Effect of N-Acetylcysteine on Acetaminophen Toxicity in Mice: Relationship to Reactive Nitrogen and Cytokine Formation

Laura P. James,*†‡ Sandra S. McCullough,*† Laura W. Lamps,‡ and Jack A. Hinson†

Departments of *Pediatrics, †Pharmacology, and ‡Pathology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72202

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The relationship between acetaminophen (APAP) reactive metabolite formation, nitrotyrosine (NT) production, and cytokine elevation in APAP toxicity was investigated. Mice were dosed with 300 mg/kg of APAP and sacrificed at 1, 2, 4, 8, and 12 h. Serum aspartate aminotransferase (AST) was elevated by 4 h. The relative amount of NT correlated with toxicity and was localized in the necrotic centrilobular cells. IL-1b was increased at 1 h, whereas IL-6, MIP-2, and MCP-1 were increased by 4–8 h. To determine the importance of the downstream events, N-acetylcysteine (NAC) was administered to mice either before APAP or 1, 2, or 4 h after APAP. The animals were sacrificed at 12 h. NAC treatment before APAP resulted in serum AST, serum nitrate plus nitrite as a measure of nitric oxide (NO) production, and hepatic cytokine levels that were similar to the controls. No APAP protein adducts or NT was present in these animals. In mice treated with NAC at 1 h, cytokines and serum AST were normal at 12 h, but APAP protein adducts were present in the hepatic centrilobular areas. No NT was present in these animals. In mice treated with NAC at 2 h and sacrificed at 12 h, serum AST was reduced by 80%, APAP adducts and NT were present in the centrilobular areas. Mice receiving NAC at 4 h had no protection from toxicity and serum nitrate plus nitrite. The NT and cytokine levels were similar to those of mice receiving APAP alone. The data suggest a relationship between metabolic events in APAP toxicity and the upregulation of NO and IL-1b. IL-6, MIP-2, and MCP-1 appear to follow the toxicity. While it is a pre-requisite event, covalent binding per se does not appear to be a toxic event in the development of toxicity.

Recent data have indicated that reactive oxygen and nitrogen species may play a role in the mediation of acetaminophen (APAP) hepatotoxicity. Our laboratory reported that nitrotyrosine formation and increased nitric oxide (NO) levels occurred in mice treated with toxic doses of APAP (Hinson et al., 1998, 2000; Michael et al., 1999, 2001). Nitrotyrosine colocalizes with APAP protein adducts in the necrotic centrilobular regions of the liver. We have previously postulated that increased nitrotyrosine in APAP toxicity is through peroxynitrite, a reactive intermediate formed by the rapid reaction of superoxide and NO (Beckman, 1996; Hinson et al., 1998). Peroxynitrite is normally detoxified by glutathione (GSH), which is depleted in APAP toxicity. Increased NO in the liver occurs through the induction of inducible nitric oxide synthase (iNOS), which has been shown to be upregulated in the APAP-treated rat (Gardner et al., 1998). The release of many cytokines in association with APAP toxicity has also been reported (Bauer et al., 2000; Blazka et al., 1995; Lawson et al., 2000). Upregulation of TNF-α and IL-1 in APAP toxicity was initially reported by Blazka et al. (1995). More recent reports have described the upregulation of various chemokines such as MIP-2 and MCP-1 in APAP toxicity (Hogaboam et al., 1999, 2000).

N-Acetylcysteine (NAC) is the standard therapy for treatment of the APAP overdose patient. The primary role of NAC in the treatment of APAP toxicity is thought to be the replacement of intracellular stores of hepatic GSH (Corcoran and Wong, 1986; Lauterburg et al., 1983), which allows for detoxification of the electrophile, N-acetyl-benzoquinone-imine (NAPQI). In this study, we examined reversible versus toxic events in the toxicity by administering the antidote NAC to mice before APAP and at various times after APAP.

MATERIALS AND METHODS

Reagents. Acetaminophen (APAP, paracetamol) was obtained from Sigma Chemical Co. (St. Louis, MO). Universal DAKO LSAB + (Labeled Streptavidin-Biotin) Peroxidase kits and DAKO protein block (serum-free) were acquired from DAKO Corporation (Carpinteria, CA). Immunoperoxidase Suppressor and Coomassie Plus Protein Assay Reagent were purchased from Pierce Chemical Co. (Rockford, IL). Gills Hematoxylin II and Permount were both from Fisher Scientific, Inc. (Pittsburgh, PA).

Animals. Six-week-old male B6C3F1 mice (mean weight, 26.5 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). All animal experimentation was in accordance with the criteria of the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences. Protocols for animal experimentation were approved by the University of Arkansas for Medical Sciences Animal Care and Use Committee. The mice were acclimatized one week prior to the planned experiments. They were fed ad libitum and were housed in individual cages on a 12-h light/dark cycle. On the day prior to the experiments, the mice were fasted overnight and dosing studies began at 0800 h the following morning. In the initial experiments, the
mice (n = 5) were dosed with APAP (300-mg/kg IP in saline) and sacrificed at 1, 2, 4, 8, and 12 h after APAP. In subsequent experiments, the mice were dosed with APAP (300-mg/kg IP in saline) and received NAC (1200-mg/kg IP in saline, pH 7.4) either immediately prior to APAP or at 1, 2, 4, or 12 h after APAP. The control mice received saline only. In these experiments, the mice were sacrificed at 12 h after APAP. At the indicated time, the animals were anesthetized with CO2. Blood was removed from the retro-orbital plexus and allowed to coagulate at room temperature. The blood was centrifuged, and the serum was removed for measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The mice were then euthanized with CO2 and the livers removed. The livers were weighed, and a portion was preserved in formalin for histological sections. The remaining livers were snap-frozen in liquid nitrogen and stored at –80°C.

Analyses. Serum AST and ALT were determined with a spectrophotometric diagnostic kit (Sigma Chemical Company, St. Louis, MO). Serum nitrate plus nitrite levels (a measure of NO synthesis) were determined by precipitation of protein, reduction of the nitrate to nitrite by cadmium, and quantitation of nitrite using the Griess reagent (Oxford Biomedical kit NB 88). Nonprotein sulfhydryls were measured by a colorimetric method using Ellman’s reagent as previously modified by Mitchell et al. (1973). Liver samples were analyzed for nitrotyrosine by an ELISA method using the kit from Oxis Research (Portland, OR) as per the manufacturer’s instructions.

Cytokine and chemokine assays. Cytokines (IL1-β, IL-6, MIP-2, MCP-1) were measured in the supernatants of liver homogenates using ELISA kits, as per the manufacturer’s instructions (R & D, Minneapolis, MN). Snap-frozen liver samples were thawed, weighed, and homogenized in solutions containing 1 ml of protease inhibitor cocktail (Complete; Boehringer Mannheim, Indianapolis, IN). The resulting supernatants were analyzed in duplicate and standardized to the weight of the homogenized liver sample.

Immunohistochemical analyses. Acetaminophen-protein adducts and nitrotyrosine adducts in the liver were analyzed by immunohistochemical procedures using rabbit antisera raised against 4-aminooxidobenzoic acid-CLH and 4-hydroxy-3-nitrobenzoic acid-CLH, respectively. Fresh liver tissues, previously trimmed to approximately 2-mm thickness, were placed in plastic cassettes and immersed in neutral buffered formalin for 24 h. Paraffin-embedded tissue sections were dewaxed in xylene (2 × 5 min, 25°C) and then rehydrated in a series of graded ethanol washes and deionized water. The sections were then placed in a Pierce Immunopure peroxidase suppressor for 5 min, 25°C. The sections were incubated with goat anti-acetaminophen antiserum (Matthews et al., 1989) or a polyclonal anti-3 nitrotyrosine antibody (Hinson et al., 1996) (1:500) or a polyclonal anti-3 nitrotyrosine antibody (Hinson et al., 2000) (data not shown). The adducts were localized in the centrilobular areas, as reported in Hinson et al., 1998, 2000; Michael et al., 1999, 2001). The regions that stained for nitrotyrosine corresponded to regions of centrilobular necrosis present by hematoxylin and eosin (H&E) stained slides, as reported in Hinson et al. (1998, 2000) and Michael et al. (1999, 2001). Elevation of nitrotyrosine in the centrilobular regions at 4, 8, and 12 h.

Elevations of Cytokines and Chemokines in the Mouse Liver

Elevations of inflammatory cytokines have been reported in association with APAP toxicity (Bauer et al., 2000; Blazka et al., 1995; Hogaboam et al., 1999a,b, 2000; Lawson et al., 2000). ELISA measurements of cytokines in the supernatants of homogenized liver samples revealed significant elevations of IFN-γ at 1 h (Fig. 2A), IL-6 at 4 h (Fig. 2B), MIP-2 at 8 h (Fig. 2C), and MCP-1 at 4 h (Fig. 2D). Elevation of IL-1β appeared early in the development of toxicity, while increased formation of IFN-γ, MIP-2, and MCP-1 appeared to be later events.

Effect of NAC on Acetaminophen Toxicity and Nonprotein Sulfhydryls

To determine the significance of reversible versus toxic events important in APAP toxicity, the mice were dosed with APAP 300-mg/kg IP and treated with NAC at varying times. The mice were sacrificed at 12 h because this has been shown previously to be a time when toxicity is maximal and APAP covalent binding has reached a minimal level as a result of hepatocyte lysis (Pumford et al., 1989). Mice received NAC as a pretreatment immediately prior to APAP or 1, 2, or 4 h after APAP. Previous data have demonstrated that covalent binding is maximal at 1 to 2 h after APAP dosing in the mouse (Pumford et al., 1989). Some animals received saline instead of NAC. As demonstrated in Figure 3, treatment with NAC resulted in significant reduction of hepatotoxicity as measured by serum AST (Fig. 3A) and ALT (data not shown). When administered as a pretreatment or at 1 h after APAP, NAC
prevented APAP toxicity (Fig. 3A). The mice that received NAC at 2 h had a reduction of AST by 80%. However, NAC was not effective in preventing hepatotoxicity in animals treated 4 h after APAP. Histopathological examination of H & E liver sections of NAC-treated mice was consistent with the AST and ALT values (data not shown).

A primary mechanism for the protective effect of NAC in APAP toxicity is the restoration of hepatic nonprotein sulfhydryls (Corcoran and Wong, 1986; Lauterburg et al., 1983). As indicated in Figure 3B, nonprotein sulfhydryl concentrations in the livers of the NAC-treated animals were elevated above those of the control animals. The APAP-only animals and animals that received NAC at 4 h had nonprotein sulfhydryls that were approximately 60–70% of the saline controls. The standard errors were large in the APAP-only group, indicating biologic variability in the rebound of GSH in these animals.

**Effect of NAC on Nitric Oxide Synthesis and Nitrotyrosine Formation**

Pretreatment with NAC and NAC treatment at 1 and 2 h after APAP lowered serum nitrate plus nitrite levels to values similar to the control animals (Fig. 3C). However, the animals receiving NAC at 4 h had increased NO formation. Similarly, nitrotyrosine levels in the animals that received NAC as a pretreatment or at 1 h were not different from the control levels (Fig. 3D). However, the mice treated with NAC at 2 and 4 h had increased levels of nitrotyrosine.

**Effect of NAC on Immunohistochemical Analyses**

Immunohistochemical analyses for nitrotyrosine were performed on liver tissues to further examine the effect of NAC on toxicity and to allow for immunohistochemical comparisons.
between APAP adduct formation and nitrotyrosine formation. Treatment of the animals with NAC resulted in decreased nitrotyrosine formation (Figs. 4B–4D). The animals pretreated with NAC (Fig. 4B) and those that received NAC 1 h (Fig. 4C) after APAP had no staining for nitrotyrosine, similar to the control animals (Fig. 4A). However, the animals that received NAC 2 h (Fig. 4D) after APAP had mild centrilobular staining for nitrotyrosine, and the animals that received NAC 4 h after APAP (Fig. 4E) had an even greater amount of nitrotyrosine staining in the centrilobular and midzonal regions. The nitrotyrosine was localized in the necrotic cells. These data are consistent with previous studies reporting the association of nitrotyrosine and toxicity (Hinson et al., 1998, 2000; Michael et al., 2001).

No staining for APAP protein adducts (Fig. 4H) or nitrotyrosine (Fig. 4B) was present in the animals that were pretreated with NAC. In the mice that received NAC at 1 h, APAP protein adducts (Fig. 4I) were present, but no staining for nitrotyrosine was apparent (Fig. 4C). These animals did not develop toxicity, suggesting that nitrotyrosine may be a more sensitive indicator of toxicity than APAP protein adduct formation in the mouse. In contrast, the animals that received NAC at 4 h and developed toxicity had comparable levels of nitrotyrosine (Fig. 4E) and APAP protein adducts (Fig. 4K). The animals treated with NAC at 2 h represented an intermediate group with respect to the development of toxicity, nitrotyrosine formation (Fig. 4D), and APAP protein adducts (Fig. 4J).

**Effect of NAC on Cytokine Formation**

Treatment with NAC resulted in significant changes in the hepatic levels of IL-1β, IL-6, MIP-2, and MCP-1 in the APAP-treated mice (Fig. 5). The animals that received NAC, either as a pretreatment or at 1 h after APAP, had hepatic IL-1β values...
similar to the controls (Fig. 5A). IL-1β levels were elevated in
the animals that received NAC at 2 and 4 h after APAP (Fig.
5B). These data are consistent with relatively early upregula-
tion of IL-1β in APAP toxicity. In contrast, the levels of IL-6,
MIP-2, and MCP-1 (Figs. 5B and 5C) remained low in the
mice that received NAC as a pretreatment and at 1 and 2 h.
These data suggest that the increased formation of IL-6, MIP-2,
and MCP-1 follow the onset of toxicity and are likely consis-
tent with their role in hepatocyte repair.

**DISCUSSION**

The primary objective of the present study was to deter-
mine the importance of reversible versus toxic events that
may be important in reactive nitrogen formation and cyto-
kine production in APAP toxicity. To this end, APAP was
administered to mice, and the time course for the formation
of covalent binding, reactive nitrogen formation, and cyto-
kine production was determined relative to toxicity. Subse-
quently, NAC was administered at early times following
APAP, and the effect of each of the above parameters was
determined at 12 h relative to toxicity. Twelve h was chosen
as a time point because of previous data showing that
maximal toxicity by histological evaluations is present by
12 h and that covalent binding has reached a minimum at
this time point as a result of hepatocyte lysis (Pumford et al.,
1989). NAC is known to replenish nonprotein sulfhydryls,
FIG. 4. Effect of NAC on nitrotyrosine and acetaminophen protein adduct formation. Mice were treated with APAP and sacrificed at 12 h. Some mice received NAC at varying times. Liver sections were stained for nitrotyrosine (A–F) and acetaminophen protein adducts (G–L). (40 ×) (A) Saline-treated mouse stained for nitrotyrosine. (B) APAP-treated mouse that was pre-treated with NAC and stained for nitrotyrosine. (C) APAP-treated mouse that received NAC at 1 h stained for nitrotyrosine. (D) APAP-treated mouse that received NAC at 2 h stained for nitrotyrosine. (E) APAP-treated mouse that received NAC at 4 h stained for nitrotyrosine. (F) APAP-treated mouse stained for nitrotyrosine. (G) Saline-treated mouse stained for acetaminophen protein adducts. (H) APAP-treated mouse that was pre-treated with NAC and stained for acetaminophen protein adducts. (I) APAP-treated mouse that received NAC at 1 h stained for acetaminophen protein adducts. (J) APAP-treated mouse that received NAC at 2 h stained for acetaminophen protein adducts. (K) APAP-treated mouse that received NAC at 4 h stained for acetaminophen protein adducts. (L) APAP-treated mouse stained for acetaminophen protein adducts.
Thus allowing more substrate for the detoxification of NAPQI (Corcoran and Wong, 1986; Corcoran et al., 1985; Lauterburg et al., 1983). Consistent with previous data (Mitchell et al., 1973; Roberts et al., 1991), nonprotein sulfhydryls were maximally depleted by 1 h following APAP administration (Fig. 1B), and significant covalent binding had occurred by this time (Fig. 4I) (Pumford et al., 1989). In agreement with previous reports, (Corcoran and Wong, 1986; Lauterburg et al., 1983), NAC replenished hepatic nonprotein sulfhydryl concentrations (Fig. 3B). Administration of NAC either immediately before APAP or at 1 or 2 h following APAP resulted in high levels of nonprotein sulfhydryls that remained elevated at 12 h (Fig. 3B). Also, consistent with previous reports (Corcoran et al., 1985), NAC prevented toxicity when administered prior to or early following APAP. Pretreatment of mice with NAC or treatment with NAC at 1 h completely prevented hepatotoxicity (Fig. 3A), while administration at 2 h only partially prevented toxicity and at 4 h NAC had no effect on toxicity. Thus, toxic events in APAP toxicity in the mouse occurred before 2 h. The efficacy of NAC in relationship to its time of administration relative to overdose in the clinical setting has been well established in APAP overdose patients (Schmidt et al., 2002; Smilkstein et al., 1988).

Analysis of covalent binding of APAP to protein indicated significant levels of covalent binding by 1 h after APAP. These data are consistent with previous reports (Pumford et al., 1989; Roberts et al., 1991). Administration of APAP and sacrifice of the animals at 12 h resulted in significant necrosis in the centrilobular areas, and, consistent with previous data, the covalent binding was localized in the necrotic areas (Fig. 4L) (Roberts et al., 1991). When NAC was administered at 1 h and the mice were sacrificed at 12 h, toxicity was not observed (Fig. 3), but covalent binding was still substantial (Fig. 4I). Since administration of NAC at 1 h completely prevented toxicity (Fig. 3A), the data suggest that, while formation of

**FIG. 5.** Effect of NAC on cytokine formation. Mice were dosed with acetaminophen (300-mg/kg IP) and received NAC as a pretreatment or at 1, 2, or 4 h after APAP. All animals were sacrificed at 12 h. (A) IL-1β remained low in animals that received NAC as a pretreatment or at 1 h after acetaminophen. (B) IL-6 remained low in animals pretreated with NAC or those receiving NAC at 1, 2, and 4 h after APAP. (C) MIP-2 remained low in animals that received NAC as a pretreatment or 1 or 2 h after APAP. (D) MCP-1 remained low in animals that received NAC as a pretreatment or at 1 h or 2 h after APAP. *p < 0.05 as compared to saline treated animals.
APAP protein adducts are a prerequisite event in the development of toxicity, covalent binding per se does not lead to toxicity. Consistent with our data, Salminen et al. (1998) demonstrated that NAC at 1 h did not change the amount of covalent binding or affect the induction of heat shock proteins but did ameliorate toxicity.

Nitrotyrosine, a marker of reactive nitrogen formation, has been previously reported to co-localize with APAP protein adducts in the necrotic centrlobular areas of the liver (Hinson et al., 1998, 2000; Michael et al., 2001). We previously postulated that elevated nitrotyrosine in APAP toxicity is from peroxynitrite, a reactive intermediate formed by the rapid reaction of superoxide and NO, that is normally detoxified by GSH, which is depleted in APAP toxicity (Hinson et al., 1998). The time course study indicated that nitrotyrosine was significantly elevated over control levels at 4 h and remained elevated at 12 h (Fig. 1D). At 12 h, the nitrotyrosine was localized in the necrotic centrilobular cells (Fig. 4F). When NAC was administered as a pretreatment (Fig. 4B), or when it was administered at 1 h after APAP (Fig. 4C), nitrotyrosine was not observed in the centrilobular hepatocytes. When NAC was administered at 2 h (Fig. 4D) or 4 h (Fig. 4E), nitrotyrosine was observed in the necrotic cells. The data clearly show that nitrotyrosine is a correlate of APAP-induced hepatocellular necrosis.

Consistent with previous reports, the present study shows that APAP increases serum levels of nitrate plus nitrite, a marker of NO synthesis (Hinson et al., 1998; Michael et al., 2001). Increased NO formation occurs through upregulation of iNOS, which has been described in a rat model of APAP hepatotoxicity (Gardner et al., 1998). In the present study, serum levels of nitrate plus nitrite were elevated at 1 h after APAP; however, the mechanism is unclear. In this regard, Kuo et al. (1997) previously showed that APAP alone did not increase NO synthesis in isolated rat hepatocytes; however, NO synthesis increased with the addition of IL-1β to hepatocytes. In the presence of IL-1β, APAP caused a concentration-dependent increase in NO synthesis. It was thus postulated that NO was dependent on the presence of IL-1β, and APAP-induced oxidative stress was thought to be important. The data of the present study are consistent with that postulation. In the present study, IL-1β was increased significantly in APAP-treated mice at 1 h and was the only cytokine studied that had an early elevation (Fig. 2A). Blazka et al. (1995, 1996) also postulated that IL-1 is of importance in the mediation of toxicity and showed that modulation of IL-1 levels in the APAP-treated mouse was hepatoprotective.

We also examined the effect of APAP on hepatic levels of IL-6, MIP-2, and MCP-1. In contrast to IL-1β, the levels of IL-6, MIP-2, and MCP-1 were not significantly altered until at least 4 h (Fig. 2), a time when significant increases in AST levels were observed. These cytokine elevations followed earlier events important in the toxicity such as hepatic nonprotein sulhydryl depletion (Fig. 1B) and elevation of serum nitrate plus nitrite (Fig. 1C). To determine the importance of reversible versus toxic events that may alter cytokine production, the effect of NAC was examined. NAC has previously been demonstrated to decrease cytokine production in murine models of inflammation (Barrett et al., 1999; Peristeris et al., 1992). The responses of IL-6, MIP-2, and MCP-1 to NAC, as well as the time course data, suggest that these cytokines are formed in response to toxicity. Pretreatment with NAC and treatment at 1 and 2 h diminished the elevation of IL-6, MIP-2, and MCP-1. While MIP-2 and MCP-1 are both chemokines, and thus are usually associated with neutrophil migration, a previous study showed that neutrophil accumulation in APAP-treated mice followed the elevation of hepatic transaminases (Lawson et al., 2000). Moreover, several reports have addressed the role of MIP-2 and MCP-1 in hepatocyte regeneration and repair in APAP toxicity (Hogaboam et al., 1999a,b, 2000). It has been suggested the MCP-1 plays a role in maintaining balance between pro-inflammatory cytokines (TNF-α and IFN-γ) and anti-inflammatory cytokines in APAP toxicity. Hogaboam et al. (2000) showed that mice that were deficient in the receptor for MCP-1 had higher levels of pro-inflammatory cytokines and more pronounced toxicity than wild type mice treated with APAP. Our data are consistent with these earlier studies.

IL-6 is a pleotropic cytokine that was initially recognized for its regulation of acute protein synthesis and the acute inflammatory response (Castell et al., 1990; Gauldie et al., 1992). IL-6 has been reported to be upregulated in several animal models of hepatic injury (Hoebe et al., 2001; Luckey and Petersen, 2001; Matuschak et al., 2001). Recent studies suggest that one role of IL-6 is the mediation of hepatocyte repair. This has been demonstrated in models of carbon tetrachloride toxicity and partial hepatectomy (Cressman et al., 1996; Koválovich et al., 2000, 2001). In the present study, significant elevation of IL-6 occurred relatively late in the development of toxicity, similar to the responses of MIP-2 and MCP-1. Similarly, in NAC-treated animals, IL-6 remained low, suggesting that it may also be important in initiating hepatocyte regeneration in APAP toxicity. Our data are similar to those of Blazka et al. (1995), who failed to detect upregulation of mRNA for IL-6 at early time points following APAP dosing to mice.

The mechanism of nitrotyrosine formation in APAP toxicity is unclear. Previously we postulated the involvement of peroxynitrite, a species formed by the rapid reaction of NO with superoxide that will nitrate proteins. Peroxynitrite was an ideal candidate for the nitrating species because it was readily detoxified by GSH and GSH is depleted in APAP toxicity (Radi et al., 1991). Oxidation of nitrite by metals or by myeloperoxidase to form the NO2 radical has been suggested as important in the nitration of tyrosine in some systems (Davis et al., 2001; Thomas et al., 2002). Since neutrophil migration is a late event in APAP toxicity (Lawson et al., 2000; Roberts et al., 1991), presumably myeloperoxidase oxidation of nitrite is not the mechanism of nitration.

In our initial report of nitrated tyrosine in necrotic cells
(Michael et al., 1999), we postulated the involvement of Kupffer cells based on previous reports showing that pretreatment with the Kupffer cell activator, gadolinium chloride, decreased APAP toxicity (Laskin et al., 1995). Kupffer cells are known to produce increased amounts of NO and superoxide. However, in a more recent study, we reported that NADPH knockout mice (which do not produce phagocytic superoxide) and wild type mice were equally susceptible to APAP and had similar amounts of nitrosated tyrosine in the centrilobular areas (James, Mayeux, in press; James, McCullough, in press). Also, data from Pohl’s laboratory showed that liposome/clodronate pretreatment completely depleted Kupffer cells in the liver and did not decrease toxicity (Ju et al., 2002). Thus, a role for Kupffer cells in APAP toxicity is questionable. Alternatively, mitochondrial permeability transition (MPT) may be an important event leading to increased superoxide and peroxynitrite (James, Mayeux, in press). MPT is known to be a lethal event in hepatocytes (Lemasters et al., 1998). A postulated role for MPT is consistent with previous in vitro data showing that the addition of the toxic metabolite NAPQI to isolated rat mitochondria caused calcium release and oxidation of reduced pyridine nucleotides. These effects could be prevented by cyclosporin A, an inhibitor of MPT (Weis et al., 1992). In addition, Grewal and Racz (1993) showed that dithiothreitol, but not NAC, stopped toxicity in isolated mouse hepatocytes when administered after the occurrence of covalent binding. Since dithiothreitol is known to prevent MPT by reduction of oxidized thiols in the MPT pore, the data are consistent with MPT being a critical event in toxicity. Thus, it may be that the oxidative effects of NAPQI are critical to the initiation of MPT in APAP toxicity and that, once MPT is initiated, peroxynitrite is formed. As a result, nitration of tyrosine is observed only in the necrotic cells. Since peroxynitrite is also known to produce MPT (Brown and Borutaite, 2001), its formation may lead to additional MPT in other mitochondria and the resulting death of the necrotic cells. Further study will be required to fully understand the role of MPT in APAP cell death.

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