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Review

Glutathione in liver diseases and hepatotoxicity

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ABSTRACT

Glutathione (GSH) is a major antioxidant as well as redox and cell signaling regulator. GSH guards cells against oxidative injury by reducing H_2O_2 and scavenging reactive oxygen and nitrogen radicals. In addition, GSH-induced redox shift with or without ROS subjects some cellular proteins to varied forms of oxidation, altering the function of signal transduction and transcription factor molecules. Increasing evidence supports the important role of ROS and GSH in modulating multiple signaling pathways. TNF-α and Fas signaling, NF-κB, JNK and mitochondrial apoptotic pathways are the focus of this review. The redox regulation either can switch on/off or regulate the threshold for some crucial events in these pathways. Notably, mitochondrial GSH depletion induces increased mitochondrial ROS exposure which impairs bioenergetics and promotes mitochondrial permeability transition pore opening which is critical for cell death. Depending on the extent of mitochondrial damage, NF-κB inhibition and JNK activation, hepatocytes may either undergo different modes of cell death (apoptosis or necrosis) or be sensitized to cell-death stimuli (i.e. TNF-α). These processes have been implicated in the pathogenesis of many liver diseases.

Contents

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1. Introduction

The tripeptide glutathione (GSH) was discovered in 1888 and its biochemistry and metabolism were established by many investigators but none made a greater contribution than Alton Meister. Because of the free thiol group in the cysteinyl residue and the abundance within cells, GSH is a major endogenous antioxidant in hepatocytes. The normal redox potential GSH/GSSG maintains functions of many cellular proteins and enzymes, including many of those involved cell death and survival cascades. In many forms of liver diseases, hepatic GSH pool is compromised through impairment of synthesis and transport and/or over-consumption. Disruption of GSH homeostasis in the liver not only gives rise to ROS that oxidize proteins, DNA and lipids, but also alters multiple signaling pathways affecting intermediary metabolism, survival and proliferation and therefore contributes to the pathogenesis of various liver diseases.

2. GSH homeostasis in hepatocytes

Hepatic GSH is maintained at a relatively constant concentration through GSH synthesis and turnover. Derived from three amino acids (cysteine, glutamate and glycine), GSH synthesis occurs in the cytosol through two ATP consuming steps. The first step, also the rate-limiting step, is the synthesis of γ-glutamylcysteine through the condensation of cysteine and glutamate with the catalysis of γ-glutamylcysteine synthetase (γ-GCS) also known as γ-glutamyl cysteinyl ligase (GCL). This is regulated by GSH feedback inhibition. GSH synthetase further catalyzes formation of GSH from γ-glutamylcysteine and glycine. GSH turnover is accounted by the efflux, inter-organelle transport, and cellular utilization.

2.1. Efflux of GSH through hepatic sinusoidal and canalicular membrane

Hepatocytes export GSH into plasma to maintain inter-organ GSH homeostasis. The overall sinusoidal efflux of GSH is electrogenic and thiol- and disulfide-sensitive. Uncharged thiols such as dithiothreitol (DTT) stimulate the efflux, while disulfides such as cystine and glutathione disulfide (GSSG) decrease the efflux (Lu et al.,1993, 1994). In addition, extracellular methionine trans-inhibits the efflux of GSH (Aw et al., 1986). An organic anion transporter polypeptide (OATP), Oatp1 has been characterized in basolateral membranes of rat hepatocytes that exchanges GSH for anions. Rat Oatp1mediates GSH efflux in an electroneutral, thiol-insensitive and disulfide-sensitive fashion. GSH transport is only seen with rat transporter. Although other organic anion transporters in human hepatocytes may mediate GSH efflux (Briz et al., 2006) their identification has been controversial (Mahagita et al., 2007). Furthermore, the specific sinusoidal GSH efflux carrier which is trans-inhibited by methionine and cystine has not been identified (Figs. 1–3).

Almost half of rat hepatic GSH efflux goes into bile through hepatic canalicular membrane. The canalicular GSH efflux involves multidrug resistance proteins (Mrps), particularly Mrp2 (Ballatori and Rebbeor, 1998). Mrp2 also exports GSH in the form of glutathione S-conjugate, a way to detoxify drugs, metals and xenobiotics and facilitates export of GSSG.

GSH efflux is an important event in Fas-induced apoptosis (Franco and Cidlowski, 2006; Franco et al., 2007; van den Dobbelsteen et al., 1996). Hammond et al observed that around 75% of cellular GSH was released from human Jurkat T lymphocytes at 2 h after FasL stimulation. During this period of time, apoptosis was initiated and reached the peak at 5 h post treatment (Hammond et al., 2007). Similar findings have also been made in HepG2 cells (Ghibelli et al., 1998; Hammond et al., 2004) and rat hepatocytes (Gumpricht et al., 2000). It is speculated that the GSH export may facilitate apoptosis by enhancing cell susceptibility to oxidative stress and the apoptotic signaling pathway. In support of this hypothesis, block of GSH export in these cells was found to delay or suppress apoptosis (Hammond et al., 2007).

2.2. Transport of GSH into mitochondria

Mitochondrial GSH (mGSH) is essential for cell survival. Of the total cellular GSH pool, 10 ~ 15% is found in the mitochondria versus 80–85% in the cytosol. As the mitochondrial volume is much smaller than the cytoplasm, mGSH concentration reaches 9–12 mmol/L, close to that of cytosol (Griffith and Meister, 1985). Due to the lack of the enzymes for GSH synthesis in mitochondria, mGSH is derived from the cytosol where GSH synthesis takes place.

Maintaining mGSH pool relies largely on a functional transport system that brings cytosol GSH into mitochondria. At physiologic pH, GSH exists as a net negative charged anionic molecule. The main barrier for GSH is the inner mitochondrial membrane (IMM) as the outer mitochondrial membrane (OMM) is permeable enough for GSH anion to move freely. Two

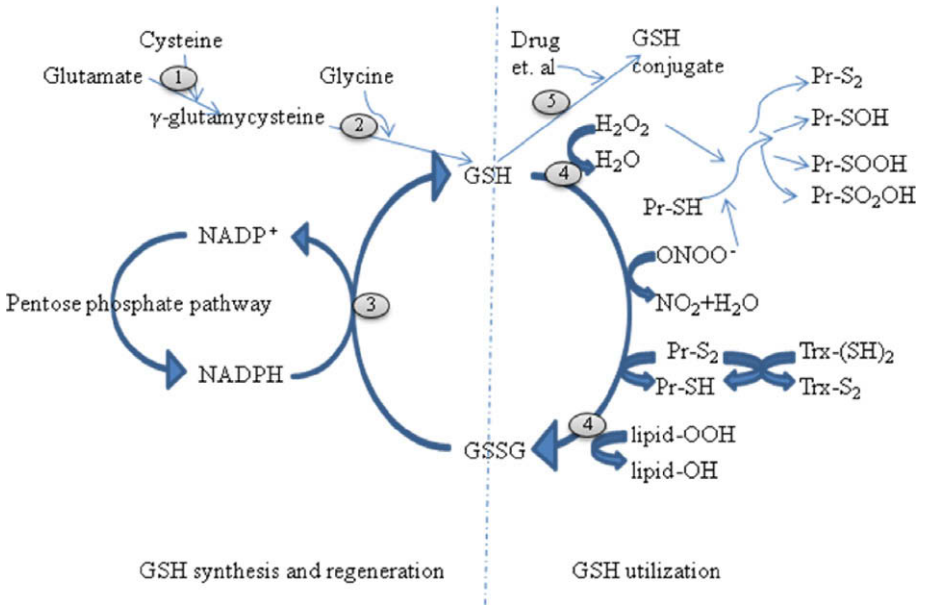

Fig. 1. The pathways for GSH synthesis, regeneration and utilization. (1) γ-glutamycysteine synthetase; (2) GSH synthetase; (3) glutathione reductase; (4) glutathione peroxidase; (5) glutathione S-transferase. Not shown, Pr–SOH (sulfenic acid) reacts with GSH to form Pr–S–S–G (glutathionylation) with is then specially reduced by glutaredoxin. Thus, glutathionylation prevents further oxidation of the sulfenic to sulfinic and sulfonic acid.

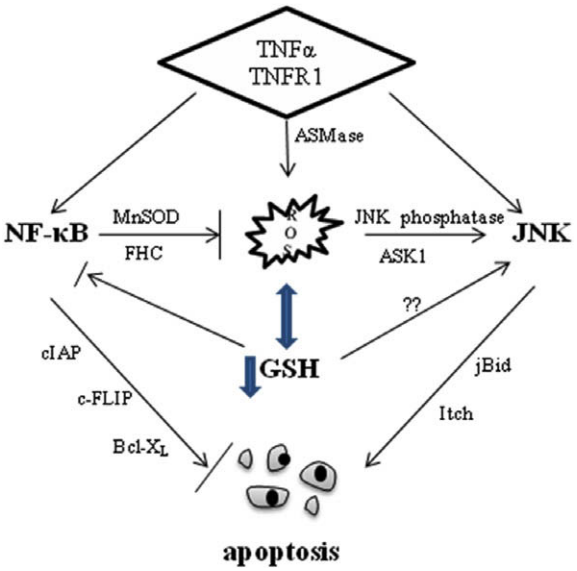

Fig. 2. ROS modulate the interplay of NF-κB and JNK. The activation of NF-κB induces MnSOD and FHC, both of which act in concert to remove TNF-induced mitochondrial ROS. When NF-κB is suppressed, ROS rises to a threshold that causes sustained JNK activation through deactivating JNK phosphatase and/or activating JNK directly or via ASK1. GSH depletion can also inhibit NF-κB. Cells survive through the fine balance of pro-apoptotic and anti-apoptotic factors. Disrupting this balance by NF-κB inhibition and JNK activation drives cells toward apoptosis or necrosis. An exogenous source of ROS derived from inflammation (not shown) which concomitantly exposes cells to TNF and ROS as well as TNF induced hepatocellular ROS production may play an important role in liver diseases, most of which are associated with primary or secondary inflammation.

anion carriers, dicarboxylate carrier (DIC) and 2-oxoglutarate carrier (2-OGC), have been implicated in GSH transport. DIC mediates the GSH anion import in exchange for inorganic phosphate Pi^{2-}. The functional reconstitution of DIC in NRK-52E cells, an immortalized cell line derived from rat proximal tubules, resulted in average 5.5-fold increase in rates of mGSH uptake (Lash, 2006). 2-OGC mediates the exchange of GSH anion for matrix 2-oxoglutarate (Colell et al., 2001, 2003).

The fluidity of the inner mitochondrial membrane influences GSH transport. The membrane fluidity is mainly determined by the percentage (un)saturation of fatty acyl groups and the molar ratio of cholesterol to phospholipids. Increasing the

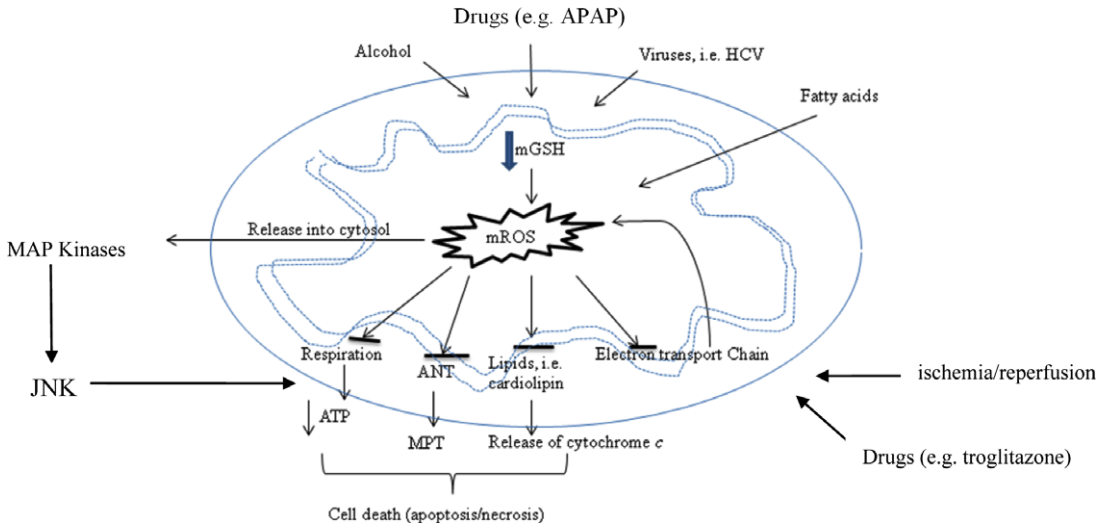

Fig. 3. Importance of mitochondrial GSH in pathogenesis of liver cell injury.

amount of cholesterol or decreasing unsaturated fatty acids, either of which enhances membrane microviscosity, have been shown to diminish GSH transport. For example, simply doubling the concentration of cholesterol in the inner membrane by incubating isolated rat liver mitochondria or mitoplasts with cholesterol/albumin complex significantly impaired the initial rate of GSH uptake, which was restored by adding A2C, a membrane fluidizing agent (Coll et al., 2003). The increased microviscosity of the inner mitochondrial membrane in hepatocytes could be induced by chronically feeding alcohol to rats, which accounts for selectively depleting mGSH pool in hepatocytes, a key feature implicated in pathogenesis of alcoholic liver disease (Colell et al., 1997; Fernandez-Checa et al., 1989).

3. The functions of GSH

GSH is critical for cellular functions and cell survival. As the most abundant reducing molecule in the hepatocyte, it maintains the redox state of the sulfhydryl groups of cellular proteins. As a major antioxidant, GSH quenches the endogenous oxidant species and combats exogenous oxidative stress. As an important regulator, GSH modulates the signaling cascades and the susceptibility of cells to different cell death stimuli.

3.1. GSH contributes to cellular redox potential

GSH is a major determinant of intracellular redox potential. Although several reducing couples such as GSH/GSSG, $NADP^+$/NADPH and thioredoxin ($TrxSS/Trx(SH)_2$) help maintain the intracellular reducing environment, GSH concentration is 100 ~ 10,000-fold greater than other couples and is therefore a determining factor of the redox potential. According to the redox reaction ($GSH + GSH \leftrightarrow GSSG + 2H^+$), cell redox potential can be derived from the Nernst equation ($\Delta E = E_0 - (RT/nF)\ln Q$) at 25 °C, pH 7.0. In Qdenotes log([GSH][GSH])/[GSSG]. Based on this equation, both GSH concentration and the molar ratio of GSH/GSSG contribute to the redox potential. Compared to GSH/GSSG ratio (~100) that is well maintained by constant reduction of GSSG from NADPH reducing equivalents through catalysis of glutathione reductase, the absolute GSH concentration may be a more sensitive factor in redox potential which is related to the square of the GSH concentration. Mild oxidative stress could consume GSH without altering GSH/GSSG, thus shifting redox potential. In different cell types, cellular GSH pool size varies by at least an order of magnitude. For example, hepatic GSH is around 10 mM, while neuronal GSH is only 1 mM. Thus, the reducing capacity of hepatocytes is much greater than that of neuron.

3.2. GSH protects cells against oxidative stress and detoxifies xenobiotics

GSH is a major antioxidant which combats oxidative stress in the cell (Fig.1). Endogenous reactive oxygen species (ROS) including superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\cdot}$) arise at very low levels as intermediates when oxygen is passed along complex I and complex III of ETC in the mitochondria. The ubiquitous NOX family of oxidases produce ROS under physiological conditions accounting for much of the ROS associated with receptor-mediated signaling. However, increased ROS commonly arises in conditions associated with infection, inflammation, xenobiotics and intracellular stress. ROS initiates cell damage by oxidizing proteins, lipids and DNA. Removal of ROS requires antioxidant enzymes including superoxide dismutase (SOD), catalase and glutathione peroxidase. SOD reduces $O_2^{\cdot}$ into H_2O_2 that is further reduced to water by catalase and glutathione peroxidase. In hepatic mitochondria where catalase is absent, glutathione

peroxidase quenches H_2O_2,although peroxiredoxins-thioredoxin2 also contribute. Glutathione peroxidase also repairs lipid oxidation by converting lipid hydroperoxides into less toxic alcohol derivatives. GSH acts as a hydrogen donor of glutathione peroxidase and its availability directly affects the enzymatic function. In addition, GSH scavenges reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$) with or without the help of glutathione peroxidase. Another important function of GSH is to conjugate with drugs and xenobiotics under the catalysis of glutathione S-transferases (GSTs). This action facilitates the export of drugs and xenobiotics through MRP family.

3.3. GSH regulates the functions of redox-sensitive proteins

Many proteins are sensitive to redox changes in the local cellular environment, especially proteins carrying a critical cysteine with a low pKa. When the sulfhydryl form (–SH) of cysteine is deprotonated into thiolate form ($–S^-$), the cysteine is more susceptible to redox modification. The accessibility of a cysteine within a three-dimensional protein structure to local redox buffer is also a contributing factor.

The protein cysteines could be oxidized into many forms. Besides forming disulfides (intra/inter-molecularly (–S–S–) or with GSH (–SG) or other low molecular weight thiols), cysteine oxidation produces a reversible sulfenic acid (S–OH), irreversible sulfinic acid ($S–O_2H$) and sulfonic acid ($S–O_3H$). GSH probably plays one of its most important redox regulatory roles in rapidly reacting with sulfenic acids, reducing them to protein-GSH mixed disulfide. This can be view as protecting the protein-SH by preventing the further irreversible oxidation of sulfenic to sulfonic acid. GSH-protein mixed disulfides are then preferentially reduced by glutaredoxin. This interplay between ROS, thiolate, sulfenic acid, GSH and glutaredoxin illustrates that in most cases it is difficult to separate effects of oxidative stress and glutathione redox.

The local milieu is complex with varied concentration of GSH and GSSG, and varied amounts and types of ROS, all of which influence the net outcome of cysteine oxidation. For example, when p50 subunit of NF-κB complex was exposed *in vitro* to reduce buffer with GSH/GSSG ratio ranging from 100 to 0.1, *S*–glutathionylation and sulfenation of Cys62 took place in varied proportions. With decreasing the ratios, oxidation of additional cysteines, such as Cys135, occurred (Pineda-Molina et al., 2001). These modifications reduced the DNA binding affinity of p50 to different degrees. The redox scenario *in vivo* is conceivably far more complex with the interplay of ROS, GSH/GSSG and redox factors causing multi-forms of protein oxidation which alter function.

The availability of other redox factors such as thioredoxin (Trx), a 12-KD protein containing CXXC motif for thiol-disulfide exchange with other proteins, affects the reduction of oxidized cysteines. For example, nucleus is a highly reducing compartment where Trx is present at high levels. Cys62 of the p50 subunit of NF-κB is oxidized in the cytoplasm while being reduced in the nucleus for optimal DNA binding. Trx is presumably translocated into the nucleus (Tanaka et al., 1997), and is responsible for reducing Cys62 of p50 (Matthews et al., 1992). Over-expression of Trx in the cytoplasm suppresses NF-κB activation, while over-expression of Trx in the nucleus enhances NF-κB activation (Hirota et al., 1999). Another important redox factor is ref-1. Ref-1 is present in the nucleus and modulates the reduction of a conserved cysteine in the leucine-zipper DNA binding motif of jun/fos heterodimer (Xanthoudakis and Curran, 1992; Xanthoudakis et al., 1992). The cysteine reduction is essential for jun/fos binding to activator protein 1 (AP-1) DNA motif. Ref-1 also regulates the transcriptional activities of p53. Over-expression of ref-1 stimulates the transcription of p21, a p53-responsive gene, while down-regulation of ref-1 markedly reduces p21 transcription (Gaiddon et al., 1999). Trx further modulates ref-1 by recruiting and reducing ref-1. The dual modulation by ref-1/Trx plays an important role in transcriptional regulation in the cell. These redox regulatory proteins undoubtedly are influenced by the GSH redox state.

4. Signaling pathways involved in molecular mechanisms of liver injury and the role of GSH

A key aspect of liver injury and the role of GSH is the response of the hepatocyte to exogenous or endogenous imposed redox stress, which activates various signal transduction and transcriptional pathways. Thus, the extent of exposure to ROS and GSH redox perturbations may be very critical in determining pro-survival versus pro-death responses and tip the balance one way or the other.

4.1. TNF-α/TNFR1 and FasL/Fas signaling and role of GSH

TNF-α is a critical cytokine mediating liver injury. The release of TNF-α mainly from liver macrophages (Kupffer cells) is an ubiquitous response during inflammation, infection, ischemia, oxidative stress and toxin exposure. The binding of soluble TNF-α to TNFR1 on the plasma membrane of hepatocytes triggers the exposure of cytoplasmic death domain (DD) of TNFR1. TRADD, RIP and TRAF2 rapidly assemble around TNFR1-DD to form complex I. Complex I activates NF-κB, JNK and P38 cascades to propagate inflammation and survival signaling. Upon dissociating from TNFR1-DD, RIP, TRAF2 and TRADD subsequently associates with FADD to form complex II. The death-effector domain of FADD recruits procaspase 8 which is then cleaved into the active form of caspase 8. FADD is also independently activated by the binding of FasL (on T cells) to Fas (also known as CD95 or Apo-1) that triggers trimerization of Fas. Fas recruits FADD and procaspase 8 to form DISC (death-inducing signal complex). In some cell types, the active caspase 8 bypasses mitochondria to initiate apoptosis through direct cleavage of procaspase 3 in cytosol. However, in hepatocytes caspase 8 activates mitochondria-mediated apoptotic pathway through cleavage of Bid into tBid. tBid further activates the Bcl-2 homology (BH)3 proapoptotic members such as Bax and Bak to

increase mitochondrial membrane permeability and release cytochrome *c* from mitochondrial intermembrane space. Cytochrome *c* forms apoptosome with Apaf-1 and procaspase 9 to initiate self-cleavage of procaspase 9. The active caspase 9 cleaves procaspase 3 into caspase 3, an apoptosis executioner.

GSH depletion alters the susceptibility of hepatocytes to TNF-α and FasL killing. Studies from our lab showed depletion of GSH sensitized primary mouse hepatocytes (PMH) to TNF-α-induced apoptosis ((Matsumaru et al., 2003; Nagai et al., 2002). Severe depletion of GSH with high doses of acetaminophen (APAP), diethylmaleate (DEM), or phorone induced necrosis of PMH, while the addition of TNF-α switched about half of cell death to apoptosis. The switch of death mode involved increased caspase activity and cytochrome *c* release. However, lower DEM and phorone doses, which minimized direct cell death while moderately depleting GSH, significantly sensitized to increased TNF-α induced apoptosis with minimal necrotic background. Therefore, altered GSH redox sensitized to TNF-induced apoptosis.

Hentze et al. (2000) showed that GSH depletion exerts a protective effect against Fas-induced apoptosis in the liver through inhibiting Fas signaling and caspase activation. In this study, a high dose of phorone injection was given to mice to induce acute and severe GSH depletion and the inhibition of cytokine release from GSH-depleted T cells played an important role in hepatocyte protection. By contrast, Haouzi et al. (2001) found that prolonged GSH depletion by a sulfur amino acid-deficient diet enhanced Fas-mediated apoptosis in mice. The major differences between these two studies at the molecular level lie in p53 and Bax expression that was unchanged in the former while significantly induced to mediate apoptosis through enhancing MPT in the latter. These studies raise an important issue with regard to the timing of GSH depletion in contributing to distinct outcomes.

4.2. NF-κB signaling pathway and the role of GSH

NF-κB mediates survival gene expression in hepatocytes. NF-κB is a homo- or hetero-dimeric transcriptional factor formed by any of the members: p50, p52, p65 (RelA) and c-Rel, with p50/p65 heterodimer being the dominant form in hepatocytes. p50/p65 is held inactive by IκB in the cytoplasm. IKK-β phosphorylates IκB for subsequent ubiquitination-proteasomal degradation. The released p50/p65 translocates into nucleus where it binds to κB site to activate the transcription of many anti-apoptotic factors including cIAPs, c-FLIP, and Bcl-X_L, and antioxidants such as Mn-SOD and ferritin-HC. TNF-*R* signaling mediates IKK activation through the assembly of RIP1, TRAF2 and TRADD. TRAF2 catalyzes the poly-ubiquitination of Lys63 of RIP and the polyUb-RIP1 recruits IKK complex consisting of the regulatory subunit IKK-γ and two catalytic subunits termed IKK-α and IKK-β. The interaction of polyUb-RIP1 and IKK eventually leads to IKK-β activation through phosphorylation.

NF-κB activation is sensitive to redox alteration in hepatocytes. The reduction of cys62 of p50 was found to be essential for the promoter binding (Nishi et al., 2002). Cys27 in c-Rel also requires Trx for reduction before being phosphorylated, a step required for c-Re1 to efficiently bind to κB sequence (Glineur et al., 2000). While these results indicate that events downstream of NF-κB activation are redox-regulated, how does IKK-IκB-NF-κB pathway, upstream of NF-κB activation, respond to redox alteration? To answer this question, Lou and Kaplowitz (2007) examined the effect of varying GSH depletion on the regulation of IKK- IκB-NF-κB activation in primary mouse hepatocytes (PMH). Moderate depletion (50%) of GSH with DEM mainly affects cytosol GSH pool. With this level of GSH depletion, mitochondrial GSH is well preserved and no increase in spontaneous ROS is induced. However, TNF induced NF- κB activation was inhibited as indicated by reduced expression of endogenous NF-κB-responsive anti-apoptotic genes including iNOS, cIAP1 and $cFLIP_L$. However, IKK activation, NF-κB nuclear translocation and p50/p65 DNA binding were all unchanged with moderate GSH depletion. Thus, moderate redox alteration in the absence of increased ROS somehow impairs NF-κB transcription but not DNA binding. Further depletion of GSH to 80% (which causes mitochondrial GSH depletion and increased ROS release), altered IKK activation by inhibiting the process of RIP1 polyubiquitination. Trolox, an antioxidant, was able to restore IKK activation, but failed to restore NF-κB transcriptional activity that appeared to be directly inhibited by decreased GSH levels. This indicated that with profound GSH depletion, both redox change and ROS participate in inhibiting NF-κB activation (Lou and Kaplowitz, 2007). A caveat here is the fact that the nuclear GSH status is poorly understood and one assumes that cytosol changes are directly reflected in the nucleus. The potential significance of this work is that liver inflammation or xenobiotic-induced GSH depletion or oxidative stress can sensitize to concomitant cytokine/death receptor mediated injury (e.g. via concomitant inflammatory cell TNF release).

4.3. JNK signaling pathway

TNF-α mediates JNK activation through phosphorylation of apoptosis signaling kinase (ASK) upon the assembly of TRAF2, RIP1 and TRADD. ASK1 is a MAP3K which phosphorylates and activates MAP2K, such as MKK4 and MKK7, which further phosphorylate and activate the MAPK, JNK and p38 kinase. The role of JNK in apoptosis has been well established in fibroblasts through phosphorylating c-Jun, ATF-2 and JunD (Davis, 2000; Weston and Davis, 2002). However, the bulk of evidence favors direct post-translational modification by JNK rather than gene expression in promoting cell death. JNK activation in response to TNF is normally transient because of NF-κB dampening of the activation. However, sustained JNK activation induces cell death. In acute liver inflammation and liver failure, JNK activation induces apoptosis by targeting distinct factors upstream of mitochondrial death pathways, among which are Itch and Bid. JNK phosphorylates and activates Itch, an E3 ubiquitin ligase. The active Itch catalyzes the ubiquitination of c-$FLIP_L$ that subsequently leads to its proteosomal degrada-

tion. Because c-FLIP$_L$ docks and inhibits caspase 8, the removal of c-FLIP$_L$ will release caspase 8 for activation. JNK also induces the cleavage of Bid to a truncated fragment "jBid". Different from tBid that activates Bak/Bax, jBid acts on mitochondria to selectively release Smac, an inhibitor of IAP. The removal of IAP by Smac will relieve the block of caspase 8. JNK also translocates to mitochondrial where it might directly trigger the release of cytochrome *c* (Aoki et al., 2002). Two JNK isoforms: JNK1 and 2, are expressed in the liver. Some functional differences of JNK1 and JNK2 in T cell differentiation have been presented through gene disruption experiments (Dong et al., 1998; Yang et al., 1998). With respect to the substrate c-Jun, JNK2 has 25-fold higher binding affinity than JNK1 (Kallunki et al., 1994). JNK1 and 2 have been shown to cooperate in p53 activation (Oleinik et al., 2007).

JNK activation induces either cell death or proliferation of hepatocytes depending on the magnitude and duration of JNK phosphorylation. Sustained JNK activation mediates TNF-α-induced apoptosis in hepatocytes and mouse liver deficient in NF-κB (Geisler et al., 2007; Maeda et al., 2003; Tang et al., 2001). Concanavalin A (ConA), a pan-T cell activator that stimulates the release of TNF-α from T lymphocytes, induces massive hepatic cell death in mice without blocking of NF-κB. Sustained JNK activation correlates with ConA-induced cell death and JNK knockout confers resistance to ConA toxicity (Maeda et al., 2003; Tang et al., 2001). Interestingly, a transient JNK activation characterized by a short period of JNK phosphorylation followed by quick termination within a few hours promotes cell proliferation necessary for liver regeneration after partial hepatoectomy (Schwabe et al., 2003; Yamada et al., 1997). This transient JNK activation involves the activation of cyclin D1 and the stimulation of G0 to G1 transition in hepatocytes (Schwabe et al., 2003).

4.3.1. ROS activate JNK signaling pathway

ROS generation appears crucial for prolonged JNK activation. TNF-α induces mitochondrial ROS (mROS) production, possibly through activation of acidic sphingomyelinase (ASMase) and the release of ceramide (Garcia-Ruiz et al., 2003). Ceramide has been suggested to block electron transport at the complex III-ubiquinone cycle, leading to ROS generation (Garcia-Ruiz et al., 1997). When NF-κB-deficient cells were challenged with TNF-α, ROS accumulated along with sustained JNK activation and necrotic cell death (Sakon et al., 2003). Removal of ROS with butylated hydroxyanisole (BHA), an antioxidant, inhibited JNK activation and cell death. Feeding mice with BHA also protected mice from ConA-induced hepatitis, a response that requires sustained JNK activation (Kamata et al., 2005).

Several mechanisms have been suggested in regard to how ROS regulate the JNK pathway. Trx regulates ASK1 activation through reduction and oxidation cycle. ASK1 is held inactive by Trx at physiologic condition and Trx oxidation allows the dissociation of ASK1 for activation (Saitoh et al., 1998). As an alternative concept, (Nadeau et al. (2007)) found that ASK1 oxidation preceded Trx oxidation upon exposure to H_2O_2, and ASK1 inter-chain disulfide formation was essential for ASK1 activation, suggesting that Trx acts as a negative regulator by reducing oxidized ASK1. JNK phosphatases are sensitive to TNF-induced ROS. The catalytic cysteine of JNK phosphatases have unusually low p$K_a$thus being highly sensitive to oxidation. When exposed to H_2O_2, the cysteine of JNK phosphatases is oxidized to sulfenic acid (–SOH) that can be reversed with reducing agent DTT or Trx reductase (Kamata et al., 2005). The oxidized JNK phosphatase loses its ability to dephosphorylate JNK and JNK activation thus becomes sustains. Deactivation of JNK phosphatase might be a major mechanism underlying sustained JNK activation in TNF-induced liver injury. GSTρ interacts directly with JNK as an inhibitor in non-stressed cells (Adler et al., 1999). ROS induces polymerization of GSTρ via intermolecular disulfides, causing dissociation of GSTρ polymer from JNK.

4.3.2. ROS mediate cross-talk between NF-κB signaling and JNK pathways

TNF-α challenge potentially activates both JNK and NF-κB cascades downstream of TNF-α/TNFR signaling. NF-κB gene expression then dampens JNK causing cells to survive. Block of NF-κB pathway leads to sustained JNK activation which then augments cell death.

ROS plays a major role in mediating the cross-talk between JNK and NF-κB. TNF induces ROS that mediates JNK activation. However NF-κB regulates ROS production through activating the transcription of several antioxidant factors including manganese-superoxide dismutase (Mn-SOD) (Wong et al., 1989) and ferritin heavy chain (FHC) (Pham et al., 2004). Mn-SOD, a mitochondrial form of SOD catalyzes the reduction of O_2^- to H_2O_2 that is further reduced to H_2O by glutathione peroxidase and FHC binds iron preventing Fenton reactions. Through these reactions, ROS are dampened to some extent and sustained JNK activation is suppressed. In addition, some of the NF-κB gene products (XIAP) may directly bind and inhibit JNK.

4.4. Mitochondrial cell death pathway and role of GSH

Mitochondria play a central role in propagating apoptotic signaling in hepatocytes. Many pro-apoptotic factors such as cytochrome *c*, AIF, Smac/Diablo and some procaspases are present in mitochondrial inter-membrane space. Their release into the cytoplasm facilitates the apoptotic cascade. The assembly of Apaf1, cytochrome *c*, and ATP with procaspase9 forms the apoptosome which activates caspase9 which then further activates caspase3. Smac/Diablo binds to IAPs and prevents IAPs from inhibiting caspases. AIF translocates into the nucleus where it elicits chromatin fragmentation by recruiting endonucleases, hence inducing so-called caspase-independent apoptotic pathway (Cande et al., 2004). The release of these factors requires sufficient permeabilization of mitochondria. Mitochondrial permeability transition (MPT) and mitochondrial outer-membrane permeabilization (MOMP) are the two models which describe mitochondrial permeability.

MPT is considered a pre-existing pore spanning the OMM and IMM. Its central components consist of voltage-dependent anion channel (VDAC) from OMM, adenine nucleotide translocase (ANT) from IMM, and cyclophilin D (CypD) from the matrix. In addition, peripheral benzodiazepine receptor, hexokinase and creatine kinase contribute to the pore structure in certain cell types. MPT opening increases the proton leak, reduces ATP generation and depolarizes mitochondrial membrane, bringing water into the matrix. Mitochondria then swell and the OMM ruptures releasing pro-apoptotic factors (Honda et al., 2005). Oxidative stress, GSH depletion and calcium influx all elicit MPT opening. Cyclosporin A inhibits the classical MPT by binding to CypD. Recent studies have questioned the role of specific MPT components in mediating cell death (Baines et al.,2005, 2007; Nakagawa et al., 2005). Mitochondria from VDAC-null mice showed the same sensitivities as mitochondria from wild mice to common stimuli that induce MTP-mediated cell death (Baines et al., 2007). CypD-deficient cells were resistant to necrotic cell death induced by oxidative stress and calcium overload (Nakagawa et al., 2005). Over-expression of CypD in the heart caused mitochondrial swelling and spontaneous cell death (Baines et al., 2005).

MPT is clearly redox-regulated. Crossing-linking the vicinal thiols on MPT increased the pore opening that was prevented by treatment with reducing agents (Costantini et al., 1995, 1996; Petronilli et al., 1994). Cys-56 of ANT is highly sensitive to redox change and cys-56 oxidation causes the cross-linking of functional ANT dimer, leading to pore opening (Costantini et al., 2000). GSH depletion causes MTP opening in a co-dependent manner with oxidative stress and calcium overload. Depletion of endogenous GSH with L–buthionine–*S*–*R*–sulfoximine (BSO) or DEM enhances ROS generation from complex III of ETC and mitochondrial and cytosol calcium overload that may originate from ER (Armstrong and Jones, 2002; Lu and Armstrong, 2007). Both ROS and calcium overload account for GSH depletion-induced MPT opening.

Selectively permeabilizing OMM allows the release of the pro-apoptotic factors from the intermembrane space without disrupting IMM. Two members of Bcl-2 family: Bax and Bak govern MOMP. Upon activation, Bax and/or Bak oligomerize and the oligomers insert into the outer mitochondrial membrane to create pores for the release of cytochrome *c* and Smac/Diablo. Bax/Bak is regulated by two groups of Bcl-2 members (Youle and Strasser, 2008): pro-survival Bcl-2 members (Bcl-2, Bcl-X_L, and Mcl1) and pro-apoptotic BH3-only members (Bim, Bid, Puma, Bad and Noxa). The pro-survival factors such as Bcl-2 and Bcl-X_L, directly inhibit Bax/Bak. Whereas, pro-apoptotic factors such as tBid and Bim directly activate Bak/Bax (Youle, 2007), or de-repress Bak/Bax through binding and inhibiting Bcl-2 and Bcl-X_L (Willis et al., 2007).

mROS can also initate MOMP through attacking the lipids. Cardiolipin, a housekeeping lipid in mitochondrial membrane, is a particularly important target of ROS. Cardiolipin collaborates with Bax polymer to produce OMM opening allowing large molecules, such as cytochrome *c* to transport during apoptosis (Kuwana et al., 2002). Recent studies (Ott et al., 2002) also showed that cardiolipin retains cytochrome *c* at IMM through electrostatic interaction and cardiolipin peroxidation abrogates this association and frees cytochrome *c*, a necessary step for the release of cytochrome c to execute apoptosis. Optimal level of mGSH is critical to minimize mROS exposure and protect cardiolipin from ROS attack.

4.5. The switch between necrosis and apoptosis

Necrosis and apoptosis are two distinct modes of cell death. Necrosis involves cell swelling and the rupture of plasma membrane. The release of cellular components from necrotic cells elicits an inflammatory response mediated by innate immune cells. Apoptosis is a programmed cell death characterized by cell shrinkage and chromatin fragmentation. The rapid removal of apoptotic cells by phagocytes or other cells minimizes the surrounding inflammation.

Although necrosis was classically considered a universal "accidental" cell death, it has recently been shown that necrosis is a type of "aborted apoptosis" that occurs in a tightly regulated and programmed fashion. Inhibition of some essential apoptotic steps can lead to a switch to necrosis. For example, the interference of caspases with peptide inhibitors sensitized murine L929 fibrosarcoma cells to TNF-α- or FasL-elicited necrosis (Vercammen et al., 1998a,b). In addition to inhibiting caspases, the broad spectrum inhibitor zVAD-fmk was found to enhance TNF-induced ROS that augmented the switch of apoptosis to necrosis. Many caspases carry a conserved cysteinyl residue in their catalytic region that is readily modified upon exposure to ROS and/or RNS (Nicotera and Melino, 2004). Oxidation of caspases has been proposed as one mechanism by which ROS and/or RNS may induce necrosis. Apoptosis is an energy-consuming process and the optimal function of caspases and particularly the apoptosome requires sufficient ATP. Therefore, as cell death progresses, profound ATP depletion promotes necrosis. In addition, poly(ADP-ribose) polymerase (PARP-1) activation, an ATP-consuming process, has been implicated inducing necrosis in some scenarios such as ischemic-perfusion damage, inflammation and drug toxicity (Ha and Snyder, 1999). PARP-1 catalyzes the cleavage of NAD^+ for the release of an ADP-ribosyl unit and ADP-ribosyl transfer to nuclear proteins and ADP-ribose polymer in response to DNA strand breaks. Over activation of this process consumes a large amount of NAD^+ which leads to consumption of ATP to produce more NAD.

5. The molecular mechanism underlying liver diseases and role of GSH

5.1. Acetaminophen-induced hepatotoxicity and the role of GSH

Acetaminophen (APAP) hepatotoxicity is the leading of acute liver failure in the United States (Lee, 2003). APAP toxicity has been the leading research focus in drug-induced hepatotoxicity for decades. APAP is metabolized *in vivo* by CYP2E1 to a toxic metabolite, *N*-acetyl-*p*-quinoneimine (NAPQI). NAPQI attacks and covalently conjugates to cysteinyl residues of cellular proteins. NAPQI in theory could arylate signal transduction proteins or transcription factors, such as Nrf-2. Another potential

NAPQI target is MPT. Enhanced MPT and cytochrome *c* release have been observed in APAP-treated mice, which was attenuated by cyclosporine A, an MPT inhibitor (Masubuchi et al., 2005). However, it has been difficult to distinguish effects mediated by NAPQI versus decreased GSH or ROS.

GSH is a major endogenous defense molecule against APAP toxicity. Hepatocytes preferentially conjugate GSH to NAPQI for detoxification. This action prompts GSH depletion when a large amount of NAPQI is produced with intake of a toxic dose of APAP. When almost all cellular GSH is exhausted, the remaining reactive NAPQI will attack cellular proteins (covalent binding).

Obviously, the state of depletion of GSH is determined by the balance between the rate and amount of NAPQI generated versus the rate of replenishment of GSH governed by the availability of cysteine and the levels for GCL. This is why N-acetylcysteine protects, namely providing cysteine for GSH synthesis. Further, the oxidative stress activates Nrf2 to activate antioxidant response element (ARE)-regulated genes, such as GCL. Thus, the status of GSH during APAP metabolism is in a dynamic state.

Profound GSH depletion (especially in mitochondria) consequently gives rise to ROS that may induce a lethal oxidative stress. Auto-oxidation of NAPQI may also contribute to ROS exposure. Oxidative stress plays a key role in inducing APAP-induced liver injury. Administration of liposome-encapsulated human recombinant SOD conferred protection to rats against toxic dose of APAP ((Nakae et al., 1990). A SOD mimetic also attenuated APAP-induced hepatotoxity by removing ROS (Ferret et al., 2001).

APAP toxicity has recently been shown to involve JNK activation. APAP induced a sustained JNK activation and necrosis that was protected by a synthetic JNK inhibitor (SP600125) in both PMH and mice (Gunawan et al., 2006; Matsumaru et al., 2003). Although JNK2 seems to play a more important role in mediating APAP toxicity than JNK1, full protection was achieved by silencing of both JNK1 and JNK2 (Gunawan et al., 2006). However, APAP seems to activate JNK independent of TNF, since sustained JNK activation has been observed in TNFR1 null mice treated with APAP (Gunawan et al., 2006). Endogenous ROS derived from mitochondria due to GSH depletion is a potential activator of JNK in the APAP situation. This is strongly supported by two lines of evidence in PMH (Hanawa et al., 2008): first, H_2O_2 was released from mitochondria upon maximal GSH depletion at 2 h post APAP dosing, and H_2O_2 release preceded JNK activation that occurred at 4 h post APAP dosing. Second, direct incubation of H_2O_2 or increasing mitochondrial H_2O_2 release with antimycin A or rotenone activated JNK in PMH. It is also possible that NAPQ1 directly modifies JNK or JNK regulators, resulting in JNK activation.

Necrosis is the major mode of cell death in APAP toxicity *in vivo*. Overwhelming oxidative stress induced by APAP could directly damage plasma membrane and membrane-bound organelles, causing acute cell lysis. Alternatively, APAP-induced necrosis may occur in a programmed fashion. The release of cytochrome *c* has been found in some studies as indicated by activation of the apoptotic cascades (Hanawa et al., 2008), while insufficient ATP due to impaired mitochondrial respiration together with caspase inhibition by the redox change and ROS likely abort the apoptotic attempt and thus switch the pro-apoptotic cells to demise from necrosis. The novelty is that a toxicant kills by activation of a lethal kinase, JNK, which bridges the biochemical effects of oxidative stress and the bioenergetic collapse. Inhibiting this bridge with JNK inhibitors or silencing the expression of JNK1 and 2 profoundly inhibits APAP toxicity without altering covalent binding or GSH depletion.

5.2. Chronic alcoholic liver disease and the role of GSH

An important pathophysiologic change in chronic alcoholic liver diseases is the selective depletion of hepatic mGSH pool by 45–60%. This has been a consistent finding in both oral ethanol feeding models (Lieber–DeCarli model) and intragastric ethanol feeding models (Fernandez-Checa et al., 2002). mGSH depletion and the consequent increased production of mROS at complexes I and III subject hepatocytes to oxidative injury. The decreased level of mGSH also renders hepatocytes susceptible to TNF-α and FasL mediated cell death (Colell et al., 1998).

Alcohol-induced mGSH depletion is partially attributed to the impaired transport of GSH through the mitochondrial inner membrane. The hepatic mitochondria isolated from the long-term ethanol-fed rats have unesterified cholesterol-enriched inner membranes with increased microviscosity. These mitochondria showed significant decrease of the initial GSH transport rate that was greatly improved by restoring the membrane fluidity with feeding of *S*–adenosylmethionine or tauroursodeoxycholic acid (Colell et al., 2001). Specifically, the membrane microvicosity impairs 2-OGC-mediated GSH transport. Moreover, A2C, a fluidizing agent, directly restored the membrane fluidity and the OGC transport in isolated mitochondria from the alcohol fed rat (Coll et al., 2003).

A topic or great interest is how alcohol stimulates lipid synthesis and/or facilitates the deposition of cholesterol in the inner mitochondrial membrane. Recent studies have focused on the regulation of sterol regulatory element-binding protein (SREBP) family of transcription factors. SREBP1c controls lipid metabolism by regulating genes involved in the synthesis and metabolism of fatty acids. SREBP2 controls cholesterol metabolism by regulating the genes involved in cholesterol synthesis such as HMG–CoA reductase and cholesterol LDL receptor. Insigs (Insig-1 and Insig-2) act as negative regulators to retain SREBPs in ER membranes through forming complexes of Insig/Scap/SREBP stabilized by cholesterol. With ubiquitin-proteasome mediated Insig turnover or in the absence of cholesterol, Scap escorts SREBPs from ER to Golgi where SREBP is processed into a mature form by two membrane-bound proteases: S1P and S2P. The mature SREBPs translocate to the nucleus for transcriptional activation. SREBPs can be activated through ER stress, a cellular pathologic response characterized by protein accumulation or sustained loss of luminal Ca^{2+} in the ER in response to extrinsic or intrinsic stress. Ethanol

induces ER stress, as indicated by up-regulation of ER stress-specific proteins such as chaperone GRP78 and CHOP/GADD153 in hepatocytes and the livers of intragastric alcohol-fed mice (Ji et al., 2006; Kaplowitz and Ji, 2006). In these alcoholic livers SREBP is up-regulated and Insigs down-regulated (Ji et al., 2006). The hypothesis is that alcohol-induced ER stress activates SREBP-2 by decreasing Insigs, leading to cholesterol accumulation in cytosol as well as in mitochondria. In support of this hypothesis, acetaldehyde, a toxic ethanol metabolite, induced ER stress that preceded mitochondrial cholesterol accumulation in HepG2 cells (Lluis et al., 2003).

The induction of CYP2E1 by ethanol produces a significant amount of ROS spontaneously and during microsomal mixed-function oxidase activity. CYP2E1 is predominantly present in the microsomal fraction. Interestingly, a 3-fold increase of CYP2E1 is found in rat liver mitochondria after chronic consumption of ethanol (Bai and Cederbaum, 2006). Mitochondrial CYP2E1 is presumably active *in situ* in producing ROS that consumes mGSH. Impaired transport of GSH into mitochondrial matrix and increased consumption of mGSH could lead to a vicious cycle of mGSH depletion.

5.3. Viral hepatitis C and the role of GSH

The progression of chronic hepatitis C may involve oxidative stress produced by HCV structural proteins. HCV core protein induces ROS specifically from complex I of ETC in mitochondria. The core protein targets the hepatic mitochondria through a mitochondrial localization motif mapped in the C-terminus, and is associated with the mitochondrial outer membrane at a specific site with a close contact with ER membrane (Schwer et al., 2004). Along with ROS production, an increase in mitochondrial Ca^{2+} and GSSG have been observed in isolated liver mitochondria of HCV transgenic mice (Korenaga et al., 2005). Consistent findings have also been obtained through direct incubation of the core protein with liver mitochondria. It remains unclear which is the initial event leading to complex I alteration and ROS production. Complex I of ETC might be directly inhibited through interaction with core protein. Alternatively, complex I may be chemically modified through Ca^{2+}-induced ROS oxidation and/or glutathionylation upon the redox change.

Alcohol exacerbates HCV-induced liver injury. One possible key point of synergy is that alcohol and HCV both act on liver mitochondria to accelerate mGSH depletion and ROS production. Co-expression of CYP2E1 and core protein in Huh-7 cell line resulted in 4-fold increase of ROS and 40% decrease of mGSH (Otani et al., 2005). The HCV core/CYP2E1 cells were sensitized to cell death induced by TNF-α- or exogenous peroxides which was completely prevented by *N*-acetylcysteine (NAC) (Otani et al., 2005). As in many forms of liver disease, the inflammation (a source of TNF, FasL and exogenous ROS) induced by viral infection accelerates the killing of liver cells which may be sensitized by redox perturbations (ROS and/or thiol-disulfide) in various subcellular compartments (cytosol, mitochondria, or nucleus).

5.4. Other liver diseases and role of GSH

Hepatic hypoxic/ischemic and/or reperfusion injury is a common clinical manifestation during circulatory shock, liver transplantation , and hepatic vein/artery occlusion. Even in chronic alcoholic liver disease, perivenous hepatocytes are exposed to low O_2 because oxidative metabolism of alcohol increases O_2 uptake along the portal vein. Hypoxia is known to induce ROS, especially mROS through blocks in ETC. Nevertheless, hypoxia-induced ROS is detoxified by intact mGSH pool so that HepG2 and PMH survive 5% O_2 condition *in vitro* (Lluis et al., 2005). Optimal mGSH level is vital for hepatocytes to survive hypoxia and depletion of mGSH to a level lower than 3 mM sensitizes HepG2 to cell death due to unopposed hypoxia-induced massive ROS generation (Lluis et al., 2005). Thus, hypoxic/ischemic injury may contribute to the pathogenesis of alcoholic liver disease due to decreased mGSH combined with low O_2 in perivenous zone as a consequence of alcohol metabolism or anemia, pneumonia, sleep apnea, etc.

Oxidative stress has been implicated in pathogenesis of nonalcoholic fatty liver disease (NAFLD), perhaps the most prevalent liver disease in United States. NAFLD represents a wide spectrum of liver injury that encompasses steatosis, steatohepatitis, fibrosis and cirrhosis. The accumulating evidence indicates that mitochondrial dysfunction characterized by damaged ETC gives rise to ROS that plays a key role in NAFLD development and progression (Pessayre, 2007) . Specifically, impairment of complex I, cardiolipin oxidation and increased ROS in rat liver mitochondria have been induced by choline-deficient diet (Petrosillo et al., 2007), a classical fatty liver model. A diminished antioxidant capacity, including GSH and SOD, was found in patients with steatosis and steatohepatitis (Videla et al., 2004). It remains unclear what initiates mitochondrial dysfunction and how it progresses through the course of NAFLD.

6. Conclusions

Besides being central to antioxidant defense, there is mounting evidence that GSH is a regulator of cell signaling. In this review, we have focused on the effect of altered GSH status on the modulation of TNF-α signaling, NF-κB, JNK and mitochondrial death pathways since these are central pathways determining cell death of hepatocytes, a key aspect of liver diseases. Experimentally, the role of GSH modulation has been examined through GSH depletion. The redox change not only turns on/off some essential events in these signaling pathways, but it also adjusts the threshold for the on/off signaling. Consequently, hepatocytes are either sensitized, or undergo cell death in the form of apoptosis, necrosis, or switch of apoptosis to necrosis. The degree of GSH depletion (with or without inducing ROS), the affected GSH pool (mitochondrial or cytosol) and the types of stimuli are all relevant to various outcomes. Perturbations of GSH status are usually a consequence of oxidative stress and

therefore the downstream effects on signaling are linked to both ROS and GSH. Oxidative stress and redox perturbations are integral to all forms of acute or chronic liver disease. The status of glutathione in terms of GSH level and the GSH/GSSG ratio may mediate functional changes in signaling either dependent on or independent of the effects of ROS. Even mild changes in GSH without increased ROS or decreased GSH/GSSG can influence redox sensitive protein thiols and more marked depletion of GSH, especially in the mitochondria, can promote ROS production (decreased removal) and sensitize to further stress on mitochondria (e.g. TNF or toxin-induced JNK toxicity). Thus, a number of potential therapeutic targets emerge which may be widely applicable, including JNK inhibitors and mitochondrial targeted antioxidants.

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