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Mechanisms of Acetaminophen-Induced Liver Necrosis

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Abstract

Although considered safe at therapeutic doses, at higher doses, acetaminophen produces a centrilobular hepatic necrosis that can be fatal. Acetaminophen poisoning accounts for approximately one-half of all cases of acute liver failure in the United States and Great Britain today. The mechanism occurs by a complex sequence of events. These events include: (1) CYP metabolism to a reactive metabolite which depletes glutathione and covalently binds to proteins; (2) loss of glutathione with an increased formation of reactive oxygen and nitrogen species in hepatocytes undergoing necrotic changes; (3) increased oxidative stress, associated with alterations in calcium homeostasis and initiation of signal transduction responses, causing mitochondrial permeability transition; (4) mitochondrial permeability transition occurring with additional oxidative stress, loss of mitochondrial membrane potential, and loss of the ability of the mitochondria to synthesize ATP; and (5) loss of ATP which leads to necrosis. Associated with these essential events there appear to be a number of inflammatory mediators such as certain cytokines and chemokines that can modify the toxicity. Some have been shown to alter oxidative stress, but the relationship of these modulators to other critical mechanistic events has not been well delineated. In addition, existing data support the involvement of cytokines, chemokines, and growth factors in the initiation of regenerative processes leading to the reestablishment of hepatic structure and function.

Keywords

Acetaminophen; Liver; Glutathione; Covalent binding; Mitochondria; Oxidative stress; JNK

1 Introduction

Acetaminophen (paracetamol, N-acetyl-*p*-aminophenol; APAP) is a widely used over-the-counter analgesic and antipyretic drug (Bessems and Vermeulen 2001; James et al. 2003b; Prescott and Critchley 1983). At therapeutic doses, it is believed to be safe, having analgesic and antipyretic effects similar to those of aspirin and ibuprofen. Unlike these other drugs, acetaminophen has only weak antiinflammatory properties.

Acetaminophen was originally introduced as an analgesic by von Mering in 1893, but was not widely used until the 1960s, following the recognition that the structural analog phenacetin was nephrotoxic in chronic abusers (Hinson 1980). More recently, concern about aspirin-mediated gastrointestinal bleeding and Rye's syndrome has further increased its popularity. According to the US Food and Drug Administration, each week approximately 50 million adults in the United States take acetaminophen-containing products.

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Although considered safe at therapeutic doses, at higher doses, acetaminophen produces a centrilobular hepatic necrosis that can be fatal. Acetaminophen poisoning accounts for approximately one-half of all cases of acute liver failure in the United States and Great Britain today (Larson et al. 2005; Ostapowicz et al. 2002) Annually, it accounts for a very high percentage of inquiries to poison control centers and deaths (Litovitz et al. 2002). The direct costs of acetaminophen overdose have been estimated to be as high as US$87 million annually (Bond and Novak 1995).

2 Acetaminophen-Induced Hepatotoxicity

Davidson and Eastham were the first to report that acetaminophen was hepatotoxic in overdose (Davidson and Eastham 1966). They described two individuals who developed hepatotoxicity following acetaminophen overdose and died on the third day following the overdose. Microscopic examination of liver sections from these individuals indicated fulminating hepatic necrosis. The necrosis was primarily in the centrilobular areas. Eosinophilic degeneration of the cells together with pyknosis of nuclear material was observed in these hepatocytes. Vacuolization and early degenerative changes were observed in the more peripheral cells surrounding the portal areas. A mild polymorphonuclear leukocytic infiltration occurred in both cases. These changes indicated fulminating necrosis confined primarily to the hepatocytes in the centrilobular regions of the liver. Necrosis in the cells of the proximal tubules of the kidney was observed in one of the patients.

Subsequent to this initial report, many cases of acetaminophen overdose were reported. Boyer and Rouff described the principal clinical symptoms as development of nausea and vomiting within 2–3 h of ingestion followed by abdominal pain in the right upper quadrant. Liver dysfunction occurred within 24 h and reached amaximum approximately 3–4 days after ingestion (Boyer and Rouff 1971). Prescott et al. summarized the clinical and biochemical changes to be a dramatic increase in serum alanine aminotransferase (ALT) and asparatate aminotransferase (AST) levels, mild hyperbilirubinemia, and increased prothrombin time (Dixon et al. 1971). In conjunction with these clinical symptoms, the elimination half-life of acetaminophen may be delayed (Schiodt et al. 2002). Also, some patients may develop nephrotoxicity in addition to hepatotoxicity (Boyer and Rouff 1971; Prescott et al. 1971).

Hepatotoxicity of acetaminophen was also observed in rodents treated with large doses of acetaminophen (Boyd and Bereczky 1966; Mitchell et al. 1973a). Whereas the rats were not very sensitive to the hepatotoxicity, both mice and hamsters proved to be very sensitive (Davis et al. 1974). Histologically the toxicity was characterized by glycogen loss and vacuolization of centrilobular hepatocytes by 2 h, resulting in a clear demarcation of the centrilobular areas from the rest of the liver. By 3 h, nuclear changes were observed in centrilobular hepatocytes and single cell necrosis with pycnotic cells. By 6 h, gross necrosis of the entire centrilobular areas were observed (Mitchell et al. 1973a). Similar changes were subsequently reported by Walker and coworkers (Walker et al. 1980). These investigators also reported the changes observed by electron microscopic analysis. In addition, they reported that toxicity was associated with hepatic congestion.

The role of apoptosis in acetaminophen liver injury is controversial. Ray and coworkers reported that following a toxic dose of acetaminophen to mice as many as 40% of the dead hepatocytes were apoptotic and 60% necrotic (Ray et al. 1996). However, in subsequent research, Gujral and coworkers (Gujral et al. 2002) used standard morphological criteria of apoptosis (cell shrinkage, chromatin condensation and margination, and apoptotic body formation) and were unable to confirm that acetaminophen caused apoptosis in livers of mice treated with toxic doses. They found that massive necrosis occurred in livers of mice treated with a toxic dose of acetaminophen (3–24 h). The number of cells meeting the morphological

criteria for apoptosis was less than 1% of all parenchymal cells. Moreover, levels of caspase 3, an effector of apoptosis, were not increased in the livers of the acetaminophen-treated mice. These data support the postulation that acetaminophen toxicity in mice occurs almost exclusively by necrosis. This review will focus on acetaminophen-induced necrosis since this is the primary toxicity in humans. However, intracellular signaling and regulatory mechanisms support the concept that there is a close relationship between apoptosis and necrosis, and alterations in mitochondria may be the key to understanding these differences (Kon et al. 2004, 2007; Malhi et al. 2006).

3 Metabolism in Acetaminophen Toxicity

In a series of four publications, Mitchell, Jollow, Potter, Gillette, and Brodie examined the role of metabolism in the hepatotoxicity in mice (Jollow et al. 1973; Mitchell et al. 1973a, b; Potter et al. 1973). Their research showed that acetaminophen was converted by drug metabolizing enzymes to a reactive metabolite that covalently bound to proteins. At nontoxic doses, the metabolite was efficiently detoxified by glutathione forming an acetaminophen-glutathione conjugate (Jollow et al. 1974). However, at toxic doses, the metabolite depleted hepatic glutathione by as much as 80–90% (Jollow et al. 1974; Mitchell et al. 1973a, b) and subsequently covalently bound to protein. The amount of covalent binding correlated with the relative hepatotoxicity (Jollow et al. 1973). Since diethylmaleate depleted hepatic glutathione without causing toxicity, it was postulated that glutathione depletion per se was not the mechanism of toxicity (Mitchell et al. 1973a, b).

Subsequently, the reactive metabolite of acetaminophen was identified to be N-acetyl-*p*-benzoquinone imine (NAPQI). It was found to be formed by cytochrome P-450 (CYP) by a direct two electron oxidation of acetaminophen, a previously unrecognized mechanism of CYP (Dahlin et al. 1984; Gillette et al. 1981; Potter and Hinson 1987). The CYP isoforms important in acetaminophen metabolism have been shown to be CYP2E1, CYP1A2, CYP3A4, and CYP2D6 (Dong et al. 2000; Raucy et al. 1989; Snawder et al. 1994; Thummel et al. 1993). Reaction of NAPQI with glutathione occurs by conjugation to form 3-glutathion-*S*-yl-acetaminophen and by reduction to acetaminophen (Dahlin and Nelson 1982; Hinson et al. 1982). The second order rate constant for the reaction of NAPQI with glutathione was found to be ($3.2 \times 10^4\ M^{-1}\ s^{-1}$). Moreover, the reaction could be catalyzed by glutathione transferase pi, and NAPQI is one of the best substrates ever described for this enzyme (Coles et al. 1988). Thus, detoxification of NAPQI is extremely rapid, and the rapid rate may explain why covalent binding to proteins was not observed in hepatocytes until glutathione was almost completed depleted (Mitchell et al. 1973a, b).

In initial work describing the importance of hepatic glutathione in acetaminophen-induced hepatotoxicity in mice, Mitchell et al. (1973a, b) showed that administration of cysteine prevented hepatotoxicity. This finding led to the development of N-acetyl-cysteine (available as Mucomyst®) as the preferred antidote (Peterson and Rumack 1977; Piperno and Berssenbruegge 1976; Prescott et al. 1977). Rumack and coworkers (Rumack et al. 1981; Rumack and Peterson 1978) analyzed toxicity data from a large number of acetaminophen overdose patients treated with N-acetylcysteine. Treatment of acetaminophen-poisoned patients by 10 h after the overdose was effective at decreasing the toxicity (Prescott et al. 1977; Rumack et al. 1981). Patients treated after 10 h of the overdose had a fourfold elevation in ALT values. The plot of serum acetaminophen levels versus time lapsed since overdose, and clinical outcome, led to the development of a treatment nomogram. This nomogram has been very effective at predicting those individuals who may be most susceptible to development of toxicity and are candidates for N-acetylcysteine treatment (Rumack et al. 1981; Smilkstein et al. 1988). The mechanism by which N-acetylcysteine inhibits acetaminophen toxicity has been

postulated to be increased detoxification of NAPQI by a direct conjugation or through increased glutathione synthesis (Corcoran et al. 1985).

Covalent binding of acetaminophen to protein was found to correlate with acetaminophen-induced hepatotoxicity (Jollow et al. 1973). Covalent binding was ascertained utilizing radiolabeled drug. Subsequently, immunochemical approaches were developed by Roberts et al. (1987) and Bartolone et al. (1987) for analysis of acetaminophen covalently bound to cysteine groups in proteins. Western blot analyzes of liver proteins from mice treated with toxic doses of acetaminophen indicated that a limited number of proteins contained acetaminophen adducts (Bartolone et al. 1989; Pumford et al. 1990). Competitive ELISA indicated that maximum levels of adducts occurred in liver at 1–2 h with subsequent lysis of hepatocytes. Acetaminophen-protein adducts were observed in the serum and immunoblot assays indicating that these adducts were of hepatic origin (Pumford et al. 1990). The appearance of acetaminophen-protein adducts in serum correlated with increases of ALT and AST in serum (Pumford et al. 1989). These data indicated that the presence of acetaminophen-protein adducts in serum was a biomarker for the formation of hepatic acetaminophen-protein adducts and acetaminophen toxicity.

Immunohistochemical analysis of liver sections from treated mice revealed a high correlation between the presence of acetaminophen-protein adducts and toxicity (Cohen and Khairallah 1997; Roberts et al. 1991). Figure 1 is a time course for formation of the acetaminophen protein adducts and development of toxicity in individual murine hepatocytes (Roberts et al. 1991). In this assay, the adducts are stained red. Adducts are visible in the liver sections within 15 min of dosing. By 1 h, staining intensity is maximal and adducts are confined to the centrilobular hepatocytes. Adducts do not occur in the periportal hepatocytes. At 2–6 h, hepatocytes containing adducts are undergoing necrotic changes as evidenced by vacuolization and pycnotic changes in the nuclei. Note that the only hepatocytes observed to develop necrosis were those containing acetaminophen-protein adducts. By 24 h, all the necrotic cells contained adducts. The majority of hepatocytes that had acetaminophen-protein adducts were reported to develop necrosis (Roberts et al. 1991). The covalent binding data suggest that the primary determinant leading to toxicity is metabolism, and that evidence for progression of toxicity subsequent to metabolism as has been suggested by other investigators (Limaye et al. 2003; Liu et al. 2004; Liu et al. 2006) was not observed in these studies (Roberts et al. 1991).

These data supported the hypothesis that acetaminophen-induced liver toxicity is mediated by covalent binding to critical proteins. In an attempt to further understand the mechanism of hepatotoxicity of acetaminophen, specific proteins to which acetaminophen was covalently bound were isolated and sequenced by our laboratory and by Cohen's laboratory (Cohen et al. 1997). The proteins that were identified by this approach were: glutamine synthase, glutamate dehydrogenase, aldehyde dehydrogenase, selenium (acetaminophen) binding protein, and N-10 formyltetrahydrofolate dehydrogenase. A toxic dose of acetaminophen to mice decreased the catalytic activity of the hepatic enzymes glutamate dehydrogenase and N-10 formyltetrahydrofolate dehydrogenase by approximately 25%. Subsequently, proteomic analyzes using mass spectral methods identified a number of additional proteins: glutathione peroxidase, thioether S-methyltransferase, aryl sulfotransferase, pyrophosphatase, topomyosin 5, proteasome subunit C8, methionine adenosyl transferase, protein synthesis initiation factor 4A, ATP synthase α subunit, carbonic anyhydrase III, urate oxidase, 2,4-dienyl Co-A reductase, osteo-blast specific factor 3, glutathione transferase π, sorbitol dehydrogenase, glycine N-methyltransferase, and 3-hydroxyanthranilate 3,4-dioxygenase (Qiu et al. 1998). Unfortunately, the percent covalent binding to the proteins and the possible effect on enzyme activities are not known. The role of these adducts in the development of acetaminophen-induced liver toxicity is unclear.

Clinical data also support the association of covalent binding and toxicity. Early studies utilized immunoassays to detect adducts in the blood samples of patients with acetaminophen overdose (Hinson et al. 1990). The highest levels of adducts were found in the patients with the most severe toxicity. The recent development of a highly sensitive and specific HPLC-EC assay for detection of acetaminophen protein adducts (3-cysteine-acetaminophen in proteins) has allowed for further study of adducts in various clinical settings. Using this assay, it has been shown that adduct levels in serum correlate with hepatic transaminase values in adults with acetaminophen-related liver failure (Muldrew et al. 2002). In addition, the assay may have value in the diagnostic examination of patients with acute liver failure of unknown etiology (Davern et al. 2006). Approximately 20% of patient serum samples from adults with acute liver failure of unknown etiology were positive for adducts, strongly suggesting that acetaminophen was the etiology of the liver failure. These patients had been previously tested for other known causes of acute liver failure. In addition, adducts were recently shown to persist in serum for at least 12 days after severe acetaminophen overdoses in adults (James et al. 2009).

Even though there is an excellent correlation between covalent binding of acetaminophen to protein and development of hepatotoxicity, there is significant evidence that suggest that covalent binding per se is not the mechanism of toxicity. Henderson and coworkers (Henderson et al. 2000) examined acetaminophen-induced glutathione depletion and hepatotoxicity in mice glutathione S-transferase Pi knockout mice, the transferase that catalyzes the conjugation of NAPQI with GSH (Coles et al. 1988). Following a toxic dose of acetaminophen, hepatic glutathione was depleted by greater than 90% in the wild-type mice but only by approximately 70% in the knockout mice. Unexpectedly, the knockout mice were much less sensitive to acetaminophen-induced hepatotoxicity than the wild-type mice; however, both groups of mice had similar levels of covalent binding. These data appear to separate covalent binding of acetaminophen to protein from development of the toxicity and are consistent with a hypothesis that 90% glutathione depletion in hepatocytes is critically necessary for the development of necrosis.

Additional data obtained using hepatocyte suspension assays suggest that covalent binding per se is not the mechanism of toxicity. Boobis and coworkers (Boobis et al. 1986; Tee et al. 1986) found that acetaminophen toxicity in freshly isolated hamster hepatocytes occurred in two phases. In these experiments, incubation of the hepatocytes with acetaminophen (2.5 mM) for 90 min resulted in glutathione depletion and covalent binding, but no toxicity. Subsequent washing of the hepatocytes to remove acetaminophen and reincubation of the hepatocytes with media alone resulted in significant toxicity in the reincubation phase. Addition of N-acetylcysteine or dithiothreitol to the reincubation media protected the hepatocytes against development of toxicity. Acetaminophen toxicity in mouse hepatocytes was similarly found to occur by a two phase mechanism (Grewal and Racz 1993; Rafeiro et al. 1994; Reid et al. 2005). These data do not rule out a role for covalent binding in toxicity but suggest that mechanism(s) downstream from GSH depletion such as oxidative stress play a role in development of toxicity.

4 Alterations in Hepatic Blood Flow in Acetaminophen Toxicity

Acetaminophen-induced hepatotoxicity has been reported to occur with hepatic congestion in humans (Rose 1969; Thompson et al. 1972) and rodents (Dixon et al. 1971; Walker et al. 1980, 1985). In mice, it occurs early and before the appearance of necrosis. Morphological studies in mice by Walker and coworkers revealed that the congestion results from the accumulation of red blood cells within endocytic vacuoles and the Space of Disse with a collapse of the sinusoidal lumens (Walker et al. 1980, 1983). They examined changes in liver weight relative to blood flow and toxicity in a time course study design. In their studies, they found that at 1.5 h after a very toxic dose of acetaminophen to mice there was a significant

increase in liver weight. The liver weight continued to increase, and at 6 h it was approximately twofold over baseline levels and subsequently decreased by 24 h. Associated with the increase in liver weight was a very large increase in liver hemoglobin (approximately fourfold at 6 h) with a subsequent decrease by 24 h. ^{125}I-albumin studies indicated a blockade of blood flow (Walker et al. 1985). The decrease in liver weight that occurred between 6 and 24 h was associated with a large increase in serum ALT and AST levels indicative of lysis of hepatocytes (Roberts et al. 1991). Associated with the large increase in liver weight and liver hemoglobin level there was a dramatic decrease in intrahepatic pressure and portal vein pressure. Administration of N-acetylcysteine at 3 h after acetaminophen administration ameliorated these observed effects (Walker et al. 1985). Subsequent work by Lim and coworkers (Lim et al. 1995) using a vascular casting technique indicated that acetaminophen toxicity in rats occurred with microvascular injury in the centrilobular areas.

DeLeve and coworkers (DeLeve et al. 1997) examined the possibility that sinusoidal hepatic endothelial cells may metabolize acetaminophen in vitro and that this may be important in toxicity. Previous data indicated that hepatic endothelial cells contain CYP enzymes (Oesch and Steinberg 1987; Steinberg et al. 1990), and that activation of acetaminophen by CYP enzymes in endothelial cells may produce toxicity. Endothelial cells were isolated from two strains of mice. Acetaminophen was not toxic to cultured endothelial cells from Swiss Webster mice but was toxic to cultured endothelial cells from C3H-HEN mice. Glutathione was depleted in the sensitive endothelial cells before the development of toxicity whereas glutathione was not depleted in endothelial cells from the Swiss Webster mice. Addition of glutathione to the incubation or the CYP inhibitor aminobenzotriazole inhibited development of toxicity in the C3H-HEN cells. However, the two strains of mice appeared to be equally sensitive to the centrilobular hepatic necrosis produced by acetaminophen *in vivo*.

McCuskey and coworkers reexamined the role of microvascular injury in acetaminophen toxicity (Ito et al. 2003; McCuskey 2006). In support of the reports by Walker and coworkers (Walker et al. 1985), they found that acetaminophen produces damage to the hepatic microvasculature (sinusoidal endothelial cells) and that these effects precede hepatocellular injury. *In vivo* microscopy indicated that the injury consisted of swelling of the endothelial cells and penetration of erythrocytes into the extrasinusoidal Space of Disse (Ito et al. 2003). There was a significant decrease at 2 and 6 h in the hepatic sinusoids containing blood (Ito et al. 2004). Utilization of an assay for the functional integrity of the endothelial cells (uptake of formaldehyde treated serum albumin) indicated impairment of function in the endothelial cells in the centrilobular regions but not in the periportal regions. These findings indicated that acetaminophen toxicity occurred with altered function of the sinusoidal endothelial cells in the centrilobular regions and confirmed the previous findings that acetaminophen toxicity is accompanied by reduced sinusoidal perfusion. These findings suggest that endothelial cell damage may play a role in the toxicity and the biochemical events associated with toxicity (Ito et al. 2003; Walker et al. 1985); however, the exact role altered blood flow plays in acetaminophen toxicity is unknown.

5 Oxidative Stress in Acetaminophen Toxicity

Early research on understanding oxidative stress in acetaminophen toxicity focused on iron-mediated oxidative stress (Fenton mechanism). This mechanism is initiated by cellular superoxide formation and its dismutation to form increased hydrogen peroxide. Superoxide may be formed by multiple mechanisms including uncoupling of cytochrome P-4502E1 or other enzymes (Koop 1992) and mitochondria (Brand et al. 2004; Casteilla et al. 2001), or activation of NADPH oxidase (Sies and de Groot 1992). Since glutathione is depleted by the metabolite NAPQI in acetaminophen-induced hepatotoxicity and glutathione is the cofactor for glutathione peroxidase detoxification of peroxides, a major mechanism of peroxide

detoxification is compromised in acetaminophen-induced toxicity. Thus, glutathione depletion may be expected to lead to increased intracellular peroxide levels and increased oxidative stress via a Fenton mechanism. This mechanism involves the reduction of peroxide by ferrous ions forming the highly reactive hydroxyl radical which may in turn oxidize lipids leading to initiation of lipid peroxidation as well as oxidation of proteins and nucleic acids. This mechanism has been implicated in various toxicities (Aust et al. 1985). In early work, Wendel and coworkers (Wendel et al. 1979) reported that acetaminophen administration to mice was accompanied by increased levels of exhaled ethane, a measure of lipid peroxidation. Younes et al. (1986) reported that acetaminophen administration to mice did not cause lipid peroxidation (ethane exhalation), but coadministration of ferrous sulfate caused an increase in lipid peroxidation without an increase in toxicity. Subsequently, Gibson et al. (1996) examined hepatic protein aldehydes in acetaminophen toxicity in mice. As with lipid peroxidation, protein aldehyde formation is also mediated by a Fenton mechanism. No evidence of increased hepatic protein aldehyde formation was observed. Thus, early findings as to the role of oxidative stress in acetaminophen-induced toxicity in animals were unclear.

However, work in hepatocytes suggested that acetaminophen toxicity may involve iron-mediated oxidative stress. Albano and coworkers (Albano et al. 1983) reported that incubation of acetaminophen with cultured mouse hepatocytes or with polycyclic aromatic hydrocarbon-induced rat hepatocytes produced oxidative stress as indicated by peroxidation of lipids (malondialdehyde formation). Moreover, the importance of iron in the toxicity of acetaminophen has been shown in both rat and mouse hepatocytes by numerous investigators (Adamson and Harman 1993; Ito et al. 1994; Kyle et al. 1987). Collectively, these data indicated that an iron chelator such as deferoxamine inhibited development of toxicity whereas addition of iron back to the incubation restored the sensitivity of the hepatocytes to acetaminophen toxicity. These data are consistent with Fenton mechanism-mediated oxidative damage playing a role in the hepatotoxicity of acetaminophen; however, the data do not rule out involvement of chelatable iron associated with a critical enzyme function or other critical protein as a mechanistic step in development of toxicity.

The discovery of nitric oxide as an important signaling molecule has led to a more in depth understanding of mechanisms of oxidative stress. Oxidative stress not only includes the classical Fenton-mediated mechanism but also involves nitric oxide. Nitric oxide reacts with superoxide at an extremely rapid rate ($\sim 9 \times 10^9\ M^{-1}\ s^{-1}$) to form peroxynitrite. Peroxynitrite is both an oxidizing agent and a nitrating agent. It is detoxified by glutathione (Sies et al. 1997) which is depleted by NAPQI in acetaminophen-induced hepatotoxicity (Mitchell et al. 1973a,b). Peroxynitrite nitrates tyrosine, leading to formation of the unique biomarker 3-nitrotyrosine, and nitrated proteins have been used as unique biomarkers of nitrogen stress (Beckman and Koppenol 1996). Livers from mice treated with toxic doses of acetaminophen develop 3-nitrotyrosine in the hepatic proteins of the centrilobular regions. Nitrated tyrosine occurs in the same cells that contain acetaminophen-protein adducts, and the development of the nitrated protein correlates with the development of necrosis (Hinson et al. 1998). Figure 2b depicts an immunohistochemical analysis of livers of acetaminophen-treated mice for 3-nitrotyrosine in proteins. Note that nitrated proteins do not occur in the periportal areas of the livers of the acetaminophen-treated mice or in the livers of saline-treated mice (Fig. 2c). Figure 2a is a section adjacent to the section in Fig. 2b but it is immunochemically stained for acetaminophen-protein adducts. Immunohistochemical comparison of slides stained for acetaminophen-protein adducts (Fig. 2a) to slides stained for 3-nitrotyrosine adducts (Fig. 2b) indicate that all cells that contained acetaminophen protein adducts also contained nitrated proteins. These were the only cells with necrotic changes (Hinson et al. 1998). These data indicate that oxidative stress is occurring in the liver following a toxic dose of acetaminophen, and that the site of oxidative stress correlates with site of the toxicity. In a previous publication, we reported that the only cells that undergo necrotic changes contained acetaminophen-protein

adducts (Fig. 1) (Roberts et al. 1991). Collectively, these data are consistent with a hypothesis that the only hepatocytes that develop necrosis are those where oxidative stress is occurring, and further suggest that reactive oxygen and nitrogen species are critical for development of acetaminophen toxicity.

The role of hepatic-inducible nitric oxide synthase (iNOS) has been investigated in the rat and in the mouse. Gardner and coworkers (Gardner et al. 1998) reported that toxic doses of acetaminophen to rats induced iNOS in the centrilobular hepatocytes. The development of toxicity correlated with the expression of iNOS. Moreover, treatment of rats with the iNOS inhibitor, aminoguanidine, decreased hepatotoxicity. The role of iNOS in the progression of acetaminophen toxicity was evaluated in iNOS knockout mice. Whereas ALT levels in acetaminophen-treated iNOS knockout mice were approximately 50% of those observed in wild-type mice, there was no difference in histological scoring of the toxic injury to the liver (Michael et al. 2001). Similar results were reported by Bourdi et al. (2002). Also, the iNOS inhibitor aminoguanidine did not alter acetaminophen toxicity in mice (Hinson et al. 2002). Protein nitration was decreased but not eliminated in iNOS knockout mice (Michael et al. 2001). These data suggested that iNOS-mediated protein nitration was not an important factor leading to acetaminophen toxicity (Michael et al. 2001). However, Gardner reported that acetaminophen toxicity was decreased in iNOS knockout mice compared to the wild-type mice (Gardner et al. 2002). Thus, iNOS appears to play a role in nitration but the role in toxicity is unclear.

The mechanism of increased reactive oxygen/nitrogen species in acetaminophen toxicity has been investigated extensively. Three different mechanisms have been suggested to account for the increased level of reactive oxygen: uncoupled CYP2E1 or other enzymes (Koop 1992), activated NADPH oxidase (Sies and de Groot 1992), and mitochondrial uncoupling (Brand et al. 2004; Casteilla et al. 2001). By each mechanism, it is envisioned that increased superoxide production is the critical event. The increased levels of superoxide can lead to increased hydrogen peroxide and peroxynitrite formation in the cell.

Work by Gonzalez and coworkers utilizing CYP2E1 null mice support the hypothesis that CYP2E1 plays a role in acetaminophen toxicity in vivo (Chen et al. 2008; Gonzalez 2007). CYP2E1 is a major CYP contributing to in the metabolism of acetaminophen to NAPQI; however, other CYPs including CYP1A2 and CYP3A4 also metabolize acetaminophen to NAPQI. CYP2E1 catalytic activity with characteristic uncoupling may be a source of increased oxidative stress in the hepatocyte. Uncoupling of oxygen consumption with NADPH oxidation produces superoxide leading to other reactive oxygen species such as hydrogen peroxide or peroxynitrite (Cederbaum 2006; Cheung et al. 2005; Gonzalez 2007). The importance of CYP2E1 in acetaminophen toxicity was demonstrated by the finding that CYP2E1 null mice were much less sensitive to acetaminophen hepato-toxicity than the wild-type mice or CYP1A2 null mice. The double null mice ($CYP2E1^{-/-}$; $CYP1A2^{-/-}$) were only mildly sensitive to the toxic effects of acetaminophen. Moreover, in CYP2E1 null mice in which the human *CYP2E1* gene was introduced as a artificial chromosome genomic clone of bacterial origin, the hepatotoxic effects of acetaminophen were restored to a significant extent (Cheung et al. 2005; Gonzalez 2007). The importance of CYP2E1-mediated oxidative stress in acetaminophen toxicity was supported in metabolomic studies where urine from acetaminophen-treated wild-type and CYP2E1 null mice were examined for the relative amounts of metabolites derived from NAPQI (acetaminophen-cysteine conjugate, acetaminophen-N-acetylcysteine conjugate, and acetaminophen-glutathione conjugate). In these studies, CYP2E1 null mice were much less sensitive to the toxic effects of acetaminophen; however, the urinary NAPQI derived metabolites at toxic doses of 200 and 400 mg kg^{-1} were not significantly different from those in wild-type mice. Moreover, maximal acetaminophen-induced hepatic depletion of glutathione was not different between the wild-

type mice and the CYP2E1 null mice. These data suggested that metabolic formation of the toxic metabolite NAPQI was not different between wild-type and CYP2E1 null mice. However, there was a substantial difference at a therapeutic dose (10 mg kg^{-1}) which indicated the importance of CYP2E1 in metabolism at therapeutic doses. Importantly, it was shown that administration of the toxic doses of acetaminophen to the wild-type mice resulted in a significant increase in hepatic hydrogen peroxide concentrations. Thus, CYP2E1 appears to be a significant mechanism leading to increased reactive oxygen species in acetaminophen toxicity. These data suggest that Fenton-mediated oxidative stress may be an important event in acetaminophen toxicity and may explain why iron chelators block toxicity (Adamson and Harman 1993; Hinson et al. 2004; Ito et al. 1994; Kyle et al. 1987; Sakaida et al. 1995; Schnellmann et al. 1999).

Available data suggest that NADPH oxidase does not play a role in acetaminophen toxicity. NADPH oxidase is the major respiratory burst enzyme that generates superoxide formation in activated Kupffer cells. Mice that were deficient in gp91*phox*, the primary subunit of NADPH, were shown to have comparable toxicity to acetaminophen as wild-type mice (James et al. 2003c). Also, the mice had similar levels of nitrated protein. In addition, treatment with the NADPH oxidase inhibitors diphenylene iodonium chloride or apocynin did not reduce toxicity in mice treated with acetaminophen (Cover et al. 2006).

Another mechanism of oxidative stress that has been investigated in acetaminophen toxicity is neutrophil-induced oxidant stress. Hypochlorite (hypochlorous acid) is produced by neutrophils by a mechanism involving myeloperoxidase utilization of hydrogen peroxide and chloride ions. The resultant hypochlorite reacts with tyrosine residues to form 3-chlorotyrosine. It was recently reported that chlorotyrosine adducts are not detected in the livers of acetaminophen-treated mice (Hasegawa et al. 2005), but these adducts are readily detected in endotoxemia, a known neutrophil-mediated hepatotoxic injury (Gujral et al. 2004). Nonetheless, Liu and coworkers reported that depletion of neutrophils in mice by treatment with anti-Gr-1 antibody (RB6-8C5) significantly protected mice against acetaminophen-induced liver injury, as evidenced by markedly reduced serum ALT levels, centrilobular hepatic necrosis, and improved mouse survival (Liu et al. 2006). However, the role of neutrophils in the development of acetaminophen toxicity has been questioned because substantial recruitment does not occur until after acetaminophen-induced liver injury in the mouse (Jaeschke and Hasegawa 2006; Lawson et al. 2000).

6 Mitochondrial Injury as a Critical Alteration in Acetaminophen Toxicity

A number of studies have examined the importance of mitochondrial dysfunction in acetaminophen toxicity. Electron microscopic examination of livers from acetaminophen treated livers by Racz's laboratory indicated alterations in mitochondrial morphology (Walker et al. 1980). Jollow et al. reported that mitochondria were a target for the acetaminophen-reactive metabolite (Jollow et al. 1973), and a number of arylated proteins were found in mitochondria (Bulera et al. 1996; Pumford et al. 1990). Functional alterations in the ability to sequester calcium have been reported (Tirmenstein and Nelson 1989). Inhibition of mitochondrial respiration at complexes I and II, but not at complex III was reported in isolated rat hepatocytes (Burcham and Harman 1991) and in vivo (Donnelly et al. 1994). In addition, ATP levels decrease in vivo and in treated hepatocytes (Burcham and Harman 1991; Vendemiale et al. 1996). Similar changes have been shown by adding NAPQI to hepatocytes (Andersson et al. 1990). Moldeus and Orrenius's laboratory reported that addition of NAPQI to isolated rat liver mitochondria caused release of sequestered calcium (Weis et al. 1992, 1994).

Mitochondrial permeability transition (MPT) has emerged as a likely mechanism in acetaminophen-induced hepatotoxicity (Kon et al. 2004; Masubuchi et al. 2005; Reid et al. 2005). Briefly, MPT represents an abrupt increase in the permeability of the inner mitochondrial membrane to ions and small molecular weight solutes. Oxidants such as peroxides and Ca^{++} promote MPT, and hepatocyte levels of peroxides (Chen et al. 2008) have been reported to increase in acetaminophen toxicity as well as alterations in calcium homeostasis (Corcoran et al. 1987; Nicotera et al. 1989; Tirmenstein and Nelson 1989; Tsokos 1989). Associated with the permeability change are inner mitochondrial membrane depolarization, uncoupling of oxidative phosphorylation, release of intramitochondrial ions and metabolic intermediates, mitochondrial swelling, and decreased ATP synthesis. As established above, MPT is promoted by oxidative stress and MPT is in turn associated with a very large increase in oxidative stress. Cyclosporine A specifically blocks MPT in a saturable manner. The block is believed to occur at a protein channel or pore that transports both anionic and cationic solutes of masses less than 1,500 Da, which may be the same structure as the voltage-dependent anion channel (VDAC), an essential element of the pore. Three proteins are believed to be in the MPT pore: the adenine nucleotide translocator, cyclophilin D, and the voltage-dependent anion channel. Whereas the adenine nucleotide translocator does not appear to be essential for development of MPT, cyclophilin D is a necessary component (Baines et al. 2005; Kokoszka et al. 2004). A number of oxidants can lead to pore opening including t-butylhydroperoxide (t-BuOOH) (Nieminen et al. 1997) and peroxynitrite (Packer et al. 1997). Addition of t-BuOOH to hepatocytes results in oxidation of pyridine nucleotides and generation of reactive oxygen species (ROS) within the cell. Subsequently, onset of MPT occurs, mitochondria depolarize, ATP levels decrease, and hepatocytes lose viability. Under normal conditions, mitochondria produce small amounts of ROS. However, after t-BuOOH treatment, mitochondrial ROS increase 15-fold as determined by confocal microscopy studies monitoring the conversion of dichlorodihydrofluorescein ($DCFH_2$) to the fluorescent product dichlorofluorescin (DCF) (Myhre et al. 2003; Nieminen et al. 1997). A major source of mitochondrial ROS may be the reaction of ubisemiquinone (generated in the cytochrome bc1 complex by the Q cycle) with oxygen leading to formation of superoxide. Importantly from an experimental perspective, Cyclosporine A, which binds avidly to cyclophilin D, will block MPT (Kowaltowski et al. 2001).

Blockade of acetaminophen toxicity both in vitro and in vivo by MPT inhibitors has been reported. Kon and coworkers (Kon et al. 2004, 2007) showed that acetaminophen toxicity in cultured mouse hepatocytes was inhibited by cyclosporine A and by the nonimmunosuppressive Cyclosporine A analog NM811. Cyclosporine A was shown not to alter acetaminophen-induced glutathione depletion indicating that the prevention of toxicity did not occur by inhibition of metabolism of acetaminophen to NAPQI. Toxicity was shown to occur with loss of mitochondrial membrane potential by using tetramethylrhodamine methyl ester (TMRM) and fluorometric analysis. Reid and coworkers (Reid et al. 2005) examined the effect of MPT inhibitors in freshly isolated mouse hepatocytes using the approach of Boobis and coworkers (Boobis et al. 1986; Tee et al. 1986). In these studies, acetaminophen was incubated with the hepatocytes for 2 h, the hepatocytes were washed free of acetaminophen, and subsequently the hepatocytes were incubated with media alone. This approach clearly separated direct metabolic effects from effects occurring downstream of acetaminophen metabolism. As previously reported, toxicity occurred in the reincubation phase (3–5 h). Inclusion of the MPT inhibitors Cyclosporine A, trifluoperazine, or dithiothreitol in the reincubation phase completely inhibited toxicity. Also, addition of N-acetylcysteine in the reincubation phase completely inhibited toxicity. Toxicity was shown to correlate with loss of mitochondrial membrane potential by utilizing the dyes TMRM and JC-1. The loss of mitochondrial membrane potential was prevented by addition of cyclosporine A and N-acetylcysteine in the reincubation phase. Utilization of the redox sensitive dye dichlorodihydrofluorescin indicated that toxicity occurred with a large increase in reactive

oxygen species in the reincubation phase. The large increase in oxidative stress was eliminated by addition of Cyclosporine A or N-acetylcysteine to the reincubation phase. These data are consistent with acetaminophen metabolism leading to glutathione depletion and covalent binding occurring in the initial 2 h incubation. Subsequently, MPT occurs with loss of mitochondrial membrane potential, a large increase in oxidative stress, and toxicity (Reid et al. 2005).

McLean and coworkers have reported that inhibitors of MPT (cyclosporine A and trifluoperazine) inhibited acetaminophen toxicity in rat liver slices and in vivo when administered as a cocktail with fructose (Beales and McLean 1996; Nieminen et al. 1997). Also, Dimova et al. (1995) reported that the MPT inhibitor trifluoperazine decreased acetaminophen-induced hepatotoxicity in the mouse. Masubuchi et al. (2005) reported that Cyclosporine A decreased acetaminophen toxicity in mice. Since hepatic glutathione depletion was the same in acetaminophen-treated and acetaminophen plus cyclosporine A-treated mice, it was concluded that the decrease in toxicity was not mediated by inhibition of NAPQI formation. Moreover, they observed that acetaminophen caused a swelling of liver mitochondria and a decrease in mitochondrial membrane potential, both of which were eliminated by cotreatment with Cyclosporine A. Collectively, the data indicate that MPT is an important mechanism leading to acetaminophen toxicity.

Peroxynitrite may be an important oxidant produced in acetaminophen-induced MPT. As discussed above, acetaminophen-induced MPT occurred with increased oxidation of the redox-sensitive dye $DCFH_2$. This dye is readily oxidized by peroxynitrite but not by superoxide, hydrogen peroxide, or hypochlorous acid; however, it may be oxidized by peroxide plus a peroxidase or a Fenton mechanism (ferrous ions plus peroxide) (Crow 1997; Myhre et al. 2003). Peroxynitrite is known to rapidly react with thiols such as N-acetylcyteine (Crow 2000), and N-acetylcysteine prevented acetaminophen-induced MPT and $DCFH_2$ oxidation (Reid et al. 2005). The finding that nitration was predominantly in mitochondria of acetaminophen-treated mice supports the hypothesis that peroxynitrite formation occurred in that organelle (Cover et al. 2005). As pointed out above, the NOS isoform was probably not iNOS, which suggests involvement of another NOS species such as mitochondrial nitric oxide synthase (mtNOS) (Ghafourifar and Cadenas 2005).

Whereas necrosis appears to be mediated by opening of the MPT pore in the inner mitochondrial membrane leading to loss of ability to produce ATP, apoptosis may also be mediated by opening of a pore or channel in the outer mitochondrial membrane, the mitochondrial apoptosis-induced channel. This pore is believed to release proapoptotic factors including cytochrome C, endonuclease G, Smac/Diablo, and apoptosis-inducing factor (AIF) from the membrane space into the cytosol (Dejean et al. 2006b; Kinnally and Antonsson 2007). The development of mitochondrial apoptosis-induced channels appear to be sensitively regulated by Bcl-2 family proteins, and consists of proapoptotic proteins such as Bax and anti-apoptotic proteins such as Bcl-2. In particular, the proapoptotic protein Bax is normally in the cytosol. Activation results in translocation of Bax to mitochondria, insertion into the outer membrane, and oligomerization to form the channels (Antonsson et al. 2000; Dejean et al. 2005, 2006a). These channels release apoptotic proteins (Boelsterli and Lim 2007).

The relative amount of ATP appears to be an important factor relative to whether the hepatocytes dies by necrosis or apoptosis. Low ATP levels are associated with necrosis whereas adequate ATP levels favor apoptosis. With acetaminophen, Kon and coworkers showed that cultured mouse hepatocytes died primarily by necrosis. However, inclusion of fructose, an ATP generating glycolytic substrate, and glycine, a membrane stabilizer, in the media decreased necrosis and promoted apoptosis (Kon et al. 2004).

7 Inflammation, Cytokines and Chemokines in Development of Acetaminophen Toxicity

The complex role of inflammatory cells and cytokines in the mediation of acetaminophen toxicity has been investigated for over 20 years. Initial investigations to understand inflammatory responses that occur in the liver during acetaminophen toxicity were performed by Laskin and coworkers. They reported that acetaminophen toxicity occurred with activation of Kupffer cells (hepatic macrophages) (Laskin and Pilaro 1986; Laskin et al. 1986). Subsequent research indicated that Kupffer cell activation led to increases in both pro-inflammatory and anti-inflammatory cytokines. Cytokines have important functions in immunity, inflammation, cell proliferation, differentiation, and cell death (Shen and Pervaiz 2006). TNF-α has been linked to increased oxidative stress, (increased formation of reactive oxygen species and reactive nitrogen species) and is known to recruit and activate other inflammatory cells (Gardner et al. 2003). Blazka and coworkers showed dramatic increases in serum levels of TNF-α and IL-1α in mice treated with acetaminophen. (Blazka et al. 1995b). Moreover, they reported that treatment of acetaminophen-intoxicated mice with either anti-TNF-α or anti-IL-1α partially prevented hepatotoxicity (Blazka et al. 1996). In further studies, it was shown that the Kupffer cell inactivators, gadolinium chloride and dextran sulfate, decreased acetaminophen toxicity in the rat (Laskin et al. 1995) and the mouse (Blazka et al. 1995a; Chen et al. 1999). These data, coupled with the proinflammatory cytokine data, suggested that Kupffer cells mediated proinflammatory responses in acetaminophen toxicity. However, more recent work brings into question the mechanistic role of Kupffer cell activation in acetaminophen toxicity. Ju et al. (2002) treated mice with liposome-encapsulated chlodronate (dichloro-methylene disphosphonate) to more completely eliminate Kupffer cells. Subsequent, treatment of the mice with acetaminophen resulted in reduced mRNA levels for TNF-α and other cytokines (IL-10, Il-6, COX-2, Il-18Bp, Clq) and there was an *increase* in liver toxicity as measured by serum ALT. Ju and colleagues postulated that these findings suggest alternative roles for Kupffer cells in the toxicity, and suggest that Kupffer cells may counteract inflammation or have a role in liver repair. Further studies are needed to elucidate the role of Kupffer cells and other cells of the innate immune system in the regulation of inflammation or the initiation of liver repair.

Other data have not supported a role for TNF-α in the development of acetaminophen toxicity. Wild-type and TNFα knockout mice had equal sensitivity to acetaminophen (Boess et al. 1998), and treatment with anti-TNF antibody or soluble TNF receptor did not alter acetaminophen toxicity in mice (Simpson et al. 2000). Subsequent work by James reported no difference in toxicity between TNFR1 knockout mice and wild-type mice (James et al. 2005). Conflicting data from these studies, compared to the findings of Blaska (Blazka et al. 1995b, 1996), may be related to strain or dose differences, or variations in repair processes among mice strains.

Other proinflammatory cytokines such as interleukin one beta (IL-1β) and interferon gamma (IFNγ) have also been examined in acetaminophen toxicity (Blazka et al. 1995b; Gardner et al. 2003; James et al. 2003d). Ishida et al. (2002) and Liu et al. (2004) reported that IFNγ mRNA was induced in livers of acetaminophen-treated mice. The importance of IFNγ in acetaminophen toxicity was investigated in wild-type and IFNγ knockout mice (Ishida et al. 2002). IFNγ knockout mice were shown to have reduced toxicity compared to wild-type mice, as indicated by serum ALT and AST levels and by histopathological evaluation of necrosis. In addition, the IFNy knockout mice had reduced mortality. The IFNγ knockout mice had significant attenuation of the mRNA transcripts for inflammatory cytokines, chemokines, adhesion molecules, Fas, and inducible nitric oxide synthase (iNOS) compared with wild-type (WT) mice. (Ishida et al. 2002). Ishida et al. further showed that treatment of mice with an anti-IFNγ antibody lowered ALT values in APAP-treated mice. Liu and coworkers examined the

role of natural killer cells (NKT) and natural killer T cells, and reported these were the primary cell type important in IFNγ production in acetaminophen toxicity. However, other investigators did not find IFNγ induction in livers of acetaminophen treated mice (Gardner et al. 2003). Pohl's laboratory reported that acetaminophen administered in a saline solution did not increase hepatic IFNg mRNA; however, when administered with DMSO there was an induction of IFNγ (Masson et al. 2008). DMSO alone was shown to increase the number of hepatic NKT cells and to activate both NKT and NK cells to produce IFNγ and granzyme B. Thus, as pointed out by Masson et al. (2008), the utilization of DMSO to facilitate dissolution may be a confounding factor.

Interleukin-6 (IL-6) has also been examined in acetaminophen toxicity. While it is known to be important in hepatocyte regeneration (discussed below), one study showed that depletion of IL-6 resulted in increased sensitivity to acetaminophen (Masubuchi et al. 2003). IL-6 knockout mice were found to have increased toxicity to acetaminophen and reduced formation of heat shock proteins (HSP) 25, 32, and 40, as well as inducible HSP70. HSPs are induced in cells by exogenous stressors, and the presence of nonnative proteins is thought to be a trigger for their induction. Salminen et al. (1998) showed that the combined treatment of mice with acetaminophen and N-acetylcysteine, protected from toxicity, and attenuated but did not prevent adduct formation or HSP induction. Pretreatment with diallyl sulfide, a CYP2E1 inhibitor, abolished HSP25 and HSP70i induction and toxicity. These data suggest that CYP2E1-mediated oxidative stress played a mechanistic role in HSP induction in acetaminophen toxicity.

The role of anti-inflammatory cytokines in acetaminophen toxicity has also been examined. Interleukin 10, 11, and 13 are anti-inflammatory cytokines that are known to modulate the pro-inflammatory response in hepatic injury (Bourdi et al. 2002; Louis et al. 1997a, b; Yee et al. 2007; Zingarelli et al. 2001). The liver is a major source of IL-10 and many cell types including activated Kupffer cells, and T and B lymphocytes produce IL-10. Bourdi et al. (2002) reported that IL-10 knock-out mice had increased toxicity to acetaminophen compared to wild-type mice. In addition, IL-10 knockout mice had increased mRNA transcripts for the pro-inflammatory cytokines TNF-α and IL-1, as well as increased mRNA transcripts for iNOS and increased serum nitrate plus nitrite, a marker of NO. The anti-inflammatory cytokine IL-11 is known to be protective in a number of organ injury models (Fiore et al. 1998; Maeshima et al. 2004; Trepicchio et al. 2001) and may mediate protection by decreasing pro-inflammatory cytokine production or decreasing macrophage activation. Trepicchio et al. (2001) showed that pretreatment of mice with recombinant human IL-11 lowered ALT values and TNF-α levels by approximately one-half the values of non-IL-11-treated mice. In addition, histologic measures of hepatotoxicity were improved. More recently, IL-13 has been examined for its hepatoprotective role in acetaminophen toxicity. Pretreatment of mice with IL-13 reduced acetaminophen toxicity- and IL-13 knockout mice were sensitive to the toxic effects of acetaminophen. In further studies, IL-13 was shown to modulate IFN-γ, nitric oxide, and inflammatory cells, including neutrophils, NK cells, and NKT cells (Yee et al. 2007).

A recent study examined acetaminophen toxicity in two strains of mice that had distinct inflammatory and cytokine profiles (Cover et al. 2006). Despite the greater predominance of neutrophils and pro-inflammatory gene expression in C3Heb/FeJ mice, and the predominance of anti-inflammatory gene expression in C57BL/6 mice, biochemical markers of toxicity and histopathology of the livers were equivalent in the two strains of mice. Cumulatively, available data suggest that a balance of pro- and anti-inflammatory cytokines is maintained in acetaminophen toxicity and that no one single pro-inflammatory or anti-inflammatory cytokine is *critical* to the mediation of the toxicity. While the animal data support the postulation that genetic variability in cytokine expression may be a contributing factor to acetaminophen

susceptibility in man (Bourdi et al. 2002; Welch et al. 2006), few clinical studies have been performed in this area.

Chemokines also play a role in acetaminophen-induced toxicity. These low molecular weight cytokines were initially recognized for their role in the chemotaxis of lymphocytes. In addition, some classes of chemokines may have angiogenic, wound healing, cell proliferative, or antiinflammatory properties. Multiple laboratories have shown that chemokines are upregulated in acetaminophen toxicity (Bone-Larson et al. 2001b; Hogaboam et al. 1999a, 2000b; James et al. 2001 ; Osawa et al. 2002).

The prototype chemokine, macrophage inhibitor protein 2 (MIP-2), is a member of the CXC chemokine family and is produced by many cell types in response to the pro-inflammatory cytokines, TNF-α and IL-1β. Jaeschke's laboratory showed that chemokine upregulation (MIP-2 and KC) and neutrophil accumulation followed the onset of acetaminophen toxicity in mice, and inactivation of B_2 integrins with an anti-CD18 antibody (e.g., neutrophil inactivation) did not alter toxicity (Lawson et al. 2000). Hepatocytes exposed to acetaminophen develop toxicity in the absence of neutrophils (Moldeus 1978; Reid et al. 2005). Mouse strains with differing degrees of neutrophil accumulation had similar toxicity to acetaminophen, suggesting that neutrophils are not mechanistically important in acetaminophen toxicity (Cover et al. 2006), but this is a controversial area. While it has been postulated that the primary role of neutrophil influx in acetaminophen toxicity is that of removal of damaged cells and cellular debris (Lawson et al. 2000), a more recent study using the anti-Gr-1 antibody (RB6-8C5) to neutrophils, showed that toxicity was significantly attenuated with neutrophil depletion in acetaminophen-treated mice (Liu et al. 2006). Several lines of evidence support the potential role of the chemokine MIP-2 as a hepatoprotective factor in acetaminophen toxicity. Hogaboam's laboratory (Hogaboam et al. 1999a) reported that treatment with MIP-2 was more effective as a "late therapy" given to acetaminophen-treated mice than the antidote N-acetylcysteine. In vitro data showed that MIP-2 maintained hepatocyte proliferation in cells exposed to acetaminophen. In addition, adenoviral vector delivery of MIP-2 reduced toxicity in acetaminophen-treated mice (Hogaboam et al. 1999a, b).

The mechanism of the protective effects of the CXC chemokines in acetaminophen toxicity is poorly understood. Some data suggest that MIP-2 may lead to the increased nuclear localization of the transcription factor signal transducer and activator of transcription 3 (STAT3), a major signal transduction factor important in hepatocyte regeneration (Hogaboam et al. 1999a, 2000a; Ren et al. 2003). Another CXC chemokine, IP-10 (Interferon – inducible protein), does not have direct mitotic effects in vitro, but may be able to induce hepatocyte growth factor (HGF), a known mitogen (Koniaris et al. 2001). Bone-Larson et al. (2001a) found that IP-10 was protective in acetaminophen toxicity and that this protection was associated with induction of the MIP-2 receptor (CXCR2) on hepatocytes. Thus, the available data suggest that MIP-2 and IP-10 are important in cell proliferation (hepatocyte regeneration) in response to acetaminophen toxicity. One study suggested that chemokines may also dampen pro-inflammatory cytokine production in acetaminophen toxicity. Mice deficient in the primary receptor for the chemokine monocyte chemoattractant protein-1 (MCP-1) were found to have increased levels of TNFα and IFNγ. Neutralization of these pro-inflammatory cytokines resulted in attenuation of toxicity to acetaminophen (Hogaboam et al. 2000b).

Very few studies have examined the role of cytokines and chemokines in acetaminophen toxicity in the clinical setting. Interleukin 8 (the human homologue of MIP-2) was shown to be increased in patients with acetaminophen toxicity and to correspond with markers of hepatic injury (James et al. 2001). Further investigation in this area may help to identify potential early determinants of severe cases of acetaminophen toxicity.

Another anti-inflammatory mechanism that has been reported to play a role in acetaminophen toxicity is cyclooxygenase-2 (COX-2) (Reilly et al. 2001). Whereas COX-1 is constitutively expressed and produces low levels of prostaglandins, COX-2 is inducible and plays a role in anti-inflammatory processes. COX-2 derived prostaglandins are recognized for their critical role in female reproduction, bone resorption, renal function, and mucosal defense. Importantly, prostaglandins have been reported to play a protective role in various hepatotoxicities (Quiroga and Prieto 1993). Reilly and coworkers showed that COX-2, but not COX-1, was induced in livers of acetaminophen-treated mice. Moreover, acetaminophen was more toxic with high lethality in COX-2 knockout mice compared to wild-type mice. Also, the COX-2 inhibitor celecoxib (Celebrex®), was found to increase acetaminophen toxicity (Reilly et al. 2001). These data are consistent with COX-2 playing an anti-inflammatory role in acetaminophen-induced hepatotoxicity.

8 Intracellular Signaling Mechanisms in Acetaminophen Toxicit

The c-Jun N-terminal kinases (JNKs), a subfamily of the mitogen-activated protein (MAP) kinases, have been shown to be activated by phosphorylation early in acetaminophen toxicity both in vitro and in vivo (Gunawan et al. 2006; Henderson et al. 2007; Latchoumycandane et al. 2006, 2007; Matsumaru et al. 2003). JNK activation may be mediated by reactive oxygen species as well as by TNF-α (Shen and Pervaiz 2006). Kaplowitz's laboratory (Gunawan et al. 2006; Matsumaru et al. 2003) reported that incubation of acetaminophen with mouse hepatocytes leads to induction of JNK activity, and that the induction of activity could be blocked by a specific JNK inhibitor (SP600125). *In vivo*, the inhibitor protected mice from the toxic effects of acetaminophen without altering acetaminophen-reactive metabolite formation. Acetaminophen-induced glutathione depletion and protein covalent binding were not altered by inhibitor treatment (Gunawan et al. 2006). Henderson and coworkers (Henderson et al. 2007) reported similar results and found that late administration of the SP600125 inhibited hepatic necrosis and was more effective than the antidote N-acetylcysteine in limiting the injury. In agreement with a role for JNK activation in acetaminophen toxicity, Latchoumycandane et al. (2007) found that the antirheumatic drug leflunomide inhibited acetaminophen-induced hepatic JNK activation and blocked development of acetaminophen toxicity in mice.

JNK activation may be a mechanism that is associated with the initiation of mitochondrial permeability transition (MPT) (Hanawa et al. 2008; Latchoumycandane et al. 2006, 2007). As discussed above, both JNK activation (Matsumaru et al. 2003) and MPT (Lemasters 1998) are known to occur as a result of increased oxidative stress. MPT leads to additional oxidative stress with loss of mitochondrial membrane potential and loss of the ability of the hepatocyte to synthesize ATP. Latchoumycandane et al. (2006, 2007) found that leflunomide protected mice from mitochondrial permeabilization. Direct evidence for a role of JNK activation in acetaminophen-induced MPT was recently reported by Hanawa et al. (2008). A time course of events indicated GSH depletion by 1–2 h, JNK activation in liver homogenate by 2–4 h, JNK translocation to mitochondria by 4 h, and increased toxicity (serum ALT by 6 h). The JNK inhibitor did not alter GSH depletion but blocked JNK activation in homogenate, JNK translocation to mitochondria, and toxicity. Mitochondria from liver of acetaminophen-treated mice showed decreased State III respiration and decreased respiratory control ratios, whereas mice treated with acetaminophen plus JNK inhibitor were partially protected from these losses. Addition of activated JNK1 or JNK2 to mitochondria from acetaminophen-treated mice plus JNK inhibitor showed a decrease in State III respiration and decreased respiratory control ratio. Addition of the MPT inhibitor cyclosporine A prevented these decreases. It was hypothesized that activated JNK is an important mediator of acetaminophen-induced MPT (Hanawa et al. 2008).

Acetaminophen-induced hepatotoxicity has also been examined in knockout mice. JNK1 knockout mice and wild-type mice were found to be equally sensitive to the toxic effects of acetaminophen in three different laboratories (Gunawan et al. 2006; Henderson et al. 2007; Bourdi et al. 2008). However, data on the role of JNK-2 are confusing. Initially, Gunawan et al. (2006) found JNK-2 knockout mice to be less sensitive to the toxicity than wild-type mice. They suggested that JNK acts downstream of metabolism in acetaminophen toxicity. However, Henderson et al. (2007) found that disruption of either JNK1 or JNK2gnenes did not protect against acetaminophen-induced liver toxicity in mice. Since administration of a JNK inhibitor blocked both JNK-1 and JNK-2, it was suggested that inhibition of both may be important in toxicity. However, Bourdi et al. recently reported JNK-2 knockout mice to be more sensitive to acetaminophen toxicity (Bourdi et al. 2008; Gunawan et al. 2006; Henderson et al. 2007). Bourdi suggested that JNK-2 modulated hepatocellular regeneration and repair. Thus, understanding the role of JNK in acetaminophen toxicity will require additional research.

DNA fragmentation is another mechanism that has been implicated in acetaminophen-induced hepatotoxicity (Salas and Corcoran 1997). Genomic DNA fragmentation in liver (TUNEL assay and DNA laddering) following hepatotoxic doses of acetaminophen in the mouse was originally reported by Corcoran's laboratory (Ray et al. 1990, 1993). They found that the rate of DNA fragmentation paralleled the rate of development of hepatotoxicity and was associated with an increase in nuclear calcium levels. Similarly, acetaminophen-induced cytotoxicity in cultured mouse hepatocytes was found to occur with DNA fragmentation and nuclear calcium accumulation (Shen et al. 1991). The presence of ladder-like DNA fragments were observed indicating the involvement of a calcium-dependent endonuclease. Aurintricarboxylic acid, a general calcium-endonuclease inhibitor, and EGTA, a chelator of calcium required for endonuclease activation, significantly decreased DNA fragmentation and toxicity (Shen et al. 1992). The calcium-calmodulin antagonist chlorpromazine and the calcium channel blocker verapamil decreased acetaminophen-induced hepatic necrosis and deceased DNA fragmentation in acetaminophen-treated mice (Ray et al. 1993).

Subsequently, it was reported that endonuclease G was important in the acetaminophen-induced nuclear fragmentation. This endonuclease is present in the mitochondria and is released under conditions of outer mitochondrial membrane permeabilization. Whereas endonuclease G was found in control mitochondria, incubation with acetaminophen resulted in trafficking of the protein to the nucleus, and the relative rate of trafficking correlated with rate of development of acetaminophen-induced loss of mitochondrial membrane potential and nuclear DNA fragmentation (Bajt et al. 2006). Subsequently, they investigated the role of Bax in acetaminophen-induced endonuclease G and apoptosis-inducing factor (AIF) trafficking to the nucleus (Bajt et al. 2008). Bax had been reported to localize in the mitochondria in acetaminophen toxicity (Adams et al. 2001; El-Hassan et al. 2003) and is well recognized to induce apoptosis-induced channels in the mitochondria with release of apoptotic proteins including endonuclease G (Antonsson et al. 2000; Dejean et al. 2005, 2006a). In Bax knockout mice, it was shown that the rate of development of acetaminophen toxicity in mouse hepatocytes was slower than in wild-type hepatocytes. At 6 h, toxicity and DNA fragmentation were much less in the Bax knockout mice than in the wild-type mice, but protein nitration was similar. However, at 12 h toxicity, DNA fragmentation and protein nitration were not different. These data suggested that in the acetaminophen toxicity model, Bax was playing a role in mitochondrial outer membrane permeabilization with formation of mitochondrial apoptosis-induced channels and release of intermembrane proteins (Bajt et al. 2008). The relationship between development of the mitochondrial apoptosis-induced channel with release of apoptotic proteins and mitochondrial permeability transition in development of necrosis is poorly understood and will require further investigations.

9 Mechanisms of Repair of Acetaminophen Liver Toxicity

Following liver injury, in an attempt to restore homeostasis, a complex series of events occur in the liver. Proliferation of all existing mature cellular populations occurs, beginning with hepatocytes (Michalopoulos and DeFrances 1997). Entry into and progression of hepatocytes through the early G_1 phase of the cell cycle, or *priming*, is mediated by TNF-α and interleukin 6 (IL6) (Diehl et al. 1994; Li et al. 2002). TNF-α has potent mitotic effects in vitro and in vivo (Gallucci et al. 2000). Diehl showed that TNF-α neutralizing antibodies administered to rats prior to partial hepatectomy decreased DNA synthesis and the activation of signaling pathways involved in hepatocyte regeneration (Diehl et al. 1994). Mice deficient in the TNF receptor one (TNFR1) have reduced DNA synthesis after partial hepatectomy and reduced activation of signal transduction factors (STAT 3) (Li et al. 2001, 2002; Yamada et al. 1997).

Several laboratories have examined the role of the TNF-α receptor 1 (TNFR1) in acetaminophen toxicity. Toxicity was increased in the TNFR1 knockout mice compared to the wild-type mice in three studies (Chiu et al. 2003a, b; Gardner et al. 2002; James et al. 2005). Chiu et al. showed that TNFR1 knockout mice had alterations in antioxidant expression following acetaminophen toxicity (Chiu et al. 2003a). Restoration of hepatic glutathione was delayed and heme oxygenase-1 and CuZn superoxide dismutase expression were reduced in the knockout mice. Two laboratories showed that TNFR1 was important in hepatocyte regeneration following acetaminophen toxicity in the mouse (Chiu et al. 2003b; James et al. 2005). In addition, TNFR1 knockout mice had higher levels of chemokines (MIP-2, IP-10) (James et al. 2005), which have been implicated to have a role in hepatocyte regeneration following acetaminophen toxicity (Hogaboam et al. 1999a, b). The data illustrate the pleotropic properties of TNF-α in acetaminophen toxicity and also suggest a redundancy of pathways for hepatocyte regeneration and recovery following acetaminophen toxicity in the mouse.

IL-6 is closely related to TNF-α, and is known to have promitotic effects on various cells types. TNF-α upregulates IL-6 via the signal transduction factor NF-kB, and IL-6 activates STAT3. STAT3 is signal transduction factor that activates a large number of genes important in hepatocyte regeneration (Li et al. 2001, 2002). IL-6 knockout mice had reduced hepatocyte regeneration, as measured by the expression of proliferating cell nuclear antigen (PCNA), following acetaminophen toxicity (James et al. 2003a). PCNA is an auxiliary protein for DNA polymerase delta and a biomarker of increased cellular proliferation (Essers et al. 2005). Treatment of the knockout mice with murine IL-6 normalized PCNA expression following acetaminophen toxicity. Similar findings were reported by Bajt et al. (2003). Decreased activation of a number of genes important in liver regeneration (e.g., c-Fos, c-Myc, LRF-1, STAT3, and JunB) have been shown in IL-6 knockout mice (Cressman et al. 1996). In addition to these studies, it is likely that multiple mechanisms for repair of the liver following acetaminophen toxicity are operative. For example, TNF-α may be linked to hepatocyte regeneration via mechanisms other than IL-6 activation. TNF-α can increase the expression of transforming growth factor-β (Kalthoff et al. 1993; Tamura et al. 1993), a known mitogen for hepatocytes and other liver-derived cells.

Growth factors are closely related to cytokines and may have overlapping effects. Vascular endothelial growth factor (VEGF) is expressed by endothelial cells and is a critical mitogen and survival factor for endothelial cells. It is also the major regulator of angiogenesis during organ development and differentiation during embryogenesis (reviewed by Ferrara 2001) and a critical mediator of angiogenesis in cancer. VEGF induces the expression of antiapoptotic proteins in human endothelial cells, suggesting that it promotes the survival of these cells. VEGF mRNA transcripts and protein are expressed by other cell types as well, including hepatocytes (Mochida et al. 1996) and hepatic stellate cells (Ankoma-Sey et al. 2000). A number of factors may regulate VEGF, including hypoxia, cytokines, iNOS, and

hyperglycemia (Ankoma-Sey et al. 2000; Yamada et al. 2003). VEGF has two primary receptors, VEGFR1 and VEGFR2, that have distinct roles. VEGFR2 appears to mediate the angiogenic properties of this growth factor, whereas emerging data suggest that VEGR1 may have promitotic effects.

VEGF has been shown to have mitogenic effects on hepatocytes by orchestrating interactions between endothelial cells and hepatocytes. LeCouter et al. (2003) showed that mice injected with a VEGF-producing cell line (Chinese hamster ovarian cells) had greater liver weights and increased mitotic counts compared to mice treated with vehicle control (Kim et al. 2002). However, VEGF itself had no direct effect on hepatocyte mitosis in vitro. Activation of VEGFR1 in vitro resulted in significant increases in the release of mitogens by endothelial cells (LeCouter et al. 2003). These mitogens included the known hepatocyte mitogens: hepatocyte growth factor (HGF) and IL-6. Treatment of mice with VEGFR1 and VEGFR2 ligands was shown to be protective in carbon tetrachloride toxicity; however, treatment with the VEGR2 ligand resulted in increased endothelial cell proliferation.

The role of VEGF in acetaminophen toxicity and repair has been examined in both the mouse and rat (Donahower et al. 2006; Papastefanou et al. 2007). Hepatic VEGF levels were increased 30-fold in mice treated with acetaminophen and followed the onset of toxicity (Donahower et al. 2006). Peak elevation of hepatic VEGF was apparent at 8 h and remained increased until 48 h. Induction of VEGFR1 peaked at 48 h and induction of VEGFR2 peaked at 8 h. Similar time course data were demonstrated in acetaminophen-treated rats. In further studies, mice were treated with SU5416, an inhibitor of VEGFR2-mediated signaling. Treatment of mice with SU5416 did not alter the metabolism of acetaminophen, but lowered ALT values and increased PCNA expression (hepatocyte regeneration) (Donahower et al. 2006). In further studies, the role of exogenous treatment with VEGF in acetaminophen toxicity in the mouse was examined. Treatment with VEGF lowered ALT values at 8, 18, 24, and 36 h and reduced the relative area of necrosis (Donahower et al. 2007). VEGF had no effect on glutathione depletion or covalent binding. In addition, VEGF enhanced the expression of PCNA in the late stages of acetaminophen toxicity (18, 24, 36 h). The mechanism for the hepatoprotection of VEGF in acetaminophen toxicity is unclear. It is possible that VEGF may have improved hepatic blood flow or altered hepatic microcirculation but further studies are needed to assess this potential effect in vivo. In addition, the VEGF-treated mice had reduced production of IL-6, possibly indicating internal regulation of the expression of this promitotic cytokine. Further studies are needed to elucidate the mechanisms of VEGF hepato-protective effects and to test the growth factor further as a potential late phase therapy for acetaminophen toxicity.

10 Conclusion

A significant impediment to a more in-depth understanding of the mechanisms of acetaminophen toxicity has been conflicting data from different laboratories regarding various modulators of toxicity. A major problem is that hepatic CYP enzymes or glutathione levels important in metabolic activation of acetaminophen and detoxification may be altered by treatments or genetic modification of the animal. For example, CYP2E1 may be inhibited by solvents such as DMSO, propylene glycol, or ethanol used in the administration of inhibitors. This results in decreased formation of NAPQI, less glutathione depletion, less covalent binding, and less toxicity. Figure 1 is included to show the relationship between acetaminophen metabolism (covalent binding) and development of toxicity. The only hepatocytes that developed necrosis had acetaminophen-protein adducts. Since covalent binding occurs only under conditions of glutathione depletion, the affected hepatocytes are believed to have very low concentrations of glutathione. This predisposes them to increased oxidative stress which is shown in Fig. 2. The only hepatocytes that had increased oxidative stress (nitrated proteins indicating peroxy-nitrite formation) contained acetaminophen-protein adducts. Thus,

understanding how modulators affect toxicity requires an understanding of their effects on metabolism and oxidative stress.

In summary, the hepatotoxicity of acetaminophen appears to occur by a complex mechanistic sequence (Fig. 3). These events include: (1) CYP metabolism to the reactive metabolite NAPQI which depletes glutathione by a conjugation reaction and covalently binds to proteins; (2) loss of glutathione causing an increased oxidative stress response (decreased detoxification of reactive oxygen and nitrogen species); (3) increased oxidative stress, possibly associated with alterations in calcium metabolism, initiation of signal transduction responses and mitochondrial permeability transition; (4) mitochondrial permeability transition occurring with an even larger increase in oxidative stress, loss of mitochondrial membrane potential, and loss of the ability of the mitochondria to synthesize ATP; and (5) loss of ATP which causes necrosis. Associated with these essential events there appears to be a number of modulators of inflammatory responses that can alter the severity of liver injury following the initiation of toxicity. Further study of the interactions of these mediators with each other and the interplay of the immune cells that produce them will help to elucidate the significance of their roles in acetaminophen toxicity. In addition, studies are needed to examine the effect of inflammatory mediators on oxidative stress and/or signal transduction responses in acetaminophen toxicity. In conjunction with these inflammatory events, apoptotic responses occur; however, the loss of ability of the hepatocyte mitochondria to produce ATP may be the single most important event causing necrosis. Finally, the liver has a very great capacity to regenerate and various cytokines and growth factors are major initiators of this process (Fig. 4). Future investigations to elucidate the signaling for these pathways may help to identify novel targets for the treatment of acetaminophen toxicity.

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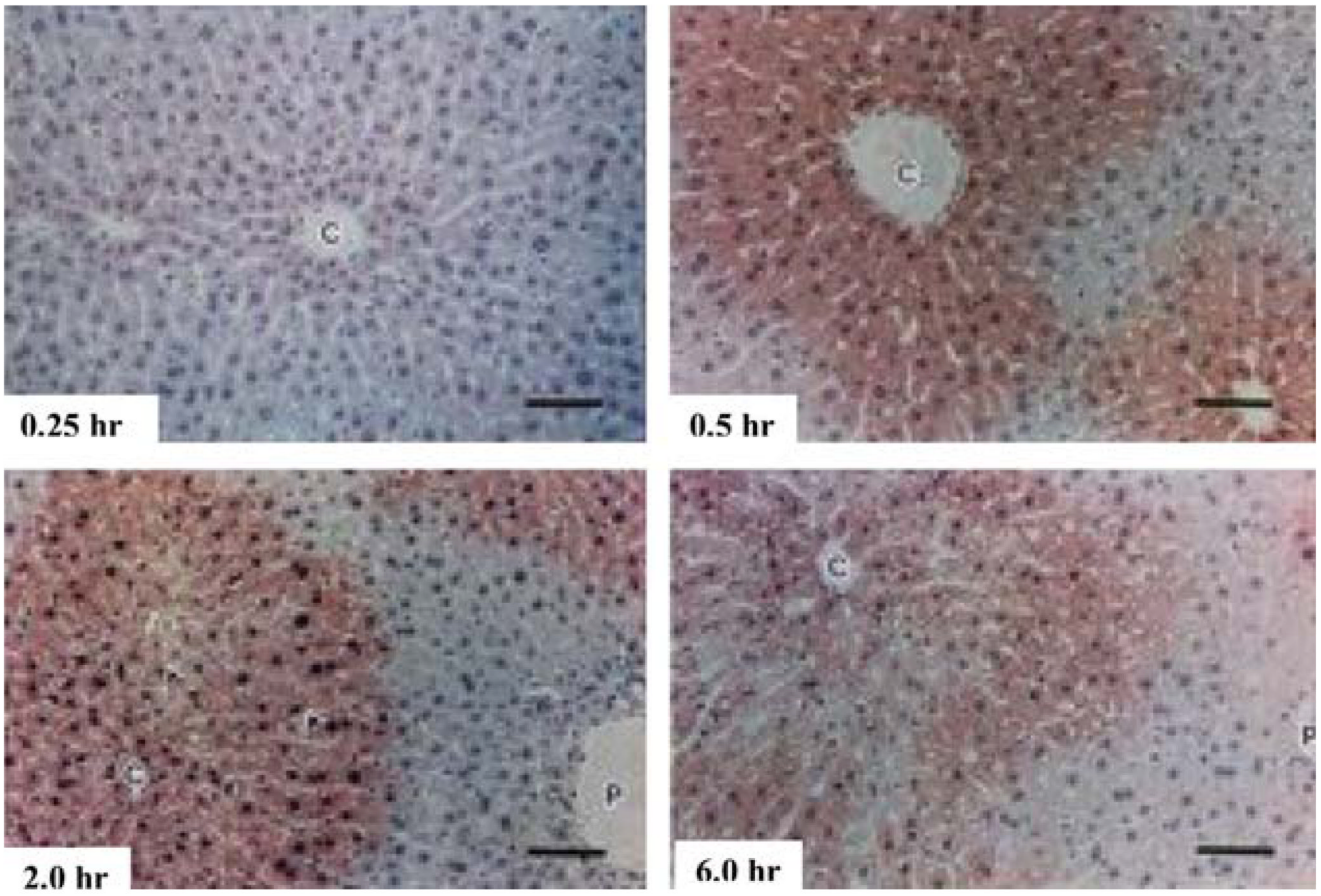

Fig. 1.

Immunohistochemical time course for acetaminophen protein adduct formation and hepatic necrosis in mice treated with a toxic dose of acetaminophen. The liver sections were immunochemically stained using antiacetaminophen-cysteine antiserum. The hepatocytes containing the adducts are stained *red*. Note the appearance of valcuoles and pycnotic nuclei indicative of necrosis only in the hepatocytes containing acetaminophen-protein adducts at 2 and 6 h. Staining was not observed in livers of saline treated mice (data not shown) (Roberts et al. 1991)

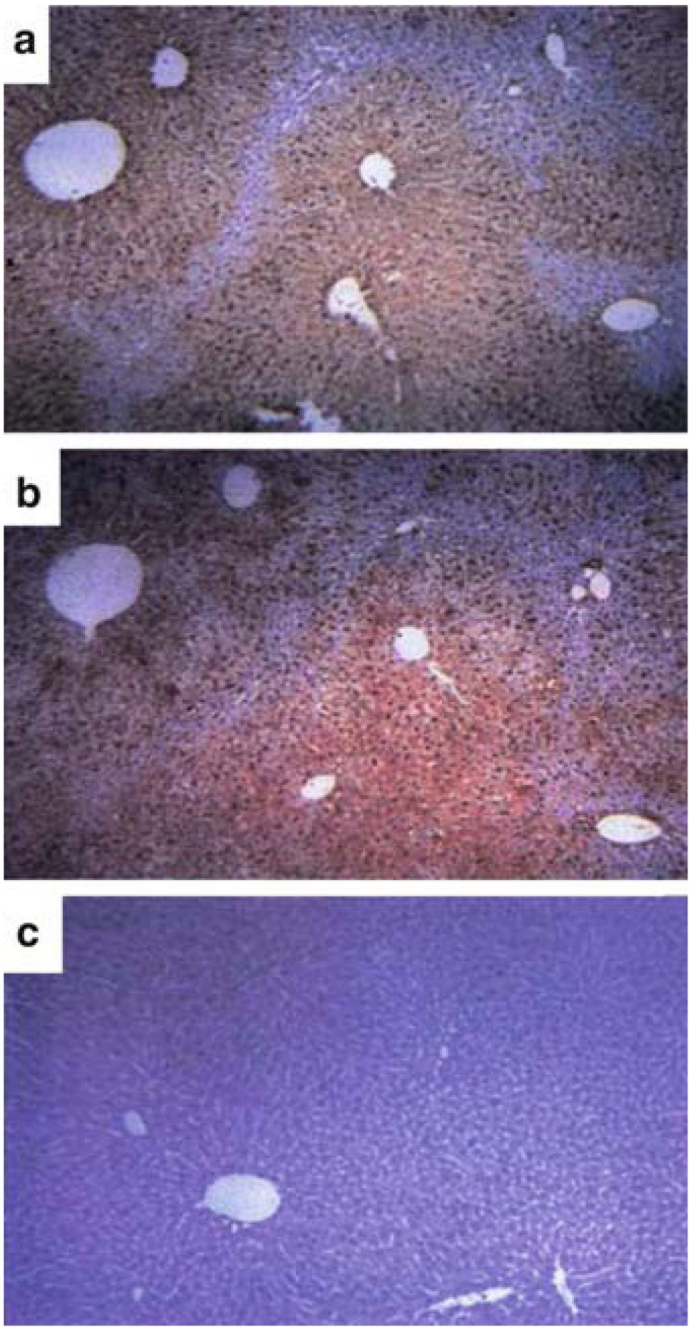

Fig. 2.

Immunohistochemical comparison of cellular localization of acetaminophen-protein adducts to nitrotyrosine in hepatic proteins of mice treated with a toxic dose of acetaminophen. Mice were treated with acetaminophen (300 mg kg^{-1}) or saline and livers removed at 4 h. (**a**) Liver section was immunochemically stained for acetaminophen protein adducts using an antiacetaminophen antiserum. (**b**) Liver section was immunochemically stained for nitrotyrosine in protein using an antinitrotyrosine antiserum. Note that the liver sections in (**a**) and (**b**) were adjacent sections from the same liver. (**c**) Liver section from a saline-treated mouse stained for nitroytrosine in protein using antinitrotyrosine antiserum (Hinson et al. 1998)

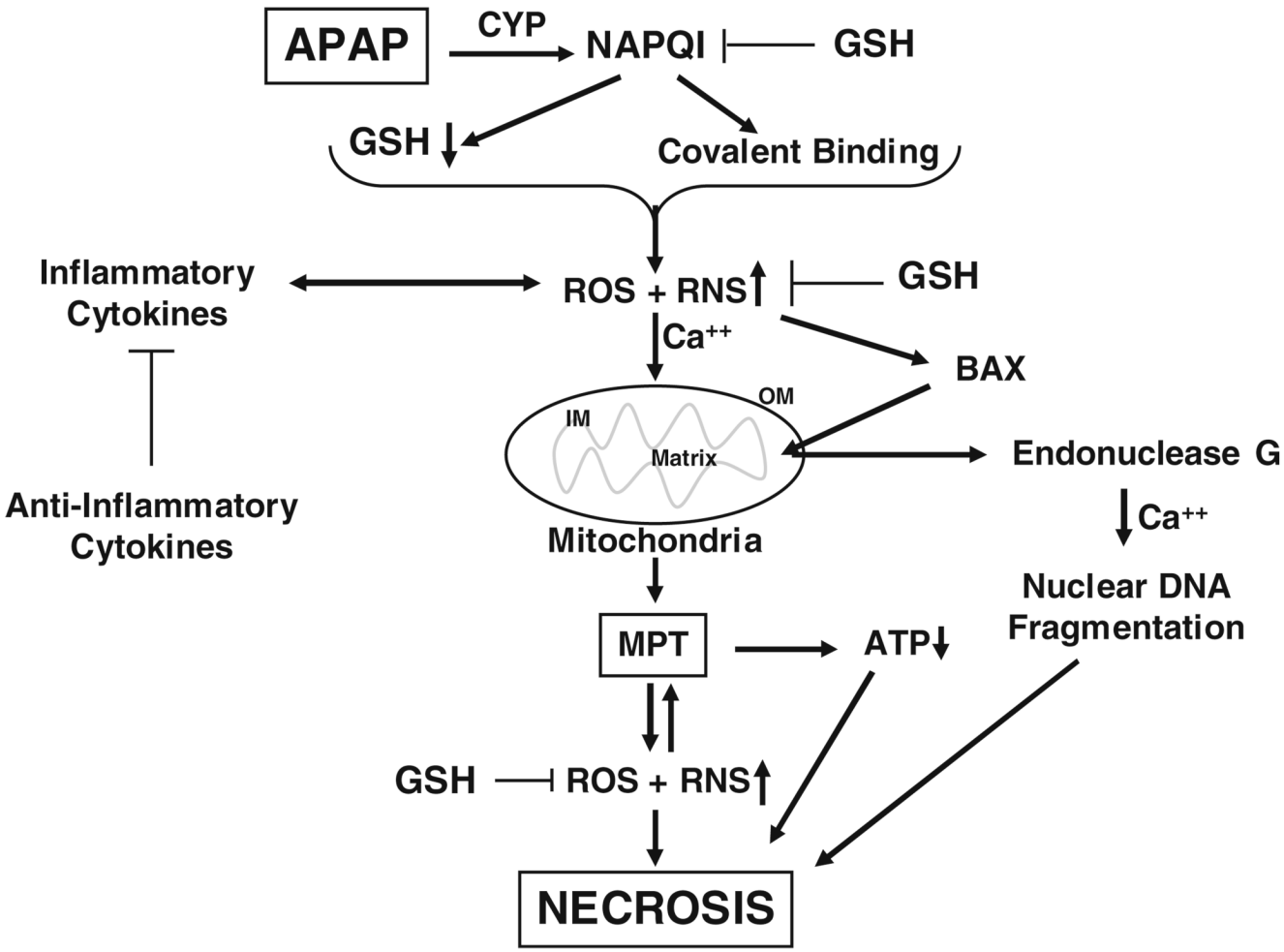

Fig. 3.

Mechanistic determinants in acetaminophen-induced hepatic necrosis. *APAP* Acetaminophen, *NAPQI* N-Acetyl-*p*-benzoquinone imine, *CYP* cytochrome P-450, *GSH* reduced glutathione, *ROS* reactive oxygen species, *RNS* reactive nitrogen species, *om* outer membrane, *im* inner membrane, *MPT* mitochondrial permeability transition, *BAX* Bcl-2-associated X protein

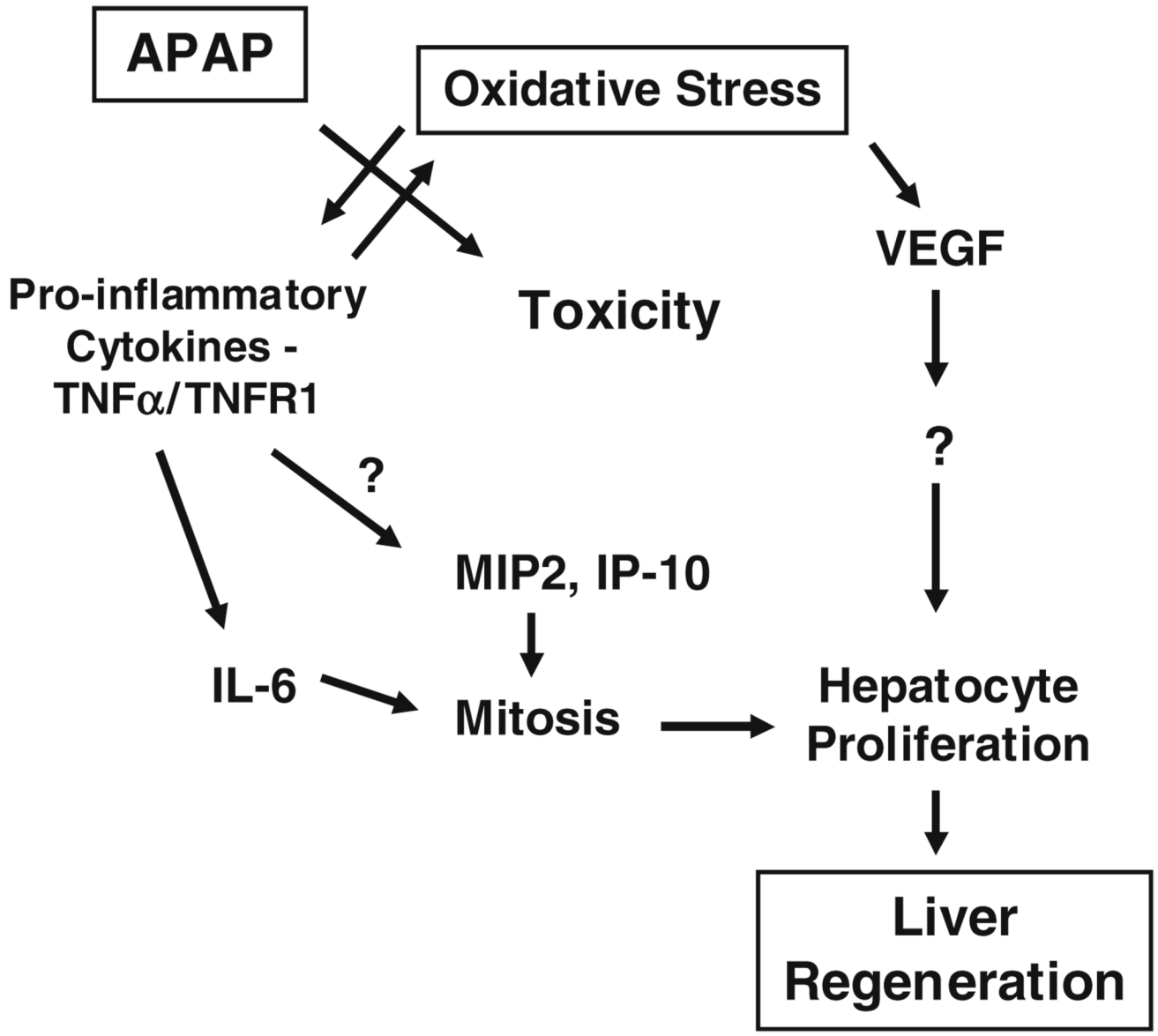

Fig. 4.

Mechanisms determinants in repair of acetaminophen-induced hepatic necrosis. *VEGF* Vascular endothelial growth factor, TNF-α tumor necrosis factor alpha, TNR1 tumor necrosis factor alpha receptor 1, MIP-2 macrophage inflammatory protein, *IP-10* Interferon-γ-inducible protein, *IL-6* Interleukin-6