Interaction among nitric oxide, reactive oxygen species, and antioxidants during endotoxemia-related acute renal failure

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During endotoxemia, reactive oxygen species (ROS) have been shown to be increased in several species (5, 16, 27). The injurious effects of ROS are attenuated, at least in part, by antioxidants. Superoxide dismutase (SOD) is an endogenous antioxidant which can scavenge superoxide (O2·−) during circumstances of stress such as endotoxemia. There are three isoforms of endogenous SOD; however, extracellular SOD (EC-SOD) is the most abundant in the vasculature as well as in the kidney in mice (17). To date it is not known whether EC-SOD is altered in the kidney during endotoxemia. This is an important question because endotoxemia-related sepsis is frequently associated with acute renal injury and ROS may play a role in this renal failure.

In addition to increased generation of ROS, endotoxemia also results in the induction of nitric oxide synthase (iNOS) and increased circulating nitric oxide (NO) (9, 29). We hypothesized that renal EC-SOD may be downregulated during endotoxemia, resulting in increased O2·− This increase in O2·− may lead to increased scavenging of NO (22, 23). The reduction in bioavailable NO in the renal vasculature may lead to renal vasoconstriction and ultimately to acute renal failure (ARF). To test this hypothesis, the present study was undertaken in a mouse model of endotoxemia ARF. We determined 1) renal EC-SOD mRNA and protein expression during endotoxemia, 2) the effect of antioxidant administration on renal function during endotoxemia, and 3) whether specific iNOS inhibition reverses any renal protective effect of antioxidant treatment during endotoxemia.

MATERIALS AND METHODS

Animals. The experimental protocol was approved by the Animal Ethics Review Committee at the University of Colorado Health Sciences Center. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Male mice aged 8–10 wk were used throughout the study. Mice were

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maintained on a standard rodent chow and had free access to water.

Materials. Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified. Manganese (III) mesotetakis[N-ethylpyridinium-2-yl] porphyrin (MnTE-2-PyP) was a kind gift of Incara Pharmaceuticals (Research Triangle Park, NC).

Measurement of renal blood flow, glomerular filtration rate, and mean arterial pressure. The animals were anesthetized with pentobarbital sodium (60 mg/kg) and placed on a thermostatically controlled surgical table. A tracheotomy was performed, at which time a steady stream of 100% oxygen was blown over the tracheal tube throughout the experiment. Catheters (custom pulled from PE-250) were placed in the jugular vein for maintenance infusion and the carotid artery for blood pressure determinations. The kidney was exposed by a left subcostal incision and was dissected free from perirenal tissue, and renal arteries were isolated for the determination of renal blood flow (RBF) using a blood flow-meter and probe (0.5; Transonic Systems, Ithaca, NY) as described by Traynor (28). Mean arterial pressure (MAP) was measured via a carotid artery catheter connected to a Transpac IV transducer and monitored continuously using Windaq Waveform recording software (Dataq Instruments). An intravenous maintenance infusion of 2.25% BSA in normal saline (NS) at a rate of 0.25 μg·body wt·min⁻¹ started 1 h before experimentation. FITC-inulin (0.75%) was added to the infusion solution for the determination of glomerular filtration rate (GFR) as described by Lorenz et al. (13). A bladder catheter (PE-10) was used to collect urine. Two 30-min collections of urine were obtained under oil and weighed for volume determination. Blood for plasma inulin determination was drawn between urine collections. FITC in plasma and urine samples was measured using a CytoFluor plate reader (PerSeptive Biosystems, Foster City, CA).

Western blot analysis. Whole kidney lysate was mixed with sample buffer containing 50 mM Tris, 0.5% glycerol, 0.01% bromophenol blue, and 0.75% SDS (pH 6.8). Identical amounts of protein were fractionated by a 4–15% Tris/glycine polyacrylamide gradient gel (EC-SOD determination) or 15% polyacrylamide separating gel (MnSOD and Cu/ZnSOD determinations) and transferred to a nitrocellulose membrane (Millipore, Bedford, MA). Membranes were blocked subsequent incubation at 4°C with 5% milk in TTBS [50 mM Tris, 150 mM NaCl, 0.1% Tween 20 (pH 7.5)] at room temperature for 60 min and were exposed to X-ray film.

Measurement of plasma NO levels. Plasma NO levels were determined by measuring plasma NO₂/NO₃ levels using a nitrate/nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, MI).

Statistical analysis. Values are expressed as means ± SE. Multiple comparisons were assessed by ANOVA using a post hoc Newman-Keuls test. Survival analysis was analyzed by the Kaplan-Meier method. P < 0.05 was considered statistically significant.

RESULTS

Normotensive endotoxemic ARF model in mice. Mice were injected intraperitoneally with 5 mg/kg LPS (Escherichia coli 026:B6, Sigma), a relatively low and nonlethal dose of LPS that permitted surgery and physiological measurements without excessive mortality. With this dose of LPS, there was no significant change in MAP (82 ± 0.8 vs. 82 ± 2.2 mmHg, n = 6, P = not significant), thus allowing measurement of renal function in the absence of hypotension.

Effect of LPS on SOD expression in the kidney. Western blot analysis was used to examine all three isoforms of SOD protein expression in the mice kidneys. In control mice, there was high expression of EC-SOD in the control kidney, but the expression was significantly decreased in mice treated with LPS at 16 h (Fig. 1A). The relative densitometry was 2.6 ± 0.13 vs. 1.2 ± 0.05 (n = 6, P < 0.01, Fig. 1B). Two bands of ~34 and 32 kDa can be observed in both groups. These represent intact and proteolytically processed forms of EC-SOD, respectively (4, 6). The top band (the intact form) is much stronger than the bottom band (proteolytic form) in vehicle-treated mice, whereas the two bands are similar in density in the kidney of LPS-treated mice. The ratio between the two bands is 2.97 ± 0.12 in the control group vs. 1.27 ± 0.07 in the LPS-treated group (n = 6/group, P < 0.01). When MnSOD and Cu/ZnSOD protein expressions in the kidney were examined, there is no difference between the control and LPS-treated group (Fig. 2, A and B).

Effect of LPS on EC-SOD mRNA expression in the kidney in mice. To examine whether the decreased EC-SOD protein expression was regulated at the transcriptional level, EC-SOD mRNA in the kidney was measured using an RNase protection assay. As shown in Fig. 3, EC-SOD mRNA was decreased in the kidney in endotoxemic mice, thus corresponding to EC-SOD protein expression.
Effect of MnTE-2-PyP on GFR, RBF, and MAP in endotoxemic mice. MnTE-2-PyP (10 mg/kg) or vehicle (NS) was injected intraperitoneally 30 min before LPS (5 mg/kg) (Fig. 4). At 16 h after LPS injection, GFR, MAP, and RBF were examined in control mice and MnTE-2-PyP-treated mice. There was no difference in MAP between vehicle- and MnTE-2-PyP-treated endotoxemic mice. Sixteen hours after LPS injection, there were significant decreases in GFR (50 ± 49 vs. 229 ± 21 μl/min, n = 8, P < 0.001) and RBF (0.61 ± 0.10 vs. 0.86 ± 0.05 ml/min, n = 8, P < 0.05) compared with vehicle-treated controls (NS). The decreased GFR and RBF were dramatically reversed by MnTE-2-PyP, which brought GFR and RBF up to 182 ± 40 μl/min (n = 6, P < 0.01 vs. vehicle-treated controls) and 1.08 ± 0.10 ml/min (n = 6, P < 0.05 vs. vehicle-treated controls), respectively. The above experiments were performed when MnTE-2-PyP was injected before LPS. Further experiments were undertaken, in which MnTE-2-PyP was administered 6 h after LPS administration. In these experiments, GFR was partially protected (85 ± 10 vs. 37 ± 12 μl/min of vehicle-treated controls, n = 8, P < 0.05).

Effect of MnTE-2-PyP on mortality in endotoxemia. A large dose of LPS (30 mg/kg) was injected intraperitoneally, and the mortality of MnTE-2-PyP (10 mg/kg ip 30 min before LPS)- vs. vehicle-treated mice was examined for 48 h. The 24-h survival rate was 0% in vehicle-treated mice (n = 18), whereas it was 50% in MnTE-2-PyP-treated mice (n = 8, P < 0.05, Fig. 5).

Effect of iNOS inhibition with L-N6-(1-iminoethyl)lysine on the protective effect of MnTE-2-PyP during...
endotoxemia. L-N\textsuperscript{6}-(1-iminoethyl)-lysine (L-NIL; 10 mg/kg ip, Alexis Biochemicals, Carlsbad, CA), a selective inhibitor of iNOS (14), was administered 30 min before LPS (5 mg/kg ip) either alone or with MnTE-2-PyP (10 mg/kg ip). As shown in our previous study (9, 29), plasma NO levels were significantly higher in endotoxemic mice compared with control mice (227 ± 16 vs. 2.5 ± 0.4 μM, n = 6; P < 0.01). This high plasma NO level decreased significantly after the treatment with L-NIL (51 ± 4 vs. 227 ± 16 μM, n = 6, P < 0.01). When L-NIL was administered with MnTE-2-PyP, it abolished the renal protective effect of MnTE-2-PyP during endotoxemia because GFR decreased from 138 ± 8 to 49 ± 9 μl/min (n = 4, P < 0.01, Fig. 6A) and RBF decreased from 1.05 ± 0.09 to 0.55 ± 0.08 ml/min (n = 4, P < 0.05, Fig. 6B), respectively.

Effect of tempol on renal function in endotoxemic mice. To examine whether the protective effect of MnTE-2-PyP was specific to superoxide scavenging, the effect of another superoxide dismutase, tempol (Calbiochem Biscience, La Jolla, CA), was also examined during endotoxemia. LPS (E. coli 0111:B4, 2.0 mg/kg, LIST Biological Laboratories, Campbell, CA) was used in this study. This LPS compound is purer and more potent than the LPS from Sigma. The 2 (LIST) and 5 mg/kg (Sigma) dosages resulted in comparable effects on GFR and RBF. Similar to what was observed with MnTE-2-PyP, tempol significantly improved both RBF (1.21 ± 0.05 vs. 0.67 ± 0.04 ml/min, n = 4, P < 0.01) and GFR (102 ± 8 vs. 58 ± 11 μl/min, n = 7, P < 0.01). These protective effects were also reversed by L-NIL because RBF (1.21 ± 0.05 vs. 0.85 ± 0.05 ml/min, n = 4, P < 0.01) and GFR (102 ± 8 vs. 60 ± 11 μl/min, n = 4, P < 0.01) decreased significantly with the administration of L-NIL (10 mg/kg) with tempol compared with tempol alone during endotoxemia.
DISCUSSION

In several studies, sepsis has been identified as the most common cause of ARF (1, 11). Moreover, the combination of sepsis and ARF is associated with mortality as high as 60–80%. However, there is no consensus either about the pathophysiology of sepsis-related ARF or the appropriate treatment.

The present study was therefore undertaken to examine the pathophysiology of endotoxemia-related ARF in a normotensive mouse model. In this model, the endotoxemia was associated with a progressive increase in NO (9, 25, 29, 30), an effect that does not occur in iNOS knockout mice (10). Sepsis is also known to increase $O_2^-$, a known scavenger of NO (22, 23).

To focus on the potential effect of ROS in endotoxemia-related ARF, the role of endogenous and exogenous ROS scavengers was examined. EC-SOD, a secreted endogenous antioxidant enzyme, is the predominant form of SOD in the vasculature and is highly expressed in mouse kidneys (8, 17, 19). In the present study, endotoxemia was associated with a decrease in renal EC-SOD, an effect that could lead to increased $O_2^-$ and enhanced scavenging of NO during endotoxemia (18). The decrease in renal EC-SOD protein was associated with diminished EC-SOD mRNA as assessed by an RNase protection assay, thus indicating an effect mediated by either decreased transcription or decreased mRNA stability. In contrast to EC-SOD, neither mitochondrial (MnSOD) nor cytosolic (Cu/ZnSOD) antioxidants were affected in our model. There was also evidence for a posttranslational effect on EC-SOD, because EC-SOD cleavage products were observed during endotoxemia.

Further study of the role of ROS during endotoxemia was undertaken by examining the effect of a potent exogenous antioxidant, MnTE-2-PyP, in endotoxemia-related ARF. The antioxidant properties of this agent include scavenging $O_2^-$, $H_2O_2$, and ONOO$^-$ (2, 3, 20). The administration of this antioxidant before LPS was associated with a highly significant improvement in both GFR and RBF during endotoxemia. Renal protection could also be demonstrated when the antioxidant was administered 6 h after LPS. A significant decrease in mortality at 24 h was also observed when the antioxidant was administered with an otherwise uniformly fatal dose of LPS (30 mg/kg).

An increase in NO, secondary to decreased scavenging by ROS, could contribute to the beneficial effect of the antioxidant against the endotoxin-induced renal vasoconstriction and ARF. To examine whether some of the protective effect of the antioxidant was due to increased bioavailability of NO, the potent antioxidant was administered in combination with the specific inhibitor of iNOS. The administration of L-NIL decreased plasma NO and reversed the renal protective effect of the antioxidant on GFR and RBF, thus supporting a vascular protective effect of NO. Similar results were also observed when tempol, a chemically dissimilar superoxide dismutase, was studied.

This role of endogenous and exogenous antioxidants in modulating endotoxemia-related renal vasoconstriction by enhancing the bioavailability of NO has implications for the early phase of endotoxemia (24). The early phase of this normotensive mouse model of endotoxin-induced ARF has been shown to be associated with activation of the sympathetic and renin-angiotensin systems (29). The systemic pressor effects of these events counterbalance the systemic vasodilatory effects of NO during endotoxemia and thereby support MAP. Nevertheless, these events occur at the expense of renal vasoconstriction. This sequence of events has been supported by demonstrating a renal protective effect of acute renal denervation in this normotensive endotoxemic model of ARF (29). The present results provide further understanding of the early events that occur during endotoxin-related ARF.

The increase in NO, which results from the endotoxin-related induction of NOS, is scavenged by the increased $O_2^-$. Although this decrease in NO may attenuate the systemic vasodilation, the present results suggest that NO bioavailability is important in countering renal vasoconstriction during early endotoxemia. This in vivo effect of iNOS-related NO may override any downregulation of constitutive nitric oxide synthase in the kidney, as suggested by in vitro studies in the rat (25). However, at a later stage, an injurious effect of iNOS-induced NO on tubules may be observed (12, 15, 21, 28). The role of the cytokine TNF-$\alpha$ has also been implicated in this early phase of endotoxemia-related ARF (10) and therefore would be expected to contribute not only to the induction of NOS but also to enhanced ROS activity (7).

In summary, the early ARF in endotoxemia involves a complex sequence of events leading to renal vasoconstriction. The present results demonstrate that the predisposition to renal vasoconstriction during endotoxemia involves a downregulation of renal EC-SOD with resultant scavenging of bioavailable NO by ROS. Antioxidant treatment by chemically dissimilar compounds exhibited an impressive amelioration of the endotoxin-mediated decrease in GFR and RBF. Increased NO bioavailability appears to be involved because the beneficial effect of antioxidants was reversed by the specific inhibition of iNOS. Scavenging of renal iNOS-related NO by ROS thus appears to be an important factor in the renal vasoconstriction associated with early (16 h) endotoxemia in mice.

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J. D. Crapo and B. J. Day are consultants for and hold equity in Incara Pharmaceuticals.

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