Acetaminophen-Induced Hepatotoxicity in Mice Occurs with Inhibition of Activity and Nitration of Mitochondrial Manganese Superoxide Dismutase

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Running Title page

a. Running Title: Hepatic MnSOD in acetaminophen toxicity in mice

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d. Abbreviations: ALT, alanine aminotransferase; APAP, acetaminophen; APAP-Cys, 3-cystein-S-yl-acetaminophen; BSA, bovine serum albumin; HPLC-EC, high pressure liquid chromatography with electrochemical detection; iNOS, inducible nitric oxide synthase (NOS2); MnSOD, Manganese Superoxide dismutase; MPT, mitochondrial permeability transition; NAPQI, N-Acetyl-p-benzoquinone imine; nNOS, neuronal nitric oxide synthase (NOS1); Nrf 2, nuclear factor erythroid 2- related factor 2; OA, ovalbumin; TCEP, tris (2-carboxyethyl) phosphine; TEAB, Triethylammonium bicarbonate buffer

e. The recommended section assignment to guide the listing in the table of contents is Toxicology
Abstract

In overdose the analgesic/antipyretic acetaminophen (APAP) is hepatotoxic. Toxicity is mediated by initial hepatic metabolism to N-acetyl-p-benzoquinone imine (NAPQI). Following low doses NAPQI is efficiently detoxified by glutathione (GSH). However, in overdose GSH is depleted, NAPQI covalently binds to proteins as APAP adducts, and oxygen/nitrogen stress occurs. Toxicity is believed to occur by mitochondrial dysfunction. Manganese superoxide dismutase (MnSOD; SOD2) inactivation by protein nitration has been reported to occur during other oxidant stress mediated diseases. MnSOD is a critical mitochondrial antioxidant enzyme that prevents peroxynitrite formation within the mitochondria. To examine the role of MnSOD in APAP toxicity, mice were treated with 300 mg/kg APAP. GSH was significantly reduced by 65% at 0.5 h and remained reduced from 1-4 h. Serum ALT did not significantly increase until 4 h and was 2290 IU/L at 6 h. MnSOD activity was significantly reduced by 50% at 1 and 2 h. At 1 h, GSH was significantly depleted by 62% and 80% at nontoxic doses of 50 and 100 mg/kg respectively. No further GSH depletion occurred with hepatotoxic doses of 200 and 300 mg/kg APAP. A dose response decrease in MnSOD activity was observed for APAP at 100, 200 and 300 mg/kg. Immunoprecipitation of MnSOD from livers of APAP treated mice followed by Western blot analysis revealed nitrated MnSOD. APAP-MnSOD adducts were not detected. Treatment of recombinant MnSOD with NAPQI did not produce APAP protein adducts. The data indicate that MnSOD inactivation by nitration is an early event in APAP-induced hepatic toxicity.
Introduction

Acetaminophen (APAP; N-acetyl-p-aminophenol), a commonly used analgesic/antipyretic drug, is the most prevalent cause of drug-induced acute liver failure in the U.S. (Lee et al., 2008). In overdose it produces a centrilobular hepatic necrosis. Toxicity is believed to occur by initial hepatic metabolism by cytochrome P450 enzymes to the highly reactive metabolite (N-acetyl-p-benzoquinone imine; NAPQI). Following a therapeutic dose NAPQI is efficiently detoxified by glutathione (GSH). When taken in overdose NAPQI leads to depletion of hepatic GSH levels (Mitchell et al., 1973) and covalent binding to hepatic cellular proteins to form 3- (cystein-S-yl)-acetaminophen adducts (Cohen et al., 1997). Covalent binding has been shown to correlate with development of necrosis (Jollow et al., 1973; Roberts et al., 1991). Subsequent events important in the toxicity are poorly understood (Jaeschke and Bajt, 2006; Hinson et al., 2010).

Our laboratory has been investigating the hypothesis that GSH depletion and covalent binding are initial events in APAP toxicity and these events lead to a subsequent oxygen/nitrogen stress. Previously we reported the presence of 3-nitrotyrosine in livers of APAP treated mice and their occurrence coincided in the centrilobular hepatocytes with APAP-protein adducts and developing necrosis (Hinson et al., 1998). It was postulated that the reactive nitrogen species peroxynitrite, formed by a rapid reaction of superoxide with nitric oxide, was important in toxicity. Peroxynitrite is both a nitrating agent and an oxidizing agent and is detoxified by GSH (Sies et al., 1997) which is depleted in APAP toxicity (Mitchell et al., 1973).

In recent work utilizing freshly isolated mouse hepatocytes we presented evidence that reactive nitrogen species was formed during mitochondrial permeability transition (MPT) and this was critical to toxicity (Reid et al., 2005; Burke et al., 2010). This mechanism has also been shown...
to occur in cultured mouse hepatocytes (Kon et al., 2004). MPT is an abrupt increase in the permeability of the inner mitochondrial membrane to ions and small molecular weight solutes. It is promoted by oxidative stress and calcium, and leads to a large increase in oxidative stress.

MPT occurs with the loss of the ability of the mitochondria to produce ATP (Byrne et al., 1999; Zorov et al., 2000), a lethal event for the cell. In our previous studies using freshly isolated hepatocytes, we showed that APAP toxicity occurs in two phases (Reid et al., 2005) as previously reported (Boobis et al., 1986) (Grewal and Racz, 1993). The initial phase (0-2 h) occurs with GSH depletion and covalent binding followed by a toxic phase (3-5 h). We found that the toxic phase occurred with MPT and nitration of protein. Toxicity and nitration could be blocked by a MPT inhibitor (cyclosporine A), an antioxidant (N-acetylcysteine), a mitochondrial nitric oxide synthase inhibitor (7-nitroindazole) and high concentrations of a peroxynitrite scavenger (acetaminophen). We postulated that MPT occurred with formation of reactive nitrogen species leading to toxicity (Burke et al., 2010).

In models of nephrotoxicity associated with renal transplantation it has been shown that the activity of the mitochondrial enzyme manganese superoxide dismutase (MnSOD; SOD2) is dramatically decreased by a mechanism involving tyrosine nitration and oxidation of the enzyme (MacMillan-Crow et al., 1996; Macmillan-Crow and Cruthirds, 2001). MnSOD is a critical enzyme in mitochondria that is important in detoxification of superoxide. MnSOD found in the mitochondrial matrix detoxifies superoxide radical ($O_2^-$) forming hydrogen peroxide ($H_2O_2$) and molecular oxygen ($O_2$). MnSOD thus limits the reaction of superoxide with nitric oxide to form the reactive nitrogen species peroxynitrite. Since the hepatotoxicity of APAP occurs with increased nitration of proteins and mitochondrial dysfunction and MnSOD heterozygote mice have been reported to be more sensitive to APAP toxicity compared to wild type mice (Fujimoto
et al., 2009), we examined hepatic MnSOD activity and possible nitration in APAP-induced hepatotoxicity.
Materials and Methods

**Reagents.** Acetaminophen (APAP, 4-Acetamidophenol, paracetamol), protein G sepharose, xanthine oxidase, were obtained from Sigma Chemical Co. (St. Louis, MO). Coomassie Plus Protein Assay Reagent and SuperSignal chemiluminescent substrate reagent were obtained from Pierce Chemical Co. (Rockford, IL). Triethylammonium bicarbonate buffer (TEAB) and tris(2-Carboxyethyl) phosphine hydrochloride (TCEP-HCl) were purchased from Pierce Biochemical (Milwaukee, WI). Peroxidase labeled goat anti-rabbit IgG, and goat anti-mouse IgG antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Thermo Scientific (Rockford, IL), respectively. Protease inhibitor cocktail was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Hybond-ECL nitrocellulose membranes for Western Blotting were obtained from Amersham Biosciences (Piscataway, NJ). Anti-3-nitrotyrosine and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Millipore (Temecula, CA). MnSOD antibodies were obtained from Assay Designs (Ann Arbor, MI). Zeba TM Desalt spin columns were purchased from Thermo Scientific, Rockford, IL. All chemicals were the highest grade commercially available. N-Acetyl-p-benzoquinone imine (NAPQI) was synthesized and purified using a method described by Dahlin and Nelson (Dahlin et al., 1984). The development and utilization of the anti-APAP antibody was previously described (Roberts et al., 1991; Michael et al., 2001).

**Animals.** Six-week old male B6C3F1 (mean weight, 24.4 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN). Mice were acclimatized for one week prior to the planned experiments. Mice were fed *ad libitum* and were housed in individual cages on a 12 h light/dark cycle. All animal experimentation and protocols were approved by the institutional animal care and use committee. Animal experiments were carried out in accordance with the Guide for the
Care and Use of Laboratory Animals as adopted by U.S. National Institute of Health. On the day prior to experiments, mice were fasted overnight. In the APAP studies, control mice received saline only and treated mice received acetaminophen in 0.4 ml saline (i.p.). In a dose-response experiment, mice were administered APAP (50, 100, 200, and 300 mg/kg) and sacrificed at 1 h after APAP (n = 3 per experimental group). In a time-course experiment, mice were administered APAP (300 mg/kg) and sacrificed at 0.5, 1, 2, 4, and 6 h after APAP (n = 3 per time point). 6 h is a time of maximal toxicity. At the designated time, mice were anesthetized in a carbon dioxide chamber; blood was withdrawn by cardiac puncture, and animals were subsequently euthanized under carbon dioxide followed by cervical dislocation. Livers were removed and weighed and 200 mg of liver was preserved in GSH homogenization buffer, while the remaining tissue was snap frozen in liquid nitrogen and stored at -80°C for later analysis.

Liver Toxicity Analysis. Toxicity was determined by the quantitation of alanine aminotransferase (ALT) in the blood which occurs as a result of liver damage. Blood was allowed to coagulate at room temperature for at least 1 h, centrifuged, and the serum was removed. ALT measurements were determined utilizing a Liquid ALT (SGPT) reagent set kit (Pointe Scientific Inc., Canton, MI) according to manufacturer’s protocol. Spectral changes were followed using Roche Cobas Mira Classic Chemistry Analyzer (Roche Diagnostic systems, Inc., Branchburg, NJ).

Hepatic Assays. MnSOD activity in liver homogenates was measured by a cytochrome c reduction method using 1mM potassium cyanide to inactivate copper-zinc SOD (SOD1) and extracellular SOD (SOD3) as previously described (McCord and Fridovich, 1969; MacMillan-Crow et al., 1996). GSH content in liver tissue was determined using a modification of the Elman procedure (Mitchell et al., 1973).
**Protein Extraction.** Liver extracts were prepared by homogenizing 0.1 g of tissue in 1 ml of 1 x PBS (pH 7.4) containing a protease inhibitor cocktail and 1 mM phenyl methanesulfonyl fluoride using a Tissue-Tearor homogenizer (BioSpec Products, Inc., Bartlesville, OK). These extracts were sonicated for two 5 s bursts in continuous mode at 30% power using a Sonic dismembrator (Fisher Scientific Inc., Florence, KY) and then centrifuged at 10,000 rpm for 5 min to remove tissue debris. Protein concentration was determined using the Bradford assay (Pierce Chemical Co., Rockford, IL).

**Immunoprecipitation.** Solubilized proteins (1 mg/sample) were pre-cleared (1 h, 4°C) with 25 μl of Protein G Sepharose. The pre-cleared supernatant was incubated with 10 μg of anti-MnSOD antibody overnight at 4°C. Immune complexes were precipitated (90 min) with 50 μl of Protein G Sepharose, washed twice with PBS, resuspended in sample loading buffer, boiled and immediately separated using SDS-PAGE followed by western blot analysis (MacMillan-Crow and Thompson, 1999; MacMillan-Crow et al., 2001).

**Western Blot Analysis.** Western blot analyses were performed using the liver homogenates of each combined treatment group (100 μg) by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes as previously described (Michael et al., 2001). Membranes were blocked 30 min for MnSOD and GAPDH while overnight for APAP and NT in blocking buffer (5% milk in TBS, 0.1% Tween-20). The blocked immunobLOTS were incubated with anti-MnSOD (1:1000), anti-GAPDH (1:1000), anti-3-nitrotyrosine (1:500) for 120 min, and with anti-APAP (1:1000) for 90 min. Membranes for MnSOD and APAP were next incubated with peroxidase labeled goat anti-rabbit IgG, while for 3-nitrotyrosine and GAPDH were next incubated with peroxidase labeled goat anti-mouse IgG for 60 min. Membranes were visualized using SuperSignal chemiluminescent substrate reagent and the relative amount of protein in the
blots was determined by densitometry analysis using a Fluorchem™ 8900 imaging densitometer (Alpha Innotech Corporation, San Leandro, CA).

**Reaction of Proteins with NAPQI and Analysis for APAP Covalent Binding to Proteins.** In preparation for covalent modification by NAPQI, proteins (BSA, OA, and MnSOD) were diluted in triethylammonium bicarbonate buffer (TEAB, 200 mM, pH 8) and then reduced for 10 min at room temperature with tris(2-Carboxyethyl) phosphine hydrochloride (final concentration 12-20 mM)(Pierce Biochemical, Milwaukee, WI). Subsequently, NAPQI in dioxane was added to the reduced protein at a molar ratio of 6 – 10 times the protein molar concentration (final concentration of dioxane <1.2% v/v) and incubated for 5 min at room temperature with gentle mixing. Small molecules were removed from the reaction mixtures by gel-filtration on centrifugal desalting columns equilibrated with PBS, pH 7.2 (exclusion volume > 6,000 daltons). The excluded, putatively modified, proteins were analyzed for the presence of APAP-protein adducts after enzymatic digestion using high pressure liquid chromatography with electrochemical detection (HPLC-EC) of APAP-Cys as previously described (Muldrew et al., 2002; James et al., 2009).

**Statistical Analysis.** All animal experiments used 3 animals per treatment group. The animal experiments were repeated twice. For statistical analyses Western blots of liver homogenates from each liver (100 μg) were performed and the density of the blots determined as described above. Numerical data are reported as mean ± S.E. The data were analyzed using one-way analysis of variance followed by the Tukey HSD post-hoc test. Statistical significance was defined as experimental being p ≤0.05 from control. PASW Statistics Student Version 18 (SPSS Inc, Chicago, IL) was used for statistical analyses.
Results

Effect of APAP on Hepatic MnSOD Activity

To determine the potential role of MnSOD in APAP toxicity, mice were treated with a toxic dose of APAP (300 mg/kg, IP). Control mice received saline only. Mice were sacrificed at 0.5, 1, 2, 4, and 6 h after APAP. Consistent with previous observations, ALT levels were significantly increased at 4 and 6 h (Figure 1A). Hepatic GSH levels were significantly depleted by 0.5 h and remained significantly depleted until 4 h. By 6 h, GSH levels had recovered to levels comparable to that of saline treated animals (Figure 1B). The effect of APAP toxicity on hepatic MnSOD was examined by measuring MnSOD activity in liver homogenates. The MnSOD activity decreased significantly (55% reduction) by 1 h and remained significantly decreased at 2 h. By 4 h MnSOD activity had recovered to levels comparable to saline treated animals and by 6 h levels were significantly increased over that observed in control animals (Figure 1C). To determine if this change in MnSOD activity was due to change in protein levels, MnSOD expression was analyzed by Western blot analysis using APAP treated liver homogenates. A single band of 24 kDa was detected in the lanes of control and APAP-treated mice. The same blot was stripped and reprobed with an anti-GAPDH antibody to ensure equal loading of protein between samples. Densitometric analysis data showed no significant changes in immunochemically detectable MnSOD levels (Figure 1D).

To further understand the effect of APAP on MnSOD activity, a dose response study was performed. Mice were injected with APAP (50, 100, 200, 300 mg/kg, IP) and sacrificed at 1 h after APAP. Since the 1 h time point is before significant toxicity (Figure 1), serum ALT remained unchanged throughout the dose response (Figure 2A). Hepatic GSH levels were
significantly reduced at all APAP doses (Figure 2B). MnSOD activity decreased by 28% following 50 mg/kg APAP and significantly decreased further to 32%, 55%, and 57% following 100 mg/kg, 200 mg/kg, and 300 mg/kg respectively (Figure 2C). MnSOD expression was analyzed by Western blot analysis using APAP treated dose-dependent liver homogenates. A single band of 24 kDa was detected in the lanes of control and APAP-treated mice. The same blot was stripped and reprobed with an anti-GAPDH antibody to ensure equal loading of protein between samples. Densitometric analysis indicated that MnSOD protein was gradually decreased at the 50, 100, 200 and 300 mg/kg doses (Figure 2D). Since APAP (100 mg/kg) significantly decreased MnSOD activity at 1 h and this dose has not been shown to be hepatotoxic (Pumford et al., 1989) another experiment was performed. APAP (100 mg/kg) was administered to mice and the mice sacrificed at 1 h and 6 h. At 1 h, MnSOD activity was significantly decreased by 33%. At 6 h, ALT levels in APAP treated mice (19.6 ± 2 IU/L) were not significantly different from saline treated controls (28 ± 10 IU/L). Therefore, the ALT data suggests that 100 mg/kg APAP was a nontoxic dose.

Examination of MnSOD for APAP Protein Adducts and/or 3-Nitrotyrosine

Two possible mechanisms were envisioned that could account for the decrease in MnSOD activity following APAP treatment. One possible mechanism was reaction of NAPQI, the reactive metabolite of APAP, with cysteine groups or other nucleophilic groups on MnSOD causing loss of activity. MnSOD has been previously reported to contain two cysteine groups (DiSilvestre et al., 1995). The second mechanism was nitration of tyrosine of MnSOD by reactive nitrogen species leading to its inactivation. Tyrosine nitration leading to inactivation of MnSOD has been shown to occur in other diseases (MacMillan-Crow et al., 1996; Xu et al., 2006; Bayir et al., 2007).
To determine if MnSOD could be adducted by NAPQI, the recombinant protein was treated with synthetic NAPQI. In control incubations, bovine serum albumin (BSA) and ovalbumin (OA) were likewise treated with NAPQI. Before treatment with NAPQI the proteins were pretreated with TCEP-HCl to reduce cysteine residues to the thiolate form. Following treatment with NAPQI, the proteins were gel filtered, hydrolyzed by protease, and analyzed for 3-cystein-S-yl-acetaminophen by HPLC-EC (Muldrew et al., 2002; James et al., 2009). As shown in Figure 3, 3-cystein-S-yl-acetaminophen was not observed in MnSOD treated with NAPQI. However, treatment of BSA and OA with synthetic NAPQI yielded a HPLC-EC peak with both proteins corresponding to authentic 3-cystein-S-yl-acetaminophen (Figure 3). These data suggest that MnSOD is not readily derivitized by NAPQI.

To confirm that MnSOD was not derivitized by NAPQI to form APAP protein adducts and to examine the possibility that the protein was nitrated, a MnSOD immunoprecipitation experiment was performed. In this experiment mice received APAP (300 mg/kg) or saline and were sacrificed at 1 h. Liver homogenates were subjected to immunoprecipitation with anti-MnSOD antibody. The immunoprecipitated protein was analyzed by SDS-PAGE and subsequently blotted and stained with polyclonal anti-APAP antibody. NAPQI treated BSA was used as a positive control. A single band of 66 kDa was found in the control lane consistent with APAP covalently bound to BSA; however, no evidence was found for APAP covalently bound to MnSOD (Figure 4A). The same blot was stripped and reprobed with monoclonal anti-MnSOD antibody to ensure immunoprecipitation occurred. Recombinant MnSOD was used as a positive control. A 24 kDa band was detected in control, saline, and APAP treated samples (Figure 4C). This result is in agreement with the HPLC/EC data suggesting that NAPQI does not covalently bind with MnSOD. Further, to determine if MnSOD was nitrated following APAP
administration, liver homogenates were subjected to immunoprecipitation with anti-MnSOD antibody, analyzed by western blot, and stained with monoclonal anti-3-nitrotyrosine antibody. Peroxynitrite treated BSA was used as a positive control. As shown in Figure 4B, 3-nitrotyrosine was detected in the immunoprecipitated MnSOD from the liver homogenates of the APAP treated mice. To ensure immunoprecipitation occurred, the same blot was stripped and reprobed with monoclonal anti-MnSOD antibody (Figure 4D). These data indicate that MnSOD was nitrated in the livers of APAP treated mice.

**APAP Time Course and Dose Response for Nitration of MnSOD**

The time course and dose response for nitration of tyrosine in proteins in the APAP treated mouse livers were determined. Western blots from figure 1D and 2D were stripped and reprobed with polyclonal anti-3-nitrotyrosine antibody. As shown in Figure 5, a nitrated protein with a molecular weight the same as MnSOD, 24 kDa, was detected in livers of APAP treated mice. Densitometric analysis showed that the relative amount of 3-nitrotyrosine in this protein significantly increased at 2, 4 and 6 h following a toxic dose of APAP (300 mg/kg) (Figure 5A). The dose response for the increase of 3-nitrotyrosine in this protein showed a significant increase at 100, 200 and 300 mg/kg by 1 h following APAP (Figure 5B). These data are consistent with nitration of MnSOD following doses of APAP (100 mg/kg, 200 mg/kg and 300 mg/kg).
Discussion

MnSOD is a critical enzyme found in the mitochondrial matrix. It catalyzes the dismutation of superoxide forming hydrogen peroxide and molecular oxygen (McCord and Fridovich, 1988; Macmillan-Crow and Cruthirds, 2001). By scavenging superoxide its activity limits the reaction of superoxide with nitric oxide to form the reactive nitrogen species peroxynitrite. Peroxynitrite is both an oxidizing agent and a nitrating agent. It can nitrate tyrosine residues forming 3-nitrotyrosine (Beckman, 1996). It is detoxified by GSH (Sies et al., 1997) which is depleted in APAP toxicity (Figures 1B and 2B). The data presented in this manuscript show that the activity of hepatic MnSOD is dramatically reduced at 1 and 2 h following a hepatotoxic dose of APAP administered to mice (Figure 1C).

The mechanism of the decrease of MnSOD activity was examined. Since a significant decrease in MnSOD activity was observed by 1 h (Figures 1 and 2), the decrease was not a result of loss of the enzyme into the blood as a result of hepatocyte lysis. Hepatocyte lysis was not observed until 4 and 6 h (Figure 1A). Also, there was no evidence that NAPQI covalently bound to the enzyme since APAP-protein adducts were not detected either upon addition of NAPQI to MnSOD (Figure 3) or in livers of APAP treated mice (Figure 4A). However, loss of MnSOD activity occurred with the formation of nitrated enzyme (Figure 4B). Western blot analysis for 3-nitrotyrosine in the 24 kDa protein suggested significant nitration of MnSOD by 1 h at doses of 100, 200, and 300 mg/kg. These data suggest that the loss of activity of MnSOD was a result of nitration and that nitration occurs at a dose of APAP that is not hepatotoxic (100 mg/kg).

We previously examined APAP toxicity in freshly isolated hepatocytes. Toxicity was shown to occur after GSH depletion and covalent binding, and to occur by MPT with increased oxidative
stress. MPT is known to be initiated by oxidative stress and leads to a large increase in oxidative stress. It occurs with loss of the ability of the mitochondria to produce ATP, a lethal event for the cell. However, in the earlier work the nature of the oxidative stress was not determined (Reid et al., 2005). In very recent work we showed that MPT occurred with increased nitration of proteins. Inhibitors of MPT inhibited both toxicity and nitration. The finding that a nNOS inhibitor, 7-nitroindazole, but not two iNOS inhibitors, inhibited both toxicity and nitration suggested involvement of a mitochondrial nitric oxide synthase as the source of nitric oxide (Burke et al., 2010). Thus, the source of both the nitric oxide and the superoxide may be from mitochondria and explain the previous finding that the nitration of proteins in APAP hepatic toxicity occurs exclusively in mitochondria (Cover et al., 2005). The finding in this manuscript that MnSOD is inhibited support the hypothesis that APAP toxicity is by mitochondrial dysfunction promoted by reactive nitrogen species.

An interesting finding in the manuscript was the large increase in hepatic MnSOD activity (Figure 1C) observed at 6 h following treatment with APAP. The increase appeared to occur without a statistically significant alteration in detectable protein levels. Whereas the decrease in the activity of MnSOD at 1 and 2 h is believed to be a result of enzyme nitration (Figure 4A), there was a doubling of activity by 6 h. Similarly, Mladenovic et al., 2009 reported that MnSOD activity increased at 6 h after a toxic dose of APAP. This increase may be an effect on the antioxidant response element. Increased oxidative stress and NAPQI are believed to increase Nrf2 translocation into the nucleus in APAP toxicity and the antioxidant response element leads to the increased synthesis of a large number of antioxidant enzymes including MnSOD and enzymes controlling GSH synthesis (Goldring et al., 2004; Okawa et al., 2006; Reisman et al., 2009). The difference between the large increase in activity without a significant increase in
protein levels is not clear, but may be related to either alterations in the immunoaffinity of the enzyme following oxidation/nitration and/or precipitation of the protein following oxidation/nitration. Thus, the Western blot analysis of the supernatant of the sonicated homogenate may not accurately measure the relative amount of MnSOD once the protein is oxidized/nitrated. This conclusion is supported by the finding that there was less immunodetectable MnSOD at 1 h at doses of 100, 200, and 300 mg/kg APAP (Figure 2D) since these losses occurred without lysis of hepatocytes (Figure 2A).

An examination of a dose response for loss of MnSOD activity at 1 h indicated that there was a significant loss of activity at APAP doses of 100, 200, and 300 mg/kg (Figure 2C). Whereas 200 and 300 mg/kg APAP are known to be hepatotoxic doses, previously a dose of 100 mg/kg was not hepatotoxic (Pumford et al., 1989). A repeat of this experiment showed that there was not a significant increase of ALT following 100 mg/kg APAP at 6 h, a time of maximal ALT levels. However, we report herein that there was a significant decrease in MnSOD activity at this dose (Figure 2C) and this was accompanied by a significant increase in nitration at 1 h (Figure 5B). Thus, the data suggest that formation of reactive nitrogen species with loss of MnSOD activity is an early event in APAP toxicity and occurs at nonhepatotoxic doses.

In conclusion, the data in this manuscript show that MnSOD activity is decreased in APAP toxicity. The decrease in activity is attributed to nitration by reactive nitrogen species. The decrease in activity is postulated to augment the developing toxicity by shunting superoxide in mitochondria to react with nitric oxide leading to increased levels of reactive nitrogen species with nitration and oxidation (Figure 6). The mechanism of how reactive nitrogen produces the toxicity is unclear, but there is a significant amount of literature that toxicity occurs with
alteration in mitochondrial activity. (Burcham and Harman, 1991; Donnelly et al., 1994; Myers et al., 1995)
Authorship contributions

Participated in research design: Rakhee, Hinson, MacMillan-Crow

Conducted experiments: Rakhee, Hinson, MacMillan-Crow, Roberts, Rafferty

Contributed reagents: Fifer

Performed data analysis: Rakhee, Hinson, Saba

Wrote or contributed to the writing of the manuscript: Rakhee, Hinson, MacMillan-Crow, James

Other: Saba helped with MnSOD activity assay experiment.
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Footnotes

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Figure Legends

Figure 1. Time course for effect of APAP on serum ALT, hepatic GSH, hepatic MnSOD activity, and hepatic MnSOD protein. Mice (n=3) were treated with 300 mg/kg dose of APAP, sacrificed at the indicated times, and serum and liver collected. The 0 time is the saline treated control mice. **(A)** ALT levels in serum (hepatotoxicity) **(B)** GSH levels in liver. **(C)** MnSOD activity in liver. **(D)** MnSOD protein levels in liver. Western blot analyses were performed on each homogenate using anti-MnSOD. GAPDH was used as a loading control. The data are presented as mean ± S.E. of the relative intensity. One representative gel is shown above the density values. * indicates significant difference from saline, \( p \leq 0.05 \).

Figure 2. Dose response for effect of APAP on serum ALT, hepatic GSH, hepatic MnSOD activity, and hepatic MnSOD protein at 1 hour. Mice (n=3) were treated with 0, 50, 100, 200 and 300 mg/kg APAP and sacrificed after 1 h. **(A)** ALT levels in serum (hepatotoxicity) **(B)** GSH levels in liver. **(C)** MnSOD activity in liver. **(D)** MnSOD protein levels in liver. Western blot analyses were performed on each homogenate using anti-MnSOD. GAPDH was used as a loading control. Data are presented as mean ± S.E. of the relative intensity. One representative gel is shown above the density values. * indicates significant difference from saline, \( p \leq 0.05 \).

Figure 3. HPLC-EC analysis of NAPQI treated MnSOD, Bovine Serum Albumin, and Ovalbumin for 3-cystein-S-yl-APAP. Bovine serum albumin (BSA), ovalbumin (OA), and recombinant MnSOD were reacted with synthetic NAPQI. The resultant proteins were gel filtered, treated with protease, and analyzed by HPLC/EC analysis. HPLC reference standards
were 3-cystein-S-yl-APAP (6.85 min, 20 pmole on column) and APAP (8.33 min, 13.2 pmole on column). Detection signal is current in nA at a potential of 180 mV versus a palladium reference electrode.

**Figure 4. Western blot of immunoprecipitated MnSOD for APAP covalent binding and tyrosine nitration.** Liver homogenates were immunoprecipitated with anti-MnSOD antibody and resolved in 10% SDS-PAGE followed by Western Blot analysis. Lanes 1, and 2 were from saline treated mouse livers and lanes 3, and 4 were from APAP treated (300 mg/kg) mouse liver at 1 h. Western blot analyses were performed using anti-APAP antibody (A), and anti-3-nitrotyrosine antibody (B). Subsequently, the blots were stripped and reprobed with anti-MnSOD antibody (C, D). BSA treated with NAPQI (A), and BSA treated with peroxynitrite (B) at 66 kDa, and recombinant MnSOD at 24 kDa (C, D) were used as positive controls. One representative experiment is shown.

**Figure 5. APAP time course and dose response for nitration of MnSOD.** The samples from the time course (Figure 1D) were stripped and reprobed with anti-3-nitrotyrosine antibody (A). The samples from the dose response (Figure 2D) were stripped and reprobed with anti-3-nitrotyrosine antibody (B). The data are presented as mean ± S.E. of the relative intensity. One representative gel is shown. * indicates significant difference from saline, $p \leq 0.05$. 

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Figure 6. Schematic representation of how reactive nitrogen can alter oxidant stress in mitochondria.
Figure 4

Liver homogenates
↓
Immunoprecipitated with anti-MnSOD antibody
↓
Western Blotting

A. Anti-APAP

66 kDa
↓
BSA + NAPQI

24 kDa
↓
Saline

B. Anti-Nitrotyrosine

BSA + PN
↓
Saline

Stripped, reprobed with Anti-MnSOD

24 kDa
↓
Recombinant MnSOD

D.

Saline

APAP
Figure 5

A. 

B. 

24 kDa

37 kDa

Relative intensity

Time (h)

0 0.5 1 2 4 6

Relative intensity

APAP (mg/kg)

0.0 0.5 1.0 1.5 2.0

0 50 100 200 300

* * *
Figure 6

APAP

\[ \cdot \text{NO} + \cdot \text{O}_2^- \rightarrow \cdot \text{O}_2^- \]

\[ \text{MnSOD} \rightarrow \text{ONOO}^- \rightarrow \text{H}_2\text{O}_2 \]

Oxidant Stress