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The interplay of glutathione-related processes in antioxidant defense

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Abstract

This review summarizes current knowledge on glutathione (GSH) associated cellular processes that play a central role in defense against oxidative stress. GSH itself is a critical factor in maintaining the cellular redox balance and has been demonstrated to be involved in regulation of cell signalling and repair pathways. Enhanced expression of various enzymes involved in GSH metabolism, including glutathione peroxidases, γ-glutamyl cysteinyl synthetase (γ-GCS), glutathione S-transferases (GST) and membrane proteins belonging to the ATP-binding cassette family, such as the multidrug resistance associated protein, have all been demonstrated to play a prominent role in cellular resistance towards oxidative stress. This review stresses the fact that *a co-ordinate interplay* between these systems is essential for efficient protection against oxidative stress. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Glutathione; Glutathione S-transferases; Multidrug resistance associated protein; Oxidative stress; Antioxidant

Nomenclature

ABC	ATP binding cassette
cMOAT	canalicular multispecific organic anion transporter
γ-GCS	γ-glutamyl cysteinyl synthetase
GPX	glutathione peroxidase
GR	glutathione reductase
GS	glutathione synthetase
GSH	glutathione
GSSG	oxidized glutathione, glutathione disulphide
GST	glutathione S-transferases
GS-X	glutathione conjugate of X
γ-GT	γ-glutamyltranspeptidase
4-HNE	4-hydroxynon-2-enal
JNK	c-jun NH_2-terminal kinase
MRP	multidrug resistance associated protein
NBD	nucleotide binding domain

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NQO	NAD(P)H:quinone oxidoreductase
PD	Parkinson's disease
PK	protein kinase
ROS	reactive oxygen species

1. Background

Reactive oxygen species (ROS) are constantly generated in cells through processes such as oxidative phosphorylation/mitochondrial electron transport, metabolism of xenobiotics and inflammation. As a result cells constantly generate ROS, such as superoxide $O_2^{-\cdot}$, hydroxyl radical $^{\cdot}OH$, and hydrogen peroxide H_2O_2, while performing their metabolic functions. The increase in intracellular levels of ROS to such a level that cellular antioxidant defenses are insufficient to maintain these harmful molecules below a toxic threshold level is generally referred to as oxidative stress. ROS are highly reactive molecules, which indiscriminately interact with essential macromolecules such as DNA, proteins and lipids, leading to the disturbance of physiological processes. Oxidative stress and oxidative damage are considered to play a role in the early stages of the pathophysiological processes of specific cancers, cardiovascular diseases, cataractogenesis and several neurodegenerative diseases (Gaté et al., 1999). In addition, ROS play a role in acute conditions such as trauma, stroke and infection and probably in physical exercise, stress and ageing (Bray, 2000; Schulz et al., 2000). Oxidative stress can be prevented by the action of enzymatic and chemical antioxidant defenses. The enzymes that provide the first line of defense against superoxide and hydrogen peroxide include superoxide dismutase, catalase and glutathione peroxidase (GPX).

Chemical small molecule antioxidant compounds including vitamins, dietary flavonoids, carotenoids, uric acid and glutathione (GSH; Gaté et al., 1999) provide a second line of antioxidant defense. GSH and GSH-related processes play a central role in this second line of antioxidant defense by contributing to a number of processes, such as free radical scavenging, reduction of peroxides, detoxification of electrophilic compounds to less toxic and more hydrophilic thioether compounds, modulation of the cellular redox status and thiol-disulphide status of proteins, the action of ATP-dependent transport proteins and regulation of cell signalling and repair pathways (Sen and Packer, 1996; Palmer and Paulson, 1997; McLellan and Wolf, 1999).

In the following paragraphs the various GSH-mediated defense mechanisms against ROS and oxidative stress induced cellular damage are reviewed.

2. Antioxidant properties of glutathione

GSH (γ-L-Glu–L-Cys–Gly) is a tripeptide and represents the most prominent low molecular weight thiol (up to 5–10 mM) present in cells (Hayes and Pulford, 1995). The cellular levels of GSH are controlled by multiple enzyme systems such as γ-glutamyltranspeptidase (γ-GT), amino acid transporters, glutathione synthetase (GS), GPX and glutathione reductase (GR), but the rate-limiting reaction in GSH synthesis is catalyzed by γ-glutamyl cysteinyl synthetase (γ-GCS) (Ishikawa et al., 1997; Wild and Mulcahy, 2000). This makes regulation of γ-GCS expression and activity critical for GSH homeostasis (Griffith, 1999). The GCS holoenzyme is a heterodimer comprising a catalytic (GCS heavy subunit) and regulatory (GCS light subunit) subunit (Lu, 2000). The kinetic properties of the heavy subunit can be significantly influenced by association with the light subunit (Huang et al., 1993; Griffith, 1999; Wild and Mulcahy, 2000). Potential signalling mechanisms mediating GCS gene induction include thiol modification of critical regulatory sensor proteins (such as the Nrf2 transcription factor) and the generation of ROS (Wild and Mulcahy, 2000). Thus, antioxidant properties of GSH, linked to its intracellular concentration, are modulated by the activity of γ-GCS.

Under oxidative stress conditions, ROS are reduced by GSH with concomitant formation of the oxidized disulphide, GSSG. Although relatively resistant to 'spontaneous oxidation', GSH reacts rapidly and non-enzymatically with hydroxyl radical, and with N_2O_3 and peroxynitrite (Griffith, 1999).

In addition to its action as a chemical antioxidant GSH also acts in the enzymatic first line antioxidant defense as a co-factor in GPX mediated reduction of peroxides, also resulting in GSSG formation.

At normal physiological circumstances, GSSG is reduced to GSH by GR at the expense of NADPH, thereby forming a redox cycle (Lu, 2000). When the reductive capacity of a cell is insufficient, leading to a decrease of the GSH/GSSG ratio, the shift in thiol redox status activates redox-sensitive transcriptional elements (Sen and Packer, 1996). The intracellular GSSG content has been assumed to be an index of oxidative stress, while the GSSG/GSH ratio reflects the cellular redox status. Many proteins have thiol moieties whose

redox status is crucial to their function and there is evidence that the DNA-binding capacity of some transcription factors is redox status dependent with sulphydryl groups on cysteine residues as the target of regulation (Kearns and Hall, 1998). Due to its capacity to regulate the redox status of transcription factors, GSH may thus be involved in the regulation of stress-response gene expression (Mulcahy and Gipp, 1995). To maintain the cellular redox balance, GSSG is exported from the cell by ATP-dependent transport proteins, implicating that severe oxidative stress depletes cellular GSH (Leier et al., 1996).

All these multiple functions of GSH point at a central and critical role of GSH in the complex cellular defense against oxidative stress.

3. Glutathione peroxidases

GPXs can be divided into two types of enzymes: the selenium-dependent GPXs and the selenium-independent GPXs. The Se-dependent GPXs are able to reduce H_2O_2 and organic hydroperoxides. The Se-dependent GPX consists of five related family members (GPX1–5) and each enzyme has antioxidant properties (recently reviewed by Hayes and McLellan (1999)). The Se-independent GPX (multiple glutathione S-transferases (GST), see below) are inactive with H_2O_2 and only catalyze reduction of organic hydroperoxides. GPX enzymes play a critical role in the defense against oxidative stress. Resistance to oxidants like H_2O_2, cumene hydroperoxide and menadione was increased as a result of overexpression of the most abundant glutathione peroxidase GPX1 (Mirault et al., 1991). In addition, a GPX(−/−) mouse strain was found to be hypersensitive to the oxidant paraquat and cortical neurones from GPX(−/−) mice have been demonstrated to be more susceptible to insult exerted by H_2O_2 (de Haan et al., 1998).

4. Glutathione S-transferases

GST (EC 2.5.1.18) are a multigene family of isoenzymes that catalyze the conjugation of electrophilic compounds to GSH. GST exhibit overlapping and broad specificities for the ability to conjugate substrates to GSH. The diversity of substrates accommodated by GST is a result of both the relatively non-specific nature of the binding site for the hydrophobic substrate and the existence of numerous isoforms of GST (Salinas and Wong, 1999). GST metabolize carcinogens, environmental pollutants, drugs and a broad spectrum of other xenobiotics.

Although microsomal forms of GST have been detected (Morgenstein and DePierre, 1988), GST activity is mainly located in the cytosol. The GST found in mammalian species appear to be a supergene family of isoenzymes and are organized into seven principle gene classes designated alpha, mu, pi, theta, sigma, zeta and kappa.

GST exist as dimeric proteins comprised of two subunits. Each of the subunits possesses a glutathione-binding site (G site) as well as an adjacent relatively hydrophobic site for the binding of the electrophilic substrate. In addition, in various isozymes (at least the alpha and pi class) a non-substrate binding site has been recognized, which may function as a transport site or a regulatory site (van Bladeren, 2000). Both homo- and hetero-dimeric proteins are found within a gene class, and each monomer has a kinetically independent active site. Intergene class heterodimers are not known.

A nomenclature has been agreed upon for the human cytosolic enzymes which is, in principle applicable to all vertebrate GST and extendable to prokaryotes and other organisms (Mannervik et al., 1992). The mammalian isoenzymes are designated as A, M, P, T, S, Z and K (for alpha, mu, pi, theta, sigma, zeta and kappa) with their subunit composition or isoenzyme type designated by Arabic numerals. Allelic variants at the same locus are entitled by lower case letters following the subunit. Species differences are assigned by small letters for the GST. For example, the human alpha class GST is designated as hGSTA.

GST participate in the defence against oxidative stress as these enzymes are able to detoxify endogenous harmful compounds like hydroxyalkenals and base propenals (breakdown products of lipid peroxidation) or DNA hydroperoxides, but also electrophilic xenobiotics and/or reactive intermediates formed during their biotransformation like epoxides and quinones (Tew, 1994; Hayes and Pulford, 1995).

At least two classes GST, the classes theta and alpha, have been shown to possess GPX activity. The GST pi form may deactivate products of lipid peroxidation such as oxidative DNA-bases, lipid hydroperoxides and their derivatives such as hydroxyalkenals, malondialdehydes and base propenals (Ketterer and Christodoulides, 1994; Berhane et al., 1994). In addition, GST pi can react directly with ROS via a sensitive SH-group, and cause inactivation by disulphide formation that can be reversed by GSH. Therefore, this enzyme has a specific role in oxidative stress (Xia et al., 1996).

5. Glutathione conjugate transport proteins

The multidrug resistance associated protein (MRP) transporter belongs to the ATP binding cassette (ABC) transporter superfamily and acts as an energy-dependent efflux pump. Characteristics of these proteins are

their nucleotide binding domains (NBD), which are essential for ATP binding and hydrolysis (Center et al., 1998). MRP transporters are present in almost all cells of the human body. MRPs have been associated with drug resistance of many malignant tumours, because of their capacity to pump (conjugates of) anticancer drugs across the plasma membrane into the extracellular space, thereby decreasing the intracellular concentration of drugs (Keppler et al., 1997, 1998a,b). Initially, it was assumed that MRP1 acts as a glutathione S-conjugate efflux pump (GS-X pump), by transporting drugs which are conjugated or co-transported with GSH. However, MRP1 is a transporter of multivalent organic anions, preferentially glutathione S-conjugates, but also of glucuronide- and sulphate conjugates. Besides organic anions, transport of a range of unconjugated chemotherapeutic agents (neutral or cationic) has also been observed to be mediated by MRP (Rappa et al., 1997; Loe et al., 1998; Keppler et al., 2000). Overexpression of MRP appears to confer resistance to a range of relatively hydrophobic anticancer agents including the vinca alkaloids (e.g. vincristine, vinblastine), the epipodophyllotoxins (e.g. etoposide), and anthracyclines (e.g. doxorubicin, daunorubicin) (Broxterman et al., 1995). Unconjugated drugs can be transported by MRP, but only in the presence of GSH. In case of vincristine, there is strong evidence that GSH is co-transported in this process (Loe et al., 1998).

The physiological function of MRPs is not only closely related to cancer drug resistance, but also with cellular detoxification, oxidative stress and inflammation (Ishikawa et al., 1997). MRP mediates the transport of GSSG, the GSH conjugate of 4-hydroxynon-2-enal (4-HNE) and GSH conjugates of epoxides which all play a role in oxidative stress. MRP also mediates high affinity transport of the cysteinyl leucotriene LTC4, an important chemical mediator of inflammatory responses (Leier et al., 1994, 1996; Hipfner et al., 1999).

The MRP family consists of at least seven members; namely the multidrug resistance-associated protein MRP1, the canalicular multispecific organic anion transporter (cMOAT) or MRP2, and four homologues MRP3–6, of which MRP3 bears the closest structural resemblance to MRP1 (Zeng et al., 2000). The existence of MRP7 has been inferred from database research and very recently structure analysis of MRP7 and its expression pattern in tissues has been investigated (Borst et al., 1999; Hopper et al., 2001). MRP1, MRP2 and MRP3 have been shown to mediate the transport of drugs implicated in drug resistance (Kool et al., 1997; König et al., 1999). MRP4 was shown to transport nucleosides (Schuetz et al., 1999). MRP1, 2, 3 and 5 have the properties of a GS-X pump (Kool et al., 1997; Borst et al., 2000). Although MRP6 was detected in anthracycline-resistant cells, no drug transport activity has been demonstrated yet (Kool et al., 1997). At present, the physiological functions of MRP3–7 have not been elucidated.

Besides efflux of drug conjugates, the phase III-multidrug transport proteins are also able to transport GSSG. The function of MRP as a GSH transporter may be a potential physiological role of MRP in protecting proliferating cells against oxidative stress. These transporters may function in the defense against oxidative stress, by exporting GSSG from cells under oxidative stress, when the reduction of GSSG becomes rate-limiting and GSSG export must be increased. Initially, it has been proposed that reduced GSH itself is not recognized as a substrate for MRP, but may undergo complex formation or serve as a co-substrate with cationic substrates. This has been demonstrated for the MRP-mediated transport of vincristine in the presence of GSH (Rappa et al., 1997). However, Rappa demonstrated that GSH transport in MRP knock out embryonic stem (ES) cells was one-half that of the wild type ES cells, while intracellular GSH levels were higher in the knock outs (Rappa et al., 1997). Indeed, Paulusma et al. (1999) recently showed that MDCKII-MRP1 and MDCKII-MRP2 cells also export reduced GSH in the absence of drugs resulting in a lowered intracellular GSH concentration in these cells. Low-affinity transport of GSH was also recently demonstrated by the yeast YCF1 transporter (homologue of MRP1; Li et al., 1996; Rebbeor et al., 1998).

Based on a high GSSG/GSH ratio in HEp-2 larynx carcinoma cells that parallels MRP pump activity, it has been proposed recently that the expression of MRP involves a mechanism related to the redox status of cells, where a sufficiently high GSSG/GSH ratio triggers the activation of redox-sensitive pathways leading to the activation of GS-X pump activity (Homem de Bittencourt et al., 1998).

6. Co-ordinate interactions between the various glutathione-related systems in oxidative stress related detoxification processes

It has become increasingly evident that a co-ordinate interaction between the cellular GSH levels, GST activity and MRP transport proteins are essential to combat cellular toxic stress.

Some typical examples in which GSH and/or GST and/or transport proteins (MRP) are involved in protection against oxidative stress such as peroxides, epoxides and quinones are described below.

6.1. Detoxification of DNA-hydroperoxides and lipid peroxidation products

GST-mediated GSH conjugation has been suggested to play a prominent role in repairing oxidatively dam-

aged DNA and detoxifying base propenals, such as adenine propenal, cytosine propenal, thymidine propenal and uracil propenal, which are the toxic products of oxidative DNA degradation (Rea et al., 1998). DNA thymine hydroperoxide (5-hydroperoxymethyluracil), a model compound representing products of oxidative damage to DNA, is a substrate for GPX and some isoforms of GSH transferase (Bao et al., 1997). Base propenals are established substrates for mammalian GST, as they contain an α,β-unsaturated aldehyde moiety, which reacts with GSH via Michael addition. Especially, GSTP1-1 is capable of inactivating these reactive compounds (Fig. 1). The high activity observed with these propenal derivatives supports the notion that GSTP1-1 serves an important role in the cellular response to oxidative stress (Danielson et al., 1987; Berhane et al., 1994).

Membrane lipid peroxidation may result in formation of the α,β-unsaturated aldehydes 4-hydroxyalkenals, which are highly electrophilic in nature and thus attack various essential cellular nucleophiles. 4-HNE is the major 4-hydroxyalkenal formed during peroxidation of arachidonic and linoleic acids, which has shown to be genotoxic and cytotoxic. The detection of 4-HNE modified proteins in several degenerative diseases (Parkinson, Alzheimer, atherosclerotic lesions) suggests a role for 4-HNE in the onset of these diseases (Renes et al., 2000). The major pathway for disposition of 4-HNE is believed to be GSH conjugation catalyzed by a group of GST with exceptionally high activity toward 4-HNE. In humans, such GST have been demonstrated to be hGST5.8 and hGSTA4-4 (Fig. 1; Singhal et al., 1994, 1999; Board, 1998; Hubatsch et al., 1998; Xiao et al., 1999). Recently, it has been demonstrated that in hepatoma cells 61% of total administered 4-HNE was converted to the GSH conjugate 3-glutathionyl-4-hydroxy-2-nonenal (Tjalkens et al., 1999). This metabolite was rapidly and efficiently exported out of the cell. Recently, it has been demonstrated that the multidrug resistance protein, MRP1, protects against the toxicity of 4-HNE by transporting its GSH conjugate out of the cell (Renes et al., 2000).

6.2. Epoxides, quinones, redox-cycling and glutathione related processes

Epoxides, also called arene oxides, are well-known reactive metabolites formed upon cytochrome P450 me-

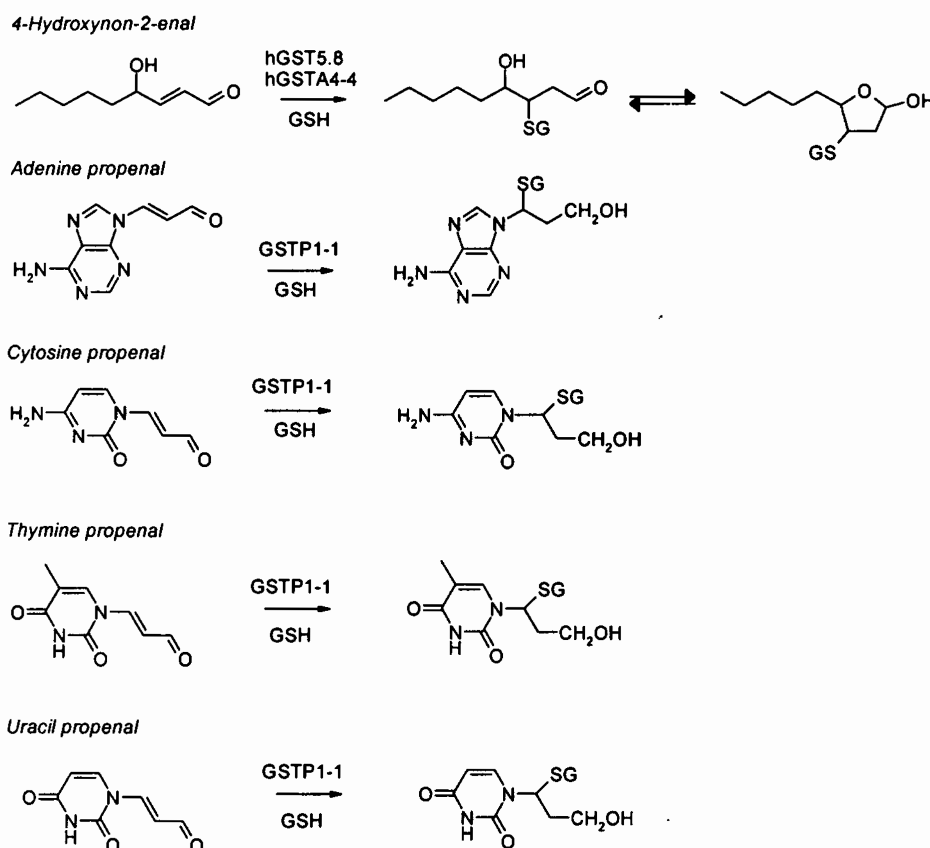

Fig. 1. GSH conjugation of GST substrates produced by oxidative stress. (a) Products of lipid peroxidation such as 4-HNE (Xiao et al., 1999); and (b) products of oxidized DNA such as adenine propenal, cytosine propenal, thymine propenal and uracil propenal (Berhane et al., 1994).

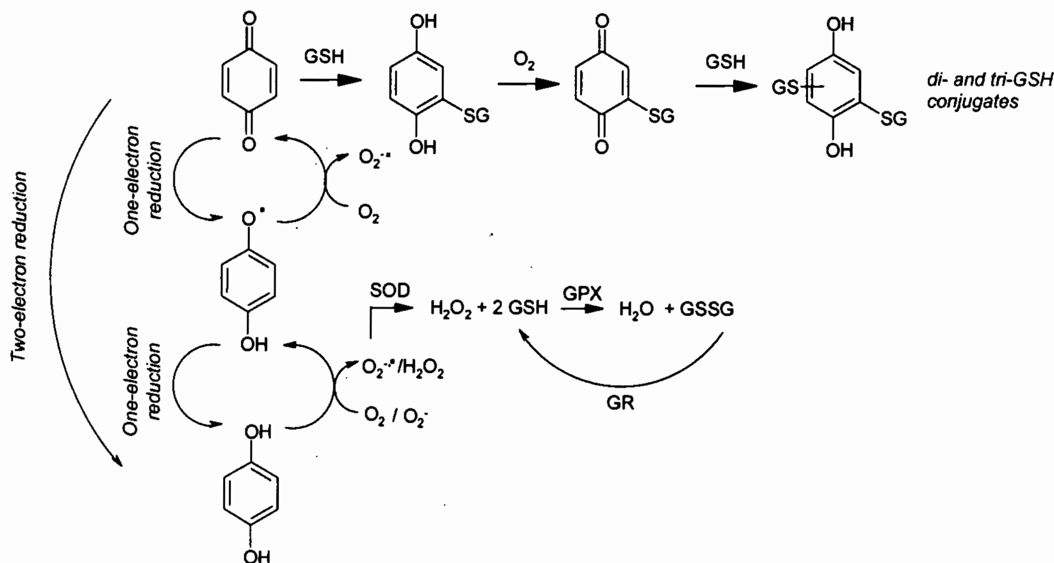

Fig. 2. The involvement of GSH-related defense mechanisms in the quinone redox-cycling process. The reactive quinones are scavenged by GSH. The superoxide anion is reduced to hydrogen peroxide by superoxide dismutase. Hydrogen peroxide is detoxified by GPX to form H_2O and oxidized glutathione. To deal with the continuous formation of H_2O_2, GSSG is reduced by GR.

diated conversion of compounds containing unsaturated carbon–carbon bonds. The alkylating properties of the epoxide moiety are responsible for the toxic effects and conversion by epoxide hydrolase and GST represent the major routes for epoxide detoxification. An example can be found in the metabolic fate of aflatoxin B1.

Aflatoxin B1, one of a group of related mycotoxins produced by the common fungal mould *Aspergillus flavus*, is a well-documented rat and human carcinogen (Eaton and Gallagher, 1994). Aflatoxin B1 is activated to the highly reactive aflatoxin 8,9-*exo*-epoxide by cytochromes P450 (Gallagher et al., 1996). This epoxide then serves as a substrate for GST, especially mGSTA3-3 has high activity toward this epoxide.

GST catalyzed conjugation of GSH to aflatoxin B1-8,9-epoxide plays an important role in preventing binding of this ultimate carcinogen to target macromolecules (Fields et al., 1999). Once formed, the glutathione S-conjugated epoxide of the potent mutagen aflatoxin B1 is actively extruded from the cell by an ATP-dependent export pump. The 190-kDa multidrug resistance protein (MRP1) is capable of energy-dependent transport of aflatoxin B1 (in the presence of GSH) and its GSH conjugates and suggest a potential protective role for MRP in mammalian chemical carcinogenesis (Loe et al., 1997).

Quinones, the so-called redox-cycling compounds induce massive radical damage upon the one-electron reduction by cytochromes P450 or by flavin dependent enzymes like NADPH-cytochrome P450 reductase and the two-electron reduction by NAD(P)H:quinone oxidoreductase (NQO). The resulting semiquinone radical and the hydroquinone form are regenerated to their parent quinone with concomitant formation of $O_2^{-\bullet}$ superoxide anions and H_2O_2 (Fig. 2).

Many drugs contain a quinone-moiety such as menadione, doxorubicin and mitomycin C, but quinones are also formed during the biotransformation of aromatic compounds. The toxicity of quinones is generally ascribed to their capacity to bind to cellular nucleophilic macromolecules and/or their capacity to support the redox-cycling process.

GSH has been demonstrated to prevent oxidative stress caused by redox-cycling of quinones. GSH conjugation of quinones prevents the electrophilic quinones reacting with cellular macromolecules. In addition, GSH and GSH-related enzymes are involved in scavenging the ROS formed during the redox-cycling process (Fig. 2). If the resulting GSH conjugate of the quinone is efficiently transported out of the cell, GSH conjugation may prevent quinones from participating in redox-cycling.

A well-known example of the involvement of GSH-related processes relates to the development of drug resistance against the relatively poor electrophilic anthracyclines such as the quinone doxorubicin. Anthracycline-resistant cells often exhibit a co-ordinate overexpression of MRP, γ-GCS and GST. For instance, overexpression of MRP and γ-GCS genes have been demonstrated to correlate with doxorubicin resistance in human malignant mesothelioma cell lines (Ogretemn et al., 1998). Overexpression of MRP results in increased resistance to anthracycline drugs and GSH seems to be required (Versantvoort et al., 1995; Zaman et al., 1995; Rappa et al., 1997). Although GSH conju-

gates have been synthesized and have been shown to interact with MRP, no compelling evidence for in vitro or in vivo formation of GSH conjugates of anthracyclines has been provided (Priebe et al., 1998; Gaudiano et al., 2000). Doxorubicin also participates in the redox-cycling pathway with production of ROS. GSH, GST and MRP may also contribute to drug resistance by another mechanism such as interacting with the ROS and oxidative stress products like 4-hydroxyalkenals (McLellan and Wolf, 1999).

Another example of the involvement of GST/GSH in quinone-induced oxidative stress is *o*-quinone formation of dopamine, an event which is believed to contribute to neurodegenerative disorder such as Parkinson's disease (PD; Schulz et al., 2000).

PD is characterized by a loss of the neurotransmitter dopamine due to degeneration of dopaminergic neurons located in the substantia nigra in the brain (Gibb and Lees, 1991), and oxidative stress has been implicated as a major factor herein (Götz et al., 1994). An important role of GSH was proposed because a decrease in total GSH concentrations in the substantia nigra has been observed already in preclinical stages, at a time when other biochemical changes (such as increased 5-S-cysteinyldopamine, catalase, GPX, and γ-GT) are not yet detectable (Schulz et al., 2000). Metabolism of dopamine (Fig. 3) both chemical and enzymatic leads to the formation of an array of dopamine-quinones, free oxygen radicals and toxic cysteine conjugates (Shen et al., 2000). GSH plays an important role in various detoxification steps. Dopamine-*o*-quinone and the cyclized form aminochrome can directly be conjugated with GSH by GST, especially by hGSTM2-2, protecting protein thiols and preventing the redox-cycling which occurs with the further reduction of aminochrome (Segura-Aguilar et al., 1997; Dagnino-Subiabre et al., 2000). GSH is also used by GPX in the detoxification of free oxygen radicals. Another interesting process in which GSH plays a role is the fact that γ-glutamyl transferase (γ-GT) is increased in PD. γ-GT is an ectoenzyme on the membrane that catalyzes the transfer of the γ-glutamyl moiety from extracellular GSH or a GSH conjugate onto an acceptor molecule. In glial cells extracellular GSH is hydrolyzed by γ-GT to cysteine and glycine, which can be taken up and used by neurons in the synthesis of GSH. Neurons cannot take up GSH directly. The release of GSH from nigral glia cells and the increased activity of γ-GT may be the initial step in pathogenesis of PD. It has been proposed by Schulz et al. (2000) that if cysteine is not used for GSH synthesis, it may react with dopamine-*o*-quinone to form 5-S-cysteinyldopamine, which in turn can be converted to highly cytotoxic dihydrobenzothiazine derivatives (Shen et al., 2000).

On the other hand, GSH conjugation of quinone-derivatives does not always represent a detoxification pathway as has been hypothesized for *p*-benzoquinone or *N*-acetyl-*p*-benzoquinone imine, because the GSH conjugate retains the electrophilic and redox properties of the parent molecule. Although conjugation with GSH and the active transport of the GSH conjugates out of the cell in which they are formed will limit their potential toxicity to those cells, once within the circulation they can be transported to tissues that are capable of accumulating these metabolites (Monks and Lau,

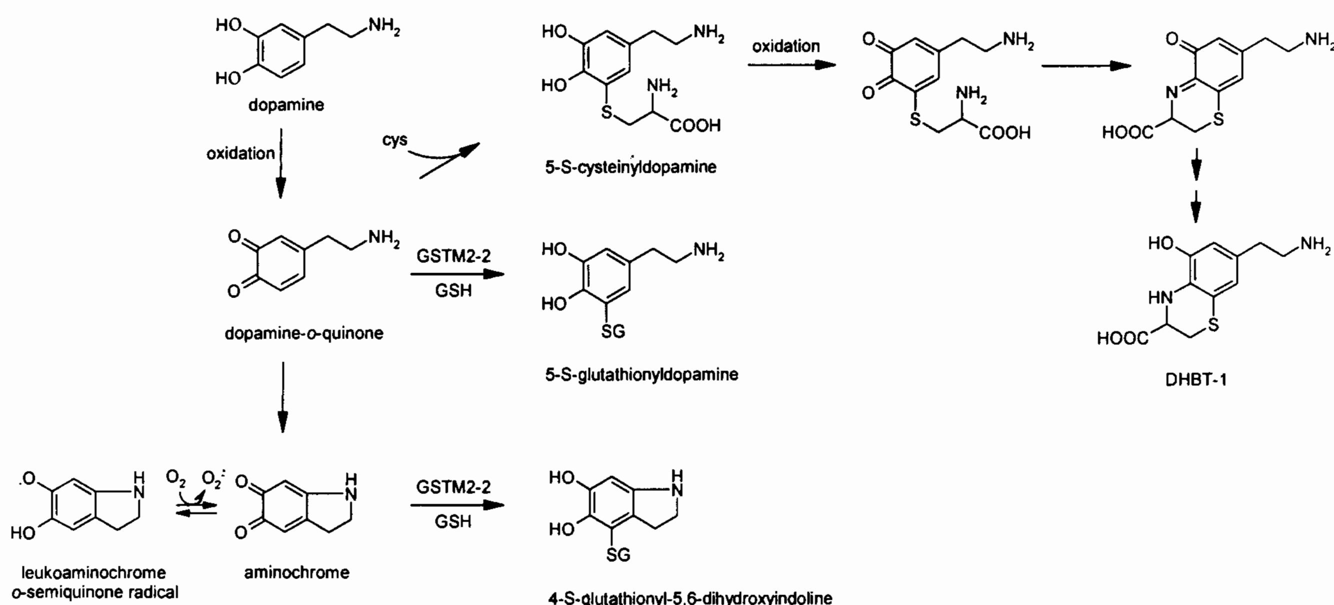

Fig. 3. The involvement of GSH in the biotransformation of dopamine.

1998). GSH targets these metabolites to the kidney, where subsequent processing by the proximal tubular brush border enzyme γ-GT are thought to be involved in the selective nephrotoxicity of these compounds (Baillie and Slatter, 1991; Fowler et al., 1994; Emeigh Hart et al., 1996; Kleiner et al., 1998; Monks and Lau, 1998).

7. Co-ordinate regulation of glutathione related enzymes and signalling pathways

Is has been reported recently by many investigators that a co-ordinate interplay between enzymes involved in drug detoxification (GST, γ-GCS, NQO) and transporters like MRP1 are essential for a good defense against toxic compounds (Laing et al., 1999). Likewise, expression of genes encoding biotransformation enzymes (a.o. GST, γ-GCS, NQO) and transporters (MRP1) are frequently observed to be co-ordinately induced already at the onset or during chemotherapy. Co-ordinate induction of γ-GCS and MRP is frequently observed by for instance cisplatin, ethacrynic acid, heavy metals and nitrosourea-based agents like ACNU (Ciaccio et al., 1996; Kuo et al., 1996; Gomi et al., 1997a,b; Kuo et al., 1998; Oguri et al., 1998). The mechanism by which genes of MRP and GSH-based enzymes (GST, γ-GCS) are upregulated is not known, but certain common intrinsic redox-sensitive factors may be involved in the expression of these genes as most inducers are compounds that can generate intracellular oxidation–reduction labile conditions and exert oxidative stress. The following observations corroborate the hypothesis that redox-sensitive regulation of the intrinsic network of detoxifying genes such as γ-GCS, GST and MRP might be involved; intracellular reactive oxygen intermediates derived from the pro-oxidants *tert*-butylhydroquinone, menadione and 2,3-dimethoxy-1,4-naphthoquinone have been shown to induce expression of γ-GCS and MRP1 in rat hepatoma H-4-II-E and human HepG2 cells. Elevated levels of GSH also down-regulated MRP1 and γ-GSC (Yamane et al., 1998).

Cellular stress can result in enhanced formation of c-fos and c-jun gene products. These form heterodimeric complexes known as AP-1. The transcription factor AP-1 binds specifically to AP-1 binding sites in the regulatory region of many detoxification genes, thereby affecting the transcriptional expression of cellular genes. Many MRP and γ-GCS overexpressing cells overexpress the transcription factor AP-1 (Powis et al., 1995). The involvement of AP-1 in the expression of MRP and γ-GCS can also be found in yeast. Yeast *GSH1* encoding γ-GCS and *YCF-1* encoding the human MRP homologue are co-ordinately regulated by *yAP-1* encoding transcription factor AP-1 (Wemmie et al., 1994; Wild and Mulcahy, 2000). In both the human MRP (Zhu and Center, 1994), γ-GCS (Mulcahy and Gipp, 1995; Mulcahy et al., 1997) and GST (Hayes and Pulford, 1995; Ainbinder et al., 1997) a putative oxidative stress-response element can be found. AP-1 binding sites are found to be present in the promoter regions of genes encoding γ-GCS and MRP (Zhu and Center, 1994; Mulcahy and Gipp, 1995).

Indeed the promoter region of GST contains an AP-1 motif suggesting that these genes may be regulated through the c-fos and c-jun gene products (Ainbinder et al., 1997). Increased expression of c-fos and c-jun is also associated with increased protein kinase (PK; Volm, 1998). In this regard it is interesting to know that MRP and GST-π are highly phosphorylated by PKs. The antioxidant *N*-acetylcysteine has been reported to inhibit tyrosine kinase-dependent induction of c-jun expression which points at a role for redox effect in the signal transduction pathway (Choi and Moore, 1993).

In addition, the antioxidant responsive element (ARE) or electrophile responsive element (EpRE) is thought to be mediating the effect of electrophilic compounds on the expression of certain biotransformation enzymes (such as GST, γ-GCS, QR). Some of these ARE/EpRE elements also include an AP-1 binding site. In the case of the regulatory subunit of γ-GCS, the constitutive expression is believed to be regulated by the AP-1 binding site, whereas the induction is regulated via the ARE/EpRE (Moinova and Mulcahy, 1998). The transcription factors that bind to the ARE/EpRE and mediate the inducible expression are believed to be heterodimers of bZIP transcription factors, such as Nrf2 and Nrf1, with small Maf family proteins or with Jun factors (Itoh et al., 1997; Venugopal and Jaiswal, 1998; Jeyapaul and Jaiswal, 2000). Probably a number of transacting factors interact; fos/jun may be involved in basal expression but heterodimers with members of the Maf family may be responsible for GST induction by monofunctional inducers (Hayes and Pulford, 1995). Under non-stressed conditions, a negative regulatory protein (Keap1), keeps Nrf2 in a silent state. Exposure to oxidative stress dissociates Nrf2 from Keap1 and allows reallocation of Nrf2 to the nucleus, where ARE responsive genes become actively transcribed (Tew and Ronai, 1999). Tew and co-workers (Tew et al., 1998) identified a DNA-PK which has been proposed to serve as an early stress response gene which may activate downstream transcription factors. The catalytic subunit of DNA-dependent PK has a high affinity for (^{35}S)azidophenacyl-GSH. In vitro data indicate that DNA-PK may influence phosphorylation of many transcription factors including SP1, p53, fos, jun and myc. Some of these transcription factors have been demonstrated to play a role in gene regulation of GST, γ-GCS and MRP (Zhu and Center, 1994; Hayes and Pulford, 1995). High levels of GSH-conjugates indicate

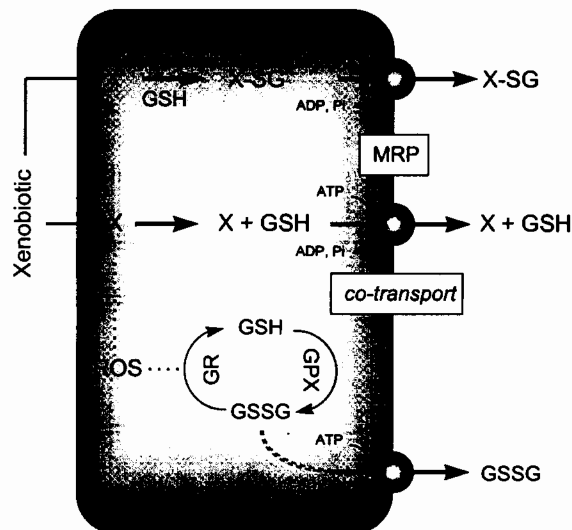

Fig. 4. The co-ordinate interaction between cellular GSH, glutathione S-transferase and glutathione S-conjugate efflux pump MRP.

cell stress and it has been speculated that DNA-dependent PK may serve as a receiver and transmitter of signals which contribute to drug resistance and maintain cell viability (Shen et al., 1997; Tew et al., 1998).

Recently, compelling evidence has been provided that GSTP1-1 has a non-enzymatic regulatory role in controlling cellular response to external stimuli (Tew and Ronai, 1999). Under conditions of oxidative stress, GSTP1-1 dissociates from c-jun NH_2-terminal kinase (JNK) and forms dimer and/or multimeric complexes. The liberated JNK regains its functional capacity to be phosphorylated and to phosphorylate c-jun, leading to enhanced transcription of AP-1 responsive genes, thereby activating the stress cascade (Adler et al., 1999a,b; Tew and Ronai, 1999).

8. Conclusion

Rather than the overexpression of the detoxification enzymes and transport proteins per se, a co-ordinate interaction between phase II GST-dependent and phase III MRP-dependent systems has been shown to be essential in the protection of cells by lowering intracellular concentrations of harmful molecules (Fig. 4). The ATP-dependent efflux protein MRP is able to transport conjugated metabolites (organic anions such as GSH conjugates) and then functions in relation to GST. MRP also transports unmodified neutral or cationic agents together with GSH (co-transport) and the level of GSH in the cell is then an important factor in MRP-mediated transport of harmful molecules. Lastly, MRP may be involved in the oxidative stress-induced gene-expression of the detoxification system. Since MRP is responsible for the release of GSSG from the intracellular environment, it functions together with various reducing pathways in maintaining the reduced status of intracellular thiols under oxidative stress conditions.

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