Role of nitric oxide in hepatic microvascular injury elicited by acetaminophen in mice

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Ito, Yoshiya, Edward R. Abril, Nancy W. Bethea, and Robert S. McCuskey. Role of nitric oxide in hepatic microvascular injury elicited by acetaminophen in mice. Am J Physiol Gastrointest Liver Physiol 286; G60–G67, 2004. First published September 11, 2003; 10.1152/ajpgi.00217.2003.—Nitric oxide (NO) is suggested to play a role in liver injury elicited by acetaminophen (APAP). Hepatic microcirculatory dysfunction also is reported to contribute to the development of the injury. As a result, the role of NO in hepatic microcirculatory alterations in response to APAP was examined in mice by in vivo microscopy. A selective inducible NO synthase (iNOS) inhibitor, L-NAME, was intraperitoneally administered to animals 10 min before APAP gavage. L-NIL suppressed raised alanine aminotransferase (ALT) values 6 h after APAP, whereas L-NAME increased those 1.7-fold. Increased ALT levels were associated with hepatic expresion of iNOS. L-NIL, but not L-NAME, reduced the expression. APAP caused a reduction (20%) in the numbers of perfused sinusoids. L-NIL restored the sinusoidal perfusion, but L-NAME was ineffective. APAP increased the area occupied by infiltrated erythrocytes into the extrasinusoidal space. L-NIL tended to minimize this infiltration, whereas L-NAME further enhanced it. APAP caused an increase (1.5-fold) in Kupffer cell phagocytic activity. This activity in response to APAP was blunted by L-NIL, whereas L-NAME further elevated it. L-NIL suppressed APAP-induced decreases in hepatic glutathione levels. These results suggest that NO derived from iNOS contributes to APAP-induced parenchymal cell injury and hepatic microcirculatory disturbances. L-NIL exerts preventive effects on the liver injury partly by inhibiting APAP bioactivation. In contrast, NO derived from constitutive isoforms of NOS exerts a protective role in liver microcirculation against APAP intoxication and thereby minimizes liver injury.

The APAP-induced hepatic necrosis is preceded by CL microvascular congestion due to collapse of the sinusoidal wall and the infiltration of blood elements into the space of Disse (25, 36). That APAP injures sinusoidal endothelial cells (SECs) is reflected in the appearance of large gaps in their cytoplasm (25, 36). These findings suggest that, in addition to direct hepatocellular damage through metabolic activation of APAP, hepatic microcirculatory disturbance participates in liver injury elicited by APAP overdose. We have recently shown (15) that hepatic microvascular derangement contributes to the development of APAP hepatotoxicity by using in vivo microscopy. Initial events occurring in the hepatic microvasculature following APAP included SEC injury exhibited by the penetration of erythrocytes into the extrasinusoidal space.

NO plays a critical role in maintaining ample blood supply into the hepatic microvasculature by affecting the expression of adhesion molecules on leukocytes, platelets, and endothelial cells (20). The NOS inhibitors exacerbated the hepatic microvascular inflammatory responses including leukocyte-endothelial interaction to endotoxin (30) and ischemia-reperfusion (13), suggesting that NO plays a protective role in stabilizing the hepatic microcirculation. However, little is known about the involvement of NO in hepatic microvascular injury elicited by APAP. As a result, the present study was conducted to examine the effects of NO inhibition on hepatic microvascular injury after APAP administration by using in vivo microscopic methods (24).

MATERIALS AND METHODS

Experimental animals. Male C57BL/6 mice (7–8 wk of age), weighing 23–25 g, were purchased from Charles River Laboratories (Charles River, Windham, ME) and were used for these experiments. All animals were allowed free access to food and water and were fasted 24 h before the experiments. The present study was performed in adherence to the National Institutes of Health guidelines for the use of experimental animals and followed a protocol approved by the Animal Care and Use Committee of the University of Arizona. APAP (300 mg/kg liquid Tylenol; McNeil-PPC, Ft. Washington, PA) was given to mice by oral gavage. N(ω)-nitro-L-arginine methyl ester (L-NAME; Sigma, St. Louis, MO; 10 mg/kg body wt) was used to inhibit nitric oxide synthase (NOS). L-NMMA (Cayman Chemical, Ann Arbor, MI; 1, 3, and 10 mg/kg body wt in 0.1 ml PBS), an inhibitor with 30- to 100-fold selectivity toward iNOS (10), and L-N(ω)-(1-iminoethyl)-lysine (l-NIL; Cayman Chemical, Ann Arbor, MI; 1, 3, and 10 mg/kg body wt in 0.1 ml PBS), an inhibitor with 30- to 100-fold selectivity toward eNOS compared with iNOS (29), were intraperitoneally administered to animals 10 min before APAP gavage. Animals receiving the same amount of vehicle (PBS) served as controls. Some of the animals were treated with l-NIL (10 mg/kg) 10 min before or simultaneously with an intraperitoneal injection of APAP (300 mg/kg; Sigma). In some

Acetaminophen, also known as paracetamol (N-acetyl-para-aminophenol; APAP), is a commonly used over-the-counter analgesic and antipyretic with few side effects when taken at therapeutic doses. However, APAP intoxication from overdosing can result in severe hepatic damage, which is characterized by hemorrhagic centrilobular (CL) necrosis and by towering the levels of transaminase in both humans and animals (34). Recently, it has been shown that nitric oxide (NO) is involved in the progression of APAP-induced liver injury. APAP hepatotoxicity is associated with increased expression of inducible NO synthase (iNOS) (7, 33) and the formation of nitrotyrosine-protein adducts (12, 19). Inhibition of iNOS with aminoguanidine is reported to minimize liver injury elicited by APAP (7, 8). However, others (11) have reported that NO inhibitors aggravated the injury.

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experiments, the NO donor 5-nitro-N-acetylpenicillamine (SNAP; Cayman) (6) was administered (20 μg/kg ip) to mice 20 min before APAP gavage.

In vivo microscopy. Animals were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium (Nembutal; Abbott Laboratories, Abbott Park, IL). The hepatic microvascular responses were examined by using established high-resolution in vivo microscopic methods (24). Briefly, a compound binocular microscope (Leitz, Wetzlar, Germany) adapted for in vivo microscopy was equipped to provide either transillumination or epi-illumination, as well as video microscopy by using a charge-coupled device camera (MTI, Michigan City, IN). The liver was exteriorized through a left subcostal incision and positioned over a window of optical-grade mica in a specially designed tray mounted on a microscopic stage. The tray provided for the drainage of irrigating fluids, and the window overlaid a long-working-distance condenser. The liver was covered by a piece of Saran Wrap (Dow Chemical, Midland, MI), which held it in position and limited movement. Homeostasis was ensured by a constant suffusion of the organ with Ringer solution maintained at body temperature. With the ×80–1.0 numerical aperture water immersion objective (Leitz) employed for these studies, the methods permitted visualization of the cells comprising the sinusoidal lining, the formed elements of the blood, the nuclei, nucleoli, mitochondria and fat droplets, and bile canaliculi. Under optimal conditions, the resolution was 0.3–0.5 μm. Microvascular events were observed and recorded for at least 30 s for subsequent offline analysis by using a Sony Betacam video tape recorder (Sony Medical Electronics, Park Ridge, NJ).

Kupffer cell (KC) function was assessed by measuring the phagoctysis of fluorescent 1.0-μm latex particles (Fluoresbrite-fluorescent monodispersed polystyrene microspheres; Polysciences, Warrington, PA) by individual cells. The latex was diluted 1:10 with sterile saline and injected in 0.1-ml volume through a mesenteric vein by using a 30-gauge lymphangiography needle (Becton Dickinson, Franklin Lakes, NJ). The distribution and relative number of phagocytic KCs was measured by counting the number of cells that phagocytosed latex particles in a standardized microscopic field 15 min after each mouse had received the injection of latex. To assess regional distribution, the number of phagocytic KCs per microscopic field was counted in 10 periporal (PP) and 10 CL regions in each animal. The relative adequacy of blood perfusion through the sinusoids was evaluated by counting the number of sinusoids containing blood flow (SCF) in the same 10 microscopic fields in which the numbers of phagocytic KCs were determined. Because reduced perfusion of individual sinusoids can limit the delivery of the latex particles to KCs in these vessels, the ratio of KCs that phagocytosed latex particles to SCF was used as an overall index of KC phagocytic activity.

To examine the interaction of leukocytes with the sinusoidal wall, quantification of leukocytes adhering to the endothelial lining of sinusoids was calculated by counting the number of leukocytes per unit length of sinusoid (adherent leukocytes/100 μm) in the same microscopic fields. A leukocyte was defined as adhering to the sinusoidal wall if it remained stationary for at least 30 s. Endothelial swelling, which is thought to be an indication of activation and/or injury, was assessed by counting the numbers of swollen cells whose nuclear regions protruded across one-third or more of the lumen in the same microscopic fields. The results were averaged, and the data were represented as the average number in each animal.

To quantify the extent of hemorrhage elicited by APAP gavage, the area occupied by extrasinusoidal erythrocytes was measured in the same microscopic fields by using a computer-assisted digital imaging processor (Scion Image; Scion, Frederick, MD). The results were expressed as extrasinusoidal area occupied by erythrocytes (μm²/10 CL regions). In addition, the diameter of SCF were measured in the same microscopic fields from video recordings.

Sampling and assays. In a separate set of experimental animals, blood was collected from the inferior vena cava and the samples were separated by centrifugation at 13,000 g for 5 min at 4°C. The samples were stored at −70°C until assays were performed. The serum activities of alanine aminotransferase (ALT) were measured by enzymatic procedures by using a diagnostic kit (Sigma). In addition, the serum concentrations of nitrite/nitrate were determined with a kit (Cayman) by means of Greiss reaction.

Histology and immunohistochemical studies. The hepatic tissues were immediately fixed with 4% paraformaldehyde in 0.1 M phosphate buffer solution (pH 7.4). After fixation, the tissues were dehydrated with a graded series of ethanol solutions and embedded in paraffin. The sections (5 μm) from the paraffin-embedded tissues were stained with hematoxylin and eosin. For immunostaining, the sections were incubated first with normal goat serum and then with rabbit anti-murine iNOS antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or with anti-nitrotyrosine antibody (1:100 dilution; Molecular Probes, Eugene, OR) for 1 h. After being washed, the sections were incubated with biotinylated anti-rabbit IgG followed by incubation with avidin-biotin-peroxidase complex (Dako kit; DakoCytomation, Carpentaria, CA). Reaction products were visualized by treating sections with 0.02% 3,3′-diaminobenzidine and 0.3% nickel ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.4). The sensitivity of the reaction was verified by using isotype-matched immunoglobulin (rabbit immunoglobulin; Sigma) in the same manner. All steps were performed at room temperature.

RT-PCR analysis. Two and six hours after APAP gavage, ~100 mg of the liver tissue were excised and stored at −20°C until being used. Total cellular RNA was extracted from the tissue by using a commercially available kit (SV total RNA isolation system; Promega, Madison, WI) in accordance with the manufacturer’s instructions. Hepatic iNOS mRNA was determined by means of semiquantitative RT-PCR (PCR ELISA kit; Roche Diagnostics, Basel, Switzerland). Amplification was performed as follows: 94°C, 45 s; 54°C, 45 s; and 72°C, 45 s for iNOS and β-actin, followed by a final extension for 10 min at 72°C and by soak at 4°C with the addition of 62.5 μm digoxigenin (DIG)-dUTP for PCR labeling. To avoid tube-to-tube variation, the primers for β-actin and iNOS were added together to each reaction. The two cDNA were coamplified. One microliter of the PCR products was denatured and incubated with commercially biotinylated probes (murine β-actin: 5′-GGGTTTGAAGTCTCATAAGTCTTGG-3′; murine iNOS: 5′-CTTGGTTGCTCCCTCTTGCCT-3′) for 4 h at 37°C on a shaker in a streptavidin-coated 96-well plate. Peroxidase-conjugated anti-DIG antibody was added and was measured by an ELISA reader. Quantitative determination of mRNA was calculated based on the slope of the standard curve and was expressed in attomoles per milliliter.

Measurement of hepatic total glutathione. Hepatic total glutathione (GSH) was determined colorimetrically by using a commercially available kit (Oxford Biomedical Research, Oxford, MI). Briefly, a portion of liver tissue was homogenized in 10 ml of cold 5% metaphosphoric acid and was centrifuged at 3,000 g for 10 min. The upper aqueous layer was collected and assayed according to the manufacturer’s protocol.

Statistical analysis. All data were expressed as means ± SE. Multiple comparisons were performed by using one-way ANOVA with a post hoc Fisher’s test. Differences were considered to be significant at P < 0.05.

RESULTS

Effect of NOS inhibitors on the levels of ALT after APAP gavage. Figure 1 shows the levels of ALT activities after oral gavage with APAP. The levels of ALT 2 h after APAP gavage did not change significantly. Neither L-NIL nor L-NAME significantly changed ALT levels in mice treated with APAP. At 6 h after APAP, the levels of ALT were markedly increased. L-NIL decreased the ALT levels by 90% in a dose-dependent manner, whereas L-NAME further elevated them. Both L-NIL...
and L-NAME by themselves resulted in no significant changes in ALT levels (data not shown). On the basis of these results, we selected a dose of 10 mg/kg L-NIL for the remaining studies. In addition, the intraperitoneal administration of APAP caused a significant increase in ALT levels 6 h after APAP (1,480 ± 193 IU/l; n = 5). Pretreatment with L-NIL (10 mg/kg) significantly reduced those (824 ± 107 IU/l; n = 5), whereas cotreatment with L-NIL and APAP did not significantly change them (977 ± 109 IU/l; n = 5).

**Effects of NOS inhibitors on liver microcirculation in response to APAP.** Figure 2 illustrates the effects of L-NIL and L-NAME on hepatic microvascular responses to APAP. The numbers of SCF in CL regions were significantly decreased 2 h after APAP when compared with untreated controls. L-NIL, but not L-NAME, restored sinusoidal perfusion (Fig. 2A). At 6 h after, the numbers of SCF in PP and CL regions were reduced by 9 and 21%, respectively. Also, L-NIL, but not L-NAME, improved the SCF. The oral gavage with APAP caused a significant increase in KC phagocytic activity in both PP and CL regions 2 and 6 h after APAP (Fig. 2B). L-NAME further raised KC phagocytic activity after APAP, whereas L-NIL inhibited this activity. APAP failed to induce any changes in the numbers of leukocytes adhering to the sinusoids in both PP and CL regions as well as to the central venules 2 and 6 h after APAP (not shown). The numbers of swollen SECs in PP regions were significantly increased (2.8-fold) 2 and 6 h after APAP. Neither L-NIL nor L-NAME changed the numbers of SECs in APAP-treated mice. Although there was no evidence of infiltrated erythrocytes into the space of Disse (namely, hemorrhage) 2 h after APAP gavage, infiltration was observed 6 h after APAP (612 ± 362 μm²/10 CL regions). L-NAME elicited hemorrhage as early as 2 h after APAP (108 ± 89 μm²/10 CL regions), although the hemorrhagic area was small. At 6 h after APAP, the hemorrhagic regions in L-NAME-treated mice (2,396 ± 1,173 μm²/10 CL regions) exceeded those in vehicle-treated mice. The diameters of PP sinusoids did not change significantly in each experimental group (not shown). In contrast, those of CL sinusoids were significantly decreased 2 and 6 h after APAP (6.7 and 12.4%, respectively). L-NAME did not change the diameters in APAP-treated mice, whereas L-NIL inhibited the reduced diameters.

**Effects of NOS inhibitors on iNOS and nitrotyrosine expression in the liver treated with APAP.** To elucidate the role of NO in APAP liver injury, we determined the hepatic expression of iNOS and nitrotyrosine. The expression of hepatic iNOS mRNA was upregulated 2 h (5.6-fold) and 6 h (9.3-fold) after APAP, respectively, when compared with controls (Fig. 3). L-NIL suppressed the increased expression of iNOS mRNA 6 h after APAP by 56.2%, whereas L-NAME did not change significantly.

**Fig. 2.** Effects of L-NIL or L-NAME on the numbers of sinusoids containing blood flow (SCF; A) and on Kupffer cell (KC) phagocytic activity (B) in mice treated with APAP. The numbers of SCF and phagocytic KCS were measured in 10 perportal (closed bars) and 10 centrilobular (open bars) regions in each animal. Mice were pretreated with L-NIL (10 mg/kg ip) or L-NAME (10 mg/kg ip) 10 min before APAP. Cont, control. Data are means ± SE from 6 mice per group. *P < 0.05 vs. untreated controls (time 0), †P < 0.05 vs. vehicle-treated mice.
The immunoreactivity with iNOS in the liver was negative 2 h after APAP (data not shown). At 6 h after APAP, the staining for iNOS was expressed in CL hepatocytes in the same area where necrosis was exhibited (Fig. 4A). L-NIL and L-NAME inhibited the expression of iNOS in CL regions (Fig. 4, B and C). Liver sections stained with hematoxylin and eosin from mice pretreated with L-NIL showed reduction in hepatic necrosis after APAP gavage (Fig. 4D).

The staining for nitrotyrosine, a convenient marker of peroxynitrite (2), in the liver from controls was negative (Fig. 5A). Two hours after APAP, the immunoreactivity with nitrotyrosine was intensely expressed in the CL sinusoids and was also shown in some individual hepatocytes in midlobular to CL regions (Fig. 5B). Six hours after APAP, a confluent staining of PP hepatocytes was seen, although there was no staining of the sinusoids or hepatocytes in the CL regions (Fig. 5C). Pretreatment with L-NIL exhibited the positive staining for nitrotyrosine in individual hepatocytes in PP regions 2 and 6 h after APAP (Fig. 5D). L-NAME induced strong positive staining for nitrotyrosine in the CL sinusoids 2 and 6 h after APAP (Fig. 5, E and F).

Table 1 summarizes the serum concentrations of nitrite/nitrate at 2 and 6 h after APAP gavage. There were no significant differences in the levels of nitrite/nitrate among the experimental groups.

Table 1. Serum Concentrations of Nitrate/Nitrite at 2 and 6 h after APAP Gavage

Effect of L-NIL on total GSH levels in the liver treated with APAP gavage. The bioactivation of APAP during the initiation phase of the liver injury causes a significant decrease in hepatic GSH levels, which correlate with APAP hepatotoxicity (28). To address the question of whether L-NIL may affect APAP bioactivation, we measured hepatic GSH levels. As shown in Fig. 6, the total GSH levels 1 and 2 h after APAP were decreased by 59.3 and 70.3%, respectively. L-NIL restored GSH levels to 75% of control levels at both time points after APAP.

**DISCUSSION**

The results of the present study demonstrated that oral gavage with APAP resulted in liver microcirculatory dysfunction including impaired sinusoidal perfusion, swelling of SECs, infiltrated erythrocytes in the extrasinusoidal space, and activated KC phagocytic function (15). The liver microcirculatory dysfunction elicited by APAP preceded parenchymal cell in-

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**Fig. 3.** Effect of L-NIL or L-NAME on hepatic inducible nitric oxide (NO) synthase (iNOS) mRNA expression after APAP gavage. The levels of iNOS mRNA in the liver were determined by RT-PCR analysis. Mice were pretreated with L-NIL (10 mg/kg ip) or L-NAME (10 mg/kg ip) 10 min before APAP. Data are means ± SE from 3 animals per group. #P < 0.05 vs. untreated controls (time 0). *P < 0.05 for vehicle vs. L-NIL at 6 h.

**Fig. 4.** Photographs of the liver sections stained with iNOS (A, B, and C) or hematoxylin and eosin (D) 6 h after APAP in combination with vehicle (A), L-NIL (B and D), or L-NAME (C). A: immunoreactivity with iNOS was shown in centrilobular hepatocytes treated with APAP. B: staining for iNOS was negative in the centrilobular hepatocytes pretreated with L-NIL. C: L-NAME reduced the expression of iNOS in centrilobular hepatocytes. D: hematoxylin and eosin-stained liver sections from mice pretreated with L-NIL showed minimal changes. PV, portal vein; CV, central vein. Original magnification, ×200.
jury as indicated by a significant increase in ALT values and hepatic necrosis. L-NIL, an inhibitor of iNOS, attenuated APAP-induced hepatocellular injury as well as hepatic microcirculatory dysfunction. In contrast, L-NAME, a nonselective NOS inhibitor that favors cNOS over iNOS, aggravated the injury. In particular, L-NAME exacerbated the extrasinusoidal area occupied by erythrocytes and KC phagocytic function in response to APAP.

NO has been implicated in the progression of APAP hepatotoxicity. APAP-induced liver injury is associated with increased iNOS protein expression (7, 8, 33) as well as increased iNOS mRNA expression (14), which is consistent with our present results. The immunohistochemical studies showed that iNOS was expressed in the damaged hepatocytes in the CL regions, suggesting that these hepatocytes are a source of iNOS (7). Increased NO production from isolated hepatocytes also has been reported (7). Furthermore, mice deficient in iNOS moderately reduce release of hepatic enzyme after APAP treatment (8). However, Michael et al. (26) reported that there is no difference in the amount of hepatic necrosis between iNOS knockout mice and wild-type mice, and Bourdi et al. (3) reported that both iNOS knockout and wild-type mice exhibited the same raised ALT levels after APAP (150 mg/kg). The discrepancy within iNOS knockout mice still remains unclear. Pretreatment with aminoguanidine, a selective iNOS inhibitor, minimizes liver injury elicited by APAP (7, 8). However, others (11) have shown that the simultaneous administration of aminoguanidine with APAP increased the initial rate of development of hepatotoxicity as indicated by ALT values (within 4 h after APAP), but there was no significant difference in ALT levels 6 and 8 h after APAP. The current study also showed that another selective iNOS inhibitor, L-NIL, attenuated APAP-induced liver injury, which was associated with reduced expression of iNOS in the liver treated with APAP. These findings suggest that NO derived from iNOS is involved in the development of APAP hepatotoxicity. In contrast, Hinson et al. (11) reported that the simultaneous administration of L-NIL (3 mg/kg ip) with APAP (300 mg/kg ip) approximately doubled ALT levels 4 h after APAP treatment in B6C3F1 mice. Therefore, we also treated mice with L-NIL (10 mg/kg ip) 10 min

![Fig. 5. Photographs of the liver sections for nitrotyrosine protein adducts after APAP in combination with vehicle, L-NIL, or L-NAME. A: untreated controls showed no staining for nitrotyrosine in the liver. B: strong positive staining was seen in the centrilobular sinusoids and centrilobular to midlobular hepatocytes 2 h after APAP. C: 6 h after APAP, a confluent staining for nitrotyrosine was observed in the perportal hepatocytes. D: immunoreactivity with nitrotyrosine was expressed in the perportal hepatocytes pretreated with L-NIL at 6 h after APAP. E: L-NAME exhibited intense expression of nitrotyrosine in the centrilobular sinusoids 2 h after APAP. F: at 6 h after APAP, pretreatment with L-NAME caused intense staining for nitrotyrosine in the centrilobular sinusoids. Original magnification, ×200 for A, D, and E and ×400 for B, C, and F.]

Table 1. Effect of L-NIL or L-NAME on the serum concentration of nitrite/nitrate in mice treated with APAP

<table>
<thead>
<tr>
<th>Control</th>
<th>2 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>L-NIL</td>
<td>L-NAME</td>
</tr>
<tr>
<td></td>
<td>73.1±7.2</td>
<td>86.7±7.6</td>
</tr>
<tr>
<td></td>
<td>63.4±5.4</td>
<td>75.4±24.1</td>
</tr>
<tr>
<td></td>
<td>104.9±5.8</td>
<td>79.7±4.8</td>
</tr>
<tr>
<td></td>
<td>89.3±21.5</td>
<td>89.3±21.5</td>
</tr>
</tbody>
</table>

Data are means ± SE (μM) from 3–6 mice. There were no significant differences in nitric oxide (NO) concentrations among the treatment groups. L-N'-[1-iminoethyl]-lysine (L-NIL) (10 mg/kg, ip) or N'-nitro-L-arginine methy ester (L-NAME) (10 mg/kg, ip) was administered 10 min before acetaminophen (APAP) gavage.
before APAP (300 mg/kg ip) or with L-NIL in combination with APAP. As shown in results, the levels of ALT 6 h after APAP were increased, and pretreatment with L-NIL slightly reduced these, whereas cotreatment with L-NIL and APAP did not significantly change them. Therefore, the discrepancy may be due to the differences in the animal species, doses of inhibitors, timing of their administration, or route of APAP administration being used.

Since it has been suggested that L-NIL inhibits iNOS by reacting with the protein at the enzyme active site, L-NIL does not appear to suppress the expression of iNOS mRNA. On the other hand, we demonstrated that L-NIL attenuated the expression of iNOS mRNA and iNOS protein in the liver. Although the mechanisms by which L-NIL prevents iNOS expression in this model remain unknown, Kang et al. (16) reported that, in mouse peritoneal macrophages, L-NIL inhibited lipopolysaccharide-induced activation of NF-kB, which regulates iNOS expression (37). Thus it may be plausible that L-NIL suppressed iNOS mRNA expression in the liver treated with APAP by inactivation of NF-kB.

The bioactivation of APAP results in the formation of the reactive metabolite, presumably N-acetyl-p-benzoquinonemine, which depletes intracellular sources of GSH (28). In the current study, L-NIL suppressed the initial drop in hepatic total GSH levels following APAP gavage, suggesting that the inhibitory effect of L-NIL on liver injury is partially mediated through a decrease in bioactivation of APAP. The possibility that L-NIL prevents APAP metabolic activation also suggests that L-NIL could inhibit covalent binding to proteins of APAP. Further studies will be required to elucidate the effect of L-NIL on APAP covalent binding. The other possibility that L-NIL maintained GSH levels is due to the reduced NO production in the liver because reactive nitrogen species are scavenged by GSH (18). Therefore, the mechanisms involved in the protective action of L-NIL against APAP-induced liver injury appear to be multifactorial in this model.

The exogenous administration of NO donor (SNAP) exacerbated ALT release but did not affect the hepatic microcirculatory dysfunction following APAP gavage. These results suggest that excess NO in the liver treated with APAP causes liver injury and that NO may act directly on hepatocytes. Recently, Liu et al. (22) reported that continuous administration of O2- and NO donor, protected the liver against APAP hepatotoxicity. The differences may be caused by different delivery of NO donor to the liver; V-PYRRO/NO is metabolized to NO preferentially in hepatocytes. Moreover, excessive extracellular NO elicited by a bolus injection of SNAP could mediate hepatocellular mitochondrial dysfunction through its actions on an enzyme such as cytochrome c oxidase, an enzyme constituting the distal end of the mitochondrial respiratory chain (4). The mitochondrial dysfunction caused by SNAP could be amplified in addition to that by APAP (14). In a preliminary study, we administered 10 mg/kg of V-PYRRO/NO (Cayman) subcutaneously to mice gavaged with APAP according to the experimental protocols by Liu et al. (23). Since V-PYRRO/NO was supplied as a solution in absolute ethanol, some animals received ethanol as vehicle. Our preliminary study revealed that not only V-PYRRO but also absolute ethanol as vehicle reduced ALT levels 6 h after APAP gavage. Since a serum level of 5 mM ethanol has been found to protect animals from APAP hepatotoxicity (38), the effect of vehicle also could contribute to protective effect of V-PYRRO/NO on APAP-induced liver injury, though we are uncertain whether Liu et al. have used ethanol as a vehicle for V-PYRRO/NO.

The toxicological effect of NO seems to occur through peroxynitrite, a potent oxidant formed by reaction of NO with superoxide. The finding of nitrotyrosine staining shown in the hepatic CL sinusoids 2 h after APAP gavage, which is consistent with those by others (12, 19), suggested that peroxynitrite formation in the sinusoids, indicating that NO derived from iNOS in the sinusoidal lining cells is involved in the formation of peroxynitrite.

Table 2. Effects of SNAP on serum ALT activity and on liver microcirculation 6 h after APAP gavage

<table>
<thead>
<tr>
<th>SNAP Gavage</th>
<th>Vehicle, n=4</th>
<th>SNAP, n=3</th>
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<tr>
<td>ALT, IUN/l</td>
<td>2.405±0.2</td>
<td>4.609±632*</td>
</tr>
<tr>
<td>No. of perfused sinusoids/field</td>
<td>4.6±0.2</td>
<td>4.9±0.2</td>
</tr>
<tr>
<td>Periportal</td>
<td>3.8±0.3</td>
<td>3.9±0.3</td>
</tr>
<tr>
<td>Centrilobular</td>
<td>3.8±0.3</td>
<td>3.9±0.3</td>
</tr>
<tr>
<td>No. of adherent leukocytes/100 μm</td>
<td>0.04±0.03</td>
<td>0.04±0.03</td>
</tr>
<tr>
<td>Periportal</td>
<td>0.04±0.04</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Centrilobular</td>
<td>0.04±0.04</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>No. of swollen SECs/field</td>
<td>0.2±0.1</td>
<td>0.3±0.0</td>
</tr>
<tr>
<td>Periportal</td>
<td>0.2±0.1</td>
<td>0.3±0.0</td>
</tr>
<tr>
<td>Centrilobular</td>
<td>0.2±0.1</td>
<td>0.3±0.0</td>
</tr>
<tr>
<td>Kupffer cell phagocytic activity</td>
<td>1.0±0.1</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>Periportal</td>
<td>1.1±0.1</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>Centrilobular</td>
<td>1.1±0.1</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>Area occupied by infiltrated erythrocytes, μm²/10 centrilobular regions</td>
<td>247±144</td>
<td>0±0</td>
</tr>
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</table>

Data are means ± SE. S-nitro-N-acetylpenicillamine (SNAP; 20 μg/kg, ip) or vehicle was administered 20 min before APAP (300 mg/kg) gavage. ALT, alanine aminotransferase; SEC, sinusoidal endothelial cells. *P < 0.05 vs. vehicle-treated mice.
trite. This finding is in agreement with that of Hinson et al. (11) showing that aminoguanidine attenuated APAP-induced nitration of tyrosine in liver tissue. In considering the results of microvascular inflammatory responses to APAP, KCs and SECs appear to be sources of NO. However, iNOS protein in the liver 2 h after APAP was not expressed in the sinusoidal lining cells, suggesting that iNOS expression was not enough to be detected by means of immunohistochemistry; even mRNA for iNOS was marginally transcripted. Pretreatment with l-NIL also minimized APAP-induced hepatic microcirculatory dysfunction, presumably by inhibiting KC function. Because inactivation of KCs with gadolinium chloride restored liver microcirculation in response to APAP (15), the inhibitory effect of l-NIL on KC activity contributes to preserving hepatic microcirculation, resulting in reduction of liver injury (21, 27). However, the mechanisms by which l-NIL suppresses KC function or whether KCs release NO in the early phase of APAP hepatotoxicity remain unknown.

At later time point (6 h after APAP gavage), nitrotyrosine staining was negative in the CL regions of the liver, whereas iNOS staining was positive in the same regions. On the other hand, nitrotyrosine staining was positive in the PP hepatocytes. The results indicate that the location for nitrotyrosine staining in the liver was shifted from the CL regions to the PP regions with time after APAP treatment. The reduced intensity of nitrotyrosine staining in CL hepatocytes 6 h after APAP may have resulted from inability to nitrate protein because of cellular lysis (31). On the other hand, the staining for nitrotyrosine was observed in the PP hepatocytes with no evidence of necrosis. These results are inconsistent with those reported by others, who demonstrated nitrotyrosine expression in the CL hepatocytes (19), but not in the PP ones (12), at 6 h after APAP. The reasons for these differences remain unclear, although it may be due to differences in animal species, response to APAP, and/or differences in immunohistochemical protocols. Our findings suggest that nitrotyrosine formation may serve as a biomarker for the effects of reactive nitrogen species and may not be relevant to the injury mechanism, because no evidence of necrosis was noticed in the PP hepatocytes. In addition, it may be speculated that peroxynitrite was reacted with hepatic glutathione (endogenous scavengers of peroxynitrite), which are still reserved in the PP hepatocytes and thereby protected against peroxynitrite-mediated oxidant stress injury in these hepatocytes.

In contrast to l-NIL, l-NAME, a nonselective NOS inhibitor, exacerbated APAP-induced liver injury. These findings are consistent with the findings by others (11) showing that N-nomonomethyl-l-arginine worsened the injury. The enhanced liver injury was associated with aggravated APAP-induced hepatic microvascular injury including KC phagocytic activity. l-NAME caused upregulated expression of nitrotyrosine in the hepatic CL sinusoids 2 and 6 h after APAP gavage, indicating that peroxynitrite could lead to SEC injury. This possibility was supported by the results of the present study showing that the extrasinusoidal area occupied by infiltrated erythrocytes was further increased by l-NAME. These results suggest that NO-mediated nitration of cellular proteins in SECs at least in part contributes to the progression of liver injury elicited by APAP and that peroxynitrite is an important mediator of injury to parenchymal cells (18) as well as SECs. However, as mentioned above, whether the presence of nitrotyrosine-protein adducts is related to the injury remains to be elucidated. The enhanced hemorrhagic necrosis by l-NAME also suggests that endogenous NO derived from cNOS plays a critical role in maintaining the functional integrity of SECs, because cNOS appears to be localized exclusively to the SECs (32). cNOS has been shown to associate with cytoskeletal proteins (1). Thus inhibition of cNOS with l-NAME could result in disruption of cytoskeleton, leading to changes in cell shape or to the formation of gaps. In addition, it has been reported that APAP increased levels of hepatic lipid peroxidation in mice treated with the iNOS inhibitor aminoguanidine (11) or in mice deficient in iNOS (26). This suggests that exacerbated liver injury elicited by APAP is mediated by lipid peroxidation during NOS inhibition. However, Knight et al. (17) recently have shown that there is no evidence of enhanced lipid peroxidation in the liver treated with APAP in combination with aminoguanidine, indicating that lipid peroxidation is not involved in the injury mechanism. Because of the antioxidant properties of cNOS-derived NO, inhibition of cNOS could increase superoxide (9). Thus it is plausible that activated KCs generated larger amounts of superoxide and thereby formed peroxynitrite in the sinusoids. However, the mechanisms by which NO from cNOS suppresses the activation of KCs remain unknown.

Recent studies reported that nonselective NOS inhibitors reduced the sinusoidal perfusion and sinusoidal diameters during endotoxemia (5, 30), indicating that cNOS inhibition caused vasoconstriction and thereby augmented liver injury. However, this is not seen in the current study, in which l-NAME did not further reduce the sinusoidal blood flow as well as sinusoidal diameter following APAP treatment. Thus aggravation of APAP-induced liver injury by l-NAME is not attributed to vasoconstriction.

In summary, selective inhibition of iNOS with l-NIL attenuated liver microcirculatory dysfunction elicited by APAP gavage. In contrast, inhibition of cNOS by l-NAME potentiated APAP-induced liver injury due to its effect on the liver microcirculation including KC function. These results suggest that NO derived from iNOS contributes to APAP-induced parenchymal cell injury and hepatic microcirculatory disturbances, whereas NO-derived cNOS exerts a protective role in liver microcirculation against APAP intoxication and prevents enhanced liver injury.

GRANTS
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