Oxidant Stress in Rat Liver after Lipopolysaccharide Administration: Effect of Inducible Nitric-Oxide Synthase Inhibition

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ABSTRACT
The role of inducible nitric-oxide synthase (iNOS) in lipopolysaccharide (LPS)-induced hepatic oxidant stress was evaluated using the iNOS inhibitor L-iminoethyl-lysine (L-NIL). Male rats were divided into three groups. One group received LPS (Salmonella minnesota) 2 mg/kg i.v. A second group received LPS plus L-NIL (3 mg/kg i.p.) at the time of LPS administration followed by a second dose 3 h later. A third group received saline i.v. At 6 h, blood and liver tissue were collected. Serum nitrate/nitrite levels were increased from 8.49 ± 0.64 nmol/mg (n = 4) in the saline group to 360 ± 48 nmol/mg in the LPS group (n = 5). Values for the LPS + L-NIL group were significantly reduced to 35 ± 7 nmol/mg. Tissue malondialdehyde levels were increased from 0.20 ± 0.02 nmol/mg (n = 4) in the saline group to 0.41 ± 0.03 nmol/mg (n = 4) in the LPS group. L-NIL significantly reduced the values in the LPS group to 0.29 ± 0.02 nmol/mg (n = 4). 4-Hydroxynonenal-protein adducts levels were increased 3.6-fold by LPS treatment as compared with saline. L-NIL significantly reversed the levels to 1.6-fold (n = 4). Intracellular GSH levels were decreased from 8.49 ± 0.64 nmol/mg (n = 4) in the saline group to 5.63 ± 0.51 nmol/mg in the LPS group (n = 7). L-NIL significantly increased the levels in the LPS group to 7.04 ± 0.46 nmol/mg (n = 7). These data indicate that LPS-induced nitric oxide generation can result in oxidant stress in the liver, and that inhibitors of iNOS may offer some protection in LPS-induced hepatic toxicity.

Lipopolysaccharide (LPS) is a component of the Gram-negative bacterial cell wall that triggers the synthesis and release of cytokines and nitric oxide (NO) (Mayeux, 1997). Septicemia and septic shock, resulting from severe bacterial infections, are associated with high mortality; current therapy is mostly supportive and largely ineffective (Wenzel et al., 1996). It is clear that systemic production of NO by inducible NO synthase (iNOS) in the vasculature is the major cause of hypotension and poor organ perfusion associated with these conditions (Titheradge, 1999). However, LPS can also cause iNOS expression in Kupffer cells and hepatocytes of the liver (Duval et al., 1996; Rockey and Chung, 1996; Roland et al., 1996). Consequently, there is the potential for large amounts of NO to be generated in the liver during sepsis; this could impair hepatic function by directly injuring cells (Darley-Usmar et al., 1995; Szabo et al., 1996; Li and Billiar, 1999).

LPS also triggers the synthesis of reactive oxygen species, such as superoxide, in the lung (Demling et al., 1986; Milligan et al., 1988), liver (Bautista and Spitzer, 1990), and kidney (Zurovsky and Gispaan, 1995; Faas et al., 1998). NO and superoxide react spontaneously to form the potent and versatile oxidant peroxynitrite (ONOO−). This highly toxic species reacts with lipids, proteins, DNA, and GSH (Stamler et al., 1992; Pryor and Squadrito, 1995). In rat kidney, LPS triggers the generation of ONOO− and the development of oxidant stress (Zhang et al., 2000). Furthermore, selective inhibition of NO synthesis from iNOS lessens the degree of oxidant stress, suggesting that iNOS-derived NO is a major player in the development of oxidant stress in the kidney after LPS administration. NO-mediated oxidant stress in the liver has not been examined in detail. The purpose of our study was to determine whether oxidant stress occurs in the liver as a result of LPS administration, and to determine the role of iNOS-derived NO in the development of oxidant stress.

Experimental Procedures

Materials and Reagents. LPS (Salmonella minnesota), phenylmethylsulfonyl fluoride, 5′-dithiobis-(2-nitrobenzoic acid), GSH, 2-thiobarbituric acid, 1,1,3,3-tetraethoxypropane, leupeptin, and an...
alanine aminotransferase (ALT) assay kit were purchased from Sigma Chemical Co. (St. Louis, MO). L-NMMA-(1-iminoethyl)-lysine hydrochloride was purchased from Alexis Corporation (San Diego, CA). Anti-iNOS rabbit polyclonal antibody, anti-4-hydroxynonenal rabbit polyclonal antibody, and anti-nitrotyrosine mouse monoclonal antibody were purchased from Cayman Chemical Co. (Ann Arbor, MI). Anti-nitrotyrosine rabbit polyclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-rabbit peroxidase-conjugated antibody and the ECL detection kit were purchased from Amersham International (Buckinghamshire, England). The NO assay kit was purchased from Oxford Biochemical Research, Inc. (Oxford, MI). Vectastain Elite peroxidase ABC kit was purchased from Vector Laboratories, Inc. (Burlingame, CA).

**LPS-Induced Injury.** All animal experiments were approved by the Animal Care and Use Committee of the University of Arkansas for Medical Sciences. Animals were housed and sacrificed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 86-23, revised 1985). Male Sprague-Dawley rats were divided into three groups. One group received 2 mg/kg LPS (S. minnesota) i.v. under ether anesthesia. A second group received the same dose of LPS plus L-arginine (100 mg/kg) (L-NIL) i.p. (two doses; one at the same time as LPS, the other 3 h later). A third group received saline as control. At 6 h, animals were anesthetized with pentobarbital sodium (50 mg/kg). Blood was collected in heparinized syringes. Liver samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Animals were sacrificed by exsanguination.

**Determination of ALT Activity in Plasma.** Plasma ALT activity was measured by using the ALT assay kit as described by the manufacturer. In brief, 5-μl plasma samples were incubated with DL-alanine and α-ketoglutaric acid in phosphate buffer for 30 min at 37°C before 2,4-dinitrophenylhydrazine was added. The absorbance was read at 490 nm. A standard curve was generated by using sodium pyruvate.

**Measurement of NO°/NO−3 in Plasma.** Plasma samples (50-μl) were deproteinized by incubation with 140 μl of deionized H2O and 10 μl of 30% ZnSO4 at room temperature for 15 min. Samples were then centrifuged at 2000g for 10 min. Nitrite was converted to nitrate using cadmium beads, and nitrate was measured spectrophotometrically using a NO assay kit as described by the manufacturer.

**iNOS Western Blot Analysis.** A rabbit polyclonal anti-iNOS antibody (Cayman Chemical) was used to detect iNOS protein. Tissue homogenates (25 μg of protein/lane) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in polyacrylamide gels, and then transferred to nitrocellulose using an electroblotting transfer apparatus. Nitrocellulose membranes were incubated in blocking buffer (10 mM Tris, 100 mM NaCl, 0.1% Tween 20, and 5% nonfat milk) overnight at 4°C. The membranes were incubated for 90 min at room temperature with rabbit polyclonal anti-iNOS antibody diluted 1:1000 in blocking buffer. The membranes were washed three times for 10 min in washing buffer (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20), then incubated with secondary antibody diluted in blocking buffer (anti-rabbit peroxidase-conjugated antibody) for 60 min at room temperature. The membranes were then washed and developed using an ECL detection kit as described by the manufacturer.

**4-Hydroxynonenal-Protein Adducts Western Blot Analysis.** A rabbit polyclonal anti-4-hydroxynonenal antibody was used to detect protein adducts (1:500 dilution). Tissue homogenates (50 μg of protein/lane) were subjected to SDS-PAGE (4-20% gradient gel) separation and transferred to nitrocellulose as described above. After Western blot analysis, each lane was analyzed using densitometry and NIH Image software.

**Determination of Lipid Peroxidation Products.** Each liver sample was homogenized in buffer containing 0.25 M sucrose, 1 mM EDTA, and 10 mM HEPES, then centrifuged for 5 min at 2000g. The protein concentration of the resulting supernatant was adjusted to 2 mg/ml, then deproteinated using 10% trichloroacetic acid and centrifuged for 5 min at 12,000g. Lipid peroxidation products were determined by measuring the levels of thiobarbituric acid-reactive substances (TBARS) as described in our previous publication (Traylor and Mayeux, 1997; Zhang et al., 2000). A standard curve was prepared from 1,1,3,3-tetraethoxypropane. Standards and samples gave the expected peak absorbance at 532 nm for TBARS adducts. Data were expressed as total TBARS per milligram of protein.

**Determination of Intracellular GSH Equivalents.** Liver tissue was homogenized and deproteinized by using homogenization buffer (125 mM NaH2PO4, 6.3 mM EDTA, 5% sulfoalicylic acid) in 1:5 (wt/v). Total GSH equivalents (GSH + GSSG) were determined using our published methods (Zhang et al., 2000). A standard curve was prepared by using GSH.

**Detection of 3-Nitrotyrosine-Protein Adducts by Immunohistochemistry.** Immunohistochemistry for 3-nitrotyrosine-protein adducts was performed on paraffin-embedded tissue sections (3-μm) as described previously (Zhang et al., 2000). Rabbit anti-nitrotyrosine antibody (1:100 dilution) was incubated with the sections for 1 h at room temperature. Primary antibody was detected using the Vectastain Elite peroxidase ABC kit with 3,3′-diaminobenzidine as the substrate. As a negative control, the antigenic binding site of the anti-nitrotyrosine antibody was blocked with 10 mM 3-nitrotyrosine for 1 h at room temperature.

**Data Analysis.** Data are reported as mean ± S.E. Each n represents one rat. All data were analyzed by one-way ANOVA followed by the Student-Newman-Keuls test. P < .05 was considered statistically significant.

**Results**

**Plasma ALT Activity.** We chose an endotoxemia model that would allow us to examine NO-mediated liver toxicity with only a modest rise in plasma ALT activity. After 6-h treatment of LPS, ALT was increased in the plasma from 5 ± 0.81 I.U/l in the saline treatment group to 19 ± 4.3 I.U/l in the LPS treatment group (P < .05). The value of LPS + L-NIL group was 17 ± 2.9 I.U/l and was not different from the LPS group.

**Hepatic Histology.** Tissue sections were stained with hematoxylin and eosin for evaluation in a blinded fashion by two pathologists.

**Data Analysis.** Data are reported as mean ± S.E. Each n represents one rat. All data were analyzed by one-way ANOVA followed by the Student-Newman-Keuls test. P < .05 was considered statistically significant.

**Western Blot Analysis of iNOS.** The presence of iNOS expression in the liver was examined using Western blot analysis (Fig. 2). The saline group did not show any detectable iNOS protein expression. LPS significantly induced
iNOS protein expression 6 h after LPS treatment. l-NIL did not change the induction of iNOS by LPS in the liver.

**Oxidant Stress.** We measured three markers of oxidant stress 6 h after administration of LPS. The first was total GSH equivalents (Fig. 3). LPS decreased GSH equivalent levels in the liver from control levels of 8.50 ± 0.64 nmol/mg (wt weight) to 5.63 ± 0.51 nmol/mg (P < .05). L-NIL reversed the effects of LPS. The l-NIL group was 7.04 ± 0.46 nmol/mg, a value not different from the saline group but significantly different from the LPS group (P < .05). Products of lipid peroxidation (TBARS) in the liver were used as a second marker of oxidant stress (Fig. 4). The TBARS assay (Draper and Hadley, 1990) measures membrane lipid peroxidation breakdown products (aldehydes such as malondialdehyde, and ketones). The basal levels of total lipid peroxidation products in the liver were 0.20 ± 0.02 nmol/mg. In the LPS group, levels increased to 0.41 ± 0.03 nmol/mg (P < .05), l-NIL treatment significantly reduced these levels to 0.29 ± 0.02 nmol/mg (P < .05 compared with LPS group), a level not statistically different from that in the saline group. The formation of 4-hydroxynonenal-protein adducts was the third marker of oxidant stress measured 6 h after administration of LPS. 4-Hydroxynonenal is a cytotoxic lipid peroxidation metabolite of omega-6-polyunsaturated fatty acids and can form covalent links with proteins (Uchida and Stadtman, 1992; Uchida et al., 1993, 1995; Eschwege et al., 1999, 2000; Zainal et al., 1999). 4-Hydroxynonenal-protein adducts were detected by Western blot analysis, then analyzed by densitometry (Fig. 5). 4-Hydroxynonenal-protein adducts significantly increased 3.6 ± 0.6-fold in the LPS treatment group compared with the saline group (P < .01) (Fig. 5B). l-NIL significantly reversed this increase to 1.7 ± 0.3-fold, a level not statistically different from saline group. A representative Western blot for detection of 4-hydroxynonenal-protein adducts is shown as in Fig. 5A.

**Evidence for ONOO− Generation.** ONOO− is formed by the reaction of superoxide and NO. The appearance of 3-nitrotyrosine-protein adducts is often used as a marker of ONOO− formation. Western blot analysis of 3-nitrotyrosine-protein adducts failed to detect any modified proteins (data not shown). Likewise, immunohistochemistry also failed to detect any 3-nitrotyrosine-protein adducts in the liver from any of the groups (data not shown).

**Discussion**

Multiple organ failures are frequent and serious complications of septicemia and septic shock (Mayeux, 1997). These conditions are associated with high mortality because current therapy is mostly supportive (Wenzel et al., 1996). Whereas vasculature-derived NO is a major contributor to systemic hypotension, induction of iNOS and a subsequent increase in NO generation can cause direct cellular damage. In the liver, LPS-activated Kupffer cells, endothelial cells, and hepatocytes are known to be a source of iNOS-derived NO (Duval et al., 1996; Rockey and Chung, 1996; Roland et
groups.

saline, LPS (2 mg/kg i.v.), or LPS plus L-NIL (3 mg/kg i.p., two doses, 3 h apart). Data are mean ± S.E. (n = 3). *P < .05 compared with all other groups.

Fig. 5. Western blot analysis of 4-hydroxynonenal-protein adducts in liver 6 h after LPS administration. A, lanes 1 and 2: Saline group; lanes 3 and 4, LPS + L-NIL (3 mg/kg i.p., two doses, 3 h apart); lanes 5 and 6, LPS group (2 mg/kg i.v.). Each lane contains 50 μg of protein from separate animals. A 4 to 20% gradient gel was used for SDS-PAGE. B, effect of LPS on 4-hydroxynonenal-protein adducts formation in the liver. 4-Hydroxynonenal-protein adducts formation were measured by Western blot and analyzed by densitometry in the liver 6 h after treatment with saline, LPS (2 mg/kg i.v.), or LPS plus L-NIL (3 mg/kg i.p., two doses, 3 h apart). Data are mean ± S.E. (n = 3). *P < .05 compared with all other groups.

al., 1996). Therefore, we used a dose of LPS (2 mg/kg) in a rat model of sepsis that would allow us to investigate the appearance of oxidant stress in the liver before the development of overt hepatic failure.

LPS caused a fall in liver GSH levels and a rise in lipid peroxidation products, including the generation of 4-hydroxynonenal-protein adducts. These three markers of oxidant stress indicated that LPS did induce oxidant stress in the liver before hepatic cell lysis. Although LPS increased plasma ALT activity at 6 h, the increase in ALT activity was quite modest (approximately 10 I.U./l greater than control animals). Furthermore, there was no histological evidence of hepatic cell necrosis. Other studies have examined the role of NO in models using higher (3-fold or greater) doses of LPS, which are associated with large increases in plasma ALT activity (200 I.U./l or greater) (Wang et al., 1998b; Wray et al., 1998; Crespo et al., 1999). Thus, our study supports the notion that oxidant stress precedes hepatic cell lysis. The fall in GSH equivalents that we observed after LPS treatment could be interpreted in a number of ways. LPS could have caused efflux of intracellular GSH, as suggested by others (Jaeschke and Farhood, 1991; Jaeschke, 1992). However, the ability of L-NIL to reverse the loss of GSH suggests that iNOS-derived NO may have contributed more directly to the loss of GSH, perhaps through augmenting oxidant stress. 4-Hydroxynonenal, a major product of membrane peroxidation, can also react with GSH to yield inactive thioether derivatives (Estabrook et al., 1991). Thus, the increase in the level of 4-hydroxynonenal may have also contributed to the decrease in intracellular GSH.

The role of iNOS-derived NO in LPS-induced oxidant stress was evaluated using the iNOS inhibitor L-NIL (Faraci et al., 1996). All three markers of oxidant stress indicated that L-NIL significantly reduced LPS-induced hepatic oxidant stress. These findings suggest that the generation of NO is a major determinant of oxidant stress. Furthermore, the source of NO may be iNOS. At 6 h after LPS administration, iNOS protein was detected in the liver. In addition, plasma levels of NO2-/NO3- were increased 100-fold. L-NIL is reported to be a selective inhibitor of iNOS (Faraci et al., 1996) and has been used to evaluated the role of iNOS in a number of in vivo studies (Connor et al., 1995; Schwartz et al., 1997; Wray et al., 1998). In our model of endotoxemia, the dosing regimen of L-NIL used inhibited the LPS-induced rise in plasma NO2-/NO3- concentration by 90% and, as expected, did not affect iNOS expression in the liver. Because other isoforms of NOS can be up-regulated by LPS (Mayeux et al., 1995), it is not possible to rule out the contribution of other isoforms in the development of oxidant stress. Nevertheless, the significant protection afforded by L-NIL suggests that the induction of iNOS was a major contributor to oxidant stress.

LPS-induced oxidant stress has been implicated as a contributor to lung, liver, and kidney injury (Demling et al., 1986; Bautista and Spitzer, 1990; Zurovsky and Gispaan, 1995; Zhang et al., 2000). Both superoxide and NO can promote the development of oxidant stress in the liver (Bautista and Spitzer, 1990; Wang et al., 1998a). Our data show that inhibition of NO synthesis greatly reduces oxidant stress after LPS administration. NO may participate in the development of oxidant stress in a number of ways. The loss of a single electron generates the highly reactive nitrosyl cation (NO+) (Stamler et al., 1992). In addition, the reaction of NO with superoxide generates ONOO-. Both NO+ and ONOO- are potent oxidants scavenged by GSH (Stamler et al., 1992). Generation of these reactive nitrogen species could also explain the loss of GSH and the appearance of markers of oxidant stress.

We recently reported that in the rat kidney, LPS administration causes a similar oxidant stress and the generation of ONOO-, as indicated by the appearance of 3-nitrotyrosine-protein adducts (Zhang et al., 2000). In contrast, we failed to detect any 3-nitrotyrosine-protein adducts in the liver using both Western blot analysis and immunohistochemistry. This may be due to the higher content of GSH in the liver compared with that in the kidney (Zhang et al., 2000). ONOO- is effectively scavenged by GSH directly (Stamler, 1994) as well as by glutathione peroxidase (Sies et al., 1997). Thus, in the liver ONOO- may be more effectively scavenged by endogenous antioxidant defenses. However, in the acetaminophen
model of hepatic injury, where GSH depletion is near complete, 3-nitrotyrosine-protein adducts are prominent in injured regions of the liver (Hinson et al., 1998; Michael et al., 1999).

In summary, at 6 h after LPS administration, the rat liver showed evidence of oxidant stress in the absence of hepatic failure. The iNOS inhibitor, L-NIL, helped to preserve intracellular GSH, and reduced lipid peroxidation. These data suggest that the generation of iNOS-derived NO was a major determinant of LPS-induced oxidant stress. Others have proposed that redox stress contributes to NO toxicity in the liver (by providing a microenvironment favoring the production of oxidants). Our studies suggest that NO itself, or through one of its metabolites, can promote oxidant stress and thus provide an environment favoring the generation of more reactive species. Consequently, reactive nitrogen species should be considered as potential therapeutic targets in other models of hepatic injury.

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References


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