Differential Effects of Nitric Oxide Synthase Inhibitors on Endotoxin-Induced Liver Damage in Rats

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See editorial on page 1405.

Background & Aims: **During endotoxemia, expression of inducible nitric oxide synthase (iNOS) and nitric oxide production in the liver is increased. NO has been suggested to have a hepatoprotective function. The aim of this study was to investigate the distribution of iNOS and the effect of different NO synthase inhibitors on liver damage and hemodynamics during endotoxemia.** ***Methods:*** **Rats were injected with lipopolysaccharide (LPS) and received the NOS-inhibitor *S*-methylisothiourea (SMT) or N^G-nitro-L-arginine methyl ester (L-NAME). iNOS induction was assessed by Western blot, immunohistochemistry, and measurement of NO metabolites in plasma and bile. Liver damage was determined by aspartate aminotransferase and alanine aminotransferase and by histology. The effects of both inhibitors on systemic and portal pressure were measured in normal and LPS-treated rats.** ***Results:*** **LPS treatment strongly induced iNOS in inflammatory cells, macrophages, bile duct epithelium, and hepatocytes, especially at the canalicular membrane. LPS-induced liver damage strongly increased after L-NAME. SMT caused a similar reduction of NO production without enhancing liver damage. In LPS-treated rats, SMT increased the systemic and portal pressure significantly more than L-NAME.** ***Conclusions:*** **During endotoxemia, administration of the NOS-inhibitor L-NAME aggravates liver damage. This liver damage does not seem to be caused by hemodynamic changes. In contrast, SMT caused significant hemodynamic changes but did not increase LPS-induced liver damage.**

Nitric oxide radical is increasingly recognized as an important mediator of physiological and pathophysiological processes.[1] NO is produced by NO synthase (NOS), an enzyme that exists in three isoforms encoded by distinct genes.[2,3] Neuronal (type I, nNOS) NOS and endothelial (type III, eNOS) NOS are Ca^{2+}- and calmodulin-dependent constitutive isoforms. nNOS has a function in neurotransmission. eNOS plays an important role in vasorelaxation, and NO produced by the endothelium has antithrombotic properties. Inducible NOS (iNOS), also known as type II NOS, is not expressed under normal conditions. However, it can be induced by cytokines and lipopolysaccharide (LPS) in many cell types, such as hepatocytes, macrophages (including Kupffer cells), neutrophils, smooth muscle cells, and chondrocytes.[4]

Harmful effects of enhanced NO production in the liver include inhibition of the mitochondrial respiratory chain enzymes and gluconeogenesis.[5] In cultured hepatocytes, NO inhibits total protein synthesis[6] and bile canalicular contraction.[7] NO has been implicated in several pathophysiological processes such as endotoxemia (sepsis), hepatic ischemia/reperfusion injury, and hepatic allograft rejection.[8] However, the exact function of iNOS and enhanced NO production under these conditions has not been elucidated yet. Most studies postulate a hepatoprotective effect of NO during endotoxemia, because inhibition of NO production increased hepatic damage.[9–12] However, in these studies the L-arginine analogues N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA) were used, which are better eNOS than iNOS inhibitors.[13–15] In contrast, *S*-methylisothiourea (SMT) has been described as a preferential iNOS inhibitor.[15–21] The aim of the present study was to compare the effects of SMT and L-NAME on liver damage and hemodynamics during endotoxemia. In addition, we investigated the cellular distribution of hepatic iNOS during endotoxemia.

Abbreviations used in this paper: eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, N^G-monomethyl-L-arginine; LPS, lipopolysaccharide; MAP, mean arterial pressure; nNOS, neuronal nitric oxide synthase; NOS, nitric oxide synthase; NOx, nitrite plus nitrate; SMT, *S*-methylisothiourea.

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Materials and Methods

Animals

Specified pathogen–free male Wistar rats (220–300g; purchased from Harlan-CPB, Zeist, The Netherlands) were kept under routine laboratory conditions at the Central Animal Laboratory of the University of Groningen. The rats received standard laboratory chow and had free access to food and water. The study as presented was approved by the Local Committee for Care and Use of Laboratory Animals.

Experimental Design

Rats were injected intraperitoneally (IP) with endotoxin LPS, *Escherichia coli*, serotype 0127:B8 (Sigma Chemical Co., St. Louis, MO), or the same volume of phosphate-buffered saline (PBS, control group; n = 6 for each experimental group). SMT (Sigma) and L-NAME (Sigma) were given together with LPS. LPS and drugs were dissolved in sterile PBS. At indicated time points after LPS injection, the rats were anesthetized with pentobarbital (60 mg/kg, IP). For determination of serum alanine transaminase (ALT) and aspartate transaminase (AST) activities and the NO metabolites nitrite plus nitrate (NOx), heparinized blood samples were obtained by cardiac puncture. The livers were perfused with PBS, removed, cut into small pieces, and snap-frozen in liquid nitrogen until further use. Bile samples were obtained by cannulation of the bile duct for 30 minutes before starting liver perfusion.

Hemodynamic Measurements

Six hours after the injection of LPS (5 mg/kg, IP) or the same volume of PBS, the effects of L-NAME, SMT, or the same volume of PBS on mean arterial pressure (MAP) and portal pressure were measured (n = 6–7 for L-NAME and SMT treated rats; n = 3 for the control groups). For blood pressure measurements, rats received sodium thiopentone (100 mg/kg, IP) and lidocaine-HCl (2%; subcutaneously locally at the site of incision). The left femoral artery was cannulated (flexible nylon tubing, Portex, Hythe, England; size 00; 0.51 mm ID; 0.64 mm OD), and the cannula was connected to a pressure transducer (Hewlett-Packard, Böblingen, Germany) for registration of the phasic and MAP. After a midline abdominal incision, the portal vein was cannulated through the ileocolic vein and this cannula was also connected to the pressure transducer. The rectal temperature was maintained at 37°C with a heating pad. On completion of the surgical procedure, rats were allowed to stabilize for 20 minutes. Six hours after LPS, L-NAME (12.5 mg/kg), SMT (12.5 mg/kg), or the same volume of vehicle (PBS) were infused via the portal-vein catheter over a 1-minute period (time 0–1). Systolic, diastolic, and portal pressure were monitored for 32 minutes. At the end of the experiment, heparinized blood samples were taken for determination of NOx. Change in MAP is expressed as the difference in MAP between time 0 (baseline MAP) and time t.

iNOS Antibodies

Specific polyclonal iNOS antibodies were raised in rabbits against a synthetic peptide (KKGNTLEEPKGTRL) comprising the C-terminal 14 amino acids 1134–1147 of the rat hepatocyte iNOS sequence.[22] An aminoterminal cysteine was added to facilitate coupling to keyhole limpet hemocyanin using *m*-maleimidobenzoyl-N-hydroxy succinimide ester chemistry.

Analytical Procedures

ALT and AST were determined by routine clinical chemistry. NOx concentrations in plasma and bile were measured according to Moshage et al.[23]

Western Blot Analysis

Livers were homogenized with a Polytron homogenizer (Kinematica GmbH, Luzern, Switzerland) in a buffer containing 20 mmol/L Tris-HCl (pH 7.2), 0.2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L ethylenediaminetetraacetic acid, and 1 mmol/L dithiothreitol. After centrifugation (30 minutes, 11,600*g*), the protein concentration in the supernatant (crude lysate) was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard. The crude lysates were fractionated on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to nitrocellulose (Amersham International, Buckinghamshire, England), using a semidry blotting system according to manufacturer's instructions (Pharmacia, Uppsala, Sweden). Prestained molecular weight standards (Bio-Rad) were used as marker proteins. The blots were incubated with a 1:5000 dilution of the polyclonal iNOS antibody in PBS containing 4% skim milk powder and 0.1% Tween-20, subsequently incubated with horseradish peroxidase labeled swine anti-rabbit immunoglobulin G (dilution 1:5000, DAKO A/S, Glostrup, Denmark) and finally developed using the enhanced chemiluminescence Western blotting system (Amersham).

NOS Activity Assay

NOS activities were measured according to Misko et al.[24] with the following modifications: each reaction included 125 μL of reaction buffer containing 20 mmol/L Tris-HCl (pH 7.2), 1 mmol/L dithiothreitol, 20 μmol/L tetrahydrobiopterin, 10 μmol/L flavin adenine dinucleotide, 10 μmol/L flavin mononucleotide, 1 mmol/L nicotinamide adenine dinucleotide phosphate (reduced), 1 mmol/L $MgCl_2$, 1 mmol/L citrulline, 25 mmol/L valine, 1 mmol/L ornithine, 10 μmol/L arginine, 0.3 μCi L-[2,3-^{3}H]arginine (44.2 Ci/mmol; NEN Dupont, Dordrecht, The Netherlands), and 25 μL of crude lysate. The reaction time was 20 minutes at 37°C. iNOS antiserum and preimmune serum were added to the reaction mixtures as indicated.

Hepatocyte Isolation and Culture

Rat hepatocytes were isolated by two-step collagenase perfusion as described previously.[25] Hepatocytes were plated at a density of 150,000 cells per cm^2 in William's medium E, supplemented with 5% fetal calf serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 20 mU/mL insulin. After 4 hours, medium was changed and

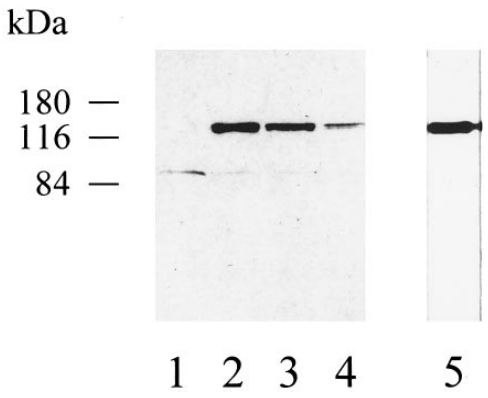

Figure 1. iNOS protein level in the liver 0 hour (lane 1), 6 hours (lane 2), 12 hours (lane 3), and 24 hours (lane 4) after LPS administration (5 mg/kg, IP) and in cultured hepatocytes incubated with LPS-conditioned medium from human monocytes (lane 5). Crude cell lysates (40 μg) were separated on a 10% polyacrylamide gel, transferred to nitrocellulose, and immunostained with antiserum against iNOS (dilution, 1:5000). Molecular weight markers are shown on the left side of the Western blot.

unattached cells were removed. Cells were used for iNOS induction 24 hours after attachment. iNOS induction in cultured rat hepatocytes was achieved by incubating the hepatocytes for 18 hours in the presence of 10% LPS-conditioned medium of human monocytes as described previously.[26] At the end of the experiments, media were collected and the cell layer was harvested in distilled water containing 0.2 mmol/L phenylmethylsulfonyl fluoride and subsequently stored at −20°C.

Histology and Immunohistology

Part of the liver tissue obtained from LPS- and PBS-treated rats was fixed by immersion in 4% buffered formalin and embedded in paraffin. Sections were stained with H&E for routine histology. For the immunohistological study, 4-μm sections were cut from frozen liver tissue. After drying overnight at room temperature, sections were fixed in acetone for 10 minutes at room temperature. The sections were incubated with 10% goat serum (50 μL per slide) for 30 minutes at room temperature. The polyclonal iNOS antibody (dilution, 1:200) was then applied in PBS containing 1% bovine serum albumin for 60 minutes at room temperature (50 μL per slide). The slides were subsequently incubated with goat anti-rabbit peroxidase (dilution, 1:100) in PBS containing 1% bovine serum albumin and 2% normal rat serum. The sections were finally incubated with filtered 3-amino-9-ethylcarbazole solution (0.2 mg/mL sodium acetate buffer containing 0.03% H_2O_2) for 15 minutes at room temperature. Nuclear counterstaining was performed with hematoxylin and the slides were covered with Kaiser's glycerin-gelatin. After each incubation, the sections were rinsed with PBS. All tissue sections were studied and scored without knowledge of the experimental manipulations. iNOS protein level was assessed for each cell type in the liver tissue (hepatocytes, bile duct epithelium, inflammatory cells, and endothelial cells) and scored semiquantitatively (0 to 3+) depending on the number of cells with positive staining, not on the intensity of staining.

Confocal Scanning Laser Microscopy

Frozen rat liver sections were stained with the following antibodies: mouse monoclonal antibody C219 against Pgp (Centocor, Antwerp, Belgium; 1:100), rabbit polyclonal antibody against iNOS (dilution, 1:5000), fluorescein isothiocyanate–labeled goat anti-mouse immunoglobulin G, and tetramethylrhodamine isothiocyanate–labeled goat anti-rabbit immunoglobulin G (both Sigma; dilution, 1:200). Images were taken with a confocal scanning laser microscope (CSLM; True Confocal Scanner Leica, Heidelberg, Germany) equipped with an argon/krypton laser and coupled to a Leitz DM IRB inverted microscope (Leica). Double-labeled images were taken sequentially at 488 and 562 nm to avoid bleed-through into the other channel.

Statistical Analysis

The data resulting from each experimental group were expressed as the mean ± SEM. The significance in differences between the presented data groups was determined using the one-way analysis of variance test with Bonferroni correction. For hemodynamic measurements, an unpaired Student's t test was used to compare means between the two groups at the same time point. A P value < 0.01 was considered significant.

Results

iNOS Protein in Rat Liver After LPS Induction

Antibodies raised against the C-terminal 14 amino acids of rat hepatocyte iNOS[22] recognized a single protein of approximately 130–135 kilodaltons in crude lysate of LPS-induced rat liver (Figure 1). Preimmune serum did not react with either untreated or LPS-induced rat liver (not shown). In untreated rats, no immunoreactivity was seen (Figure 1, lane 1). Six hours after LPS administration (5 mg/kg, IP), iNOS protein was significantly induced (Figure 1, lane 2). After 12 hours, iNOS protein had declined, and it was barely detectable 24 hours after LPS treatment (lanes 3 and 4, respectively).

NOS activity (Table 1) was measured by the conver-

Table 1. Inhibition of NOS Enzyme Activity by iNOS Antibody

Crude lysate	Addition	NOS activity
Control		254
LPS, 6 h		1881
LPS, 6 h	2 μL preimmune serum	1947
LPS, 6 h	10 μL preimmune serum	1943
LPS, 6 h	2 μL anti-iNOS	1271
LPS, 6 h	10 μL anti-iNOS	1089
LPS, 12 h		721
LPS, 24 h		454

NOTE. NOS enzyme activities were determined as described in the Materials and Methods section. NOS activity was expressed as disintegrations per second in total reaction mixture.

sion of [^{3}H]arginine into [^{3}H]citrulline. It was assumed that the NOS activity in control liver is due to constitutive eNOS and that the increase in enzyme activity after LPS administration is predominantly due to iNOS. After LPS administration, the NOS enzyme activity (Table 1) correlated with iNOS protein level (Figure 1). The antibody against iNOS was able to inhibit about 50% of the total NOS enzyme activity. Preimmune serum did not inhibit NOS activity (Table 1).

Rat hepatocytes incubated with LPS-conditioned medium from monocytes contained significant amounts of iNOS, shown by Western blotting (Figure 1, lane 5). This was accompanied by a sevenfold increased NOx level in culture medium.

Histology

Liver tissue of control animals showed a normal architecture. There were no inflammatory cells in the portal tracts nor in the parenchyma and there were no signs of cellular damage. Six hours after LPS administration, there was a slight increase in inflammatory activity (Figure 2*A*). Inflammatory cells consisted mainly of neutrophilic granulocytes, lymphocytes, and sinusoidal macrophages. These cells were evenly distributed in the sinusoidal spaces, whereas portal inflammation was inconspicuous. Single cell acidophilic necrotic hepatocytes (Councilman's bodies) were present sporadically in the parenchyma (Figure 2*A*).

Twenty-four hours after LPS administration, circumscribed necrotic areas were seen with inflammatory cells in addition to intralobular infiltrates (Figure 2*B*).

Immunohistology

There was no iNOS staining in liver sections of control animals. In the liver of all three experimental groups (LPS, LPS + L-NAME, and LPS + SMT), positive iNOS staining was seen 6 hours after LPS treatment in inflammatory cells, hepatocytes, and bile duct epithelium (Figure 2*C* and *D*). The iNOS staining of hepatocytes showed a scattered pattern with positive hepatocytes randomly distributed throughout the parenchyma (Figure 2*C*). These positive hepatocytes showed a weak cytoplasmic staining and a strong membranous staining. The chicken-wire pattern of iNOS immunoreactivity (Figure 2*C*) suggests association with canalicular membranes. This was confirmed by confocal scanning laser microscopy: iNOS staining of membranes predominantly colocalized with the canalicular membrane marker Pgp (Figure 3). Semiquantitative scoring of iNOS staining in the various cell types did not result in significant differences between the three experimental groups (n = 6 for each experimental group).

Twenty-four hours after LPS treatment, iNOS staining on hepatocytes had completely disappeared. Only inflammatory cells in necrotic areas stained positive at this time point (Figure 2*E*).

Nitrite/Nitrate (NOx) Concentrations in Plasma and Bile

NOx concentrations in plasma and in bile were measured at various times after LPS treatment (Figure 4). NOx concentrations in plasma and bile were nearly identical at 6 and 12 hours after LPS induction. In contrast, 24 hours after LPS treatment the NOx concentration in bile had returned to control levels, whereas the plasma NOx level still was significantly increased. NOS inhibitors reduced NOx concentrations in plasma and bile to the same extent (Figure 5).

Inhibition of Nitrite/Nitrate Production by SMT and L-NAME

Figure 6 shows the effects of the inhibitors SMT and L-NAME on NOx, AST, and ALT levels in plasma at 6 hours after LPS administration. LPS treatment (5 mg/kg) resulted in an increase of plasma NOx concentration from 6 ± 0.7 to 351 ± 46 μmol/L after 6 hours. Administration of the inhibitor SMT (25 mg/kg), given together with LPS, led to a more than two-thirds decrease in plasma NOx. Administration of L-NAME (25 mg/kg) reduced plasma NOx levels by 50%. It is obvious that neither L-NAME nor SMT led to a complete inhibition.

AST and ALT were measured as indicators of liver damage. Rats treated with LPS alone showed an approximately twofold increase in AST and ALT. This was statistically not significant. After administration of L-NAME, liver damage was strongly increased. In contrast, SMT did not increase the LPS-induced liver damage (Figure 6).

Hemodynamic Effects of L-NAME and SMT

We measured the effects of L-NAME and SMT on hemodynamic parameters in normal rats and in rats 6 hours after LPS treatment (n = 6–7 for each of the four experimental groups). In control animals, no iNOS was detected with Western blot and plasma NOx level was normal (NOx = 25 ± 1.6 μmol/L). In LPS-treated animals, iNOS protein was induced as detected by Western blot, and plasma NOx levels were increased (NOx = 262 ± 32.5 μmol/L). However, baseline MAP of untreated rats and rats 6 hours after LPS was not significantly different: 103.5 ± 2.2 and 101.2 ± 1.4 mm Hg, respectively (n = 15 for the two groups).

In control animals and LPS-treated animals, MAP and portal pressure were stable during the entire monitoring period after injection of vehicle (PBS, n = 3 for each

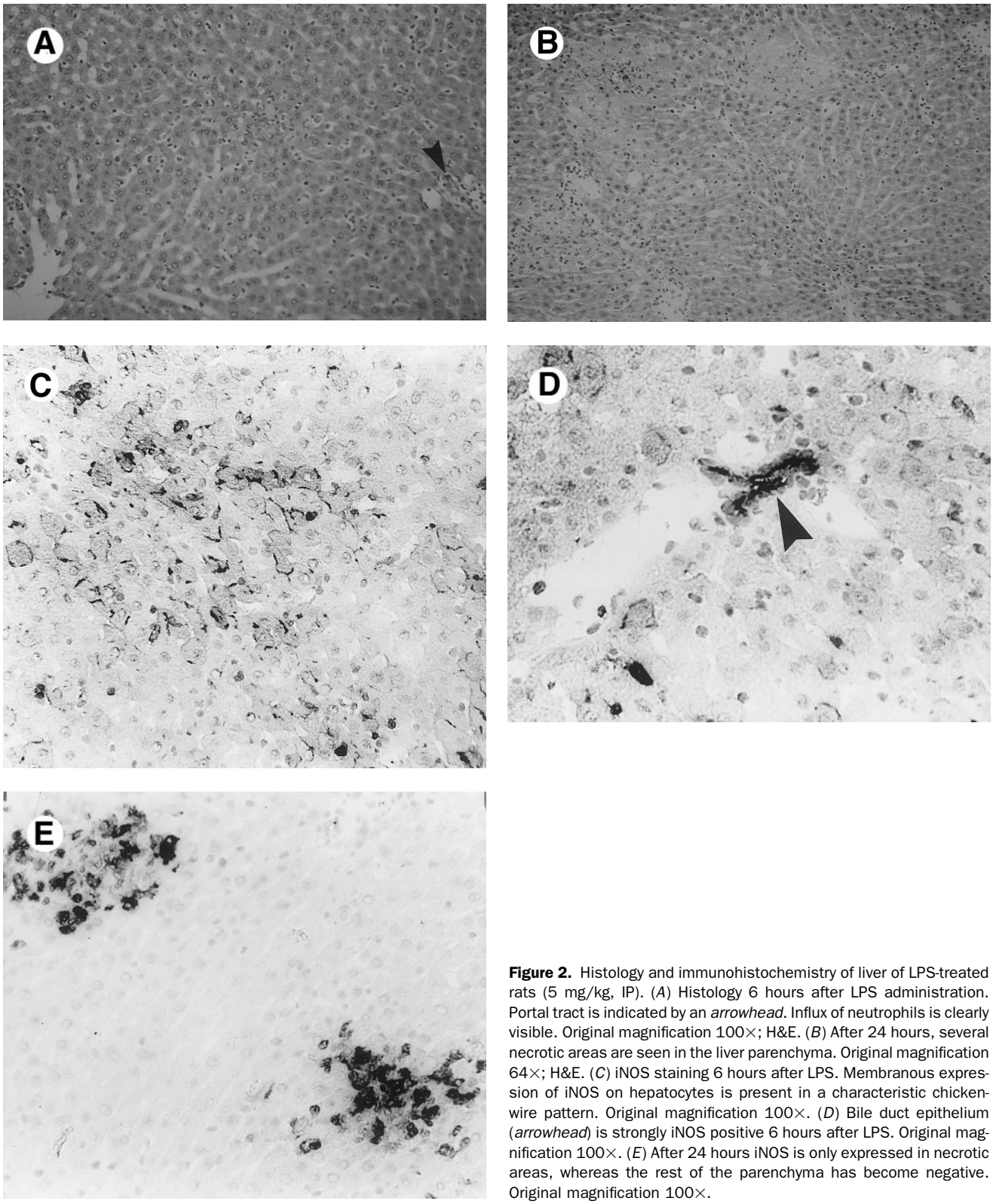

Figure 2. Histology and immunohistochemistry of liver of LPS-treated rats (5 mg/kg, IP). (*A*) Histology 6 hours after LPS administration. Portal tract is indicated by an *arrowhead*. Influx of neutrophils is clearly visible. Original magnification 100×; H&E. (*B*) After 24 hours, several necrotic areas are seen in the liver parenchyma. Original magnification 64×; H&E. (*C*) iNOS staining 6 hours after LPS. Membranous expression of iNOS on hepatocytes is present in a characteristic chicken-wire pattern. Original magnification 100×. (*D*) Bile duct epithelium (*arrowhead*) is strongly iNOS positive 6 hours after LPS. Original magnification 100×. (*E*) After 24 hours iNOS is only expressed in necrotic areas, whereas the rest of the parenchyma has become negative. Original magnification 100×.

group, data not shown). L-NAME (12.5 mg/kg, IV) increased MAP in normal rats significantly more than in LPS-treated rats (Figure 7). Initially, SMT (12.5 mg/kg, IV) caused a significantly higher increase of MAP in normal rats than in LPS-treated rats. The SMT effect was only transient and lasted about 17 minutes (Figure 8).

We also monitored the portal pressure in these experimental groups. Baseline values of normal rats and rats 6

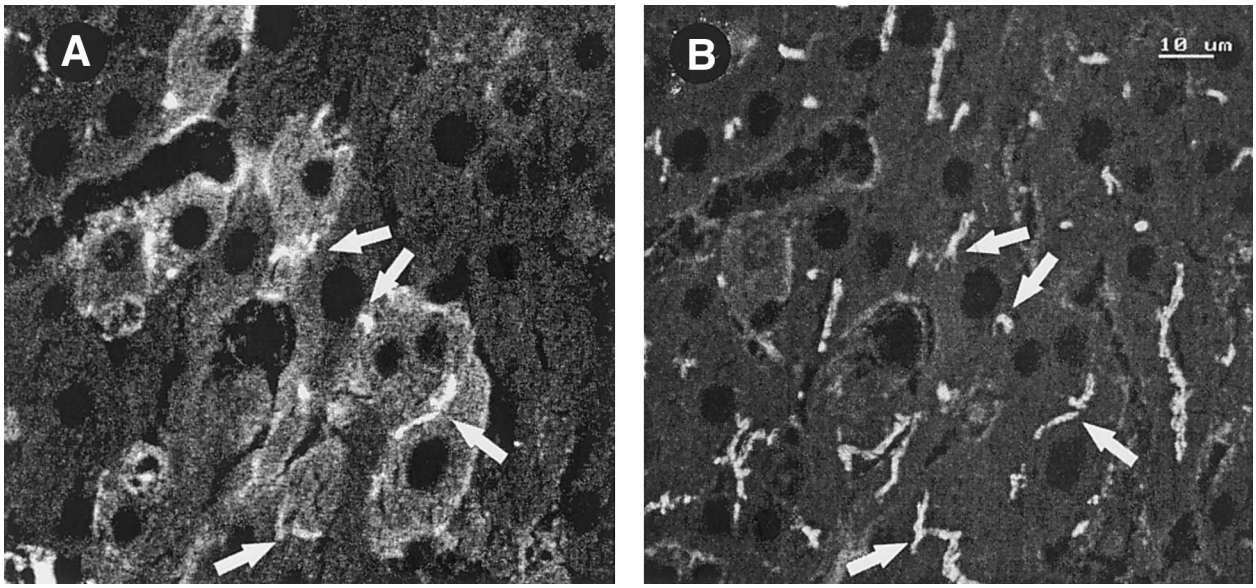

Figure 3. Canalicular localization of iNOS in rat liver 6 hours after LPS (5 mg/kg, IP). Double-labeling with antibodies against (*A*) iNOS and (*B*) Pgp (C219 monoclonal antibody), assessed with confocal scanning laser microscopy. As indicated by *arrows*, membrane staining of iNOS-positive hepatocytes colocalizes with Pgp, a canalicular membrane marker.

hours after LPS were 6.7 ± 0.2 and 6.8 ± 0.3 mm Hg, respectively (n = 15 for each group). L-NAME and SMT had no effect on portal pressure in normal rats (Figure 9). In LPS-treated rats, L-NAME had no effect on portal pressure, whereas SMT caused a significant increase (Figure 9).

Discussion

In the present study, we investigated the cellular distribution of iNOS and studied the effect of the NOS inhibitors L-NAME and SMT on LPS-induced liver damage.

A polyclonal antibody was raised against the C-terminal 14 amino acids of rat hepatocyte iNOS. This antibody recognizes a single protein in LPS-induced liver homogenates and cytokine-stimulated cultured hepatocytes and shows no immunoreactivity with control liver homogenates. The apparent molecular weight of this protein is

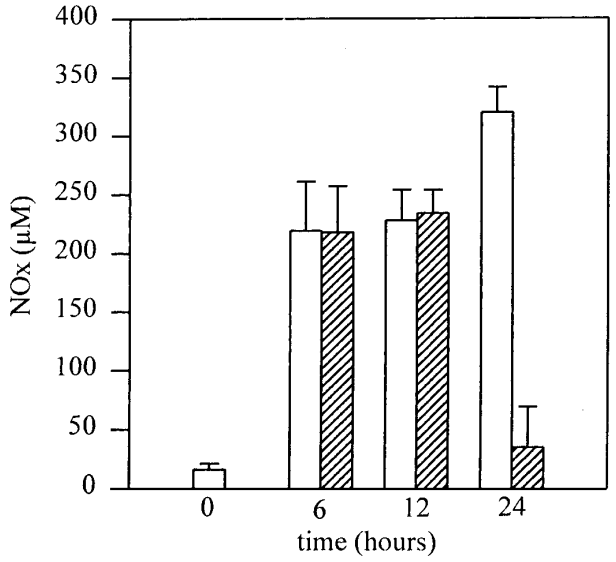

Figure 4. NOx levels in plasma (□) and bile (▨) 0, 6, 12, and 24 hours after LPS administration (1 mg/kg, IP). The data represent the mean ± SEM of duplicate samples of a typical experiment.

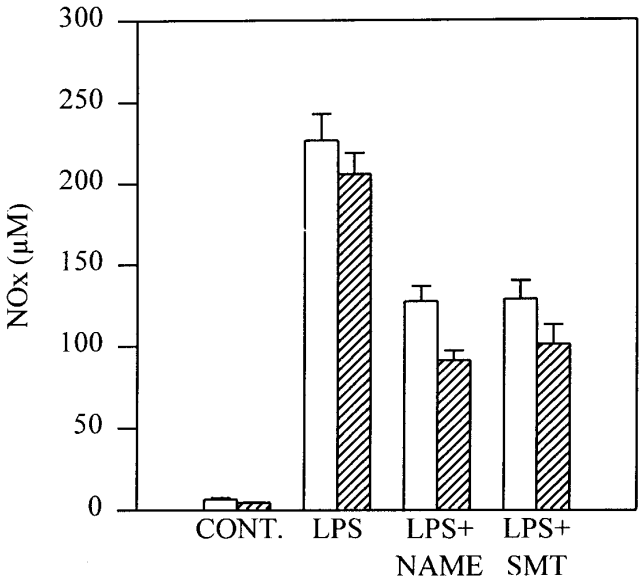

Figure 5. NOx levels in plasma (□) and bile (▨) 6 hours after administration of LPS (1 mg/kg, IP). The inhibitors L-NAME and SMT were given together with LPS at a dose of 25 mg/kg. The data represent the mean ± SEM of n = 2–4 per group of a typical experiment.

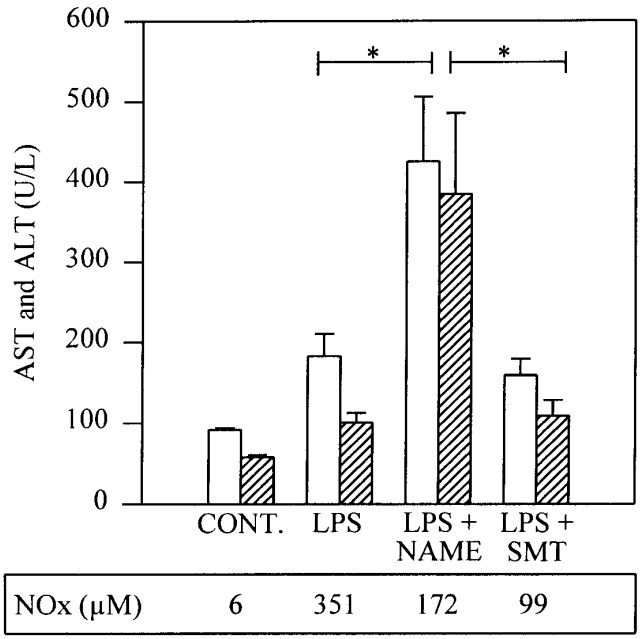

Figure 6. Effect of L-NAME and SMT on LPS-induced hepatic damage assessed by AST (□) and ALT (▨) levels in plasma. The control group received PBS (1 mL/kg, IP). The inhibitors SMT and L-NAME were injected (25 mg/kg, IP) together with LPS (5 mg/kg, IP). Six hours later, the rats were killed. Data are expressed as the mean ± SEM of n = 6–8 rats per group. $*P < 0.01$.

130–135 kilodaltons, corresponding to the molecular weight of rat iNOS. Furthermore, the antibody inhibits NOS enzyme activity in liver homogenates. The antibody was used to study the cellular distribution of iNOS in rat liver. iNOS is found in inflammatory cells, Kupffer cells, bile duct epithelium, and arterial endothelial cells. This is in agreement with other reports.[27–30] We also found a very clear staining of iNOS in a substantial proportion of the hepatocytes. Although the iNOS localization is usually assumed to be cytoplasmic, in our study, the iNOS-positive hepatocytes stain more intensely at the plasma membrane than in the cytoplasm. Double-labeling of iNOS and Pgp using confocal scanning laser microscopy showed iNOS immunoreactivity to be localized predominantly at the canalicular membrane. In macrophages, iNOS is associated with a 105,000*g* particulate fraction.[31,32] This also suggests that iNOS is associated with a membrane-containing fraction. The iNOS-positive hepatocytes seem to be randomly distributed throughout the liver. There is neither a colocalization of iNOS-positive Kupffer cells and iNOS-positive hepatocytes nor a clear zonal distribution.

Based on the staining of a few scattered hepatocytes in endotoxin-treated rats, some investigators have suggested that LPS is a weak inducer of hepatocyte iNOS and that most iNOS in LPS-treated liver is derived from inflammatory cells and Kupffer cells.[27,28,33] We now show that a considerable number of hepatocytes are strongly iNOS positive. Moreover, in homogenates of cytokine-exposed hepatocytes, a high level of iNOS is detectable by Western blot. The reason for this difference in interpretation may be explained by the use of different antibodies and fixation procedures. Our antibody is directed against the C-terminal amino acids 1134–1147 of rat iNOS, whereas most antibodies used so far are directed against the N-terminal amino acids 47–71 of mouse iNOS[27,28] or monoclonal antibodies against an unknown epitope of rat iNOS.[29] In addition, some investigators used formalin or paraformaldehyde-fixed material for immunostaining.[27–29] In our hands, these fixation procedures markedly reduced iNOS positivity compared with acetone fixation. Our results show that hepatocytes are an important source of iNOS and, consequently, of NO radical production during endotoxemia.

The induction of iNOS in rat liver after LPS administration is maximal at 6 hours and has almost returned to undetectable levels after 24 hours. In accordance, NOS enzyme activity in rat liver homogenates correlates with the iNOS protein levels as detected by Western blot. Plasma NOx levels are highly elevated at all time points studied.

In addition to NOx in plasma, we also determined NOx in bile. The biliary NOx levels were similar to those in plasma 6 and 12 hours after LPS treatment. Biliary NOx levels have almost normalized 24 hours after

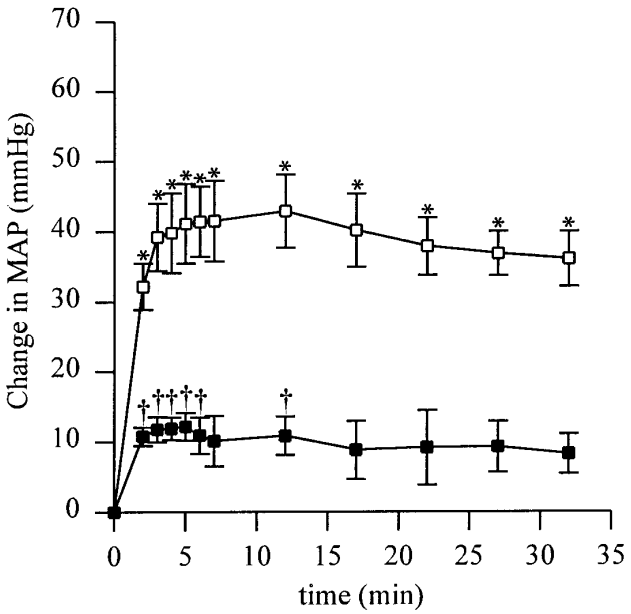

Figure 7. Changes in MAP in normal rats (□) and rats 6 hours after LPS administration (5 mg/kg, IP; ■) in response to L-NAME (12.5 mg/kg, IV) on time 0. Data are expressed as the mean ± SEM of n = 6–7 rats per experimental group. $*P < 0.01$, LPS + L-NAME vs. L-NAME and $^{\dagger}P < 0.01$, LPS + L-NAME vs. LPS.

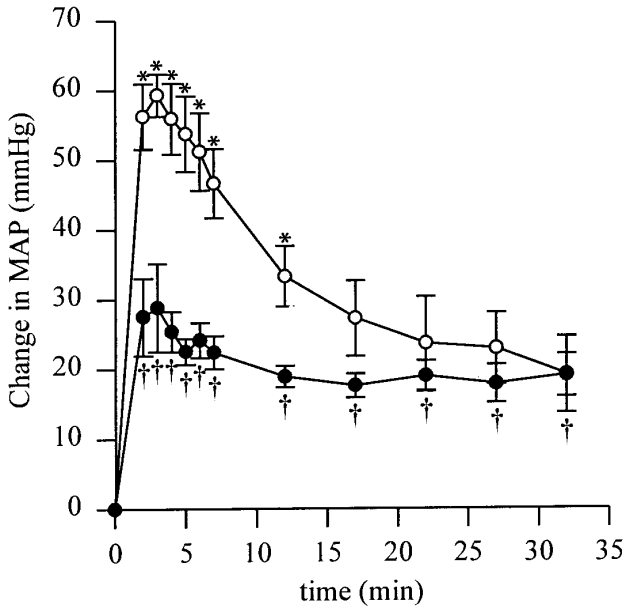

Figure 8. Changes in MAP in normal rats (○) and rats 6 hours after LPS administration (5 mg/kg, IP; ●) in response to SMT (12.5 mg/kg, IV) on time 0. Data are expressed as the mean ± SEM of n = 6–7 rats per experimental group. *$P < 0.01$, LPS + SMT vs. SMT and †$P < 0.01$, LPS + SMT vs. LPS.

treatment, at which time point plasma NOx levels still are highly elevated. Two mechanisms could explain these findings: NOx in bile measured 6 and 12 hours after LPS administration could result from an increased permeability of tight junctions during the first 12 hours after LPS. This is supported by equal NOx concentrations in plasma and bile during the first 12 hours after LPS administration. Increased tight junctional permeability for horseradish peroxidase has been observed 18 hours after LPS administration in an isolated perfused rat liver model.[34] Moreover, NO itself can increase tight junctional permeability, as has been shown in cultured intestinal epithelial cells treated with the NO donor sodium nitroprusside.[35] Apparently, 24 hours after LPS administration, the tight junctions are impermeable to NOx again and NOx cannot enter bile. Alternatively, NOx in bile may originate exclusively from iNOS in hepatocytes and/or bile duct epithelial cells. In this case, biliary NOx correlates with hepatic iNOS expression.

Many studies have suggested that during endotoxemia NO in the liver has a protective function, because inhibition of NO production leads to increased hepatic damage. In contrast, Laskin et al.[36] showed an improvement in liver histology when NO production was inhibited during endotoxemia. Because these studies were performed with L-arginine analogues that show some selectivity towards eNOS, in vitro and in vivo,[13–15] we investigated the effects of SMT, a preferential iNOS inhibitor,[15–21] and L-NAME on endotoxin-induced liver damage.

In LPS-treated rats, plasma ALT and AST are approximately twofold elevated, indicating minor liver damage. Recently, Thiemermann et al.[19,37] reported similar results, although in their study the liver damage induced by LPS alone was much higher and SMT actually decreased liver damage. We used various dosages and schemes of LPS administration but were not able to induce substantial liver damage with LPS alone. This is in accordance with recent studies using iNOS knockout mice.[38–40] In these studies, LPS administration to both iNOS-positive and -negative mice led to minor liver damage.

Liver damage is dramatically increased when the NO production is inhibited with L-NAME. In contrast, when SMT is used, liver damage does not increase, although plasma NOx is reduced to the same extent. It has been reported that liver endothelial cells produce increased amounts of NO in response to LPS.[41] However, endothelial cells produce less NO than Kupffer cells and hepatocytes on a per cell basis.[41,42] Furthermore, the percentage of endothelial cells in the liver is about 12.5%, whereas hepatocytes and Kupffer cells together account for 78%.[43] This leads to the conclusion that the contribution of endothelial cells to the increased NO production after LPS administration is low. Therefore, increased plasma NOx mainly originates from iNOS and the NOx reduction after administration of the NOS inhibitors L-NAME and SMT mainly results from iNOS inhibition. Thus, in

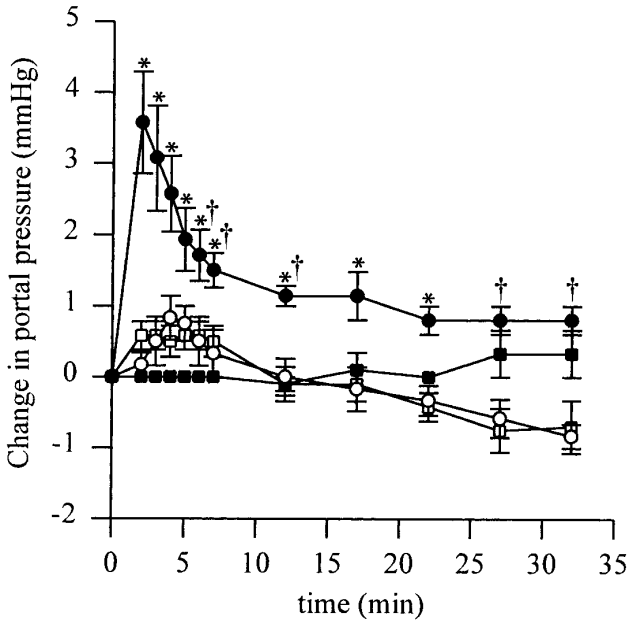

Figure 9. Changes in portal pressure in normal rats (○, □) and rats 6 hours after LPS administration (5 mg/kg, IP; ●, ■) in response to L-NAME (12.5 mg/kg, IV; *squares*) or SMT (12.5 mg/kg, IV; *circles*) on time 0. *$P < 0.01$ LPS + SMT vs. LPS + L-NAME and †$P < 0.05$ LPS + SMT vs. SMT.

this in vivo model L-NAME and SMT inhibit iNOS to the same extent, but only L-NAME causes increased liver damage.

It has been suggested that inhibition with L-NAME causes vasoconstriction and thrombosis due to inhibition of eNOS.[10] Our results show a stronger effect of L-NAME on MAP in normal rats than in LPS-treated rats, suggesting that L-NAME is a stronger eNOS than iNOS inhibitor. This is in agreement with Wu et al.[44] SMT administration causes a significant but transient rise of MAP in normal compared with LPS-treated rats.

Like MAP, portal pressure in normal and LPS-treated animals is not significantly different, in agreement with the findings of Pastor et al.[45] We found no effect of L-NAME on portal pressure in control rats. This lack of effect of L-NAME on portal pressure is in agreement with recent observations in intact pigs[46] and rats[47] and in isolated perfused rat liver.[48] In endotoxemic rats, L-NAME also did not affect portal pressure. This is in agreement with Gundersen et al., who used a porcine model of endotoxemia.[49] In contrast, Ayuse et al. observed an increase in portal pressure after administration of L-NAME in endotoxemic pigs; the difference between this study and the present study is a different time interval between LPS and L-NAME administration.[46] In contrast to L-NAME, SMT had no effect on portal pressure of control rats but significantly increased portal pressure in LPS-treated rats.

In conclusion, our study shows that L-NAME is hepatotoxic in LPS-treated rats, although this agent does not seem to affect portal pressure. On the other hand, SMT causes no liver damage, but it increases portal pressure in LPS-treated rats. It is important to note that we measured portal pressure and not portal flow or hepatic perfusion. Therefore we cannot rule out a possible effect of L-NAME on hepatic perfusion. For example, this agent could increase intrahepatic vascular resistance and at the same time decrease splanchnic and portal blood flow without any change in portal pressure. Effects of L-NAME on portal flow or sinusoidal resistance have been reported by several groups in control[47,50] and in LPS-treated animals.[10,48,50] On the other hand, Suematsu et al. observed no effect of L-NAME on hepatic vascular resistance and on sinusoidal hemodynamics in normal isolated perfused rat liver.[51] Similarly, the increased portal pressure, observed after SMT administration to LPS-treated rats, could result from either an increase in intrahepatic vascular resistance with unchanged portal flow or from increased portal flow with unchanged resistance. If hemodynamic changes form the basis for the differences in hepatotoxicity observed after L-NAME and SMT, one would have to postulate a decreased hepatic perfusion after L-NAME, which does not occur after SMT.

Apart from possible hemodynamic consequences of NOS inhibition, strong inhibition of the NO production during endotoxemia may lead to an increased generation of reactive oxygen species within endothelial cells and mast cells.[52,53] Moreover, Bautista and Spitzer[54] reported on stimulation of superoxide anion release in the liver after L-NAME in normal and LPS-treated rats. When the more specific iNOS inhibitor L-NMMA was used, this effect was absent in normal rats and less pronounced in LPS-treated rats.

Our study suggests that NO radical production can be inhibited in the endotoxemic rat without increasing liver damage. The damaging effect of L-NAME is not due to an increased portal pressure.

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