Disruption of renal peritubular blood flow in lipopolysaccharide-induced renal failure: role of nitric oxide and caspases

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Tiwari, Manish M., Robert W. Brock, Judit K. Megyesi, Gur P. Kaushal, and Philip R. Mayeux. Disruption of renal peritubular blood flow in lipopolysaccharide-induced renal failure: role of nitric oxide and caspases. Am J Physiol Renal Physiol 289: F1324–F1332, 2005.—Acute renal failure (ARF) is a frequent and serious complication of endotoxemia caused by lipopolysaccharide (LPS) and contributes significantly to mortality. The present studies were undertaken to examine the roles of nitric oxide (NO) and caspase activation on renal peritubular blood flow and apoptosis in a murine model of LPS-induced ARF. Male C57BL/6 mice treated with LPS (Escherichia coli) at a dose of 10 mg/kg developed ARF at 18 h. Renal failure was associated with a significant decrease in peritubular capillary perfusion. Vessels with no flow increased from 7 ± 3% in the saline group to 30 ± 4% in the LPS group (P < 0.01). Both the inducible NO synthase inhibitor L-N6-1-iminoethyl-lysine (L-NIL) and the nonselective caspase inhibitor benzoxycarbonyl-Val-Ala-Asp fluoromethylketone (Z-VAD) prevented renal failure and reversed perfusion deficits. Renal failure was also associated with an increase in renal caspase-3 activity and an increase in renal apoptosis. Both L-NIL and Z-VAD prevented these changes. LPS caused an increase in NO production that was blocked by L-NIL but not by Z-VAD. Taken together, these data suggest NO-mediated activation of renal caspases and the resulting disruption in peritubular blood flow are an important mechanism of LPS-induced ARF.

Acute renal failure; inducible nitric oxide synthase; intravital videomicroscopy; benzoxycarbonyl-Val-Ala-Asp fluoromethylketone; L-N6-1-iminoethyl-lysine; apoptosis

Acute renal failure (ARF) is a frequent and serious complication of endotoxemia caused by lipopolysaccharide (LPS) and contributes significantly to mortality (29). Despite the advances in supportive treatment, mortality rates remain unchanged (21). Hence, there is an urgent need for novel therapeutic targets to improve outcomes.

LPS-induced ARF is associated with hemodynamic changes that are strongly influenced by the overproduction of nitric oxide (NO) through the cytokine-mediated upregulation of inducible NO synthase (iNOS) (29). These hemodynamic changes alter renal blood flow, intrarenal hemodynamics, and glomerular filtration rate (GFR) (24). Intravital videomicroscopy has made it possible to study microcirculatory changes in the kidney at the level of the individual peritubular capillary. This technique has revealed that changes in renal microcirculatory flow can impact renal function in a major way (10, 39). Neither peritubular blood flow nor its regulation by NO has ever been specifically examined in LPS-induced ARF. In addition to hemodynamic effects, elevated levels of NO can have a cytotoxic effect in the kidney through the generation of reactive nitrogen species (5, 32). Despite these studies, the role of NO in LPS-induced ARF is still unclear.

Recently, caspases have been shown to play a role in LPS-induced ARF. Nonselective inhibition of caspase activity protects against LPS-induced ARF and apoptosis in the kidney (13). Also, caspase-1 knockout mice show reduced susceptibility to ARF (34). Thus caspases appear to be capable of modulating renal injury through at least two distinct signaling pathways: 1) activation of proinflammatory cytokines and/or 2) promotion of apoptotic cell death (8).

NO and caspases have been shown to play important roles in the pathogenesis of ARF (7, 17, 18, 26, 32, 40). The interrelationships between NO and caspases have not been examined in LPS-induced renal failure despite the fact that both occur in the kidney (9, 42) and NO can activate or inhibit caspase activities depending on the level of NO generated (15, 28, 30). We hypothesized that LPS-induced ARF is caused by NO-mediated activation of caspases. To address this, we examined the roles of NO and caspase activation on renal peritubular blood flow and apoptosis in a murine model of LPS-induced ARF.

MATERIALS AND METHODS

Materials

LPS (Escherichia coli serotype 055:B5) and fluorescein isothiocyanate-dextran (FITC-dextran; 150,000 MW), 7-amino-4-methylcoumarin (AMC), and all other chemicals (unless noted otherwise) were purchased from Sigma (St. Louis, MO). L-N6-1-iminoethyl-lysine (L-NIL) was purchased from Alexis Biochemicals (San Diego, CA). Benzoxycarbonyl-Val-Ala-Asp fluoromethylketone (Z-VAD-fmk) was purchased from Enzyme Systems Products (Aurora, OH). Ac-YVAD-AMC and Ac-DEVD-AMC were purchased from Peptide Institute (Osaka, Japan).

Model of ARF

All animals were housed and killed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with approval of the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee. Male C57BL/6 mice (Harlan Laboratories, Indianapolis, IN) were studied at 8 wk of age (20–22 g). At 18 or 24 h following treatment, mice were anesthetized under CO2 anesthesia and blood was collected via the orbital sinus. This was followed immediately by cervical dislocation. Kidneys were then rapidly harvested. One kidney was snap-frozen in liquid nitrogen and used for later determination of caspase activities.

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The other kidney was fixed in 10% phosphate-buffered formalin for histology. The collected blood was allowed to stand for 30 min at room temperature and then centrifuged at 10,000 g for 30 min at 4°C. The resulting serum was used for blood urea nitrogen (BUN), serum creatinine, nitrate/nitrite, and cytokine measurements.

**Caspase inhibition studies.** All animals received the same total injection volume. Animals received saline (2 ml/kg ip) or LPS (10 mg/kg in saline, 2 ml/kg ip) and a single injection of Z-VAD-fmk vehicle (12% DMSO in saline, 3 ml/kg ip). To examine the role of caspases, separate groups of animals received Z-VAD-fmk (2 mg/kg in 12% DMSO in saline, 3 ml/kg ip) alone or along with LPS at time 0. Preliminary studies revealed no statistically significant difference in the development of renal failure between mice administered LPS alone and those administered LPS + DMSO/saline vehicle.

**iNOS inhibition studies.** These studies included different saline- and LPS-treated groups of animals because of the multiple dosing schedule. All animals received the same total injection volume. At time 0, either saline (2 ml/kg ip) or LPS (10 mg/kg in saline, 2 ml/kg ip) was administered to mice. Three dosing schedules of L-NIL (3 mg·kg⁻¹·dose⁻¹·ip, 2 ml/kg in saline) were studied. The 1× L-NIL group received a single dose of L-NIL at time 0. The 2× L-NIL group received doses at time 0 and 9 h. The 3× L-NIL group received doses at time 0, 6, and 12 h. Control and LPS-treated mice received saline at an equivalent dosing schedule.

**BUN and Serum Creatinine Determination**

Estimations of BUN and creatinine concentrations were performed using a Beckman Synchron CX7 Analyzer (Fullerton, CA).

**Cytokine Measurements**

Serum concentrations of TNF-α and IFN-γ were determined by cytometric bead analysis using a commercially available kit (BD Biosciences, San Diego, CA) as described by the manufacturer.

**Serum Nitrate/Nitrite Levels**

Serum nitrate/nitrite (NOx) levels were determined as previously described with modifications. Briefly, serum samples (25 μl) were diluted in H₂O (165 μl) and deproteinized by treatment with 30% ZnSO₄ (10 μl) for 15 min at room temperature. Samples were then centrifuged at 2,000 g for 5 min, and supernatants were collected. Nitrate was converted to nitrite by treating supernatants with cadmium beads overnight with agitation at 4°C. Samples were then centrifuged, and nitrite levels were estimated in supernatants using the Griess reagent. Griess reagent was made of equal volumes of 1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylenediamine in water and 0.5 N HCl. Supernatants and Griess reagent were mixed in a ratio of 1:1 and incubated at room temperature for 15 min, and absorbance was read.
at 543 nm. Results were compared against a NaNO₂ standard curve, and nitrite concentration was calculated in μM.

**Immunohistochemistry**

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining for apoptotic nuclei was carried out in paraffin-embedded tissue sections (3 μm) using a commercially available kit (Calbiochem, Darmstadt, Germany) with modifications as described below. Paraffin-embedded kidney tissue sections were deparaffinized by two treatments with xylene for 5 min at room temperature. Sections were then rehydrated and washed in phosphate-buffered saline. Following this, sections were permeabilized with proteinase K treatment (20 μg/ml) for 20 min at room temperature and incubated with 3% H₂O₂/methanol for 5 min at room temperature to block endogenous peroxidase activity. Incubation with terminal deoxynucleotidyl transferase labeling reaction mixture for 60 min at 37°C was carried out. Labeling reaction was terminated, and labeled fragments were detected by the diaminobenzidine reaction for 2 min.
Methyl green was used as counterstain. Sections were examined under light microscopy at ×400 magnification, and the total number of apoptotic nuclei was counted by a blinded observer. Data are expressed as percent of total vessels per 200 μm.

**Renal Histology**

Renal tissue injury was assessed in tissue sections stained by the periodic acid-Schiff (PAS) reaction. A semiquantitative score for tubulointerstitial injury and acute tubular necrosis (ATN score) was assigned to each animal by a blinded observer as described by Wang et al. (34). For each animal, at least 10 high-power fields were examined. The percentage of tubules that displayed cellular necrosis, loss of brush border, cast formation, vacuolization, and tubule dilation were scored as follows: 0 = none, 1 = <10%, 2 = 11–25%, 3 = 26–45%, 4 = 46–75%, and 5 = >76%.

**Statistical Analysis**

Data were analyzed with Prism 4.0 software for Mac (GraphPad Software, San Diego, CA). Data are expressed as means ± SE. One-way ANOVA followed by Student-Newman-Keuls test was used to compare groups. A P value <0.05 was considered significant. Each n represents data or tissue obtained from one mouse.

**RESULTS**

**Caspase Inhibition Protects Against LPS-Induced ARF**

Renal function was assessed 18 and 24 h after administration of LPS (10 mg/kg; Fig. 1). Renal failure occurred at 18 h as evidenced by a rise in BUN (saline 28 ± 2 vs. LPS 83 ± 6 mg/dl) and serum creatinine levels (saline 0.25 ± 0.02 vs. LPS 0.56 ± 0.06 mg/dl; n = 8–9; P < 0.001). LPS-induced rise in BUN (77 ± 17 mg/dl) and creatinine levels (0.38 ± 0.05 mg/dl) at 24 h were not significantly different from values at 18 h. The role of caspas was examined using Z-VAD-fmk, an irreversible inhibitor of caspases. Z-VAD (2 mg/kg) significantly reduced BUN (50 ± 7 mg/dl; P < 0.05) and creatinine levels (0.23 ± 0.01 mg/dl; P < 0.001) at 18 h compared with the LPS-treated group. This suggests that caspases may be involved in endotoxin-induced renal failure.

**NOS Inhibition Protects Against LPS-Induced ARF**

The selective iNOS inhibitor, L-NIL (25), was used to examine the role of iNOS. A single dose of L-NIL (1 × L-NIL; 3 mg/kg) administered along with LPS failed to protect against...
renal failure (Fig. 2). Two doses of L-NIL (2 × L-NIL) administered at time 0 and 9 h produced only a partial protection against ARF. Three doses of L-NIL (3 × L-NIL) administered at time 0, 6, and 12 h produced a complete protection. Both indexes of renal function in animals treated with LPS + Z-VAD-fmk alone were not different from saline-treated animals, showing that Z-VAD-fmk alone does not affect NOx levels. In contrast, the rise in NOx concentration was blocked dose dependently by L-NIL (Fig. 3B). These data suggest that NO generation is not dependent on caspase activation.

Role of Peritubular Blood Flow in LPS-Induced ARF

Renal cortical peritubular blood flow was examined 18 h following LPS administration using intravital videomicroscopy. Representative video frames captured from a saline- and LPS-treated animal at 18 h are shown in Fig. 4. Data from the analyzed videos are presented in Fig. 5, A–C. Vessel density (vessels/200 μm) was unchanged in the four groups. Vessels with continuous flow decreased significantly from 88 ± 6% in the saline group to 63 ± 3% in the LPS-treated group (n = 5; P < 0.01; Fig. 5B). In addition, LPS produced a dramatic
increase in vessels with no flow (saline 7 ± 3% vs. LPS 30 ± 4%; n = 5; P < 0.01; Fig. 5C). These changes in peritubular blood flow were prevented by Z-VAD-fmk (2 mg/kg) and l-NIL (3 doses of 3 mg/kg at times 0, 6, and 12 h). These data indicate that inhibition of either caspase activity or iNOS preserves peritubular capillary perfusion and protects against renal failure.

Role of Cytokines

To examine whether a change in inflammatory cytokine profile could explain the protection seen with Z-VAD and l-NIL, serum levels of TNF-α and IFN-γ were measured (Fig. 6). LPS produced a 7-fold increase in serum TNF-α levels and a 13-fold increase in IFN-γ levels (n = 5; P < 0.001 compared with saline). Neither the combination of LPS + Z-VAD-fmk nor LPS + 3× l-NIL affected the ability of LPS to raise TNF-α and IFN-γ levels. These data suggest that changes in TNF-α and IFN-γ levels do not contribute to the protective effects of Z-VAD-fmk and l-NIL.

Renal Histology

PAS-stained kidney tissue sections revealed relatively mild morphological damage 18 h after LPS administration (Fig. 7, A–D). Morphological changes observed on LPS treatment were characterized by mild brush-border loss, tubular degeneration, and vacuolization in the early segments of proximal tubules. Occasional tubular dilatation and epithelial flattening in the distal nephron segment of the cortex could also be seen. There was no red blood cell extravasation, tubular necrosis, or tubular cast formation. Figure 7E shows the cortical injury score. Administration of either Z-VAD-fmk or l-NIL along with LPS significantly reduced the cortical injury score compared with LPS alone (n = 4–5, P < 0.05).

Role of Apoptosis in LPS-Induced Renal Failure

TUNEL staining of kidney tissue sections was carried out to quantitate the level of apoptosis. Representative stained sections are presented in Fig. 8, A–D. At 18 h following LPS...
administration, total apoptotic nuclei counted over 10 high-power fields increased more than threefold (n = 4–5; P < 0.05) compared with all other groups (Fig. 8E). Both Z-VAD-fmk and 3× l-NIL prevented the increase induced by LPS. These data suggest that iNOS-derived NO increases apoptosis.

Caspase Activation Dependent on NO

Because LPS caused an increase in apoptosis in the kidney, we examined whether caspase activity is elevated in the kidney following LPS administration. Both caspase-1, a proinflammatory caspase, and caspase-3, the major executioner caspase, have been implicated in LPS-induced renal failure (13, 34). None of the treatments changed caspase-1 activity (Fig. 9A). In contrast, the activity of caspase-3, the principal executioner caspase, was significantly increased in kidney tissue homogenates following LPS treatment (saline 4.3 ± 0.5 vs. LPS 9.0 ± 1.2 nmol min⁻¹ mg⁻¹ protein⁻¹; n = 6–9; P < 0.01; Fig. 9B). The LPS-induced rise in caspase-3 activity was blocked by Z-VAD (5.8 ± 0.3 nmol min⁻¹ mg⁻¹ protein⁻¹; P > 0.05 compared with saline). Three doses of l-NIL also prevented the rise in LPS-induced caspase-3 activity.

Taken together, these results indicate that inhibition of caspases or iNOS can protect against LPS-induced renal failure. Furthermore, caspase activation is a downstream event of NO generation.

DISCUSSION

LPS caused a major disruption of renal cortical peritubular perfusion, renal apoptosis, and renal failure. The ability of the iNOS inhibitor, l-NIL, to prevent these effects indicates that iNOS induction is a critical event in the development of ARF in this model. Furthermore, the mechanism by which increased NO generation results in ARF appears to be through the activation of caspases. Despite the well-recognized role of NO (11, 26, 32, 33, 42) and the emerging role of caspases in the pathophysiology of ARF (2, 13, 17, 18, 34), interactions between NO and caspases have never been studied. To our knowledge, these studies are the first to suggest a link between iNOS-induced NO generation and caspase activation in the development of ARF.

The role of iNOS-derived NO in LPS-induced ARF has been controversial. In rat models of LPS-induced ARF, iNOS inhibition is generally protective (16, 24, 42). However, in the mouse, iNOS inhibition has not been shown to offer protection (19, 35) and iNOS knockout mice are equally susceptible to LPS-induced ARF as wild-type (19). While species differences and model differences may explain some of the disparity regarding the role of iNOS, our data indicate that dose and dosing schedule of the iNOS inhibitor are critically important. l-NIL provided complete protection in our model but only when three doses of l-NIL were administered 6 h apart. This was most likely due to the fact that three doses of l-NIL were required to completely block overproduction of NO.

A recent report by Guo et al. (13) found that Z-VAD was protective against LPS-induced renal failure indicating that caspases may be involved. We also found protection with Z-VAD. Surprisingly, Z-VAD offered protection against renal failure despite the fact that NO generation was unchanged. These data support the notion that caspase activation is downstream of NO generation.

Tubular epithelial cell function and renal blood flow are the two most important regulators of GFR (36). Intravital videomicroscopy has revealed that changes in renal microcirculatory flow can impact renal function in a major way (10, 39). Our studies clearly show that LPS causes a major disruption of cortical peritubular microvascular flow. Specifically, vessels with no flow dramatically increased and vessels with continuous flow decreased after LPS administration. The potential for NO to impact both tubular function and GFR is well known (22, 24, 37, 41). We found that specific iNOS inhibition with l-NIL preserved renal cortical perfusion. The ability of l-NIL to prevent the decrease in peritubular perfusion suggests that iNOS-derived NO is, in some way, responsible for changes in flow. Furthermore, preserving perfusion may be one mechanism through which iNOS inhibition is protective. In the rat, laser Doppler studies showed that LPS produces a decrease in cortical blood flow and an increase in renal medullary perfusion (24). Interestingly, the nonselective NOS inhibitor, N⁵G⁶-guanylnitroso-L-arginine (l-NMMA), did not improve flow characteristics and only partially improved renal function. This is consistent with the notion that inhibition of both constitutive NOS and iNOS with the use of nonselective NOS inhibitors offers little protection and has been shown in clinical studies to be detrimental (20, 27).

Like l-NIL, Z-VAD prevented LPS-induced alterations in renal perfusion and function. Studies with Z-VAD suggest that caspase activation and perhaps apoptosis are major contributors to the disruption of peritubular perfusion. These data are also the first to link caspase activation to changes in peritubular blood flow. In LPS-induced ARF, vascular endothelial cell
injury (6, 12, 14, 23), neutrophil migration (1, 9), or intravascular coagulation (29) could potentially play a role in mechanical obstruction of small blood vessels in the kidney and contribute to renal failure. Additional studies are needed to explain how caspase inhibition could prevent cortical perfusion changes. The anti-inflammatory activity of Z-VAD may offer a possible explanation (13). Another explanation could be preservation of endothelial cell integrity and function (23, 31).

Endotoxemia produces a proinflammatory state. Both TNF-α and IFN-γ were elevated at the time of renal failure. However, neither L-NIL nor Z-VAD altered the levels of these cytokines. The role of TNF-α in LPS-induced ARF has been shown using TNF receptor-1 knockout mice (9) and TNF neutralization by a soluble receptor (19). Our data suggest that the protective effects of L-NIL and Z-VAD are not due to changes in TNF-α and IFN-γ levels. Both caspase-1 and caspase-3 have also been implicated in the development of LPS-induced ARF (9, 13, 34). We observed an increase in renal apoptosis and caspase-3 activity but not caspase-1 activity, confirming the findings of a recent report in a similar model (13). Z-VAD prevented the increase in caspase-3 activity and apoptotic nuclei as reported by others (13). This shows that effective caspase inhibition was achieved at the dose of Z-VAD used. The most significant finding was that the LPS-induced rise in both caspase-3 activity and renal apoptosis was blocked by L-NIL. The ability of iNOS-derived NO to activate caspase-3 and trigger apoptosis in vivo is consistent with in vitro studies (15). NO can activate caspases through its effects on mitochondria (4) or through generation of reactive nitrogen species (5).

In summary, LPS caused a catastrophic disruption of renal cortical peritubular perfusion, caspase-dependent renal apoptosis, and renal failure. Inhibition of iNOS not only decreased renal caspase-3 activity and apoptosis but also preserved renal perfusion and function. Caspase inhibition also preserved renal cortical perfusion, prevented apoptosis, and protected against renal failure. However, this effect was downstream of iNOS. Taken together, these data suggest NO-mediated activation of renal caspases and the resulting disruption in peritubular blood flow are an important mechanism of LPS-induced ARF.

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