Evidence for the Role of Reactive Nitrogen Species in Polymicrobial Sepsis-Induced Renal Peritubular Capillary Dysfunction and Tubular Injury

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Acute kidney injury (AKI) remains a frequent and serious complication of human sepsis that contributes significantly to mortality. It is estimated to occur in 20 to 50% of patients with sepsis, and the mortality rate for these patients with renal failure approaches 70% (1,2). Sepsis-induced AKI is most common in the elderly patient in the intensive care unit, for whom current therapy is, for the most part, still primarily supportive (3). Therefore, there is a critical need to uncover new therapeutic approaches because the incidence of sepsis-induced AKI is predicted to increase as the population ages (4).

The timing of therapy in the patient with sepsis clearly affects outcome (3,5), particularly the development of AKI (1). A major hindrance to the advance of new therapeutic approaches is a lack of understanding regarding the temporal relationships between the evolution of the inflammatory response, mediator signaling, and end-organ failure. This is especially important because treatment in the patient with sepsis is generally begun only after the onset of symptoms (e.g., systemic inflammatory response syndrome, hypotension) (3).

Acute kidney injury (AKI) remains a frequent and serious complication of human sepsis that contributes significantly to mortality. For better understanding of the development of AKI during sepsis, the cecal ligation and puncture (CLP) murine model of sepsis was studied using intravital video microscopy (IVVM) of the kidney. IVVM with FITC-dextran was used to determine the percentage of capillaries with continuous, intermittent or no flow at 0 (sham), 10, 16, and 22 h after CLP. There was a dramatic fall in capillary perfusion as early as 10 h after CLP that persisted through 22 h. The percentage of vessels with continuous flow at 16 h decreased from 73 ± 2% in shams to 16 ± 2% (P < 0.05), whereas the percentage of vessels with no flow increased from 4 ± 1% in shams to 42 ± 2% (P < 0.05). The capillary perfusion defect preceded the rise in serum creatinine. IVVM with dihydorhodamine-123 was used to quantify in real time reactive nitrogen species (RNS) generation by renal tubules, and the inducible nitric oxide synthase inhibitor L-iminoethyl-lysine (mg/kg) was used to examine the role of inducible nitric oxide synthase inhibitor on capillary dysfunction and RNS generation. Tubular generation of RNS was significantly elevated at 10 h after CLP and was associated with tubules that were bordered by capillaries with reduced perfusion. L-iminoethyl-lysine significantly reversed the capillary perfusion defect, blocked RNS generation, and reduced AKI. These data show that capillary dysfunction and RNS generation contribute to tubular injury and suggest that RNS should be considered a potential therapeutic target in the treatment of sepsis-induced AKI.


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(iNOS)-derived NO is an important mediator of the hemodynamic failure that is associated with sepsis, but its role in CLP-induced AKI has never been examined. It has also been proposed that reactive nitrogen species (RNS) derived from iNOS may contribute to the development of sepsis-induced AKI. This notion is supported by the appearance of nitrated plasma proteins in the patient with sepsis and kidney injury (13) and the appearance of nitrated proteins and oxidation products in the kidney during LPS-induced renal injury in rats (14,15). Although the generation of RNS in the kidney after CLP has never been examined, the protective effects of anti-inflammatory antioxidants in CLP suggest that oxidants may play a role in CLP-induced AKI (10,16). Accordingly, these studies were undertaken to monitor, in real time, peritubular capillary perfusion using intravitral videomicroscopy (IVVM) and to establish whether RNS generation occurs in the peritubular capillary/tubular microenvironment during the development of CLP-induced AKI.

Materials and Methods
FITC-dextran 500,000 Da conjugate was purchased from Sigma-Aldrich (St. Louis, MO). Dihydorhodamine-123 (DHR) was purchased from Invitrogen (Eugene, OR). Rabbit polyclonal anti-nitrotyrosine and anti-iNOS antibodies were purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). 1-N^6-(1-iminoethyl) lysine (L-NIL) was purchased from Axxora (San Diego, CA).

CLP
CLP-induced AKI was established essentially as described by Miyaji et al. (10). 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 C57/BL6 mice (Harlan, Indianapolis, IN) at 39 to 40 wk of age were acclimated from 11 to 25%; 3, 26 to 45%; 4, 46 to 75%; and 5, >76%.

Assessment of Kidney Morphology
Paraffin-embedded sections (3 μm) were prepared from kidneys that were fixed in 10% phosphate-buffered formalin. The periodic acid-Schiff (PAS) stain was used for the analysis of morphology with light microscopy (Nikon E800; Melville, NY) by a blinded observer. A semi-quantitative score for tubular injury and acute tubular necrosis was assigned as described by Wang et al. (19). For each mouse, 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 dilatation were scored as follows: 0, none; 1, <10%; 2, 11 to 25%; 3, 26 to 45%; 4, 46 to 75%; and 5, >76%.

Immunohistochemistry
Paraffin-embedded tissue sections were cleared in xylene, rehydrated, and washed in PBS. Endogenous peroxidase activity and non-specific protein binding were blocked using reagents that were supplied in the DakoCytomation LSAB+ System-HRP kit (Dako, Carpinteria, CA). Sections were then incubated with rabbit anti-iNOS antibody (1:1000 dilution) or anti-nitrotyrosine antibody (1:400 dilution) overnight at 4°C. After washing in PBS, sections were incubated with the second antibody supplied by the kit at room temperature for 30 min. Sections were then incubated with streptavidin peroxidase for 30 min, then with chromagen solution as described by the manufacturer. Gill’s hematoxylin II was used as a counterstain. The negative control for detection of nitrotyrosine was preincubation of the antibody with 10 mM nitrotyrosine for 1 h before use.
Serum Nitrate/Nitrite Levels

Serum nitrate/nitrite (NOx) levels were determined using a Kamiya Biomedical assay kit (Seattle, WA) as directed by the manufacturer. Briefly, nitrate in serum samples was first converted to nitrite using nitrate reductase. Data were expressed as serum NOx concentration in μM.

Statistical Analyses

Data were analyzed with Prism 4.0 software for Mac (GraphPad Software, San Diego, CA). Data are expressed as means ± SEM. A one-way ANOVA followed by the Student-Newman-Keuls test was used to compare groups. For analysis of tissue injury scores, a one-way ANOVA followed by the Kruskal-Wallis test was used to compare groups. *P < 0.05 was considered significant. Each n represents data from one mouse.

Results

Time Course of Kidney Injury

Representative images of PAS-stained sections from sham-treated mice and mice at 10, 16, and 22 h after CLP are shown in Figure 1, A through D, respectively. Morphologic changes including loss of brush border, tubular cell sloughing, tubular dilation, and tubule vacuolization increased in severity over time. Serum creatinine concentration increased over time to a significant level at 16 h and remained elevated through 22 h (Figure 1E), indicating the development of functional renal injury after CLP.

Time Course of Disruption of Capillary Perfusion

Mice were subjected to IVVM with FITC-dextran to assess cortical peritubular capillary perfusion at 10, 16, or 22 h after CLP. These data are presented in Figure 2. Sham controls are indicated as 0 h. The percentage of vessels designated as having continuous flow, intermittent flow, or no flow at each time point (see the Materials and Methods section for details). Data are means ± SEM (n = 6 to 7 mice per time point). *P < 0.05 versus the sham group. †P < 0.05 versus 10 h.

10 h (*P < 0.05 compared with sham) and decreased further to 16 ± 2% at 16 h (*P < 0.05 compared with 10 h). The decrease in the percentage of vessels with continuous flow was accompanied by a significant increase in the percentage of vessels with no flow. At 16 h after CLP, the percentage of vessels with intermittent flow was significantly elevated also. The decrease in cortical peritubular capillary perfusion persisted through 22 h.

Imaging of Reactive Oxygen Species/RNS Generation

DHR is oxidized to fluorescence rhodamine by hydroxyl radical, nitrogen dioxide, peroxynitrite, and peroxidase-derived species (20). Therefore, the oxidation of DHR to rhodamine was used as an indicator of reactive oxygen species (ROS)/RNS generation. Rhodamine fluorescence was visualized in real time and localized using IVVM. Representative images of FITC-dextran and rhodamine fluorescence from the same field of view from the kidney of a mouse 16 h after CLP are shown in Figure 3. Capillaries with no flow were defined as having no detectable RBC movement during the 10-s video capture. In single-frame images, as in Figure 3, these capillaries may appear white (because of the lack of RBC movement), collapsed, or to contain stacked RBC. Single-frame images of capillaries with continuous perfusion generally appear as dark because of the movement of RBC that obscure FITC-dextran fluorescence. Rhodamine fluorescence was increased in tubules that were bordered by capillaries with compromised perfusion. Moreover, rhodamine fluorescence seemed to be most intense in discrete areas within the tubular epithelium. For further examination of this, the same fields of view from the kidneys of mice 16 h after CLP were subjected to IVVM to detect rhodamine fluorescence and epi-illumination to reveal surface contour. Shown in Figure 4 are representative images from the same field of view of rhodamine fluorescence (Figure 4A) and...
epi-illumination (Figure 4B) at 16 h after CLP. Increased rho-damine fluorescence was observed in what seemed to be surface tubule vacuoles. Figure 4C shows a representative PAS-stained kidney section from a mouse 16 h after CLP. The circle encloses to vacuoles in the tubular epithelium.

**Time Course of Systemic NO Generation and Tubular ROS/RNS Generation**

The time course of changes in serum NOx concentration and tubular rhodamine fluorescence are presented in Figure 5. Serum NOx concentration, an indicator of systemic NO synthesis, was significantly elevated at 10 h after CLP and remained elevated through 22 h (Figure 5A). Tubular rhodamine fluorescence was also significantly elevated at 10 h after CLP, paralleling systemic NO generation (Figure 5B). Values for mice that were subjected to sham surgery at each time point are presented to rule out the effects of surgery trauma on these parameters. Immunohistochemistry was performed to detect iNOS protein and nitrotyrosine-protein adducts, a marker of peroxynitrite generation, in kidney sections from sham mice and mice at 16 h after CLP. Specific staining for iNOS and nitrotyrosine was very weak in sham mice (Figure 5, C and E, respectively). In contrast, iNOS and nitrotyrosine staining were observed in the kidneys from mice at 16 h after CLP (Figure 5, D and F, respectively) in both glomeruli and tubules. Sections from the kidneys of mice at 16 h after CLP did not show staining for iNOS in the absence of primary antibody or staining for nitrotyrosine when the anti-nitrotyrosine antibody was preincubated with 10 mM nitrotyrosine before use (data not shown). Both IVVM and immunohistochemistry indicated that proximal tubules were the major site of RNS generation. Although our IVVM studies were not capable of imaging murine cortical glomeruli because they reside too deep within the cortex, immunohistochemistry revealed some staining for nitrotyrosine in the glomeruli and very weak staining in some distal tubules of the cortex.

**L-NIL Protects Against Capillary Dysfunction and Tubular ROS/RNS Generation**

To assess the effects of pharmacologic inhibition of iNOS on capillary perfusion and ROS/RNS generation after CLP, mice were treated with L-NIL (3 mg/kg, intraperitoneally) at the time of surgery and received a second dose at 6 h. Four groups of mice (sham, sham + L-NIL, CLP 16 h, and CLP 16 h + L-NIL) underwent IVVM with FITC-dextran to assess peritu-

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*Figure 3.* Imaging of reactive oxygen species/reactive nitrogen species (ROS/RNS) generation in renal tubules. Shown are representative images of perfusion (A) and rhodamine fluorescence (B) captured from videos of the same field of view from the kidney of a mouse 16 h after CLP. Arrows indicate capillaries with no perfusion. A pseudocolored image of B is shown in C to highlight changes in pixel intensity. Intense regions of rhodamine fluorescence are localized to discrete regions of the tubular epithelium bordered by capillaries with reduced perfusion.

*Figure 4.* ROS/RNS generation in injured tubules after CLP. Rhodamine fluorescence in A is localized in tubules with vacuoles and possible blebbing observed in the same field of view (B) using epi-illumination to reveal surface contour. Light microscopy in C from the PAS-stained kidney cortex at 16 h after CLP shows extensive vacuolization (within circle). Magnification, ×400.
bular capillary perfusion and DHR to assess ROS/RNS generation. The effects of L-NIL on peritubular capillary flow at 16 h after CLP is presented in Figure 6. The percentage of vessels with continuous flow in the CLP/L-NIL group was significantly higher compared with the CLP group (42/18 versus 18/3; \( P < 0.05 \)). L-NIL treatment also significantly reduced the percentage of vessels with no flow compared with the CLP group (21/40 versus 40/1; \( P < 0.05 \)) but did not affect the percentage of vessels with intermittent flow. L-NIL treatment did not affect the distribution of flow in sham mice.

For confirmation that the dosing regimen for L-NIL inhibited NO synthesis, serum NOx levels were measured. At 16 h after CLP, L-NIL significantly blocked the rise in serum NOx levels (Figure 7A), indicating induction of iNOS activity and the effectiveness of L-NIL treatment. The effects of L-NIL on rhodamine fluorescence are presented in Figure 7B. L-NIL treatment blocked the increase in rhodamine fluorescence that was observed in CLP-treated mice. These data suggest that after CLP, DHR oxidation (rhodamine fluorescence) is predominantly due to iNOS-derived RNS.

Figure 8 shows representative images that were captured from the kidney of mice after sham surgery (Figure 8, A and D), 16 h after CLP (Figure 8, B and E), and 16 h after CLP and treatment with L-NIL (Figure 8, C and F). Images of perfusion (Figure 8, A through C) and rhodamine fluorescence (Figure 8, D through F) are from the same fields of view, respectively. Arrows indicate capillaries with no perfusion. These images provide additional evidence to suggest a link between iNOS-derived NO and RNS generation and the increased generation of RNS by tubules in areas that were bordered by capillaries with reduced perfusion.

L-NIL Protects Against Renal Injury

Serum creatinine concentration and morphologic scoring were used to assess the effects of L-NIL treatment on renal injury. Mice that were treated with CLP + L-NIL had significantly lower serum creatinine than the CLP group, indicating that L-NIL treatment preserved renal function (Figure 9A). In addition, L-NIL showed protection as based on tubular injury score (Figure 9B).

Discussion

Microvascular dysfunction is believed to be a key feature of sepsis that contributes to end-organ failure (21). These studies establish that peritubular capillary perfusion is dramatically decreased during the course of CLP-induced sepsis. Furthermore, these studies are the first to demonstrate real-time generation of RNS by renal tubules and link decreased peritubular capillary perfusion to the generation of RNS and tubular injury. Moreover, studies with the iNOS inhibitor L-NIL suggest that iNOS-derived NO/RNS is critical to the development of capillary dysfunction and AKI after CLP.

It is recognized that endothelial injury can have detrimental effects on peritubular capillary flow and permeability barrier (22). Although several reports indicate that endothelial cells are targets of bacterial toxins (6) and RNS (23), the role of the peritubular capillary in the development of renal injury after sepsis is suspected but not fully elucidated. Studies by Yasuda et al. (7) were the first to suggest the involvement of the peritubular capillary in CLP-induced AKI. Peritubular capillary leakage, as assessed by Evans blue dye leakage, occurs as early as 6 h after CLP. Moreover, evidence of hypoxia (increase in pimonidazole-protein adducts) observed at 24 h after CLP...
might have resulted from decreased capillary flow (7). The striking decrease in peritubular capillary perfusion revealed by IVVM supports the notion that regional hypoxia may contribute to mitochondrial dysfunction and oxidant generation.

Decreased capillary perfusion coupled to capillary leak (7) suggests the occurrence of direct injury to the renal microvasculature after CLP. A unique feature of IVVM is the ability to monitor both capillary perfusion and tubular RNS generation in real time. These studies are the first to examine specifically the renal peritubular microenvironment and implicate RNS as a possible mediator of peritubular capillary dysfunction and tubular injury that are associated with CLP-induced sepsis. Pathophysiologic effects of NO are observed when excessive amounts are generated. For example, during iNOS induction, NO reacts with oxygen or superoxide to produce RNS that can initiate protein oxidation, lipid oxidation, and DNA damage (24,25). One RNS, peroxynitrite (ONOO\textsuperscript{-}), is formed from the chemical reaction between NO and superoxide (26) and can damage not only membranes but also nitrate protein tyrosine and change the activities of a number of important enzymes (27,28). Moreover, decreased capillary perfusion can lead to parenchymal hypoxia (29) and superoxide generation to fuel RNS synthesis (30). Therefore, it is likely that increased NO generation (as a result of iNOS induction) in the setting of a pro-oxidant, hypoxic microenvironment caused by decreased perfusion, exacerbates renal injury (31).

In models of AKI associated with iNOS induction, the contribution of RNS has drawn recent attention, particularly in ischemia/reperfusion injury and LPS-induced injury (32–35). These studies are the first to examine the potential therapeutic benefits of pharmacologic inhibition of iNOS in CLP-induced AKI. In other models of CLP-induced sepsis, pharmacologic inhibition of iNOS prevents the fall in BP and other hemodynamic changes in the rat (36), and transgenic mice that are deficient in iNOS maintain arteriolar responsiveness to catecholamines and show improved survival (37). The appearance of nitrated proteins and oxidation products in the kidney during LPS-induced renal failure in rats and the ability of iNOS
inhibitors to reduce these products and preserve renal function (14,15) suggest that iNOS-derived RNS are important mediators of renal injury. In these studies, L-NIL treatment ameliorated CLP-induced capillary dysfunction, morphologic injury, and renal dysfunction, suggesting a role for iNOS. Nevertheless, pharmacologic studies with iNOS inhibitors must be interpreted with caution because there are no absolutely selective iNOS inhibitors, and constitutive NOS isoforms may be induced during sepsis as well (38). L-NIL has been used extensively as an iNOS inhibitor and displays a 14-fold selectivity for iNOS compared with endothelial NOS (39). The dosage used in these studies was chosen because it did not significantly reduce basal serum NOx levels, suggesting a minimal effect on constitutive isoforms. Still, additional studies with more selective inhibitors such as 1400W (39) are needed to establish iNOS fully as an important therapeutic target.

Although induction of iNOS in inflammatory cells occurs during sepsis, sepsis-induced AKI is not associated with a significant influx of activated inflammatory cells to the kidney (10). Rather, kidney epithelial cells seem to be the major source of iNOS-derived NO in the kidney during sepsis (40). These studies extend these finding to suggest that renal tubular epithelial cells are perhaps the major source of NO-derived oxidants. Two independent methods (IVVM and immunohistochemistry) for detecting the generation of RNS both indicated the generation of RNS by renal tubules after CLP. Furthermore, both IVVM and immunohistochemistry revealed RNS generation in tubular vacuoles. The potential clinical relevance of these findings is supported by the detection of nitrated plasma proteins in patients with sepsis and renal failure (13). Although these studies do not establish cause–effect, it is reasonable to speculate that RNS generation contributed to tubular injury. Additional support for the role of oxidants comes from the findings that the anti-inflammatory antioxidant ethyl pyruvate is protective against CLP-induced AKI (10). It must be acknowledged that oxidation of DHR to rhodamine is not solely dependent on RNS. However, the ability of L-NIL to block completely the rise in rhodamine fluorescence in CLP-treated mice compared with sham mice suggests that RNS were the major oxidants generated after CLP. It is interesting that sham mice showed a basal DHR oxidation localized to tubules that were bordered by capillaries with reduced flow that was not inhibited by L-NIL. The absence of nitrotyrosine staining in sham mice suggests that generation of ROS rather than RNS was responsible for DHR oxidation in the absence of iNOS induction.

A rapid decline in peritubular capillary perfusion accompanied by tubular injury is also a feature of LPS-induced AKI (8,17). However, in the CLP model, the decline in capillary perfusion seems to be greater than in the LPS model, and tubular morphologic changes are more severe. Still, studies in both types of sepsis models show that a change in the peritubular microenvironment is a common feature. Sepsis-induced AKI has an extremely complex etiology. While mediators of the early response by the kidney may be different in the two models (9), iNOS-mediated peritubular capillary dysfunction and tubular injury occur in both models of sepsis-induced AKI (17). This suggests that common pathways are activated in the microenvironment after inflammatory stimuli that mediate tubular injury. From a clinical perspective, therapeutic agents that
target later events might be the most beneficial because downstream mediators may be relatively independent of upstream events and because therapy is generally started after initiation of sepsis (3).

Our findings have implications beyond sepsis-induced AKI. It is becoming increasingly clear that endothelial injury and peritubular capillary dysfunction may initiate and extend the pathogenesis of both acute and chronic renal injury (41–44) by compromising renal vascular responsiveness and tubule function (41, 45, 46). In addition, reductions in peritubular capillary perfusion causes localized hypoxia, which over time may lead to tubulointerstitial injury (47). We found that even in the absence of disease, localized decreases in peritubular capillary perfusion are associated with localized areas of oxidant generation. Therefore, even transient localized oxidant generation may sensitize the kidney to injury.

Conclusion
We have uncovered a previously unrecognized role for capillary dysfunction and tubular RNS generation in CLP-induced AKI. The close association between capillary dysfunction, RNS generation, and tubular damage suggests that the peritubular capillary/tubular microenvironment plays a critical role in sepsis-induced AKI. Furthermore, studies with the iNOS inhibitor L-NIL suggest that pharmacologic inhibition of iNOS and/or RNS should be considered as potential therapeutic approaches for the prevention of sepsis-induced AKI.

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Disclosures
None.

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