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Acetaminophen-Induced Hepatotoxicity: Role of Metabolic Activation, Reactive Oxygen/Nitrogen Species, and Mitochondrial Permeability Transition

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ABSTRACT

Large doses of the analgesic acetaminophen cause centrilobular hepatic necrosis in man and in experimental animals. It has been previously shown that acetaminophen is metabolically activated by CYP enzymes to N-acetyl-p-benzoquinone imine. This species is normally detoxified by GSH, but following a toxic dose GSH is depleted and the metabolite covalently binds to a number of different proteins. Covalent binding occurs only to the cells developing necrosis. Recently we showed that these cells also contain nitrated tyrosine residues. Nitrotyrosine is mediated by peroxynitrite, a reactive nitrogen species formed by rapid reaction between nitric oxide and superoxide and is normally detoxified by GSH. Thus, acetaminophen toxicity occurs with increased oxygen/nitrogen stress. This manuscript will review current data on acetaminophen covalent binding, increased oxygen/nitrogen stress,

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and mitochondrial permeability transition, a toxic mechanism that is both mediated by and leads to increased oxygen/nitrogen stress.

Key Words: Acetaminophen; Peroxynitrite; Nitrotyrosine; Oxidative stress; Hepatotoxicity.

INTRODUCTION

At therapeutic doses acetaminophen is a very safe analgesic/antipyretic. However, in overdose acetaminophen may produce a fulminating hepatic necrosis that can be fatal (Bessems and Vermeulen, 2001; James et al., 2003a; Prescott, 2000). While many of the initial biochemical and metabolic events that occur in the early stages of toxicity have been known for many years, the mechanism by which hepatocytes die is still not understood.

Early work by Mitchell, Jollow, Gillette, and Brodie established the importance of metabolic activation in acetaminophen toxicity (Mitchell et al., 1973a). It was shown that in rodents acetaminophen was metabolized to a reactive metabolite by cytochrome P450 (Jollow et al., 1973; Potter et al., 1973). At therapeutic doses the metabolite was detoxified by glutathione conjugation, but following a large dose total hepatic glutathione was depleted by as much as 90% and the metabolite covalently bound to protein (Jollow et al., 1973, 1974). The covalently bound adduct was subsequently identified as an acetaminophen-cysteine adduct (Axworthy et al., 1988; Hoffmann et al., 1985). Covalent binding was shown to correlate with toxicity under a variety of conditions (Jollow et al., 1973). The reactive metabolite was identified to be the two electron oxidation product N-acetyl-p-benzoquinone imine (NAPQI) (Dahlin et al., 1984; Potter and Hinson, 1987). Its formation was not by N-hydroxylation (Hinson et al., 1979b) or 3,4-epoxidation (Hinson et al., 1977, 1979a) but by a direct two electron oxidation (Dahlin et al., 1984; Guengerich and Liebler, 1985; Potter and Hinson, 1987). Using purified CYP enzymes, it has been established that formation is by CYP 2E1 (Raucy et al., 1989), 1A2 (Raucy et al., 1989), 3A4 (Patten et al., 1993; Thummel et al., 1993), 2A6 (Chen et al., 1998), and 2D6 (Dong et al., 2000). Of these various enzymes, CYP2E1 may be the most important in human metabolism (Manyike et al., 2000).

Recent work in our laboratory indicates that not only is metabolic activation leading to GSH depletion and covalent binding an important mechanistic factor in acetaminophen toxicity, but formation of reactive oxygen/nitrogen species may play a decisive role in the toxicity. Mitochondrial permeability transition appears to be a key event. It is initiated by reactive oxygen/nitrogen stress and leads to additional oxygen/nitrogen stress. Mitochondrial permeability transition is a lethal event for the cell. This manuscript will review the relationship between toxicity, covalent binding, reactive oxygen, and nitrogen species, and mitochondrial permeability transition as it relates to acetaminophen toxicity.

PROTEIN ADDUCTS IN ACETAMINOPHEN HEPATOTOXICITY

Initial research to study acetaminophen protein adducts utilized radiolabeled protein. These data were used to establish quantitative relationships between adduct

formation and toxicity (Jollow et al., 1973). Autoradiography studies showed that covalent binding was localized in the necrotic cells (Jollow et al., 1973). The acetaminophen protein adduct was identified by mass spectral methods to be 3-(cystein-S-yl)acetaminophen (Axworthy et al., 1988; Hoffmann et al., 1985). To further understand the importance of acetaminophen covalent binding to protein, our laboratory developed specific antisera that specifically recognized the 3-(cystein-S-yl)acetaminophen protein adducts (Potter et al., 1989; Roberts et al., 1987). Using antisera, the relationship between acetaminophen covalent binding and toxicity in mice was studied. Immunohistochemical analyses showed that acetaminophen adducts developed only in the centrilobular hepatocytes, the site of the ensuing necrosis (Roberts et al., 1991). The hepatocytes in the periportal regions of the liver where toxicity does not occur were spared. Zonation of the toxicity is believed to be related to the localization of specific cytochrome P450 isoforms important in metabolism and in particular the role of CYP 2E1 (Hart et al., 1995). Adducts were observed as early as fifteen minutes following toxic doses of acetaminophen to mice and by one hour adducts reached a maximum level in all centrilobular hepatocytes. Subsequently, adduct intensity decreased as a result of the necrotic process (lysis) starting from the central vein and proceeding toward the periportal areas. Adduct loss from hepatocytes correlated with the appearance of cytosolic enzymes in the serum (Roberts et al., 1991). Competitive ELISAs were performed to determine the relative amounts of adducts under various conditions and in various fractions. Hepatic adducts peaked at 1 to 2 hr and subsequently declined with adducts appearing in the serum as a result of hepatocyte lysis. Adduct levels were determined to be highest in the plasma membrane with lesser amounts in the cytosol and mitochondria and the lowest level in the nucleus (Pumford et al., 1990b). Also, Western blot assays were performed to determine the molecular weight profile of adducts. Adducts were found to be in all subcellular fractions with the major adduct being cytosolic with a molecular weight of approximately 55 kDa. Importantly, it was shown that adducts appearing in the serum were of hepatic origin (Pumford et al., 1990a). Thus, analysis of serum was shown to serve as a source for determining specific hepatic proteins. In addition, Cohen, Khrairallah, and coworkers used a similar approach to study the relationship between acetaminophen toxicity and adduct formation (Cohen and Khairallah, 1997; Cohen et al., 1997).

A number of acetaminophen protein adducts have been identified. The initial approach was isolation and identification by sequence analysis (Cohen et al., 1997). These proteins which have been identified by this approach are listed in Table 1. The activities of two of the enzymes, N-10-formyltetrahydrofolate dehydrogenase (Pumford et al., 1997) and glutamate dehydrogenase (Halmes et al., 1995), were decreased approximately 20–25% in the acetaminophen intoxicated livers. More recently, laser desorption ionization mass spectrometry has been utilized to identify 20 additional proteins containing covalently bound acetaminophen (Qiu et al., 1998) (Table 1).

Even though covalent binding has been shown to be an excellent correlate of toxicity, there are a number of reports that suggest covalent binding per se does not produce toxicity. Henderson and coworkers examined acetaminophen toxicity and covalent binding in GSH transferase pi knockout mice (Henderson et al., 2000). N-Acetyl-p-benzoquinone imine is the best substrate ever reported for GSH transferase pi (Coles et al., 1988). They found that administration of a large dose of acetaminophen produced significant hepatotoxicity in wildtype mice, but the drug was only slightly

Table 1. Proteins that covalently bind acetaminophen.

Mass kDa	Fraction	Protein
16	Cytosol	Aryl sulfotransferase[b]
22	Cytosol, mitochondria	Glutathione peroxidase[b]
22	Cytosol, mitochondria	GSH peroxidase[b]
22	Cytosol (macrophages)	Osteoblast-specific factor 3[b]
23	Cytosol, mitochondria	Glutathione transferase π[b]
28	Mitochondria	House keeping protein[b]
28	Cytosol	Proteasome subunit C8[b]
29	Cytosol	Carbonic anhydrase III[b]
29	Cytosol, microsomes	Thioether *S*-methyltransferase[b]
29	Cytoskeleton	Tropomyosin 5[b]
32	Not known	Pyrophosphatase[b]
32	Cytosol	Glycine *N*-methyltransferase[b]
32	Cytosol	3-Hydroxyanthranilate 3,4-dioxygenase[b]
35	Peroxisomes	Urate oxidase[b]
36	Mitochondria, peroxisomes	2,4-Dienoyl-CoA reductase[b]
40	Cytosol	Sorbitol dehydrogenase precursor[b]
44	Microsomes	Glutamine synthetase[a]
45	Cytosol	Methionine adenosyl transferase[b]
46	Ribosomes	Protein synthesis initiation factor 4A[b]
50	Mitochondria	Glutamate dehydrogenase[a]
52	Cytosol	Selenium (acetaminophen) binding protein[a,b]
54	Mitochondria	Aldehyde dehydrogenase[a,b]
56	Mitochondria	Aldehyde dehydrogenase
59	Mitochondria	ATP synthetase α-subunit[b]
74	Nucleus	Lamin-A[a]
100	Cytosol	N-10 formyltetrahydrofolate dehydrogenase[a]

[a]Proteins identified by isolation and sequence analysis (Cohen et al., 1997).
[b]Proteins identified by mass spectrometry (Qiu et al., 1998).

toxic in the knockout mice. GSH was maximally depleted in the livers of the wildtype mice; however, the depletion was less in the knockout mice. Covalent binding of radiolabeled acetaminophen to the mice indicated significant levels in both the knockout mice and the wildtype mice (Henderson et al., 2000). Our laboratory recently examined the effect of early administration of the N-acetylcysteine following toxic doses of acetaminophen to mice (James et al., 2003c). N-Acetylcysteine leads to increased levels of GSH and is used as an antidote in overdose patients (Prescott and Critchley, 1983). We showed that administration of N-acetylcysteine to mice at 1 hour following a large dose of acetaminophen resulted in complete protection from toxicity; however, significant levels of acetaminophen covalently bound to protein were observed in the centrilobular hepatocytes of the liver. Administration of N-acetylcysteine at subsequent times did not protect from acetaminophen toxicity or covalent binding. Roberts and coworkers reported similar data (Salminen et al., 1998). Taken together, these findings suggest that acetaminophen adducts per se do not cause toxicity.

REACTIVE OXYGEN SPECIES IN ACETAMINOPHEN HEPATOTOXICITY

The importance of oxidative stress in acetaminophen hepatotoxicity is controversial. Since GSH is depleted in acetaminophen toxicity (Mitchell et al., 1973b; Roberts et al., 1991) the major peroxide detoxification enzyme, GSH peroxidase, would be expected to be inhibited. Thus, increased formation of superoxide, which is formed by a number of enzymatic reactions including acetaminophen oxidation to NAPQI by cytochrome P450, may lead to increased hydrogen peroxide and may initiate oxidative stress via a Fenton mechanism leading to lipid peroxidation (Dai and Cederbaum, 1995; Samokyszyn et al., 1988). By this mechanism ferrous ions react with peroxide(s) to form the hydroxyl radical. This radical is a potent oxidant and may oxidize lipids leading to lipid peroxidation as well as oxidize nucleic acids and proteins. These reactions are indicative of oxidative stress (Aust et al., 1985, 1986). Some investigators have reported that oxidative stress as measured by lipid peroxidation may be mechanistically important in acetaminophen hepatotoxicity (Noriega et al., 2000; Wendel and Feuerstein, 1981; Wendel et al., 1979). However, others have found no evidence for oxidative stress in acetaminophen toxicity (Knight et al., 2003; Lauterburg et al., 1984; Mitchell et al., 1981a,b; Smith and Jaeschke, 1989; Smith and Mitchell, 1985). Likewise, Younes and coworkers using ethane exhalation as a measure of lipid peroxidation found that acetaminophen toxicity did not increase ethane exhalation; however, prior glutathione depletion followed by a toxic dose of acetaminophen doubled ethane exhalation. In an attempt to further understand the role of oxidative stress in acetaminophen toxicity, they determined the effects of ferrous sulfate on acetaminophen toxicity. Ferrous sulfate did not increase ethane exhalation, but co-administration of ferrous sulfate plus acetaminophen produced a nine-fold increase in ethane exhalation. Prior glutathione depletion followed by a toxic dose of acetaminophen plus ferrous sulfate resulted in a twenty six-fold increase in ethane exhalation. However, histological analysis of livers indicated that ferrous sulfate did not increase the hepatotoxicity of acetaminophen (Younes et al., 1986). Thus, co-administration of ferrous sulfate with acetaminophen increased oxidative stress but had no effect on hepatotoxicity. Our laboratory developed an assay for protein oxidation (Keller et al., 1993) to determine its importance in acetaminophen toxicity. Using this assay, we did not find any evidence that protein oxidation was increased in acetaminophen toxicity (Gibson et al., 1996). Ferrous sulfate did not increase the degree of hepatotoxicity of acetaminophen, but did increase the rate of development of hepatotoxicity (Gibson et al., 1996).

Even though ferrous sulfate did not increase the toxicity of acetaminophen, a significant amount of research using iron chelators has suggested that iron may play a critical role in acetaminophen-induced hepatotoxicity. Administration of the iron chelator deferoxamine to acetaminophen intoxicated rats and mice has been shown to prevent or delay development of the hepatotoxicity (Nakae et al., 1988; Sakaida et al., 1995; Schnellmann et al., 1999). In addition, it has been shown in multiple labs that the iron chelators deferoxamine (Adamson and Harman, 1993; Gerson et al., 1985; Ito et al., 1994; Kyle et al., 1988; Nakae et al., 1988) and phenanthroline (Ito et al., 1994) dramatically decreases acetaminophen toxicity in isolated hepatocytes. Moreover, addition of ferrous or ferric salts to the hepatocyte incubations restored the sensitivity

of the hepatocytes to the toxic effects of acetaminophen (Kyle et al., 1987). Thus, these early studies were contradictory. Some studies suggested that oxidative stress played an important role in acetaminophen toxicity, while other studies indicated that oxidative stress was not important in acetaminophen toxicity.

REACTIVE NITROGEN SPECIES IN ACETAMINOPHEN HEPATOTOXICITY

In 1998, our laboratory showed nitrotyrosine residues in proteins of the centrilobular hepatocytes of acetaminophen treated mice (Hinson et al., 1998). Immunohistochemical analyses revealed that the nitrated tyrosine was localized in necrotic cells, the same cells that contained acetaminophen-protein adducts; however, acetaminophen-protein adducts occur before toxicity whereas nitration of tyrosine occurs at the same time as toxicity (Hinson et al., 1998, 2002). Nitrotyrosine is believed to be formed by reaction of tyrosine with peroxynitrite ($ONOO^-$), a species formed by a superoxide with nitric oxide at a rate near the diffusion controlled limit (7×10^9 $M^{-1}s^{-1}$) (Beckman, 1996; Pryor and Squadrito, 1995). Peroxynitrite is not only a nitrating agent, but it is also a potent oxidant and readily catalyzes both one- and two-electron oxidation reactions. It has been reported to react with lipids, proteins, and DNA and has been postulated to be important in various toxic effects (Pryor and Squadrito, 1995; Rubbo, 1998). We postulated that peroxynitrite was a toxic species in acetaminophen liver necrosis (Hinson et al., 1998, 2000). Moreover, peroxynitrite is normally detoxified by GSH/GSH peroxidase and GSH is depleted in acetaminophen toxicity (Sies et al., 1997). Thus, in the absence of GSH, detoxification of peroxynitrite was postulated to be impaired and this was a major factor important in the increased nitration.

We postulated that Kupffer cells (hepatic macrophages) were important in peroxynitrite formation (Hinson et al., 2000; Michael et al., 1999) since a number of laboratories had reported that compounds that suppress Kupffer cell function and activation (gadolinium chloride and dextran sulfate) decreased acetaminophen liver toxicity in both the rat and the mouse (Blazka et al., 1995; Goldin et al., 1996; Laskin et al., 1995). When activated, Kupffer cells release numerous cytokines and signaling molecules, including nitric oxide and superoxide which we envisioned would lead to increased peroxynitrite and tyrosine nitration. Kupffer cell inactivators would be expected to decrease peroxynitrite formation by this mechanism. As was reported by others, we found that pretreatment of mice with either gadolinium chloride or dextran sulfate decreased acetaminophen toxicity in the mouse (Michael et al., 1999). However, recent work from Pohl's laboratory indicated that this conclusion may be incorrect (Ju et al., 2002). They found that pretreatment of mice with gadolinium chloride resulted in only a partial decrease in hepatic Kupffer cells, but consistent with previous reports acetaminophen toxicity was decreased in the pretreated mice. However, they also found that pretreatment of mice with the suicide inactivator, dichloromethylene diphosphonate, completely eliminated the Kupffer cells, but hepatotoxicity was increased. These data suggest that the mechanism of the hepatotoxicity of acetaminophen was not dependent on the activation of Kupffer cells (Ju et al., 2002).

The importance of tyrosine nitration in acetaminophen toxicity was investigated in mice genetically deficient in inducible nitric oxide synthase (iNOS knockout mice) (Michael et al., 2001). Laskin laboratory had previously reported that iNOS was induced in the rat hepatocytes following acetaminophen (Gardner et al., 1998). An examination of the time course for development of acetaminophen toxicity in iNOS knockout mice and wildtype mice indicated that acetaminophen produced the same level of toxicity in the two groups of mice (Michael et al., 2001). However, there was much less nitration in the iNOS knockout mice and much less nitric oxide synthesis (serum nitrate plus nitrite). These data suggested that tyrosine nitration per se was not the mechanism of toxicity. However, the data clearly showed that acetaminophen induced nitric oxide synthesis and tyrosine nitration was mediated by iNOS (Michael et al., 2001). Further evidence that tyrosine nitration per se did not cause toxicity was obtained using the iNOS inhibitor aminoguanidine (Hinson et al., 2002). This inhibitor did not decrease acetaminophen induced hepatotoxicity; however, aminoguanidine did decrease tyrosine nitration and did decrease nitric oxide synthesis.

The interrelationship between oxidative stress and nitric oxide synthesis in acetaminophen toxicity has been investigated. We found that acetaminophen caused an increase in nitric oxide synthesis and in tyrosine nitration in two different mouse strains (Hinson et al., 1998, 2002; James et al., 2003c; Michael et al., 2001). In both cases there was no increase in oxidative stress (hepatic malondialdehyde levels did not increase). However, when the iNOS inhibitor aminoguanidine was co-administered with acetaminophen there was a decrease in nitric oxide synthesis, a decrease in tyrosine nitration, and a significant three-fold increase in hepatic malondialdehyde levels (Hinson et al., 2002). These data supported the chemical mechanism that nitric oxide reacts with superoxide to form peroxynitrite with nitration of tyrosine. In the absence of nitric oxide superoxide leads to oxidative stress. Studies in the iNOS knockout mice yielded similar results. Again, acetaminophen did not cause an increase in hepatic malondialdehyde levels in the wildtype mice but caused a significant increase in nitric oxide formation and tyrosine nitration. In the iNOS knockout mice, acetaminophen did not produce tyrosine nitration but caused a significant increase in malondialdehyde levels (oxidative stress) (Michael et al., 2001). However, the effect of acetaminophen on nitric oxide synthesis may be strain specific. Using a different mouse strain, Knight and coworkers found that acetaminophen did not increase nitric oxide synthesis (Knight et al., 2001), but there was an increase in tyrosine nitration. In this mouse strain neither acetaminophen nor acetaminophen plus aminoguanidine, caused a significant increase in hepatic malondialdehyde levels (Knight et al., 2003). Thus, nitric oxide synthesis may be highly influenced by strain differences in mice.

Laskin and coworkers reported that a toxic dose of acetaminophen induced iNOS in hepatocytes (Gardner et al., 1998, 2002). Also, we reported that toxic doses of acetaminophen increased nitric oxide synthesis in the mouse (serum levels of nitrate plus nitrite) (Hinson et al., 1998; Michael et al., 2001). The mechanism by which acetaminophen induced iNOS activity in hepatocytes is unclear, but it appears to involve both cytokines and oxidative stress. Oxidative stress and cytokines were shown to be important in iNOS induction in rat hepatocytes treated with benzenetriol, a compound that increased superoxide generation (Kuo et al., 1997a, 2000). These investigators found that benzenetriol alone did not induce nitric oxide synthesis. Interleukin-1 (IL-1β) did induce nitric oxide synthesis; however, in the presence of

IL-1β plus benzenetriol (oxidative stress) there was a very large increase in nitric oxide synthesis indicating the importance of oxidative stress in this mechanism. These investigators also examined the effect of acetaminophen in rat hepatocytes (Kuo et al., 1997b). Their data indicated that IL-1β induced nitric oxide synthesis and acetaminophen did not; however, in the presence of IL-1β plus acetaminophen there was an even larger induction of nitric oxide synthesis that occurred in an acetaminophen concentration-dependent manner over that observed with IL-1β alone. Acetaminophen toxicity in the mouse is associated with an increase in IL-1 (Blazka et al., 1995; James et al., 2003c). Thus, it appears that acetaminophen induction of nitric oxide synthesis may involve a cytokine effect and an oxidative stress effect; however, the rapidity of increased NO synthesis suggests that other factors may be involved.

The increased superoxide leading to increased tyrosine nitration in acetaminophen may be formed via a number of mechanisms (Sies and de Groot, 1992). Cytochrome P4502E1, the major enzyme in acetaminophen bioactivation, can form superoxide (Dai and Cederbaum, 1995; Koop, 1992; Raucy et al., 1993). We evaluated the potential role of NADPH oxidase in acetaminophen toxicity. This enzyme is associated with Kupffer cells, macrophages, or neutrophils, and is the enzyme associated with the respiratory burst. Activation of cells such as Kupffer cells in the liver would lead to reduction of oxygen producing superoxide on the outer surface of the plasma membrane (Knight, 2000) and this could be the source of the nitrated tyrosine. We thus examined acetaminophen toxicity, nitration, and GSH metabolism in mice genetically altered not to express Gp91 phox, a critical subunit of NADPH oxidase (James et al., 2003b). We found that NADPH oxidase knockout mice had similar toxicity to acetaminophen as wildtype mice at 4 hr and 24 hr. Also, the knockout mice and wildtype mice had comparable levels of hepatic tyrosine nitration. Lastly, we found that depletion of mitochondrial GSH was the same in the two groups (James et al., 2003b). From these data, we concluded that acetaminophen toxicity was not mediated by activation of NADPH oxidase. These data are consistent with the data from Pohl's laboratory that elimination of Kupffer cells by the suicide inactivator liposome-entrapped chlodronate did not decrease acetaminophen toxicity (Ju et al., 2002).

MITOCHONDRIAL PERMEABILITY TRANSITION IN ACETAMINOPHEN HEPATOTOXICITY

Mitochondrial permeability transition is postulated to be occurring in acetaminophen-induced hepatotoxicity. Mitochondrial permeability transition (MPT) is an abrupt increase in the permeability of the inner mitochondrial membrane to small molecular weight solutes. MPT occurs with membrane depolarization, uncoupling of oxidative phosphorylation, release of intramitochondrial ions and metabolic intermediates, and mitochondrial swelling. MPT also occurs with release of superoxide. MPT is a lethal event for the cell. We postulate that this is the source of superoxide leading to peroxynitrite and tyrosine nitration, and a critical, toxic event in the cell (Kim et al., 2003; Lemasters, 1998, 1999).

MPT is mediated by reactive nitrogen and oxygen species such as peroxynitrite and peroxide as well as certain ions such as Ca^{2+} and P_i. Mg^{2+}, ADP, acidic matrix pH, and

high membrane potential favor the closed state. Of particular note is that MPT is mediated by oxidant stress and MPT causes increased oxidant stress. Cyclosporin A specifically blocks the onset of MPT in a saturable manner implying a protein channel or pore. This pore is believed to transport both anionic and cationic solutes of mass less than 1500 Da and is the previously identified multiple conductance channel. The pore is believed to consist of three proteins: cyclophilin D (a cyclosporin A binding protein), the adenine nucleotide transporter, and the voltage-dependent anion channel. Oxidation of vicinal thiols in the pore promotes an open conductance state and can be blocked by dithiol reducing agents such as dithiothreitol (Kim et al., 2003; Lemasters, 1998, 1999).

Several studies indicate a role for MPT in acetaminophen hepatotoxicity. McLean and coworkers reported that administration of cyclosporine A decreased acetaminophen toxicity in rat liver slices and in vivo (Beales and McLean, 1996). Moldeus and Orrenius's laboratory found that addition of NAPQI to isolated rat liver mitochondria decreased synthesis of ATP and increases release of sequestered Ca^{2+}. This release was blocked by cyclosporin A (Weis et al., 1992, 1994). Dithiothreitol, a reducing agent that blocks MPT, has been shown to prevent toxicity of acetaminophen and N-acetyl-p-benzoquinone imine in cultured rat hepatocytes (Andersson et al., 1990; Birge et al., 1991; Nicotera et al., 1989; Porubek et al., 1987; Rundgren et al., 1988). Recently, Lemasters and coworkers, using cultured mouse hepatocytes, showed that cyclosporin A prevented toxicity. Also, it was shown, using the dyes calcein and tetramethylrhodamine methyl ester, that acetaminophen produced MPT and loss of mitochondrial membrane potential, respectively (Kon et al., 2004). Our laboratory has obtained similar data. Using the dye calcein and tetramethylrhodamine methyl ester we showed that acetaminophen toxicity in freshly isolated mouse hepatocytes occurs with MPT and loss of mitochondrial membrane potential (Reid et al., 2004).

ACETAMINOPHEN TOXICITY IN HEPATOCYTES OCCURS IN TWO PHASES

Research from Boobis's laboratory indicated that acetaminophen toxicity in freshly isolated hamster hepatocytes occurred by a two step mechanism. They showed that incubation of the freshly isolated hepatocytes with acetaminophen for 1.5 hrs resulted in minimal toxicity; however, GSH was depleted and acetaminophen covalently bound to protein. Subsequent washing the hepatocytes to remove acetaminophen and reincubation of the hepatocytes in media alone resulted in hepatocyte toxicity over the ensuing 4.5 hrs. Addition of dithiothreitol to the hepatocyte in the reincubation phase resulted in a decrease in toxicity (Boobis et al., 1986; Tee et al., 1986). Racz's laboratory presented similar data using mouse hepatocytes. They showed that GSH depletion and covalent binding occurred in the initial phase and toxicity occurred upon reincubation of hepatocytes in the absence of acetaminophen. Addition of dithiothreitol to the hepatocytes in the reincubation phase resulted in decreased toxicity (Grewal and Racz, 1993; Rafeiro et al., 1994). We have reinterpreted their data in light of current theories of MPT. We conclude that the two phases of acetaminophen represent a metabolic phase where acetaminophen is metabolized by CYP enzymes to NAPQI. This species reacts with GSH leading to its depletion and covalent binding to protein (metabolic phase). The second phase is an oxidative phase that occurs with MPT and

cell death (Fig. 1). Our laboratory investigated this hypothesis. We incubated freshly isolated mouse hepatocytes with acetaminophen for 2 hrs during which GSH depletion and covalent binding was shown to occur, but minimal toxicity was detected. Subsequently, the hepatocytes were washed twice to remove acetaminophen and reincubated in media alone. Toxicity (necrosis as determined by propidium iodide fluorescence and ALT release) occurred in the subsequent 3 hr reincubation of hepatocytes with media alone. Inclusion of cyclosporin A or dithiothreitol in the reincubation phase eliminated toxicity. These data are consistent with MPT occurring in the reincubation phase leading to cell toxicity (Reid et al., 2004). When the hepatocytes were incubated with acetaminophen for only one hour in the initial phase, washed free of acetaminophen, and reincubated with media alone, toxicity was not detected over the next 4 hr. Taken as a whole, the data are consistent with a hypothesis where acetaminophen induced liver toxicity occurs in two phases, an initial metabolic phase where NAPQI formation by CYP enzymes leads to GSH depletion and covalent binding, and a subsequent late oxidative phase where MPT occurs leading to toxicity (Fig. 1).

The mechanistic events leading to MPT are unclear. As discussed above, oxidation of critical dithiol at the MPT pore appears to be a prerequisite for MPT (Kim et al., 2003; Lemasters, 1998, 1999). The acetaminophen metabolite NAPQI could possibly initiate MPT. Other quinones have been reported to produce MPT by redox cycling and by non-redox cycling mechanisms (Henry and Wallace, 1995; Palmeira and Wallace, 1997). Since acetaminophen does not undergo redox cycling, this mechanism is not viable (Potter and Hinson, 1987; Ramakrishna et al., 1990), but NAPQI will oxidize thiols to disulfides and may initiate MPT (Tirmenstein and Nelson, 1990). However, the role of NAPQI in the direct production of MPT is questionable since the freshly isolated hepatocytes were washed to remove the acetaminophen (Boobis et al., 1986; Grewal and Racz, 1993; Rafeiro et al., 1994; Reid et al., 2004; Tee et al., 1986). Also, we recently found using freshly isolated mouse hepatocytes that addition of the iron chelator deferoxamine in the reincubation phase prevented toxicity (Reid et al., 2004). These data suggest that Fenton oxidation mechanisms are important in MPT and the toxicity. Moreover, these findings and the previous findings discussed above that iron chelators decrease acetaminophen toxicity in vivo as well as in cultured hepatocytes, suggest that the mechanism by which iron chelators inhibit acetaminophen toxicity is

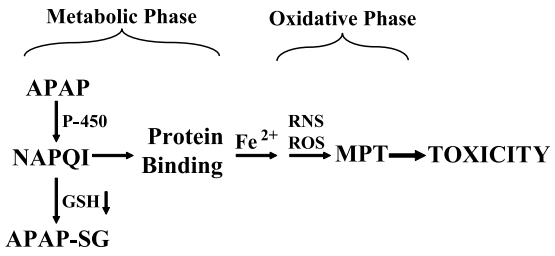

Figure 1. Postulated mechanism of acetaminophen (APAP) hepatotoxicity. RNS and ROS are reactive nitrogen species and reactive oxygen species, respectively. MPT is mitochondrial permeability transition.

by prevention of MPT. Thus, the data suggest a critical role for MPT in acetaminophen-induced liver necrosis in vivo.

POSTULATED MECHANISM OF ACETAMINOPHEN-INDUCED LIVER TOXICITY

From the data presented above, we postulate that acetaminophen toxicity occurs in two phases. The metabolic phase consists of CYP mediated formation of the reactive metabolite NAPQI. This metabolite is detoxified by GSH/GSH transferase leading to GSH depletion. This is a critical event leading to the second phase, the oxidative phase and toxicity. GSH depletion in the metabolic phase is postulated to impair detoxification of peroxides and peroxynitrite (Sies and de Groot, 1992). Various enzymatic reactions including mitochondria normally generate small amounts of superoxide which may lead to increased peroxide levels. The source of the presumed increase in iron levels in the Fenton oxidation reactions is unclear. Free iron in the cell is tightly controlled and is present primarily in ferritin (Lash and Saleem, 1995). One potential mechanism that may be important in increase in free iron levels in the hepatocytes is covalent binding of acetaminophen to an iron containing protein causing release of the iron either as free iron or as a heme iron. Unfortunately, no suitable candidate protein has yet been identified (Table 1). By the above mechanism, increased cytosolic iron coupled with increased peroxide would lead to increased Fenton oxidation and MPT. Since MPT occurs with release of superoxide once MPT is initiated, increased amounts of peroxide and peroxynitrite may be formed leading to additional MPT in other mitochondria in the same cell. Peroxynitrite would also nitrate tyrosine residues in cells undergoing MPT and cell death. In the absence of nitric oxide, the increased superoxide would lead to increased oxidative stress and lipid peroxidation. Thus, the toxicity is postulated to be mediated by GSH depletion and covalent binding leading to increased oxygen/nitrogen stress and MPT. This mechanism is presented in Fig. 1.

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