Peritubular capillary dysfunction and renal tubular epithelial cell stress following lipopolysaccharide administration in mice

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Submitted 11 July 2006; accepted in final form 15 August 2006

Wu L, Tiwari MM, Messer KJ, Holthoff JH, Gokden N, Brock RW, Mayeux PR. Peritubular capillary dysfunction and renal tubular epithelial cell stress following lipopolysaccharide administration in mice. Am J Physiol Renal Physiol 292: F261–F268, 2007. First published August 22, 2006; doi:10.1152/ajprenal.00263.2006.—The mortality rate for septic patients with acute renal failure is extremely high. Since sepsis is often caused by lipopolysaccharide (LPS), a model of LPS challenge was used to study the development of kidney injury. Intravital video microscopy was utilized to investigate renal peritubular capillary blood flow in anesthetized male C57BL/6 mice at 0, 2, 6, 10, 18, 24, 36, and 48 h after LPS administration (10 mg/kg ip). As early as 2 h, capillary perfusion was dramatically compromised. Vessels with continuous flow were decreased from 89 ± 4% in saline controls to 57 ± 5% in LPS-treated mice (P < 0.001), and vessels with intermittent flow were increased from 6 ± 2% to 31 ± 5% (P < 0.01). At 2 h, mRNA for intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 were elevated 50- and 27-fold, respectively, suggesting that vascular inflammation is an early event that may contribute to capillary dysfunction. By 10 h, vessels with no flow increased from 5 ± 2% in saline controls to 19 ± 3% in LPS-treated mice (P < 0.05). By 48 h, capillary function was returning toward control levels. The decline in functional capillaries preceded the development of renal failure and was paralleled by induction of inducible nitric oxide synthase in the kidney. Using NAD(P)H autofluorescence as an indicator of cellular redox stress, we found that tubular cell stress was highly correlated with the percentage of dysfunctional capillaries (r² = 0.8951, P < 0.0001). These data show that peritubular capillary dysfunction is an early event that contributes to tubular stress and renal injury.

intravital video microscopy; sepsis; inducible nitric oxide synthase; intercellular adhesion molecule-1; vascular cell adhesion molecule-1

SEPSIS IS A PROGRESSIVE SYNDROME associated with a disseminated inflammatory response that leads to multiorgan failure (10). Acute renal failure (ARF) occurs in ~20–50% of septic patients diagnosed by positive blood culture (26), and the mortality rate for septic patients with ARF is extremely high, approaching 70% (3). Septic patients have elevated plasma levels of soluble adhesion molecules [intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)], which indicates endothelial activation (2), and elevated cytokine levels, which may predict mortality (28). Lipopolysaccharide (LPS) released from the gram-negative bacterial cell wall is one of the major initiators of the inflammatory response during sepsis.

Although they do not replicate all the manifestations of human sepsis (7, 25), murine models of LPS challenge do produce ARF and are used to study the role of the LPS-induced inflammatory response in renal injury (8, 9, 34). LPS produces an early rise in cytokines through activation of extrarenal Toll-like receptor 4 (9). Subsequently, cytokines such as tumor necrosis factor mediate renal injury (8) directly or through the actions of nitric oxide (NO), reactive nitrogen species, and caspases (11, 29, 35). Cytokines also contribute to LPS-induced vascular inflammation and endothelial damage (12), which may lead to reduced organ perfusion. Endothelial injury and capillary dysfunction are recognized as important factors in the development of multiple causes of ARF (19, 22, 33).

LPS produces a peripheral vasodilation but a paradoxical renal vasoconstriction (17, 26). We recently reported that peritubular capillary perfusion is dramatically reduced at the time of LPS-induced renal failure in the mouse, and inhibitors of inducible NO synthase (iNOS) or caspases prevent capillary dysfunction and renal injury (29). These findings suggested a possible relation between capillary dysfunction and the development of renal injury. To examine this, we performed a detailed time-course study of the development of peritubular capillary dysfunction and, using intravital video microscopy (IVVM), examined the relation between capillary dysfunction and tubular epithelial cell stress in real time. Understanding of the development of LPS-induced renal injury and how capillary dysfunction impacts epithelial stress could uncover new therapeutic approaches.

MATERIALS AND METHODS

Lipopolysaccharide (Escherichia coli 055:B5 strain) and FITC-labeled dextran (FITC-dextran, 150,000 mol wt) were purchased from Sigma-Aldrich (St. Louis, MO); RNase easy minikit and QiaShredder column from Qiagen (Valencia, CA); iScript eDNA synthesis kit and iQ SYBR Green Supermix from Bio-Rad (Hercules, CA); DNase Treatment & Removal Kit from Ambion (Austin, TX); antibodies used in Western blot and immunohistochemistry from Upstate Cell Signaling Solutions (Lake Placid, NY); Supersignal West Pico chemiluminescence detection kit from Pierce Biotechnology (Rockford, IL); and DakoCytomation LSAB+ System-HRP kit from Dako North America (Carpentaria, CA).

Mouse Model of Endotoxin-Induced Renal Injury

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, 4301 West Markham St., #611, Little Rock, AR 72205 (e-mail: prmayeux@uams.edu).
Sciences Institutional Animal Care and Use Committee. Male C57BL/6 mice (8 wk of age) were acclimated for 1 wk with free access to food and water. At the start of the experiment, mice were injected with saline or LPS (10 mg/kg ip). After blood collection via the orbital sinus under CO₂ anesthesia, the animals were killed by cervical dislocation and the kidneys were rapidly harvested: one kidney was snap frozen in liquid nitrogen and used for real-time PCR, and the other was fixed in 10% phosphate-buffered formalin. Paraffin-embedded sections (3 μm) were stained by the periodic acid-Schiff reaction for analysis of morphology and neutrophil infiltration by a blinded observer.

Serum Blood Urea Nitrogen and Creatinine Determination

Blood urea nitrogen (BUN) and creatinine concentrations in serum were estimated using a Synchront Cx7 Analyzer (Beckman, Fullerton, CA).

Serum Nitrate/Nitrite Levels

Serum nitrate/nitrite (NOx) levels were determined as previously described (29). Briefly, serum samples diluted in water were deproteinized by treatment with 30% ZnSO₄ (1:100 dilution) 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 treatment of the supernatants with cadmium beads overnight with room temperature. Samples were then centrifuged at 2,000 g. Nitrite levels were estimated in supernatants using the Griess reagent [equal volumes of a solution of 1% sulfanilamide-0.8% N-(1-naphthyl)-ethylenediamine in water and 0.5 N HCl]. After 15 min of incubation at room temperature, absorbance was read at 543 nm. Results were compared against an NaNO₂ standard curve, and nitrite concentration (μM) was calculated.

IVVM

At 2, 6, 10, 18, 24, 36, or 48 h after LPS treatment, mice were anesthetized with isoflurane, and the left kidney was exposed by laparotomy. The kidney was positioned on a glass stage above an inverted fluorescent microscope (Axiovert 200, Zeiss, Jena, Germany) equipped with a digitizing camera (Hamamatsu, Bridgewater, NJ), kept moist with saline, and covered. The entire IVVM procedure, including surgery, was completed within 30 min. During this time, core temperature was monitored and maintained at 36–37°C with an infrared heat lamp.

Evaluation of peritubular capillary dysfunction. At ~15 min before IVVM, FITC-dextran (150,000 mol wt) was administered (2 μmol/kg) via tail vein. The renal intravascular space and red blood cell (RBC) movement were visualized at 470-nm excitation and 520-nm emission. For each animal, five 10-s videos were captured at ~15 frames/s from five randomly selected fields of view (~200). Capillary function was analyzed as previously described (29). Briefly, ~150 randomly selected vessels per animal were classified into 3 categories of blood perfusion: "continuous flow," where RBC movement in the vessel was not interrupted during the video; "intermittent flow," where RBC movement stopped or reversed at any time during the video; and "no flow," where no RBC movement was detected. Data are expressed as percentage of vessels in each of the three categories.

Evaluation of renal tubular epithelial cell stress. Reduced forms of nicotinamides [NAD(P)H] are highly fluorescent (16). IVVM was used to assess cellular stress by monitoring NAD(P)H autofluorescence, an indicator of cellular redox state (23, 32). Because NADH and NADPH emit the same spectra with the same extinction coefficients at 365-nm excitation, together, they are designated NAD(P)H. Under the same fields of view used to assess capillary function, NAD(P)H was visualized at 365-nm excitation and 420-nm emission. To minimize photo bleaching, a <3-s exposure was used to capture videos of five randomly selected fields of view per animal. Gain and other contrast-enhancement settings were identical for all fields of view. Fluorescence intensity was quantified densitometrically (arbitrary units/μm²) using AxioVison Imaging Software (Zeiss).

Real-Time PCR

Real-time PCR was used to estimate mRNA levels of inflammatory markers in kidney cortex. Total RNA was isolated from the kidney cortex using the RNeasy minikit and QiaShredder column as described by the manufacturer. After removal of genomic DNA using DNase treatment, RNA was reverse transcribed using the iScript cDNA synthesis kit. Specific primers for ICAM-1, VCAM-1, iNOS, and GAPDH were used for amplification of cDNA using qSYBR Green Supermix. The expression ratio of target molecules (ICAM-1, VCAM-1, and iNOS) relative to GAPDH was analyzed using an i-Cycler (Bio-Rad). The fold increase compared with saline control animals was calculated. The primers were as follows: 5'-CAG CTC GGC TGT ACA AAC CTT-3' (forward) and 5'-TGA ATG TGA TGT TTG CTG CGG-3' (reverse) for iNOS, 5'-CCA AGG AGA TCA CAT TCA CGG-3' (forward) and 5'-GGA TGG TAG CTG GAA GAT CGG-3' (reverse) for ICAM-1, 5'-CTT CCG TAG GTT ACA CAG TGG-3' (forward) and 5'-GAG CAG GTC AGC TTC ACA GGC GG-3' (reverse) for VCAM-1, and 5'-GGG AAC CTC ACT GGC ATG G-3' (forward) and 5'-CTT CTT GAT GTC ATC ATA CTT GGC AGG-3' (reverse) for GAPDH.

iNOS Western Blot Analysis

Tissue homogenates [in 0.5% Triton X-100, 20 mM EDTA, and 5 mM Tris-HCl (pH 8.0)] were separated by 10% SDS-PAGE (50 μg protein/lane) and then transferred to nitrocellulose membranes. Membranes were incubated in 5% nonfat milk blocking buffer for 90 min at room temperature, washed, and incubated with a rabbit polyclonal anti-iNOS antibody (1:200 dilution) night at 4°C with rabbit anti-iNOS antibody (1:200 dilution in 5% nonfat milk) overnight at 4°C with agitation. Subsequently, membranes were washed and incubated with a goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:20,000 dilution in 5% nonfat milk) for 90 min at room temperature. Finally, membranes were washed and developed using Supersignal West Pico chemiluminescence detection kit as described by the manufacturer. The same membrane was also probed for α-tubulin using a mouse monoclonal anti-tubulin antibody (1:1,000 dilution) and a goat anti-mouse HRP-conjugated secondary antibody (1:20,000 dilution) as described above.

Immunohistochemistry

Paraffin-embedded tissue sections were cleared in xylene, rehydrated, and washed in PBS. For antigen retrieval, slides in 10 mM citrate (pH 6.0) were subjected to high temperature using microwave energy. Endogenous peroxidase activity and nonspecific protein binding were blocked using reagents supplied in the DakoCytomation LSAB + System-HRP kit. Sections were then incubated overnight at 4°C with rabbit anti-iNOS antibody (1:125 dilution), washed in PBS, and incubated at room temperature for 30 min with the secondary antibody supplied with the kit. Sections were incubated with streptavidin-peroxidase for 30 min and then with chromagen solution as described by the manufacturer. Gill’s hematoxylin II was used as a counterstain. For negative control, the section was incubated without primary antibody.

Statistical Analysis

Data were analyzed with Prism 4.0 software for Mac (GraphPad Software, San Diego, CA). Values are means ± SE; n represents data or tissue obtained from one mouse. A one-way ANOVA followed by Student-Newman-Keuls test was used to compare groups. Pearson’s correlation test was used to characterize the relations between capillary dysfunction and NAD(P)H level. P < 0.05 was considered significant.
RESULTS

Time Course of Renal Failure

Renal function was assessed 6, 10, 18, 24, 36, and 48 h after LPS administration (10 mg/kg ip). Serum BUN and creatinine levels were used as markers for evaluation of renal function (Fig. 1, A and B). BUN and creatinine levels were elevated by 10 h after LPS administration and peaked at 18 h. BUN levels for saline and LPS-treated animals at 18 h were 26 ± 8 mg/dl (n = 5) and 90 ± 8 mg/dl (n = 8), respectively (P < 0.001). Serum creatinine levels for saline and LPS-treated animals at 18 h were 0.20 ± 0.03 mg/dl (n = 6) and 0.51 ± 0.05 mg/dl (n = 8), respectively (P < 0.001). BUN and creatinine levels remained elevated through 24 h and returned to saline control levels by 36 h. Renal histology revealed mild morphological damage, including tubular cell sloughing (black arrows), loss of brush border, and appearance of neutrophils (white arrow). Original magnification ×400.

Serum NOx Levels

LPS is known to induce iNOS and increase systemic NO synthesis. Serum NOx, metabolites of NO, indicate NO generation. NOx levels were significantly elevated by 10 h after LPS administration (Fig. 2). At 18 h, NOx levels were elevated ∼15-fold from 33 ± 6 μM (n = 11) in the saline group to 476 ± 25 μM (n = 12) in the LPS group (P < 0.001). NOx levels then began to decrease by 24 h and returned to the saline group level by 36 h.

Time Course of Renal Capillary Dysfunction

We recently reported that cortical peritubular capillary function was severely compromised at the time of renal failure in LPS-treated mice (29). To help clarify the role of capillary perfusion in the development of renal injury, IVVM was used to determine the time course of capillary dysfunction. IVVM showed that renal peritubular capillary perfusion was significantly compromised as early as 2 h after LPS administration.

Fig. 1. Time course of renal failure. A and B: serum blood urea nitrogen and creatinine levels 6–48 h after LPS administration (10 mg/kg ip). Time 0, saline-treated mice. Renal failure peaked between 18 and 24 h and then returned toward basal levels. Values are means ± SE (n = 5–10). *P < 0.05 vs. time 0. C and D: periodic acid-Schiff-stained kidney sections treated with saline and LPS, respectively, at 18 h. LPS caused mild morphological damage, including tubular cell sloughing (black arrows), loss of brush border, and appearance of neutrophils (white arrow). Original magnification ×400.

Fig. 2. Time course of serum nitrate/nitrite (NOx) levels. Serum levels of NOx were measured 2–48 h after LPS administration (10 mg/kg ip). Time 0, saline-treated animals. LPS caused a significant elevation in serum NOx levels by 10 h. NOx levels peaked at 18 h and then returned to basal levels. Values are means ± SE (n = 4–12). *P < 0.001 vs. time 0.
Changes in cortical peritubular capillary flow after administration of saline (time 0) or LPS (10 mg/kg ip) were assessed by intravital video microscopy using FITC-labeled dextran. Percentage of continuous-flow, intermittent-flow, and no-flow vessels is plotted at 2–48 h after LPS. Values are means ± SE, (n = 4–8 animals per group). *P < 0.05 vs. time 0.

Induction of Inflammatory Markers

Since LPS can cause vascular inflammation and compromise vascular function (24), we determined whether renal inflammation might be an early event. To examine this, the change in mRNA expression in the kidney cortex encoding three inflammatory markers, ICAM-1, VCAM-1, and iNOS, was determined at 2, 6, 10, and 18 h after LPS administration (Fig. 4). ICAM-1 and VCAM-1 were elevated 50- and 27-fold, respectively, compared with saline-treated mice at 2 h after LPS administration. In contrast, iNOS mRNA expression was delayed, reaching an ~90-fold increase over saline-treated mice at 18 h.

Induction of iNOS Expression

Induction of iNOS was examined further by Western blot and immunohistochemistry. The time course of iNOS protein expression (Fig. 5) paralleled that of iNOS mRNA expression (Fig. 4). Immunohistochemistry performed on tissue from saline-treated animals showed relatively little specific staining for iNOS (Fig. 6A). In contrast, staining for iNOS protein in the kidneys of LPS-treated mice at 18 h was abundant and localized to renal tubules, with minimal staining in glomeruli (Fig. 6B).
and the corresponding NAD(P)H level from the same field of view were determined in saline-treated (n = 5, 2 fields of view per animal) and LPS-treated (18 h, n = 5, 2 fields of view per animal) mice (Fig. 8C). Pearson’s correlation test indicated a high degree of correlation between NAD(P)H level and the percentage of dysfunctional capillaries (r² = 0.8951, P < 0.0001). This finding suggests that peritubular capillary dysfunction may contribute to tubular epithelial cell stress.

Renal Tubular Epithelial Cell Stress After LPS Administration

To examine the time course of the development of cellular stress, NAD(P)H autofluorescence was measured at 2, 6, 18, and 48 h after LPS administration (Fig. 9). In contrast to capillary dysfunction 2 h after LPS administration (Fig. 3), NAD(P)H autofluorescence intensity was not significantly elevated at 2 h (Fig. 9). However, at 6 and 18 h, NAD(P)H autofluorescence intensity was significantly elevated compared with saline-treated animals (P < 0.001, n = 5). By 48 h, NAD(P)H autofluorescence intensity had returned to saline control levels.

Taken together, these results show that peritubular capillary dysfunction is an early event that precedes renal failure following LPS administration. Furthermore, capillary dysfunction is correlated with cellular stress and may contribute to the development of tubular injury and renal failure.

DISCUSSION

The present study is the first to document the time course of renal responses to LPS in the mouse and uncover a link between peritubular capillary function and tubular epithelial cell stress. LPS administration produced a gradual increase in...
serum BUN and creatinine concentrations that peaked at 18 h and returned to saline control levels by 36 h. LPS also produced a rapid decline in cortical peritubular capillary perfusion that preceded the development of renal failure. Importantly, the degree of cortical peritubular capillary dysfunction was positively correlated with renal tubular epithelial cell stress, suggesting that capillary dysfunction and the resulting cellular stress are major contributors to the development of renal injury and failure after LPS administration.

Microcirculatory dysfunction is a key feature of sepsis that contributes to end-organ failure (14). It is becoming clear that endothelial injury and microvascular dysfunction play important roles in multiple causes of ARF (19, 22, 33). Nevertheless, the underlying cause of renal capillary dysfunction following LPS administration remains unknown. LPS is recognized to cause endothelial cell injury and even apoptosis, which could contribute to microvascular dysfunction (1, 4, 12, 20, 24). Two inflammatory adhesion molecules, ICAM-1 and VCAM-1, were significantly elevated in the kidney by 2 h after LPS administration. Adhesion molecules propagate the inflammatory response and facilitate the adherence of leukocytes to the microvascular endothelium (15). Plasma levels of soluble ICAM-1 and VCAM-1 increase in patients with sepsis (2) and may predict outcome (31). Neutrophil infiltration, although relatively mild, is reported in some LPS models of sepsis (8, 9, 21). However, the role of neutrophil accumulation in LPS-induced renal injury remains unclear. The rapid induction of adhesion molecules indicates an early inflammatory response initiated by LPS and suggests that vascular inflammation may initiate peritubular capillary dysfunction, leading ultimately to renal injury. The importance of the inflammatory response in LPS-induced renal injury is supported by studies showing that Toll-like receptor 4- and tumor necrosis factor receptor type 1-knockout mice are resistant to LPS-induced renal failure (8, 9).

We recently reported that pharmacological inhibition of iNOS protected mice from capillary dysfunction and renal injury following LPS administration (29). The present studies provide additional support for the role of iNOS and help explain the need for continuous iNOS inhibition over 18 h to achieve full protection (29). The time course of capillary dysfunction was paralleled by the induction of iNOS in the
kidney, as well as the systemic increase in iNOS-derived NO generation. Despite peripheral vasodilation, LPS produces a paradoxical renal vasoconstriction (17, 26) that is thought to be due, at least in part, to iNOS-derived NO-mediated inactivation of endothelial NOS (6, 27). In addition, iNOS serves as a major source of NO for the generation of reactive nitrogen species in the kidney (5, 27, 30, 35). Thus these effects of NO can act synergistically to compromise renal function.

The present studies also examined the impact of capillary dysfunction on tubular stress. We found increased cellular levels of NAD(P)H in tubules adjacent to poorly perfused capillaries compared with tubules bordered by well-perfused capillaries. Although it has been proposed that decreased peritubular capillary flow could lead to tubular injury (18, 33), these data are the first to demonstrate in any model of renal injury the relation between capillary perfusion and redox stress in neighboring tubules. NADH and NADPH are electron donors that transfer electrons to oxygen by means of an electron transport chain in the mitochondrial inner membrane. Both species are involved in numerous oxidative-reductive reactions and are indicators of redox stress (16, 23, 32). The increase in NAD(P)H levels is a likely consequence of hypoxia caused by reduced capillary perfusion. For example, hepatic NAD(P)H levels are highly correlated with hypoxia in the liver (23, 32). Under hypoxic conditions, intracellular levels of NAD(P)H accumulate and redox potential increases. The decrease in capillary perfusion preceded the increase in NAD(P)H levels, suggesting that capillary dysfunction led to cellular stress. This was further supported by the finding that NAD(P)H levels returned toward basal levels as capillary perfusion tended toward recovery. Hypoxia due to capillary dysfunction is a likely explanation for these findings, because sluggish or stagnant microvascular flow can produce regions of hypoxia (23). These data demonstrate that peritubular capillary perfusion is a critical regulator of renal tubular stress. Three lines of evidence support this relation: 1) a significant shift in the percentage of vessels with continuous flow to vessels with intermittent or no flow occurred before the rise in NAD(P)H levels, 2) the degree of redox stress in tubular epithelial cells was directly correlated to the percentage of dysfunctional vessels, and 3) redox stress recovered as the percentage of vessels with compromised flow returned toward control levels. Although not specifically addressed by these studies, tubular redox stress may contribute to a fall in glomerular filtration rate, because tubular stress can reduce tubular transport and, thereby, activate the tubuloglomerular feedback system to reduce glomerular filtration rate.

Peritubular capillary dysfunction following LPS administration was a progressive process. The time-dependent shifts in the percentage of vessels with continuous flow to vessels with intermittent or no flow were greatest at 10 h. By 48 h, functional capillary density was only partially recovered. Delayed recovery of peritubular capillary function may be a characteristic of this microvascular bed, since delayed recovery is also observed after renal ischemia-reperfusion (33).

The contention that peritubular capillary dysfunction plays a major role in LPS-induced renal injury is supported by the observations that peritubular capillary dysfunction preceded the development of tubular redox stress and renal failure and that both tubular redox stress and renal failure recovered as functional peritubular capillary density returned toward control levels. However, despite a return to normal renal function at 48 h (as assessed by serum BUN and creatinine), overall capillary function had not fully recovered. The percentage of vessels with continuous flow was still decreased, and the percentage of vessels with intermittent flow was still increased, but the percentage of vessels with no flow had returned to control levels. Although our studies were limited to superficial cortical vessels, the data suggest that vessels with no flow appear to have the greatest impact on the development of renal failure, because vessels with no flow would be expected to produce most of the regional hypoxia.

Although LPS models of sepsis do not reproduce the hyperdynamic state associated with human sepsis, microvascular dysfunction has been reported in other tissues in polymicrobial models of sepsis (13, 14). Understanding the relations between the kidney microcirculation and tubular cell stress during sepsis may uncover new therapeutic targets for preventing sepsis-induced renal failure. The present studies are the first to demonstrate that renal microvascular defects contribute to renal epithelial cell stress following LPS exposure and provide compelling evidence that the renal microvasculature may play a critical role in the development of sepsis-induced renal injury.

ACKNOWLEDGMENTS

The authors thank Sandra McCullough for technical assistance.

GRANTS

This work was supported by a University of Arkansas for Medical Sciences Pilot Study Grant (P. R. Mayeux) and by the University of Arkansas for Medical Sciences Graduate Student Research Fund (L. Wu).

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