Role of heme oxygenase-1 in endotoxemic acute renal failure

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APPROXIMATELY 700,000 PATIENTS develop sepsis each year with 210,000 fatalities (2). When sepsis is associated with acute renal failure (ARF), the mortality rate approaches 70% (8). The pathogenesis of septic acute renal failure (ARF) involves systemic vasodilatation with compensatory upregulation of vasoconstrictors. This can lead to renal vasoconstriction and ARF. Heme oxygenase (HO) is the rate-limiting step in heme metabolism and produces carbon monoxide (CO) and biliverdin. HO-1 is an inducible form of the enzyme and is expressed in response to cell injury. It was hypothesized in endotoxemia, induction of HO-1 would lead to increased production of the vasodilator CO, lower blood pressure, and decrease renal function. The role of HO-1 was therefore examined in a mouse model of endotoxemia. One group of mice received LPS alone and were compared with mice that received LPS in addition to an inhibitor of HO-1, zinc protoporphyrin (ZnP). Treatment of mice with LPS resulted in significant increases in the protein expression of HO-1 compared with controls treated with vehicle. Immunohistochemical analysis localized this upregulation to both the proximal and distal tubules as well as the vasculature. Hemodynamic studies were performed during endotoxemia and the mean arterial pressure (MAP) was found to be significantly higher in the HO-1 inhibitor-treated compared with vehicle-treated mice (78 ± 3 vs. 64 ± 2 mmHg, P < 0.01). It was found that the inhibitor group had higher renal blood flows (RBF) also during endotoxemia and the glomerular filtration rate (GFR), as measured by inulin clearance, was 80262 (e-mail: Robert.schrier@uchsc.edu).

Heme oxygenase (HO) is the rate-limiting step in the metabolism of heme-containing proteins (13, 24, 25). There are two isoforms of HO with HO-1 being the inducible form that is upregulated by varied stimuli including lipopolysaccharide and nitric oxide (NO) (4, 5, 9, 11, 20, 23, 29). HO-2 is constitutively expressed and involved in heme metabolism. The action of HO leads to the production of biliverdin, a known potent antioxidant and carbon monoxide (CO) (23). Production of CO is known to induce vasodilation via its binding soluble guanylate cyclase that results in production of cGMP (16). Furthermore, CO has been shown to decrease the production of vasoconstrictors such as endothelin-1 as well as decrease sensitivity to vasoconstrictors (15). Metalloporphyrins such as zinc protoporphyrin competitively inhibit the action of HO and have been used to investigate the role of HO in toxic and ischemia-related ARF.

In models of ARF including toxic injury with glycerol-induced ARF and ischemia reperfusion-related ARF, the induction of HO-1 has been shown to be protective as inhibition of the enzyme exacerbated the injury (18, 21). However, in these models, it is likely that the cytoprotective effects of HO predominated. In contrast, in a hemodynamically mediated model of ARF, the vascular effects of CO may prove to be more important. In a model of normotensive, endotoxemic ARF, the upregulation of vasoconstrictors, including the rennin-angiotensin and norepinephrine, and the protective effect of renal denervation of renal function implicated the importance of renal vasoconstriction in this model of ARF (27). We therefore hypothesized that inhibiting the hemodynamic effects induced by the generation of CO with ZnP in endotoxemia-related ARF would result in decreased renal vasoconstriction and improved renal function.

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

Materials. Lipopolysaccharide (LPS) was purchased from Liposan (Campbell, CA). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Western blot analysis of HO-1 expression. Kidneys were homogenized in 250 mM sucrose, 25 mM imidazole, 1 mM ethylenediaminetetraacetic acid, and 1/10 vol of a protease solution consisting of 25 μg/ml antipain, 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 0.1 mg/ml soybean trypsin inhibitor, and 200 μM phenyl-

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methylsulfonyl fluoride. After homogenization, samples were sonicated and spun at low speed to clear debris, and the supernatant was frozen at −80°C until assayed for protein content. SDS-PAGE/immunoblotting was performed on 25 μg of extract. Samples were electrophoresed through a 12% acrylamide gel for detection of HO-1 (StressGen Biotechnologies, Victoria, British Columbia, Canada; molecular mass = 30 kDa). HO-1 protein was detected using a rabbit polyclonal antibody (StressGen Biotechnologies) diluted 1:1,000 in Tris-buffered saline + 0.1% Tween 20 containing 5% dry milk. The secondary antibody (donkey anti-rabbit conjugated to horseradish peroxidase) was diluted 1:1,000 in Tris-buffered saline + 0.1% Tween 20 containing 5% dry milk. Antigenic detection was made by enhanced chemiluminescence (Amersham, Arlington Heights, IL) with exposure to X-ray film.

**Immunohistochemical detection of HO-1.** Tissue samples were fixed in 4% formaldehyde solution for 24 h, dehydrated, embedded in paraffin, and cut into 3-μm-thick slices. Immunohistochemical staining was performed using the avidin-biotinylated peroxidase method. After deparaffinization and rehydration, sections were pretreated with 0.3% hydrogen peroxidase in 70% methanol to exhaust endogenous peroxidase activities. Sections were preincubated with 10% horse serum, then incubated with the rabbit HO-1 antibody at a 1:200 dilution at 37°C for 1 h. Slides were washed and incubated with biotinylated secondary antibody, goat anti-rabbit immunoglobulin G, at a 1:400 dilution in phosphate-buffered saline. After treatment of the slides with an Elite ABC kit (Vector Laboratories, Burlingame, CA), antigens were visualized with the Sigma fast 3,3-diaminobenzidine tablet system and applied on the slides for 5 min. Counterstaining was performed with hematoxylin.

**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, and an endotracheal tube was inserted. 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 flowmeter and probe (0.5v; Transonic Systems, Ithaca, NY). Mean arterial pressure (MAP) was measured continuously using Windaq Waveform recording software. An intravenous maintenance infusion of 2.25% BSA in normal saline (NS) at a rate of 0.25 μL·g body wt−1·min−1 was 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 and Gruenstein (12). 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 (Perceptive Biosystems, Foster City, CA).

**RESULTS**

**Renal iNOS and HO-1 during endotoxemia.** To assess the expression of HO-1 in the kidney during endotoxiaemia, C57BL/6 mice received intraperitoneal injections of LPS (2.5 mg/kg). At 16 h, the expression of HO-1 as well as iNOS was found to be significantly greater in the kidneys of endotoxemic mice compared with control animals treated with vehicle (Fig. 1). The administration of the HO-1 inhibitor, ZnPP, had no effect on iNOS but upregulated HO-1 protein expression.

**Immunohistochemical localization of HO-1.** To further define the role of HO-1 in endotoxemic ARF, immunohistochemical studies were undertaken to localize the upregulation of HO-1 anatomically as well as determine the effect of treatment with the inhibitor of HO-1, ZnPP. It was found that during endotoxiaemia, HO-1 is upregulated throughout both proximal and distal tubules in the cortex as well as the medulla. However, there was inadequate renal vasculature in the samples to make any definitive statement about HO-1 protein expression in the renal vasculature. As with the HO-1 protein expression (Fig. 1), the immunohistochemical studies demonstrated an upregulation of HO-1 with ZnPP treatment (Fig. 2).

**Effect of inhibition of HO-1 on systemic and renal hemodynamics.** To determine the role of HO-1 in endotoxemic ARF, a metalloporphyrin inhibitor of HO-1 was employed. ZnPP (25 mg/kg) was injected intraperitoneally 30 min before the administration of LPS. This dose of metalloporphyrin was chosen because it has been found to have minimal effects on the activity of nitric oxide synthase (NOS) (17, 30). At 16 h of endotoxiaemia it was found that the MAP was significantly elevated in the mice treated with ZnPP (78 ± 3 vs. 64 ± 2 mmHg, P < 0.01; Fig. 3A). Furthermore, the ZnPP-treated group exhibited a significant increase in RBF compared with

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**Fig. 1.** Renal HO-1 (A) and iNOS (B) protein expression during endotoxemia. Western blot was used to examine HO-1 and iNOS protein expressions in the kidney in the control and endotoxemic mice with or without ZnPP. Kidneys were harvested at 16 h after LPS (2.5 mg/kg) administration. Rabbit polyclonal antibodies were used as the primary antibody and protein bands were detected using ECL method.

**Fig. 2.** Immunohistochemical detection of HO-1 in the kidney. Kidneys from vehicle control (left), LPS treated (middle), and LPS plus ZnPP-treated mice (right) were harvested 16 h after LPS treatment. Tissues were fixed in 4% formaldehyde solution and paraffin embedded. They were then stained using the avidin-biotinylated peroxidase method. A rabbit HO-1 antibody was used as primary antibody and a biotinylated goat anti-rabbit IgG was used as secondary antibody. Counterstaining was performed with hematoxylin.
vehicle-treated, endotoxemic mice (1.83 ± 0.21 vs. 0.68 ± 0.08 ml/min, P < 0.01; Fig. 3B). In keeping with these results, when the RVR was calculated, there was a significant decrease in the ZnPP-treated group (43.5 ± 3.4 vs. 95.9 ± 11.3 mmHg·ml⁻¹·min⁻¹, P < 0.01; Fig. 3C). The plasma renin activities were significantly lower in the hemeoxygenase inhibitor group (22.7 ± 1.9 vs. 32.5 ± 3.0 ng·ml⁻¹·h⁻¹, P < 0.05) and thus could have contributed to the renal vasodilation. A functional significance of these renal hemodynamic differences was supported by comparing the GFR of the two groups. In the ZnPP-treated group, the GFR was elevated at 111.7 ± 19.5 µl/min compared with the control group with a GFR of 66.2 ± 3.5 µl/min (P < 0.05; Fig. 3D).

**DISCUSSION**

The pathogenesis of experimental ARF secondary to endotoxemia involves impaired hemodynamics with compensatory upregulation of vasoconstrictor systems and renal vasoconstriction. However, the ARF also has been shown to be mediated by oxidant injury and can be ameliorated by a superoxide mimetic (28). Thus both hemodynamic and prooxidant injury of the kidney appear to be involved in experimental ARF secondary to endotoxemia. In this regard, HO-1 could affect both of these aspects of endotoxemia-related renal injury. HO-1 has been shown in toxic (1, 22) and ischemic (14, 21) models of ARF to have cytoprotective effects by the degradation of the toxic, prooxidant heme moiety as well as the production of the antioxidant bilirubin (23). Conversely, the induction of HO-1 results in the production of CO which has vasodilatory properties. CO binds to and activates the enzyme sGC resulting in cyclic GMP generation and vasodilation. The hemodynamic effects of CO however are complicated, as high levels of CO may bind to NOS and inhibit the production of NO. CO may also decrease the vascular reactivity to vasoconstrictors. On the other hand, lower concentrations of CO may induce release of NO from intracellular stores and thus mimic the vascular effects of NO (26). In either case, the results indicate that inhibiting HO-1 with ZnPP would be expected to decrease CO and its associated vasodilatory effect. This interpretation is supported by the increase in MAP, and thus renal perfusion pressure, with the associated rise in RBF and GFR during ZnPP administration. We can, however, not exclude a direct effect of CO on RBF.

The present study was undertaken to define whether the inhibition of HO-1 may be involved in endotoxemia-related ARF by providing cytoprotection or worsening the injury by effects on systemic hemodynamics. In previous studies, HO-1 knockout mice were more predisposed to organ injury with LPS than with the use of HO-1 inhibitor in wild-type mice (19). In the present study, the beneficial hemodynamic effects of HO-1 inhibitor predominated over the cellular protective effect of HO-1 during endotoxemia in wild-type mice. Results with inhibition of HO-1 indicated that induction of the HO-1 enzyme during endotoxemia led to a series of hemodynamic changes that culminated in the deterioration of renal function. Specifically, endotoxemic mice treated with a metalloporphyrin inhibitor of HO-1 exhibited significantly higher MAP as well as higher levels of RBF and decreased RVR. These hemodynamics were associated with improved renal function as assessed by GFR. Therefore, in this mouse model of endotoxemic ARF, the deleterious effects of further hemodynamic compromise by HO-1 were more important than any cytoprotective effects HO-1 may confer. The capacity of this HO-1 inhibitor has also been shown to reverse the hemodynamic perturbations associated with experimental cirrhosis (6).

In addition, the immunohistochemical studies have localized the upregulation of HO-1 throughout the cortex and medulla in both proximal and distal tubules as well as the vasculature. Despite the significant upregulation of HO-1 in the tubules, any cytoprotection was masked by the increase in renal vasoconstriction. In the HO-1 inhibitor-treated group, there was also an increase in the level of HO-1 protein expression that may be
related both to decreased renal injury associated with improved renal and systemic hemodynamics and a further upregulation of HO-1.

In conclusion, in experimental endotoxemic ARF, inhibiting HO-1 with the metalloporphyrin ZnPP resulted in protection of renal and systemic hemodynamics and a further upregulation of HO-1 with the metalloporphyrin ZnPP. Protection was associated with significantly improved systemic hemodynamics, less renal vasoconstriction, and a higher GFR.

GRANTS

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REFERENCES