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Acetaminophen Toxicity in Mice Lacking NADPH Oxidase Activity: Role of Peroxynitrite Formation and Mitochondrial Oxidant Stress

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Previous data have indicated that activated macrophages may play a role in the mediation of acetaminophen toxicity. In the present study, we examined the significance of superoxide produced by macrophages by comparing the toxicity of acetaminophen in wild-type mice to mice deficient in gp91*phox*, a critical subunit of NADPH oxidase that is the primary source of phagocytic superoxide. Both groups of mice were dosed with 300 mg/kg of acetaminophen or saline and sacrificed at 1, 2, 4 or 24 h. Glutathione in total liver and in mitochondria was depleted by approximately 90% at 1 h in wild-type and knock out mice. No significant differences in toxicity (serum transaminase levels or histopathology) were observed between wild-type and mice deficient in gp91*phox*. Mitochondrial glutathione disulfide, as a percent of total glutathione, was determined as a measure of oxidant stress produced by increased superoxide, leading to hydrogen peroxide and/or peroxynitrite. The percent mitochondrial glutathione disulfide increased to approximately 60% at 1 h and 70% at 2 h in both groups of mice. Immunohistochemical staining for nitrotyrosine was present in vascular endothelial cells at 1 h in both groups of mice. Acetaminophen protein adducts were present in hepatocytes at 1 h in both wild-type and knock out animals. These data indicate that superoxide from activated macrophages is not critical to the development of acetaminophen toxicity and provide further support for the role of mitochondrial oxidant stress in acetaminophen toxicity.

Keywords: Acetaminophen; Peroxynitrite; Nitrotyrosine; NADPH oxidase; Superoxide; Glutathione

INTRODUCTION

In overdose the commonly used analgesic acetaminophen (APAP) produces centrilobular necrosis in the livers of humans and experimental animals. The mechanism has been studied extensively. Briefly, it has been shown that the initial step is metabolism by cytochrome P450 enzymes to the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI). With therapeutic doses of acetaminophen, NAPQI is efficiently detoxified by glutathione (GSH) to form 3-(glutathione-*S*-yl)acetaminophen which is further metabolized and excreted. However, in overdose total hepatic GSH is depleted and NAPQI covalently binds to cysteine groups on proteins as APAP-cysteine adducts. Immunohistochemical analyses have revealed that the large majority of cells that develop APAP-cysteine adducts subsequently become necrotic.[1]

Even though metabolic activation leading to GSH depletion and covalent binding is well recognized as an important step in APAP-induced liver necrosis, a significant body of work indicates that oxidant stress is also important. Thus, Nakae *et al*.[2] reported that administration of encapsulated superoxide dismutase delayed the development of hepatotoxicity in rats. Also, Jaeschke[3] reported that oxidant stress occurred in mitochondria of APAP treated mice. He showed that glutathione disulfide (GSSG) as a percent of total glutathione (GSH + GSSG) dramatically increased in mitochondria. However, APAP does not produce lipid peroxidation in treated mice or rats, and GSSG biliary efflux is not increased in mice or rats treated with toxic doses of APAP.[3,4] In contrast, increased biliary efflux for GSSG and

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hepatic lipid peroxidation have been demonstrated for diquat, a compound that undergoes redox cycling.[5]

More recently, several laboratories have suggested that Kupffer cells are involved in APAP toxicity. In the activated form these resident macrophages produce superoxide, nitric oxide, and inflammatory cytokines.[6] Pretreatment of rats with gadolinium chloride, dextran sulfate, or LPS before the administration of APAP decreased the subsequent toxicity of APAP.[6] Likewise, pretreatment of mice with gadolinium chloride, dextran sulfate, or dichloromethylene disphosphonate significantly decreased the susceptibility of mice to the hepatotoxicity of APAP.[7,8] Mechanistically, these data suggest that metabolism of APAP is associated with activation of Kupffer cells, which results in increased superoxide production, leading to oxidant stress and toxicity.

Our laboratory presented evidence that peroxynitrite is mechanistically important in APAP-induced toxicity. Peroxynitrite is formed by a very rapid reaction between superoxide and nitric oxide. It is both an oxidant and a nitrating agent and has been shown to cause nitration of proteins.[9] Using an antiserum that recognizes 3-nitrotyrosine, we showed that 3-nitrotyrosine and APAP protein adducts co-localize in the centrilobular cells of the liver following toxic doses of APAP.[10,11] It was postulated that GSH depletion by NAPQI was a significant factor since GSH is a detoxification mechanism for peroxynitrite. Also, data were presented that Kupffer cell inactivators decreased tyrosine nitration following a toxic dose of APAP and a role for Kupffer cells in peroxynitrite formation was postulated.[12]

The purpose of the present study was to investigate whether superoxide generated by NADPH oxidase is essential to the development of APAP toxicity. NADPH oxidase is the major source of superoxide produced by activated macrophages, monocytes and neutrophils, as well as some nonphagocytic cells.[13,14] NADPH oxidase is a multi-component electron transport system that reduces oxygen to superoxide with NADPH as a cofactor. Mutations in any of the essential subcomponents result in dysfunction of NADPH oxidase, the enzyme associated with chronic granulomatous disease,[15] a disorder characterized by recurrent bacterial and fungal infections. In the following study, we evaluated the role of NADPH oxidase in APAP toxicity through the use of mice deficient in the gene encoding gp91*phox*, the large membrane-bound subunit of the NADPH respiratory burst oxidase complex.[16] We hypothesized that the absence of NADPH oxidase, and therefore the lack of superoxide production by Kupffer cells, would decrease peroxynitrite production and consequently, nitrotyrosine formation and APAP hepatotoxicity. Contrary to our hypothesis, we found no difference in nitrotyrosine formation and APAP hepatotoxicity in mice deficient in NADPH oxidase, as compared to wild-type mice.

MATERIALS AND METHODS

Reagents

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

Animals

B6.129S6-*Cybb*^{tm1} mice (previously referred to as C57BL/6-*Cybb*^{tm1}),[17] also known as X-CGD (chronic granulomatous disease) mice, have a targeted disruption of the gp91^{phox} subunit of the NADPH-oxidase complex (phox), and were derived from a C57BL/6 × 129/Sv background and backcrossed six times with C57BL/6 mice.[17] Peripheral blood neutrophils[17] and macrophages[18] from mice deficient in pg91phox fail to generate superoxide and have an increased susceptibility to infections with *Staphylococcus aureus* and *Aspergillus fumigatus*. Male B6.129S6-*Cybb*^{tm1} (knockout, or KO) and C57BL/6J (wild-type, or WT) mice (average weight, 22.9 g) were obtained from Jackson Laboratories (Bar Harbor, ME). Mice (6 weeks of age) were acclimatized for 1 week before the experiment. The day before the experiment, food was removed from the animals at 4:00 P.M., and at 8:00 A.M. the next morning animals were injected with 300 mg/kg APAP i.p. in 0.4 ml saline. In initial experiments, mice were sacrificed at 4 and 24 h after APAP. In subsequent experiments, mice were sacrificed at 1 and 2 h after APAP. At the indicated time, the mice were anesthetized with CO_2 and blood was taken from the retro-orbital sinus.

The blood was allowed to coagulate at room temperature and the samples were centrifuged. The serum was removed and stored at 4°C prior to analysis. Immediately after bleeding, the mice were sacrificed and the livers were removed. A portion of each liver was weighed and homogenized in a 5:1 v/w of 20 mM Tris base, 1.15% potassium chloride, 20 mM butylated hydroxyanisole (pH 7.4) buffer.

Analyses

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined

spectrophotometrically through the use of a Sigma Chemical Co. kit (St Louis, MO). Hepatic GSH was measured by a colorimetric method using Ellman's reagent as previously modified by Mitchell *et al*.[19] Mitochondrial GSSG and GSH were measured with a modified method of Tietze.[3] Isolated mitochondria (described below) were homogenized at 0°C in 3% sulfosalicylic acid containing 0.1 mM EDTA. An aliquot of the homogenate was added to 10 mM NEM in phosphate buffer (KPP) and another aliquot was added to 0.01 M HCl. The NEM-KPP sample was centrifuged and the supernatant was passed through a C18 cartridge to remove free NEM and NEM-GSH adducts (Sep-pak; Waters Associates, Waltham, MA). The HCl sample was centrifuged and the supernatant was diluted with KPP. All samples were assayed using dithionitrobenzoic acid (DTNB). All data are expressed in GSH-equivalents.

Acetaminophen-protein adducts and nitrotyrosine adducts in liver were analyzed by immunohistochemical procedures using rabbit antisera raised against 4-acetamidobenzoic acid-KLH and 4-hydroxy-3-nitrobenzoic acid-KLH.[18] Briefly, fresh liver tissues were cut to approximately 2-mm thickness and fixed in 10% buffered formalin for 24 h. Paraffin-embedded liver tissue sections were deparaffinized in xylene (2 × 5 min, 25°C), and then rehydrated through a series of graded ethanol washes and deionised water. To inhibit the endogenous peroxidase activity, the sections were immersed in Immunopure peroxidase suppressor for 30 min, followed by a 3 min wash in phosphate buffered saline (PBS). Then 0.15% Triton-X 100 in PBS was added to each tissue section for 20 min to permeablize the cells. Sections were washed in PBS. Next, DAKO protein block was placed on each section to inhibit nonspecific binding. The sections were washed in PBS and exposed to the primary antibodies of APAP (1:500) and nitrotyrosine (1:200) for 1 h at room temperature. The suggested protocol in DAKO LSAB + kit was followed for color development. The slides were counterstained with Gills Hematoxylin II for 2 min, then rinsed in deionized water and immersed in ammonia blue for 2 min. The slides were dehydrated and mounted with Permount.

Mouse liver samples were also analyzed for nitrotyrosine by ELISA, using the kit from Oxis Research (Portland, OR) as per the manufacturer's instructions.

Isolation of Mitochondria

A portion of the liver was homogenized in ice-cold isolation buffer (pH 7.4) containing 220 mM mannitol, 70 mM sucrose, 2.5 mM Hepes, 10 mM EDTA, 1 mM EGTA, and 0.1% bovine serum albumin. The liver homogenate was centrifuged at 600*g* for 8 min at 4°C to remove nuclei and cellular debris. The supernatant was removed and centrifuged at 10,000*g* for 10 min at 4°C to pellet the mitochondria; the pellet was washed once with 2 ml of isolation buffer. The mitochondrial pellet was resuspended in 3% sulfosalicylic acid containing 0.1 mM EDTA, vigorously vortexed, and centrifuged to sediment the precipitated protein. A part of the supernatant was diluted in 100 mM potassium phosphate buffer (pH 6.5) for the determination of total glutathione (GSH + GSSG) and another part was added to 10 mM *N*-ethylmaleimide (NEM) in potassium phosphate buffer for the determination of GSSG.

Statistical Analysis

Results are expressed as means ± SE. Comparisons between multiple groups were by one-way analysis of variance followed by the Tukey HSD post-hoc test; $p \leq 0.05$ was considered statistically significant. SPSS Version 10.0 (SPSS Inc., Chicago, IL) was used for statistical analyses.

RESULTS

Previous data suggested a role for activated Kupffer cells in the hepatotoxicity of APAP.[7] Kupffer cells utilize NADPH oxidase to generate reactive oxygen species. To determine the importance of this enzyme in toxicity, wild-type mice (WT) and NADPH oxidase knockout mice (KO) were administered 300 mg/kg of APAP or saline and sacrificed at 1, 2, 4 or 24 h. One and 2 h are times before serum elevations of hepatic transaminases occur. Four hour is an early time point in the development of toxicity and 24 h is associated with the full development of the toxicity. Relative toxicity was determined by increases in serum transaminase levels and histological examination of slides from treated mice. The administration of APAP resulted in significant elevation of serum AST and ALT by 4 h in both WT and KO mice and there was no significant difference between the two groups of mice (Fig. 1). Also, at 24 h there was no significant difference between serum transaminase levels in the WT and KO mice (Fig. 1).

Histological analysis of necrosis in H&E stained slides in the WT and KO mice at all time points indicated the same degree of toxicity (Fig. 2). At 1 h, scattered cells in the centrilobular regions had cytoplasmic vacuoles and increased eosinophilia. By 2 h, the majority of cells in the centrilobular regions contained large vacuoles. By 4 h, nuclei of cells were pyknotic or absent and by 24 h, evidence of necrosis encompassed the entire centrilobular regions of the livers of both WT and KO animals

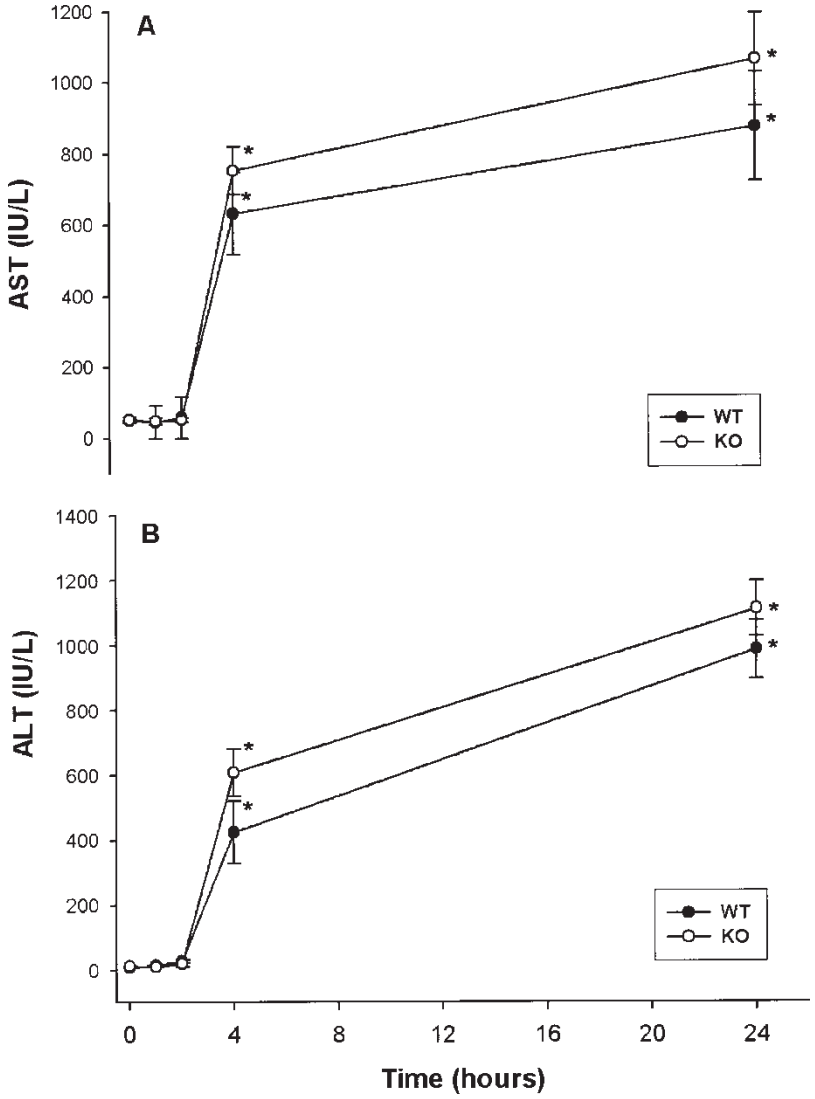

FIGURE 1 Time course for APAP-induced hepatotoxicity in WT and KO mice. (A) Serum levels of aspartate aminotransferase. (B) Serum levels of alanine aminotransferase. Mice ($n = 4$) were treated with APAP (300 mg/kg) and sacrificed at 1, 2, 4, and 24 h after APAP. No significant differences were present between WT and KO mice. (*$p < 0.05$, compared to controls, represented as time 0).

(Fig. 2). These data indicated that WT and KO mice were equally susceptible to the toxic effects of APAP.

To determine if there were differences between the WT and KO animals in the relative amount of peroxynitrite formed, immunohistochemical assays for 3-nitrotyrosine were performed. Significant nitration of tyrosine was detected in the livers of WT and KO animals at 4 (Fig. 3) and 24 h (data not shown). Thus, both the WT and KO mice were equally susceptible to nitration of tyrosine in proteins following toxic doses of APAP. These data indicate that NADPH oxidase from activated macrophages is not the source of superoxide leading to peroxynitrite and tyrosine nitration in APAP toxicity.

It has been previously reported that the percentage of GSSG in the total GSH pool (GSH + GSSG) is increased particularly in mitochondria, following toxic doses of APAP.[3,20] This has been referred to as

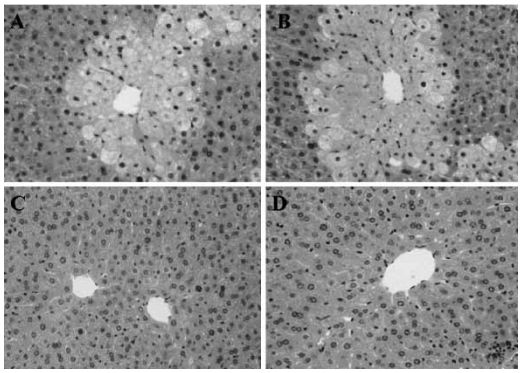

FIGURE 2 Liver pathology in WT and KO mice treated with APAP and saline (200×). (A) H&E stained section from APAP treated WT mouse at 24 h. (B) H&E stained from APAP treated KO mouse at 24 h. (C) H&E stained section from saline treated WT mouse. (D). H&E stained section from KO mouse. Mice were treated with APAP (300 mg/kg) or saline and animals were sacrificed at 24 h.

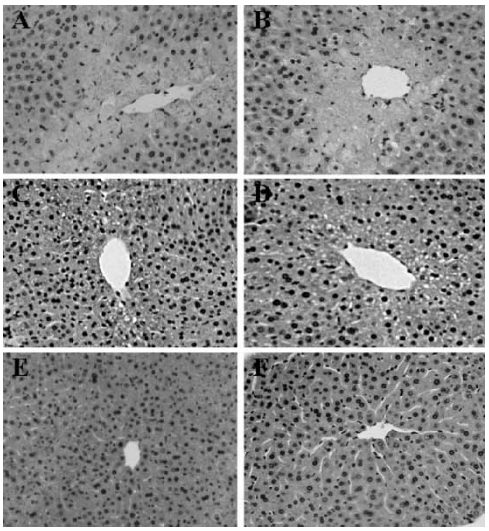

FIGURE 3 Nitrotyrosine in livers of APAP treated WT and KO mice at 24 and 4 hours (200 ×). (A) WT mouse at 24 hours. (B) KO mouse at 24 hours. (C) WT mouse at 4 hours. (D) KO mouse at 4 hours. (E) WT saline treated mouse. (F) KO saline treated mouse. Mice were treated with APAP (300 mg/kg) or saline and sacrificed at 24 or 4 hours.

mitochondrial oxidant stress. To determine if NADPH oxidase is an important source of superoxide leading to mitochondrial oxidant stress, WT and KO mice were treated with APAP and sacrificed at 1 and 2 h. The total hepatic glutathione (GSH + GSSG) in mitochondria was determined. Hepatic GSH was significantly depleted in liver homogenates of both WT and KO mice by approximately 90% at 1 and 2 h (Fig. 4A). No differences in hepatic GSH depletion were apparent between WT and KO mice. Similarly, mitochondrial GSH + GSSG was significantly reduced in both WT and KO mice at 1 and 2 h (Fig. 4B) and no differences were found between WT and KO mice. Mitochondrial oxidant stress, as indicated by the percent GSSG in the total mitochondrial glutathione pool, was significantly increased in both WT and KO animals at 1 and 2 h (Fig. 4C) and no differences were found between WT and KO animals at either time point. A repeated experiment gave similar data. Thus, the oxidant stress observed in mitochondria of APAP treated animals is not a result of NADPH oxidase.

In a previous study, we reported staining for 3-nitrotyrosine in hepatic sinusoids as a very early event in APAP-induced hepatotoxicity.[20] Nitrotyrosine staining in vascular lining cells has previously been detected as early as 1 h after APAP. These data suggested the possible involvement of activated Kupffer cells as a source of superoxide in APAP-induced hepatotoxicity. To determine if nitration of proteins in sinusoidal cells was the result of NADPH oxidase, we stained liver sections for 3-nitrotyrosine from the WT and KO mice that were sacrificed at 1 and 2 h after APAP. As shown in Fig. 5, nitration was observed in the sinusoids of treated mice at 1 h and the relative amount was similar in WT and KO mice. By 2 h following APAP, nitrotyrosine staining was present in sinusoids and in some hepatocytes (data not shown).

To further study nitrotyrosine formation in APAP toxicity, liver samples from mice treated with APAP and sacrificed at 1 and 2 h were analyzed for nitrotyrosine by ELISA. The mean (±SE) nitrotyrosine level at 2 h for WT animals was 26.3 ± 6.5 pmol/mg protein, compared to 25.6 ± 3.2 pmol/mg protein for KO animals and comparable values were found at 1 h between the KO and WT animals as well (data not shown). Thus, these data indicated that nitrotyrosine formation was similar between the two groups of mice and are consistent with the immunohistochemical studies above (Fig. 5).

Previous studies have reported APAP protein adduct formation in both centrilobular and midzonal regions by 1 h after APAP dosing in WT animals.[1] Immunohistochemical analyses for APAP covalently bound to protein were conducted to compare the extent of APAP protein binding between WT and KO mice. No staining for APAP was present among control animals. By 1 h, staining for APAP protein adducts was detectable in hepatocytes in the centrilobular regions of the livers of WT and KO animals (Fig. 6). The intensity of staining did not differ between the two groups of animals. By 2 h, staining for APAP protein adducts involved the majority of the centrilobular hepatocytes in both WT and KO animals, consistent with previous data (data not shown).[1]

DISCUSSION

Previous studies have demonstrated that nitrotyrosine co-localizes with APAP protein adducts and that its formation correlates with the development of APAP toxicity.[10,11] Nitrotyrosine is formed by peroxynitrite, which is produced by the spontaneous reaction of superoxide and nitric oxide. Superoxide and nitric oxide may be produced by activated macrophages[21] and several laboratories have reported that pretreatment of animals with macrophage inactivators (e.g. gadolinium chloride, dextran sulfate, or LPS) reduces APAP toxicity.[7,8] To further understand the role of superoxide and activated macrophages in the mediation of APAP toxicity, we studied APAP toxicity in KO mice with impaired superoxide production from macrophages. Mice deficient in gp91*phox* lack the catalytic subunit of NADPH oxidase, the major oxidant-generating enzyme in activated macrophages.[22] Peripheral blood neutrophils[17] and macrophages[18]

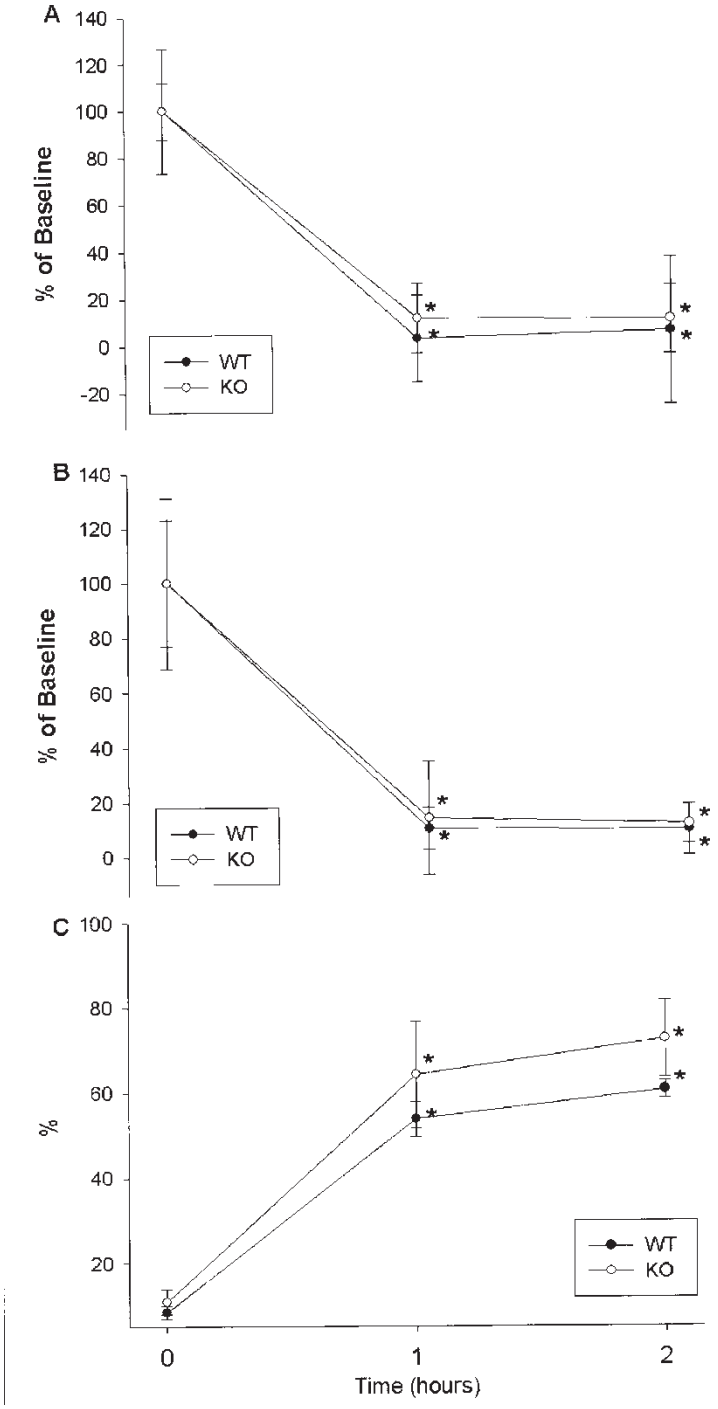

FIGURE 4 Effect of APAP on glutathione in total liver and hepatic mitochondria. (A) Hepatic GSH depletion. Baseline WT, 1.23 ± 0.16 umol/g; Baseline KO, 1.02 ± 0.27 umol/g. No statistical differences were present between baseline hepatic GSH of WT and KO mice. (B) Depletion of total mitochondrial glutathione (GSH + GSSG) content. Baseline WT, 3.95 ± 0.91 nmol GSH equivalents/mg protein; Baseline KO, 2.89 ± 0.90 nmol GSH equivalents/mg protein. No statistical differences were present between baseline hepatic GSH + GSSG of WT and KO mice. (C) Mitochondria GSSG as percent of the total (GSH + GSSG) mitochondrial glutathione pool. Mice were treated with APAP (300 mg/kg) and sacrificed at 1 and 2 h after APAP. No differences were present between WT and KO mice. (*$p < 0.05$, compared to controls, represented as time 0).

from mice deficient in pg91phox fail to generate superoxide after stimulation with PMA, as measured by the nitroblue tetrazolium test and cytochrome c reduction assay. As a result, these mice have increased susceptibility to bacterial, fungal, and parasitic infections.[17,23] Mice deficient in gp91 phox are frequently referred to as CGD mice, because they serve as a phenotypic model for the clinical syndrome, chronic granulomatous disease, a genetic disorder characterized by defective phagocytic respiratory burst oxidase and life-threatening infections. In addition, mice deficient in

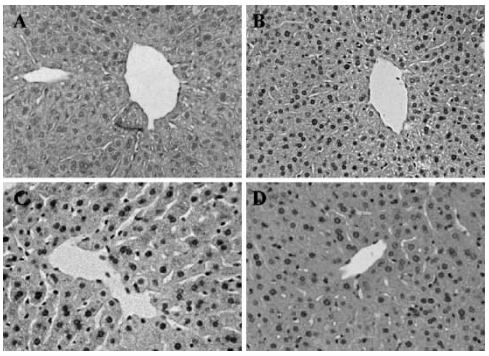

FIGURE 5 Nitrotyrosine staining at early times in livers of APAP treated WT and KO mice (200 ×). (A) WT mouse at 1 h. (B) KO mouse at 1 h. (C) WT saline treated mouse. (D) KO saline treated mouse. Mice were treated with APAP (300 mg/kg) or saline and sacrificed at 1 h.

pg91phox have been previously utilized in a study of superoxide-related liver injury. Harada recently assessed the role of NADPH oxidase-derived superoxide in an ischemia reperfusion model of liver injury and reported that the knock out mice were protected from liver injury.[24]

In the present study, we hypothesized that decreased superoxide formation in KO mice would lead to decreased peroxynitrite formation and therefore reduced nitrotyrosine staining and APAP hepatotoxicity, as compared to WT mice. In contrast, we found that mice deficient in gp91*phox* and WT mice had equal hepatotoxicity to APAP. WT and KO mice had equal levels of serum hepatic transaminases and comparable immunohistochemical staining for nitrotyrosine. In addition, no differences in nitrotyrosine formation measured by ELISA were found between the WT and KO mice. These data suggest that superoxide formation from NADPH oxidase in kupffer cells does not contribute to peroxynitrite formation and to the mediation of APAP toxicity.

Previous data have demonstrated that mitochondrial oxidant stress is important in APAP toxicity.[3]

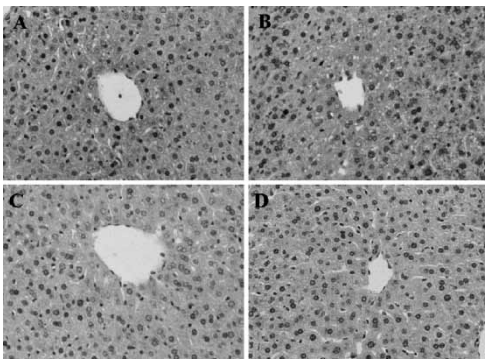

FIGURE 6 APAP-protein adducts in the livers of APAP treated WT and KO mice (200 ×). (A) WT mouse at 1 h. (B) KO mouse at 1 h. (C) WT saline treated mouse. (F) KO saline treated mouse. Mice were treated with APAP (300 mg/kg) and sacrificed at 1 h.

Knight and coworkers[20] found that the percentage of mitochondrial GSSG increased 8 fold in mice 6 h after APAP dosing. These results are consistent with the findings of the present study showing 6 and 7 fold elevation in mitochondrial %GSSG at 1 and 2 h after APAP. Mitochondrial dysfunction occurs early after the administration of toxic APAP doses.[25] Moreover, mitochondrial proteins are covalently bound by APAP.[26]

The immunohistochemical data of the present study are also consistent with the previous finding of early staining (i.e. 1 h) for nitrotyrosine in the vascular endothelial cells of the centrilobular regions of the liver.[20] In the present study, staining for nitrotyrosine was present by 1 h in the vascular endothelial cells of both WT and KO mice. Sinusoidal endothelial cells have previously been demonstrated to be susceptible to APAP under *in vitro* conditions.[27] DeLeve *et al.* reported that GSH was depleted by 60% in sinusoidal endothelial cells of C3H-HEN mice exposed to APAP, and that the susceptibility of these cells to APAP was reversed by pre-treatment with aminobenzotriazole, a suicide inhibitor of P450 isozymes.[27] Sinusoidal endothelial cells have been shown to possess cytochrome P450 activity[28] and to be targets of metabolic activation.[29] In addition, direct infusion of sources of superoxide and nitric oxide (i.e. the precursors of peroxynitrite) in an isolated perfused rat liver system resulted in morphological and structural alterations in liver sinusoids, consistent with the *in vivo* changes found in APAP toxicity.[30] Collectively, these studies suggest the APAP metabolism to NAPQI and peroxynitrite formation occur in sinusoidal endothelial cells a very early event in APAP toxicity.

The development of equal toxicity in KO and WT mice suggests that superoxide generated from Kupffer cells does not play a critical role in the mediation of APAP toxicity. The data from the present study are interesting in light of previous studies[7,8] showing that treatment of mice with macrophage inactivators decreases acetaminophen toxicity. However, increased superoxide formation may arise from sources other than resident macrophages, such as recruited macrophages, neutrophils and xanthine oxidase. Both macrophages[1] and neutrophils[31] are recruited to the liver during APAP toxicity. Since accumulation of these inflammatory cells[1] follows the increase of hepatic transaminases, it is unlikely that these cells are unlikely involved in the mechanism of injury. Moreover, functional inactivation of neutrophils with antibodies against β_2 integrins did not protect against APAP hepatotoxicity.[31] Our current data with KO mice are consistent with this previous study[31] and suggest that neutrophil-derived reactive oxygen species are not involved in vascular or hepatocellular peroxynitrite formation and the initiation of liver cell injury.

Increased conversion of xanthine dehydrogenase to the superoxide-generating enzyme xanthine oxidase has been observed during APAP hepatotoxicity.[3] Treatment with high doses of allopurinol, an inhibitor of xanthine oxidase, prevented nitrotyrosine formation and protected effectively against APAP-induced liver injury.[3,20] The mechanism of decreased toxicity was by inhibition of covalent binding of the reactive metabolite to the protein. Lower doses of allopurinol, which completely inhibited xanthine oxidase activity, did not affect APAP hepatotoxicity.[3] These data suggest that xanthine oxidase is not a relevant source of superoxide formation after APAP treatment. Since high dose allopurinol prevented the increase in mitochondrial GSSG levels and hepatocellular nitrotyrosine staining, we postulated that mitochondria are the main source of the intracellular oxidant stress.[3,20] Our current data confirm the increased mitochondrial GSSG formation in APAP toxicity and demonstrate that oxidant stress is independent of NADPH oxidase.

Recent data indicate that homologues of gp91*phox* are present in nonphagocytic cells. The first described homologue, Nox1, formerly referred to as Mox1, has been found to be present in human, rat and mouse vascular smooth muscle cells as well as other cells such as colonic epithelium and fibroblasts.[18,32] Nox1 is thought to be of importance in mitogenic signaling, cancer, and angiogenesis.[33] Nox1-transfected cells have increased superoxide generation.[32] Mice deficient in gp91 *phox* and WT mice[18] were found to have equal amounts of Nox1 mRNA, suggesting that Nox1 is regulated independently of gp91*phox*[18] Whether or not Nox1 or other homologues of gp91*phox* are present in the liver and/or the sinusoidal endothelium is unknown. Therefore, in the present study, we cannot completely rule out that some superoxide may have been generated from nonphagocytic homologues of NADPH oxidase, which would be present in both WT and KO animals. However, the fact that allopurinol prevented mitochondrial dysfunction and oxidant stress[20] suggest that homologues of NADPH oxidase are not a major source of superoxide in APAP hepatotoxicity.

In conclusion, we found that mice lacking NADPH oxidase activity and WT mice had equal toxicity to APAP. The data of the present study are consistent with the hypothesis that mitochondria are the major source of increased superoxide formation, leading to peroxynitrite and the resulting toxicity. Sinusoidal endothelial cells appear to be early targets of APAP toxicity by NAPQI and peroxynitrite.

Acknowledgements

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