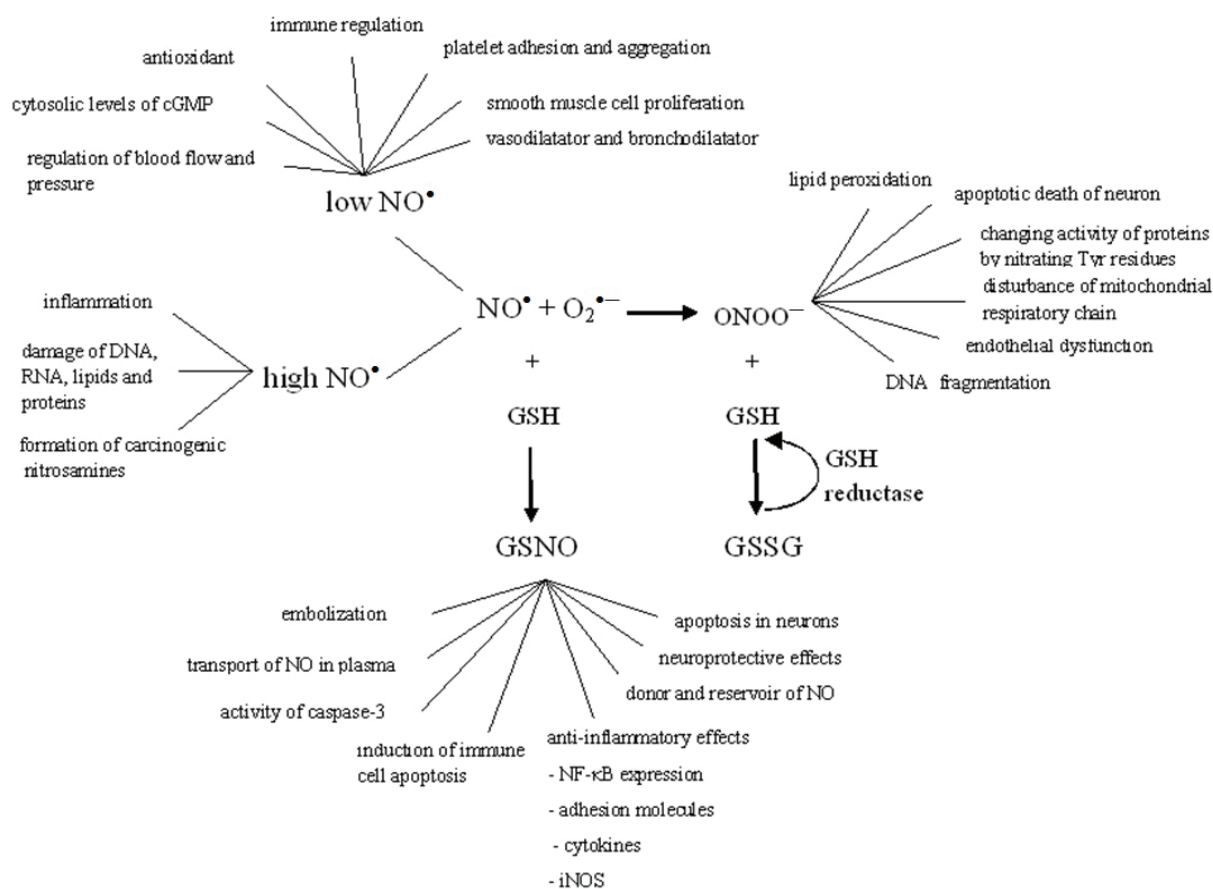


Figure 5 NO[•] adduct formation, including ONOO⁻ and GSNO, and their physiological roles

GSSG is formed in a reaction between ONOO⁻ and GSH, when it is a two-electron oxidation process, which happens only in normal physiological conditions.

GSH – reduced glutathione; GSSG – oxidised disulphide form of glutathione; NO – nitric oxide; NO[•] – nitrosonium ion; ONOO⁻ – peroxynitrite; GSNO, S-nitrosoglutathione

Modifications can be detected using reducing agents, such as ascorbate for S-nitrosylation, Na-arsenite for sulphenic acid, recombinant GLRX for S-glutathionylation, and DTT or tris(2-carboxyethyl) phosphine for intra- and inter-protein disulphides (196, 197).

Other promising agents for identifying new glutathionylation targets are biotinylated GSH esters, which are also commercially available (196, 198). S-glutathionylated proteins can also be detected directly with commercially available monoclonal antibodies against GSH covalently bound to target proteins. This method, however, is limited by antibody sensitivity and the need of large quantities of the proteins, whose signal is low. Glutathionylated proteins can be purified using an affinity matrix and then identified with Western blotting for specific targets.

Other methods include N-ethyl maleimide and dimedone (5,5-dimethyl-1,3-cyclohexadione (NEM) or isobaric tag (iTRAQ) labelling as well as tandem- or liquid chromatography-mass spectrometry (9) or fluorescent protein sensors, such as redox-sensitive yellow and green fluorescent protein variants (rxYFP and roGFPs) (199). Knockout mice or siRNA for enzymes important in glutathionylation/deglutathionylation reactions are also available to study protein glutathionylation (32).

Bioinformatics can also provide valid directions for further experimental research of certain proteins *in vitro* and *in vivo*. For example, Pal et al. (200) described 254 glutathionylated proteins, some of which have already been proven to be glutathionylated experimentally in living cells. Bioinformatics can predict glutathionylated and non-glutathionylated cysteines and help to focus experiments on certain proteins and create databases with experimentally validated information about the physiological effects of glutathionylation (198, 199, 204).

New arrays are being developed (201, 202) with increasing sensitivity (203). Advancements in affordable and broadly used methods such as modified HPLC (205, 206) will definitely influence future research. As an epigenetic control mechanism, glutathionylation of regulatory and signalling proteins and transcription factors is a growing research field with clinical relevance that will probably expand over the years.

Conflicts of interest

None to declare.

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Glutationilacija – regulacijska uloga glutationa u fiziološkim procesima

Glutation (γ -glutamil-cisteinil-glicin) stanični je tripeptid, tiolni spoj i jaki antioksidans koji sudjeluje u metabolizmu otrova i biotransformaciji ksenobiotika faze II. Može se vezati na različite proteine u procesu poznatom pod nazivom glutacionilacija. Proteinska glutacionilacija dokazano je jedan od važnih posttranslacijskih upravljačkih mehanizama u fiziologiji stanica i tkiva. Izravne i neizravne upravljačke uloge u fiziološkim procesima uključuju glutacionilaciju glavnih transkripcijskih faktora, eikozanoida, citokina i dušikova oksida (NO). U ovom se preglednom radu razmatraju navedeni upravljački mehanizmi na primjerima regulacije glutationom u apoptozi, vaskularizaciji, metaboličkim procesima, mitohondrijskom integritetu, imunološkom sustavu i fiziologiji živčanog sustava. Težište je rada na novim opisanim fiziološkim ulogama glutationa, pored uobičajeno opisane uloge u biotransformacijskom metabolizmu.

KLJUČNE RIJEČI: *epigenetika; GSH; GSSG; imunost; transkripcijski čimbenici*