Involvement of Toll-like receptor 4 in acetaminophen hepatotoxicity

Herbert C. Yohe,1,5 Kimberley A. O’Hara,6 Jane A. Hunt,1 Tamar J. Kitzmiller,1 Sheryl G. Wood,1 Jenna L. Bement,1 William J. Bement,1 Juliana G. Szakacs,2 Steven A. Wrighton,3 Judith M. Jacobs,1,6 Vsevolod Kostrubsky,2 Peter R. Sinclair,1,5,7 and Jacqueline F. Sinclair1,5,7

1Veterans Administration Medical Center, White River Junction, Vermont; 2Department of Pathology, Harvard Vanguard Medical Associates, Boston, Massachusetts; 3Department of DrugDisposition, Lilly Research Laboratories, Indianapolis, Indiana; 4Drug Safety Evaluation, Pfizer Research and Development, Ann Arbor, Michigan; and 5Department of Pharmacology and Toxicology, Department of Immunology, and 7Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire

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YOHE, Herbert C., KIMBERLEY A. O’HARA, JANE A. HUNT, TAMAR J. KITZMILLER, SHERYL G. WOOD, JENNA L. BEMENT, WILLIAM J. BEMENT, JULIANA G. SZAKACS, STEVEN A. WRIGHTON, JUDITH M. JACOBS, VSEVOLOD KOSTRUBSKY, PETER R. SINCLAIR, and JACQUELINE F. SINCLAIR. Involvement of Toll-like receptor 4 in acetaminophen hepatotoxicity. Am J Physiol Gastrointest Liver Physiol 290: G1269–G1279, 2006. First published January 26, 2006; doi:10.1152/ajpgi.00239.2005.—The objective of this study was to determine whether Toll-like receptor 4 (TLR4) has a role in alcohol-mediated acetaminophen (APAP) hepatotoxicity. TLR4 is involved in the inflammatory response to endotoxin. Others have found that ethanol-mediated liver disease is decreased in C3H/HeJ mice, which have a mutated TLR4 resulting in a decreased response to endotoxin compared with endotoxin-responsive mice. In the present study, short-term (1 wk) pretreatment with ethanol plus isopentanol, the predominant alcohols in alcoholic beverages, caused no histologically observed liver damage in either C3H/HeJ mice or endotoxin-responsive C3H/HeN mice, despite an increase in nitrotyrosine levels in the livers of C3H/HeN mice. In C3H/HeN mice pretreated with the alcohols, subsequent exposure to APAP caused a transient decrease in liver nitrotyrosine formation, possibly due to competitive interaction of peroxynitrite with APAP producing 3-nitroacetaminophen. Treatment with APAP alone resulted in steatosis in addition to congestion and necrosis in both C3H/HeN and C3H/HeJ mice, but the effects were more severe in endotoxin-responsive C3H/HeN mice. In alcohol-pretreated endotoxin-responsive C3H/HeN mice, subsequent exposure to APAP resulted in further increases in liver damage, including severe steatosis, associated with elevated plasma levels of TNF-α. In contrast, alcohol pretreatment of C3H/HeJ mice caused little to no increase in APAP hepatotoxicity and no increase in plasma TNF-α. Portal blood endotoxin levels were very low and were not detectably elevated by any of the treatments. In conclusion, this study implicates a role of TLR4 in APAP-mediated hepatotoxicity.

endotoxin; nitrotyrosine

CHRONIC ALCOHOL CONSUMPTION is associated with increased risk of acetaminophen (APAP) hepatotoxicity (reviewed in Ref. 75). In humans, necrosis, steatosis, and inflammation are observed in APAP hepatotoxicity associated with alcohol consumption (75). This steatosis in humans has been attributed to the alcohol consumption, because in the cases of APAP overdose with no alcohol consumption, only necrosis and inflammation have been observed (75). Several studies have implicated bacterial endotoxin involvement in experimental alcoholic liver disease. Many bacterial products trigger an inflammatory innate immune response by pattern recognition receptors called Toll-like receptors (TLR). One member of the Toll-like receptor family, TLR4, is the receptor for bacterial endotoxin (3). Macrophages, including the Kupffer cells of the liver, respond to bacterial endotoxin via TLR4, resulting in the release of reactive oxygen species and proinflammatory cytokines. Although acute ethanol administration can result in downregulation of TLR4 (47), chronic administration of ethanol enhances TLR4 levels and TLR4-based signals in rodents, suggesting that TLR4 activity contributes to the development of alcohol-induced liver disease (44, 76). Chronic treatment of rats with ethanol alone increased plasma levels of endotoxin (10, 45), whereas inclusion of antibiotics during the alcohol treatment decreased endotoxin, inflammation, and steatosis (1, 10). In addition, human hepatic stellate cells, the main fibrogenic cell type arising in injured liver, contain TLR4 and respond to endotoxin, upregulating pathways leading to elevated levels of chemokines and adhesion molecules that can further aggravate liver injury (48).

C3H/HeJ (HeJ) mice display normal CD14 and bind endotoxin but respond weakly (62). In a genetic comparison with the near-congenic C3H/HeN strain, Poltorak et al. (50) found that HeJ mice have a missense mutation in TLR4 that causes their decreased response to endotoxin. Even in the presence of LPS (endotoxin)-binding protein, macrophages from HeJ mice, including Kupffer cells of the liver, fail to respond to endotoxin (61). After 1 mo of intragastric ethanol feeding, hepatic steatosis, inflammation, and necrosis were dramatically decreased in HeJ mice compared with endotoxin-responsive mice (68). However, despite the association between TLR4 and alcohol-induced liver injury, an association with alcohol-enhanced APAP hepatotoxicity and TLR4 has not yet been made. Therefore, we investigated the role of TLR4 in APAP-mediated hepatotoxicity, comparing TLR4 mutant HeJ mice with endotoxin-responsive C3H/HeN (HeN) mice.

METHODS

Animals. Female HeN and HeJ mice were obtained from the National Cancer Institute and housed in a controlled environment with a 12:12-h light-dark cycle. Mice weighed 18–26 g and were 3 mo old. HeN and HeJ mice originated from the C3H/He strain (54) and are

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used extensively for comparison of endotoxin responsiveness (reviewed in Refs. 18, 46).

**Alcohol and APAP treatments.** Animal treatment protocols were approved by the Institutional Animal Care and Use Committee of the Veterans Administration Medical Center. Mice were fed liquid control diet (Lieber-DeCarli) containing maltose-dextrin for an initial 2-day period. Alcohol-treated animals were then administered 2.8% (wt/vol) ethanol and 0.4% (wt/vol) isopentanol, the predominant alcohols in alcoholic beverages (53), for 7 days in liquid diet, as described previously (58).

Liquid diets were substituted with water 11 h before APAP administration to eliminate alcohols from blood, because serum levels of 5 mM ethanol protect animals from APAP hepatotoxicity (66). APAP was delivered by intragastric intubation as described previously (59) at doses indicated. One and seven hours after APAP dosing, CO2 was used to anesthetize the mice. Blood was removed by cardiac puncture for measurement of plasma alanine aminotransferase (ALT) and TNF-α.

**Effect of alcohol treatment on cytochrome P-450 and inducible nitric oxide synthase.** Animals were euthanized immediately after the 7-day alcohol treatment. A 20% (wt/vol) liver homogenate was prepared in buffer containing 0.1 M Tris (pH 7.4), 0.1 M KCl, 0.1 mM EDTA, 10 μM phenylmethylsulfonyl fluoride, 10 μg/ml antipain, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 20 μg/ml aprotonin (modification of Ref. 40). Homogenates were centrifuged at 12,000 g for 10 min at 4°C. Supernatant was removed for analysis of inducible nitric oxide synthase (iNOS), and the remainder was used for preparing microsomes (59).

Cytochrome P-450 (CYP) forms 2E and 3A were measured in hepatic microsomes by Western blotting (59), using an antibody against human CYP3A that detects mouse CYP3A (59) and an anti-human CYP2E1 antibody that detects mouse CYP2E1 (Oxford Biomedical Research, Oxford, MI). CYP2E1 was quantified by densitometry (ONE-Scan; Scanalytics, Fairfax, VA).

iNOS in 12,000 g supernatants was measured by Western blotting (40). Antibody to mouse iNOS and iNOS standard were purchased from Upstate Biotechnology (Lake Placid, NY). As a positive control, endotoxin-responsive mice were injected intraperitoneally with killed Escherichia coli K-235 (1.4 mg/0.5 ml sterile PBS) and then killed 24 h later, and liver supernatants were prepared as described above.

**Histology.** Four-micron sections of formalin-fixed liver embedded in paraffin were stained with hematoxylin and eosin. Two to three complete transsections of each liver were reviewed at four levels by a board-certified pathologist who was blinded to treatments and strains. Steatosis, congestion, and necrosis were graded separately over the entire submitted tissue as previously described (59). The grading system used to measure the extent of damage was the same for steatosis, necrosis, and congestion as follows: normal, no abnormality; mild, ≤30% of cells or lobule affected; moderate, 30–60% of cells or lobule affected; and severe, >60% of cells or lobule affected. Criteria for necrosis included karyorrhexis (loss of nucleus) and/or degeneration of cytoplasm with either coagulative or liquefactive changes. Steatosis was defined as either macrovesicular, if the vacuoles were multiple within the cytoplasm and did not indent the nucleus, or macrovesicular, if there was a single vacuole with displacement and distortion of the nucleus. Steatosis progresses from macrovesicular to macrovesicular with severity; however, our criteria for evaluation were the number of cell affected, not the size of the vacuole within the cell. Congestion was identified by the expansion of the sinusoids with blood cellular elements. Inability to view hepatocytes because of congestion was not equated with necrosis.

**Nitrotyrosine.** Nitrotyrosine was measured immunohistochemically on 4-μm sections of formalin-fixed paraffin-embedded liver sections with the use of an anti-nitrotyrosine rabbit polyclonal antibody (Molecular Probes, Eugene, OR). Tissue sections were deparaffinized, rehydrated, and incubated with 2% H2O2 to block endogenous peroxidase activity. Nonspecific binding was blocked with 10% normal goat serum before overnight incubation at 4°C with the primary antibody. Bound antibodies were visualized using the Vector Elite rabbit ABC peroxidase kit (Vector Laboratories, Burlingame, CA) according to manufacturer’s instructions with 3,3’-diaminobenzidine (Vector Laboratories) as the substrate. Sections were counterstained with hematoxylin QS (Vector Laboratories). Rabbit IgG (Dako, Carpinteria, CA) was used as the negative control, and the antibody specificity was verified by the absence of staining in liver sections exposed to antibody preincubated with 10 mM 3-nitro-l-tyrosine. Brown-stained areas indicate nitrotyrosine-protein adducts. The intensity of the staining was scored blinded to the treatment and the strain, with four fields analyzed per slide.

**Adipophilin.** Adipophilin was measured immunohistochemically on 4-μm sections of formalin-fixed paraffin-embedded liver sections with the use of a guinea pig polyclonal antibody to adipophilin (Pregen, Heidelberg, Germany). Tissue sections were deparaffinized, rehydrated, and incubated with 2% H2O2 to block endogenous peroxidase activity. Tissue sections were microwaved (2 × 5 min) in 10 mM sodium citrate buffer (pH 6.0) to unmask antigens. Nonspecific binding was blocked with 10% normal goat serum before 1-h incubation at room temperature with the primary antibody. Bound antibodies were visualized using the Vector goat ABC peroxidase kit (Vector Laboratories) according to the manufacturer’s instructions with 3,3’-diaminobenzidine (Vector Laboratories) as the substrate. Sections were counterstained with hematoxylin QS (Vector Laboratories). Brown-stained areas indicate adipophilin protein.

**Lipid staining.** Lipid staining was done with oil red O, using a modification of the original method by Lillie and Ashburn (31). Frozen liver sections (10 μm) were air-dried in the cryostat to anneal the sections to the slide and then fixed for 1 min in 10% formalin. Sections were rinsed in distilled water, stained with oil red O for 6 min, rinsed in tap water, and counterstained with hematoxylin QS (Vector Laboratories). Sections were then mounted in glycerol mounting medium (Aqua mount; Polysciences, Warrington, PA).

**TNF-α.** TNF-α was measured in plasma and liver by ELISA (R&D Systems, Minneapolis, MN). TNF-α levels in freshly prepared plasma and liver supernatants were compared with samples that had been stored at −80°C and thawed once. No differences were observed.

**Reduced glutathione.** Reduced glutathione (GSH) was measured in whole liver homogenates and in cytosolic and mitochondrial fractions. Mice were administered Lieber-DeCarli diet, with and without the alcohols, as described in Alcohol and APAP treatments. After the fast, mice were killed either immediately or 1 h after the administration of APAP. The 20% liver homogenates were prepared in a Tris-sucrose buffer (pH 7.2) containing 0.25 M sucrose, 1 mM EDTA, 10 mM KCl, and 10 mM Tris·HCl. Homogenates were centrifuged at 1,000 g for 10 min at 4°C. The supernatants were removed and centrifuged at 10,000 g for 10 min at 4°C, and resulting supernatants were designated as the cytosolic fractions and the pellets as the mitochondrial fractions. The whole liver homogenates and the cytosolic fractions were extracted with an equal volume of 10% TCA; 5% TCA (500 μl) was added directly to the mitochondrial pellets. All TCA extracts were then centrifuged at 4°C for 10 min at 10,000 g. GSH was analyzed in the TCA supernatants by using a modification of the procedure of Boyne and Ellman (60). For measurement of protein, pellets from the TCA extracts were solubilized in 0.1 N NaOH-0.1% SDS with the use of an anti-nitrotyrosine rabbit polyclonal antibody (Molecular Probes, Eugene, OR). Tissue sections were deparaffinized, rehydrated, and incubated with 2% H2O2 to block endogenous peroxidase activity. Nonspecific binding was blocked with 10% normal goat serum before overnight incubation at 4°C with the primary antibody. Bound antibodies were visualized using the Vector Elite rabbit ABC peroxidase kit (Vector Laboratories, Burlingame, CA) according to manufacturer’s instructions with 3,3’-diaminobenzidine (Vector Laboratories) as the substrate. Sections were counterstained with hematoxylin QS (Vector Laboratories). Rabbit IgG (Dako, Carpinteria, CA) was used as the negative control, and the antibody specificity was verified by the absence of staining in liver sections exposed to antibody preincubated with 10 mM 3-nitro-l-tyrosine. Brown-stained areas indicate nitrotyrosine-protein adducts. The intensity of the staining was scored blinded to the treatment and the strain, with four fields analyzed per slide.
used did not interfere with the assay, and no samples inhibited the assay of endotoxin standards. Hemoglobin-NO was measured in the blood by using electron paramagnetic resonance spectroscopy (6). Microsomal formation of the active metabolite of APAP was measured using HPLC as described previously (59).

**Statistical analysis.** Values are presented as means ± SE or SD, as indicated. Data were analyzed for normalcy and statistical significance using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical analyses were done using either Student’s t-test or one-way ANOVA and the post hoc Tukey-Kramer multiple comparisons test for intrastrain comparisons. Two-way ANOVA was used for all interstrain comparisons for strain and treatment effects combined. Where appropriate, nonparametric analyses were done using the Mann-Whitney or Wilcoxon test. P values < 0.05 indicate significance as noted in the text.

**Chemicals.** APAP and isopentanol (a mixture of 70% 3-methyl butanol and 30% 2-methyl butanol) were obtained from Sigma Chemical (St. Louis, MO), absolute ethanol (USP) from PharmCo Products (Brookfield, CT), and Lieber-DeCarli diets from BioServ (Frenchtown, NJ).

**RESULTS**

**APAP hepatotoxicity in HeN vs. HeJ mice.** In these studies, hepatotoxicity was determined in two ways: by histological examination of the livers (Table 1; Fig. 1) and by plasma ALT (Fig. 2). As assessed using both methods (Table 1; Figs. 1 and 2A), APAP treatment was more hepatotoxic to endotoxin-responsive HeN mice than to endotoxin-hyporesponsive HeJ mice. APAP-mediated liver damage for each of the three different criteria (steatosis, congestion, and necrosis) was significantly greater in HeN mice compared with HeJ mice (Table 1, P < 0.001). At an APAP dose of 300 mg/kg, plasma ALT

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**Table 1. Histological analysis of acetaminophen-mediated liver damage in C3H/HeN vs. C3H/HeJ mice**

<table>
<thead>
<tr>
<th>Strain: Pretreatment</th>
<th>APAP, mg/kg</th>
<th>Total no. of Animals</th>
<th>Normal</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
<th>Congestion</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
<th>Necrosis</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
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<tr>
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<td>17</td>
<td>17</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>HeJ: None</td>
<td>0</td>
<td>12</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>HeN: None</td>
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<td>14</td>
<td>2</td>
<td>1</td>
<td>9</td>
<td>11</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>HeJ: None</td>
<td>300</td>
<td>12</td>
<td>6</td>
<td>1</td>
<td></td>
<td>5</td>
<td>1</td>
<td>6</td>
<td></td>
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<tr>
<td>HeN: EIP</td>
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<td>12</td>
<td>11</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>HeJ: EIP</td>
<td>0</td>
<td>16</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>HeN: EIP</td>
<td>300</td>
<td>8</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>12</td>
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<tr>
<td>HeJ: EIP</td>
<td>300</td>
<td>12</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td></td>
<td>4</td>
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*Animals were pretreated for 7 days with control Lieber-DeCarli diet or diet containing 2.8% (wt/vol) ethanol along with 0.4% (wt/vol) isopentanol for 7 days (EIP). At the end of 7 days, the diet was replaced with water for 11 h, and acetaminophen (APAP) was administered intragastrically in saline. The animals were euthanized 7 h after APAP administration. Slices of liver were fixed in formalin, stained with hematoxylin and eosin, and examined histologically. Grading of damage: mild, in <30% of lobule; moderate, in 30–60% of lobule; severe, in >60% of lobule. The numbers in each column indicate the number of mice with noted degree of damage. Data were analyzed by comparing strain and treatment for 1) percentage of normal vs. abnormal liver pathology independently using the 3 different criteria steatosis, congestion, and necrosis, and 2) severity of total liver damage (see RESULTS).*
levels also were significantly greater in HeN mice compared with HeJ mice (Fig. 2A, P < 0.02). APAP alone has not been shown previously to cause steatosis in experimental animals (38), yet steatosis was observed histologically in these mice. To confirm that the vesicles actually contained fat, we analyzed liver sections for lipids with oil red O and for the presence of adipophilin. Adipophilin is a protein located on the surface of lipid droplets, including lipid droplets observed in fatty liver (15). In C3H/HeN mice treated with APAP alone (300 mg/kg), the macrovesicular and microvesicular vesicles identified as being fatty droplets in the histological analysis (Table 1) contained adipophilin on the surface of the droplets (Fig. 3) and stained with oil red O (Fig. 4), thus confirming the presence of steatosis in these mice.

Effect of alcohol pretreatment on APAP hepatotoxicity in HeN vs. HeJ mice. A 7-day treatment with ethanol plus isopentanol (EIP) caused essentially no liver damage in either strain of mice, as analyzed histologically (Table 1) and by plasma ALT (results not shown), similar to our previous findings in rats (28, 58). However, pretreatment with EIP increased APAP hepatotoxicity compared with APAP alone in HeN mice (Table 1, P < 0.01) but not in HeJ mice (Table 1).

In mice treated with APAP, plasma ALT levels were significantly elevated by the alcohol pretreatment in HeN mice (P < 0.001) but not in HeJ mice (Fig. 2B). There is a good correlation between the degree of liver damage measured histologically and by plasma ALT levels in individual animals treated with 300 mg/kg APAP (Fig. 5A, HeN mice, r² = 0.76; Fig. 5B, HeJ mice, r² = 0.5). The values for liver damage and ALT cluster at the top of the range for HeN mice (Fig. 5A) and near the bottom of the range for HeJ mice (Fig. 5B), illustrating greater severity of liver damage in HeN mice compared with HeJ mice.

Effect of alcohols and APAP on hepatic levels of nitrotyrosine in HeN mice. Liver damage from alcohol or APAP has been associated with increased formation of nitrotyrosine in the liver because of the formation of highly reactive peroxynitrite generated from the reaction of NO with superoxide (reviewed in Refs. 19, 20, 23). However, recent studies have shown that the reaction of NO with superoxide is a protective mechanism (12, 16, 41). In animals pretreated with the alcohols followed by an 11-h fast, hepatic nitrotyrosine was increased in HeN mice but not in HeJ mice (Fig. 6, P < 0.03). In a time-course study in HeN mice treated with alcohols and APAP, APAP
caused a decrease in nitrotyrosine at 1, 2, and 4 h (Fig. 7, A–F, \( P < 0.01 \)). By 7 h after APAP, the nitrotyrosine levels appeared to increase compared with the earlier time points (Fig. 7G). Hepatic levels of nitrotyrosine in both control and alcohol-treated HeJ mice were similar to the alcohol-induced levels in HeN mice (Fig. 6) despite the lower levels of liver damage from APAP in HeJ mice (Fig. 2; Table 1).

Effect of alcohols and APAP on endotoxin, nitrite, and iNOS. Alcohol-mediated increases in nitrotyrosine in HeN mice may have arisen from induction of iNOS by endotoxin (reviewed in Ref. 23). Therefore, we investigated whether EIP...
increased liver iNOS and blood levels of endotoxin and nitrite. Both strains of mice had low levels of endotoxin in portal blood (<12 pg/ml), determined immediately at the end of the alcohol treatment. Hepatic levels of iNOS protein and plasma nitrite levels were not increased by any of the treatments, nor was whole blood hemoglobin-bound NO (results not shown).

**Effect of alcohols and APAP on hepatic and plasma TNF-α in HeN and HeJ mice.** We investigated whether short-term treatment of the mice with EIP or APAP, or both, increased liver and plasma TNF-α. Hepatic TNF-α protein was greater (5.4-fold) in untreated HeN mice compared with untreated HeJ mice (Fig. 8A, P < 0.001). The amount of hepatic TNF-α protein observed in untreated HeN mice was similar to that
reported for endotoxin-responsive mice of other strains (17). Treatment with EIP alone caused twofold increases in hepatic TNF-α in HeJ mice (P < 0.05) but not in HeN mice (Fig. 8A). However, even with this increase, the hepatic levels of TNF-α in HeJ mice were still lower than levels in HeN mice.

Plasma TNF-α was detected in HeN mice treated with APAP and was increased 14-fold in animals treated with the alcohols before APAP (Fig. 8B, P < 0.01). Although these findings suggest that TNF-α may have been transiently increased in the liver and subsequently released into the blood, there was no increase in hepatic TNF-α measured at 1, 2, and 4 h after administration of APAP (data not shown). In HeJ mice, plasma TNF-α was below the level of detection of the assay in all treatment groups (Fig. 8B).

Effect of the alcohol pretreatment on hepatic CYP2E1 and CYP3A. Pretreatment with EIP has been shown to induce CYP2E1 and CYP2E2 in rodents (32, 58), two forms of CYP that activate APAP (49, 65). In both strains of mice, basal levels of CYP2E1 were similar and were induced by EIP to similar levels (elevated ~50%; Fig. 9, P < 0.01). Thus our findings that alcohol potentiates APAP hepatotoxicity in HeN but not HeJ mice suggest that the alcohol effect is not solely mediated by induction of CYP2E1. Basal levels of CYP3A forms also were similar in both strains. Treatment with EIP increased the higher molecular weight CYP3A protein, with a greater increase in this CYP3A form in HeN compared with HeJ mice (Fig. 10). However, microsomal activities for APAP activation, measured as the APAP glutathione adduct (APAPSG), were similar in both strains (5.2 ± 1.6 vs. 4.9 ± 1.8 nmol APAPSG·min⁻¹·mg protein⁻¹ for HeN vs. HeJ mice, respectively; n = 6 mice/strain).

Comparison of hepatic glutathione in HeN and HeJ mice. Because GSH plays a critical role in inactivating the reactive metabolite of APAP (reviewed in Refs. 19, 20), we investigated whether differences in hepatic GSH in HeN and HeJ mice could account for the differences in APAP hepatotoxicity. Hepatic levels of GSH were not markedly different between HeN and HeJ mice (Fig. 11, A–C). Mitochondrial (Fig. 11B) and cytosolic levels of GSH (Fig. 11C) also were similar in untreated mice of both strains. At 1 h after APAP administration, hepatic GSH levels were decreased in both mitochondrial and cytosolic fractions, but the decreases were similar for C3H/HeN and C3H/HeJ mice (Fig. 11, B and C). Thus hepatic GSH levels at the time of administration of APAP cannot account for the differences in APAP hepatotoxicity in the two strains.

DISCUSSION

The current study was undertaken to determine whether TLR4 plays a role in APAP-dependent hepatotoxicity in the presence and absence of pretreatment with alcohols. Comparing TLR4-defective, endotoxin-hyporesponsive HeJ mice with normal HeN mice, we found that APAP-dependent steatosis, congestion, and necrosis all were greater in HeN mice (Table 1). Furthermore, pretreatment with the alcohols in-

![Fig. 8](http://example.com/figure8.png)

Fig. 8. Effect of alcohols and APAP on hepatic and plasma levels of TNF-α in HeN vs. HeJ mice. Mice were treated as described in the legend to Fig. 1. TNF-α was measured in liver (A) and plasma (B) by ELISA. Each value represents the mean ± SE of samples from 5–8 mice per treatment. nd, Not done; u, below the limit of detection of the assay. Data were analyzed using 2-way ANOVA, omitting the data for APAP alone from the analysis (see RESULTS).

![Fig. 9](http://example.com/figure9.png)

Fig. 9. Effect of the alcohol pretreatment on hepatic cytochrome P-450 (CYP) form CYP2E1. Mice were treated with control liquid diet or EIP for 7 days. At the end of this time, the animals were euthanized liver microsomes were prepared and analyzed immunochemically for CYP2E1, as described in METHODS. Each value represents the mean ± SE of the densitometric scans of immunoblots from 4 animals per treatment. Data were analyzed using 2-way ANOVA (see RESULTS).

![Fig. 10](http://example.com/figure10.png)

Fig. 10. Effect of the alcohol pretreatment on hepatic levels of CYP3A. Hepatic microsomes from mice described in the legend to Fig. 7 were analyzed for CYP3A, as described in METHODS.)
creased APAP-dependent steatosis, congestion, and necrosis in HeN mice but not in HeJ mice (Table 1). These findings strongly suggest that TLR4 is involved in APAP-mediated liver damage.

In experimental studies on alcohol-mediated increases in APAP hepatotoxicity, we and others in the field continue to use the Lieber-DeCarli diet as a way to investigate the effect of chronic alcohol consumption on APAP hepatotoxicity (2, 27, 28, 34, 58, 63, 67, 73, 74). This diet was developed to overcome the aversion that most strains of rodents have to consume ethanol. This model would be similar to the alcohol abuser, because the animal is continually consuming ethanol as part of the diet. In rodents on this diet, blood alcohol levels can be as high as 50 mM (32). Thus, in experimental models using the Lieber-DeCarli diet to investigate alcohol-mediated APAP hepatotoxicity, the alcohols are withdrawn 11–24 h before the administration of APAP to allow the clearance of ethanol from the blood, because blood ethanol concentrations as low as 5 mM (equivalent to an acute exposure) inhibit APAP hepatotoxicity, probably because of decreases in NADPH, the cofactor for CYP (66).

The doses of APAP used in our studies are high compared with clinical doses. However, high doses of APAP are used in rodent models because rodents are more resistant to APAP than humans. Yet, rodent studies identified the role of CYP in APAP activation and the protective role of GSH and N-acetylcysteine, leading to the clinical treatment of APAP overdose (8, 21, 22, 38, 39, 51, 52).

The 7-day treatment with alcohols used in our study did not increase hepatic or plasma TNF-α or portal blood endotoxin. In contrast, Uesugi et al. (68) reported that treatment of endotoxin-responsive mice with ethanol intragastrically for 1 mo resulted in increased plasma endotoxin and hepatic TNF-α mRNA. The lack of alcohol-mediated increases in endotoxin in our study may be due to the alcohol feeding method, the shorter treatment time, or the intestinal flora of the mice in our facility. In another study, no increases in hepatic TNF-α mRNA were observed in C57BL/6 mice fed ethanol in the Lieber-DeCarli diet for 14 days (13), suggesting that portal blood endotoxin also was not elevated by this treatment.

Ethanol alters the sensitivity of liver Kupffer cells to bacterial endotoxin (LPS) (71). The presence of ethanol reduces the transfer of the LPS-CD14 complex to the lipid raft-based TLR4 (7). However, although the presence of ethanol is inhibitory, an enhancement of the LPS response associated with increased CD14 expression has been observed 24 h after ethanol administration (72). These findings may explain, in part, why ethanol has a transient inhibitory effect on LPS signaling (71, 72). These studies involved the administration of a pharmacological dose of endotoxin (71, 72). In our study, endogenous endotoxin levels were low and similar in both C3H/HeJ and C3H/HeN mice. The 7-day treatment with ethanol in combination with isopentanol did not elevate endotoxin levels in either portal or peripheral blood. However, the alcohol pretreatment may have increased the sensitivity of TLR4 to basal levels of endotoxin, possibly by increasing expression of CD14 (71, 72).

Alternatively, our finding of greater APAP injury in the TLR4-competent HeN strain, with no difference in endotoxin levels between the two strains, suggests that a mechanism independent of endotoxin may be working through TLR4. It now has been shown that low-molecular-weight hyaluronic acid, released from extracellular matrix during chemical or mechanical injury, also initiates proinflammatory signals through TLR4 (64). Such injuries can further amplify the systemic inflammatory immune responses by enhancing TLR4
reactivity and also can result in leukocyte sequestration in the lungs and liver along with increased proinflammatory cytokine and chemokine levels in these tissues (43). Serum hyaluronic acid is elevated in APAP-mediated liver injury in humans and even has been suggested as a prognostic indicator of survivability in APAP overdose (70). In eosinophils and lymphocytes, hyaluronic acid signaling through TLR4 increases the levels of granulocyte monocyte-colony stimulating factor (GM-CSF) by stabilization of GM-CSF mRNA, resulting in rapid initiation of an inflammatory cascade (11). In a recent study of the genomics and proteomics of APAP injury in mouse liver, a threefold increase of GM-CSF mRNA occurred within 15 min of APAP administration (55). Cultured vascular smooth muscle cells from normal mice release low levels of GM-CSF, but the levels in cells from the C3H/HeJ strain are barely detectable (56). These findings are consistent with the lower APAP-mediated liver injury in C3H/HeJ mice, compared with that in TLR4-competent C3H/HeN mice, in both the presence and absence of alcohol (Table 1); a hyaluronic acid-enhanced TLR4 response would not occur in C3H/HeJ mice. Whether GM-CSF is involved through a hyaluronic acid-mediated TLR4 signal in APAP-mediated liver injury is now under investigation in this laboratory.

Our findings that exposure to APAP for 7 h was associated with increases in plasma TNF-\(\alpha\) in alcohol-pretreated HeN mice with no increase in hepatic TNF-\(\alpha\) (Fig. 8) suggest that hepatic TNF-\(\alpha\) may have been transiently increased by APAP in these mice and then released into the blood. However, we found that hepatic TNF-\(\alpha\) was not increased at 1, 2, and 4 h after the administration of APAP, similar to findings reported by Simpson et al. (57). Hepatic TNF-\(\alpha\) may have been elevated at another time point. Alternatively, because plasma levels of TNF-\(\alpha\) are dramatically less than hepatic levels (Fig. 8), a small relative increase in liver TNF-\(\alpha\) may have preceded the increase in the plasma levels. The increase in plasma TNF-\(\alpha\) likely results from, rather than causes, liver damage, because liver damage from APAP can occur in mice without increases in hepatic TNF-\(\alpha\) (4, 9). In addition, APAP hepatotoxicity is not decreased by deletion of the genes for both TNF-\(\alpha\) and lymphotoxin-\(\alpha\) in a mouse model (4) or by treatment of mice with soluble TNF-\(\alpha\) receptor (57).

In APAP hepatotoxicity, induction of iNOS is thought to have a critical role in development of liver damage (14, 25, 26, 36). In our studies, iNOS was not detectably increased, despite extensive liver damage by APAP, similar to the findings of Knight et al. (26). In two other studies, deletion of the iNOS gene had no effect on APAP hepatotoxicity (4, 35), suggesting that induction of iNOS is not essential for development of APAP-mediated liver damage. However, Gardner et al. (14) reported that deletion of iNOS resulted in protection from APAP hepatotoxicity. In that study, the iNOS knockout mice developed liver damage from APAP before nitrotyrosine was detected in the liver (14), suggesting that the early damage was independent of formation of nitrotyrosine.

In another recent study, treatment with inhibitors of iNOS was found to enhance APAP hepatotoxicity, suggesting a protective role of NO in inactivating superoxide (16). The inhibitors of iNOS used in that study have been shown by others not to inhibit activities of CYPs involved in APAP hepatotoxicity (29, 42). In a different study (12), treatment of mice and cultured mouse hepatocytes with a NO derivative of ursodeoxycholic acid (NCX-1000), which releases NO in the liver, afforded protection from APAP toxicity. However, NO has been shown to inhibit the activities of CYPs as well as their formation (24, 69), and that inhibition may have prevented APAP toxicity.

In HeN mice treated with the alcohols alone, increases in hepatic nitrotyrosine (Figs. 6 and 7, B vs. A) imply that there was increased formation of superoxide and peroxynitrite. Although decreases in hepatic GSH are hypothesized to have a role in formation of nitrotyrosine (reviewed in Refs. 19, 20), hepatic levels of GSH were not decreased by the alcohol treatment alone in HeN mice (Fig. 11). The superoxide generated after exposure to the alcohols was probably inactivated by NO, because there was no histologically observable liver damage (Fig. 7B, Table 1). The concept of peroxynitrite formation being a protective action appears perplexing, because peroxynitrite is so reactive. However, superoxide is more reactive. In addition, peroxynitrite is inactivated directly by GSH through glutathione peroxidase (5). Our findings and those of Hinson et al. (16) that low levels of nitrotyrosine are detected in the liver with no histological evidence of liver damage and no elevation in serum levels of ALT suggest that low amounts of tyrosine nitrosylation are tolerated. This possibility, along with the inactivation of peroxynitrite by GSH, may explain why there are levels of peroxynitrite generated from the interaction of NO with superoxide that actually transiently protect the liver from superoxide toxicity.

Our findings that APAP caused a transient decrease in hepatic nitrotyrosine (Fig. 7) in alcohol-pretreated animals, even though liver damage was increased (Fig. 2), are consistent with the hypothesis that APAP reacts in vivo with peroxynitrite and competitively inhibits formation of nitrotyrosine (16). Peroxynitrite reacts with APAP in vitro to form 3-nitroacetaminophen (30). The transient decrease in nitrotyrosine levels after administration of APAP (Fig. 7) may be due to the transient presence of APAP in the liver. In 129SV mice, we have found that plasma APAP levels are highest 1 h after intragastric administration, but by 4 and 6 h there is no detectable APAP remaining in plasma (results not shown). We hypothesize that by 4 h, when APAP is cleared from the blood and no longer competing for peroxynitrite, nitrotyrosine formation would increase, which is what we observed at 7 h compared with 1 and 4 h (Fig. 7G). Increases in APAP hepatotoxicity (Figs. 1 and 2; Table 1) may be due to increased oxidative damage resulting from the combination of low levels of GSH (Fig. 11) along with increased levels of superoxide that are too high to be inactivated by NO.

In summary, our results suggest TLR4 plays a role in APAP-dependent steatosis, congestion, and necrosis, because these effects were greater in endotoxin-responsive HeN compared with endotoxin-hyporesponsive TLR4 mutant HeJ mice. Alcohol pretreatment increased APAP hepatotoxicity in HeN mice but not in HeJ mice, also implicating a role for TLR4 in alcohol-mediated APAP hepatotoxicity.

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