Differential Effects of Nonselective Nitric Oxide Synthase (NOS) and Selective Inducible NOS Inhibition on Hepatic Necrosis, Apoptosis, ICAM-1 Expression, and Neutrophil Accumulation during Endotoxemia

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The roles of nitric oxide derived from either the constitutive endothelial NO synthase (eNOS or NOS3) or the inducible NOS (iNOS or NOS2) in hepatic injury during endotoxemia remain controversial. To investigate this further, rats received a bolus of lipopolysaccharide (LPS) following implantation of osmotic pumps containing one of two nonselective NOS inhibitors (NMA or NAME), one of two inducible NOS inhibitors (NIL or AG), or saline. The inhibitors were infused continuously into the liver via the portal vein. Treatment of LPS-injected rats with NMA and NAME resulted in 106 and 227% increases, respectively, in circulating hepatic enzyme levels compared to LPS-treated control rats. In contrast, infusion of the iNOS-selective inhibitors had no effect on the LPS-induced hepatic necrosis. In rats receiving NAME, LPS induced greater neutrophil infiltration and ICAM-1 expression than in the LPS + saline group, whereas NIL infusion did not. The increased hepatic necrosis and PMN infiltration in the LPS + NAME group was partially prevented by a simultaneous infusion of a liver-selective NO donor. Inhibition of PMN accumulation using an anti-ICAM-1 antibody or by PMN depletion using vinblastine pretreatment, however, did not reverse the increased necrosis with NAME infusion during endotoxemia. In contrast to the assessment for necrosis, increased apoptosis was observed in the livers of LPS-treated rats receiving infusions of either NAME or NIL, but not with LPS alone. These data indicate that NO produced by eNOS may be adequate to prevent necrosis by a mechanism independent of PMN, while induced NO appears to prevent apoptosis.

Key Words: Nitric oxide; NOS inhibitors; liver; necrosis; apoptosis; neutrophil; adhesion molecule.

Administration of endotoxin (lipopolysaccharide, LPS) has been used experimentally to simulate the

Abbreviations used: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α; NOS, nitric oxide synthase; nNOS, neuronal NOS; iNOS, inducible or inflammatory NOS; eNOS, endothelial NOS; NAME, N'G-nitro-L-arginine-methyl ester; NMA, N'G-monomethyl-L-arginine; NIL, L-N6-(1-iminoethyl)lysine; AG, arginine; PMN, neutrophil; V-PYRRO/NO, O2- vinyl 1-(pyrroli-din-1-yl)diazen-1-ium-1,2-dioate; OCT, ornithine carbamoyltransferase; DEPC, diethyl carbonate; PBS, phosphate-buffered saline; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
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systemic inflammatory response and organ injury characteristic of sepsis. Hepatic responses induced by experimental endotoxemia include the activation of reticuloendothelial cells followed by degenerative changes in hepatocytes (1, 2). Cytotoxicity has also been described following exposure of hepatocytes to endotoxin in vitro (3) and nonparenchymal - hepatocyte cocultures (4). The mechanisms of hepatocellular toxicity in endotoxemia appear to involve cytokines such as tumor necrosis factor \( \alpha \) (TNF-\( \alpha \)) (5) and toxic radicals (6). Recent work has focused on nitric oxide as a pivotal cellular mediator in the liver during endotoxemia. Whereas some studies have suggested that reactive nitrogen intermediates induce tissue injury, including in hepatocytes (4, 7, 8), others support the concept that NO plays a hepato-protective role (9–14). We have suggested that the end result of NO formation may be dependent on the rate of NO synthesis, the cellular source of the NO, the quantity and species of other radicals that are produced, and the antioxidant status of the liver (10, 11, 15).

The quantity of NO synthesized in a tissue and the temporal pattern of production are dependent on the types of NOS isoforms expressed. Three NOS isoforms are known to exist and include the neuronal NOS (nNOS or NOS1), the inducible or inflammatory NOS (iNOS or NOS2), and the endothelial NOS (eNOS or NOS3) (reviewed in 16, 17). nNOS and eNOS are calcium/calmodulin-dependent enzymes that are constitutively expressed, while iNOS activation is independent of elevations in intracellular calcium and is typically expressed in cells exposed to signals which induce iNOS gene expression. eNOS is constitutively expressed in the endothelium in the liver, while iNOS can be induced in hepatocytes (18–20), Kupffer cells (21), fat-storing cells (22), and endothelial cells (23, 24) under inflammatory conditions such as endotoxemia. Since nonselective NOS inhibitors increase hepatic injury in endotoxemia (9, 10, 25) and inhibitors with some selectivity toward iNOS have been reported to reduce damage (26), it is possible that the consequence of NO production will be determined by the enzymatic source. However, in these previous studies the inhibitors were administered systemically and as a bolus, raising concerns about the effective delivery of the inhibitor to the liver. To establish the consequences of eNOS or iNOS inhibition within the liver, we undertook this study in which either nonselective NOS inhibitors (\( \text{N}^6 \)-guanidino-L-arginine methyl ester [NAME] or \( \text{N}^6 \)-monomethyl-L-arginine [NMA]) or inhibitors with some selectivity toward iNOS (\( \text{L-N}^6 \)-[1-iminoethyl]lysine [NIL] or aminoguanidine [AG] (27–29)) were continuously infused directly into the portal vein of rats treated with LPS. We examined not only hepatic necrosis but also apoptosis, and report the novel observation that nonselective inhibitors increase both hepatic necrosis and apoptosis, whereas iNOS-selective inhibitors promote only apoptosis. Furthermore, nonselective inhibitors increased ICAM-1 expression and PMN accumulation but iNOS-selective inhibitors did not. However, the increased hepatic necrosis with nonselective NOS inhibitors was not dependent on PMN accumulation. These results suggest specific and separate actions for NO produced by eNOS and iNOS in the liver in endotoxemia.

MATERIALS AND METHODS

Materials

NMA was from CHEM-BIOCHEM Research Inc. (Salt Lake City, UT). AG was from Aldrich Chemical Co. (Milwaukee, WI). NIL was provided by P. Davies of Merck Research Laboratories (Rahway, NJ). Alzet osmotic pumps, Model 2001D, were from Alzet Co. (Palo Alto, CA). Lipopolysaccharide from Escherichia coli 0111:B4 (LPS) was obtained from Difco Laboratories (Detroit, MI). Vinblastine was from Bedford Laboratories (Bedford, OH). Anti-neutrophil monoclonal IgM antibody (RP3) was prepared as described by Sekiya et al. (30). Anti-rat neutrophil monoclonal IgG antibody and isotype-matched control antibody were from Pharmingen Co. (San Diego, CA), and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

We have previously reported the design, synthesis, and specificity of a compound, \( \text{O}^2 \)-vinyl -1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (V-PYRRO/NO), which concentrates NO release in the liver in vivo (31).

Experimental Protocol

Experimental procedures were approved by the Institutional Animal Care and Use Committee, Uni-
University of Pittsburgh. Male Sprague–Dawley rats (250–300 g, from Harlan Sprague–Dawley, Madison WI) were fasted overnight prior to the initiation of the experiments but allowed free access to water. Prior to the injection of LPS, Alzet osmotic pumps were implanted for the continuous infusion of NOS inhibitors, V-PYRRO/NO (NO donor), or vehicle controls. For NOS inhibitor infusion, PE-10 catheter tubing was inserted into the left gastric vein for direct portal vein infusion with the pump positioned in the peritoneal cavity. In some rats a second pump containing V-PYRRO/NO was implanted subcutaneously on the back of the animal with the catheter inserted into the jugular vein. Animals were anesthetized with inhaled isoflurane for the procedure, the PE-10 catheter tubing was inserted into the vein through a venotomy, and the vein was ligated around the catheter. The catheter was flushed with the same solution that was used to fill the pump and then connected to the pump. All pumps were placed in saline solution at 37°C for 4 h before use so that the flow into the vein would start immediately after implantation. LPS (12 mg/kg) or saline was injected intravenously after the completion of pump placement, and no other pharmacologic reagents were given during the infusion period until the animals were anesthetized again with isoflurane just prior to sacrifice for plasma and liver tissue isolation, 16 h following the injection of the LPS. AG was delivered at a dose of 125 μmol/kg/h; NMA was delivered at 20.8 μmol/kg/h; NAME at 6.9 μmol/kg/h; NIL at 20.8 μmol/kg/h; NAME + NIL at 3.4 + 10.4 μmol/kg/h, respectively; and V-PYRRO/NO at 11 μmol. The doses of the NOS inhibitors were selected by first establishing the lethal dose of NAME (10.4 μmol/kg/h) in rats given LPS and then reducing the dose to a sublethal amount. The doses of the other NOS inhibitors were then calculated based on the published IC₅₀ values for these compounds against iNOS and eNOS relative to NAME (27–29). The dose of V-PYRRO/NO was based on our published data showing hepatic NO release without systemic hypotension (31).

**Immunohistochemistry**

For immunohistochemical analysis, the liver was perfused with 30 ml saline, embedded in ornithine carbamoyltransferase (OCT), and frozen in liquid nitrogen. Liver sections were stained using the avidin–biotin-complex method (32). Briefly, frozen specimens were sectioned with a cryostat, air-dried overnight, fixed in acetone for 10 min, air-dried for 15 min, rehydrated in 1% bovine albumin–phosphate-buffered saline for 10 min, and then incubated with the primary antibody (1:200 RP3, an anti-rat PMN monoclonal antibody (33), or anti-ICAM-1) for 1 h. The biotinylated secondary antibody and avidin–biotin complex (Vector Lab, Burlingame, CA) were applied for 30 and 45 min, respectively. A peroxidase chromogen kit (Biomeda Co., Foster City CA) was used to visualize antibody binding. The substitution of the isotype-matched control antibody for the primary antibody was used as a negative control. Neutrophils were identified by RP3-positive staining and morphology, and the average number was determined by counting 40 high-power fields at 400× magnification in a blinded fashion. Only those neutrophils that were present within sinusoids or extravasated into the tissue, and not neutrophils present in large hepatic vessels, were counted.

**DNA Fragment Assay**

Enriched, low-molecular-weight DNA from whole liver tissue was isolated and extracted according to modifications of the methods of Wyllie (34) as well as Hughes and Gorospe (35). Whole frozen liver tissue was homogenized in ice-cold lysis buffer (5 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8.0). Following the addition of diethyl pyrocarbonate (DEPC, final concentration = 0.2% v/v) to inhibit endogenous nuclease activity, homogenates were incubated on ice for 90 min with occasional mixing. The homogenate was then centrifuged at 27,000g for 20 min to
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Separate fragmented DNA and subjected to proteinase K digestion (0.5 mg/ml, 1 h at 37°C) followed by repeated phenol:chloroform extraction. Nucleic acids were precipitated with 100% ethanol at −20°C, collected by centrifugation at 15,000 g, and subjected to ribonuclease A (2.5 μg/ml) treatment. Samples were extracted, precipitated as above, and dried, and nucleic acid pellets were resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The concentration and purity of DNA were estimated spectrophotometrically by the ratio of absorbencies at 260 and 280 nm. Samples (~50 μg DNA) were mixed with gel loading buffer (0.05% w/v bromophenol blue, 40% w/v sucrose, 0.1 M EDTA, pH 8.0) and electrophoresed on 2% agarose gels at 50 V in TAE buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.6). Gels were stained with ethidium bromide and visualized by ultraviolet transillumination. A double-stranded 123-bp DNA ladder served as a standard.

Biochemical Analysis

Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma were determined with a chemistry autoanalyzer (Bayer, Pittsburgh, PA).

Statistical Analysis

Results are expressed as means ± SE with the number of different experiments given in the figure legends. Statistical significance between groups was determined using ANOVA, with significance established at P < 0.05.

RESULTS

We examined the effects of nonselective or selective NOS inhibition 16 h following the injection of LPS. This time point was chosen based on previous data showing that iNOS expression peaked between 12 and 16 h (37) and preliminary data showing that both injury and neutrophil infiltration peaked at this time point (data not shown). LPS injection has been shown to transiently elevate circulating NO2/NO3 levels in rats (18). Even though plasma NO2/NO3 would not be expected to selectively measure hepatic NO production, acute changes in levels can be used as an estimate of increased systemic NO production. In agreement with previous studies, we found that intravenous administration of LPS resulted in an increase in plasma levels of NO2/NO3 from a baseline of 9.3 ± 4.5 to 367.2 ± 47.6 μM (Fig. 1) at 16 h in animals implanted with Alzet pumps containing only saline. Portal vein infusion of NIL, AG, NMA, NAME, or NIL/NAME reduced the LPS-induced increases in plasma NO2/NO3 levels by 96.7, 88.8, 70.7, 67.0, and 96.4%, respectively, compared to the LPS-treated animals with pumps containing saline (Fig. 1). Thus, all the inhibitors exhibited significant activity toward the LPS-induced NO production.

LPS injection induced hepatocellular necrosis as Light Microscopic Detection of Apoptosis

Cryosections of fixed, frozen livers were cut, mounted on slides, and labeled using the TUNEL assay. This assay specifically detects laddered DNA by terminal tailing of DNA with fluorescently tagged bases. DNA strand breaks were directly labeled by incubating sections with 10 M fluorescein-12-dUTP and 200 U terminal deoxynucleotidyltransferase/ml in reaction buffer. Cobalt was added to enhance labeling of protruding, recessed, and blunt DNA breaks containing a free 3'-OH. After 45 min at 37°C, the reaction was terminated by washing with PBS and the specimen was counterstained with Hoechst 33258, 2 mg/ml for 3 min. This stain specifically stains DNA and allows all nuclei in the section, or the entire section, to be examined. The cells were then mounted in Gelvatol and observed using a Nikon FXL photomicroscope. Random images using a 60× objective were collected using a 3 Chip Sony color camera. The apoptotic nuclei in each field were counted and the types of apoptotic cells were classified.

Nitrite and Nitrate Assay

For the NO2/NO3 assay, plasma was collected and stored at −80°C. Samples were thawed and deproteinized by the addition of 400 μl 0.5 N sodium hydroxide and 400 μl 10% zinc sulfate solution to each 200 μl sample, vortexed for 30 s, and centrifuged to pellet the protein. Total NO2 + NO3 was determined using an automated HPLC system based on the Griess reaction, in which NO2 is first converted to NO3 using copper-coated cadmium, as described (36).

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trated in the liver because this prodrug is metabolized to NO preferentially in hepatocytes (31). Infusion of V-PYRRO/NO in LPS-treated rats in the absence of NAME had no effect on AST levels, but when given with NAME, the donor attenuated the increase in liver damage, as measured by a reduction in plasma AST levels (Fig. 3). Thus, the NAME-induced increase in injury appears to be due, at least in part, to an inhibition of NO synthesis.

It has been proposed that NO protects the liver from damage by blocking neutrophil infiltration (38, 39). To assess the effects of NOS inhibition on PMN infiltration, the number of infiltrating neutrophils at 16 h following LPS treatment was determined. As shown in Fig. 4, LPS injection in the absence of NOS inhibitors resulted in a 4.25-fold increase in neutrophil infiltration over controls (saline-containing saline) was injected intravenously immediately after pump placement and 16 h before plasma samples were taken. Plasma NO\textsubscript{3} + NO\textsubscript{2} levels were measured as described under Materials and Methods. Data are the mean ± SE from seven animals per group (*P < 0.002 vs saline + LPS).

Increased ICAM-1 expression occurs in the liver following endotoxemia (40) and contributes to neutrophil-mediated injury in liver ischemia/reperfusion injury (41). Therefore, we examined the level of ICAM-1 protein expression in liver sections by immunohistochemistry using an anti-ICAM-1 monoclonal antibody. Faint constitutive ICAM-1 protein expression was detected on surfaces of the sinusoid endothelial cells in rats that received pumps with saline (Fig. 5A), which was not different from livers studied from control rats without pumps (not shown). Infusion of NAME or NIL without LPS did not cause a detectable change in ICAM-1 expression (Figs. 5B and 5C). ICAM-1 expression was increased after LPS injection in rats with an intraportal infusion of saline (Fig. 5D). Portal vein infusion of NAME following LPS injection resulted in an even greater increase in the LPS-induced increase in ICAM-1 expression (Fig. 5E). In contrast, infusion of NIL had no effect on LPS-induced changes in ICAM-1 expression levels (Fig. 5F). The combination of NIL with NAME appeared identical to using only NAME with LPS, whereas the infusion of V-PYRRO/NO with
FIG. 2. NMA and NAME aggravate hepatocellular necrosis in LPS-treated animals. Rats were injected with LPS (A) or saline (B) following implantation with osmotic pumps for portal vein infusion of either the NOS inhibitors indicated or saline. Plasma AST and ALT levels were determined 16 h after LPS injection. Data are the mean ± SE from seven animals per group (* P < 0.03 vs saline / LPS).

FIG. 3. Exogenous NO partially prevents the NAME-induced liver injury in LPS-treated rats. Rats were injected with LPS intravenously following implantation of two pumps—one osmotic pump containing NAME or saline with the catheter positioned in the portal vein, and another pump containing V-PYRRO/NO or saline with the catheter positioned in the jugular vein. Blood samples were taken just before sacrificing the animals, 16 h after the LPS injection. Data are the mean ± SE from six animals per group (* P < 0.05 vs jugular saline, portal NAME).

FIG. 4. Effect of NOS inhibition on neutrophil accumulation in the liver. Rats received a single dose of LPS or saline intravenously following implantation of osmotic pumps containing saline, NIL, NAME, or NIL + NAME for portal infusion. Some of the rats with NAME pumps received a second pump containing V-PYRRO/NO for simultaneous jugular vein infusion. Neutrophils in liver tissue were stained and counted as described under Materials and Methods. Data represent the mean ± SE for six animals per group (* P < 0.03 vs saline + LPS).
FIG. 5. Effect of NOS inhibition on hepatic ICAM-1 expression. Rats received a single dose of LPS or saline intravenously following implantation with osmotic pumps containing saline, NIL, or NAME for portal infusion. ICAM-1 expression in liver tissue samples was detected, as described under Materials and Methods. Groups include: (A) Pump with saline/saline injection; (B) pump with NAME/saline injection; (C) pump with NIL/saline injection; (D) pump with saline/LPS injection; (E) pump with NAME/LPS injection; (F) pump with NIL/LPS injection. Tissue sections are representative of one of three identical experiments.
NAME attenuated the increase in ICAM-1 expression in LPS-treated rats (not shown).

We next carried out experiments to determine if the increase in PMN accumulation caused by NAME infusion during endotoxemia contributed to the increase in hepatocellular damage. Injection of a blocking anti-ICAM-1 antibody but not an isotype control prevented the NAME-induced increase in PMN accumulation in the liver (Fig. 6A), but did not reduce circulating PMN counts (Fig. 6B). We also inhibited PMN accumulation by inducing neutropenia using vinblastine pretreatment. As shown in Fig. 6B, a single injection of vinblastine 5 days prior to LPS injection resulted in a 99.3% drop in circulating PMN counts. This treatment also completely prevented the NAME-induced increase in PMN infiltration in the liver. However, no change in liver damage was detected with either anti-ICAM-1 antibody or vinblastine pretreatment (Fig. 7). Thus, the NAME-induced hepatocellular injury in the LPS-treated animals is associated with, but occurs independent of, increased PMN accumulation.

Increases in circulating hepatic enzyme levels is a measure of hepatocellular necrosis but may not detect cell death due to apoptosis because cells undergoing apoptosis do not release their contents. NO exposure has been shown to cause apoptosis in some cell types (42-44) but inhibits apoptosis in other cell types (45-47), including hepatocytes (31). To determine if NOS inhibition altered the level of apoptosis in the liver following LPS treatment, DNA isolated from liver was analyzed for fragmentation characteristic of apoptosis. In the absence of LPS, intraportal infusion of saline, NAME, NIL, NIL + NAME, or the NO donor V-PYRRO/NO did not result in detectable DNA fragmentation (Fig. 8). Injection of LPS with portal saline infusion also was not associated with detectable apoptosis. In contrast, infusion of any of the NOS inhibitors with LPS resulted in the appearance of DNA fragmentation, indicating increased levels of apoptosis in these livers. Coinfusion of V-PYRRO/NO with NAME prevented the appearance of DNA fragmentation. Thus, nonspecific NOS inhibition increased both necrosis and apoptosis, whereas infusion of an iNOS-specific inhibitor with LPS increased only apoptosis.

To identify the distribution of apoptotic cells within the liver of LPS-treated rat infused with NIL, liver sections were subjected to the TUNEL assay. No apoptosis was detectable in hepatocytes from the animals treated with LPS or NIL alone (not shown). In LPS-treated animals infused with NIL, apoptosis was observed predominantly in endothelial cells and other sinusoidal cells, though it could also be de-
neutrophil infiltration. The failure of the iNOS-selective inhibitors to mimic the effects of the nonselective inhibitors argues that the doses of NIL and AG that we used did not significantly inhibit eNOS. NIL infusion was, however, associated with the appearance of apoptosis in the livers of LPS-treated animals. Taken together, these data suggest that NO derived from eNOS functions in protecting the liver from inflammation-induced necrosis, whereas NO produced by iNOS or a combination of iNOS and eNOS functions to prevent apoptosis.

Our results are consistent with previous reports examining the effect of nonselective NOS inhibitors on liver injury in rodent endotoxemia (9, 10, 25) but inconsistent with a study which examined selective iNOS inhibitors (26). All of these previous studies examined the effect of bolus injection of the inhibi-

**DISCUSSION**

Although it is well-established that NO production increases in the liver in acute inflammatory conditions such as endotoxemia (18), the consequences and functions of local hepatic sources of NO remain controversial. This study was undertaken to examine the effects of the direct and constant intraportal infusion of NOS inhibitors with differing potencies against eNOS (NOS3) and iNOS (NOS2). We show here that nonselective inhibitors increase hepatic necrosis following LPS injection. This increased necrosis was associated with an increase in neutrophil infiltration into the liver and hepatic ICAM-1 expression; however, the injury was independent of the neutrophil accumulation. In clear contrast to the nonselective inhibitors, inhibitors with 30- to 100-fold selectivity toward iNOS compared to eNOS (NIL and AG) (27–29) caused no change in LPS-induced hepatic necrosis, ICAM-1 expression, or apoptosis.
**FIG. 9.** Detection of apoptosis in liver sections by TUNEL. (A) Low-power image of TUNEL staining in NIL-treated liver, probed for fragmented DNA, as described under Materials and Methods. Abundant apoptotic figures may be seen; the majority of these are in sinusoidal cells, including Kupffer cells, leukocytes, and endothelial cells. Apoptotic hepatocytes may also be seen (arrow). Bar, 20 μm. (B, C) These images show the typical morphology of apoptotic figures found in the liver. The DNA has fragmented and condensed into small cytoplasmic inclusions (B, arrow); the identity of the condensed fragments was confirmed with Hoechst dye, which binds specifically to DNA (not shown). The identity of the apoptotic cell as a hepatocyte is confirmed in C (arrow). Bar, 20 μm.

tors in models of endotoxin-induced liver injury. In our own previous work, we showed that bolus injection of NMA at the time of LPS injection in a model of endotoxin hypersensitivity significantly increased liver damage (9) through mechanisms which appear to involve oxygen radical production and platelet deposition (10). NO was shown to act in concert with prostacyclin to attenuate liver damage (11). We also showed that bolus injection of sufficient doses of NMA 6 h after LPS infusion caused a modest increase in liver injury (25). Others have since verified our observations using nonselective NOS inhibitors (48). In contrast to the studies using nonselective inhibitors, Szabo et al. (26) recently reported that bolus administration of an isothiourea, which exhibits selectivity toward iNOS, prevents liver damage in a model of rat endotoxemia. To overcome any untoward effects of bolus infusion and to assure that the inhibitors reached the liver in adequate concentrations to inhibit NOS activity, we devised a method that delivers a constant infusion of NOS inhibitors directly into the portal vein by cannulating the left gastric vein with a catheter connected to an Alzet osmotic pump. Our results confirm the results of previous studies showing increased necrosis with nonselective NOS inhibition but failed to demonstrate a protective effect with selective iNOS inhibitors. Several potential explanations exist for the difference between our study and the previous study using isothiourea. First, the reported benefit from iNOS inhibition on hepatic necrosis could be due to improved systemic hemodynamic effects and not due to inhibition of NO production specifically in the liver. This possibility seems unlikely because we would expect that even though we infused inhibitors directly into the liver, it is probable that enough of the infused material escaped metabolism in the liver to have systemic actions. This is supported by the near-complete inhibition of elevations in plasma NO x levels by NIL and AG infusion and based on the assumption that extrahepatic sources of NO also contribute to elevations in plasma NO x levels. Second, differences in timing of the administration of the inhibitors may be important. Finally, our observations using nonselective NOS inhibitors (48). In contrast to the studies using nonselective some isothioureas have been shown to block not only iNOS enzyme activity but also iNOS expression (49), and therefore this class of compounds could have salutary actions unrelated to iNOS inhibition.

The increase in hepatocellular necrosis with inhibitors effective toward both eNOS and iNOS but not inhibitors selective for iNOS suggests that NO derived from eNOS is critical to preventing hepatocellular necrosis. Our results also raise the possibility that eNOS regulates ICAM-1 expression in the inflamed liver. The observation that anti-ICAM antibodies block neutrophil accumulation also suggests but does not prove that the increased ICAM-1 expression promotes neutrophil accumulation associated with NOS inhibition. The capacity of the liver-specific NO donor to reduce the damage, ICAM-1 expression levels (data not shown), and neutrophil influx points to the importance of NO in all three changes. Even though activated neutrophils have been shown to kill hepatocytes (50), the failure to

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link neutrophil accumulation with necrosis indicates that the enhanced hepatocellular injury is not mediated by neutrophils. This conclusion is additionally supported by the experiments using vinblastine, which assures that PMN were depleted throughout the experimental period. The cause of the hepatocellular necrosis is unclear, but may include the unopposed action of oxygen radicals or alterations in liver perfusion. It is likely that the combined inhibition of iNOS and eNOS causes severe disturbances in the hepatic microcirculation through an increase in intrahepatic resistance. Using an isolated perfused liver system, we have shown that nonselective NOS inhibitors reverse the hyporeactivity to phenylephrine caused by LPS pretreatment (51).

NAME infusion alone has been shown to increase PMN infiltration into the mesentery (52, 53) through a process in which the inhibition of NO synthesis causes mast cell degranulation (54). Inhibition of endothelial NO production results in increased ICAM-1 expression and neutrophil adhesion to endothelial monolayers in vitro (55), whereas both NO (56) and peroxynitrite (57) have been shown to suppress surface P-selectin expression on the endothelium of mesenteric venules in vivo. We saw no increase in neutrophil accumulation in the liver with NOS inhibition alone, indicating differences between the mesentery and the liver.

Our observation that NOS inhibition resulted in detectable apoptosis in the liver following LPS injection suggests that induced NO may function to block apoptosis. Because both selective and nonselective inhibitors increase apoptosis to a similar degree, it is likely that the quantities of NO generated by iNOS are necessary to block apoptosis in both endothelial cells and hepatocytes. Although NO can cause susceptible cells to undergo apoptosis (42–44), a number of other cell types are protected from apoptosis by NO through either cGMP-dependent (46, 47) or cGMP-independent (45) mechanisms. We and others have shown the same to be true for hepatocytes. Large quantities of NO can protect hepatocytes from TNF-α-induced apoptosis through the induction of heat shock protein 70 (14), whereas small quantities of sustained NO production blocks TNF-α-induced apoptosis via mechanisms involving the inhibition of caspase-3-like protease activation and activity (58). The experiments presented here do not discern the mechanism of protection from apoptosis or the stimulus for the apoptosis, although TNF-α is a likely candidate (5). Furthermore, the increase in apoptosis was seen with all the NOS inhibitors, whereas the increase in necrosis was detected only with inhibitors which effectively blocked eNOS. Because both NMA and NAME have activity toward iNOS, it cannot be determined if the increase in apoptosis with these inhibitors was due to iNOS or eNOS inhibition. Nonetheless, these studies reveal another potential mechanism of protection for NO in the liver as an antiapoptotic agent.

It is important to point out that the doses and route of administration of the inhibitors used in these experiments were chosen to permit the study of the actions of NO in the liver in endotoxemia and not to determine the efficacy of NOS inhibitors to treat clinical septic shock. Significantly lower doses of NMA have been used to reverse hypotension without leading to hepatocellular injury based on plasma enzyme levels (59, 60).

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