

Review

Apoptosis and glutathione: beyond an antioxidant

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Apoptosis is a conserved homeostatic process critical for organ and tissue morphogenesis, development, and senescence. This form of programmed cell death also participates in the etiology of several human diseases including cancer, neurodegenerative, and autoimmune disorders. Although the signaling pathways leading to the progression of apoptosis have been extensively characterized, recent studies highlight the regulatory role of changes in the intracellular milieu (permissive apoptotic environment) in the efficient activation of the cell death machinery. In particular, glutathione (GSH) depletion is a common feature of apoptotic cell death triggered by a wide variety of stimuli including activation of death receptors, stress, environmental agents, and cytotoxic drugs. Although initial studies suggested that GSH depletion was only a byproduct of oxidative stress generated during cell death, recent discoveries suggest that GSH depletion and post-translational modifications of proteins through glutathionylation are critical regulators of apoptosis. Here, we reformulate these emerging paradigms into our current understanding of cell death mechanisms.

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Apoptosis or programmed cell death is a ubiquitous homeostatic process involved in numerous biological systems. Under physiological conditions it is critical not only in the turnover of cells in tissues but also during normal development and senescence. Moreover, its deregulation has been widely observed to occur as either a cause or consequence of distinct pathologies including cancer, autoimmune, and neurodegenerative diseases. Apoptosis is a highly organized program induced by a myriad of stimuli that are characterized by the progressive activation of precise pathways leading to specific biochemical and morphological alterations in individual cells without involving an inflammatory response. Early stages of apoptosis are characterized by initiator caspase activation, cell shrinkage, loss of plasma membrane lipid asymmetry, and chromatin condensation. The execution phase of apoptosis is characterized by activation of executioner caspases and endonucleases, apoptotic body formation, and cell fragmentation¹ (Figure 1). Interestingly, recent studies have shown that changes in the intracellular milieu of the cells, such as alterations in the redox environment, are important regulators of the progression to apoptosis.² These discoveries have led to the concept of a permissive apoptotic environment necessary for the activation of the proper signaling pathways regulating apoptosis. Glutathione (GSH) depletion is an early hallmark in the progression of cell death in response to a variety of apoptotic stimuli in numerous cell types^{3,4} (Figure 2), although the exact role of GSH depletion in apoptosis is still controversial. We

review recent data that show new insights into the understanding of the causes and consequences that alterations in the redox environment of the cell have on apoptosis.

The Role of GSH in the Regulation of Apoptosis

GSH synthesis, depletion, and apoptosis. GSH (L- γ -glutamyl-L-cysteinyl-glycine) in its reduced form is a tripeptide enzymatically formed by glycine, cysteine, and glutamate, and is the most abundant non-protein thiol in mammalian cells. GSH acts as a reducing agent and as a major antioxidant within cells by maintaining a tight control of the redox status. GSH is also involved in many distinct physiological reactions including cellular signaling, metabolism of xenobiotics, thiol disulfide exchange reactions, and as an important reservoir of cysteine. GSH is a ubiquitous molecule that is produced intracellularly attaining mM concentrations whereas plasma concentrations of GSH are at low μ M levels because of its rapid catabolism. GSH is 85–90% freely distributed in the cytosol, but can also be compartmentalized in organelles including the mitochondria, the peroxisomes, the nuclear matrix, and the endoplasmic reticulum (ER) after its cytosolic synthesis. Synthesis of GSH is initiated by the synthesis of γ -glutamylcysteine from glutamate and cysteine by the γ -glutamylcysteine synthetase (γ -GCS). Glycine is subsequently added by the activity of GSH synthetase (GS).^{4,5}

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Abbreviations: GSH, reduced glutathione; GSSG, glutathione disulfide; GS \bullet , thyl radical; ROS, reactive oxygen species; RNS, reactive nitrogen species; RS, reactive species; γ -GCS, γ -glutamylcysteine synthetase; GPX, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; GSNO, nitrosoglutathione; G6PD, glucose-6-phosphate dehydrogenase; NAC, N-acetyl-L-cysteine; NO, nitric oxide; protein-SSG, protein glutathionylation; protein-SNO, protein nitrosylation

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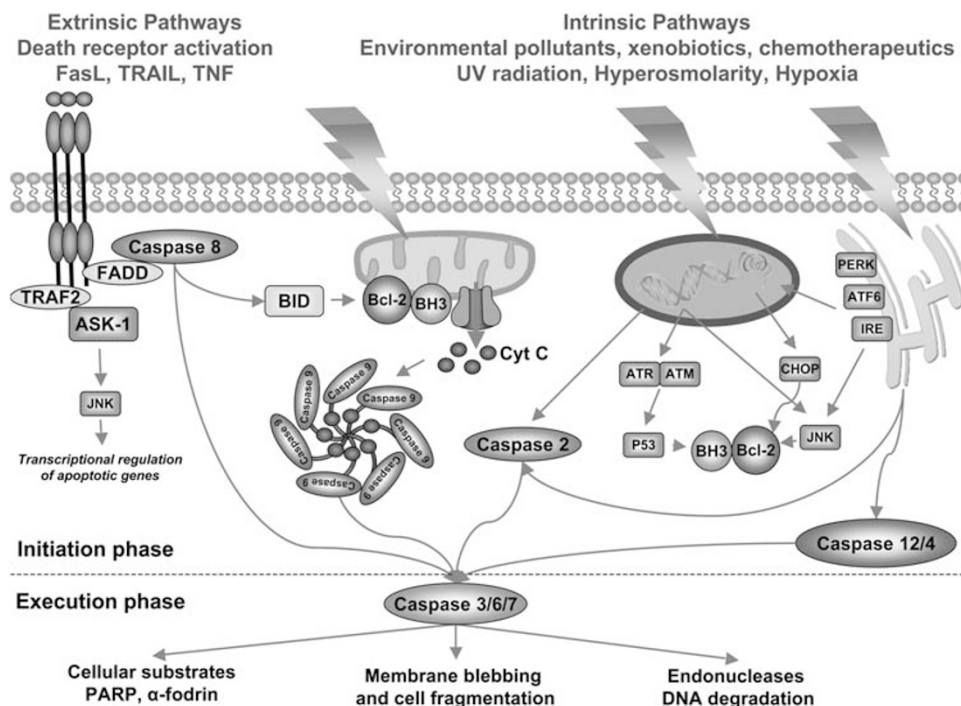


Figure 1 Apoptotic signaling pathways (see text for further details)

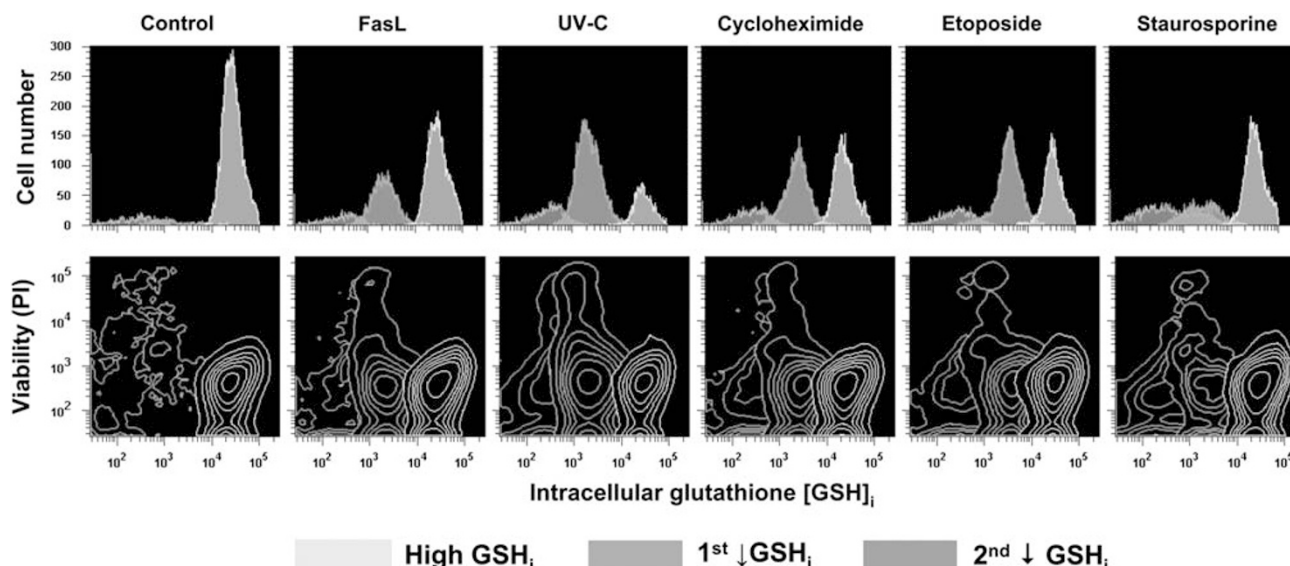


Figure 2 GSH depletion is a hallmark of apoptosis. Apoptosis was induced in the human lymphoma cell line (Jurkat) by activation of Fas death receptors (FasL), stress (UV radiation), or cytotoxic drugs. Briefly, Jurkat cells were exposed for 8 h to either 25 ng/ml FasL, 15 mJ/cm² UVC radiation, 35 μM cycloheximide, 100 μM etoposide, or 20 nM staurosporine. GSH content and cell viability was assessed by FACS using a BD-LSRII and BD-FACSDiva software. Cells were preloaded with monochlorobimane (a GSH-binding dye; 10 μM, 15 min) and propidium iodide (PI) (for plasma membrane integrity; 10 μg/ml, 5 min) before FACS analysis; 10 000 cells per sample were analyzed and results are represented as histograms or contour plots showing the distribution of cells with differences in GSH content and cell viability. For further details refer to Ref.^{20,21} Apoptosis induces a decrease in the population of cells with high intracellular GSH content (GSH_i) with a concomitant increase in the populations of cells with depleted GSH_i. Two populations of cells with depleted GSH content can be identified based on other biochemical characteristics. The first decrease in GSH_i is associated with the initiation phase of apoptosis, whereas the secondary decrease in GSH_i is observed after the execution phase is triggered (upper panels).^{20,21} GSH depletion is an early event during apoptosis, which occurs before the loss of cell viability, measured by PI uptake (lower panels)

GSH is essential for cell survival as the GSH-depleted knock-out mouse of γ-GCS dies from massive apoptotic cell death.⁶ As the availability of cysteine is the rate-limiting factor

in GSH formation, cysteine starvation has been shown to induce apoptosis by GSH depletion.⁷ Several studies have shown a correlation between GSH depletion and the

progression of apoptosis, and knockdown of γ -GCS induces time-dependent apoptosis in various cell types.⁸ Conversely, overexpression of γ -GCS protects against apoptosis induced by stimuli that activate both extrinsic and intrinsic cell death pathways.^{9,10} γ -GCS is a heterodimer of a catalytic subunit and a regulatory subunit. The γ -GCS promoter contains many potential *cis*-acting elements, including consensus recognition sites for binding of activator protein-1 (AP-1 or TRE), activator protein-2 (AP-2), Sp-1, nuclear factor κ -B (NF- κ B), and the electrophile responsive element EpRE (also called the antioxidant response element). EpREs are located in the 5'-flanking sequences of both subunit genes and are thought to mediate gene induction following xenobiotic exposure.¹¹ The EpRE is the *cis*-acting DNA element that mediates the NF-E2-related factor-2 (Nrf2)-dependent regulation of gene expression.¹² Downregulation of Nrf2 has been shown to reduce γ -GCS gene expression and decrease GSH levels¹³ and GSH-dependent enzymes.¹⁴ Interestingly, Nrf2 levels have been directly associated with resistance to apoptosis.¹⁵ Nrf2 has also been shown to protect against oxidative stress-induced cell death by upregulation of enzymes involved in GSH biosynthesis, use, and export.¹⁶

High intracellular GSH levels have been associated with apoptotic resistant phenotypes in several models of apoptosis,^{17,18} and GSH depletion by itself has been observed to either induce or stimulate apoptosis.^{17,19} For example, buthionine sulfoximine (BSO)-induced GSH depletion does not trigger apoptosis, but potentiates death receptor-induced apoptosis in T cells.^{17,19} GSH supplementation with *N*-acetyl-L-cysteine (NAC) also prevents apoptosis^{20–22} Replenishment of intracellular GSH pools with sulfur amino acids including *S*-adenosyl-methionine, which is a precursor of GSH through its sequential conversion from *S*-adenosylhomocysteine (SAH) to cysteine, has also been shown to protect against apoptosis.^{18,23}

At present, the precise contribution of cytosolic *versus* mitochondrial GSH pools in apoptosis is not fully understood. Some reports suggest that apoptosis correlates directly with depletion of cytosolic GSH rather than with mitochondrial GSH depletion.²⁴ However, other studies have shown that mitochondrial GSH depletion is important in triggering the cell death cascade. Accordingly, promotion of cell death by diseases or treatments that deplete cellular GSH seems to correlate closely to the extent of depletion of mitochondrial GSH rather than the changes in the cytoplasmic pool.^{25,26} Mitochondrial thiols have been clearly shown to act as regulators of apoptosis.²⁷ Selective depletion of mitochondrial GSH sensitizes cells to oxidative stress- and nitrate stress-induced apoptosis.²⁸ Mitochondria depend on GSH for the prevention of oxidative damage because of aerobic respiration. Salvage of glutathione-disulfide (GSSG) by GSH reductase (GR) and the uptake of cytosolic GSH across the outer membrane are required for the maintenance of mitochondrial GSH levels because these organelles are incapable of *de novo* synthesis. It has also been proposed that GSH must be actively transported into the mitochondria or exchanged for another anion. This observation likely reflects the fact that the matrix space of the mitochondria possesses a negative membrane potential relative to the cytoplasm, and also that GSH is a negatively charged molecule at

physiological pH. Some of the possible candidates involved in the mitochondrial import of cytosolic GSH include the monocarboxylate, dicarboxylate, 2-oxoglutarate, tricarboxylate, glutamate-hydroxide, and glutamate-aspartate transporters.²⁵ Accordingly, overexpression of the mitochondrial glutathione transporters (dicarboxylate and 2-oxoglutarate carriers) has been shown to protect cells from chemically induced apoptosis.^{29,30} Ethanol toxicity has been shown to deplete mitochondrial GSH by inhibition of its uptake from the cytosol, which potentiates acetaminophen- and tumor necrosis factor (TNF- α)-induced apoptosis.^{31,32} Recently, inhibition of mitochondrial GSH transport has been reported to exacerbate oxidant-induced cytochrome *c* (Cyt *c*) release, caspase 9 activation, and apoptosis.³³

Intracellular GSH depletion is an early hallmark in the progression of cell death in response to different apoptotic stimuli^{3,4} (Figure 2). GSH depletion during apoptosis induced by cytotoxic agents, which by themselves induce oxidative stress such as xenobiotics, chemotherapeutics, and metals, has been reported to be mediated by GSH oxidation to GSSG by reactive species (RS) of both oxygen (ROS) and nitrogen (RNS), or by its conjugation to highly reactive compounds^{34,35} (Figure 3). In contrast, apoptosis induced by distinct stimuli, such as activation of death receptors, has been reported to be mediated a plasma membrane efflux transport of GSH.²¹ Inhibition of GSH depletion under these conditions is able to rescue cells from apoptosis.²¹ Several studies have suggested that both multidrug resistance proteins (MRP)³⁶ and/or GSH/organic anion (OA⁻) exchangers might be involved in GSH efflux during apoptosis.²¹ The MRPs act as transporters of GSH, GSSG, and GSH-adducts and require the hydrolysis of ATP for its transport activity.³⁷ Pharmacological activation of MRPs induces apoptosis by GSH depletion.³⁸ In clear contrast, other studies have shown that inhibition of MRP-mediated transport accelerates apoptosis.^{20,21} Conversely, overexpression of MRP proteins inhibit cell death induced by death receptor activation and cytotoxic drugs.³⁹ These data suggest that MRP-mediated transport might act in a protective manner against apoptosis. The organic anion transporting polypeptides (OATP) have also been proposed to mediate GSH efflux by a GSH/OA⁻ exchange,⁴⁰ although recent studies have suggested that GSH/OA⁻ exchange is not mediated by this family of transporters.⁴¹ Thus, there is a strong possibility that GSH efflux is mediated by a different and still uncharacterized entity. Whether GSH is depleted by its efflux, intracellular oxidation, or conjugation seems to depend on the nature and dose of the apoptotic stimuli. Interestingly, recent findings have suggested that γ -GCS is a direct target of caspase 3,⁴² which may represent another mechanism for preventing GSH replenishment ensuring apoptosis. The cystic fibrosis transmembrane conductance regulator has also been suggested to mediate transport of GSH during apoptosis, which modulates the activation of Bax.⁴³ Connexins, which when unapposed form hemichannels, have been recently suggested to mediate the efflux of GSH but their role in the progression of apoptosis has not been studied yet.⁴⁴

GSH depletion by its efflux has been thought to regulate intracellular apoptotic signaling. However, it has also been suggested that efflux of GSH and/or its extracellular accumu-

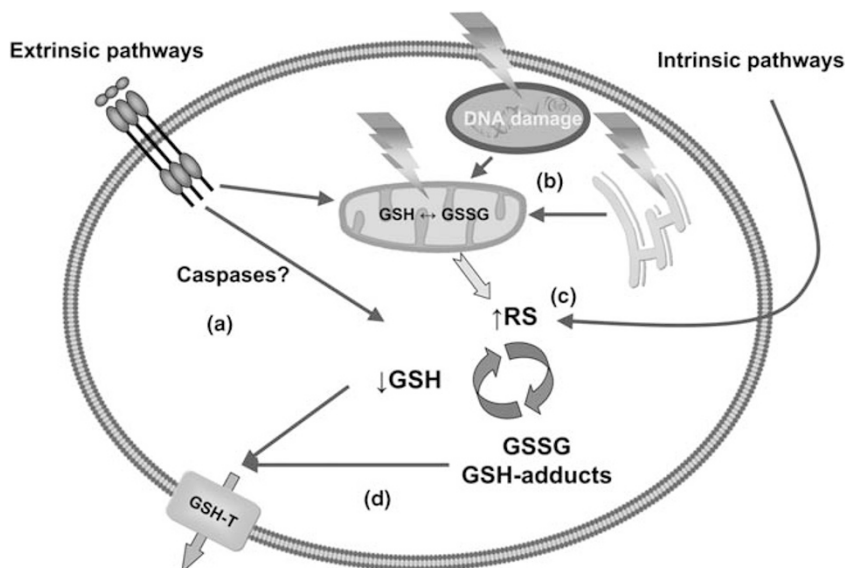


Figure 3 Mechanisms involved in GSH depletion during apoptosis. GSH depletion during apoptosis can occur by distinct mechanisms. (a) Activation of death receptors induces GSH loss by its extrusion across the plasma membrane through GSH transporters or pumps (GSH-T). Recent evidence suggests that caspases might be modulating this process. (b) Activation of intrinsic pathways of apoptosis might mediate GSH depletion by its oxidation to GSSG because of the generation of reactive species (RS) of both oxygen (ROS) and nitrogen (RNS) that arise from impairment of mitochondrial function. A crosstalk between transport- and RS-mediated GSH depletion might exist according to the apoptotic conditions. (c) Apoptotic signals arising from pro-oxidant stimuli mediate GSH depletion by both its direct oxidation and/or conjugation. (d) GSSG and GSH-adducts formed are further reduced or extruded to avoid their deleterious effects inside the cell. Other mechanisms such as impairment of GSH synthesis and recycling might also contribute to preventing GSH replenishment during apoptosis

lation might regulate apoptosis by different signaling events mediated by GSH pro-oxidant catabolism through the γ -glutamyl transpeptidase (γ -GT).^{45,46} The γ -GT, expressed mainly on the apical surface of cells, initiates the catabolism of not only GSH, but of glutathione S-conjugates and glutathione-complexes by removal of the γ -glutamyl moiety from GSH and GSH-conjugated compounds, producing cysteinylglycine or cysteinylglycine-conjugates. These products are subsequently hydrolyzed by ectoprotein dipeptidases and then, cysteine, together with the γ -glutamyl-amino acids formed, are uptaken by the activity of specific transporters. Once in the cytosol, γ -glutamyl-derivatives are substrates for the γ -glutamyl cyclotransferase that forms 5-oxoproline, which is finally converted to glutamate by the activity of the 5-oxoprolinase.⁴⁶ Thus, by cleavage of GSH and the subsequent recapture of cysteine, cystine, and γ -glutamyl-amino acids, γ -GT facilitates replenishment of intracellular GSH pools. Accordingly, overexpression of γ -GT has been shown to protect cells from apoptosis induced by oxidative stress.⁴⁷

GSH and apoptotic signaling cascades. The signaling cascades that regulate the progression of apoptosis have been extensively studied and characterized; and both extrinsic and intrinsic pathways have been described for the activation of apoptosis (Figure 1). Induction of apoptosis through extrinsic pathways is triggered by death receptors such as those activated by Fas ligand or FasL (Fas, CD95/Apo-1); by TNF-related apoptosis-inducing ligand or TRAIL (DR4, DR5); and by TNF- α (TNFR1). Activation of CD95, DR4, and DR5 leads to the formation of the death-inducing signaling complex (DISC) through the recruitment of the Fas-associated death domain (FADD), caspase 8 (and in some

cases caspase 10), and the cellular FLICE-inhibitory protein (FLIP). Initiator caspase 8 is activated and further amplifies the apoptotic cascade by activation of executioner caspases (3, 6, and 7). In cells that have lower levels of DISC formation and thus, reduced caspase 8 activation (Type II cells), the progression of apoptosis relies on an amplification loop induced by caspase 8-dependent cleavage of the Bcl-2 family protein, Bid, translocation to the mitochondria, and subsequent release of Cyt c. In contrast, TNFR1 signaling results in the formation of two signaling complexes. TNF-induced complex I formation mediates the recruitment of receptor-interacting protein, TRADD (TNFR-associated death domain protein), and TRAF-1/2 (TNFR-associated factor). This complex lacks FADD and procaspase 8, but it is reported to translocate to the cytosol in which FADD, caspase 8/10, and FLIP are recruited to form the traddosome or complex II in which activation of caspase 8 takes place. In addition, complex I has been shown to mediate other pro-apoptotic signaling cascades including the activation of the apoptosis signal-regulating kinase 1, and the stress activated kinase (SAPK) JNK leading to the transcriptional/post-transcriptional regulation of apoptotic genes.¹

The intrinsic pathways of apoptosis involve the mitochondria, the ER, and the DNA damaging pathways. These pathways are activated by a wide variety of stimuli including chemotherapeutic and cytotoxic agents (environmental pollutants, xenobiotics, drugs), stress (radiation, hyperglycemia, hypoxia, oxidative, and osmotic stress), and cytokine withdrawal. Activation of the mitochondria pathway mediates the release of Cyt c that is associated with the opening of the mitochondrial permeability transition pore (MPTP) and loss of the mitochondrial membrane potential. Although MPTP was

initially proposed to be formed by the voltage-dependent anion channel, the adenine-nucleotide translocator, and the cyclophilin D, recent studies have suggested that mitochondrial apoptosis is independent of these complex, which in contrast might be involved in cell death by necrosis. The intrinsic mitochondrial pathway is regulated by Bcl-2 family proteins. The BH3-only proteins (members of the Bcl-2 family) Bad, Bid, Bim, NOXA, and PUMA regulate the anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-xl) to promote apoptosis. Bcl-2 and Bcl-xl inhibit Bax and Bak. Induction and/or activation of BH3-only proteins de-repress Bax and Bak by direct binding and inhibition of the Bcl-2 anti-apoptotic family members. Bax and Bak are crucial for inducing the permeabilization of the outer mitochondrial membrane and the release of Cyt c. This leads to the recruitment of Apaf1 into the apoptosome and activates caspase 9 to further regulate execution caspases.¹

GSH depletion has been shown to regulate both extrinsic and intrinsic apoptotic signaling cascades at distinct checkpoints. GSH depletion can predispose cells to apoptosis or directly trigger cell death by modulation of both the permeability transition pore formation and the activation of execution caspases.^{48,49} *In vitro* studies have shown that a reduction in the GSH content is necessary for the formation of the apoptosome.⁵⁰ GSH depletion activates the intrinsic apoptotic pathway initiator Bax by its oxidation-dependent dimerization.⁵¹ GSH depletion has also been shown to trigger Cyt c release and it has been proposed that released Cyt c from the mitochondria needs to be oxidized for its pro-apoptotic action, which would require cytosolic GSH levels to be depleted.⁵² Recently, it was shown that in healthy neurons and cancer cells, Cyt c is reduced and held inactive by increased GSH content, generated as a result of glucose metabolism through the pentose phosphate pathway.⁵³ TNF- α -induced apoptosis has also been shown to depend on caspase-dependent GSH depletion that further regulates ceramide production by the neutral sphingomyelinase.⁵⁴ In addition, GSH depletion has also been shown to impair TNF- α -induced NF- κ B signaling sensitizing cells to apoptosis.⁵⁵

The anti-apoptotic role of Bcl-2 has been widely linked to GSH content. Bcl-2 has been shown to regulate GSH content in different cellular compartments.⁵⁶ Overexpression of Bcl-2 increases GSH levels and inhibits mitochondrial-induced cell death elicited by GSH-depleting reagents.⁵⁷ Recent studies suggest that Bcl-2 can regulate the mitochondrial GSH pool by a direct interaction with GSH through the BH3 groove, which mediates its antioxidant function.⁵⁸ Accordingly, depletion of intracellular GSH has been reported to overcome Bcl-2-mediated resistance to apoptosis.⁴⁸ However, these effects seem to be cell type-specific and context-dependent.⁵⁹ The anti-apoptotic effect of Bcl-xl has also been attributed to the regulation of GSH homeostasis by preventing GSH loss.⁶⁰

Other intrinsic pathways of apoptosis are also regulated by GSH homeostasis. ER stress is induced by alterations in cellular energy levels, the redox state, or Ca²⁺ concentration, which results in the accumulation and aggregation of unfolded proteins. Severe ER stress has been proposed to lead to the activation of JNK kinase and induction of the C/EBP homologous protein (CHOP), which impairs the anti-apoptotic function of Bcl-2, leading to the activation of Bim, Bax, and Bak. Activation of these pro-apoptotic Bcl-2 family members

transmits the apoptotic signal from the ER to the mitochondria leading to the execution of death by activation of caspases and/or ER-mediated Ca²⁺ overload. ER stress has also been proposed to lead to the activation of caspase 12/4 but this observation is still controversial. In addition, a role for caspase 2 and ASK-1 in ER stress-induced apoptosis has been proposed.¹ ER stress-induced apoptosis has been shown to trigger oxidative stress and to mediate GSH depletion by its oxidation.⁶¹ The lumen of the ER contains a relatively high concentration of GSSG, which is maintained in part by the selective transport of GSH from the cytosol. This gradient allows the formation of disulfide bonds and their isomerization through the activity of protein disulfide isomerases (PDI) and oxidoreductases. Reduced PDI is further oxidized by the flavoprotein Ero1 that generates ROS as byproducts, which are then detoxified by GSH, thereby generating high levels of GSSG. PDI can be either reduced by GSH or oxidized by GSSG. Finally, high luminal GSSG concentrations are then secreted from the ER.^{62,63} Thus, GSH serves as a redox buffer against ROS generated in the ER. The accumulation of unfolded proteins in the ER has been recently shown to induce PERK-dependent activation of Nrf2 that contributes to the maintenance of GSH levels. Enhanced GSH content prevents the accumulation of ROS during the unfolded protein response. Thus perturbations in cellular GSH concentrations have been shown to sensitize cells to ER-induced apoptosis.⁶⁴ ER stress also induces CHOP expression, which has been shown to promote GSH depletion and oxidative injury that is counteracted by the action of Bcl-2.⁶⁵

Specific DNA lesions or damage, including cross-links and double-strand breaks (DSBs) are well known to trigger apoptosis. DSBs are detected by ataxia telangiectasia mutated gene, ataxia telangiectasia, and Rad3-related proteins, which signal downstream to the CHK1 and CHK2 checkpoint kinases, and p53. p53 induces transcriptional activation of pro-apoptotic factors such as FAS, PUMA, and BAX. Apoptosis on DNA damage has also been shown to be mediated by activation of caspase 2 and JNK.¹ Oxidative DNA damage and impaired cellular repair mechanisms contribute to apoptosis. Recent reports have suggested a critical role of nuclear GSH pools in protecting DNA from oxidative modifications that may alter the efficiency of the DNA repair machinery. The nuclear GSH pool is thought to be maintained by the diffusion of GSH into the nucleus across nuclear pores.^{66,67} Conjugation of GSH is an essential aspect of xenobiotic and normal physiological metabolism. The glutathione S-transferases (GST) form a class of enzymes with overlapping substrate specificities that generate a large set of thioesters, called glutathione S-conjugates. GSTs can also serve as peroxidases, isomerases, and thiol transferases. GSTs protect against DNA damage-induced apoptosis by scavenging the formation of lipid-peroxide-modified DNA on oxidative stress.⁶⁸ Mitochondrial DNA damage is also modulated by GSH which reduces its susceptibility to oxidant-induced damage and apoptosis.⁶⁹ DNA cross-linker agents, such as cisplatin, have been reported to induce apoptosis paralleled by GSH depletion. In addition, apoptosis induced by the DNA intercalating agent doxorubicin was shown to be potentiated by BSO-induced GSH depletion.¹⁷

Redox signaling in apoptosis by GSH. As summarized above, GSH content and its depletion have been widely reported to regulate a variety of apoptotic signaling pathways, however, the precise molecular mechanisms involved in this process are still unclear. Changes in the intracellular thiol-disulfide (GSH/GSSG) balance are considered major determinants in the redox status/signaling of the cell.⁷⁰ GSH constitutes the major intracellular antioxidant defense against RS and oxidative stress. GSH has been shown to scavenge directly a wide variety of RS, including superoxide anion ($\text{O}_2^{\bullet-}$), hydroxyl radical (OH^{\bullet}), singlet oxygen ($^1\text{O}_2$), protein-, and DNA radicals, by donating electrons and becoming oxidized to thyl radical (GS^{\bullet}). The generation of disulfide bonds between two GS^{\bullet} leads to the further formation of GSSG. GSH also catalytically detoxifies cells from peroxides such as hydroperoxides (H_2O_2), peroxynitrite (OONO^-), and lipid peroxides (LOO^{\bullet}) by the action of GSH peroxidases (GPX) and peroxiredoxins (PXR). Accumulation of GSSG on oxidative stress has been observed to be toxic to the cell. GSSG has been shown to directly induce apoptosis by the activation of the SAPK/MAPK pathway.⁷¹ An early and transient rise in intracellular GSSG has been shown to precede Cyt *c* release and caspase 3 activation during apoptosis. In these studies GSH/GSSG ratio was observed to return to normal levels 1 h after the apoptotic stimuli, however, it did not influence the progression of cell death.⁷² GSSG is reduced back to GSH by the action of GR, which requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor reductant. Under physiological conditions GSSG is maintained at $\sim 1\%$ of the total cellular glutathione concentration and increases in GSSG are counteracted by its rapid reduction by GR. GR requires reduced NADPH as the electron donor reductant. Glucose-6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme of the oxidative pentose phosphate cycle, is indispensable for the regeneration of NADPH from NADP^+ .⁷³ G6PD also helps in the maintenance of protein mixed disulfides (protein-SG) in the cell.⁷⁴ Impairment of G6PD sensitizes cells to oxidant-induced apoptosis.⁷⁵ GSH depletion during apoptosis is also paralleled by decreases in NADPH availability, which might contribute to a sustained cellular redox imbalance by impairing GSSG reduction by GR.⁷⁶ Increased NADPH formation through the pentose pathway has been shown to protect against apoptosis induced by excitotoxicity, death receptors, and serum deprivation.^{77,78} Inhibition of GR has also been shown to induce GSH depletion and oxidative stress, which sensitizes cells to undergo apoptosis.⁷⁹ In addition, GSSG has also been shown to be detoxified by its efflux transport across the plasma membrane.⁸⁰

Cellular stress has been widely reported to induce apoptosis primarily through oxidant toxicity in which depletion of GSH by its oxidation has been considered to be just a byproduct of oxidative stress. As GSH depletion has been shown to sensitize cells to agents that induce oxidative cell death, apoptosis under these conditions has been assumed to be largely regulated by the signaling action of RS of oxygen and nitrogen. Indeed the role of RS in apoptosis has been extensively studied,⁸¹ and several GSH-dependent

antioxidant enzymes protect cells from undergoing apoptosis. Glutathione peroxidases (GPX) are selenoproteins that reduce peroxides with different substrate specificity. GPX1 whose preferred substrate is hydrogen peroxide is the most abundant isoform found in the cytoplasm of nearly all mammalian tissues whereas GPX2 is an intestinal and extracellular enzyme.⁸² GPX1 and GPX2 have been reported to protect against apoptosis induced by oxidative stress^{83,84} ischemia/reperfusion injury⁸⁵ and doxorubicin⁸⁶ and to reduce pro-apoptotic Bax expression.⁸⁷ GPX4 (also known as phospholipid hydroperoxide glutathione peroxidase, PHGPX) overexpression has been reported to protect against oxidative stress-induced apoptosis.⁸⁸ GPX4 also protects against oxidative stress and amyloid-induced apoptosis.⁸⁹ More recently, GPX4 downregulation has been shown to induce 12/15 lipoxygenase-dependent lipid peroxidation and apoptosis-inducing-factor-mediated cell death.^{90,91} Overexpression of the mitochondrial GPX4 was also shown to protect against apoptosis induced through the intrinsic mitochondrial pathway by reducing mitochondrial hydroperoxide accumulation.⁹²

The role of GSH depletion and ROS in apoptosis induced by physiological stimuli such as death receptor activation, which does not induce oxidative stress by itself, remains more controversial.⁹³ GSH depletion has been shown to occur at earlier stages during the progression of the cell death program and it is followed by a delayed accumulation of ROS, which requires GSH depletion.²⁰ Several reports have shown contradictory roles including both inhibitory and stimulatory effects of ROS on apoptosis.^{2,94} As GSH is the major antioxidant within the cells, it has been largely thought that GSH regulates apoptosis by preventing the accumulation of ROS. GSH peroxidase (GPX) has been shown to protect against apoptosis induced by Fas activation.⁹⁵ However, death receptor (Fas and TNF)-induced cell death was shown to be similar in animals deficient in GPX compared with WT.⁹⁶ Many studies have linked ROS formation with the progression of Fas-induced apoptosis by the protective effect of extracellular thiols such as GSH and NAC, effects ascribed to their known capacities as antioxidants.⁹⁴ However, protective effects of thiol compounds on apoptosis in the absence of excessive ROS formation are also observed.⁹⁷ Additionally, apoptosis has been suggested to occur under anaerobic conditions in the absence of ROS formation,⁹⁸ or in cells lacking mitochondrial DNA, which are deficient in respiration and mitochondrial oxidative phosphorylation, the source of ROS during apoptosis.⁹⁹ These results imply a more active and widespread role for GSH in the regulation of apoptosis rather than simply acting as an antioxidant/scavenger against oxidative stress and ROS. GSH depletion seems to be required for ROS formation during apoptosis. For example, we and others have recently shown that GSH depletion is necessary for the generation of ROS during FasL-induced apoptosis,²⁰ and specifically observed that GSH content, but not the excess in ROS formation and oxidative stress, regulates apoptosis induced by Fas activation.²⁰ Other studies have also shown that apoptosis seems to be actively regulated by GSH content and not by excessive oxidative stress and ROS generation.¹⁰⁰ These findings support the idea of a direct role of GSH in the regulation of the apoptotic machinery independent from oxidative stress. Perhaps,

excessive ROS overload might well be just an epiphenomena associated with the depletion of GSH.

Protein glutathionylation and apoptosis. Recently, oxidative post-translational modifications (OPMs) in proteins have been shown to regulate the activity of a wide variety of proteins. OPMs in general can be classified as reversible and irreversible modifications. Reversible modifications include cysteine residues modified through alternative redox-based modifications (nitrosylation, SNO; hydroxylation, SOH; glutathionylation, SSG; disulfide bond formation, S-S) that may enable differential effects on protein function. Protein (S-) glutathionylation (protein-SSG, also known as [S]-glutathiolation) refers to the formation of a protein mixed disulfide between the cysteine of GSH and a cysteine moiety of a protein (thiol exchange reactions). One of the most accepted mechanisms of protein-SSG formation is the reaction of GSSG with protein sulfhydryls (given by exposed protein cysteine residues). Under non-stressed conditions, the intracellular environment favors a fully reduced thiol state. It has been shown that a significant basal level of protein-SSG (~1%) is found within cells, which is proportionally upregulated by increases in GSSG content. S-glutathionylation of proteins has a dual role in protecting against irreversible protein thiol oxidation and in regulating protein function.¹⁰¹ Oxidative stress and ROS formation during apoptosis induced by pro-oxidant conditions might regulate protein-SSG formation. However, depletion of GSH in response to non-pro-oxidant apoptotic stimuli (activation of death receptors) and as a consequence, a decrease in the GSH/GSSG ratio should by itself be able to

promote protein-SSG formation in the absence of excessive oxidative stress (Figure 4). Thus, a high intracellular concentration of GSH under basal conditions should act as a protective mechanism against GSSG-induced protein-SSG formation. GSH can also induce protein-SSG formation, but only in the presence of oxidized cysteines (sulfenic acids), which might be a plausible mechanism under pro-oxidant apoptotic stimuli.¹⁰² Apoptosis is accompanied by increased protein-SSG formation.¹⁰³ TNF- α -induced apoptosis is reported to be paralleled by increased protein-SSG formation, which is inhibited by overexpression of Bcl-2.¹⁰³ Recently, FasL-induced apoptosis has also been reported to increase protein-SSG, which amplifies the apoptotic signaling cascade by glutathionylation of the Fas receptor.¹⁰⁴ In contrast, caspases have been reported to be glutathionylated under basal conditions and to become deglutathionylated upon the induction of apoptosis.¹⁰⁵ Accordingly, GSSG-induced caspase 3 glutathionylation has been recently reported to inhibit its proteolytic activity.¹⁰⁶

Glutathione content has been shown to regulate the activation of ionic conductances during apoptosis. Alterations in the intracellular ionic homeostasis of the cell have been clearly linked to the progression of apoptosis.¹⁰⁷ Recent reports show that during apoptosis, cell shrinkage or apoptotic volume decrease, associated with the impairment in the Na⁺-K⁺-ATPase activity,¹⁰⁸ and activation of K⁺ and Cl⁻ conductances^{109,110} are also regulated by alterations in GSH homeostasis. A wide variety of ion channels and transporters have been shown to be regulated by ROS.¹¹¹ In addition, sulfhydryl groups within cytoplasmic cysteine domains of ion channels directly modulate channel activity.¹¹² However, it is

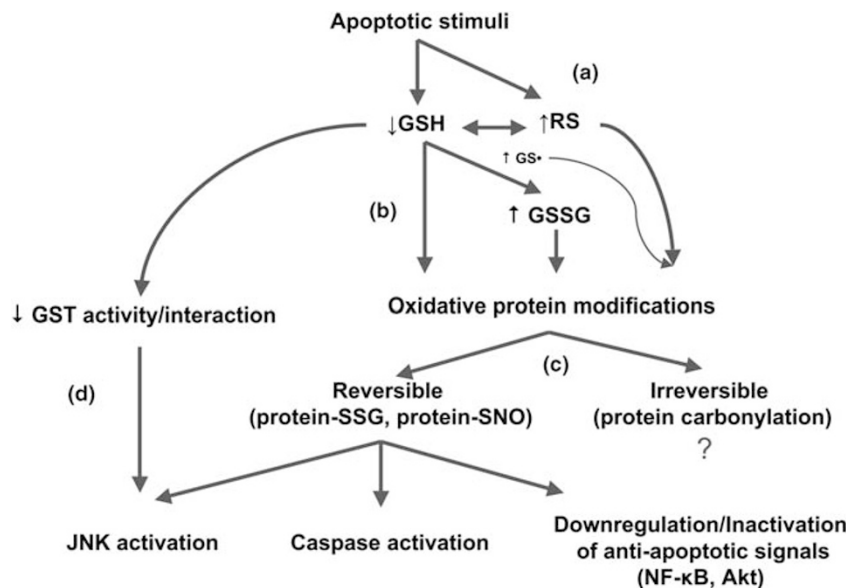


Figure 4 Molecular mechanisms involved in the regulation of apoptosis by GSH. GSH has been reported to regulate apoptosis by distinct mechanisms. (a) GSH might be a prerequisite for the generation of RS. In some cases RS induced by pro-oxidant apoptotic conditions lead directly to GSH depletion by its oxidation to GS● and further formation/accumulation of GSSG. (b) GSH depletion and the concomitant decrease in GSH/GSSG ratio might increase the availability of GSSG to exert its redox signaling, even in the absence of excessive RS formation and oxidative stress. (c) Alterations in GSH, GSSG, and RS concentrations and equilibrium have been shown to mediate oxidative post-translational modifications (OPMs) in proteins that may regulate the progression of apoptosis. The oxidated intermediate of GSH, GS●, might also be involved in the direct generation of OPMs. GSH and GSSG are known to promote reversible OPMs such as protein-SSG (glutathionylation) and protein-SNO (nitrosylation) formation that regulate different signaling proteins involved in apoptotic cell death. (d) Changes in GSH content also modulate glutathione S-transferases (GST) activity and its interaction with other apoptotic signaling proteins such as JNK

still unclear whether during apoptosis GSH depletion regulates ionic pathways indirectly through ROS or directly by protein glutathionylation.

Protein-S-SG linkages are removed by changes in the intracellular GSH/GSSG balance and/or the activities of glutaredoxin (GRX) enzymes.¹¹³ GRXs are versatile oxidoreductases that use the reducing power of GSH to catalyze reversible protein glutathionylation (reduction of mixed disulfides). Inhibition of GRXs by cadmium induces apoptosis in the absence of any other stimuli, which is associated to increased protein-S-SG formation.¹¹⁴ Human cells contain four GRXs. Of these, the cytosolic dithiol GRX1 and the mitochondrial monothiol GRX2 have been implicated in the regulation of apoptosis.¹¹⁵ GRX1 has been shown to protect against apoptosis induced by Fas activation by reducing Fas glutathionylation, which potentiates its aggregation and recruitment into lipid rafts.¹⁰⁴ Overexpression of GRX1 also protects against doxorubicin-induced cell death.¹¹⁶ Conversely, GRX1 downregulation sensitizes lens epithelial cells to oxidative stress-induced apoptosis.¹¹⁷ Mitochondrial GRX2 is also involved in the regulation of apoptosis, and its downregulation sensitizes cells to apoptosis.¹¹⁸ GRX2 has been shown to protect against apoptosis by reducing Cyt c release and cardiolipin oxidation.¹¹⁹ Recently GRXs have been shown not only to deglutathionylate specific proteins but also to catalyze the glutathionylation of several proteins in the presence of GS• radicals. Hence, GRXs are capable of catalyzing both glutathionylation and deglutathionylation of proteins through distinct mechanisms.¹²⁰ Thus, the role of both GRXs and protein-S-SG formation needs to be simultaneously assessed to understand their specific involvement during apoptosis. Although GRXs have been shown to have a protective effect against apoptosis by decreasing protein-S-SG formation, knockdown of GRX1 was recently reported to significantly inhibited TNF- α -induced cell death because of attenuated caspase 3 cleavage and increased glutathionylation.¹⁰⁵ Therefore, tissue specific and spatio-temporal regulation of protein glutathionylation in apoptosis might be having a function in cell death progression.

Non-enzymatic reaction of nitric oxide with GSH results in the formation of nitrosoglutathione (GSNO), which can induce protein-S-SG formation by reaction with protein-thiyl radicals (protein-S•).¹²¹ GSNO has been shown to induce glutathionylation caspases.¹²² GSNO can also be directly conjugated inactivated by its direct conjugation to proteins by protein nitrosylation (protein-SNO). Nitrosylation has been suggested to act as an intermediate for glutathionylation because of the fact that protein-SNO can readily react with GSH leading to protein-S-SG formation.¹²³ As GSH can promote protein-SNO, through GSNO protein-SNO might be one of the mechanisms by which changes in GSH content regulate apoptosis. Protein nitrosylation has been clearly shown to regulate apoptosis.¹²⁴ For example, caspases have been shown to be nitrosylated under basal conditions and to become denitrosylated on apoptosis.¹²⁵

The role of GSH in other types of cell death pathways. Cell death is often classified by both biochemical and morphological criteria. According to the classification of cell death recommended by the Nomenclature Committee on

Cell Death,¹²⁶ three distinct routes of cellular catabolism can be defined according to morphological criteria, which are apoptosis, autophagy, and necrosis although there are numerous examples in which cell death displays mixed features. Autophagy is a major catabolic pathway by which eukaryotic cells degrade and recycle macromolecules and organelles. It has an essential role in differentiation and development, as well as in cellular response to stress. Autophagy can be activated during amino-acid deprivation and has been associated with neurodegenerative diseases, cancer, pathogen infections, and myopathies. Autophagy is initiated by the surrounding of cytoplasmic constituents by the crescent-shaped isolation membrane/phagophore, which forms a closed double-membrane structure, called autophagosome. Finally, the autophagosome fuses with a lysosome to become an autolysosome, and its content is degraded by acidic lysosomal hydrolases. Autophagic cell death is morphologically defined by massive autophagic vacuolization of the cytoplasm in the absence of chromatin condensation.¹²⁶ Replenishment of intracellular GSH levels with NAC has been shown to abolish ROS-induced formation of autophagosomes and the consequent degradation of proteins during starvation-induced autophagy.¹²⁷ Lipopolysaccharide-induced autophagy was also shown to be paralleled by ROS formation and GSH depletion, which was prevented by NAC.¹²⁸ Treatment with gamma-glutamyl-cysteinyl ethyl ester, a precursor for *de novo* GSH formation, also prevents autophagy after traumatic brain injury.¹²⁹

Necrotic cell death is characterized by a gain in cell volume, swelling of organelles, plasma membrane rupture, and subsequent loss of intracellular contents. It is now recognized that execution of necrotic cell death may be finely regulated by a series of signal transduction pathways and catabolic processes.¹²⁶ Excessive GSH depletion and oxidative stress have also been reported to switch apoptotic to necrotic cell death.¹³⁰ Earlier work has shown that GSH-depleting agents such as DEM at doses that deplete mitochondrial GSH levels will induce necrosis. However, modest doses of GSH-depleting agents resulting mainly in cytoplasmic GSH depletion and minimal change in cell viability sensitize hepatocytes to TNF- α -induced apoptosis.⁷⁹ Ceramide has been implicated as a secondary messenger for TNF- α -induced cell necrosis and NAC or GSH-monoethylester have been shown to delay the onset of ceramide-induced necrosis.¹³¹

GSH, apoptosis, and disease progression

Alterations in intracellular GSH have been shown to occur during the progression of distinct pathological situations many of which are also related to alterations in apoptosis rates.⁴ We next summarize the evidence suggesting a role for GSH in the regulation of some of these human diseases.

Cancer. Glutathione and GSH-metabolism participate in both cancer prevention and progression. For example, under stress conditions, GSH scavenges harmful molecules such as electrophiles (by GSH conjugation), ROS, and RNS (by GSH antioxidant properties), preventing tumor initiation. Glutathione metabolism has been reported to participate in chemotherapy-induced tumor resistance to

apoptotic cell death. Increased expression and polymorphisms of GST and GSH-transporters as well as high GSH concentration are common features of transformed cells that, in turn, are associated with high resistance to chemotherapy-induced apoptosis.¹³² High intracellular GSH content in tumor cells is a consequence of high γ -GCS expression and can promote tumor cell survival by inhibition of apoptosis. Modulation of intracellular GSH content has been largely studied for potential anti-cancer therapies. GSH depletors such as BSO, DEM, ethacrynic acid, and diamide have been shown to sensitize Bcl-2 overexpressing cell to undergo apoptosis.¹³³ Another potential role for anti-neoplastic therapy is the modulation of GSH efflux by acting on its transporters or pumps. Recently, it has been shown that transformed cells are sensitized to cell death when intracellular GSH is depleted through stimulation of GSH efflux pumps,^{21,134} whose expression varies in different cancer cell lines.¹³⁵

Neurodegenerative Diseases. In the nervous system, GSH is necessary for the defense against oxidative stress and the progression of distinct pathologies including those of Parkinson's disease (PD) and Alzheimer's disease (AD). Neurodegenerative diseases are associated with selective neuron loss by apoptosis. Alterations in intracellular GSH homeostasis have been observed in different experimental models of neurodegenerative diseases.¹³⁶ Recent reports have shown a direct link between a reduction in GSH content and apoptosis in neuronal cells associated with neurodegeneration.^{8,137} Glutathione metabolism is reported to be altered in affected brain regions and peripheral cells from AD patients while its levels are depleted in experimental models of AD.¹³⁸ AD is associated with the formation of amyloid plaques in the brains of AD patients. Amyloid β -peptide is the main constituent of amyloid plaques. Amyloid β -peptides induce GSH depletion in cultured neurons and its cytotoxicity and accumulation is stimulated by GSH loss.¹³⁹ Cultured neurons from GSH peroxidase 1 GPX1(−/−) mice are more susceptible to cell death induced by amyloid β -peptides¹⁴⁰ whereas overexpression of GPX1 in the PC12 pheochromocytoma cells or rat embryonic cultured cortical neurons renders these cells more resistant to these peptides.¹⁴¹ In addition, increased protein glutathionylation (protein-SSG) has been reported during AD, which is associated with increased oxidative stress.¹⁴² Accordingly, GRX1 overexpression protects against amyloid β -induced toxicity.¹⁴³

PD progression is associated with a depletion of GSH levels and an increase in ROS formation in the substantia nigra. In fact GSH depletion has been proposed as one of the early biochemical events associated with neuronal apoptosis in PD.¹³⁷ Decreases in total GSH concentrations in the substantia nigra have been observed in preclinical stages of PD, at a time at which other biochemical changes are not yet detectable.¹⁴⁴ Thiol antioxidants, such as GSH and NAC, have been reported to prevent apoptosis induced by dopamine in neural cultures, which is proposed to mediate oxidative stress during PD by its auto-oxidation.¹⁴⁵ Overexpression of GPX1 in neuroblastoma cells *in vitro* and in nigral dopaminergic neurons *in vivo* attenuates apoptosis in

experimental models of PD.¹⁴⁶ Glutathionylation of mitochondrial NADP-dependent isocitrate dehydrogenase has been observed in an animal model of PD¹⁴⁷ and overexpression of GRX2 protected against apoptosis in distinct experimental models of PD.¹⁴⁸

Liver Toxicity. Drugs, infections, and inflammation in the liver increase the generation of ROS and RNS with concomitant decreases in GSH levels causing a shift in the cellular redox status of hepatocytes. This shift alters several cell signaling pathways in hepatocytes leading to the pathogenesis of many liver diseases. Liver injury induced by acetaminophen involves severe GSH depletion of both cytosolic and mitochondrial pools, ROS generation, and cell death mediated by JNK and ASK-1 activation. It has been shown that Nrf2 is activated by acetaminophen, which in turn increases GSH synthesis in hepatocytes. In this way, NAC has been proven to be effective in treating patients with acetaminophen-induced liver injury that, conversely, is potentiated by GSH depletors.¹⁴⁹ Alcohol-induced liver injury has often been associated with oxidative stress and decreases in hepatic antioxidant defense, especially GSH. GSH precursors have also been shown to protect against ethanol-induced toxicity in rat models. Chronic ethanol treatment is believed to deplete mitochondrial GSH by ethanol-induced changes in the inner membrane fluidity associated with cholesterol accumulation, which decreases GSH transporter activity. As liver mitochondria lack catalase, mitochondrial GSH and GSH peroxidases (GPX) are the major mechanisms for H₂O₂ in detoxification. Thus, ethanol toxicity is also paralleled by increased GSSG accumulation.¹⁵⁰

Conclusions and Perspectives

Recent studies have shown an active role for GSH in redox regulation of different processes. GSH depletion during apoptosis has been observed to occur at early stages during the cell death program. GSH depletion was initially ascribed to its oxidation by RS generated during oxidative stress. However, it is now recognized that under more physiological stimulation of apoptosis, such as activation of death receptors, GSH depletion occurs as an active process involving its extrusion across the plasma membrane. This phenomenon has also been shown to precede oxidative stress generated by the accumulation of RS and to be necessary for the progression of apoptosis. Indeed, GSH depletion has been shown to induce or potentiate apoptosis, and excessive oxidative stress. Although the exact mechanisms involved in the regulation of apoptosis by GSH remain elusive, recent reports show that oxidative post-translational modifications in proteins regulated by GSH content such as glutathionylation (protein-SSG) and nitrosylation (protein-SNO) are important regulators of apoptosis. Thus, it is time to look beyond GSH as a simple antioxidant and define its precise role in programmed cell death signaling.

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