

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/44800683>

Reactive Nitrogen Species in Acetaminophen-Induced Mitochondria! Damage and Toxicity in Mouse Hepatocytes

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · JULY 2010

Impact Factor: 3.53 · DOI: 10.1021/tx1001755 · Source: PubMed

CITATIONS

35

READS

50

3 AUTHORS, INCLUDING:



[Lee Ann MacMillan-Crow](#)

University of Arkansas for Medical Sciences

58 PUBLICATIONS 3,165 CITATIONS

SEE PROFILE

Published in final edited form as:

Chem Res Toxicol. 2010 July 19; 23(7): 1286–1292. doi:10.1021/tx1001755.

Reactive Nitrogen Species in Acetaminophen-Induced Mitochondrial Damage and Toxicity in Mouse Hepatocytes

Angela S. Burke, Lee Ann MacMillan-Crow, and Jack A. Hinson*

Department of Pharmacology and Toxicology College of Medicine, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

Abstract

Acetaminophen (APAP) toxicity in primary mouse hepatocytes occurs in two phases. The initial phase (0–2 h) occurs with metabolism to *N*-acetyl-*p*-benzoquinoneimine which depletes glutathione, and covalently binds to proteins, but little toxicity is observed. Subsequent washing of hepatocytes to remove APAP and reincubating in media alone (2–5 h) results in toxicity. We previously reported that the reincubation phase occurs with mitochondrial permeability transition (MPT) and increased oxidative stress (dichlorodihydrofluorescein fluorescence) (DCFH₂). Since DCFH₂ may be oxidized by multiple oxidative mechanisms, we investigated the role of reactive nitrogen species (RNS) leading to 3-nitrotyrosine in proteins by ELISA and by immunoblots. Incubation of APAP with hepatocytes for 2 h did not result in toxicity or protein nitration; however, washing hepatocytes and reincubating in media alone (2–5h) resulted in protein nitration which correlated with toxicity. Inclusion of the MPT inhibitor, cyclosporine A, in the reincubation media eliminated toxicity and protein nitration. The general nitric oxide synthase (NOS) inhibitor L-NMMA and the neuronal NOS (NOS1) inhibitor, 7-nitroindazole, added in the reincubation media decreased toxicity and protein nitration; however, neither the inducible NOS (NOS2) inhibitors L-NIL (N⁶-(1-iminoethyl)-L-lysine) nor SAIT (*S*-(2-aminoethyl)isothiourea) decreased protein nitration or toxicity. The RNS scavengers, *N*-acetylcysteine, and high concentrations of APAP, added in the reincubation phase decreased toxicity and protein nitration. 7-Nitroindazole and cyclosporine A inhibited the APAP-induced loss of mitochondrial membrane potential when added in the reincubation phase. The data indicate a role for RNS in APAP induced toxicity.

Introduction

Acetaminophen (APAP¹) is a commonly used analgesic/ antipyretic that produces necrosis of the liver when taken in overdose (1). Hepatic necrosis occurs as a result of the metabolism of APAP to the highly reactive metabolite (*N*-acetyl-*p*-benzoquinone imine) (NAPQI) via cytochrome P450 metabolism (2, 3, 4). At therapeutic doses, NAPQI is detoxified by glutathione; however, in APAP overdose it leads to depletion of glutathione, covalent binding of NAPQI to proteins, and necrosis of hepatocytes (5). Covalent binding correlates with toxicity and has been postulated to be the mechanism of cell death. A

© 2010 American Chemical Society

*Corresponding author. Department of Pharmacology and Toxicology, Mail Slot 638, University of Arkansas for Medical Sciences, 4300 West Markham Street, Little Rock, AR 72205. Tel: 501-686-7036. Fax: 501-686-8970. hinsonjacka@uams.edu. .

¹Abbreviations: APAP, acetaminophen; DCFH₂, dichlorodihydrofluorescein; MPT, mitochondrial permeability transition; JC-1, 5,5', 6,6' tetrachloro-1,1',3,3' -tetraethyl-benzimidazolylcarbocyanine chloride; L-NMMA, N^G-methyl-L-arginine; L-NIL, L-N⁶-(1-iminoethyl)-lysine; NAC, *N*-acetylcysteine; NAPQI, *N*-acetyl-*p*-benzoquinone imine; 7-NI, 7-nitroindazole; eNOS, endothelial nitric oxide synthase, NOS3; iNOS, inducible nitric oxide synthase, NOS2; nNOS, neuronal nitric oxide synthase, NOS1; RNS, reactive nitrogen species; ROS, reactive oxygen species; SAIT, *S*-(2-aminoethyl) isothiourea.

number of proteins have been identified to which APAP binds; however, little is known about how protein modification leads to liver cell necrosis (1, 6).

We previously reported nitrated tyrosine in necrotic hepatocytes of mice treated with a toxic dose of APAP (7). The finding that APAP toxicity involved the formation of 3-nitrotyrosine residues in proteins led to the postulation that toxicity was mediated by a reactive nitrogen species (RNS) (1, 7). However, understanding the importance of RNS in APAP toxicity has remained elusive. Since inducible nitric oxide synthase (iNOS; NOS2) is the principal NOS in hepatocytes, its role in toxicity was investigated. iNOS knockout mice were found to be equally sensitive to APAP toxicity as the wild type mice which suggested that this enzyme did not play an important role in toxicity (1, 8). Also, iNOS inhibitors did not alter APAP-induced toxicity in mice (9). In addition, James et al. investigated the role of the respiratory burst in APAP toxicity and found that NADPH oxidase knockout mice were equally sensitive to APAP as the wild type mice and had equal levels of 3-nitrotyrosine, which suggested that the oxidative stress in APAP toxicity is not from the activation of Kupffer cells (1).

Previous work has shown that APAP toxicity in freshly isolated mouse hepatocytes occurs in two phases (10–12). The initial phase (0–2 h) involves the metabolism of APAP, depletion of glutathione, and covalent binding of NAPQI to proteins as APAP adducts; however, little toxicity is involved. Toxicity occurs in the second phase (3–5 h) in the absence of APAP. Our laboratory recently reported that the latter, second phase involves mitochondrial permeability transition (MPT), a lethal event for the cell, and increased oxidative stress (13). MPT is an increase in the permeability of the mitochondrial membranes to molecules of less than 1500 Da and occurs with the loss of mitochondrial membrane potential and uncoupling of oxidative phosphorylation (14). It occurs as a result of oxidative stress and leads to a large increase in oxidative stress (15, 16); however, the biochemical mechanism for this remains unclear. To determine the role of mitochondrial damage in APAP toxicity, we incubated APAP (1 mM) for 2 h with freshly isolated mouse hepatocytes, followed by washing to remove APAP and subsequently reincubating in media alone or with the media containing the potential inhibitor (2–5 h). Toxicity was determined by ALT release from the hepatocytes into the media and by increased propidium iodide fluorescence. Cells incubated with APAP showed little toxicity at 2 h, but GSH was maximally depleted as early as 0.5 h (17). Subsequent incubation in media alone resulted in a time-dependent increase in toxicity at 3, 4, and 5 h (13). These data confirmed the previous finding that the toxicity in the reincubation phase was occurring independently of the presence of APAP (10–12) and indicated a nonmetabolic role in APAP-induced hepatotoxicity in the second phase. MPT inhibitors (cyclosporine A or trifluoperazine), when added in the second phase, blocked toxicity as did the antioxidant *N*-acetylcysteine (NAC). Confocal microscopy studies utilizing the dyes calcein AM and tetramethylrhodamine methyl ester (TMRM) indicated that MPT occurred and was blocked by cyclosporine A and trifluoperazine (13). The rate of mitochondrial dysfunction in the form of membrane depolarization was determined utilizing the dye JC-1. The APAP-mediated mitochondrial membrane depolarization was blocked by cyclosporine A and NAC (13). Increased oxidative stress was observed using the redox-sensitive fluorometric dye, 2, 7-dichlorodihydrofluorescein (DCFH₂), and was found to be blocked by the MPT inhibitor cyclosporine A and the antioxidant *N*-acetylcysteine. In other work, Kon et al. (18) showed that APAP toxicity occurred with MPT in mouse hepatocytes. Last, we reported that the hepatotoxicity of chloroform also occurs in two phases. The initial phase occurs with GSH depletion, and the second phase occurs with MPT and oxidative stress in the absence of chloroform (19).

In this article, we have examined a role of RNS in APAP-induced mitochondrial damage and toxicity in freshly isolated mouse hepatocytes. A potential role for RNS in APAP toxicity was previously suggested by the occurrence of 3-nitrotyrosine in proteins of necrotic hepatocytes in the livers of APAP-treated mice (7, 20) and in mitochondria of APAP-treated hepatocytes (7, 20). Thus, we have measured protein nitration and toxicity in APAP-treated hepatocytes assayed under various toxicity conditions and compared protein nitration to toxicity. Also, we have included DCFH₂ oxidation data as Supporting Information. DCFH₂ oxidation may occur by RNS but can also occur by other reactive oxygen intermediates (21, 22).

Experimental Procedures

Chemicals and Suppliers

Acetaminophen (APAP, 4-acetamidophenol), HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethane-sulfonic acid]), heparin sodium salt grade 1-A from porcine intestinal mucosa, penicillin G sodium salt, RPMI-1640 modified media with L-glutamine and without sodium bicarbonate or phenol red, *N*-acetyl-L-cysteine (NAC), trifluoperazine, *N*^G-methyl-L-arginine (L-NMMA), Percoll, and trypan blue 0.4% solution were obtained from Sigma Chemical Co. (St. Louis, MO, USA). L-*N*⁶-(1-Iminoethyl)-lysine (L-NIL⁵), *S*-(2-aminoethyl) isothiurea (di-hydrobromide) (SAIT⁹), and 7-nitroindazole (7-NI) were obtained from Cayman Chemical Co. (Ann Arbor, MI). Collagenase A from *Clostridium histolyticum* was acquired from Roche Laboratories (Nutley, NJ). Cyclosporine A was obtained from Bedford Laboratories (Bedford, OH). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH₂) and 5,5', 6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimid-azolylcarbocyanine iodide (JC-1) were purchased from Molecular Probes (Eugene, OR). All chemicals were of the highest grade commercially available. ALT (SGPT) colorimetrics was obtained from TECO Diagnostics (Anaheim, CA). Four to 20% precast protein gels for Western blotting were obtained from Pierce Biotechnology (Rockford, IL, USA). Hybond-ECL nitrocellulose membranes for Western blotting were obtained from Amersham Biosciences (Piscataway, NJ, USA). All chemicals were of the highest grade commercially available.

Animals

Six-week old male B6C3F1 mice were obtained from Harlan Laboratories (Indianapolis, IN, USA). All animal experimentation and animal protocols were approved by University of Arkansas for Medical Sciences 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 the U.S. National Institutes of Health. Mice were acclimatized one week prior to the experiments and fed *ad libitum* until the time of sacrifice.

Hepatocyte Isolation and Incubations

Freshly isolated hepatocytes were obtained from 25 g male B6C3F1 mice by collagenase perfusion following a modification of the method of Grewal and Racz (12, 13, 23). Briefly, for each individual experiment, hepatocytes were isolated from a single mouse as previously described, followed by centrifugation at 140g for 8 min in a 90% Percoll gradient to purify the cells, followed by a wash in media, and a 3 min centrifugation at 140g to wash the Percoll from cells. Preparations yielding >40 million cells and cell viability >90% as determined by Trypan blue exclusion were used for the experiments. The hepatocytes were incubated at a concentration of 1,000,000 cells/mL in RPMI-1640 (supplemented with 25 mM HEPES, 10 IU heparin/mL, and 500 IU penicillin G/mL) in 125 mL Erlenmeyer flasks at 37 °C under an atmosphere of 95% O₂–5% CO₂. APAP (1 mM), at a concentration similar to that occurring in animals treated with a toxic dose of APAP, was added to experimental hepatocytes, but no APAP was added to control flasks. At 2 h following drug

addition, the hepatocytes were centrifuged for 2 min at 140g, and the supernatants were discarded. Fresh media were added to the cells, and the procedure was repeated. This washing of the cells removes greater than 99% of the unbound APAP (10). Following the final wash, some cells were resuspended in fresh media containing either 1 mM NAC, 10 μ M cyclosporine A, 10 μ M trifluoperazine, 1 mM L-NMMA, 1 mM L-NIL, 10 μ M 7-nitroindazole, or 1 mM SAIT. The toxicity data were obtained from three to four separate incubations that were performed on hepatocytes from separate mice on different days.

Toxicity Assays

Toxicity was quantified by the presence of alanine aminotransferase (ALT) in the media which occurs as a result of lysis from hepatocytes. ALT in the media was determined with a colorimetric end point method using a commercial kit (TECO Diagnostics, Anaheim, CA). Briefly, the hepatocytes were separated from the media by centrifugation. ALT substrate (50 μ L) was added to each sample of media (10 μ L) followed by incubation in a heating bath at 37 °C for 30 min. Subsequently, ALT Color Reagent (50 μ L) was added to each sample and reincubated for 10 min. ALT Color Developer (200 μ L) was added to each sample and the mixture placed in a heating bath for 5 min. The absorbance of samples was determined spectrophotometrically in a Bio-Rad 550 plate reader at a wavelength of 490 nm and ALT values determined as described.

Fluorescence Assays

The relative mitochondrial membrane potential was determined by a mitochondrial membrane specific cationic dye JC-1 (13, 24). JC-1 enters the mitochondria based on high negative membrane potential. JC-1 emits fluorescence as a monomer at 535 nm or as an aggregate at 590 nm. The monomer indicates a low membrane potential, whereas the aggregate indicates a high membrane potential. Briefly, hepatocytes of 2 mL aliquots were centrifuged at 140g for 2 min and the supernatants discarded. Cells were resuspended with 6.5 μ M JC-1 in 3 mL of JC-1 buffer (containing 137 mM NaCl, 3.6 mM KCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, and 10 mM Hepes) and incubated for 25 min at 37 °C in atmosphere of 95% O₂–5% CO₂. Following incubation, cells were centrifuged and washed of excess dye, resuspended in 2 mL of JC-1 buffer, and excited at 490 nm in a Hitachi F-2500 fluorescence spectrophotometer. Membrane potential was determined by using Hitachi Fluorescence Spectrophotometer FL Solutions software to calculate the area under the curve of the monomer and aggregate peak, and a 590 nm/535 nm ratio of the peaks was determined using DataQ-Windaq software (13). Fluorometric assays for oxidative stress (RNS and ROS) presented as Supporting Information were performed using the dye 2',7'-dichlorodihydrofluorescein diacetate (DCFH₂-DA) as previously described using a Hitachi F-2500 fluorescence spectrophotometer (13).

Western Blot Analyses

Nitrotyrosine was determined using SDS-PAGE (40 μ g protein/lane). Proteins from 4–20% precast gels were transferred onto nitrocellulose membranes at 150 V for 1 h and blocked for 48 h in blocking buffer (5% milk, TBS, 135 mL water, and 0.1% Tween-20). The blocked immunoblots were incubated with 1:500 antinitrotyrosine antibody for 2 h (25). Bound primary antibody was detected by treatment with 1:4000 HRP-conjugated antirabbit for 1 h followed by exposure of the membranes to a SuperSignal chemiluminescent substrate reagent. The relative amount of nitrated proteins in the blots were determined by densitometric analysis of each entire lane.

Nitrotyrosine ELISA

Nitrotyrosine was also measured using a commercial ELISA kit and performed as described by Upstate Cell Signaling Solutions (Charlottesville, VA, USA). Nitrotyrosine was detected by reading streptavidin peroxidase absorbencies at 450 nm indicative of nitrotyrosine levels present.

Statistical Analyses

Analysis of variance was performed with a Tukey post-hoc test using the SPSS 9.0 program. Statistical significance was defined as experimental being $p < 0.05$ from the control.

Results

Protein Nitration in APAP Toxicity

To determine the potential role of RNS in APAP toxicity, freshly isolated mouse hepatocytes were incubated with APAP (1 mM). At 2 h, the hepatocytes were washed and subsequently incubated with media alone. At 5 h, incubations were stopped and proteins assayed by Western blot analysis for 3-nitrotyrosine. Figure 1 shows the presence of nitrated proteins at 5h in APAP-treated hepatocytes compared to those of the control. Control hepatocytes were not incubated with APAP but otherwise treated identically. Each lane contains hepatocyte proteins from a separate incubation obtained from a separate mouse.

A time course for increasing levels of 3-nitrotyrosine in APAP-treated hepatocytes and control hepatocytes was performed by ELISA. There was a linear increase in protein nitration from 2 to 5h in APAP-treated hepatocytes (Figure 2), and the relative increase in nitration correlated with the relative increase in APAP toxicity (Figure 3). Control incubations did not show significant protein nitration (Figure 2) or toxicity (Figure 3).

Further experiments (Supporting Information) were carried out to assess the extent of RNS using the oxidation of 2',7'-dichlorodihydrofluorescein (DCFH₂), which can occur by peroxynitrite as well as other oxidants (21, 22). Figure 1S (Supporting Information) shows that there was a significant increase in DCFH₂ oxidation in APAP-treated hepatocytes when compared to that of the control hepatocytes. The increases in DCFH₂ oxidation correlated with the relative increases in APAP-induced toxicity and with the relative protein nitration.

Effect of MPT Inhibitors on APAP Toxicity and Protein Nitration

We previously reported that the addition of the MPT inhibitors, cyclosporine A and trifluoperazine, inhibited toxicity (13). To determine whether the MPT inhibitor, cyclosporine A, inhibits protein nitration, the experiment was repeated. Consistent with the previous data, both cyclosporine A (10 μ M) and trifluoperazine (10 μ M) were found to inhibit APAP toxicity (ALT release into the media, Figure 3). Cyclosporine A (CSP) inhibited protein nitration as determined by ELISA (Figure 2) and by Western blot analysis. Table 1 compares the percent decrease between 2 and 5h in APAP-induced toxicity and protein nitration (determined by ELISA and by Western blot). In addition, Supporting Information (Figure 1S) shows that APAP-mediated increase in DCFH₂ oxidation was inhibited by cyclosporine A and by trifluoperazine. These data are summarized in Table 1.

Effect of NOS Inhibitors on APAP Toxicity and Protein Nitration

Since nitric oxide or its metabolites contribute to RNS, we evaluated the effects of nitric oxide synthase (NOS) inhibitors on APAP-mediated toxicity. Two inducible NOS (iNOS; NOS2) inhibitors (L-NIL and SAIT) were used, one nonspecific NOS inhibitor (L-NMMA) and one neuronal NOS (nNOS; NOS1) inhibitor (7-nitroindazole). When added in the

reincubation phase, both L-NMMA and 7-nitroindazole decreased toxicity (Figure 4A) and protein nitration (Figure 2 and Table 1). However, neither of the iNOS inhibitors, L-NIL nor SAIT, showed protection against APAP toxicity (Figure 4B). Control cells showed no significant increases in toxicity. The iNOS inhibitor SAIT did not significantly alter protein nitration (Figure 2). In Supporting Information (Figure 2S), we show that the iNOS inhibitors had no effect on DCFH₂ oxidation, whereas L-NMMA and 7-nitroindazole both inhibited DCFH₂ oxidation suggesting a role for nitric oxide in the observed DCFH₂ oxidation.

Effect of Scavengers of Reactive Nitrogen Species on APAP Toxicity and Protein Nitration

The effect of the RNS scavengers *N*-acetylcysteine (NAC) and APAP on toxicity and protein nitration was also evaluated. In these experiments, APAP (1 mM) was incubated with hepatocytes, washed for 2 h, and subsequently reincubated with media containing *N*-acetylcysteine (NAC) (1 mM) or APAP (50 mM). *N*-Acetylcysteine was previously reported to inhibit DCFH₂ oxidation (13). *N*-Acetylcysteine inhibited protein nitration (Figure 2 and Table 1). APAP, a phenol-like tyrosine, has also been reported to react with the RNS peroxynitrite (26, 27). Thus, APAP (50 mM) was found to decrease toxicity (Figure 5) and protein nitration (Figure 2 and Table 1). In Supporting Information, it is shown that NAC and APAP (50 mM) also inhibited DCFH₂ oxidation (Figure 3S). In other data not shown, APAP (1, 10, and 50 mM) was added after the initial 1 mM APAP incubations at 2 h. Addition of 1 mM, 10 mM, and 50 mM APAP at 2 h decreased toxicity at 5 h by 14% (not significant), 60% (significant), and 73% (significant), respectively. DCFH₂ oxidation was decreased by 24% (not significant), 61% (significant), and 75% (significant), respectively (data not shown), at 5 h. Toxicity and DCFH₂ oxidation were not significantly different between incubations where 1 mM APAP was maintained for 5 h (no wash) compared to incubations which were incubated with APAP for 2 h and subsequently washed and then incubated in media alone to 5 h (data not shown).

Effect of NOS1 Inhibitors on APAP-Induced Loss of Mitochondrial Membrane Potential

We previously reported that APAP toxicity occurred with MPT. Since MPT occurs with the loss of mitochondrial membrane potential, the effect of NOS inhibition on APAP-induced loss of mitochondrial membrane potential was determined utilizing the dye JC-1. The loss of mitochondrial membrane potential was blocked with the addition of the MPT inhibitor cyclosporine A (Figure 6) as previously reported (13). Figure 6 also shows that the nNOS inhibitor, 7-nitroindazole (7-NI), inhibited the APAP-induced loss of mitochondrial membrane potential as effectively as the MPT inhibitor cyclosporine A (CSP).

Discussion

The primary objective of this study was to better characterize the role that RNS have in APAP-induced toxicity using freshly isolated mouse hepatocytes. Protein nitration was used as a biomarker of RNS production. In mice, we previously observed nitrated proteins in the centrilobular hepatocytes which were undergoing necrotic damage (7). In freshly isolated hepatocytes, we previously observed that APAP-induced toxicity occurred with increased fluorescence of the oxidant-sensitive dye DCFH₂, which is indicative of increased RNS (13). Cover et al. observed nitrated protein in hepatic mitochondria of APAP treated mice (20). These data suggested a role for RNS in APAP toxicity. Thus, in freshly isolated hepatocytes, we determined the relationship between the nitration of proteins and toxicity under conditions where RNS formation was modulated.

Western blot analysis (Figure 1) and ELISA for 3-nitrotyrosine in proteins (Figure 2) showed significant levels of protein nitration at 2-5 h after APAP exposure. A time-

dependent increase in nitration was observed (Figure 2), and the relative rate correlated with the relative increase in the development of toxicity (Figure 3). These data suggest a role for RNS in APAP toxicity in the isolated hepatocytes. We also obtained oxidation data utilizing the dye DCFH₂. RNS readily oxidize DCFH₂; however, DCFH₂ oxidation may also occur by a number of other reactive species such as peroxide/peroxidase reactions, hypochlorous acid, or by Fenton oxidation (21, 22). Since DCFH₂ oxidation is not specific for RNS, these data are included as Supporting Information; however, a noteworthy correlation between DCFH₂ oxidation and nitration was evident.

The effect of the NOS inhibitors on APAP-induced toxicity and nitration was very revealing. Even though the presence of iNOS in hepatocytes is well described (28), the iNOS inhibitors L-NIL or SAIT did not alter toxicity (Figure 4C) or nitration (Figure 2 and Table 1). These data are consistent with the previous *in vivo* findings that APAP toxicity is the same in wild type and iNOS knockout mice (8, 29). Even though the iNOS inhibitors did not inhibit toxicity, the nonspecific NOS inhibitor L-NMMA decreased toxicity (Figure 4A) and protein nitration (Table 1). Moreover, the nNOS inhibitor 7-nitroindazole decreased toxicity (Figure 4A) and protein nitration significantly at 5 h (Figure 2). Thus, these data suggest that a NOS isoform other than iNOS is important in APAP toxicity. 7-Nitroindazole has been used extensively as a selective nNOS inhibitor (30). *In vivo*, it does appear to be selective for nNOS, but *in vitro*, it has been reported to be an inhibitor of endothelial nitric oxide synthase (eNOS; NOS3) (31). However, the presence of nNOS and eNOS in hepatocytes is controversial. nNOS has been reported to be present in hepatic mitochondria, which would be consistent with it having a role in mitochondrial toxicity (32, 33); however, its presence in mitochondria has been questioned (34). McMillan utilized immunoblot assays and did not find eNOS in rat hepatocytes (35); however, McNaughton et al. reported that eNOS was present in human hepatocytes (36). Thus, the specific NOS isoform important in APAP toxicity will require further investigation. However, to understand the role of RNS in APAP toxicity, the important factor to note is that two nitric oxide synthase inhibitors inhibited toxicity, protein nitration, and DCFH₂ oxidation. These data strongly suggest that APAP toxicity is mediated via a mechanism involving a nitric oxide-mediated production of RNS.

Several studies suggest the involvement of mitochondrial dysfunction in APAP toxicity (13, 18, 37, 38). Mitochondrial dysfunction, in the form of MPT, can be induced by oxidative stress or Ca²⁺ (15, 16). MPT has been shown to be an important factor involved in many toxicities. MPT-induced cell necrosis occurs with mitochondrial swelling, loss of mitochondrial membrane potential, decreased oxidative phosphorylation, ATP depletion, and cell death (15, 16). When the MPT inhibitor, cyclosporine A was added in the reincubation phase, not only was toxicity decreased (Figure 3) but also protein nitration (Figure 2; Table 1) was decreased. An alternative MPT inhibitor, trifluoperazine, also decreased toxicity (Figure 3). Both MPT inhibitors decreased DCFH₂ oxidation (Figure 1S, Supporting Information). Moreover, the nNOS inhibitor 7-nitroindazole was as effective at inhibiting the APAP-induced loss of mitochondrial membrane potential as the MPT inhibitor cyclosporine A (Figure 6).

To further investigate the role of RNS in APAP toxicity in hepatocytes, the effect of the scavengers *N*-acetylcysteine and APAP were investigated. *N*-Acetylcysteine, a thiol, is a very efficient RNS scavenger (39) but is a nonspecific antioxidant that can be converted to GSH. *N*-Acetylcysteine is the antidote used to treat APAP overdose in humans. The antidotal mechanism is believed to be more efficient detoxification of the reactive metabolite NAPQI or detoxification of S-thiolated NAPQI (1, 40). In the present study, *N*-acetylcysteine, when added in the reincubation phase, decreased toxicity (Figure 5) and protein nitration (Figure 2 and Table 1). Thus, detoxification of RNS by *N*-acetylcysteine

may be another mechanism to explain the antidotal properties of *N*-acetylcysteine in APAP overdose. Also, since APAP is a phenol, its effect on toxicity was determined when added to the hepatocytes after the initial 2 h incubation with 1 mM APAP. Thus, the addition of APAP (50 mM) at 2 h, decreased subsequent toxicity and protein nitration (Figures 2 and 5). These data are consistent with the previous reports that APAP is a RNS scavenger (26, 27). Lakshmi et al. (28) showed that RNS reacts with APAP to form 3-nitroAPAP; however, APAP may scavenge RNS by the reduction of nitrating radicals. Moreover, the decrease in toxicity and DCFH₂ oxidation by the addition of various concentrations of APAP after the 2 h wash indicated that the decrease was concentration-dependent.

The RNS that are important in the nitration of tyrosine in proteins with APAP toxicity are unclear. It has been previously postulated to be peroxynitrite (7). Peroxynitrite is formed by the very rapid reaction of superoxide and nitric oxide (NO) (41). However, other mechanisms of nitration have been described. Nitration of tyrosine can be achieved by the incubation of myeloperoxidase or horseradish peroxidase with hydrogen peroxide and sodium nitrite (42). While neither of these enzymes is present in hepatocytes, it cannot be ruled out that another peroxidase is active in hepatocytes. Also, heme iron catalyzes the nitration of tyrosine residues by using hydrogen peroxide and nitrite (43). However, it should be pointed out that the nitrate or peroxide concentrations at 2 h would be very low since the hepatocytes were washed at that time. Relative to the RNS, the nitrite radical has been suggested as an important nitrating intermediate formed both by the peroxidation of nitrite (43) and from peroxynitrite (44).

In conclusion, the data presented in this article suggest that increased RNS and mitochondrial damage play a pivotal role in APAP toxicity in mouse hepatocytes. Importantly, it appears that both MPT inhibitors and NOS1 inhibitors block RNS production, mitochondrial damage, and toxicity. These data suggest an important role for RNS in APAP-induced mitochondrial damage and toxicity. The data are consistent with the hypothesis that RNS causes mitochondrial damage and that mitochondrial damage induces additional RNS (Figure 7). However, the mechanism of RNS production and how RNS produces mitochondrial damage leading to toxicity will require further research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

A.S.B. was supported by a training grant from the National Institute of Environmental Health Science (T-32 ES010952) (PI: J.A. Hinson). The following NIH research grants are acknowledged: R01 DK079008 (PI: J.A.H.) and R01 DK059872 (PI: L.A.M.-C.). The authors declare that there are no conflicts of interest.

Supporting Information Available: Oxidation of DCFH₂ and the effect of inhibitors. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1). James LP, Mayeux PR, Hinson JA. Acetaminophen-induced hepatotoxicity. *Drug Metab. Dispos.* 2003; 31:1499–1506. [PubMed: 14625346]
- (2). Dahlin DC, Miwa GT, Lu AY, Nelson SD. N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. *Proc. Natl. Acad. Sci. U.S.A.* 1984; 81:1327–1331. [PubMed: 6424115]
- (3). Gonzalez FJ. The 2006 Bernard B. Brodie Award Lecture. Cyp2e1. *Drug Metab. Dispos.* 2007; 35:1–8. [PubMed: 17020953]

- (4). Hinson, JA.; Roberts, DW.; James, LP. Handbook of Experimental Pharmacology. Springer; New York: 2010. Mechanisms of Acetaminophen-Induced Liver Necrosis; p. 369-405.
- (5). Mitchell JR, Jollow DJ, Potter WZ, Gillette JR, Brodie BB. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J. Pharmacol. Exp. Ther.* 1973; 187:211–217. [PubMed: 4746329]
- (6). Qiu Y, Benet LZ, Burlingame AL. Identification of the hepatic protein targets of reactive metabolites of acetaminophen in vivo in mice using two-dimensional gel electrophoresis and mass spectrometry. *J. Biol. Chem.* 1998; 273:17940–17953. [PubMed: 9651401]
- (7). Hinson JA, Pike SL, Pumford NR, Mayeux PR. Nitrotyrosine-protein adducts in hepatic centrilobular areas following toxic doses of acetaminophen in mice. *Chem. Res. Toxicol.* 1998; 11:604–607. [PubMed: 9625727]
- (8). Bourdi M, Masubuchi Y, Reilly TP, Amouzadeh HR, Martin JL, George JW, Shah AG, Pohl LR. Protection against acetaminophen-induced liver injury and lethality by interleukin 10: role of inducible nitric oxide synthase. *Hepatology.* 2002; 35:289–298. [PubMed: 11826401]
- (9). Hinson JA, Bucci TJ, Irwin LK, Michael SL, Mayeux PR. Effect of inhibitors of nitric oxide synthase on acetaminophen-induced hepatotoxicity in mice. *Nitric Oxide.* 2002; 6:160–167. [PubMed: 11890740]
- (10). Boobis AR, Tee LB, Hampden CE, Davies DS. Freshly isolated hepatocytes as a model for studying the toxicity of paracetamol. *Food Chem. Toxicol.* 1986; 24:731–736. [PubMed: 3781426]
- (11). Tee LB, Boobis AR, Huggett AC, Davies DS. Reversal of acetaminophen toxicity in isolated hamster hepatocytes by dithiothreitol. *Toxicol. Appl. Pharmacol.* 1986; 83:294–314. [PubMed: 3961817]
- (12). Grewal KK, Racz WJ. Intracellular calcium disruption as a secondary event in acetaminophen-induced hepatotoxicity. *Can. J. Physiol. Pharmacol.* 1993; 71:26–33. [PubMed: 8513431]
- (13). Reid AB, Kurten RC, McCullough SS, Brock RW, Hinson JA. Mechanisms of acetaminophen-induced hepatotoxicity: role of oxidative stress and mitochondrial permeability transition in freshly isolated mouse hepatocytes. *J. Pharmacol. Exp. Ther.* 2005; 312:509–516. [PubMed: 15466245]
- (14). Byrne AM, Lemasters JJ, Nieminen AL. Contribution of increased mitochondrial free Ca^{2+} to the mitochondrial permeability transition induced by tert-butylhydroperoxide in rat hepatocytes. *Hepatology.* 1999; 29:1523–1531. [PubMed: 10216138]
- (15). Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, Cascio WE, Bradham CA, Brenner DA, Herman B. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. *Biochim. Biophys. Acta.* 1998; 1366:177–196. [PubMed: 9714796]
- (16). Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. *J. Exp. Med.* 2000; 192:1001–1014. [PubMed: 11015441]
- (17). James LP, Donahower B, Burke AS, McCullough S, Hinson JA. Induction of the nuclear factor HIF-1 α in acetaminophen toxicity: evidence for oxidative stress. *Biochem. Biophys. Res. Commun.* 2006; 343:171–176. [PubMed: 16530163]
- (18). Kon K, Kim JS, Jaeschke H, Lemasters JJ. Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. *Hepatology.* 2004; 40:1170–1179. [PubMed: 15486922]
- (19). Burke AS, Redeker K, Kurten RC, James LP, Hinson JA. Mechanisms of chloroform-induced hepatotoxicity: oxidative stress and mitochondrial permeability transition in freshly isolated mouse hepatocytes. *J. Toxicol. Environ. Health, Part A.* 2007; 70:1936–1945. [PubMed: 17966065]
- (20). Cover C, Mansouri A, Knight TR, Bajt ML, Lemasters JJ, Pessayre D, Jaeschke H. Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. *J. Pharmacol. Exp. Ther.* 2005; 315:879–887. [PubMed: 16081675]

- (21). Crow JP. Dichlorodihydrofluorescein and dihydrorhodamine 123 are sensitive indicators of peroxynitrite in vitro: implications for intracellular measurement of reactive nitrogen and oxygen species. *Nitric Oxide*. 1997; 1:145–157. [PubMed: 9701053]
- (22). Myhre O, Andersen JM, Aarnes H, Fonnum F. Evaluation of the probes 2',7'-dichlorofluorescein diacetate, luminol, and lucigenin as indicators of reactive species formation. *Biochem. Pharmacol.* 2003; 65:1575–1582. [PubMed: 12754093]
- (23). Rafeiro E, Barr SG, Harrison JJ, Racz WJ. Effects of N-acetylcysteine and dithiothreitol on glutathione and protein thiol replenishment during acetaminophen-induced toxicity in isolated mouse hepatocytes. *Toxicology*. 1994; 93:209–224. [PubMed: 7974515]
- (24). Reers M, Smiley ST, Mottola-Hartshorn C, Chen A, Lin M, Chen LB. Mitochondrial membrane potential monitored by JC-1 dye. *Methods Enzymol.* 1995; 260:406–417. [PubMed: 8592463]
- (25). Hinson JA, Michael SL, Ault SG, Pumford NR. Western blot analysis for nitrotyrosine protein adducts in livers of saline-treated and acetaminophen-treated mice [In Process Citation]. *Toxicol. Sci.* 2000; 53:467–473. [PubMed: 10696795]
- (26). Whiteman M, Kaur H, Halliwell B. Protection against peroxynitrite dependent tyrosine nitration and alpha 1-antiproteinase inactivation by some anti-inflammatory drugs and by the antibiotic tetracycline. *Ann. Rheum. Dis.* 1996; 55:383–387. [PubMed: 8694578]
- (27). Lakshmi VM, Hsu FF, Davis BB, Zenser TV. Nitrating reactive nitric oxygen species transform acetaminophen to 3-nitroacetaminophen. *Chem. Res. Toxicol.* 2000; 13:891–899. [PubMed: 10995262]
- (28). BATTERY LD, Evans TJ, Springall DR, Carpenter A, Cohen J, Polak JM. Immunochemical localization of inducible nitric oxide synthase in endotoxin-treated rats. *Lab. Invest.* 1994; 71:755–764. [PubMed: 7526041]
- (29). Michael SL, Mayeux PR, Bucci TJ, Warbritton AR, Irwin LK, Pumford NR, Hinson JA. Acetaminophen-induced hepatotoxicity in mice lacking inducible nitric oxide synthase activity. *Nitric Oxide*. 2001; 5:432–441. [PubMed: 11587558]
- (30). Wolff DJ, Gribin BJ. The inhibition of the constitutive and inducible nitric oxide synthase isoforms by indazole agents. *Arch. Biochem. Biophys.* 1994; 311:300–306. [PubMed: 7515613]
- (31). Reiner A, Zagvazdin Y. On the selectivity of 7-nitroindazole as an inhibitor of neuronal nitric oxide synthase. *Trends Pharmacol. Sci.* 1998; 19:348–350. [PubMed: 9786021]
- (32). Tatoyan A, Giulivi C. Purification and characterization of a nitric-oxide synthase from rat liver mitochondria. *J. Biol. Chem.* 1998; 273:11044–11048. [PubMed: 9556587]
- (33). Ghafourifar P, Richter C. Nitric oxide synthase activity in mitochondria. *FEBS Lett.* 1997; 418:291–296. [PubMed: 9428730]
- (34). Lacza Z, Snipes JA, Zhang J, Horvath EM, Figueroa JP, Szabo C, Busija DW. Mitochondrial nitric oxide synthase is not eNOS, nNOS or iNOS. *Free Radical Biol. Med.* 2003; 35:1217–1228. [PubMed: 14607521]
- (35). McMillan JM. Galactosamine decreases nitric oxide formation in cultured rat hepatocytes: mechanism of suppression. *J. Biochem. Mol. Toxicol.* 1999; 13:143–148. [PubMed: 10098899]
- (36). McNaughton L, Puttagunta L, Martinez-Cuesta MA, Kneteman N, Mayers I, Moqbel R, Hamid Q, Radomski MW. Distribution of nitric oxide synthase in normal and cirrhotic human liver. *Proc. Natl. Acad. Sci. U.S.A.* 2002; 99:17161–17166. [PubMed: 12482944]
- (37). Burcham PC, Harman AW. Mitochondrial dysfunction in paracetamol hepatotoxicity: in vitro studies in isolated mouse hepatocytes. *Toxicol. Lett.* 1990; 50:37–48. [PubMed: 2296776]
- (38). Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ. Mechanisms of hepatotoxicity. *Toxicol. Sci.* 2002; 65:166–176. [PubMed: 11812920]
- (39). Crow JP. Peroxynitrite scavenging by metalloporphyrins and thiolates. *Free Radical Biol. Med.* 2000; 28:1487–1494. [PubMed: 10927173]
- (40). Chen W, Shockcor JP, Tonge R, Hunter A, Gartner C, Nelson SD. Protein and nonprotein cysteinyl thiol modification by N-acetyl-p-benzoquinone imine via a novel ipso adduct. *Biochemistry*. 1999; 38:8159–8166. [PubMed: 10387061]
- (41). Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am. J. Physiol.* 1996; 271:C1424–1437. [PubMed: 8944624]

- (42). Sampson JB, Ye Y, Rosen H, Beckman JS. Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide. *Arch. Biochem. Biophys.* 1998; 356:207–213. [PubMed: 9705211]
- (43). Bian K, Gao Z, Weisbrodt N, Murad F. The nature of heme/iron-induced protein tyrosine nitration. *Proc. Natl. Acad. Sci. U.S.A.* 2003; 100:5712–5717. [PubMed: 12709594]
- (44). Daiber A, Mehl M, Ullrich V. New aspects in the reaction mechanism of phenol with peroxynitrite: the role of phenoxy radicals. *Nitric Oxide.* 1998; 2:259–269. [PubMed: 9851367]

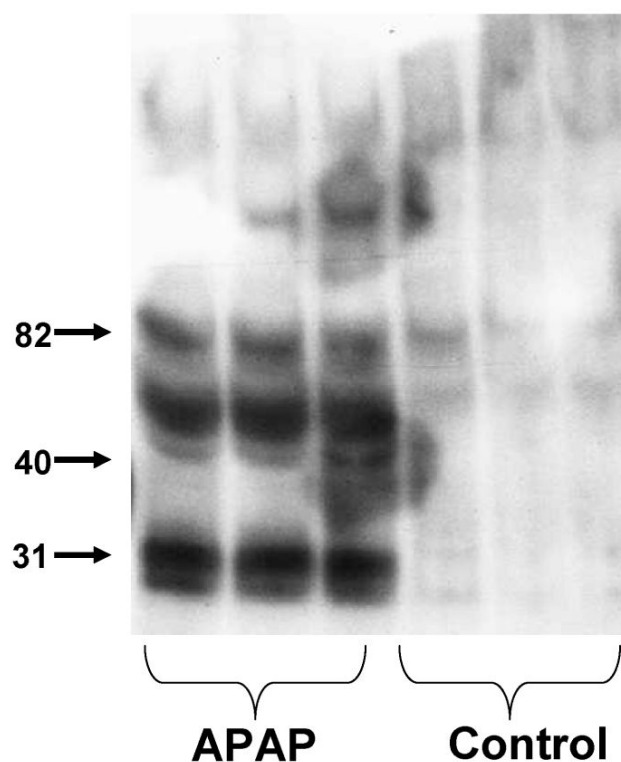


Figure 1.

Western blot analysis for nitrotyrosine in proteins of APAP-treated hepatocytes. Freshly isolated mouse hepatocytes were incubated with APAP (1 mM) as described in Experimental Procedures. Controls were incubated with media alone. At 5 h, incubations were terminated, and 3-nitrotyrosine levels (protein nitration) were determined using Western blot analysis as described in Experimental Procedures. Each lane contains protein from a separate incubation performed on hepatocytes obtained from a separate mouse.

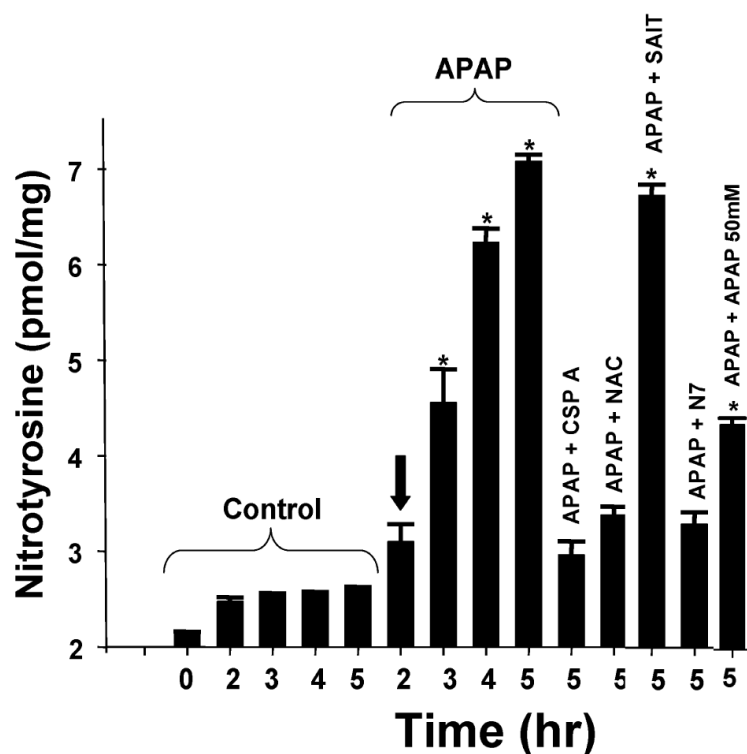


Figure 2.

ELISA determination for nitrotyrosine in proteins of APAP-treated hepatocytes: time course and effect of various inhibitors of toxicity. Freshly isolated mouse hepatocytes were incubated with media alone or with APAP (1 mM) for 2 h. Subsequently, hepatocytes were washed to remove APAP (arrow) and incubated with media. To some incubations, cyclosporine A (10 μ M) (CSP), *N*-acetylcysteine (1 mM) (NAC), SAIT (1 mM), or 7-nitroindazole (10 μ M) (N7) was added to the pretreated cells for the remaining 5 h of incubation. Control cells were reincubated with media alone. At the indicated time, aliquots were taken, and 3-nitrotyrosine was analyzed using a commercial ELISA kit. Samples ($n = 3$ from separate mice) which significantly increased from the 2 h wash are indicated by * ($p \leq 0.05$).

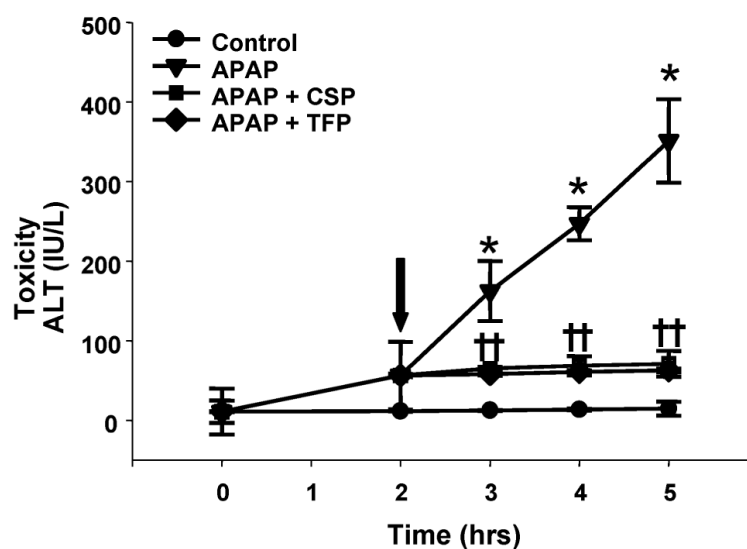


Figure 3.

Effect of MPT inhibitors on APAP-induced toxicity in freshly isolated hepatocytes. Hepatocytes were incubated with APAP (1 mM). Subsequently, hepatocytes were washed to remove APAP (arrow) and incubated in media alone (▼), media containing cyclosporine A (10 μ M) (CSP) (■), or media containing trifluoperazine (10 μ M) (TFP) (◆) for 2–5 h. Control hepatocytes were incubated with media alone for 2 h, washed, and subsequently incubated with media alone for 2–5 h (●). Time points were taken, and relative toxicity was measured using ALT release. Samples which significantly increased from the 2 h wash are indicated by * ($p \leq 0.05$). Samples ($n = 3$ from separate mice) which are significantly decreased from APAP alone at the same time point are designated by †† (* ($p \leq 0.05$)).

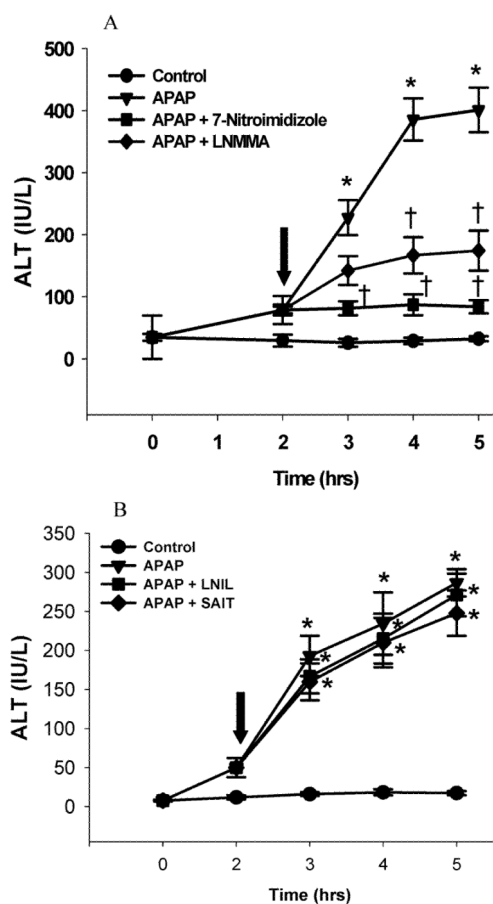


Figure 4.

Effect of NOS inhibitors on APAP-induced toxicity in freshly isolated hepatocytes. Hepatocytes were incubated with APAP (1 mM) for 2 h, washed to remove APAP (arrow), and subsequently incubated in media alone (2–5 h) (▼). Control hepatocytes were incubated in media alone for 2 h, washed, and subsequently incubated in media alone (2–5 h) (●). Following washing to remove APAP, some hepatocytes (A) were incubated with the general NOS inhibitor L-NMMA (1 mM) (◆) or the nNOS inhibitor 7-nitroindazole (10 μ M) (■) (2–5 h). Following washing to remove APAP, other hepatocytes (B) were incubated with the iNOS inhibitors L-NIL (1 mM) (■) or SALT (1 mM) (◆) (2–5 h). Aliquots were taken and toxicity determined by ALT release. Samples (n = 3 from separate mice) which significantly increased from the same 2 h incubation are indicated by * ($p \leq 0.05$).

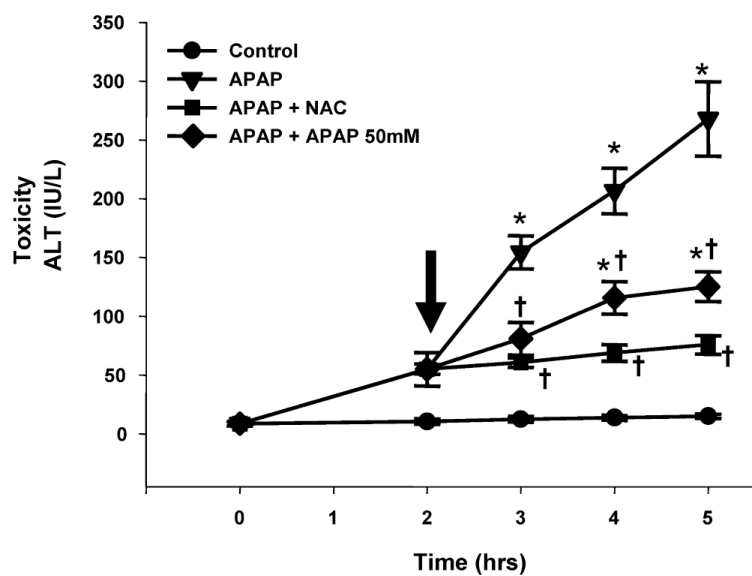


Figure 5.

Effect of scavengers of reactive nitrogen species on APAP-induced APAP toxicity in freshly isolated hepatocytes. Hepatocytes were incubated with APAP (1 mM) for 2 h, washed to remove APAP (arrow), and subsequently incubated in media alone (2–5h) (▼). Control hepatocytes were incubated in media alone for 2 h, washed, and subsequently incubated in media alone (2–5h) (●). Following washing to remove APAP, some hepatocytes were incubated with the RNS scavengers *N*-acetylcysteine (1 mM) (■) or acetaminophen (50 mM) (◆) (2–5 h). Aliquots were taken and toxicity determined by ALT release. Samples ($n = 3$ from separate mice) which significantly increased from the same 2 h incubation are indicated by * ($p \leq 0.05$).

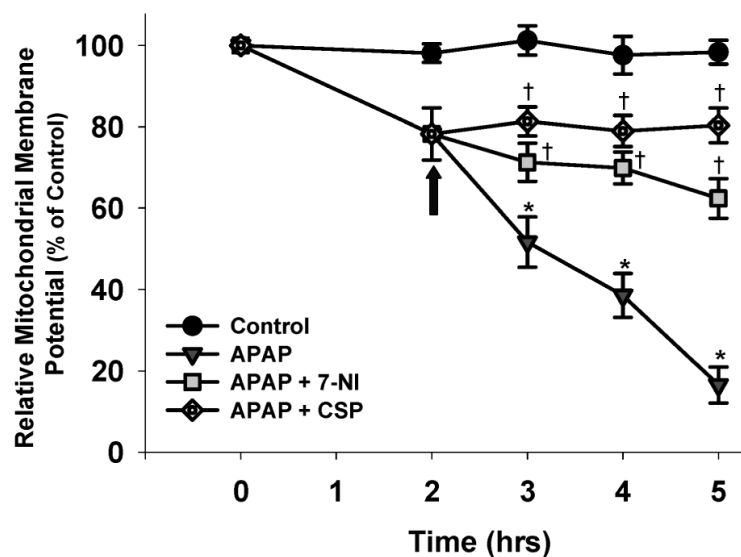


Figure 6.

Effect of NOS1 inhibitor on APAP-induced loss of mitochondrial membrane potential in freshly isolated hepatocytes. Hepatocytes were incubated with APAP (1 mM) or media alone for 2 h. Subsequently, hepatocytes were washed to remove APAP (arrow) and incubated in media alone (▼), media containing cyclosporine A (10 μ M) (diamond), or media containing 7-nitroindazole (7-NI) (10 μ M) (square). The control was incubated with media alone, washed for 2 h, and subsequently incubated with media alone (●). At the designated time mitochondrial membrane potential was determined using JC-1 as described in Experimental Procedures. *Significantly different from APAP treated at 2 h (hepatocytes from separate mice, $n = 3$, $p < 0.05$). †Significantly different from APAP-treated mice (hepatocytes from separate mice, $n = 3$, $p \leq 0.05$).

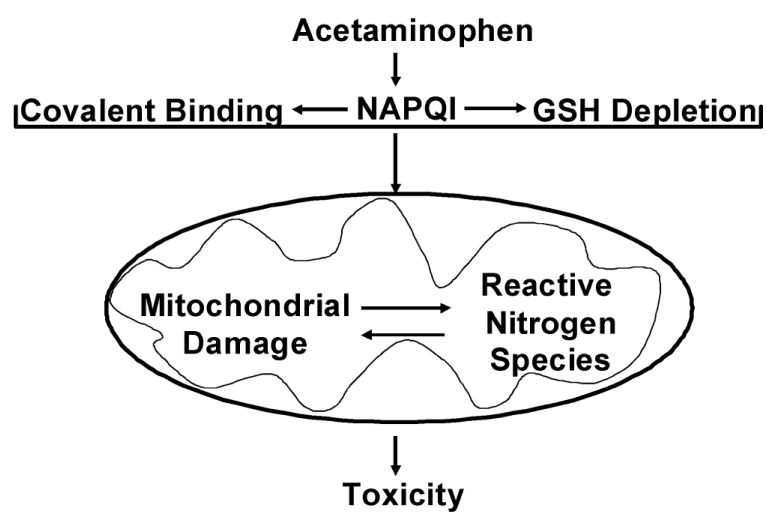


Figure 7. Postulated mechanism of acetaminophen toxicity. It is postulated that mitochondrial damage is a result of RNS and leads to additional RNS which causes additional mitochondrial damage.

Effect of MPT Inhibitors, Nitric Oxide Synthase Inhibitors, and Reactive Nitrogen Species Scavengers on APAP Toxicity, Protein Nitration, and DCFH₂ Oxidation^a

Table 1

test agent	inhibitor type	inhibition of toxicity (ALT) (5 h)	inhibition of nitration (Western Blot) (5 h)	inhibition of nitration (ELISA) (5 h)	inhibition of DCFH ₂ oxidation (5 h)
cyclosporine A	MPT inhibitor	98% ^b	97% ^b	100% ^b	90% ^b
trifluoperazine	MPT inhibitor	97% ^b	n/a	n/a	90% ^b
L-NMMA	NOS inhibitor	68% ^b	68% ^b	n/a	63% ^b
7-nitroindazole	nNOS inhibitor	95% ^b	n/a	94% ^b	91% ^b
L-NIL	iNOS inhibitor	10%	n/a	n/a	22%
SAIT	iNOS inhibitor	17%	4%	11%	17%
N-acetyl-cysteine	peroxynitrite scavenger	92% ^b	100% ^b	93% ^b	89% ^b
acetaminophen (50 mM)	peroxynitrite scavenger	69% ^b	n/a	70% ^b	68% ^b

^aThe decrease in protein nitration by ELISA between 2 and 5 h was calculated from the data in Figure 2. For the decrease in protein nitration by Western blot, analyses were performed in two separate blots as reported in Figure 1. Incubations contained APAP (1 mM), control, and APAP (1 mM) plus test agent added at 2 h after washing hepatocytes (*n* = 3 analyses/agent performed using hepatocytes from separate mice). The blots were analyzed by densitometric analyses of the whole lanes, and data are the percent decrease in densities compared to the absence of test agents. The decrease in toxicity by the test agents was calculated between 2 and 5 h. The decrease in DCFH₂ oxidation by the test agents was calculated between 2 and 5 h from Supporting Information. Statistical analyses were performed to determine if significant decreases occurred upon the addition of inhibitors.

^b *p* ≤ 0.05 indicates a significant decrease from APAP alone.