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Glutathione and Glutathione-dependent Enzymes Represent a Co-ordinately Regulated Defence Against Oxidative Stress

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Increases in the intracellular levels of reactive oxygen species (ROS), frequently referred to as oxidative stress, represents a potentially toxic insult which if not counteracted will lead to membrane dysfunction, DNA damage and inactivation of proteins. Chronic oxidative stress has numerous pathological consequences including cancer, arthritis and neurodegenerative disease. Glutathione-associated metabolism is a major mechanism for cellular protection against agents which generate oxidative stress. It is becoming increasingly apparent that the glutathione tripeptide is central to a complex multifaceted detoxification system, where there is substantial inter-dependence between separate component members. Glutathione participates in detoxification at several different levels, and may scavenge free radicals, reduce peroxides or be conjugated with electrophilic compounds. Thus, glutathione provides the cell with multiple defences not only against ROS but also against their toxic products. This article discusses how glutathione biosynthesis, glutathione peroxidases, glutathione *S*-transferases and glutathione *S*-conjugate efflux pumps function in an integrated fashion to allow cellular adaption to oxidative stress. Co-ordination of this response is achieved, at least in part, through the antioxidant responsive element (ARE) which is found in the promoters of many of the genes that are inducible by oxidative and chemical stress. Transcriptional activation through this enhancer appears to be mediated by basic leucine zipper transcription factors such as Nrf and small Maf proteins. The nature of the intracellular sensor(s) for ROS and thiol-active chemicals which induce genes through the ARE is described. Gene activation through the ARE appears to account for the enhanced antioxidant and detoxification capacity of normal cells effected by many cancer chemopreventive agents. In certain instances it may also account for acquired resistance of tumours to cancer chemotherapeutic drugs. It is therefore clear that determining the mechanisms involved in regulation of ARE-driven gene expression has enormous medical implications.

Keywords: Antioxidant responsive element, cancer chemoprevention, γ-glutamylcysteine synthetase, glutathione peroxidase, glutathione *S*-conjugate efflux pump, glutathione *S*-transferase, glutathione synthetase, multidrug resistance-associated protein

Abbreviations: AFB_1, aflatoxin B_1; ALDH, aldehyde dehydrogenase; AP-1, activator-protein-1; ARE, antioxidant responsive element; BHQ, *tert*-butylhydroquinone; βNF, β-naphthoflavone; bZIP, basic leucine zipper; COX, cyclooxygenase; CYP, cytochrome P450; DGR, double glycine repeats; GPX, glutathione peroxidase; GCS, γ-glutamylcysteine synthetase; GCS_h, γ-glutamylcysteine synthetase heavy subunit;

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GCS$_l$, γ-glutamylcysteine synthetase light subunit; GPEI, glutathione transferase P enhancer I; GS, glutathione synthetase; GSH, reduced glutathione; GSSG, oxidised glutathione; GST, glutathione *S*-transferase; γGT, γ-glutamyl transpeptidase; HO-1, heme oxygenase-1; KGF, keratinocyte growth factor; MAPEG, membrane associated proteins involved in eicosanoid and glutathione metabolism; MRP, multidrug resistance-associated protein; NQO1, NAD(P)H : quinone oxidoreductase 1; PAH, polycylic aromatic hydrocarbon; PB, phenobarbital; PBREM, phenobarbital-responsive enhancer module; ROS, reactive oxygen species; SOD, superoxide dismutase

BACKGROUND

Reactive oxygen species (ROS; e.g. the superoxide radical, H_2O_2, and the hydroxyl radical) are produced continuously within the cell as a result of mitochondrial electron transfer processes or as a byproduct of the actions of the enzymes xanthine oxidase, lipoxygenase and cyclooxygenase (COX).[1] Furthermore, ROS can also be generated as a consequence of intracellular metabolism of foreign compounds by cytochrome P450 (CYP) monooxygenases or because of exposure to environmental factors such as excessive iron salts or UV irradiation.[1,2] Reactive oxygen species are highly cytotoxic, and can cause damage to DNA, impairment of protein function and peroxidation of lipids. Several different antioxidant enzymes exist, including superoxide dismutase (SOD), catalase, thioredoxin peroxidase, peroxiredoxin and glutathione peroxidase, which convert ROS into less noxious compounds. Collectively, these enzymes provide a first line of defence against superoxide and H_2O_2 (Figure 1). Whilst such enzymes are of enormous importance in limiting ROS-mediated damage to intracellular macromolecules, they are clearly not 100% effective at performing this task as under normal physiological conditions, lipid and DNA oxidation products can be detected in blood and urine.[3] Since certain of the chemicals generated following interaction of ROS with macromolecules are highly reactive, there is an equal necessity to detoxify these secondary oxidation products in order to prevent them from also

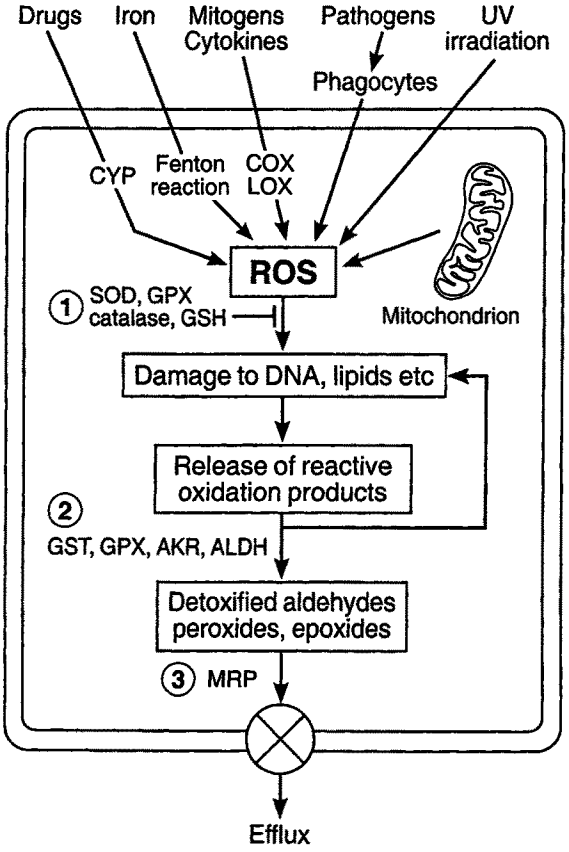

FIGURE 1 *Multiple levels of defence against ROS.* The cell possesses at least three tiers of defence against ROS. The first of these is provided against the superoxide anion and H_2O_2 by SOD, GPX, catalase and glutathione. The second tier of defence is provided by the detoxication enzymes GST, GPX, AKR and ALDH, and the third tier is provided by the efflux pump MRP. The glutathione-associated components of these defences are inducible and therefore allow adaption to situations in which the cell is subjected to increased levels of ROS.

damaging DNA, proteins and lipid; without the adequate detoxification of such products, an extended chain reaction will occur resulting in the degradation of cellular components and the ultimate death of the cell. This second line of defence against ROS is provided by enzymes such as glutathione peroxidase, glutathione *S*-transferase, aldo–keto reductase and aldehyde dehydrogenase (Figure 1). Finally, the detoxified metabolites produced by these enzymes are eliminated from the cell by energy-dependent

efflux pumps such as the glutathione *S*-conjugate transporter, also called the multidrug resistance-associated protein (MRP). It is apparent from this brief description that reduced glutathione (GSH) plays a central role in intracellular antioxidant metabolic processes, as it is involved in not only the first and second lines of defence against ROS, but is also involved in the ultimate removal of detoxified oxidation products from the cell. Most importantly, many of the GSH-dependent proteins are inducible and therefore represent a means whereby cells can adapt to oxidative stress.

OXIDATIVE STRESS AND GLUTATHIONE

Glutathione is the principal intracellular non-protein thiol.[4] It is present in concentrations up to 10 mM in many cells and provides a primary defence against oxidative stress by its ability to scavenge free radicals or participate in the reduction of H_2O_2(Figure 2). Mechanisms whereby GSH synthesis can be up-regulated in response to increased utilisation of the thiol are an essential component of adaptive cellular response to oxidative stress. Evidence suggests that regulation of glutathione synthesis is achieved through γ-glutamylcysteine synthetase (GCS), the enzyme catalysing the penultimate step in the pathway.[4]

Glutathione is synthesised in the cell cytosol by the sequential actions of GCS and glutathione synthetase (GS). GCS activity appears to be rate-limiting as it is subject to feed-back inhibition by physiological concentrations of glutathione.[5] GCS is a heterodimer comprising a catalytic (heavy subunit, GCS_h, 73 kDa) and regulatory (light subunit, GCS_l, 30 kDa) polypeptide.[6–8] The larger GCS_h is responsible for the catalytic activity, whereas the smaller GCS_l modulates the K_m of the enzyme for glutamate and its sensitivity to feed-back inhibition by glutathione. The two subunits interact through both hydrophobic and covalent interactions, the latter being in the form of a reversible disulphide bridge. Formation or reduction of the disulphide bridge has been postulated to represent a mechanism whereby GCS activity is regulated by intracellular redox status *in vivo*.[7]

In addition to kinetic mechanisms for the regulation of GCS activity and glutathione synthesis, the levels of the heavy and light polypeptides also appear to play a role in GSH homeostasis. The GCS subunits are subject to tissue-specific expression, with the greatest levels being found in kidney which has a high rate of glutathione synthesis.[7] Expression of GCS_h and GCS_l may not be co-ordinated as the relative amounts of mRNA encoding each of the subunits varies between different tissues, suggesting that molar ratios of GCS_h and GCS_l are not constant.[9] Possibly variation in the relative amounts of the heavy and light subunits represents a further means by which GCS activity can be regulated. Recent studies have demonstrated that heterologous stable overexpression of GCS_l in HeLa cells increases GCS activity, and provides the cells with an enhanced ability to resynthesise glutathione following its depletion by diethylmaleate.[10]

Both GCS_h and GCS_l have been shown to be inducible by agents which can generate an oxidative stress, such as *tert*-butylhydroquinone (BHQ) and β-naphthoflavone (βNF).[11–16] This has been shown to be in part due to transcriptional activation of the genes encoding GCS_h (*GLCLC*) and GCS_l (*GLCLR*). The involvement of an antioxidant responsive element (ARE; discussed in more detail below) has been proposed in βNF-mediated induction of *GLCLC*.[12] Also, Moinova and Mulcahy[15] found that an ARE was important for induction of *GLCLR* by βNF. Further studies have shown that induction of *GLCLR* expression by BHQ does not depend on an ARE, but requires a 42 bp nucleotide sequence upstream of the ARE.[16]

Glutathione synthetase does not appear to represent a regulatory step in glutathione biosynthesis. Whilst GS catalyses the final stage in glutathione synthesis, it is not subject to product inhibition, and there is no evidence to suggest that GS activity is rate-limiting.[4] Nevertheless, GS is

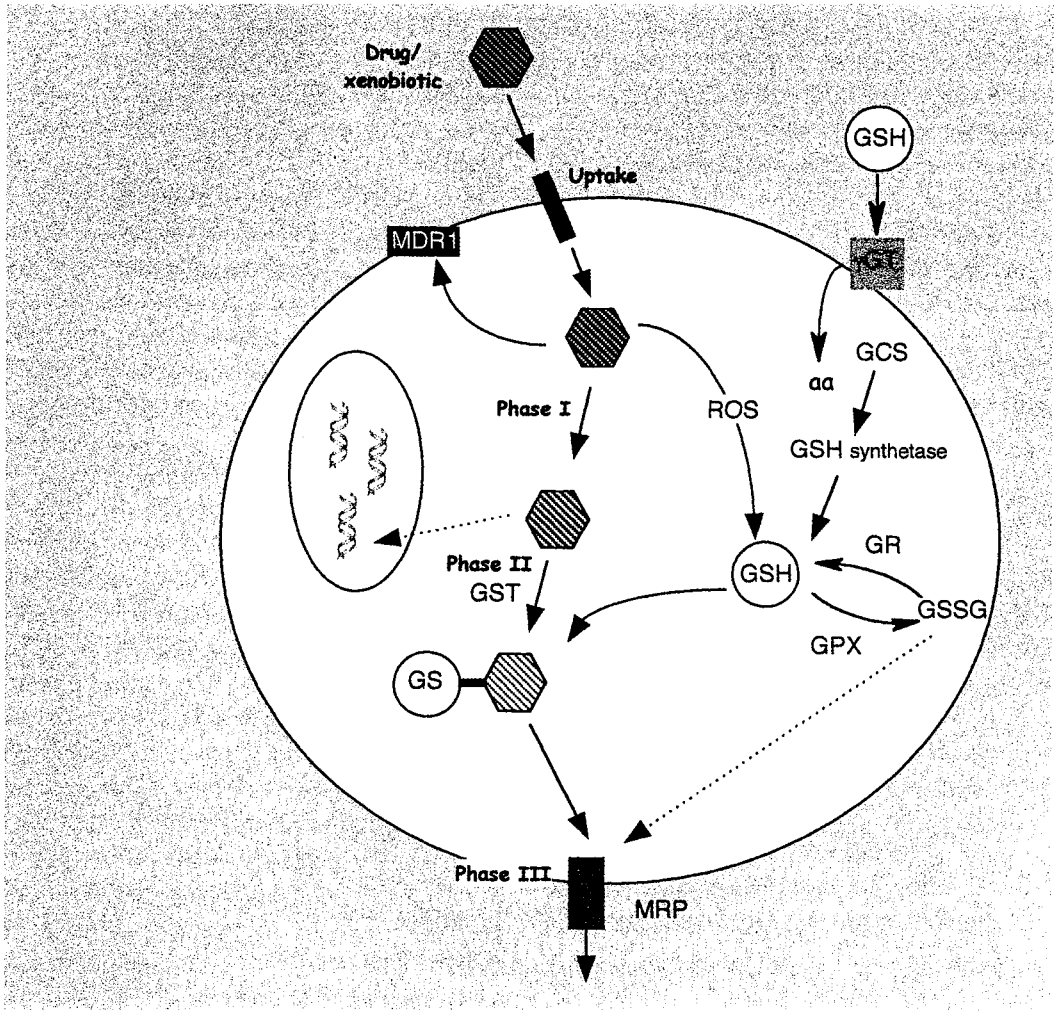

FIGURE 2 *Glutathione-dependent cellular defence mechanisms.* Environmental toxins or drugs can undergo metabolism by cytochrome P450s (phase I) to generate electrophilic cytotoxic intermediates as well as ROS. Glutathione associated detoxification mechanisms play a major role in their detoxification. GST (phase II) catalyse glutathione conjugation reactions, which allows xenobiotics to be recognised by MRP family members which transport conjugates from the cell (phase III), whereupon they can be metabolised further and excreted from the organism. MRP can also transport certain unmodified agents in the presence of glutathione. Glutathione is also important in the reduction of peroxides and the scavenging of free radicals. This can result in the production of GSSG which is either reduced by GR or exported by MRP. Glutathione is synthesised in the cytosol by GCS and GS. γGT may play a role in salvage of amino acids (aa) from extracellular glutathione.

subject to tissue-specific expression, with high levels being found in kidney.[17] This suggests that in cells with a requirement for a large capacity to synthesise glutathione it is necessary for the levels of GCS and GS to be appropriately elevated. It is interesting to note that levels of γ-glutamytranspeptidase (γGT) are also high in kidney, and it has been proposed that under certain circumstances glutathione synthesis can take place in the presence of GS and γGT without a requirement for GCS. This would require firstly the transfer of the γ-glutamyl moiety of glutathione by γGT to cystine, the import of γ-glutamylcystine and its subsequent reduction to γ-glutamylcysteine plus cysteine, followed by the addition of glycine to γ-glutamylcysteine by GS.[4]

Glutathione is central to the detoxification of ROS, but in the absence of an enzyme system to catalyse the many different detoxification reactions, as well as the ability to catalyse the reduction of oxidised glutathione (GSSG), this thiol could not function as an intracellular antioxidant. The production of GSSG by the reduction of peroxides or as a consequence of free radical scavenging is potentially highly cytotoxic. In this regard, the activity of glutathione reductase, which utilises NADPH as a reductant, represents one of the most important determinants of cellular protection against oxidative stress. Alternatively, if the capacity of glutathione reductase is exceeded, members of the MRP family of transport proteins can act to export GSSG from the cell.[18] The presence of glutathione is, therefore, not in itself sufficient to prevent the cytotoxicity of ROS, and the glutathione-dependent enzymes which participate in the first and second lines of defence against mediators of oxidative stress are discussed below.

GLUTATHIONE PEROXIDASES

Members of the GPX Family of Selenium-dependent Enzymes

Glutathione peroxidases (GPX) catalyse the reduction of H_2O_2 and organic hydroperoxides to water and alcohols respectively, with the generation of GSSG. Several distinct families of enzymes have evolved which display GPX activity, and these have simply been classified as being selenium-dependent or selenium-independent. Until fairly recently, only one Se-dependent glutathione peroxidase (GPX1) was known, whereas it was recognised that multiple glutathione *S*-transferases exhibit Se-independent peroxidase activity; these enzymes can be functionally distinguished because GPX1 can reduce both H_2O_2 and organic peroxides, whilst the transferases are inactive with H_2O_2 and only exhibit activity with organic hydroperoxides. Within the past decade however, it has become apparent that the Se-dependent GPX is not a unique enzyme but is a member of a structurally-related family of peroxidases, not all of which require Se for catalysis (Table I). In addition, other unrelated enzymes have also been shown to possess GPX activity.

The most extensively studied GPX is the selenium-dependent enzyme discovered by Mills in 1957.[19] With the subsequent discovery of additional glutathione peroxidases, this enzyme has since been named GPX1, or cytosolic (or cellular) GPX. Molecular characterization of peroxidases has resulted in the addition of a further four evolutionary-related proteins to the GPX multigene family (Table I).

Biological Function and Regulation of GPX1

In the human, GPX1 is ubiquitously expressed and is particularly abundant in erythrocytes, kidney and liver. In addition to being cytosolic, GPX1 is also found in mitochondria.[20] Several *Gpx* gene knockouts have been performed.[21,22] Contrary to expectations, the first *Gpx* (−/−) mouse reported appeared to have no phenotype in that neither development nor fertility was affected by the mutation, nor was any abnormal histopathology found.[21] Also, the *Gpx* (−/−) mouse was not found to have increased sensitivity to hyperbaric oxygen, leading to the suggestion that contribution of GPX1 activity to cellular protection in both normal and hyperoxic conditions is limited. However, it has subsequently been shown that GPX1 is of critical importance in protection against oxidative stress generated by paraquat and H_2O_2.[22] These latter studies described the generation of a separate *Gpx* (−/−) mouse strain, and in addition to showing that they were hypersensitive to the lethal effects of the oxidant paraquat, it was demonstrated that cortical neurones from the *Gpx* (−/−) mice were more susceptible to killing by H_2O_2.

GPX1 may also be important in protection from certain pathogens. Beck *et al.*[23] have found the

TABLE I Human glutathione peroxidases

Glutathione peroxidase	Nomenclature	Localisation	Predicted subunit size (kDa)*	Selenium dependency	Substrates	Electron donors	Identities (%) with GPX1
Cytosolic GPX	GPX1	Intracellular, cytosolic (partly mitochondrial)	21.9	Yes	H_2O_2, t-BHP	GSH	100
Gastrointestinal GPX‡	GPX2	Intracellular, cytosolic	21.9	Yes	H_2O_2, t-BHP	GSH	68
Extracellular GPX (plasma GPX)	GPX3	Plasma	25.5	Yes	H_2O_2, t-BHP, phospholipid hydroperoxides	GSH, thioredoxin, glutaredoxin	47
Phospholipid hydroperoxide GPX	GPX4	Intracellular, partly cytosolic, partly mitochondrial, partly membrane-bound	22.1	Yes	Phospholipid hydroperoxides, thymine hydroperoxide, H_2O_2	GSH, DTT, 2-ME, L-Cys	36
Epididymal GPX†,‡	GPX5	Epididymal fluid, epididymis	25.2	No	Low activity towards H_2O_2 and organic hydroperoxides	GSH (physiological electron donor unidentified)	50
Selenoprotein P	Not assigned	Plasma	43.2	Yes	Phospholipid hydroperoxides	GSH, DTT, 2-ME, L-Cys, DL-Homocys	Not significant

Abbreviations: GPX, Glutathione peroxidase; t-BHP, tert-Butylhydroperoxide; DTT, 1,4-Dithiothreitol; 2-ME, 2-Mercaptoethanol; L-Cys, L-Cysteine; DL-Homocys, DL-Homocysteine.

*Molecular weights were calculated from the predicted open reading frame from the GPX cDNAs and do not take post-translational modifications nor the potential use of alternative translational start sites into account.

†GPX5 protein has been detected in rat and macaque epididymal and sperm extracts, but not in human. It has been predicted that the majority of human GPX5 transcripts are incorrectly spliced.

‡Data on substrates and location are for rodent or porcine GPX.

Gpx1 (−/−) mice to be sensitive to myocarditis following infection with an avirulent strain of coxsackievirus B3. The myocarditis was found to be associated with an increased mutation frequency of the benign coxsackivirus in infected *Gpx* (−/−) mice, resulting in virulence; transformation from avirulence to virulence in the GPX1-deficient mice is proposed to be a result of oxidative RNA damage, increased mutagenesis of oxidised viral RNA and impairment of immune function. The observation that the *Gpx* (−/−) mutation predisposes to the transformation of benign viruses to virulent strains is of particular interest with regard to Keshan disease, a cardiomyopathy prevalent in Se-deficient regions of China. Although Keshan disease has a viral component, coxsackivirus being the principal candidate, infection is not sufficient for cardiomyopathy, and it is postulated that Se deficiency is required for the deleterious effect of the virus. As selenium deficiency can result in diminished GPX activity, the *Gpx* (−/−) mouse study supports a mechanism whereby impaired GPX activity in Se-deficient individuals may promote virulence of coxsackiviruses leading to the cardiomyopathy found in Keshan disease. It is feasible that antioxidant status and GPX activity may also be of importance in other human viral diseases.

Transcriptional upregulation of GPX1 as an adaptive response to oxidative stress has been demonstrated *in vitro*. Using reporter constructs with the human *GPX1* promoter, it has been shown that paraquat can induce transcriptional activity by between 2- and 3-fold.[22] Furthermore, transcriptional activity of *GPX1* has been shown to be increased by hyperoxia.[24] The mechanism for this hyperoxia-mediated induction is unknown and it does not appear to be dependent on an oxygen response element or AP-1 sites in the 5′-flanking sequence of the *GPX1* gene. The induction of GPX1, however, does not appear to be part of a co-ordinately regulated adaptive response to xenobiotics that can be metabolised to electrophiles *in vivo*. Previous studies from our laboratory have shown that GPX activity towards H_2O_2 is either decreased or unchanged in the livers of rodents treated with compounds that induce GST activity.[25,26]

Contribution of GPX Isoenzymes to Resistance to Oxidative Stress

In addition to GPX1, four further related family members have been identified to date that possess at least 36% amino acid sequence identity with GPX1. These additional peroxidases have been called GPX2–GPX5, and their properties are summarised in Table I. Although there is uncertainty about the physiological role of certain of these proteins, there is evidence that phospholipid hydroperoxide glutathione peroxidase (GPX4) has antioxidant properties *in vivo*.[27]

GPX4 is widely expressed, but is most abundant in renal epithelial cells and testes where it has been found to be present in the cytosol and mitochondria.[28] In addition, GPX4 has been found to be associated with other intracellular membrane fractions, and it has been proposed that the principal function of GPX4 is the reduction of lipid hydroperoxides within membranes and lipoproteins, thereby protecting the cell membranes from oxidative damage.

The regulation of GPX4 expression and activity is of particular interest as the presence of multiple transcriptional start sites has been shown to result in two distinct populations of mRNAs that have different translational start sites.[28] In the rat, it has been shown that the alternative translational start sites give rise to a long form (approx. 23 kDa) and a short form (approx. 20 kDa); the long form contains a leader sequence that is required for transport into the mitochondria, whereas the short form lacks the leader sequence.[27,28] The GPX4 long form was found to be synthesised principally in the testes, whereas the short form is located in somatic cells. The mechanism for the differential regulation of transcription is unknown. Overexpression of both the long and

short form of GPX4 has been carried out in rat basophilic leukaemia 2H3 cells (RBL 2H3), and it was found that the localisation of GPX4 in mitochondria is of critical importance in protection of cells from mitochondrial injury caused by KCN (an inhibitor of complex IV of the mitochondrial respiratory chain). Treatment of the RBL 2H3 cells with KCN was shown to cause the rapid generation of hydroperoxides, followed by lipid peroxidation and cell death. The heterologous expression of mitochondrial GPX4 inhibited the generation of hydroperoxides by KCN, inhibited lipid peroxidation and prevented cell death. By contrast, expression of cytosolic GPX4 was not found to prevent KCN-mediated cell death. The mitochondrial expression of GPX4 was also found to be more effective than cytosolic GPX4 in protecting cells from *tert*-butylhydroperoxide. It was proposed that mitochondria might be a target for both intracellular and extracellular oxidative stress, and that the mitochondrial form of GPX4 therefore may play a primary role in protecting cells from oxidative stress.

Gastrointestinal GPX (GPX2)[29,30] has the highest degree of amino acid sequence identity to GPX1, and is highly expressed in mucosal epithelial cells in the gastrointestinal tract. Its substrate specificity appears to be similar to GPX1. The biological function of GPX2, as well as the physiological requirement for its expression in the GI tract, remain to be determined.

Two secreted GPX isoenzymes have been identified, extracellular (plasma) GPX (GPX3)[31–33] and epididymal GPX (GPX5).[34,35] Unlike the other GPX family members, the latter is not a selenoenzyme, and purified porcine GPX5 has been shown to have little glutathione peroxidase activity towards H_2O_2 or organic hydroperoxides.[34] The physiological electron donor remains unidentified, but the location of GPX5 in the epididymis suggests that it may function to protect spermatazoa, which are rich in polyunsaturated fatty acids, from oxidative damage. Despite the potential importance of GPX5 in preventing lipid peroxidation in spermatazoa, it has recently been demonstrated that, unlike rat, pig and monkey, most human GPX5 transcripts are incorrectly spliced.[35] The GPX5 protein was found to be undetectable in human sperm extract or seminal plasma suggesting that GPX5 does not have a major role in protecting spermatazoa from oxidative damage in the human. It is possible that either GPX3, which is also found in the epididymis, or certain members of the glutathione *S*-transferase family carry out this function.

In addition to GPX3, an additional plasma protein with GPX activity has recently been identified. This protein is designated selenoprotein P and was shown to possess phospholipid hydroperoxide GPX activity *in vitro*.[36] Selenoprotein P contains between 6 and 10 selenocysteine residues per molecule (the amino acid sequence predicted from the cDNA suggests the presence of 10 selenocysteines, but analysis of the amino acid composition of purified human selenoprotein P suggests that only 6 selenocysteine residues are present in the protein molecule), and has no amino acid sequence identity with the GPX isoenzyme family described above. Selenoprotein P appears to have no activity towards H_2O_2 and its activity towards phospholipid hydroperoxides is substantially lower than that found for GPX4. Like GPX4, thiols other than glutathione could also serve as electron donors. Several isoforms of selenoprotein P exist in plasma, and it will be of interest to discover whether they also possess GPX activity.

A further novel glutathione peroxidase was recently identified as potentially being involved in wound healing.[37] It was shown to be regulated by keratinocyte growth factor (KGF), and was identified as a GPX on the basis of its high level of amino acid sequence homology (95% identical) with a GPX which had been identified in bovine eye.[38,39] Neither of these GPX forms contain a selenocysteine, nor is there any significant amino acid sequence identity with GPX1-5. Whilst these enzymes are certainly likely to possess a peroxidase function, there is some

uncertainty, at least for the KGF-regulated human form, regarding their identification as glutathione peroxidases. The bovine eye GPX was cloned recently,[39] and shown to have GPX activity with H_2O_2 as substrate. Its human counterpart, however, appears to be identical to a recently described human peroxiredoxin (a family of enzymes with amino acid sequence similarity to thioredoxin peroxidase), which has peroxidase activity towards H_2O_2 in the presence of dithiotheitol.[40] Neither glutathione nor thioredoxin were found to be able to support the peroxidase activity under the assay conditions used.

GLUTATHIONE *S*-TRANSFERASES

Role of Glutathione *S*-Transferase in Oxidative Stress

The GST are structurally highly diverse enzymes which protect against reactive α,β-unsaturated carbonyls, epoxides and hydroperoxides produced *in vivo* as the breakdown products of macromolecules during periods of oxidative stress.[41,42] They also detoxify noxious electrophilic metabolites of xenobiotics which are produced intracellularly following exposure to air-borne products of combustion, from consumption of either over-cooked or mycotoxin-contaminated food, or from drinking polluted water.[41] Although the activation of carcinogenic foreign compounds is usually perceived as being catalysed exclusively by CYP monooxygenases, it can also occur in extrahepatic tissues by oxidative reactions catalysed by peroxidases and hydroperoxidases.[43] In addition to metabolising harmful compounds of endogenous and exogenous origin, GST are involved in the biosynthesis of eicosanoids and prostanoids.[44–46] Furthermore, they have been proposed to serve in a non-catalytic capacity as intracellular transporters of lipids and steroids, and can sequester carcinogens and β-lactam antibiotics.[47–50]

That GST confer a measure of protection against oxidative stress is certain because particular isoenzymes are extremely efficient at catalysing the conjugation of GSH with 4-hydroxynonenal,[51,52] a major genotoxic and cytotoxic α,β-unsaturated aldehyde formed from n-6 polyunsaturated fatty acids during lipid peroxidation.[53] The physiological relevance of this reaction is supported by the fact that mercapturic acid conjugates of 4-hydroxynonenal can be identified in rat urine.[54] Although there is no evidence that GST detoxify malondialdehyde, another important product of lipid peroxidation,[1] certain transferases can catalyse the conjugation of GSH with cholesterol α-oxide,[55] a mutagenic compound which is similarly generated during oxidation of membranes.[56] The transferases also conjugate GSH with adenine and thymine propenals,[57] reactive purine and pyrimidine bases formed during oxidative damage to DNA caused, for example, by γ-irradiation or the anticancer drug bleomycin.[58] Furthermore, the transferases catalyse the conjugation of GSH with acrolein,[57] a noxious compound formed in smog by photo-oxidation, and also endogenously from metabolism of allyl alcohol and cyclophosphamide.[58]

In addition to catalysing the conjugation of GSH with the above α,β-unsaturated carbonyls and cholesterol α-oxide, a number of GST exhibit Se-independent glutathione peroxidase activity towards organic hydroperoxides. For example, fatty acid, cholesteryl and phospholipid hydroperoxides are reduced in a GSH-dependent fashion by the transferases.[59–61] Esterified fatty acid hydroperoxides are not good substrates for GST,[59] though a few isoenzymes are active with phospholipid hydroperoxides suggesting that these transferases might be able to reduce membrane phospholipids *in situ*.[60,62] By contrast, many GST are active with free fatty acid hydroperoxides that are liberated from membranes by the actions of phospholipase A_2.[63,64] Once released from the membrane, the fatty acid hydroperoxide is reduced by the transferase and may be transported intracellularly by GST for reutilisation. The ability of GST to reduce

lipid hydroperoxides to their respective alcohols is of biological significance, because in the presence of transition-metal complexes hydroperoxides are vulnerable to conversion to peroxy radicals, thereby becoming involved in free radical propagation reactions leading to membrane decomposition. It would therefore be envisaged that the reductase activity of GST could arrest lipid peroxidation, and there is good experimental evidence that GST can act *in vivo* to inhibit this process.[65,66] It should be recognised that GPX4 has more activity towards phospholipid hydroperoxides than GST, and therefore the transferases are not unique in their proposed function in protecting membranes against oxidative damage.[62] However, the transferases are likely to be of physiological importance in reducing peroxidized lipids as they are considerably more abundant than GPX4 in most tissues. Most significantly, during Se-deficiency, when GPX4 fails to be synthesised, the expression of GST in mouse and rat liver is induced.[67,68]

In DNA, thymine residues are most likely to be the target of free radical damage as they have the highest electron affinity, and will give rise to thymine hydroperoxide.[69] The biological significance of the ability of GST to reduce DNA hydroperoxides, and thymine hydroperoxide in particular,[70] is uncertain because oxidised purine and pyrimidine bases are not repaired *in situ* but are excised by DNA glycosylases, to yield apurinic/apyrimidinic (AP) sites.[71] The AP site is recognised by an endonuclease which cleaves the nucleic acid sugar–phosphate backbone to generate a 3′ OH priming site for a DNA polymerase. The lesion is finally repaired by deoxyribose phosphodiesterase and a ligase.[71] The value of GST reducing thymine hydroperoxide lies in possible prevention of this compound from either modifying directly thiols present in critical nuclear proteins or decomposing to radicals that further damage DNA; a number of basic leucine zipper (bZIP) transcription factors have cysteine residues in their DNA-binding domains, and as these require to be in the reduced state for maximal activity it is likely that ROS will decrease the activity of such factors possibly causing downregulation of many genes.[72] In addition to catalytic actions, GST may sequester DNA hydroperoxides and thereby prevent them from interacting with the genome or with transcription factors. As noted above for phospholipid hydroperoxides, GPX4 also exhibits higher activity for thymine hydroperoxide than GST, but again the high abundance of the transferases suggests that they will help inhibit secondary reactions associated with oxidative damage to DNA. This proposal is supported by the fact that in rat liver, GST appear to translocate to the nucleus during periods of drug-induced oxidative stress.[73]

In considering the role of GST in oxidative stress it is appropriate to first concentrate on their detoxification of lipid peroxidation products and oxidised DNA bases. However, besides metabolising these compounds, GST also detoxify other endogenous oxidation products including *o*-quinones formed from catecholamines[74] and estrogen-3,4-quinones.[75] Increased generation of ROS can result not only in the generation of noxious compounds from endogenous macromolecules, but it can also result in the activation of foreign chemical carcinogens. For example, aflatoxins, aromatic amines and polycyclic aromatic hydrocarbon dihydrodiols can be converted into their respective ultimate carcinogenic forms by COX during prostaglandin H_2 synthesis or by leukocytes undergoing an oxidative respiratory burst.[43]

These biochemical data indicate that GST do not provide a first line of defence against free radicals, this is provided by SOD, catalase and GPX. However, GST represent a second line of defence against the highly toxic spectrum of substances produced by ROS-mediated reactions, and because of their broad substrate specificity they are well suited for this task.

Families of Glutathione *S*-Transferase

Two apparently evolutionary separate multigene families encode these enzymes. One superfamily

of GST comprises soluble proteins,[41] whereas the other is composed of membrane-bound transferases.[76] The former family is highly complex and in mammals at least 7 classes of transferase, designated Alpha, Mu, Pi, Sigma, Theta, Zeta and Kappa, have been characterized.[77–90] These enzymes used to be called cytosolic GST, but as Kappa is located in the mitochondrion they are best referred to collectively as soluble GST.[90] An eighth family of GST, class X (chi), has been identified in the mouse as a stress-responsive protein.[90a] Class X GST are probably also represented in other species.[90b] In bacteria, insects and plants, further GST have been identified and designated Beta, Delta, Phi and Tau,[91–96] making a total of 12 classes of soluble GST in nature (Table II). The microsomal transferase family has recently been called MAPEG, Membrane Associated Proteins involved in Eicosanoid and Glutathione metabolism.[76] This family has not been as thoroughly studied as the soluble GST, but recent advances in molecular cloning have revealed that it contains significantly more members than was thought even just a couple of years ago. Thus, as Table II shows, the human alone possesses at least 21 GST genes, of which 15 encode soluble transferases[47,78,82,86,89,90] and 6 are for membrane-bound transferases and related proteins.[97,99–103]

The two superfamilies of GST are not only structurally separate but are also functionally distinct. In circumstances where there is an increased intracellular concentration of ROS, the soluble transferases act primarily as detoxication enzymes to prevent cytotoxic and genotoxic damage caused by electrophiles generated as breakdown products of macromolecules (Table III).[41,104–115] During oxidative stress, members of the MAPEG

TABLE II Glutathione *S*-transferase gene families: structure and distribution

Superfamily	Class	Subunit* structure	Active site residue	Species	Enzyme or subunits†
Soluble	Alpha	Dimer	tyr and arg	Mammals	hGSTA1, A2, A3, A4
Soluble	Mu	Dimer	tyr	Mammals, helminths	hGSTM1, M2, M3, M4, M5
Soluble	Pi	Dimer	tyr	Mammals, fish, toad, insects	hGSTP1
Soluble	Sigma	Dimer	tyr	Mammals, cephalopods, chicken	hGSTS1
Soluble	Theta	Dimer	ser	Mammals	hGSTT1, T2
Soluble	Zeta	Dimer	tyr	Mammals, plants, fungi	hGSTZ1
Soluble	Kappa	Dimer	nd	Mammals	hGSTK1
Soluble	X (chi)	nd	nd	Mouse, plants	—
Soluble	Phi	nd	nd	Plants	—
Soluble	Tau	nd	nd	Plants	—
Soluble	D (delta)	Dimer	nd	Drosophila	—
Soluble	Beta	Dimer	cys	Bacteria	—
MAPEG	Microsomal	Dimer or trimer	nd	Mammals, drosophila	MGST-I, MGST-II, MGST-III, LC_4S, FLAP, PIG12

The data for this table are from Refs. [77–103]. *Abbreviation*: nd, not determined.
*Whilst MGSTM-I is trimeric, leukotriene C_4 synthase has been reported to be dimeric.
†All the enzymes and subunits listed are from the human. PIG12 is also called microsomal glutathione *S*-transferase 1-like 1 (MGST1-L1).

TABLE III Classes of soluble glutathione *S*-transferase enzymes and their functions

Family	Chromosomal location of human gene	Subcellular localisation	Size* (amino acids)	Substrates of biological importance or used *in vitro* for identification	
				Compounds conjugated with GSH	Substrates not conjugated with GSH (i.e. reduced, oxygenated, isomerised or dehydrochlorinated)
Alpha	6p12	Cytosolic	221, 222	Cholesterol α-oxide; CDNB; NBD-Cl; 4-hydroxynonenal; EA; aflatoxin B_1-8,9-epoxide; bay- and fjord-region epoxides of PAH	PLPC-OOH; cholesterol 7-hydroperoxide CuOOH prostaglandin H_2 (PGE_2 and $PGF_{2\alpha}$ synthase) Δ^5 androstene-3, 17-dione
Mu	1p13	Cytosolic	217, 225	CDNB; DCNB; bay- and fjord-region epoxides of PAH aminochrome; dopchrome; noradrenochrome	DNA hydroperoxide — —
Pi	11q13	Cytosolic, nuclear	207, 209	Acrolein; CDNB; EA; bay- and fjord-region epoxides of PAH; sulphoraphane	— — —
Sigma	nd	Cytosolic	199	CDNB	Prostaglandin H_2 (PGD_2 synthase)
Theta	22q11.2	Cytosolic, nuclear	239, 244	1,2-epoxy-3-(4′-nitrophenoxy)propane; dichloromethane; diepoxybutane 1-menaphthyl sulphate; hydroxymethyl-chrysene sulphate	Thymine hydroperoxide arachidonic acid 15-hydroperoxide linolenic acid 13-hydroperoxide CuOOH
Zeta	14q24.3	Cytosolic	216	NBD-Cl; EA; 4-nitrophenyl acetate; 2-chloropropionic acid	Dichloroacetic acid; bromofluoroacetic acid; maleylacetoacetic acid; cyanidin-3-glucoside
Kappa	nd	Mitochondrial	226	EA	—
D (delta)	(Insect)	nd		CDNB	CuOOH; DDT
Beta	(Bacteria)	Cytosolic	203	Non-catalytic binding of β-lactam antibiotics	—

The data in this table are from Refs. [41] and [104–115].
Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; DCNB, 1,2-dichloro-4-nitrobenzene; DDT, dichlorodiphenyltrichloroethane; EA, ethacrynic acid; nd, not determined; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; PLPC-OOH, 1-palmitoyl-2-(13-hydroperoxy-cis-9,trans-11-octadecadienoyl)-L-3-phosphatidylcholine.
*Calculation of size includes the translational initiator methionine.

family serve to inhibit lipid peroxidation and possibly influence the mobility of arachidonic acid within membranes. Although evidence suggests that several microsomal GST isoenzymes do not detoxify xenobiotics, but are involved exclusively in leukotriene C_4 synthesis, it remains to be seen whether such activities are modulated by oxidative stress (Table IV).

The soluble class Alpha GSTA4-4 is highly efficient *in vitro* at catalysing the conjugation of 4-hydroxynonenal with GSH, and the class Pi GSTP1-1 catalyses the conjugation of acrolein and adenine propenal with GSH. On the basis of these biochemical findings it was proposed that class Alpha and class Pi GST act *in vivo* to detoxify α,β-unsaturated aldehydes, and this hypothesis has been supported by transfection experiments. It has been shown that HeLa and HepG2 cells which contain elevated levels of human GSTP1-1 are protected against the cytotoxic effects of adenine propenal and acrolein, respectively.[57] Overexpression of mouse GSTA4-4 in human liver HepG2 cells has been demonstrated to protect against H_2O_2, organic hydroperoxides and phosphatidylcholine hydroperoxide during plating and allow attachment of cells to the culture dish.[116] The rat class Alpha GSTA1-3 and A2-3 can catalyse the conjugation of cholesterol α-oxide with GSH, but to date no experiments have been conducted to determine the biological effect(s) of this activity in cell lines.

Class Mu GSTM2-2 has been found to be highly efficient at catalysing the conjugation of GSH with aminochrome, dopachrome and noradrenochrome. It has been postulated that this activity of GST will prevent redox-cycling of *o*-quinones, an event which is believed to contribute to neurodegenerative disorders such as Parkinson's disease.[74]

The work of Christophersen led to the recognition that rat liver microsomes and mitochondria contain an endogenous factor which inhibits lipid peroxidation and is both GSH-dependent and requires vitamin E.[117] Using reconstituted rat liver microsomes, Morgenstern and his colleagues provided evidence that MGST-I represents the peroxidation-inhibitory factor by showing a good correlation exists between inhibition of formation of malonaldehyde and transferase activity following treatment of the enzyme with cystamine (an activator of MGST-I) and diethylpyrocarbonate (an inhibitor of MGST-I).[66]

TABLE IV The human family of membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG)

Name	Chromosomal location of gene	Size* (amino acids)	Substrates of biological importance or used *in vitro* for identification	
			Componds conjugated with GSH	Compounds reduced
Microsomal GST-I	12	154	CDNB (activated by N-ethylmaleimide) 4-hydroxynon-2-enal hexachlorobutadiene	Cholesteryl linoleate hydroperoxide, dilinoleoyl phosphatidylcholine hydroperoxide, linoleic acid ethylester hydroperoxide
Microsomal GST-II	4q28–30	147	CDNB leukotriene A_4	5-hydroxyeicosatetraenoic acid —
Microsomal GST-III	1q23	152	Leukotriene A_4	5-hydroxyeicosatetraenoic acid
Leukotriene C_4 synthase	5q35	150	Leukotriene A_4	—
5-lipoxygenase-activating protein (FLAP)	13q12	160	Non-catalytic binding of arachidonic acid	—
p53-inducible gene 12 (PIG12 or MGST1L1)	nd	152	nd	nd

The data presented are from Refs. [96–103].
Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; nd, not determined.
*Calculation of size includes the translational initiator methionine.

Furthermore, incubation of microsomes with GSH analogues which can serve as nucleophilic substrates for MGST-I (glutaryl-L-Cys-Gly and α-L-Glu-L-Cys-Gly) as well as those which do not serve as nucleophilic substrates (α-D-Glu-L- Vys-Gly, γ-D-Glu-L-Cys-Gly, β-L-Asp-L-Cys-Gly, α-L-Asp-L-Cys-Gly and α-D-Asp-L-Cys-Gly) also supported the hypothesis that inhibition of lipid peroxidation could be ascribed to this transferase.[66] In addition, Mosialou *et al.* also used inhibitors of GPX1 (mercaptosuccinate) and GPX4 (iodoacetate) to exclude the possibility that either of these glutathione peroxidases represents the endogenous microsomal inhibitor of lipid peroxidation.[66]

The possible role of other members of the MAPEG family, MGST-II, MGST-III, leukotriene C_4 synthase, 5 lipoxygenase-activating protein and p53-inducible gene 12, in inhibiting lipid peroxidation has not been explored. However, several MAPEG enzymes, MGST-II and MGST-III, can catalyse the reduction of (*S*)-5-hydroperoxy-8,11,14-cis-6-trans-eicosatetraenoic acid (5-HPETE) to 5-HETE. As 5-HPETE is an intermediate in the 5-lipoxygenase-catalysed conversion of arachidonic acid to leukotriene A_4, it is possible that MGSTM-II and MGSTM-III can inhibit production of leukotrienes. Alternatively, MGST-II and MGST-III may be able to reduce oxidised lipids. It is interesting to note that leukotriene C_4 is a strong inhibitor of MGST-I, and therefore the protective actions of this enzyme might be impaired in cells exposed to high levels of leukotriene.[118]

Involvement of the Antioxidant Responsive Element in Mediating Cellular Adaption to Oxidative Stress

Certain soluble GST in rodent and human liver are highly inducible by treatment with a range of xenobiotics, suggesting that they are part of one or more adaptive stress response mechanisms.[119] Interestingly, a substantial number of compounds which induce GST are pro-oxidants either because their metabolism results in the generation of free radicals or they deplete intracellular GSH levels. In such instances GST induction appears to be a form of adaption to oxidative stress. Consistent with this hypothesis, hepatic and dermal GST have been found to be inducible by 4-hydroxynonenal[120] and UVB irradiation,[121] respectively. The mammalian GST which have been shown to date to be inducible are members of class Alpha, Mu, Pi and Theta families; Figure 3 shows induction of these enzymes in rat liver by a range of xenobiotics. Significantly, not only are mammalian GST inducible by pro-oxidants, but plant GST also appear to be inducible by ROS.[122] This conservation emphasises the unique contribution made by GST to cellular physiology.

The agents which induce GST are diverse and can be divided into several groups according to the *cis*-acting element responsible for transcriptional activation. The largest group of inducing agents represent pro-oxidant and/or electrophiles, and includes α,β-unsaturated carbonyls, catechols and hydroquinones, dithiolethiones, organic isothiocyanates and oxidisable phenolic compounds and polycyclic aromatic hydrocarbons (PAH).[119] It should be appreciated that the task of identifying precisely the compounds in this group is difficult as some members are direct GST inducers whereas others require biotransformation in order to effect induction, presumably by either inducible or constitutively expressed CYP (see Figure 4). Further complexity in the task of identifying the ultimate effective inducing agent arises from the fact that whilst biotransformation of phenolic antioxidants and PAH will give rise to an active inducing agent (e.g. metabolism of butylated hydroxyanisole to *tert*-butyl hydroquinone) both CYP-mediated catalysis and CYP induction can give rise to free radicals which also generate substances which may cause gene induction.[123–125] In other words, the involvement of CYP activities can result in the initiation of metabolic cascades which can activate gene expression.

Work from the laboratory of Talalay first pointed out that this disparate group of inducers all possess, or acquire, a common chemical

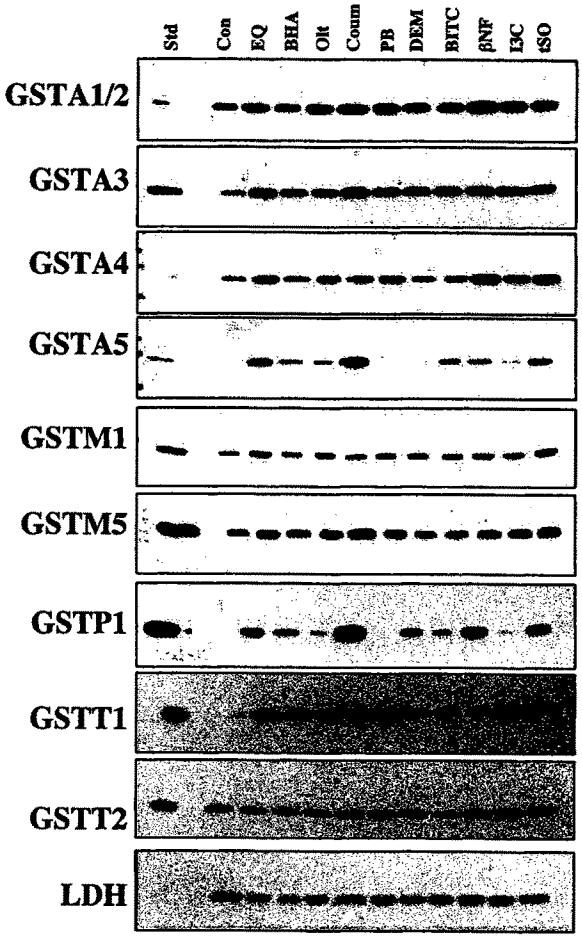

FIGURE 3 *Inducible expression of GST subunits in livers of rats treated with cancer chemopreventive agents and xenobiotics.* Portions of soluble hepatic extract (4 μg of protein) were subject to SDS/PAGE before transfer to Immobilon-P membranes. The treatment group (control [lane 2] or drug-treated [lanes 3–12]) from which the different samples were obtained is indicated at the top of the gel. The following abbreviations are used: Con, control; EQ, ethoxyquin; BHA, butylated hydroxyanisole; Olt, oltipraz; Coum, coumarin; PB, phenobarbital; DEM, diethyl maleate; BITC, benzyl isothiocyanate; βNF, β-naphthoflavone; I3C, indole-3-carbinol; tSO, *trans*-stilbene oxide. Each panel in the figure shows the immunoblot ECL stain obtained with a separate antibody, and the identity of the cross-reacting rat subunit is indicated in the left hand margin. Lactate dehydrogenase (LDH) was used as a loading control. The figure is based on data from Ref. [73].

signature (Michael reaction acceptor or other electrophile).[126] It was therefore argued that these agents affect transcriptional activation by a receptor-independent mechanism. Subsequently, it has been recognised that these compounds transcriptionally activate GST genes through the antioxidant responsive element (ARE), an enhancer originally described by Pickett and his colleagues in the 5′-flanking region of the rat GSTA2 gene as 5′-GTGACnnnGC-3′.[127,128] In addition to the induction of GST genes, the ARE is involved in the regulation of NAD(P)H: quinone oxidoreductase (NQO1), heme oxygenase-1 and GCS.[119] More recently the consensus sequence for the ARE has been extended to 5′-RTGACnnnGCR-3′ (where R = A or G) to account for the induction of these additional genes.[129]

In human HepG2 cells, transfection of the bZIP transcription factors Nrf1 and Nrf2 augments both basal and inducible ARE-driven transcription.[130] From examining a Nrf2 gene knockout mouse, it was found that this factor is essential for GST and NQO1 induction by BHA in murine liver and small intestine.[131] As all of the compounds which work through the ARE appear to be capable of interacting with cysteine residues,[41] it has been speculated that the presence of this type of inducing agent is recognised by protein phosphatases which contain an active-site cysteine.[132] According to this hypothesis, the inactivation of such phosphatases results in autophosphorylation events proceeding unchecked, allowing the activation of signal transduction pathways directed by receptor tyrosine kinases such as epidermal growth factor receptor, platelet-derived growth factor receptor and the insulin receptor. The notion that protein phosphorylation is responsible for activation of transcription factors recruited to the ARE may not be correct (see below). However, this does not imply that Michael reaction acceptors and electrophiles will not influence signal transduction. For example, the SAP kinase pathway is activated by isothiocyanates and polyphenolic compounds.[133,134]

Recently, Keap1 has been identified as a negative regulator of Nrf2 that also acts as a possible intracellular sensor for thiol-active xenobiotics.[135] Keap1 is related to the Drosophila cytoskeleton-binding protein Kelch, and similarly contains a BTB protein interaction domain and six double glycine repeat (DGR) modules.

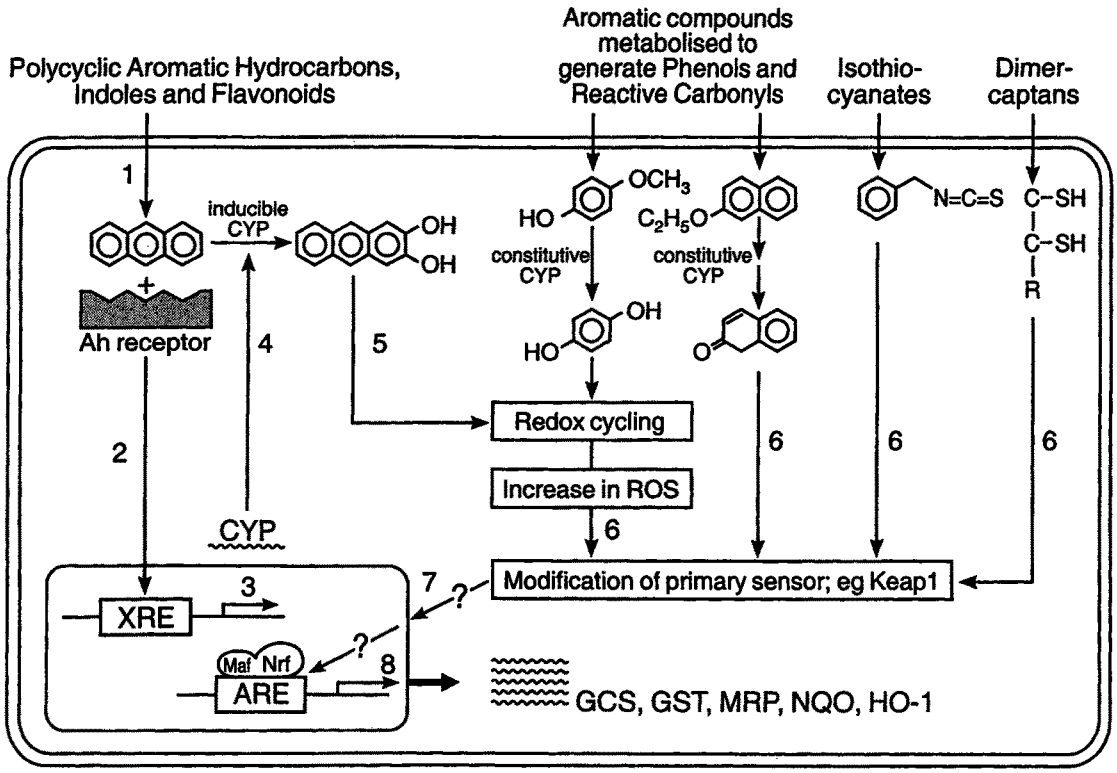

FIGURE 4 *Role of intracellular metabolism in gene induction by cancer chemopreventive agents.* The chemical structures shown are representative of the types of inducers involved in chemoprevention, and are not intended to represent specific compounds. Inducers which transcriptionally activate genes through the ARE may function directly (e.g. isothiocyanates and dimercaptans). Alternatively, they may require metabolic activation, and are either converted to active inducing agents by CYP isoenzymes that are normally constitutively expressed (e.g. BHA and ethoxyquin), or are converted to active inducing agent by CYP isoenzymes that are not normally expressed and therefore require prior induction themselves (e.g. polycyclic aromatic hydrocarbons, flavonoids and indoles). Chemopreventive agents which require to be oxidised by inducible CYP1A enzymes before they can activate gene expression through the ARE trigger the following steps in order to be effective: 1, binding of chemopreventive agent to the Arylhydrocarbon (Ah) receptor; 2, translocation of the Ah receptor–chemopreventive agent complex to the nucleus; 3, transcriptional activation of xenobiotic responsive element (XRE)-regulated *CYP1A* genes; 4, oxidation of the agent by CYP1A; 5, redox-cycling of metabolites generated by CYP1A. As shown in step 6, the presence of all inducing agents (whether formed by inducible CYP, constitutive CYP or direct-acting) is detected by one or more intracellular sensors, possibly in a thiol-dependent fashion. The presence of such inducing agents is signalled to the nucleus (step 7) causing Nrf2 and small Maf protein to be recruited to ARE and increased transcription of certain antioxidant and detoxification genes (step 8). The figure is adapted from data presented in Ref. [119].

Yamamoto and his colleagues have produced good evidence in support of a model in which Keap1 is irreversably anchored to the cytoskeleton of the cell, and under reducing conditions Keap1 binds and retains Nrf2 in the cytoplasm where it is unable to function as a transcription factor.[135] During transition to oxidising conditions (brought about using diethyl maleate), Nrf2 dissociates from Keap1 allowing it to translocate to the nucleus where it heterodimerises with small Maf proteins and activates ARE-driven transcription. Itoh *et al.* showed that the DGR modules in the C-terminal portion of Keap1 are responsible for binding Nrf2 through a lysine–glutamine–glutamic acid-rich region (KEYELEKQKKLEKERQEQLQKE) in its N-terminus.[135] The basis for the putative ability of Keap1 to sense oxidative stress and/or alkylating agents is unknown, but Keap1 contains a total of 25 cysteines of which 10 are likely to be more reactive than the

remainder as they are located immediately adjacent to basic histidine, arginine or lysine residues. Provocatively, 3 cysteines are located within the final 12 C-terminal amino acids of Keap1. It remains to be established whether all inducing agents relieve the negative regulation of Nrf2 by Keap1 in an identical fashion. For example, do all α,β-unsaturated carbonyls, isothiocyanates and oxidised PAH, despite large differences in their size, interact with a single unique cysteine in Keap1, do they modify different cysteine residues, or are their effects on Keap1 indirect?

A significant number of questions remain to be answered about the function of the ARE, and there is reason to believe that other factors besides Nrf2 are involved in regulating ARE-driven gene expression. According to the model proposed by Yamamoto and his colleagues[135] in which oxidative stress causes release of Nrf2 from cytoskeleton-bound Keap1 and its translocation to the nucleus, the resulting ARE-driven transcription would be expected to occur relatively quickly. However, contrary to expectations, a time delay of possibly 12 h occurs before a transcriptional response is mounted, suggesting a requirement for the prior synthesis of additional proteins before ARE-driven gene induction. It should also be noted that ARE-binding proteins of 28 and 40 kDa which appear to be distinct from Nrf2 and small Maf, have been shown to be constitutively present in nuclear extracts from HepG2 cells,[136,137] and it remains to be clarified what role they play in induction of genes through this enhancer.

Finally, uncertainty exists surrounding the role of Nrf1 in induction of antioxidant and detoxification genes. It is not known what function Nrf1 plays in adaptive responses because although it is widely expressed, and can activate transcription from the ARE in HepG2 cells, its presence was insufficient to allow induction by butylated hydroxyanisole of GST and NQO1 in the liver and small intestine of the Nrf2 knockout mouse.[132] In this context it would be helpful to know whether Nrf1 is exclusively nuclear or whether it can also exist in the cytoplasm. If Nrf1 exists in the cytoplasm, it probably will not interact with Keap1 as its sequence corresponding to that region of Nrf2 which interacts with Keap1 shares little homology (in Nrf1 the sequence is KEqdvEKelr-dggeQdtwagE, where capitals represent conserved residues). Assuming Nrf1 is a nuclear protein, it would be interesting to know whether it heterodimerizes with small Maf proteins, and whether the fact that it can exist as a shortened form (p47/49) influences its activity.[138,139]

Involvement of Enhancers Related to the ARE in Mediating Adaption to Oxidative Stress

Certain of the compounds which activate gene expression through the ARE also appear to transcriptionally activate the rat GSTP1 gene through its glutathione transferase P enhancer I (GPEI) *cis*-acting element.[140] In rat liver GSTP1 is induced by the α,β-unsaturated carbonyls coumarin and diethyl maleate, the phenolic antioxidants ethoxyquin and butylated hydroxyanisole and by the metabolisable flavonoid βNF (Figure 3). However, by contrast with rat GSTA2, GSTP1 is not inducible in the liver to any significant extent by the dithiolethione oltipraz or by benzyl isothiocyanate. It is difficult to be certain whether the apparent variations in the inducibility of rGSTA2 and rGSTP1 can be ascribed to functional differences between the ARE and GPEI, or metabolic differences in the types of liver cells where the transferases are induced (e.g. centrilobular vs periportal hepatocytes). The ARE and GPEI enhancers are related but are not identical; the latter enhancer does not contain a perfect ARE consensus, but comprises two inverted repeat imperfect AREs.[119] Work from the laboratory of Muramatsu has implicated a heterodimer between a c-Jun-related protein and a novel large Maf protein in the binding of GPEI.[141] This work suggests that bZIP proteins other than Nrf2 are responsible for the responsiveness of at least the rat GSTP1 gene to oxidative stress. Furthermore, it

appears possible that there exists several closely related ARE-type enhancers which recruit different *trans*-acting factors and therefore can respond in several distinct ways to stress.

Regulation of Antioxidant Genes by the Aryl Hydrocarbon Receptor

Certain class Alpha GST are inducible by dioxin, 3-methylcholanthrene, flavonoids and indoles, all of which are ligands for the Ah receptor.[41] The rat GSTA2 gene contains a single functional xenobiotic responsive element in the 5′-flanking region, and therefore planar aromatic compounds can induce this transferase directly.[142] Co-induction of CYP1A1 induces oxidative stress, and this may be sufficient to increase the basal expression of ARE-regulated genes.[125] Furthermore, induction of CYP1A1 results in oxidative metabolism of 3-methylcholanthrene and the flavonoid βNF which in turn cause induction of ARE-driven gene expression.

Regulation of GST by Phenobarbital and Dexamethasone

Class Alpha and class Mu GST genes are transcriptionally activated by phenobarbital (PB) in livers of rats, mouse and human.[41,143] Although the mechanisms involved in PB regulation of GST are unknown, a phenobarbital-responsive enhancer module (PBREM) has been identified in the murine cytochrome P450 Cyp2b10 gene.[144] The PBREM binds the nuclear orphan receptors CAR and RXR as a heterodimer. Furthermore, transfection of CAR and RXR into HepG2 cells resulted in synergistic activation of a PBREM reporter construct. Although likely, it requires to be established whether CAR and RXR are involved in the regulation of antioxidant genes.

Dexamethasone has been found to induce hepatic GST in the mouse.[41] By contrast, dexamethasone has been found to repress the basal expression of the rat GSTA2 gene as well as inhibiting its induction by PAH.[145] The upstream region of the rat gene contains a canonical glucocorticoid consensus sequence and the inhibitory effects of dexamethasone appeared to be mediated by the glucocorticoid receptor.

Nuclear Localisation of GST as an Adaption to Oxidative Stress

Transcriptional activation of antioxidant genes appears to be a major adaptive response to oxidative stress. Immunohistochemistry of rat liver has revealed that in addition to protein overexpression, GST are able to translocate to the nucleus in response to drug treatments.[73] From an experimental point of view this phenomenon is most obvious with the class Theta GSTT2-2, though the same trend is apparent with class Alpha and class Pi transferases. It has been found that treatment of rats with oltipraz (Figure 5), ethoxyquin or diethyl maleate is effective at causing relocation of GSTT2-2 from the cytoplasm to the nucleus. The mechanism responsible for the migration of GST to the nucleus is unknown. However, it should be noted that GSTT2-2 is not inducible, and therefore translocation is separate from enzyme induction.

The possibility that GST may possess nuclear functions that are separate from that of detoxification has been the subject of speculation for several years. Bennett *et al.* have reported that class Alpha and class Mu GST are identical to non-histone protein BA isolated from rat liver nuclei.[146] On the basis of this finding, these workers speculated that within the nucleus GST are associated with U-snRNPs and may therefore be involved in the maturation of hnRNA to mRNA.

More recently, Adler and colleagues have reported that the class Pi GST is an endogenous inhibitor of JNK signalling.[147] These workers demonstrated that the inhibitory form of the transferase was the GSTP1 monomer. It was proposed that oxidative stress caused dimerisation and oligomerisation of the P1 subunit and resulted in loss of inhibition of JNK by GST. It remains to be established whether nuclear GST

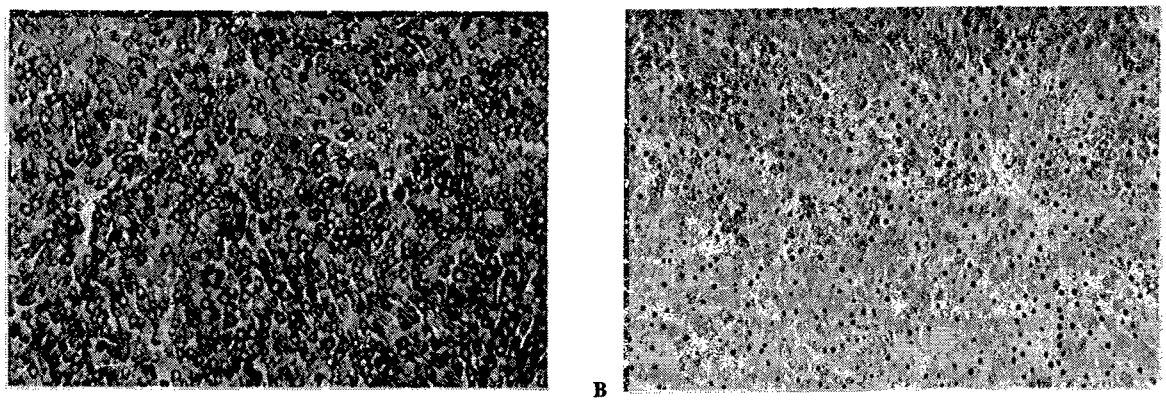

FIGURE 5 *Nuclear localisation of GSTT2-2 in oltipraz-treated rat livers.* Panel A shows the predominantly hepatic cytoplasmic immuno-staining for the T2 subunit in rats fed a control diet. Panel B shows the nuclear localization of GSTT2-2 in hepatocytes of animals fed on an oltipraz-containing diet.

exist as monomers, dimers or oligomers, and whether they modulate protein kinase activity in the nucleus.

GLUTATHIONE *S*-CONJUGATE EFFLUX PUMPS

An accumulating body of evidence suggests that conjugation with glutathione is not, in itself sufficient for detoxification of many electrophilic compounds. The class Alpha, Mu and Pi GST are sensitive to product inhibition, and unless the conjugates are eliminated from the cell these transferases will be ineffective at catalysing detoxification reactions.[148] Furthermore, glutathione *S*-conjugates require to be excreted from the cell in order to undergo further metabolism and removal from the organism. The relatively recent discovery and characterisation of MRP1 has highlighted the importance of this critical final phase of glutathione-dependent detoxification (Table V).

The cDNA encoding MRP1 was first isolated from a doxorubicin-selected small cell lung cancer cell line, and its overexpression was found to be associated with drug resistance.[149] MRP1 was later shown to be capable of transporting a variety of substrates that are conjugated to GSH, including 2,4-dinitrophenyl *S*-glutathione, ethacrynic acid *S*-glutathione, glutathione conjugates of aflatoxin B_1 (AFB_1), prostaglandin A_1 and the cysteinyl leukotriene, LTC_4.[18,150–153] Oxidised glutathione has also been shown to be a substrate for the efflux pump, as well as glucuronated or sulphated compounds.[18,153] In addition to transportation of glutathione conjugates, certain drugs have been shown to be transported by MRP1 in the presence of GSH without being required to form conjugates. For example, the natural product drug, vincristine, is transported in a glutathione-dependent manner in the absence of vincristine glutathionylation or other redox changes.[154] Similarly, MRP1 also transports AFB_1 in the presence of glutathione, without the requirement of formation of conjugates.[151]

Recent experiments examining drug-resistance using heterologous overexpression of different GST family members in MRP1-overexpressing cells have highlighted the importance of interplay between different phases of glutathione-dependent drug metabolism. Transfection of human GSTA1-1 into a MCF7/VP breast carcinoma cell line which overexpresses MRP1 results in resistance to the alkylating anticancer drug, chlorambucil. By contrast, transfection of GSTA1-1 into the parental MCF7/WT line which does not overexpress MRP1, fails to confer resistance to chlorambucil.[155] Using the same cell lines, Morrow

TABLE V Human multidrug resistance proteins

MRP family member	Identified substrates	Identity (at C-terminal 124 amino acids) with MRP1 (%)*	Tissue distribution*	Association with anticancer drug resistance
MRP1	Glutathione conjugates of aflatoxin B_1, 2,4-dinitrophenyl and ethacrynic acid, oxidised glutathione, LTC4, glucuronidated and sulphated metabolites, unconjugated vincristine and aflatoxin B_1 in the presence of glutathione	100	Expressed in many tissues including lung, kidney, bladder, spleen, thyroid, testis, colon, adrenal gland	Doxorubicin, vincristine, etoposide
MRP2 (cMOAT)	Glutathione conjugates of 2,4-dinitrophenyl and ethacrynic acid, oxidised glutathione, LTC4, glucuronidated and sulphated metabolites	67	Highest levels found in liver, with a lesser amount in duodenum	Cisplatin
MRP3	Unknown	75	Highest levels found in liver. Also highly expressed in duodenum, colon and adrenal gland	None to date
MRP4 (MOAT-B)	Unknown	60	Low levels of expression in lung, kidney, bladder, gall bladder and tonsil	None to date
MRP5	Unknown	55	Expressed in many tissues with highest levels being found in skeletal muscle and brain	None to date
MRP6	Unknown	58	Highest levels found in liver and kidney	None to date

*Data from Kool *et al.*[158,175]

et al.[156] have also shown synergism in protection against 4-nitroquinoline 1-oxide toxicity when GSTP1-1 is overexpressed in MCF7/VP cells. The requirement for both GST and drug-conjugate transport systems to carry out effective detoxification, has also been demonstrated for the anticancer alkylating agent thiotepa. In this case, human breast carcinoma MCF-7 cells stably transfected with GSTP1-1 have an enhanced ability to form monoglutathionylthiotepa.[157] Inhibition of MRP1 by probenecid or verapamil, however, was found to increase the cytotoxicity of thiotepa, indicating that the monoglutathionylthiotepa derivative is still cytotoxic and export is required before glutathione conjugation is a fully effective detoxification process.

MRP1 has been shown to be overexpressed in several different anticancer drug-resistant cell lines.[158] Gene amplification may be a cause of increased MRP1 levels in several drug-resistant cell lines, but the transcriptional regulation of this gene is poorly understood. It is of interest, however, that a coordinate regulation of MRP1 and GCS_h appears to occur. Elevations in levels of both proteins have been shown in cisplatin-resistant cells, and frequent co-ordinate levels of expression have been observed in other cancer cell lines and tumour samples.[159–162] A recent study

has shown that both GCS_h and MRP1 can be induced by compounds which generate oxidative stress.[163] This suggests that similar enhancer elements may be operational in the adaptive regulation of expression of the separate genes. Transcriptional activation of the gene encoding GCS_h in response to βNF has been shown to be mediated by an ARE.[12] Furthermore, an activator-protein-1 (AP-1) binding site has also been shown to be important in regulation of GCS_h levels in both drug-resistant cell lines and in induction of gene expression by TNF.[164,165] It is therefore possible that an ARE or an AP-1 site also has a role in the regulation of MRP1 expression, and it is pertinent that the 5′ flanking sequence of the *MRP1* gene contains core consensus sequences for the ARE and an AP-1 binding site although their involvement in gene induction has not yet been established. The 5′ flanking sequence of *MRP1* also contains a consensus sequence for NFκB binding, and as NFκB has been shown previously to be activated in response to oxidative stress,[166] it may also have a role in the regulation of *MRP1*. Interestingly, a recent report suggests that expression of *MRP1* can be suppressed by wild-type p53.[167] Wild type p53 was found to inhibit the transactivating effect of Sp1 on the *MRP1* promoter, but it has yet to be demonstrated whether there is a direct association between non-functional p53 expression and MRP1 activity in human cancer.

MRP1 is a member of a multigene family of ATP-binding cassette (ABC) transport proteins, which appears to comprise at least 6 family members including MRP2 (cMOAT), MRP3, MRP4 (the cDNA encoding MRP4 has been isolated by Lee *et al.*[168] and named MOAT-B), MRP5 and MRP6.[158] MRP1 has been characterised in the greatest detail and has been shown to be involved in resistance to anthracyclines such as doxorubicin and daunorubicin, etoposide and vincristine.[169,170]

MRP2 is the major organic anion transporter in the canalicular membrane of hepatocyte[171] and it has been suggested that overexpression of MRP2 may contribute to cisplatin resistance in cancer cell lines.[153,158,172] Taniguchi *et al.*[172] showed that MRP2 is overexpressed in a human cisplatin-resistant head and neck cancer cell line, and Kool *et al.*[158] found that, unlike MRP1, expression of MRP2 in drug-resistant cell lines correlates with cisplatin resistance. MRP-1, however, may also have a role in cisplatin resistance as a GS-X pump overexpressed in cisplatin-resistant HL-60/R-CP cells has been identified as MRP1 by Ishikawa *et al.*[173] It has been shown that glutathione can complex with cisplatin to form a bis-(glutathionato)-platinum chelate; this is exported from the cell by an ATP-dependent glutathione *S*-conjugate export pump.[174] It is probable that, like the GST family of enzymes, MRP family members have broad and overlapping substrate specificities, and a similar spectrum of substrate specificities has been observed for MRP1 and MRP2.[18,171] The physiological functions of MRP3-6 are, at present, unknown, although MRP6 has been recently cloned and characterised in more detail.[175]

CONCLUDING COMMENTS

It is clear that cells require to mount effective defences against oxidative stress, and that failure to counter the deleterious effects of ROS increases the likelihood of developing degenerative disease. This overview, has provided a brief insight into the ways in which GSH and the glutathione-dependent enzymes protect the cell against ROS. In essence, GSH provides a first line of defence against ROS as it can scavenge free radicals and reduce H_2O_2. By contrast, glutathione-dependent enzymes provide a second line of defence as they primarily detoxify noxious byproducts generated by ROS and also help prevent propagation of free radicals. Many of the GSH-dependent proteins are co-ordinately induced through the ARE in response to oxidative stress, suggesting that they function in an inter-dependent integrated fashion. Certain soluble GST can translocate to the

nucleus in response to drugs which are likely to elevate levels of ROS, suggesting that they perform some specialised nuclear function. Lastly, it is apparent that GST and MRP are required to be co-ordinately regulated because overexpression of GST alone, without increased capacity to transport conjugates out of the cell, is not sufficient to confer resistance to oxidative and chemical stress.

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