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Mechanisms of Hepatotoxicity

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This review addresses recent advances in specific mechanisms of hepatotoxicity. Because of its unique metabolism and relationship to the gastrointestinal tract, the liver is an important target of the toxicity of drugs, xenobiotics, and oxidative stress. In cholestatic disease, endogenously generated bile acids produce hepatocellular apoptosis by stimulating Fas translocation from the cytoplasm to the plasma membrane where self-aggregation occurs to trigger apoptosis. Kupffer cell activation and neutrophil infiltration extend toxic injury. Kupffer cells release reactive oxygen species (ROS), cytokines, and chemokines, which induce neutrophil extravasation and activation. The liver expresses many cytochrome P450 isoforms, including ethanol-induced CYP2E1. CYP2E1 generates ROS, activates many toxicologically important substrates, and may be the central pathway by which ethanol causes oxidative stress. In acetaminophen toxicity, nitric oxide (NO) scavenges superoxide to produce peroxynitrite, which then causes protein nitration and tissue injury. In inducible nitric oxide synthase (iNOS) knockout mice, nitration is prevented, but unscavenged superoxide production then causes toxic lipid peroxidation to occur instead. Microvesicular steatosis, nonalcoholic steatohepatitis (NASH), and cytolytic hepatitis involve mitochondrial dysfunction, including impairment of mitochondrial fatty acid β-oxidation, inhibition of mitochondrial respiration, and damage to mitochondrial DNA. Induction of the mitochondrial permeability transition (MPT) is another mechanism causing mitochondrial failure, which can lead to necrosis from ATP depletion or caspase-dependent apoptosis if ATP depletion does not occur fully. Because of such diverse mechanisms, hepatotoxicity remains a major reason for drug withdrawal from pharmaceutical development and clinical use.

Key Words: **bile acids; cytochrome P4502E1; cholestasis; Kupffer cells; microvesicular steatosis; mitochondrial permeability transition; neutrophils; nitric oxide; oxidative stress; peroxynitrite.**

Drugs continue to be pulled from the market with disturbing regularity because of late discovery of hepatotoxicity. Such unexpected toxicities appear to be the consequence of the unique vascular, secretory, synthetic, and metabolic features of the liver. About 75% of hepatic blood comes directly from the gastrointestinal viscera and spleen via the portal vein. Portal blood brings drugs and xenobiotics absorbed by the gut directly to the liver in concentrated form. Drug-metabolizing enzymes detoxify many xenobiotics but activate the toxicity of others. Hepatocytes are highly reliant on ATP for ureagenesis, gluconeogenesis, and fatty acid metabolism among many other metabolic processes. In fasted individuals with low hepatic glycogen content especially, hypoxia, mitochondrial inhibition and damage to mitochondrial DNA lead to hepatocellular necrosis.

The liver synthesizes, concentrates, and secretes bile acids and excretes other toxicants, such as bilirubin. Drug-induced injury to hepatocytes and bile duct cells can lead to cholestasis. Cholestasis, in turn, causes intrahepatic accumulation of toxic bile acids and excretion products, which promotes further hepatic injury. Fortunately, the liver has enormous regenerative capacity, but regeneration of hepatocytes lost by necrotic and apoptotic cell death may mask detection of drug-induced injury. Furthermore, the active proliferative response of hepatocytes makes the liver an important target of carcinogens.

Hepatic nonparenchymal cells, the Kupffer, sinusoidal endothelial, and stellate (fat-storing or Ito) cells, and newly recruited leukocytes, i.e., monocytes and neutrophils, also contribute to the pathogenesis of hepatic toxicity. Kupffer cells and neutrophils are a source of proinflammatory cytokines and chemokines and of reactive oxygen and nitrogen species, which promote oxidative stress in injury induced by toxicants and ischemia/reperfusion. Kupffer cells also play a key role in hepatic injury due to ethanol consumption. The uniquely fenestrated sinusoidal endothelial cell is selectively vulnerable to cold ischemia/reperfusion injury to cause graft failure after

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transplantation and to cancer chemotherapy agents to cause veno-occlusive disease. Activated stellate cells synthesize collagen whose overproduction leads to hepatic fibrosis and cirrhosis.

The goal of this brief review is to discuss new developments in our understanding of the mechanisms of liver toxicity from drugs and other xenobiotics in the context of hepatic physiology, metabolism, and cell biology. The sections that follow emphasize important injury mechanisms, which can be a consequence of metabolism and/or direct cell toxicity of chemicals. These mechanisms include bile acid-induced liver cell injury during cholestasis, pathophysiological effects of mitochondrial dysfunction, and cell damage by reactive oxygen and nitrogen species. The importance of vascular (Kupffer cells, neutrophils) and intracellular generation of reactive oxygen by mitochondria and xenobiotic-inducible enzymes (e.g., CYP 4502E1) will be discussed.

Bile Acid-Induced Hepatocyte Apoptosis

Bile formation is an essential function of the liver, and failure of bile formation is a pathophysiologic process termed cholestasis. Retention of bile constituents within the hepatocyte during cholestasis is associated with hepatocyte apoptosis (Patel *et al.,* 1998). Although the mechanisms of cholestasis associated with hepatocyte apoptosis are likely complex and multifactorial, hydrophobic bile acids are especially hepatotoxic, and they accumulate in the liver in cholestatic disorders (Rodrigues *et al.,* 1998). The intrinsic hepatotoxicity of these hydrophobic, sterol-derived molecules is apparent in children who have a mutation in the bile salt excretory pump in the canalicular membrane (Strautnieks *et al.,* 1998). The failure to secrete bile acids into bile results in liver injury, cirrhosis, and death from liver failure (Strautnieks *et al.,* 1998). This unfortunate human disease highlights the toxicity of bile acids in humans.

In cultured rat hepatocytes, the hydrophobic bile acid glycochenodeoxycholate, GCDC, at pathophysiologically relevant concentrations (20–100 μM) induces apoptosis, as documented by cell shrinkage, nuclear condensation and lobulation, caspase activation, DNA fragmentation, and phosphatidylserine externalization (Patel *et al.,* 1994). Thus, bile acids provide a valuable model to dissect the mechanisms of liver cell apoptosis and the role of apoptosis in liver injury from endogenous toxicants.

Apoptosis occurs by one of two pathways: (1) a death-receptor pathway, and (2) the mitochondrial pathway (Green, 1998). To determine if death-receptor pathways contribute to bile acid-mediated apoptosis, hepatocytes from tumor necrosis factor-receptor 1 (TNF-R1) and Fas-deficient mice were exposed to GCDC. TNF-R1 and Fas are the predominant death receptors expressed by hepatocytes (Faubion and Gores, 1999). Hepatocytes from Fas-deficient lpr mice were resistant to GCDC-mediated apoptosis, whereas TNF-R1-deficient hepatocytes readily underwent apoptosis. Unexpectedly, hepatocytes from Fas ligand-deficient mice were also sensitive to GCDC-stimulated apoptosis (Faubion *et al.,* 1999). These data implicate ligand-independent Fas-mediated apoptosis as a contributing mechanism for bile acid-related liver injury. To further test this concept, the bile ducts of wild type and Fas-deficient mice were ligated to produce severe extrahepatic cholestasis. Caspase 8, an initiator cysteine-aspartate protease in apoptosis, was activated in wild type animals but not Fas-deficient mice. Bile duct ligated Fas-deficient animals also had less apoptosis, decreased liver injury, and improved survival as compared to wild type mice (Miyoshi *et al.,* 1999). Thus, Fas activation appears to play a dominant role in bile acid cytotoxicity.

How do bile acids cause Fas activation? Potential mechanisms include alterations in Fas synthesis, Fas compartmentation, and Fas trimerization in the plasma membrane. However, toxic bile acids did not increase Fas synthesis. Rather, bile acids promoted rapid transport of cytoplasmic vesicular Fas to the plasma membrane in a microtubule-dependent manner (Sodeman *et al.,* 2000). Bile acid-induced apoptosis was dependent upon this translocation of Fas to the plasma membrane. Whether Fas translocation is sufficient to trigger spontaneous association of Fas receptor death domains is unclear. Nonetheless, toxicant-induced transport of intracellular death receptors to the plasma membrane is a new paradigm for cell death. In summary, bile acids accumulate in the liver when canalicular transport is impaired, which results in translocation of cytoplasmic Fas to the plasma membrane where these receptors self-aggregate and trigger cell death by apoptosis (Fig. 1).

Adhesion Molecules and Oxidant Stress in Inflammatory Liver Injury

Sepsis/endotoxemia, alcoholic hepatitis, ischemia-reperfusion injury, and certain drug-induced liver toxicities are characterized by systemic and local inflammation with recruitment of macrophages and neutrophils into the liver vasculature (Jaeschke and Smith, 1997; Jaeschke *et al.,* 1996; Laskin and Laskin, 2001). The main function of these phagocytes is to destroy invading microorganisms and to remove dead cells and cell debris in preparation for tissue regeneration. Because of the nature of the toxic mediators generated by these phagocytes, healthy cells may also be affected, which can aggravate the original liver injury. Therefore, it is important to understand the mechanisms involved in the activation, recruitment, and cytotoxicity of these phagocytes in the liver.

Previous work during the last 10 years characterized a role for neutrophils in the pathophysiology of inflammatory liver injury, and many aspects that are relevant for neutrophil-mediated cytotoxicity also apply to mononuclear cells. Neutrophils can be recruited into the hepatic vasculature by local tissue injury and CXC chemokine generation (Lawson *et al.,* 2000b; Maher *et al.,* 1997) or the systemic exposure to inflammatory mediators, including tumor necrosis factor-α (TNF-α),

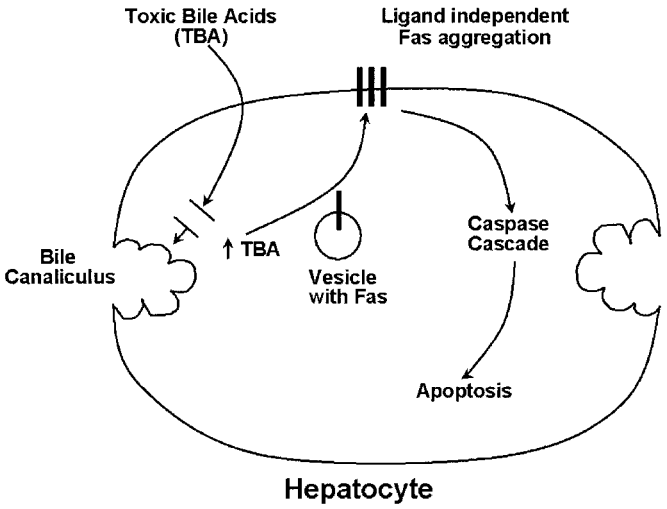

FIG. 1. Bile acid-induced hepatocyte apoptosis. Bile acids are normally secreted rapidly from hepatocytes by transporters located in the canalicular membrane. In cholestasis, secretion is impaired, resulting in elevated concentrations of toxic bile acids (TBA) within hepatocytes. At pathophysiologic concentrations, toxic bile acids trigger translocation of intracellular Fas bearing vesicles to the plasma membrane where they self-aggregate in the absence of ligand. Activated Fas receptor complexes on the plasma membrane then cause caspase 8 activation and an apoptotic cascade.

IL-1, complement factors, platelet activating factor, and CXC chemokines (Jaeschke, 1997). Each of these mediators upregulates β_2 integrins on neutrophils (Jaeschke, 1997). In liver, neutrophils accumulate in sinusoids and adhere to venular endothelial cells (Chosay *et al.*, 1997). In general, recruitment of neutrophils into sinusoids does not depend on cellular adhesion molecules (CAMs; Jaeschke, 1997) but appears to result from mechanical trapping due to rheological changes in neutrophils, active vasoconstriction in sinusoids and swelling of the sinusoidal lining cells (Jaeschke, 1997). However, subsequent steps of firm adhesion to endothelial cells, transmigration and adherence to hepatocytes are dependent on CAMs, including ICAM-1 and VCAM-1 (Essani *et al.*, 1995, 1997). Expression of E-selectin on endothelial cells activates neutrophils during transmigration (Lawson *et al.*, 2000a). Neutrophil adhesion and extravasation in sinusoids do not involve PECAM-1 or P- or L-selectin. In contrast, neutrophil rolling and adhesion in postsinusoidal venules are dependent on P- and L-selectin and ICAM-1, respectively (Jaeschke, 1997; Lawson *et al.*, 2000a). CAMs are differentially expressed and are cytokine-inducible on all liver cell types (Jaeschke, 1997). On leukocytes, members of the β_2-(CD18)-integrin family are critical for neutrophil-mediated injury (Jaeschke *et al.*, 1993). LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) are involved in transmigration and adhesion to hepatocytes (Jaeschke and Smith, 1997). Upregulation of Mac-1 is a prerequisite for neutrophil cytotoxicity (Jaeschke *et al.*, 1993). Adherence of neutrophils to target cells through Mac-1 triggers release of proteases and prolonged reactive oxygen formation. Neutrophils are rarely cytotoxic when present in sinusoids and must transmigrate into the subsinusoidal space to cause tissue injury (Chosay *et al.*, 1997). In order to transmigrate and attack, neutrophils must receive a chemotactic signal. CXC chemokines generated by hepatocytes can trigger a neutrophil-induced injury (Maher *et al.*, 1997). Furthermore, lipid peroxidation products are highly chemotactic (Curzio *et al.*, 1986) and may be responsible for the continuation and amplification of the injury (Liu *et al.*, 1994). Recently, apoptotic cell death of hepatocytes was identified as a potent stimulus for neutrophil extravasation and enhancement of endotoxin-induced injury (Jaeschke *et al.*, 1998; Lawson *et al.*, 1998). In human alcoholic hepatitis, apoptotic hepatocytes colocalize with neutrophils, which correlates strongly with the severity of tissue damage (Ziol *et al.*, 2001). Thus, hepatocyte apoptosis and neutrophil extravasation may be important events in alcoholic liver injury.

Despite the improved understanding of neutrophil-mediated hepatotoxicity, the molecular mechanism of cell death remains controversial (Jaeschke, 2000). *In vitro* studies using neutrophil-hepatocyte cocultures have identified proteases as the critical mediators of cell injury (Jaeschke *et al.*, 1996; Jaeschke and Smith, 1997). In support of this concept, protease inhibitors attenuate neutrophil hepatotoxicity *in vivo* (Jaeschke and Smith, 1997). Recent data also suggest that neutrophil-derived reactive oxygen species can induce an intracellular oxidant stress in hepatocytes that triggers necrotic cell injury in less than 1 h (Jaeschke *et al.*, 1999). Similar results can be obtained with a macrophage-derived oxidant stress in the liver (Bilzer *et al.*, 1999). The mechanism of injury does not involve gross lipid peroxidation (Jaeschke *et al.*, 1999) but may be caused by the opening of the membrane permeability transition pore and the collapse of the mitochondrial membrane potential (Nieminen *et al.*, 1995). In addition to causing cell injury, reactive oxygen species promote inflammation by enhancing the activation of the transcription factor NF-κB, which controls the formation of cytokines, chemokines, and adhesion molecules (Jaeschke, 2000).

In summary, drug toxicity, tissue trauma, ischemia-reperfusion, sepsis, and other pathophysiological events activate both neutrophils and Kupffer cells directly or through activation of complement (Fig. 2). Kupffer cells release cytotoxic mediators, such as reactive oxygen species, and proinflammatory mediators, such as cytokines and chemokines. Complement factors (e.g., C5a) and cytokines prime and activate neutrophils to promote their recruitment into the hepatic vasculature. If chemotactically stimulated, neutrophils extravasate and adhere to parenchymal cells, which induces necrotic cell death through release of reactive oxygen and proteases. Adhesion molecules on neutrophils (β_2 integrins, especially CD11b/CD18) and ICAM-1 on endothelial cells and hepatocytes are essential for neutrophil margination, extravasation, and oxidant production. Cytokines can induce hepatic adhesion molecule and chemokine formation, which in turn is modulated by oxidant stress.

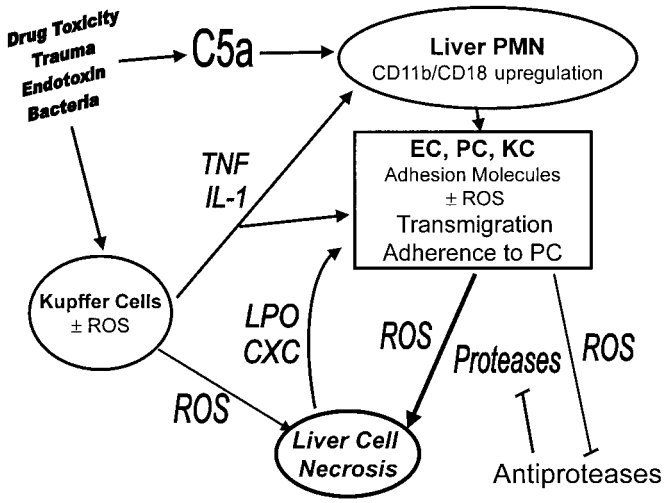

FIG. 2. Mechanisms of neutrophil-induced liver injury. Tissue trauma, endotoxin, and bacteria trigger formation of inflammatory mediators such as complement factors (C5a), cytokines (TNF-α, IL-1) and CXC chemokines. Each of these factors can upregulate expression of β_2 integrin (CD11b/CD18) and prime neutrophils (PMN) for ROS formation. C5a also stimulates Kupffer cells (KC) to release ROS. In addition, cytokines activate expression of adhesion molecules on endothelial cells (EC) and hepatocytes (PC). If primed neutrophils receive a chemotactic signal from the parenchyma, they will transmigrate and adhere to hepatocytes. This leads to the final activation of neutrophil with degranulation (protease release) and adherence-dependent oxidant stress, which causes cell necrosis. Mediators generated during cell injury, such as lipid peroxidation products (LPO) and chemokines, become chemotactic signals for further neutrophil activation and transmigration.

The growing insight into mechanisms of neutrophil cytotoxicity *in vivo* should lead to new therapeutic strategies to prevent neutrophil-induced tissue injury without paralyzing host-defense functions.

CYP2E1-Dependent Toxicity in HepG2 Cells

Cytochrome P4502E1 (CYP2E1), the ethanol-inducible form, metabolizes and activates many toxicologically important substrates, including ethanol, carbon tetrachloride, acetaminophen, and N-nitrosodimethylamine, to more toxic products (Guengerich *et al.*, 1990; Koop, 1992). CYP2E1-dependent ethanol metabolism produces oxidative stress through generation of reactive oxygen species (ROS), a possible mechanism by which ethanol is hepatotoxic (Bondy, 1992; Dianzani, 1985). Induction of cytochrome P4502E1 by ethanol is a central pathway by which ethanol generates oxidative stress, and in the intragastric model of ethanol feeding a prominent induction of CYP2E1 occurs along with significant alcohol liver injury (Morimoto *et al.*, 1994; Nanji *et al.*, 1994). Lipid peroxidation also occurs, and ethanol-induced liver pathology correlates with CYP2E1 levels and elevated lipid peroxidation, which is blocked by inhibitors of CYP2E1.

An approach to understand the effects and actions of CYP2E1 is to express human CYP2E1 in cell lines. CYP2E1 overexpressing HepG2 cell lines were established by retroviral infection methods (E9 cells) and by plasmid transfection methods (E47 cells; Chen and Cederbaum, 1998; Dai *et al.*, 1993). E9 and E47 cells express CYP2E1 at levels of about 10 and 45 pmol/mg microsomal protein, respectively. Compounds actively metabolized by CYP2E1 to reactive intermediates, such as acetaminophen or carbon tetrachloride, were toxic to CYP2E1-overexpressing HepG2 cells but not to control cells, which validates the model for study of CYP2E1-dependent toxicity (Dai and Cederbaum, 1995a,b).

Ethanol, iron, and polyunsaturated fatty acids, such as arachidonic acid (but not monoenoic acids such as oleic acid), were considerably more toxic to CYP2E1-overexpressing E9 cells than MV5 control cells (Chen *et al.*, 1997; Sakurai and Cederbaum, 1998; Wu and Cederbaum, 1996). Toxicity was concentration- and time-dependent and associated with lipid peroxidation. Antioxidants, especially inhibitors of lipid peroxidation, prevented toxicity. The toxicity correlated with CYP2E1 levels and was enhanced after transfection with a sense CYP2E1 plasmid and diminished after transfection with an antisense CYP2E1 plasmid. CYP2E1-dependent cell killing was apoptotic, associated with activation of caspase 3 (a major effector caspase in apoptosis), and blocked by pancaspase inhibition (Chen *et al.*, 1997; Sakurai and Cederbaum, 1998; Wu and Cederbaum, 1999). Bcl-2 is a proto-oncogene that blocks cytochrome *c* release during apoptotic signaling through mitochondria, and transfection with a plasmid containing Bcl-2 prevented the apoptosis, implicating a mitochondrial pathway for apoptosis (Chen *et al.*, 1997). Iron and arachidonic acid decreased mitochondrial membrane potential in the CYP2E1-overexpressing cells and lowered cellular ATP levels. HepG2 cells were also infected with adenoviruses containing catalase cDNA and catalase cDNA directed to mitochondria by the 27-amino acid peptide leader sequence of manganese superoxide dismutase. Both catalase constructs protected CYP2E1-overexpressing E47 cells against iron and arachidonic acid toxicity (Bai and Cederbaum, 2001).

Glutathione (GSH) is a critical cellular antioxidant. After GSH depletion with buthionine sulfoximine (BSO), the toxicity of ethanol, iron, arachidonic acid, and acetaminophen was strikingly enhanced (Chen and Cederbaum, 1998; Chen *et al.*, 1997; Sakurai and Cederbaum, 1998; Wu and Cederbaum, 1996, 1999). BSO treatment of CYP2E1-overexpressing E47 cells caused toxicity even in the absence of an added toxicant (Chen and Cederbaum, 1998). CYP2E1 inhibitors and antioxidants prevented cell killing, which was partly apoptotic and partly necrotic. Surprisingly, GSH in E47 cells was increased compared to C34, E9, and MV control cells. Increased GSH represented increased GSH synthesis due to transcriptional activation of the gamma-glutamyl cysteinyl synthase gene and was blocked by antioxidants (Mari and Cederbaum, 2000). Activity, protein, and mRNA levels for other antioxidant enzymes, such as catalase, α- and microsomal glutathione transferases, were also increased in E47 cells (Mari and Cederbaum,

2001). Upregulation of these antioxidant genes may reflect an adaptive mechanism to detoxify CYP2E1-derived oxidants.

Hepatic stellate cells are central to the fibrotic response of the liver to injury, and ROS activate stellate cells (Friedman, 2000). Since CYP2E1 produces ROS, ethanol-induced CYP2E1 expression may promote collagen type I biosynthesis by stellate cells. However, CYP2E1 is mostly present in hepatocytes, whereas stellate cells contain low levels of CYP2E1. Accordingly, a coculture model involving HepG2 cells and stellate cells was developed (Mari *et al.,* 2001, Nieto *et al.,* manuscript in preparation). A time-dependent increase in collagen type I was observed when stellate cells were coincubated with C34 control cells, which was further elevated when stellate cells were coincubated with CYP2E1-overexpressing E47 cells. However, little type I collagen was released into the incubation medium from the C34 plus stellate cell coculture. By contrast, E47 plus stellate cell cocultures secreted much more type I collagen protein. These experiments suggest that CYP2E1-overexpressing E47 cells generate diffusible mediators that promote type I collagen synthesis and release by stellate cells. Catalase and vitamin E markedly decreased type I collagen synthesis by both cocultures and completely blocked the increased collagen production by the E47 coculture. These results suggest that E47 cells release ROS, such as H_2O_2 and lipid peroxidation products, that stimulate type I collagen synthesis by stellate cells.

HepG2 cells expressing CYP2E1 have proven to be a valuable model to characterize the biochemical and toxicological properties of CYP2E1. Induction of CYP2E1 by ethanol appears to be one of the central pathways by which ethanol generates a state of oxidative stress. Figure 3 depicts a working hypothesis of the role of CYP2E1 in ethanol-induced oxidative stress and hepatotoxicity. While several mechanisms likely contribute to alcohol-induced liver injury, the linkage between CYP2E1-dependent oxidative stress, mitochondrial injury, and increased collagen formation by stellate cells may make an important mechanistic contribution to the toxic action of ethanol on the liver.

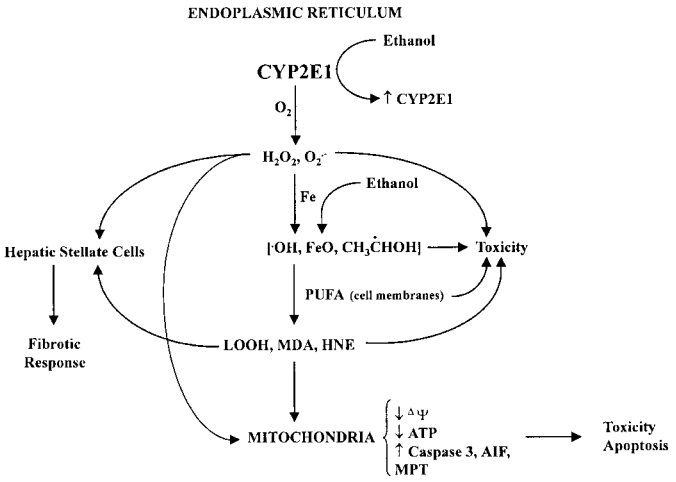

FIG. 3. Role of cytochrome P4502E1 in oxidative stress after ethanol. Ethanol increases levels of CYP2E1, largely by a posttranscriptional mechanism involving stabilization against degradation. CYP2E1, a loosely coupled enzyme, generates reactive oxygen species such as superoxide radical and hydrogen peroxide during its catalytic cycle. In the presence of iron, which is increased after ethanol treatment, more powerful oxidants including hydroxyl radical, ferryl species, and 1-hydroxyethyl radical are produced. These various oxidants can promote toxicity by protein oxidation and enzyme inactivation and by damage to cell membranes via lipid peroxidation and production of reactive lipid aldehydes, such as malondialdehyde and 4-hydroxynonenal. Mitochondria appear to be among the critical cellular organelles damaged by CYP2E1-derived oxidants. A decrease of mitochondrial membrane potential and perhaps the mitochondrial membrane permeability transition causes release of proapoptotic factors resulting in apoptosis. Some CYP2E1-derived reactive oxygen species, e.g., H_2O_2, LOOH, MDA, HNE, are diffusible and may exit hepatocytes and enter other liver cell types, such as stellate cells, and stimulate these cells to produce collagen and elicit a fibrotic response.

Peroxynitrite in Drug-Induced Hepatotoxicity

In overdose, the analgesic/antipyretic acetaminophen produces centrilobular hepatic necrosis (Mitchell *et al.,* 1973a). Cytochrome P450 metabolism to N-acetyl-*p*-benzoquinone imine (NAPQI) is a critical step. NAPQI reacts with hepatic glutathione (GSH) leading to its depletion by as much as 90% (Mitchell *et al.,* 1973b). Additionally NAPQI covalently binds to proteins as acetaminophen-cysteine adducts (Cohen *et al.,* 1997). Immunochemical studies indicate that the cellular site of covalent binding correlates with the toxicity (Hart *et al.,* 1995; Roberts *et al.,* 1991).

Recent work shows that nitrated tyrosine occurs in hepatic centrilobular cells. These adducts colocalize in cells containing the acetaminophen-protein adducts (Hinson *et al.,* 2000, 1998). Peroxynitrite, a highly reactive nitrating and oxidizing species formed by the rapid reaction of nitric oxide (NO) and superoxide, produces nitrated tyrosine (Beckman, 1996; Pryor and Squadrito, 1995). Since acetaminophen-protein adducts correlate with development of necrosis (Hart *et al.,* 1995; Roberts *et al.,* 1991), it follows that nitration of tyrosine correlates with necrosis.

Recent evidence suggests that activated Kupffer cells are mechanistically important in NO and superoxide formation. Pretreatment of rats and mice with macrophage inactivators (gadolinium chloride, dextran sulfate, LPS, or dichloromethylene diphosphonate) dramatically decreased acetaminophen toxicity (Blazka *et al.,* 1995; Goldin *et al.,* 1996; Laskin *et al.,* 1995; Laskin and Pendino, 1995; Michael *et al.,* 1999; Winwood and Arthur, 1993). Neither gadolinium chloride nor dextran sulfate decreased acetaminophen protein binding, but both decreased nitration of tyrosine (Michael *et al.,* 1999). However, other cellular sources of NO and superoxide may be important. Hepatocytes and stellate cells express inducible nitric oxide synthase (iNOS; Muriel, 2000), and acetaminophen induces iNOS in rat hepatocytes (Gardner *et al.,* 1998). Endothelial cells constitutively express eNOS (Muriel, 2000).

Various sources produce superoxide, including damaged mitochondria (Knight *et al.,* 2001).

The importance of iNOS in acetaminophen toxicity was investigated by utilizing iNOS knockout mice (Michael *et al.,* 2001). Although serum ALT levels (a biomarker of liver toxicity) was less in iNOS knockout mice than in wild type mice after acetaminophen treatment, histology showed no significant differences in hepatotoxicity. Acetaminophen induced an approximate 5-fold induction of NO synthesis (serum nitrate plus nitrite) in wild type mice, and the increase in serum nitrate plus nitrite paralleled increases in serum ALT. Increased NO synthesis was not observed in iNOS knockout mice, although a small increase in nitrotyrosine residues was observed. Nitrotyrosine in the knockout mice was in centrilobular areas, which suggested involvement of constitutively expressed NOS. Consistent with previously reported data, acetaminophen did not increase lipid peroxidation in wild type mice (Kamiyama *et al.,* 1993). By contrast, hepatic lipid peroxidation (malondialdehyde) increased in iNOS knockout mice (Michael *et al.,* 2001).

It is hypothesized that the initial step in acetaminophen toxicity is metabolism to NAPQI, leading to depletion of GSH and covalent adduct formation, as previously proposed. In wild type mice, induction of NO synthesis and superoxide generation occurs subsequently, leading to peroxynitrite formation. Ordinarily, GSH detoxifies peroxynitrite (Sies *et al.,* 1997). However, after GSH depletion by NAPQI, peroxynitrite nitrates protein tyrosine and may oxidize other macromolecules. *In vitro* acetaminophen competes with tyrosine for reaction with peroxynitrite, but *in vivo* peroxynitrite reacts rapidly with protein tyrosine in wild type mice. In iNOS knockout mice, superoxide increases after acetaminophen but not NO synthesis. Superoxide then causes lipid peroxidation. Thus acetaminophen toxicity may be mediated by nitration in wild type mice and by lipid peroxidation in iNOS knockout mice (Fig. 4). Indeed, by reacting with superoxide NO may prevent lipid peroxidation in wild type mice (Rubbo *et al.,* 1994).

These data indicate the importance of peroxynitrite as a mediator of hepatotoxicity and suggest that nitric oxide is important in controlling superoxide levels. Depending on GSH status, nitric oxide may induce a toxification or detoxification mechanism. With hepatotoxins like acetaminophen, bromobenzene, chloroform, and allyl alcohol that deplete hepatic GSH, peroxynitrite formation promotes toxicity. However with hepatotoxins that cause lipid peroxidation but do not deplete GSH, such as carbon tetrachloride, NO may scavenge superoxide by forming peroxynitrite, which is then detoxified by GSH.

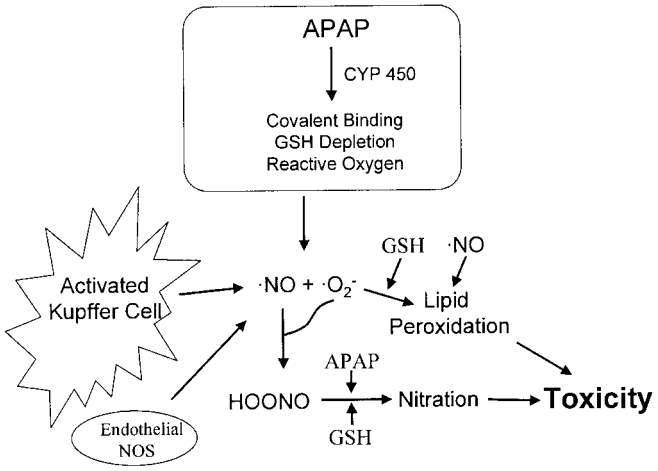

FIG. 4. Postulated mechanism of acetaminophen (APAP)-induced hepatotoxicity. Hepatocytes, Kupffer cells, and endothelial cells all participate in the production of reactive nitrogen and oxygen species. The relative levels of nitric oxide (NO) and superoxide (O_2-) determine whether the mechanism of hepatic necrosis is dependent on protein nitrosylation or lipid peroxidation. GSH, glutathione; HOONO, peroxynitrite; NOS, nitric oxide synthase; CYP 450, cytochrome P450.

Hepatotoxicity Due to Mitochondrial Dysfunction

Microvesicular steatosis. Primary and secondary mitochondrial dysfunction is an important mechanism of drug-induced microvesicular steatosis, nonalcoholic steatohepatitis (NASH), and cytolytic hepatitis (Fromenty and Pessayre, 1995). Severe impairment of mitochondrial fatty acid β-oxidation causes microvesicular steatosis, characterized by accumulation of tiny lipid vesicles in the cytoplasm of hepatocytes (Fromenty and Pessayre, 1995). Because of poor mitochondrial oxidation, nonesterified fatty acids (NEFAs) accumulate in the liver and become esterified into triglycerides. Hepatic triglycerides, perhaps emulsified by a rim of amphiphilic NEFAs, amass as small lipid vesicles (Fromenty and Pessayre, 1995). The sudden onset or aggravation of mitochondrial dysfunction leaves no time for the progressive coalescence of tiny lipid droplets into the large fat inclusions of macrovacuolar steatosis.

Microvesicular steatosis is the histological hallmark of severe metabolic perturbations causing energy shortage. Inhibition of β-oxidation itself deprives cells of their most important source of energy during fasting. Furthermore, NEFAs and their dicarboxylic acid metabolites directly impair mitochondrial energy production (Fromenty and Pessayre, 1995). Finally, disruption of hepatic mitochondrial β-oxidation decreases delivery of hepatic ketone bodies and glucose to peripheral tissues (Fromenty and Pessayre, 1995). The resulting deficiency of energy substrates may cause renal failure, pancreatitis, coma, and death (Fromenty and Pessayre, 1995).

Damage to mitochondrial DNA (mtDNA) and direct inhibition of mitochondrial respiration also inhibit β-oxidation (Fromenty and Pessayre, 1995). β-Oxidation consumes NAD^+ and transforms it into NADH. Mitochondrial respiration reoxidizes NADH into the NAD^+ that is required for β-oxidation. Therefore, impairment of respiration inhibits β-oxidation. Thus, various endogenous and exogenous substances impair mitochondrial β-oxidation to cause microvesicular steatosis through

different mechanisms. Oxidative stress after ethanol causes damage to mitochondrial proteins, lipids, and DNA. mtDNA depletion occurs in ethanol-treated mice (Mansouri *et al.,* 1999). In humans, these oxidative lesions cause mtDNA deletions (Mansouri *et al.,* 1997). Interferon-α and nucleoside analogs (dideoxynucleosides, fialuridine) impair mtDNA transcription and replication, respectively (Lewis and Dalakas, 1995; Shan *et al.,* 1990). DNA polymerase γ incorporates nucleoside reverse transcriptase inhibitors into mtDNA, an event that blocks mtDNA replication, eventually causing mtDNA depletion.

Salicylic acid and valproic acid sequester CoA, which is needed to form thio esters with fatty acids (Deschamps *et al.,* 1991; Kesterson *et al.,* 1984). 2,4-Diene-valproyl-CoA, a reactive metabolite of valproic acid, may also inactivate β-oxidation enzymes (Kassahun and Abbott, 1993). Several drugs inhibit β-oxidation, including tetracycline derivatives (Labbe *et al.,* 1991), glucocorticoids (Lettéron *et al.,* 1997), the nonsteroidal antiinflammatory drugs ibuprofen and pirprofen (Fréneaux *et al.,* 1990; Genève *et al.,* 1987), the antidepressant drugs amineptine and tianeptine (Fromenty *et al.,* 1989; Le Dinh *et al.,* 1988), the antianginal cationic amphiphilic drugs amiodarone, perhexiline, and diethylaminoethoxyhexestrol (Berson *et al.,* 1998; Deschamps *et al.,* 1994; Fromenty *et al.,* 1990), as well as female sex hormones or pregnancy (Grimbert *et al.,* 1993).

These metabolic effects may combine to block mitochondrial β-oxidation and trigger microvesicular steatosis. For example, Reye's syndrome occurs after a viral infection in children taking aspirin and/or having a latent inborn defect in β-oxidation enzymes (Fromenty and Pessayre, 1995). Likewise, acute fatty liver of pregnancy is more frequent in women whose fetus has a genetic deficiency in long-chain 3-hydroxyacyl-CoA dehydrogenase (Ibdah *et al.,* 1999).

Nonalcoholic steatohepatitis (NASH). NASH develops progressively in patients with chronic, macrovacuolar, or microvesicular steatosis, leading to liver cell death, Mallory bodies, polynuclear cell infiltrates, fibrosis, and cirrhosis (Pessayre *et al.,* 2001). NASH occurs in patients with the obesity/hypertriglyceridemia/insulin resistance syndrome, or can be induced by chronic amiodarone, perhexiline, or diethylaminoethoxyhexestrol administration (Pessayre *et al.,* 2001). These cationic amphiphilic drugs concentrate electrophoretically into mitochondria to inhibit β-oxidation and respiration (Berson *et al.,* 1998). Respiratory inhibition leads to ROS formation by mitochondria to cause lipid peroxidation of fat deposits (Berson *et al.,* 1998). Similarly in alcohol abuse, increased ROS formation causes lipid peroxidation and steatohepatitis (Pessayre *et al.,* 2001). Both lipid peroxidation and ROS-induced cytokine release (TGF-β, TNF-α, IL-8) may contribute to the development of NASH (Pessayre *et al.,* 2001).

Cytolytic hepatitis. Cytolytic hepatitis is a severe liver lesion that can cause liver failure and may involve mitochondrial uncoupling or respiratory inhibition (Berson *et al.,* 1996, 2001). Another mechanism is onset of the mitochondrial permeability transition (MPT) caused by opening of permeability transition (PT) pores in the mitochondrial inner membrane. PT pore opening causes mitochondrial depolarization, uncoupling, and large amplitude swelling and can lead to both necrotic and apoptotic cell death (Lemasters *et al.,* 1998; Pessayre *et al.,* 1999). PT pore opening in all mitochondria of a cell causes ATP depletion, which prevents apoptosis (an energy-requiring process) and causes necrosis (Lemasters *et al.,* 1999; Pessayre *et al.,* 1999). In contrast, PT pore opening in only some mitochondria permits ATP synthesis by the unaffected mitochondria, thus preventing necrosis. In mitochondria undergoing the MPT, however, matrix swelling and outer membrane rupture causes release of mitochondrial cytochrome *c*, which activates caspases in the cytosol to cause apoptosis (Bradham *et al.,* 1998; Feldmann *et al.,* 2000; Hatano *et al.,* 2000).

Drugs cause the MPT through diverse mechanisms. Some compounds, such as ROS (Nieminen *et al.,* 1995), thio crosslinkers, bile acids (Botla *et al.,* 1995), atractyloside (Halestrap and Davidson, 1990), betulinic acid (Fulda *et al.,* 1998), and lonidamide (Ravagnan *et al.,* 1999), may directly induce PT pore opening, whereas other drugs, such as salicylic acid and valproic acid, may facilitate PT pore opening by calcium (Lemasters *et al.,* 1998). Other drugs, such as anticancer drugs, cause Fas ligand expression in hepatocytes to initiate Fas- and MPT-dependent fratricidal killing (Müller *et al.,* 1997).

The most frequent mechanism of cytolytic hepatitis is cytochrome P450-dependent formation of reactive metabolites that cause direct toxicity or immune reactions. Reactive metabolites may cause DNA damage and overexpression of p53 and Bax, as well as glutathione depletion, protein thiol oxidation, and increased cytosolic Ca^{2+} (Haouzi *et al.,* 2000). Bax overexpression, disulfide formation, and increased mitochondrial Ca^{2+} all promote MPT and cell death (Haouzi *et al.,* 2000). Covalent binding of reactive metabolites to hepatic proteins can also trigger an immune response. Cytotoxic T lymphocytes kill their targets by 3 mechanisms: cell surface Fas ligand expression, formation of TNF-α, and release of granzyme B (Pessayre *et al.,* 1999). All 3 events trigger the MPT to cause death of target cells (Bradham *et al.,* 1998; Feldmann *et al.,* 2000; Hatano *et al.,* 2000; Pessayre *et al.,* 1999).

CONCLUSION

In summary, several mechanisms initiate liver cell damage and aggravate ongoing injury processes. Mitochondria are prominent targets for the hepatotoxicity of many drugs. Dysfunction of these vital cell organelles results in impairment of energy metabolism and an intracellular oxidant stress with excessive formation of reactive oxygen species and peroxynitrite. In addition to mitochondria, induction of cytochrome P450 isoenzymes such as CYP2E1 also promote oxidant stress

and cell injury. Once hepatocellular function is impaired, accumulation of bile acids causes additional stress and cytotoxicity. Cell injury, gut-derived endotoxin or a combination of both also activate Kupffer cells and recruit neutrophils into the liver. Although responsible for removal of cell debris and part of the host-defense system, under certain circumstances these inflammatory cells initiate additional liver injury. However, cell injury and death is not only determined by the nature and dose of a particular drug but also by factors such as an individual's gene expression profile, antioxidant status, and capacity for regeneration. Because of the many direct and indirect mechanisms of drug-induced cell injury in the liver, hepatotoxicity remains a major reason for drug withdrawal from pharmaceutical development and clinical use.

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