

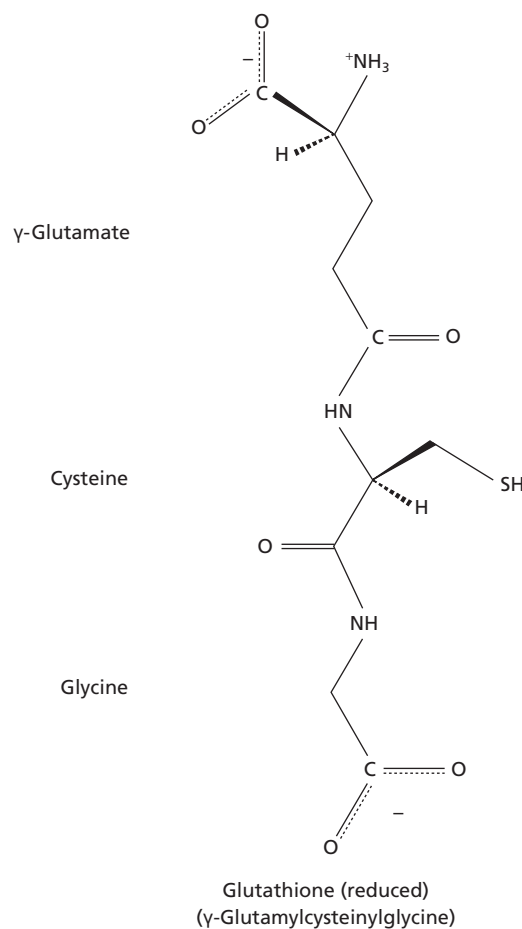
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### 2.3.9 Glutathione

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#### Introduction

The tripeptide glutathione (GSH) is one of the most intensively studied intracellular non-protein thiols on account of the critical role that it plays in cell biochemistry and physiology. GSH is a tripeptide found in high concentrations in all cells. It is synthesized from glutamate, cysteine and glycine in the cytosol in two steps, each requiring adenosine triphosphate (ATP) hydrolysis. GSH has certain structural features responsible for its stability and biological functions. For instance, the peptide bond linking the amino-terminal glutamate and the cysteine residue of GSH is through the  $\gamma$ -carboxyl group of glutamate rather than



**Fig. 1** Structure of GSH and its constituent amino acids. The bond between glutamate and cysteine determines the stability and resistance of GSH to hydrolysis, while its functions are mainly determined by the SH group of cysteine in its reduced form.

the conventional  $\alpha$ -carboxyl group (Fig. 1). This unusual arrangement resists degradation by intracellular peptidases and is subject to hydrolysis by only one known enzyme,  $\gamma$ -glutamyltranspeptidase (GGT). Furthermore, the carboxyl-terminal glycine moiety of GSH protects the molecule against cleavage by intracellular  $\gamma$ -glutamylcyclotransferase. As a consequence, GSH is only metabolized extracellularly by GGT, which acts on the external face of certain cell types. Moreover, the sulphhydryl group of cysteine is required for GSH's functions, in particular the regulation of disulphide bonds of proteins and the disposal of electrophiles and oxidants.

Despite its exclusive synthesis in the cytosol, GSH is distributed in intracellular organelles, including endoplasmic reticulum (ER) and mitochondria. GSH is found predominantly in its reduced form except in the ER, where it exists mainly as oxidized glutathione (GSSG) to provide the adequate environment necessary for protein metabolism. A tight connection between ER and cytosol is necessary to ensure an appropriate secretory function of the ER, as GSH in the cytosol reduces protein

disulphide isomerase and restores normal disulphide formation and secretory rates. In mitochondria, however, GSH is mainly found in reduced form and represents a minor fraction of the total GSH pool (10–15%). Considering the volume of the mitochondrial matrix, the concentration of mitochondrial GSH (mGSH) may be similar to that of cytosol (10–14 mM). Owing to the widespread roles of GSH in cell physiology, changes in GSH homeostasis have been implicated in the aetiology and progression of a number of human diseases. In this chapter, we review its regulation and functions and its role in oxidative stress, hepatocyte death and liver diseases.

## GSH homeostasis

### GSH biosynthesis

GSH exists in high concentrations in all types of cells and is synthesized from precursor amino acids in the cytosol in two steps. The regulation of GSH biosynthesis is dependent mainly on cysteine availability and enzymatic activity [1,2]. The liver has unique features regarding GSH regulation, as hepatocytes are able to convert methionine to cysteine through the trans-sulphuration pathway. In addition, the rate of GSH biosynthesis in the hepatocyte is balanced by its rate of export, mainly into plasma and bile. The synthesis of GSH from its constituent amino acids, L-glutamate, L-cysteine and L-glycine, involves two ATP-requiring enzymatic steps (Fig. 2). The first step in GSH biosynthesis is rate limiting and catalysed by  $\gamma$ -glutamylcysteinyl synthetase (GCS), which exhibits an absolute requirement for either  $Mg^{2+}$  or  $Mn^{2+}$ . GCS is composed of a heavy (GCS-HS) and a light (GCS-LS) subunit, and each is encoded by different genes in both rat and human. The heavy subunit exhibits all the catalytic activity of the isolated enzyme and feedback inhibition by GSH. The light subunit, on the other hand, plays an important regulatory role in the overall function of the enzyme and allows the holoenzyme to be catalytically more efficient and less subject to inhibition by GSH than the heavy subunit alone.

GCS is regulated physiologically by two important factors: GSH inhibition and cysteine availability. Thus, inhibition by GSH is non-allosteric and involves binding of GSH to the glutamate site of the enzyme. A second binding site for GSH has been described and involves interaction with the thiol moiety of GSH. On the other hand, the availability of its precursor, L-cysteine, also regulates GCS. The apparent  $K_m$  values of GCS for glutamate and cysteine are 1.8 and 0.1–0.3 mM respectively. The

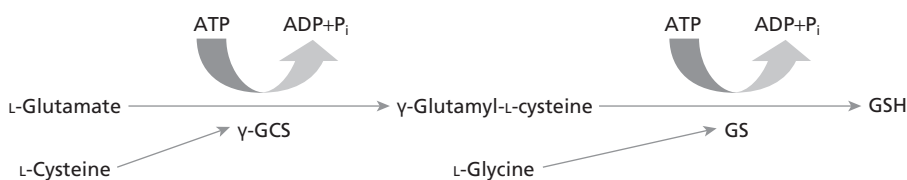
intracellular glutamate concentration is severalfold higher than the  $K_m$  value of GCS for glutamate, but the intracellular cysteine concentration approximates the apparent  $K_m$  value of GCS for cysteine. Hence, both the availability of intracellular cysteine and the activity of GCS greatly influence the rate of GSH synthesis.

The second step in the synthesis of GSH is catalysed by GSH synthetase (GS) (Fig. 2). This enzyme has not been studied as extensively as GCS. However, GS is not subject to feedback inhibition by GSH. Overexpression of GS fails to increase GSH levels as opposed to overexpression of GCS, which increases GSH. However, GS deficiency in humans can result in dramatic metabolic consequences because the accumulated  $\gamma$ -glutamylcysteine is converted to 5-oxoproline, which can cause severe metabolic acidosis [3].

Because the availability of cysteine is a critical determinant of GSH synthesis, factors that regulate intracellular cysteine levels ultimately contribute to GSH biosynthesis and, hence, cellular GSH levels. Among these, the transport of cysteine or cystine (and its subsequent reduction to cysteine intracellularly) or methionine transport modulate the intracellular levels of GSH. The transport of methionine is effective in regulating GSH biosynthesis through the subsequent conversion of methionine into cysteine by the trans-sulphuration pathway (Fig. 3), which operates only in hepatocytes. In addition to the intracellular cysteine availability, the regulation of GCS is a critical step in GSH homeostasis. The activity of GCS is regulated at both the transcriptional and the posttranscriptional levels, and hormones may modulate hepatic GSH biosynthesis through GCS regulation [4].

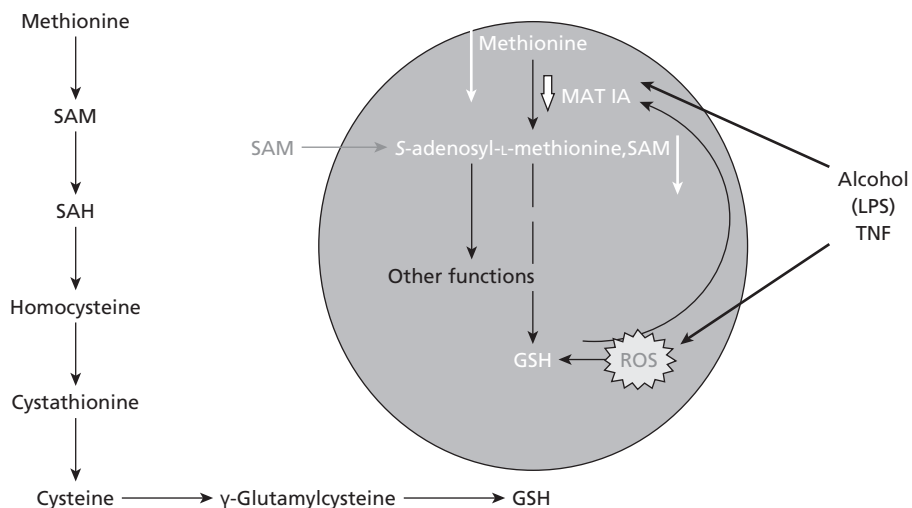
### GSH degradation

In contrast to GSH synthesis, GSH degradation occurs exclusively in the extracellular space and only in cells that express the enzyme GGT. GGT, which is abundant on the apical surface of most transporting epithelia, including liver canalicular and bile ductular membranes, is the only enzyme that can initiate catabolism of GSH, glutathione-S-conjugates and glutathione complexes under physiological conditions. Because GGT is a plasma membrane-bound enzyme with its active site on the extracellular surface of the membrane, export of GSH and its adducts into the extracellular space is the initial, and presumably regulated, step in their turnover in all mammalian cells. GGT removes the glutamyl moiety from GSH and GSH-containing compounds to yield cysteinylglycine or cysteinylglycine-S-



**Fig. 2** GSH synthesis. The biosynthesis of GSH needs glutamate, cysteine and glycine, which are bound in two steps catalysed by  $\gamma$ -GCS and GS, which adds glycine to  $\gamma$ -glutamylcysteine. Both enzymes require ATP hydrolysis and, hence, the synthesis of GSH is energy consuming. In addition to  $\gamma$ -GCS, which is the rate-limiting enzyme, the availability of cysteine regulates GSH synthesis.

**Fig. 3** The trans-sulphuration pathway and role of *S*-adenosyl-L-methionine (SAM) in alcohol-induced GSH regulation. In addition to the limited availability of cysteine in the biosynthesis of GSH, methionine meets the need for cysteine. It is channelled for GSH biosynthesis through the trans-sulphuration pathway, in which the sulphur of methionine is converted into cysteine. A key component of this process is SAM, which acts as a GSH precursor in hepatocytes and, hence, prevents GSH depletion induced by agents such as alcohol. LPS, lipopolysaccharide; ROS, reactive oxygen species; TNF, tumour necrosis factor.



conjugates. These compounds are substrates for dipeptidases, which hydrolyse the peptide bond between cysteine and glycine. Dipeptidases are also ectoproteins; thus, these reactions also occur in the extracellular space. The hepatic transport of GSH into plasma and bile thus contributes to its extrahepatic metabolism, which has been reviewed elsewhere [5].

## Biological functions

### Detoxification of Michael electrophiles and metals

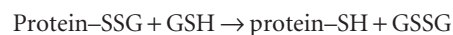
Detoxification of xenobiotics or their metabolites is one of the major functions of GSH. GSH, being a nucleophile, targets electrophiles with  $\alpha\beta$  unsaturated carbonyl moieties or Michael electrophiles. These compounds form conjugates with GSH either spontaneously or enzymatically in reactions catalysed by GSH *S*-transferases [1]. This reaction results not only in the removal of the electrophile via its subsequent metabolism or transport out of the cell, but also in the potential depletion of GSH, particularly if GSH biosynthesis does not match the rate of electrophile elimination. The metabolism of GSH conjugates begins with cleavage of the  $\gamma$ -glutamyl moiety by GGT, leaving a cysteinyl-glycine conjugate. The cysteinyl-glycine bond is cleaved by dipeptidase, resulting in a cysteinyl conjugate. This is followed by *N*-acetylation of the cysteine conjugate, forming a mercapturic acid. The metabolism of GSH conjugates to mercapturic acid begins in the biliary tree, intestine or kidney, but the formation of the *N*-acetylcysteine conjugate usually occurs in the kidney.

In addition to exogenous compounds, many endogenously formed compounds also follow similar metabolic pathways. Some examples include estradiol-17- $\beta$ , leukotrienes and prostaglandins [4,6]. GSH also forms metal complexes via non-enzymatic reactions. GSH is one of the most versatile and pervasive metal-binding ligands and plays an important role in metal transport, storage and metabolism. GSH functions in

the mobilization and delivery of metals between ligands, in the transport of metals across cell membranes, as a source of cysteine for metal binding and as a reductant or cofactor in redox reactions involving metals. The sulphhydryl group of the cysteine moiety of GSH has a high affinity for metals, forming thermodynamically stable but kinetically labile mercaptides with a number of metals, including mercury, silver, cadmium, arsenic, lead, gold, zinc and copper.

### Maintenance of thiol status

As the dominant non-protein thiol in mammalian cells, GSH is essential in maintaining the intracellular redox balance and the essential thiol status of proteins [1,4,6]. To achieve this, GSH undergoes thiol-disulphide exchange in a reaction catalysed by thiol-transferase, as follows:



As this reaction is reversible, the equilibrium is determined by the redox state of the cell, which depends on the concentrations of GSH and GSSG [proportional to the log of  $(\text{GSH})^2/(\text{GSSG})$ ]. Normally, cellular GSSG is kept very low (< 1% of the total GSH pool) so that protein mixed disulphide formation is limited. The thiol-disulphide equilibrium within the cell is known to regulate a diverse number of metabolic processes, including enzyme activity, transport activity and gene expression, via alteration of redox-sensitive *trans*-activating factors.

### Antioxidant function

As a consequence of aerobic metabolism, all aerobic organisms are subject to a certain level of physiological oxidative stress. Mitochondrial respiration of xenobiotic transformation through cytochrome P450s yields reactive oxygen species (ROS) such as superoxide ( $\text{O}_2^-$ ) and hydrogen peroxide, and can lead to the further production of toxic oxygen radicals that can cause lipid peroxidation and cell injury. The endogenously produced

hydrogen peroxide is reduced by GSH in the presence of selenium-dependent GSH peroxidase. As a result, GSH is oxidized to GSSG, which in turn is reduced back to GSH by GSSG reductase at the expense of nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH), forming a redox cycle. Either GSH peroxidase or GSH *S*-transferase can reduce organic peroxides. Hydrogen peroxide can also be reduced by catalase, which is present only in the peroxisome. In the mitochondria, GSH is particularly important because there is no catalase and, therefore, GSH is responsible for maintaining a functionally competent organelle [7,8]. Because mitochondria are essential for cell function, mitochondrial GSH is a vital antioxidant that regulates cell death pathways and, hence, is of significance in disease progression. In addition, GSH also works as a coenzyme for several other antioxidant enzymes such as glutaredoxins and thioredoxins.

### Cysteine storage and the $\gamma$ -glutamyl cycle

One of the most important functions of GSH is to store cysteine. Cysteine is extremely unstable in the extracellular space and rapidly auto-oxidizes to cystine, in a process producing potentially toxic ROS. The  $\gamma$ -glutamyl cycle, first described by Meister in the early 1970s, allows GSH to serve as a continuous source of cysteine [6]. In this cycle, GSH is released from the cell by carrier-mediated transporter(s), and the ectoenzyme GGT then transfers the  $\gamma$ -glutamyl moiety of GSH to an amino acid (the best acceptor being cystine), forming  $\gamma$ -glutamyl amino acid and cysteinylglycine. The  $\gamma$ -glutamyl amino acid can then be transported back into the cell to complete the cycle. Once inside the cell, the  $\gamma$ -glutamyl amino acid can be metabolized further to release the amino acid and 5-oxoproline, which can be converted to glutamate and used for resynthesis of GSH. Cysteinylglycine is broken down by dipeptidase to generate cysteine and glycine, and cysteine is then taken up and converted back to GSH, a process that occurs in most types of cells. Once inside the cell, the majority of cysteine is incorporated into GSH; some is incorporated into protein, depending on the need of the cell, and some is degraded into sulphate and taurine. For most cells, this mechanism provides a continuous source of cysteine. Thus, the  $\gamma$ -glutamyl cycle allows the efficient utilization of GSH as cysteine storage.

### GSH compartmentation: mitochondrial GSH

GSH is synthesized exclusively in the cytosol of cells; however, it is distributed intracellularly, including in the ER and mitochondria. While GSH in the ER is important for the assembly and secretory pathway for proteins, GSH in mitochondria (mGSH) plays an essential role in the maintenance of a functionally competent organelle and has been recognized more recently to regulate cell death/survival pathways [8]. Mitochondria are the main consumers of molecular oxygen

in the cell, and this process functions as a transducing device to provide the energy required for ATP synthesis in oxidative phosphorylation. Most of the oxygen consumed during oxidative phosphorylation is fully reduced to water; however, a minor percentage of oxygen is partially reduced by a single electron, generating superoxide anion that acts on the matrix side of mitochondria and from which other ROS and oxidants (e.g. hydrogen peroxide) arise as byproducts of aerobic respiration. Although mitochondria are exposed to the constant generation of oxidant species, the organelle remains functional because of the existence of an antioxidant defence system, starting with transformation of superoxide anion into hydrogen peroxide by manganese superoxide dismutase (MnSOD). If the accumulation of hydrogen peroxide is not limited, it may either oxidize mitochondrial components (proteins, lipids, DNA) or participate in a chain of reactions (Haber–Weiss–Fenton) that generate more reactive free radicals, e.g. hydroxyl radical.

The task of controlling endogenous ROS in mitochondria is mainly accomplished by GSH, because mitochondria lack catalase, and because GSH is an integral component of the redox cycle with the participation of the GSH peroxidase and NADPH-dependent GSSG reductase. Moreover, a balance between the activity of MnSOD and the GSH redox cycle must exist to ensure the efficient disposal of hydrogen peroxide [7]. As mGSH concentration is high, moderate depletion of mGSH would not be expected to have a negative impact on the disposal of hydrogen peroxide by GSH peroxidase or on mitochondrial function. However, the depletion of mGSH below a critical level would compromise the adequate reduction of hydrogen peroxide, particularly in conditions of stimulated ROS generation from the mitochondrial electron transport chain. Thus, under complex 3 inhibition by antimycin A, stimulated hydrogen peroxide generation is only observed when GSH is depleted to 2–3 nmol/mg protein [9], which corresponds to the range of the  $K_m$  of GSH peroxidase for GSH (3 mM). Furthermore, because of the existence of GSH *S*-transferases (GST) in mitochondria, GSH also ensures the reduction of organic hydroperoxides, including products of lipid peroxidation [7].

Thus, as inferred from its versatility in reducing oxidants and in conjugating electrophiles, mGSH plays an essential role in maintaining mitochondria in a healthy state, and its depletion may be a key event in disease pathogenesis and sensitization of cells to oxidant and drug-induced cell injury. In addition, by regulating the redox environment, mGSH influences the mitochondrial cell death pathway through mitochondrial membrane permeabilization (MMP), which is recognized to play a role in pathophysiology.

### mGSH transport

The concentration of mGSH is in the range of cytosolic GSH. However, unlike cytosol, mitochondria do not contain the enzymatic machinery to synthesize GSH from its constituent amino acids. Therefore, mGSH arises from the cytosol by

carrier-mediated transport located in the inner membrane that overcomes the unfavourable entry against an electrochemical gradient [10]. The earlier characterization of the transport of GSH into rat liver mitochondria indicated an active, energy-dependent process stimulated by ATP and inhibited by glutamate and upon collapse of the protonmotive force with protonophores [7,10].

Although hepatic mGSH transport has been functionally expressed in *Xenopus laevis* oocytes, the identification of the carrier(s) responsible had remained elusive until recently [11]. Indeed, the functional expression of the hepatic 2-oxoglutarate carrier in mitochondria from *Xenopus laevis*, demonstrating the transport of reduced GSH in a phenylsuccinatesensitive manner, further confirmed the suggestion that the 2-oxoglutarate carrier contributes to the transport of GSH in liver mitochondria [11]. The initial rate of 2-oxoglutarate transport in rat liver mitochondria was reduced following depletion of mGSH, suggesting a 2-oxoglutarate/GSH exchange. In contrast to the transport of GSH in kidney mitochondria, which occurs with a single kinetic component, the kinetics of GSH in rat liver mitochondria display a high-affinity and a low-affinity component. Moreover, the kinetics of 2-oxoglutarate transport in rat liver mitochondria exhibited a single Michaelis–Menten component with sensitivity to phenylsuccinate and GSH. Interestingly, the transport of 2-oxoglutarate was dependent on mitochondrial membrane fluidity, a characteristic feature of the hepatic mitochondrial transport of GSH (see below).

Thus, these studies suggest that the 2-oxoglutarate carrier may be responsible for the low-affinity transport of GSH in rat liver mitochondria in exchange for matrix 2-oxoglutarate. However, the identification of alternative carriers that account for the high-affinity transport of GSH in liver mitochondria remains to be established. In this regard, uncoupling protein 2 (UCP2) has been suggested to favour the mitochondrial transport of GSH. Although it may be conceivable that the transport of protons back into the matrix by UCP2 may favour the movement of GSH, the role of UCP2 in the transport of GSH into mitochondria remains speculative.

### Regulation of mGSH transport

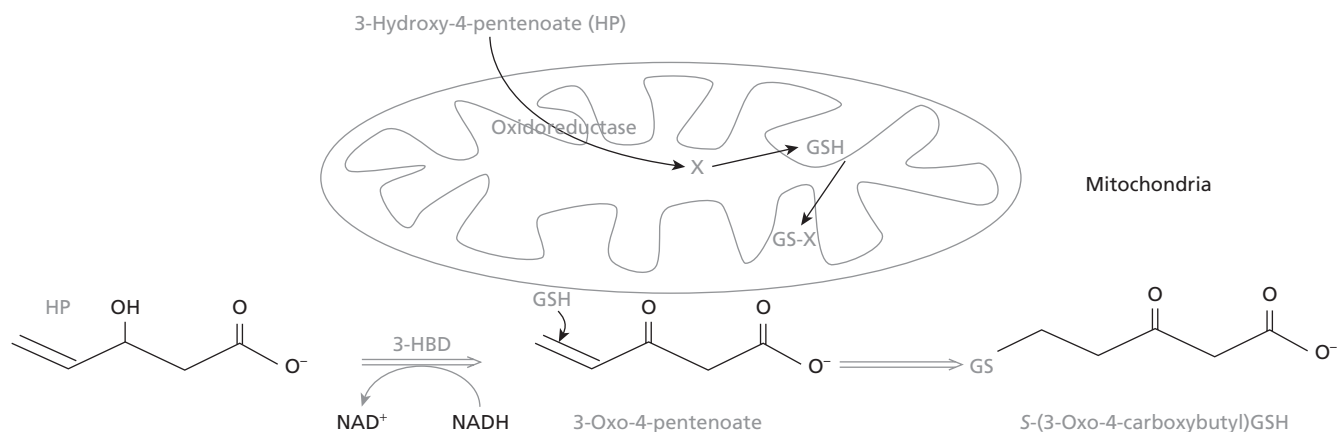
Recent cumulative evidence in hepatocytes and isolated mitochondria indicates that the transport of mGSH is highly dependent on appropriate membrane physical properties [8]. The function of membrane proteins including carriers can be modulated by the microenvironment of the membrane in which they are inserted, and lipid composition determines the dynamic properties of biological membranes. Both the cholesterol/phospholipid molar ratio and the (un)saturation of fatty acyl groups in phospholipids contribute to the membrane fluidity changes that correspond to the transition of membranes from gel to a liquid crystalline state. Cholesterol enrichment of cellular membranes or large multilamellar liposomes have been shown to decrease membrane fluidity monitored by fluores-

cence polarization of specific probes without an effect on the lifetime of the excited state of the probe. On the other hand, highly unsaturated fatty acyl chains (e.g. 20:4 and 22:6) in phospholipids are unlikely to associate with cholesterol and may thus create fluid membrane domains.

As the function of the electron transport chain can be affected by the lipid composition of the mitochondrial inner membrane, the regulation of mitochondrial GSH transport by membrane fluidity has been examined in isolated rat liver mitochondria enriched in cholesterol (reviewed in [8]). Cholesterol incorporated into mitochondria by incubation with a cholesterol–bovine serum albumin (BSA) mixture results in increased cholesterol content (two- to threefold). Although the bulk of cholesterol (70–80%) is found in the outer membrane after exposure to the cholesterol–BSA complex, the cholesterol incorporated in the inner membrane is twice as great as that present in control mitoplasts. The measurement of fluorescence polarization of fluorescent probes indicates decreased membrane fluidity in the cholesterol-enriched mitochondria. Cholesterol-enriched mitochondria display impaired initial rates of GSH uptake at different GSH concentrations, whereas the uptake of adenosine diphosphate (ADP) through the adenine nucleotide translocator remains unaffected. Moreover, the fluidization of cholesterol-enriched mitochondria by  $A_2C$  restored the mitochondrial transport of GSH despite increased cholesterol content. Thus, in addition to its regulation by the protonmotive force, ATP hydrolysis and glutamate, the mitochondrial transport of GSH is dependent on appropriate membrane fluidity.

### GSH and cell death regulation

Through the maintenance of protein sulphhydryls in the appropriate redox state, GSH can regulate important death/survival pathways, which modulate the fate of cells in response to apoptotic stimuli. A number of studies have shown that intracellular GSH loss, such as that induced by stimulated efflux out of the cell or its consumption, sensitizes different cell types to a variety of apoptotic stimuli (reviewed in [12]). For instance, epithelial HeLa cells expressing mutated cystic fibrosis transmembrane conductance regulator (CFTR) display resistance to hydrogen peroxide-mediated apoptosis accompanied by higher intracellular GSH stores and lower mitochondrial Bax levels. Recent studies in mouse hepatocytes in which the GSH levels were depleted by diethylmaleate or paracetamol indicated a sensitization to tumour necrosis factor (TNF)-induced apoptosis. In examining the activation of stress kinases and NF- $\kappa$ B-dependent survival genes, GSH depletion in the cytosol/nuclei resulted in sustained activation of Jun N-terminal kinase (JNK) by TNF. Intriguingly, while GSH depletion did not impair the nuclear DNA binding of NF- $\kappa$ B induced by TNF, it did prevent the induction of NF- $\kappa$ B-dependent survival genes such as inducible nitric oxide synthase (iNOS). These data show a differential dependence on GSH levels between the NF- $\kappa$ B DNA-binding activity and its transactivation, with the latter showing a requirement for critical GSH



**Fig. 4** Depletion of mitochondrial GSH. The biotransformation of HP into a Michael electrophile, 3-oxo-4-pentenoate, occurs selectively in the mitochondrial matrix thanks to the 3-HBD. This  $\alpha\beta$  unsaturated carbonyl is then conjugated with GSH yielding *S*-(3-oxo-4-carboxybutyl)GSH, resulting in the depletion of mitochondrial GSH. This pharmacological approach has been useful to learn specifically about the relevance of mitochondrial GSH in cell death regulation.

levels. Although the DNA binding of NF- $\kappa$ B can be modulated by the redox environment, recent findings indicate a novel pathway for the redox regulation of NF- $\kappa$ B based on mass spectrometry and molecular modelling of the *S*-glutathionylation of cysteine 62 of the p50 subunit of NF- $\kappa$ B. Thus, depending on the extent and mechanism of GSH depletion (efflux from cytosol of cells vs. decreased GSH/GSSG ratio), a limited storage of cell GSH can promote cell death through sustained JNK activation and/or suppression of NF- $\kappa$ B-dependent survival pathways.

In addition to the redox regulation of these death-promoting components, the engagement of the death-inducing signalling complex (DISC) and caspase-8 activated by ligation of death receptors relies on the appropriate redox environment for proper function. Through this mechanism, adequate GSH levels are required to ensure active DISC and caspase-8 activity after Fas stimulation in CEM and H9 cells. However, this dependence has not been observed in HepG2 or Hepa1-6 cells, indicating that the requirement of GSH for active DISC is cell-type specific. In hepatocytes, for instance, the role of GSH in sensitization to Fas or TNF is controversial. The length (prolonged vs. acute) and extent of GSH depletion (cytosolic vs. mitochondrial) may determine the fate of hepatocytes after Fas or TNF challenge. Thus, the existence of additional factors, e.g. the presence of redox-active thioredoxin in different cell types, may determine the outcome of the delicate balance between physiological antioxidants (GSH) and endogenously produced oxidants (ROS and nitric oxide) in the progression of apoptosis signalling.

### Role of mGSH and susceptibility to cell death

Recently, pharmacological approaches have been used selectively to deplete mGSH in order to examine its role in the susceptibility of primary hepatocytes to death stimuli. By exploiting the selective biotransformation of (R,S)-3-hydroxy-4-pentenoate (HP) into a Michael acceptor within mitochondria by the (R)-3-hydroxybutanoate NAD<sup>+</sup> oxidoreductase (3-HBD) (Fig. 4),

it has been possible selectively to deplete the mGSH pool in intact hepatocytes while sparing cytosolic GSH, underscoring the specific role of mGSH in apoptosis induced by TNF [13]. Preincubation of primary cultured rat hepatocytes with HP results in sensitization to TNF-mediated cell death in the absence of any other sensitization approach such as blocking protein or total RNA synthesis or NF- $\kappa$ B activation. mGSH-depleted hepatocytes exposed to TNF exhibit a significant and early generation of peroxides that precedes the loss of mitochondrial membrane potential, release of cytochrome *c* and apoptotic and necrotic demise. Similar findings have been observed when the depletion of mGSH is induced by chronic ethanol intake, which are reversed upon repletion of mGSH. On the other hand, overexpression of a mitochondrial GSH transporter, the dicarboxylate carrier, which results in substantially higher mGSH levels, protects NKK-52E cells from oxidant-induced apoptosis. In addition, mGSH depletion by triterpenoid derivatives in pancreatic cancer cells enhances apoptosis by a redox-dependent mechanism, thus further validating the crucial role of mGSH in controlling cell survival [14]. Moreover, mGSH depletion by HP has been shown to sensitize cultured hepatocytes to hypoxia-induced cell death [15]. Thus, mitochondria are both a source and a target of ROS. Through the maintenance of superoxide anion-induced hydrogen peroxide generated on the matrix side of the mitochondrial inner membrane, the pool of mGSH serves as a critical line of defence that controls the fate of cells in response to apoptosis stimuli.

In addition to ensuring the appropriate redox state of critical mitochondrial proteins (e.g. components of the mitochondrial permeability transition pore complex), GSH in mitochondria may also be vital in guarding the integrity of lipids. A recent study demonstrated the critical role of cardiolipin, in collaboration with proapoptotic Bcl-2 family members, in the formation of supramolecular openings in the outer mitochondrial membrane [16]. In addition to its fundamental role as a housekeeping lipid in the organization of individual complexes into functional

units of the respiratory chain, cardiolipin has a defined distribution pattern within mitochondria and may serve as a functional link in the action of BH1–3 multidomain proteins, e.g. Bax, or BH3-only Bid, to elicit the release of cytochrome *c*. Cytochrome *c* is bound to the inner membrane by cardiolipin. Recent studies in rat liver mitochondria have characterized a two-step process in the release of cytochrome *c*, consisting of the detachment of this protein from its membrane-anchoring lipid, cardiolipin, followed by permeabilization of the outer membrane allowing the release of cytochrome *c* into the extramitochondrial environment. As the peroxidation of cardiolipin contributes to the transition from tight to loose conformation of cytochrome *c*, an additional aspect of the protective role of mGSH might involve protection of cardiolipin from ROS attack. In this regard, hepatocellular mGSH depletion by HP leads to intact cardiolipin loss and subsequent accumulation of peroxidized cardiolipin in mitochondrial membranes. Whether this step is essential for mitochondrial membrane permeabilization (MMP) and, hence, apoptosome assembly by TNF is currently being investigated in liposomes reconstituted with intact vs. peroxidized cardiolipin. Thus, either through the maintenance of vital mitochondrial proteins and/or cardiolipin, mGSH depletion associated with disease states (such as hypoxia–reperfusion injury, toxic bile acid-induced damage and chronic alcohol intake) will favour conditions for cell damage.

### GSH depletion in liver diseases

Because GSH is a critical antioxidant, and ROS are recognized in the pathophysiology of liver diseases, GSH depletion is associated with, and contributes to, the progression of liver diseases. In this capacity, GSH depletion may contribute to bile acid-induced liver damage, ischaemia–reperfusion liver injury and hepatitis B and C-induced liver damage [17,18].

Alcohol-induced liver damage is among the best characterized of diseases associated with GSH depletion (see also Section 12, Alcoholic liver disease). Hepatic mitochondria are recognized targets of ethanol metabolism in the liver. Studies in animal models of chronic ethanol feeding demonstrate functional alterations in oxidative phosphorylation, while patients with alcoholic hepatitis display mitochondria with morphological aberrations. Therefore, we examined the regulation of mGSH status and transport in experimental models of chronic alcohol feeding (reviewed in [19]). Our earlier observations more than a decade ago indicated that chronic ethanol intake selectively reduced the levels of mGSH in hepatocytes. Furthermore, similar findings have been observed in rats fed ethanol intragastrically, reproducing some of the pathological findings seen in patients ranging from steatosis to inflammation and fibrosis. These studies demonstrate a progressive depletion of mGSH with ethanol intake that precedes signs of liver injury in the ethanol-fed mice. Moreover, intragastric alcohol-fed mice also exhibit mGSH depletion that is prevented by MnSOD overexpression, indicating that mGSH levels are regulated by oxidative

stress. The depletion of mGSH caused by chronic alcohol feeding is seen preferentially in perivenous hepatocytes and results from a defective transport of GSH from cytosol into mitochondria, as demonstrated in *in vitro* and *in vivo* tracer kinetic studies. Consistent with the observations that mGSH depletion caused by alcohol intake is secondary to impaired transport of GSH, we have documented decreased membrane fluidity in alcohol-fed livers [19]. Furthermore, acetaldehyde stimulates *de novo* synthesis of cholesterol in HepG2 cells, which then traffics to the mitochondrial inner membrane resulting in increased order parameter and impaired transport of GSH into mitochondria [20]. The fluidization of mitochondria from alcohol-fed livers or from acetaldehyde-treated HepG2 cells with A<sub>2</sub>C restores the ability of mitochondria to transport GSH. In agreement with the impairment of mitochondrial GSH transport by alcohol intake, raising cytosolic GSH levels with N-acetylcysteine, a GSH precursor, does not increase the mGSH level in hepatocytes from alcohol-fed livers. However, S-adenosyl-L-methionine (SAM), which, in addition to its role in promoting GSH synthesis, can prevent alterations of membrane lipid composition including cholesterol/phospholipid increase, prevents alcohol-induced membrane fluidity loss in alcohol-fed rats and normalizes the mGSH levels. Exogenous SAM prevents TNF-induced MAT1A downregulation [21], which can also contribute to the depletion of SAM and subsequent GSH levels (Fig. 3). Interestingly, treatment of alcohol-fed rats with tauroursodeoxycholic acid replenishes mGSH levels through restoration of mitochondrial membrane fluidity.

Thus, alcohol-stimulated cholesterol increase and subsequent deposition in mitochondrial membranes accounts for the impairment of GSH transport in mitochondria, leading to depleted mGSH levels. In addition to the observations in hepatocytes, the depletion of mGSH has also been observed in alveolar type 2 cells from alcohol-fed rats resulting from impaired transport of GSH into mitochondria.

In cirrhosis, which is also associated with mitochondrial dysfunction, similar findings of mGSH dysregulation may occur. For instance, secondary biliary cirrhosis in rats induced by bile duct ligation will deplete mGSH, which occurs earlier than the observed decrease in GSH levels in liver homogenates. Although the mechanism is not entirely clear, mitochondria from bile duct-ligated rats exhibit altered lipid composition, with a two- to threefold increase in the cholesterol/phospholipid ratio in the mitochondrial inner membrane, thus raising the possibility of impaired transport of GSH into mitochondria.

Thus, as cholesterol influences the mitochondrial transport of GSH via the modulation of membrane physical properties, the transport of cholesterol to mitochondria may be significant in pathological states such as alcohol-induced liver disease (ALD). Most of the cholesterol in membranes is located in the plasma membrane, representing 65–80% of the total cellular cholesterol. Mitochondria, however, are cholesterol-poor organelles, with the outer mitochondrial membrane containing more cholesterol than the inner membrane.

Although the transport to and function of cholesterol in mitochondria is best understood in steroidogenic cells, the transport of cholesterol to hepatic mitochondria may be important in the bile acid synthesis pathway through its conversion into 27-hydroxycholesterol by the sterol 27-hydroxylase (Cyp27). In addition to its function as a precursor for bile acid synthesis, 27-hydroxycholesterol regulates liver steatosis by acting as a ligand of the liver X receptor (LXR) transcription factor via induction of sterol regulatory element-binding protein (SREBP)-1c. While steroidogenic acute regulatory protein (StAR) is key for the transport of cholesterol in steroidogenic cells, a recently purified protein from rat liver mitochondria has been shown to transport cholesterol to the mitochondrial inner membrane [22]. An intermembrane protein of 57.5 kDa, which transports cholesterol between mitochondrial membranes, has been purified by cholesterol affinity chromatography. In addition, a recent study has reported the detection of StAR in human liver cells, correlating its presence with the 27-hydroxylation of cholesterol, a process that occurs in the mitochondria and is considered the rate-limiting step in bile acid synthesis via the CYP27A1-initiated 'acidic' pathway [23]. A better understanding of this process and its regulation may identify novel therapeutic targets to maintain mitochondrial function and improve mGSH transport in liver diseases.

There is also a novel mechanism for cholesterol enrichment of mitochondrial inner membrane. Acetaldehyde can stimulate *de novo* cholesterol synthesis in HepG2 cells through ER stress [20], a process characterized by the accumulation of unfolded or misfolded proteins in the ER, which signals the induction of responsive genes [24]. Acetaldehyde increases the levels of GADD153 and the ER-associated transcription factor SREBP, which regulates cholesterol synthesis by activation of the rate-limiting enzyme hydroxymethylglutaryl-CoA reductase (HMGCoAR). The acetaldehyde-stimulated mitochondrial cholesterol content in HepG2 is preceded by increased levels of GADD153 and SREBP1, mimicked by the ER stress-inducing agents tunicamycin and homocysteine and prevented by the HMGCoAR inhibitor lovastatin. In addition, homocysteine, a toxic non-protein sulphur-containing amino acid formed exclusively upon demethylation of the essential amino acid methionine, contributes to alcohol-induced liver injury through ER stress [25]. Indeed, feeding mice betaine to promote methylation of homocysteine into methionine ameliorates alcohol-induced ER stress in mouse livers, indicating that homocysteine-induced ER stress contributes to alcohol-induced liver injury. Hence, alcohol intake leads to hyperhomocysteinaemia, which in turn may elicit ER stress, thereby promoting alcohol-induced liver injury.

### **GSH and hepatotoxicity** (see also Section 14, Toxic liver injury)

mGSH is of critical relevance in hepatotoxicity because of its role in downregulating ROS and oxidants generated in the

mitochondrial electron transport chain and maintaining overall mitochondrial function. For instance, patients taking LipoKinetix, a dietary supplement marketed as a weight loss agent, developed acute hepatotoxicity, which improved spontaneously after its use was discontinued. Usnic acid, a metabolite found in lichen, is a key constituent of LipoKinetix; usnic acid induces hepatocyte necrosis associated with disruption of mitochondrial respiration and energy metabolism, leading to ATP depletion and GSH depletion [26]. Furthermore, usnic acid increases hydrogen peroxide production in mitochondria, indicating that the depletion of mGSH is necessary for the sensitization of hepatocytes to usnic acid cytotoxicity. These findings suggest that usnic acid may be responsible, at least in part, for hepatotoxicity and liver failure seen in patients taking LipoKinetix.

Paracetamol (*N*-acetyl-*p*-aminophenol, APAP) is a widely used analgesic that is considered safe when taken at therapeutic doses. However, hepatocellular necrosis occurs at higher doses or under conditions that enhance the susceptibility to APAP. APAP hepatotoxicity is mediated by its metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is generated by liver cytochrome P450, particularly cytochrome P450 2E1 (CYP2E1), and is detoxified by conjugation with GSH. NAPQI initiates its toxicity by first attacking mitochondria, followed by mGSH depletion. Although APAP can decrease GSH in both cytosol and mitochondria, the kinetics of GSH depletion in hepatocytes exposed to APAP indicate that the depletion of mGSH precedes that of cytosolic GSH, establishing the relevance of this GSH pool in the hepatotoxicity of APAP [27]. Interestingly, *N*-acetyl-*m*-aminophenol (AMAP), a non-hepatotoxic regioisomer of APAP, depletes cytosolic GSH but not mGSH. Like many other drugs, APAP cytotoxicity in hepatocytes is mediated through MMP, and agents that block it, such as ciclosporin A, protect hepatocytes against APAP-induced cell death. The synergism between alcohol intake and susceptibility to APAP further illustrates the sensitization to APAP hepatocellular toxicity by mGSH depletion. Selective depletion of mGSH caused by chronic ethanol feeding contributes to the enhanced susceptibility to APAP toxicity. Furthermore, ethanol induces CYP2E1, and the enhanced APAP toxicity induced by chronic ethanol is closely associated with the magnitude of both CYP2E1 induction and mGSH depletion, as the APAP toxicity disappears when CYP2E1 and the mGSH pool return to control levels after alcohol withdrawal [28]. Thus, these observations not only explain the likely mechanisms by which ethanol potentiates the toxicity of APAP, but also help to explain the low frequency with which the interaction is observed given the high incidence of concomitant use of alcohol and APAP.

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### 2.3.10 Haem biosynthesis and excretion of porphyrins

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#### Introduction

Synthesis of porphyrins occurs in nearly all living cells: in animals for haem production and in plants for chlorophyll production. Porphobilinogen (PBG) and  $\delta$ -aminolaevulinic acid (ALA) are linear porphyrin precursors, whereas porphyrins are molecules that are cyclic precursors of haem. In mammalian cells, haem synthesis occurs mostly in liver and erythropoietic tissues. Eight enzymes are involved in haem synthesis from succinyl CoA and glycine; the biosynthetic pathway starts in the mitochondrion and, after passing through three cytoplasmic stages, re-enters the mitochondrion for the final steps of haem formation. Therefore, the first and last three enzymes are found in mitochondria and the others in the cytosol (Fig. 1). All these enzymes are encoded by nuclear genes, and their full-length human cDNA and genomic sequences have been isolated and characterized [1]. Haemoproteins, including haemoglobin or myoglobin, mitochondrial or microsomal cytochromes, catalase, peroxidase, nitric oxide synthase, prostaglandin endoperoxide