Scavenging nitric oxide reduces hepatocellular injury after endotoxin challenge

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Nadler, Evan P., Eva C. Dickinson, Donna Beer-Stolz, Sean M. Alber, Simon C. Watkins, David W. Pratt, and Henri R. Ford. Scavenging nitric oxide reduces hepatocellular injury after endotoxin challenge. Am J Physiol Gastrointest Liver Physiol 281: G173–G181, 2001.—Sustained upregulation of inducible nitric oxide (NO) synthase in the liver after endotoxin (lipopolysaccharide [LPS]) challenge may result in hepatocellular injury. We hypothesized that administration of a NO scavenger, NOX, may attenuate LPS-induced hepatocellular injury. Sprague-Dawley rats received NOX or saline via subcutaneous osmotic pumps, followed 18 h later by LPS challenge. Hepatocellular injury was assessed using biochemical assays, light, and transmission electron microscopy (TEM). Interleukin (IL)-6 mRNA was measured by RT-PCR. Tumor necrosis factor (TNF)-α protein expression was determined by immunohistochemistry. NOX significantly reduced serum levels of ornithine carbamoyltransferase and aspartate aminotransferase. TNF-α and IL-6 expression were increased in the livers of saline-treated but not NOX-treated rats. Although there was no difference between groups by light microscopy, TEM revealed obliteration of the space of Disse in saline-treated but not in NOX-treated animals. Electron paramagnetic resonance showed the characteristic mononitrosyl complex in NOX-treated rats. We conclude that NOX reduces hepatocellular injury after endotoxemia. NOX may be useful in the management of hepatic dysfunction secondary to sepsis or other diseases associated with excessive NO production.

inducible nitric oxide synthase; endotoxemia; dithiocarbamate; interleukin-6; ornithine carbamoyltransferase

THE LIVER PERFORMS a variety of important host defense and metabolic functions that include synthesis of acute phase proteins, gluconeogenesis, detoxification, and clearance of endogenous mediators, as well as secretion of proinflammatory cytokines (41). Hepatic dysfunction after sepsis is a frequent event that is characterized by loss of synthetic function, hepatocellular necrosis, and release of inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, prostanoglandins, and nitric oxide (NO) (3, 7, 13, 25). The specific role of these various cytokines in the pathogenesis of hepatocellular dysfunction or necrosis after endotoxemia is still undefined.

Whereas sustained production of NO in the gut has been shown to induce derangement in intestinal barrier function (9, 51, 55), the role of NO as a putative mediator of hepatic injury after endotoxic shock remains controversial. Several authors have shown that nonspecific inhibition of all three isoforms of NO synthase (NOS) during endotoxemia may augment hepatocellular injury (12, 18, 39). NO donors have been shown to preserve hepatic perfusion during endotoxemia and to prevent inflammatory changes in the microcirculation (37, 42). However, a growing body of evidence suggests that sustained production of NO resulting from upregulation of inducible NOS (iNOS) after lipopolysaccharide (LPS) challenge may cause hepatocellular injury, either directly (57), or indirectly, by forming reactive nitrogen intermediates (35). Menzies et al. (33) recently demonstrated that a NO scavenger, NOX, decreased hepatocellular injury and improved survival after hemorrhagic shock. We hypothesized that NOX may prevent hepatocellular injury after endotoxic shock.

MATERIALS AND METHODS

Experimental Design

The experimental protocol was approved by the Animal Research and Care Committee of the Children’s Hospital of Pittsburgh, in accordance with the National Institutes of Health guidelines for animal care. Male Sprague-Dawley rats (Halar Sprague Dawley, Indianapolis, IN) weighing between 250 and 300 g were acclimatized for a minimum of 1 wk before experimentation. After pentobarbital sodium (50 mg/kg) was administered intraperitoneally to the animals, osmotic pumps (Alzet model 2ML1, 10 ml/h, 7-day pump, Alza, Palo Alto, CA) were placed subcutaneously in the back of each rat, and the delivery catheter was tunneled subcutaneously in the neck. The animals were randomized to receive either 2 ml of normal saline (NS) or NOX (450 mg) in a final volume of 2 ml via the osmotic pumps. NOX was a kind gift of Dr. Ching-San Lai (Medinox, San Diego, CA). Additional...
doses of NOX (112.5 mg) or NS (0.5 ml) were given concomitant with LPS administration and at 4-h intervals thereafter for a total of three doses. A dose-response curve was generated for NOX before the final dose used in these experiments was selected (9). Eighteen hours later, the animals were challenged with 10 mg/kg ip of LPS (Escherichia coli 0111: B4, Difco, Detroit, MI). In our posttreatment groups, the same protocol was used except that the delivery catheter was not tunneled until 4 and 8 h after LPS administration. All animals were killed 24 h after LPS challenge.

**Electron Paramagnetic Resonance Spectrometry**

Sprague-Dawley rats were randomized to receive either NOX or NS as described for 24 h before LPS challenge (10 mg/kg ip). Additional doses of NOX or NS were given as described. At 6, 8, and 24 h after LPS challenge, the rats were killed, and their livers were harvested and perfused with NS via the portal vein until the perfusate in the right atrium was free of any blood. Sections of the perfused liver weighing 1–2 g were then placed in capillary tubing and stored at −80°C until electron paramagnetic resonance (EPR) studies were performed.

The EPR spectra were recorded with an E4 spectrometer (Varian Associates, Palo Alto, CA) at 77 K to allow detection of nitrosyl-hemoglobin signals. The scans were run at full microwave power (~100 mW), a modulation amplitude of 10 G, a microwave frequency of ~9.10 GHz, a scan time of 4 min, and a time constant of 0.3 s. Identification of the carrier of each spectrum was made by measuring its $g$ value. This measurement was performed by using an electronic counter to determine the microwave frequency to one part in 10$^8$ and comparing the field position of the sample with that of 1,1-diphenyl-2-picrylhydrazyl, which has a known $g$ value (2.0036). A standard solution of 5 mM S-nitroso-N-acetyl-d,l-penicillamine (SNAP), 1 mM FeSO$_4$, and 10 mM NOX dissolved in water was used to verify the spectrum of a mono-nitrosyl-iron-NOX complex in our system. The spectrum for each rat was then determined by EPR and assessed by a single investigator (D. W. Pratt) who was blinded to the treatment groups. The spectra were evaluated for the presence or absence of a nitrosyl-hemoglobin signal or a mononitrosyl-iron complex signal.

**Biochemical Assays**

Plasma was harvested by cardiac puncture. Ornithine carbamoyltransferase (OCT) was measured directly from the serum according to the method published by Ohshita et al. (38). Serum glucose and aspartate aminotransferase (AST) levels were measured by routine clinical chemistry. Fibronogen levels were determined by the modified heat precipitation reaction (30). Briefly, capillary tubes were filled with blood specimens and centrifuged for 3 min in an international microhematocrit (model MB, Inter Equipment, Needham, MA) and then placed in a water bath at 56°C for 3 min. The tubes were centrifuged for 5 min, and the height of the plasma column and the layer of fibrinogen sedimented on the packed red blood cells were measured to the nearest 0.1 mm and expressed as volumes per milliliter.

**RNA Preparation and RT-PCR**

Individual livers were homogenized in guanidinium isothiocyanate using a Polytron homogenizer (Kinematica, Switzerland), and total RNA was extracted according to the method of Chomczynski and Sacchi (8). The amount of RNA was determined spectrophotometrically. Two micrograms of RNA from each sample were subjected to first-strand cDNA synthesis using oligo(dT) primer and 100 µl murine Maloney leukemia virus (MMLV). Samples were incubated at 37°C for 60 min. To test the efficacy of reverse transcriptase, RT-PCR was performed for β-actin mRNA. All PCR reactions were carried out in a total volume of 50 µl containing 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM MgCl$_2$, and 1.25 U of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). Amplification was performed for 18–32 cycles, each of which consisted of 1 min denaturation at 94°C, 1 min annealing at 58°C, and 1 min primer extension at 72°C. PCR products were visualized on 2% agarose gel containing ethidium bromide. Semiquantitation was performed using $\gamma^{32}$P end-labeled 5′ primer. Fifteen microliters of the PCR reaction were separated on 10% polyacrylamide gel. The gel was dried and exposed to a PhosphorImager screen (Molecular Dynamics PhosphorImager, Sunnyvale, CA), and the relative radioactivity of the bands was determined by band density volume integration using laser scanning densitometry (31). Primers for iNOS, IL-6, and TNF-α were synthesized by the University of Pittsburgh DNA Synthesis Facility. The sequence of the iNOS 3′ primer was CGAACGGGGACCT-TCAA. The sequence for the iNOS 5′ primer was GCTTGC-CCTGGAACTT. The sequence of the IL-6 3′ primer was ATGTTCTTGTCCTTACCCAC. The sequence of the IL-6 5′ primer was ACAGCGATGATGCAGTCTGAC. The sequence for the TNF-α 3′ primer was ATGGTCTTTTACGTGAG. The sequence for the TNF-α 5′ primer was TGTCTACTGAATTCCGGT.

**Light Microscopy**

The tissue was fixed in 10% neutral buffered Formalin and stained with hematoxylin and eosin (H + E). Ten specimens from each group were reviewed by a Children’s Hospital of Pittsburgh pathologist (M. Parizhkaya) who was blinded to the treatment group.

**Transmission Electron Microscopy**

The portal vein was cannulated, and retrograde fixation of the liver was performed with 2.5% glutaraldehyde. The liver was fixed in 2.5% glutaraldehyde overnight and then washed with 0.1 mol/l PBS. The tissue was sectioned in 1 mm blocks and postfixed for 6 h in 1% osmium tetroxide, dehydrated through graded alcohols, and embedded in epoxy resin (Epon) (Energy Beam Sciences, Agawam, MA). After embedding, thin sections (60 nm) were cut by a microtome (Reichert Ultracut S, Lieca, Deerfield, IL), mounted on copper grids, counterstained with 2% uranyl acetate for 10 min and 1% lead citrate for 7 min, dried, and analyzed using a transmission electron microscope (TEM; JEOL 100CX). Thick sections were cut (300 nm) and stained with 1% toluidine blue. The TEM pictures were reviewed by two investigators (D. Beer-Stolz and S. C. Watkins) who were blinded to the experimental groups.

**TUNEL Assay**

The TdT-mediated dUTP nick end labeling (TUNEL) protocol was conducted as per standard published procedure and is briefly described below (10). After the liver specimens were sectioned, three washes in PBS were performed, followed by fixation in cold methanol for 30 min. The specimens were washed twice more in PBS. Ten microliters of terminal deoxynucleotidyl transferase (TdT) reaction mixture containing cobalt chloride and biotinylated dUTP were added to the slides and incubated at 37°C for 90 min. Slides were then washed with PBS three
more times and labeled with Streptavidin-conjugated Alexa 488 (Molecular Probes, Eugene, Oregon) for detection of DNA strand break. Gelvatol (1% gelatin) (Monsanta, St. Louis, MO) was then added, and the slides were coverslipped.

**Immunocytochemical Labeling for Kupffer cells, TNF-α, and iNOS**

Frozen sections were washed three times in PBS containing 0.5% bovine serum albumin and 0.15% glycine, pH 7.5 (buffer A), followed by a 30-min incubation with purified goat IgG (50 mg/ml) at 25°C and three additional washes with buffer A. Sections were then incubated for 60 min with a primary monoclonal anti-rat TNF-α antibody (R&D Systems, Minneapolis, MN) or primary polyclonal iNOS antibody (Transduction Laboratories, Lexington, KY) or primary monoclonal antimurine ED1 (Kupffer cells). These antibodies cross-react with the rat protein. Antibody incubations were then followed by three washes in buffer A and a 60-min incubation with biotinylated antimurine IgG secondary antibody (Vector Laboratories, Burlingame, CA) for iNOS, and the other primary antibodies were labeled with antimurine indocarbocyanine (Cy3) (Vector Laboratories). Specimens underwent three washes in buffer A and were then mounted in Gelvatol and coverslipped for fluorescent microscopy.

**Statistical Analysis**

Data are presented as means ± SE. Analyses were discrete comparisons between groups, and significance was determined using the unpaired Student’s t-test for normally distributed data. A P value of <0.05 was considered statistically significant.

**RESULTS**

**EPR Spectrometry**

To confirm that NOX scavenged excess NO produced in the liver, we evaluated liver samples from rats challenged with LPS for the presence of nitrosylated hemoglobin or mononitrosyl-iron-NOX complexes by EPR. Mixture of the NO donor SNAP, FeSO₄, and NOX in vitro resulted in a characteristic mononitrosyl-iron spectrum by EPR (Fig. 1A). Specimens from rats challenged with LPS and treated with NOX consistently demonstrated the presence of nitrosylated hemoglobin, but without the mononitrosyl-iron complex. The strongest nitrosylated hemoglobin signals were detected in rats killed at 6 or 8 h after LPS administration (Fig. 1B). In contrast, specimens from NOX-treated rats showed marked suppression or total absence of the nitrosylated hemoglobin signal and an EPR spectrum similar to that generated by the mixture of SNAP, FeSO₄, and NOX (Fig. 1C). The standard solution had a calculated g value of 2.037 ± 0.010, whereas the signal in the NOX-treated rats had a g value of 2.030 ± 0.010. These two values are equal within experimental error.

**Effect of NOX on LPS-Mediated Hepatocellular Injury**

Hepatocellular injury was evaluated in vivo by measuring the serum levels of AST and OCT. Endotoxin administration resulted in increased AST levels in animals receiving NS compared with controls (P < 0.001, Student’s t-test); however, this effect was substantially diminished by NOX pretreatment (Table 1). OCT is an enzyme that is located in the mitochondria of hepatocytes and is highly specific for hepatocellular injury. Endotoxemia also resulted in a notable increase in OCT levels over control values (P = 0.002, Student’s t-test); NOX administration reduced serum OCT to near baseline (Table 1).

In a separate set of experiments, we attempted to determine whether NOX could prevent hepatocellular injury when administered after LPS challenge (Table 2). There was a trend toward decreased AST levels (P = 0.10) when NOX was administered 4 h after LPS challenge. By 8 h, NOX was ineffective in preventing...
Effect of NOX pretreatment on serum AST, OCT, glucose, and fibrinogen levels after LPS challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>OCT, IU/l</th>
<th>AST, IU/l</th>
<th>Glucose, mg/dl</th>
<th>Fibrinogen, vol/300 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.8 ± 0.3</td>
<td>40.7 ± 4.2</td>
<td>191.3 ± 22.1</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td></td>
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<tr>
<td>NS</td>
<td>58.3 ± 14.6</td>
<td>508.1 ± 102.8</td>
<td>127.7 ± 6.7</td>
<td>6.4 ± 0.5</td>
</tr>
<tr>
<td>(n = 14)</td>
<td>(n = 12)</td>
<td>(n = 8)</td>
<td>(n = 8)</td>
<td></td>
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<tr>
<td>NOX</td>
<td>19.1 ± 2.8</td>
<td>265.8 ± 39.8</td>
<td>165.4 ± 14.5</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td>(n = 14)</td>
<td>(n = 12)</td>
<td>(n = 8)</td>
<td>(n = 8)</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.009</td>
<td>0.039</td>
<td>0.006</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Data represent mean values ± SE; n = no. of animals. Rats were randomized to receive either NOX or normal saline (NS) followed 18 h later by lipopolysaccharide (LPS) challenge (10 mg/kg ip). Plasma was obtained 24 h after LPS administration via cardiac puncture. Ornithine carbamoyltransferase (OCT), aspartate aminotransferase (AST), glucose, and fibrinogen were measured using specific assays. Control animals did not receive LPS or any other treatment. P values are for NOX vs. NS treatment. A P value <0.05 was considered statistically significant.

AST elevation. Although OCT levels after LPS challenge were slightly higher in this set of experiments, NOX was effective in reducing serum OCT levels when administered 4 h, but not 8 h, after LPS challenge (Table 2).

Hepatocellular injury after endotoxemia may be associated with decreased serum glucose and impaired gluconeogenesis (21). LPS-challenged animals had serum glucose levels that were significantly decreased compared with those found in control animals (P = 0.002). NOX pretreatment reduced the fall in serum glucose (Table 1). Because endotoxemia has been shown to increase levels of fibrinogen and the acute-phase reactants (47, 50), we measured serum fibrinogen levels 24 h after LPS challenge. Fibrinogen levels were significantly elevated in saline-treated animals challenged with LPS compared with controls (P = 0.001). NOX pretreatment significantly reduced serum fibrinogen levels after endotoxemia (Table 1).

Effect of NOX on Expression of Proinflammatory Mediators

Inflammatory mediators are released in response to LPS challenge and may impair hepatic function (6). We examined iNOS, IL-6, and TNF-α mRNA expression in the liver by semiquantitative RT-PCR at 24 h. There was no significant difference in the expression of TNF-α or iNOS mRNA between the groups, although TNF-α mRNA expression trended toward decreased expression in the NOX-treated animals (P = 0.10) (Table 3). The NOX-treated rats had significantly lower levels of IL-6 mRNA than their NS counterparts. Further studies revealed that there was no difference in TNF-α mRNA expression at 4 or 8 h between the groups; however, there was a trend toward decreased IL-6 mRNA expression at these time points (data not shown).

Immunohistochemical analysis of freshly harvested sections of liver from animals in both groups revealed that iNOS protein was found in Kupffer cells and in hepatocytes in all LPS-challenged animals regardless of treatment. There was no difference in expression of iNOS protein between the NOX and NS groups 24 h after LPS challenge (data not shown). Immunohistochemical analysis revealed that control animals that did not receive LPS demonstrated low baseline levels of TNF-α expression (Fig. 2A). At 24 h after LPS challenge, significant TNF-α expression can be detected in the NS-treated group (Fig. 2B). However, NOX-treated animals showed relatively little expression of TNF-α (Fig. 2C).

Morphological Analysis

Random sections of liver obtained from NS-treated and NOX-treated rats 24 h after LPS challenge were stained with H + E and examined by light microscopy. Patchy areas of hepatocellular necrosis, accompanied by hemorrhage and neutrophil infiltration, were detected in both NS-treated and NOX-treated animals without any apparent difference between the two groups (data not shown).

When examined by TEM, the necrotic areas from NS-treated rats after LPS challenge revealed swelling of the mitochondria and endoplasmic reticulum, obliteration of the hepatic sinusoids, and necrosis of endothelial cells and hepatocytes (Fig. 3D). We also examined normal-appearing areas by H + E staining in the
same liver using TEM. The space of Disse, an area that is normally filled with extracellular matrix, is important for communication between hepatocytes and sinusoids. In the NS group, the space of Disse was significantly reduced because of an increased number of microvilli on the basolateral surfaces of the hepatocytes (Fig. 3C). These changes were not evident in the NOX-treated (Fig. 3B) or control (Fig. 3A) groups.

Neutrophil and Kupffer Cell Response to LPS Challenge

Neutrophils and Kupffer cells have been implicated in hepatocellular injury after LPS challenge. Kupffer cells were identified by immunohistochemistry. At 24 h there were few Kupffer cells in the control animals (Fig. 2D) and the NOX-treated group (Fig. 2F). The NS-treated rats had a significant increase in the number of Kupffer cells (Fig. 2E). Histochemical staining for myeloperoxidase showed relatively few neutrophils in the liver under normal physiological conditions (Fig. 4A). Endotoxemia resulted in a significant increase in neutrophils in the hepatic sinusoids of the NS-treated group (Fig. 4B), but not in the NOX-treated group (Fig. 4C). Quantitative analysis showed $2.7 \pm 0.6$ neutrophils per field (40× magnification) in the control animals and $3.2 \pm 1.8$ in the NOX-treated group, compared with $8.9 \pm 2.4$ neutrophils per field (40× magnification) in the NS-treated group ($P < 0.001$).

To determine if apoptosis plays a role in the mechanism of hepatocellular injury after LPS challenge, we qualitatively measured apoptosis in the liver by the TUNEL assay. There was no increased rate of apoptosis in the liver in animals treated with NS after LPS challenge (Fig. 2, G and H), suggesting that necrosis was the predominant mechanism of injury. NOX administration had no effect on the low rate of apoptosis (Fig. 2I).

DISCUSSION

Multiorgan dysfunction represents a major cause of morbidity and mortality in sepsis (11). Numerous studies suggest that proinflammatory mediators such as
TNF-α, IL-6, or NO may play an important role in the systemic response to infections in general, and in hepatic dysfunction during sepsis in particular (15, 28, 56). The present study demonstrates that NO mediates hepatocellular injury after endotoxic shock. Administration of the NO scavenger, NOX, reduced hepatocellular injury after LPS challenge as evidenced by a reduction in serum levels of the liver enzymes OCT and AST. These findings were associated with diminished Kupffer cell proliferation, decreased neutrophil infiltration, and attenuation of morphological changes seen on TEM, although not by light microscopy. There was a concomitant decrease in the mRNA levels of IL-6 and in immunoreactivity to TNF-α. The protective effect of NOX on serum liver enzymes was seen even when administered up to 4 h after the onset of sepsis.

Fig. 3. Electron micrographs (×1,000 magnification). Control group (A; no LPS) and NOX-treated group (B) appear normal. C: an area from the liver of a NS-treated rat that appeared normal by light microscopy. However, by TEM, the space of Disse has been obliterated because of the proliferation of microvilli on the basolateral surfaces of hepatocytes. D: an area of necrosis from a NS-treated rat showing obliteration of the sinusoid, organelle swelling (mitochondria and endoplasmic reticulum), and necrosis of the hepatocyte. L, sinusoidal lumen; EC, endothelial cell; SD, space of Disse; H, hepatocyte; M, mitochondria; RER, rough endoplasmic reticulum. Bar, 1 μm.

Fig. 4. Rats were randomized to receive either NOX or NS before LPS challenge as described. Myeloperoxidase staining of liver sections demonstrates few neutrophils (black arrows) in control (A) and NOX-treated rats (C). NS-treated group (B) shows a significant increase in the number of neutrophils within the sinusoids. Magnification, ×20.
NOX is a 300-Da water-soluble dithiocarbamate derivative that chelates reduced iron and binds NO in vitro and in vivo (9, 23, 27). Several classes of dithiocarbamate derivatives that differ in their NO binding properties exist, including diethylidithiocarbamate, which is hydrophobic, as well as N-methyl-D-glucamine dithiocarbamate (MGD) and proline dithiocarbamate, which are hydrophilic (26, 34, 40). Dithiocarbamate-iron complexes effectively bind NO, resulting in the formation of a paramagnetic mononitrosyl-iron complex that can be detected by EPR (26, 27). NOX, which most closely resembles MGD in its physical and biochemical properties, is less likely to bind peroxynitrite or superoxide in vitro (44). We used EPR to verify that NOX scavenged excess NO produced in the liver. After LPS challenge, liver sections from rats treated with NS demonstrated the characteristic nitrosylated hemoglobin signals by EPR. However, specimens from NOX-treated rats revealed diminution or absence of the nitrosylated hemoglobin signal and formation of a characteristic mononitrosyl-iron dithiocarbamate signal. The morphology of the EPR spectra in NOX-treated rats is similar to that of other mononitrosyl-iron dithiocarbamate signals published by other investigators (34), which suggests that NOX indeed acted as a NO scavenger in our system. When dissolved in water, NOX produces a yellow solution that interferes with the Greiss reagent, which detects nitrite and nitrate by a colorimetric reaction; thus we did not assess NO production in the serum (9).

NO production is normally regulated by three isoforms of NO synthase (NOS). NOS-1 (nNOS) and NOS-3 (eNOS) are calcium dependent and are produced constitutively in tissues at low levels. Inhibition of eNOS leads to decreased hepatic perfusion and increased hepatocellular injury in a model of hemorrhagic shock (14). NOS-2 (iNOS) is calcium independent and may be induced in large quantities by inflammatory stimuli, including LPS (2, 53). Small quantities of NO derived from eNOS may exert a protective role in the liver by 1) preserving hepatic arterial and portal blood flow (42, 43), 2) preventing inflammation in the hepatic microcirculation (37), or 3) inhibiting reactive oxygen intermediate release and limiting TNF-α-mediated liver injury (4, 20, 24). However, excess NO produced in inflammation may be deleterious, as we have previously shown in the gut. Although there have been conflicting reports regarding the role of NO in hepatocellular damage, our findings corroborate those of several authors who have shown that NO, or its reactive nitrogen intermediates, may promote liver injury after endotoxemia, ischemia/reperfusion, or hemorrhagic shock (32, 33, 36). Mustafa et al. (36) used platelet-activating factor receptor antagonists to inhibit NO formation and prevent hepatic injury in LPS-challenged livers and in Kupffer cell culture. Menezes et al. (33) showed that NOX prevented hepatic dysfunction in a model of hemorrhagic shock. Although these authors did not show that the NOX effect was due to local scavenging of NO in the liver, NOX reduced serum OCT elevation when infused continuously during the hypotensive insult. The hepatocellular injury attributed to NO may be due to its direct cytotoxicity or its diffusion-limited reaction with superoxide to produce the toxic nitrogen metabolite peroxynitrite (45). Ma et al. (32) pretreated mice with endotoxin to induce hepatic NO production before ischemia/reperfusion, which resulted in increased hepatocellular injury, implicating peroxynitrite as a causative agent (32). However, we could not demonstrate evidence of peroxynitrite-mediated injury in our study because of marked background autofluorescence in the liver tissue. Nonetheless, given this limitation, we cannot exclude the possibility that NOX prevented hepatocellular injury by decreasing peroxynitrite generation, consistent with our observations in the rat intestine after LPS challenge (9, 51).

The mechanism of NOX-mediated hepatocellular injury also remains somewhat controversial. Early reports suggested that LPS-induced hepatic dysfunction was primarily due to necrosis rather than apoptosis (56). However, Redmond et al. (46) used LPS in conjunction with antioxidants to induce hepatocellular apoptosis. Inhibition of NO production reduced both hepatocyte necrosis and apoptosis in this model. Wang et al. (57) confirmed these results by illustrating that the NO donor, SNAP, could induce hepatocellular apoptosis. However, in the presence of reactive oxygen intermediates, NO led to hepatic necrosis. In our study, LPS challenge induced hepatic injury via necrosis rather than apoptosis. We were unable to detect apoptosis in the liver in any of our treatment groups.

Both TNF-α and IL-6 have been implicated in hepatocellular dysfunction and necrosis associated with sepsis (48, 49, 58). Our data show that NOX downregulates IL-6 mRNA expression and TNF-α protein production in the liver (although TNF-α mRNA was only mildly suppressed). These findings are consistent with
those of Hierholzer et al. (16), who demonstrated that upregulation of these cytokines in hemorrhagic shock was dependent on iNOS (16). The decreased levels of TNF-α and IL-6 in animals receiving NOX may reflect in part a decrease in Kupffer cell proliferation or a decrease in neutrophil infiltration because both types of cells are capable of secreting these inflammatory mediators (29). Furthermore, neutrophils have been shown to promote hepatocellular injury in vitro and in vivo (17). In our study, we observed a threefold increase in neutrophils in the hepatic sinusoids of NS-treated rats after LPS challenge. Again, NOX abrogated this effect. We cannot exclude the possibility that posttranscriptional modification of TNF-α mRNA in the NOX-treated animals resulted in decreased TNF-α protein production, although we did not test this hypothesis. Alternatively, because NOX is a dithiocarbamate that binds nuclear factor-kB, it may block signaling via this transcription factor, which has been shown to upregulate both TNF-α and IL-6 production (1, 22). In contrast to TNF-α and IL-6, neither iNOS mRNA nor protein expression was affected by NOX. However, these findings are not surprising because a NO scavenger would not be expected to affect iNOS mRNA or protein expression. The EPR data are consistent with NOX acting as a NO scavenger in the liver.

In addition to decreased serum liver enzyme levels and morphological damage, further evidence that NOX maintained hepatocyte viability during endotoxemia includes decreased serum fibrinogen levels and preservation of normal serum glucose levels. Serum fibrinogen levels were increased during endotoxemia, similar to observations in humans (5, 47). NOX administration reduced fibrinogen levels, although not to baseline. Glucose levels are normally decreased during sepsis (21). Titheradge et al. (54) reported that the inhibition of gluconeogenesis after LPS challenge is the result of the inhibition of phosphoenolpyruvate carboxykinase due to the sustained production of NO (54). Thus NOX therapy may reduce synthesis of the acute-phase proteins and improve gluconeogenesis.

In conclusion, our data corroborate previous reports suggesting that excess NO production after LPS challenge results in increased hepatocellular injury. Hepatic injury was associated with sustained upregulation of iNOS, TNF-α, and IL-6 mRNA, as well as increased serum fibrinogen levels and decreased serum glucose. The novel NO scavenger NOX attenuated LPS-induced hepatocellular dysfunction. Thus NOX may have important implications for the treatment of septic shock and other inflammatory conditions associated with sustained production of NO.

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