Attenuation of renal ischemia-reperfusion injury in inducible nitric oxide synthase knockout mice

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Recent studies suggest that nitric oxide (NO) is involved in ischemic and hypoxic tubular injury in vivo and in vitro (4, 14, 16, 29). NO may react with superoxide to generate peroxynitrite, which is capable of nitratating tyrosine residues of proteins and enzymes (4, 16). In this regard, there is support for a role of inducible nitric oxide synthase (iNOS)-generated NO and peroxynitrite formation in the pathogenesis of ischemic acute renal failure (ARF). However, these studies were performed in different species with somewhat different results. Noiri et al. (16) demonstrated in a rat model of ischemic ARF that iNOS was induced in the kidneys along with a subsequent increase of renal cortical tubule nitrite/nitrate production. These increases were effectively blocked by antisense deoxynucleotides targeting iNOS. In studies by Chiao et al. (4), the prevention of iNOS induction by α-melanocyte-stimulating hormone during renal ischemia-reperfusion injury in mice was also associated with functional protection. In this mouse model of ischemia, most of the tubular necrosis and iNOS induction occurred in the proximal straight tubule in the outer stripe of the medulla. In contrast to the study by Noiri et al. (16), this study could not detect iNOS protein in the renal cortex during ischemia.

Based on these reports implicating iNOS and NO in ischemic ARF, we proposed that animals lacking the gene for iNOS may be protected from ischemia-reperfusion injury. Therefore, the present study was designed to examine the protection against ischemic ARF in iNOS knockout mice and to further define the mechanisms of this protection.

MATERIALS AND METHODS

Animals. iNOS knockout mice were generously provided by Drs. John Mudgett (Merck Research Laboratories, Rahway, NJ) and Carl Nathan (Cornell University, New York, NY). The proximal 585 bases of the iNOS promoter, a region required for iNOS expression in macrophages, plus exons 1–4, including the ATG translational start site in exon 2, had been deleted (15). Northern and Western blots confirmed the successful deletion of the specific iNOS gene (15). We also have used RT-PCR analysis to examine iNOS mRNA expression in aortic tissues from both wild-type and iNOS knockout mice treated with lipopolysaccharide (LPS) (14) and confirmed the absence of iNOS gene expression in these iNOS knockout mice. The same background mice (B6/129-F2) served as wild-type controls (Jackson Laboratories, Bar Harbor, ME).

Surgery and experimental protocol. Mice 6–8 wk of age and weighing 16–18 g were anesthetized with an intraperitoneal injection of Avertin (2,2,2-tribromoethanol; Aldrich, Milwaukee, WI). The mice were placed on a micropuncture heating pad kept at 39°C to maintain constant body temperature. A midline incision was made, and renal arteries and veins were bilaterally occluded for 26 min with microaneurysm clamps. The time of ischemia was chosen to maximize reproducibility of renal functional impairment, while minimizing mortality in these animals. To help maintain thermoregulation during surgery, the abdominal contents were replaced and the abdomen was temporarily closed with several sutures. After 26 min of renal ischemia, the abdomen was reopened and clamps were removed. The kidneys were again inspected for restoration of blood flow, and 0.5 ml of prewarmed (37°C) normal saline was instilled into the abdominal cavity. The abdomen was closed in two layers. Sham-treated animals underwent the same surgical procedure as the ischemic mice, including dissection of the renal pedicle; however, renal clamps were not applied to the pedicle.
Animals were killed at 24 and 48 h after renal ischemia-reperfusion injury or sham surgery. At the time of death, blood was collected by heart puncture for measurement of serum creatinine. Both kidneys were harvested for histological study and immunoblot analysis.

Creatinine level. Creatinine was measured using an Astra Autoanalyzer (Beckman Instruments, Fullerton, CA).

Histological examination. Paraformaldehyde (4%)-fixed and paraffin-embedded kidney sections were stained with both hematoxylin-eosin and periodic acid-Schiff (PAS). Histological changes were mainly evaluated by quantitative measurement of tubular necrosis, which was assessed by counting the number of necrotic cells, loss of brush border, cast formation, and tubule dilation as follows: 0 = none, 1 = <10%, 2 = 11–25%, 3 = 26–45%, 4 = 46–75%, and 5 = >76%. At least 10 fields were reviewed for each slide. All evaluations were made on coded sections without knowledge of the experimental group to which the mice belonged.

Immunoblot analysis. Renal cortex was homogenized in lysis buffer (in mM: 5 Na2HPO4, 5 NaH2PO4, 150 NaCl, 1 EDTA, 1 EGTA, 0.1% Triton X-100, 50 NaF, and 0.2 Na3VO4, and 0.1% β-mercaptoethanol, pH 7.2) plus protease inhibitors (100,000 μM/ml aprotinin, 20 μM leupeptin, 20 μM pepstatin A, and 200 μM phenylmethylsulfonyl fluoride). The tissue was subjected to three freeze-thaw cycles followed by homogenization with a low-clearance Teflon pestle. The homogenates were centrifuged (3,000 g at 4°C for 5 min) to remove unbroken cells and debris. Supernates were mixed with sample buffer containing 50 mM Tris-base (pH 6.8), 0.5% glycerol, 0.01% bromphenol blue, and 0.75% SDS and heated at 95°C for 5 min. Equal amounts of protein (50–450 μg/lane) were fractionated by Tris-glycine-SDS-7.5% PAGE. Proteins were then transferred to a nitrocellulose membrane (Millipore, Bedford, MA) by wet electroblotting for 90 min. The membranes were blocked with 5% milk in TBST (50 mM Tris pH 7.5, 150 mM NaCl, and 0.1% Tween 20) buffer at pH 7.5 for 1 h at room temperature. Immunoblot analyses were performed with the following antibodies: two anti-iNOS polyclonal antibodies (one was a gift from Dr. Bruce Kone at the University of Texas-Houston Trauma Research Center and the other was purchased from Upstate Biotechnology, Lake Placid, NY), an anti-iNOS monoclonal antibody (Transduction Laboratories, Lexington, KY), anti-heat shock protein (HSP)-72 and anti-HSP-73 monoclonal antibodies (StressGen, Victoria, Canada), and anti-nitritrosylsine polyclonal and tubudinal antibodies (Upstate Biotechnology). The membranes were incubated with the antibodies at 1:1,000 dilution in TBST buffer at 4°C for 12 h, washed in TBST buffer, and further incubated with goat anti-rabbit IgG or anti-mouse IgG coupled to horseradish peroxidase (Amersham, Arlington Heights, IL) at 1:1,000 dilution in TBST buffer for 1 h at room temperature. Subsequent detection was carried out by enhanced chemiluminescence (Amersham), according to the manufacturer’s instructions. Prestained protein markers (BioRad, Temecular, CA) were used for molecular mass determination. Protein extracts from rat renal cortex stimulated with LPS (15 mg/kg; Sigma, St. Louis, MO) plus Propionibacterium acneus (100 μg/kg; Vetoquinol USA, Tampa, FL) were used as positive controls for iNOS.

Immunofluorescent staining. Immunofluorescent detection and localization of HSP-72 and HSP-73 proteins were performed by an indirect immunofluorescent technique (22). Briefly, transverse sections (5 μm thick) of kidneys were cut with a cryotome (Minotome Plus, Needham Heights, MA) and then dried at room temperature for 1 h. Sections were fixed in a mixture of 30% methanol and 70% acetone at −20°C for 10 min and then washed with PBS. Unless indicated, all incubations were performed at room temperature. To block nonspecific binding, sections were incubated for 30 min with 10% goat serum in PBS. Sections were then incubated for 90 min with mouse and rat monoclonal antibodies against HSP-72 and HSP-73, respectively (5 μg/ml each in PBS containing 1% BSA). After three washes with PBS, sections were incubated for 45 min with Cy3-labeled goat anti-mouse or anti-rat IgG (Amersham, 1:250 dilution with PBS containing 1% BSA). After thorough washing with PBS, specimens were counterstained with fluorescein-labeled wheat germ agglutinin (5 µg/ml, for cell surface staining) and bisbenzimide (2 µg/ml, for nuclear staining). The sections were then mounted with aqueous anti-quenching medium. To assess the specificity of the immunostaining, adjacent sections were incubated with nonimmune mouse or rat IgG (5 μg/ml each in PBS containing 1% BSA) instead of the primary antibodies and then processed under identical conditions. Microscopic observation and photography were performed with a Leica DMRXA confocal microscope (Heidelberg, Germany).

Statistics. Values are expressed as means ± SE. Multiple group comparisons were done using ANOVA. A P value of <0.05 was considered statistically significant. Animal survival was assessed using Fisher’s exact test.

RESULTS

Renal functional determination. Ischemia-induced renal dysfunction was assessed at 24 and 48 h of postischemic reperfusion. In wild-type mice subjected to bilateral renal pedicle clamping for 26 min, serum creatinine levels (mg/dl) increased from 0.25 ± 0.03 to 2.28 ± 0.38 after 24 h of reperfusion and remained elevated at 2.0 ± 0.18 at 48 h (Fig. 1). As depicted in Fig. 2, iNOS knockout mice were protected against renal ischemia-reperfusion injury. At 24 h, serum creatinine concentration (mg/dl) was 1.21 ± 0.25 in the knockout mice vs. 2.28 ± 0.38 in the wild-type mice (P < 0.05); similarly, at 48 h, the serum creatinine concentration (mg/dl) was 1.47 ± 0.21 in the knockout mice vs. 2.75 ± 0.37 in the wild-type mice (P < 0.05).
concentration (mg/dl) was 0.96 ± 0.25 in the knockouts vs. 2.0 ± 0.18 in the wild-type mice (P < 0.01).

Renal histological evaluation. Histological examination of the kidneys from both wild-type and iNOS knockout mice was undertaken at 24 h after renal ischemia-reperfusion. The kidneys of wild-type mice had significantly more tubular epithelial necrosis in the outer medulla and cortex (mostly S3, some S1 and S2) than iNOS knockout mice (Fig. 3, A and B). This injury included widespread loss of brush border (dramatically decreased PAS staining) and cellular nuclei, tubular dilation, and sloughing of proximal tubules with obstructing granular casts (S3). There was also ischemic necrosis of medullary thick ascending limbs in the outer medulla. Regenerative activity, as evidenced by tubular epithelial cell mitosis, was also prominent in wild-type mice. In contrast, kidneys of iNOS knockout mice displayed almost normal tubular structure after renal ischemia-reperfusion injury with intact brush border or, at most, patchy necrosis with less cast formation and loss of nuclei (Fig. 3, C and D). Thus iNOS knockout mice were significantly protected with respect to renal histology (histological scores were 3.33 ± 0.3 for wild-type mice, n = 3, vs. 0.875 ± 0.2 for knockouts, n = 4; P < 0.01).

In parallel to the protection of both renal function and tubular damage, the survival rate at 24 h after renal ischemia-reperfusion injury was also significantly higher in iNOS knockout mice in comparison with that for ischemic wild-type mice (Fig. 4).

Determination of iNOS protein expression and tyrosine nitration. iNOS protein expression was determined by immunoblot analysis using highly specific polyclonal and monoclonal antibodies against iNOS. Neither renal cortex nor outer medulla displayed an induction of iNOS after ischemia-reperfusion at 12 and 24 h. Even up to 48 h, there was no induction of iNOS protein expression after ischemia-reperfusion (data not shown).

A positive control for iNOS protein in mouse kidney was performed. Mice were treated with 30 mg/kg sc LPS. Whole kidney was removed and immunoblotted for iNOS protein as described in MATERIALS AND METHODS. The polyclonal iNOS antibody obtained from the University of Texas-Houston Trauma Research Center was used. iNOS protein was not detected basally. There was an induction of iNOS protein detected at 12 h after LPS injection, reaching a maximum at 24 h (data not shown).

To evaluate whether peroxynitrite was generated during ischemia-reperfusion, tyrosine nitration was examined in the kidneys of wild-type mice after ischemia-reperfusion. There was no significant increase of tyrosine nitration after ischemia-reperfusion injury using either polyclonal or monoclonal antibodies. Hence, it is unlikely that renal ischemia-reperfusion stimulated an activation of iNOS in the kidneys to a significant extent and unlikely that subsequent formation of peroxynitrite contributed to the tubular damage observed after ischemia-reperfusion in these wild-type mice.

Immunoblot and immunofluorescent staining of HSP. In an attempt to determine what mechanism may be contributing to the protection seen in the iNOS knockout mice, we examined renal expression of two HSPs, the inducible isoform, HSP-72, and constitutive isoform, HSP-73, using immunoblot analysis. As shown in Fig. 5, HSP-72 expression was enhanced in wild-type mice after ischemia-reperfusion injury, which is consistent with several earlier reports (1, 25). This increased expression of HSP-72 with ischemia-reperfusion was also seen in the knockout group. However, and possibly more importantly, under basal (sham) conditions, renal cortical HSP-72 was dramatically upregulated in iNOS knockout mice compared with wild-type mice. HSP-73 remained unchanged under both basal and ischemia-reperfusion conditions in iNOS knockout mice compared with wild-type mice (data not shown).

Immunofluorescent staining was performed to confirm the increased basal expression and localization of HSP-72 in the kidneys from both wild-type and iNOS knockout mice. As shown in Fig. 6, the staining was barely visible in the kidneys of wild-type mice, whereas, in iNOS knockout mice, HSP-72 was dramatically upregulated and the staining was concentrated in the proximal tubules. The constitutive isoform, HSP-73, showed no differences between the wild-type and iNOS knockout mice (Fig. 6).

Effect of iNOS inhibition on the expression of HSP-72. It was determined whether iNOS inhibition results in the upregulation of HSP-72 in wild-type mice. The iNOS inhibitor, N-3-(aminomethyl)benzyl acetamide (1400W), obtained from Alexis Biochemicals (San Diego, CA), was used. 1400W is a highly selective inhibitor of iNOS both in vitro and in vivo. In a rat model of endotoxemia, it prevented endotoxin-induced vascular
leakage with a half-maximal effective dose of <1 mg/kg sc (8). Wild-type mice were injected with 1400W (15 mg/kg sc) or vehicle (PBS) three times a day for 4 days. There were four mice in each group. The renal cortex was homogenized and immunoblotted as described in MATERIALS AND METHODS. There was no upregulation of HSP-72 in iNOS inhibitor-treated mice compared with vehicle-treated controls.

Effect of ketamine on ischemia-reperfusion injury. Ketamine is considered a potent inducer of the HSP-70 family (2, 23). Therefore, to test the direct effect of HSP on renal function, further experiments were conducted in which ketamine (Fort Dodge Laboratories, Fort Dodge, IA, 5 mg/kg ip) was administrated to wild-type mice 4 h before renal ischemia-reperfusion injury. Compared with nontreated mice, serum creatinine concentrations were lower by almost 50% in the ketamine-treated mice at 24 h after the ischemic insult (Fig. 7).

DISCUSSION

Alterations in NO synthesis have been incriminated in pathophysiological changes of ischemia-reperfusion injury in several key organs (9, 10, 20). For example, neurotoxicity in animal models of focal ischemia is
mediated at least in part by NO, since this toxicity is blocked by inhibitors of NOS (17). Furthermore, in mutant mice deficient in neuronal NOS (nNOS) activity, infarct volumes decreased significantly 24 and 72 h after middle cerebral artery occlusion, and the neurological deficits were less than those in wild-type mice (11, 20). The role of NO in ARF of experimental animals is also of special importance (13, 18). Recently, several groups have reported that iNOS is the injurious isoform involved in ischemic and endotoxin-induced ARF, based on in vivo studies in both rats and mice (4, 16, 21).

In the present study, we examined the effect of a selective knockout of the iNOS gene on renal ischemia-reperfusion injury. Our studies demonstrated that the iNOS knockout mice were protected against the renal dysfunction associated with ischemia-reperfusion. There was also a dramatic attenuation in these mice of the tubular epithelial cell damage induced by ischemia-reperfusion, a histological hallmark of ischemic ARF. Moreover, the renal protection was associated with a higher survival rate in iNOS knockout mice after ischemia-reperfusion. These results therefore supported a role for iNOS in ischemia-reperfusion injury. Surprisingly, however, when iNOS protein expression was measured in either the renal cortex or outer medulla of wild-type mice, iNOS protein was undetectable at all time points measured between 12 and 48 h of
postischemic reperfusion. Moreover, these findings were confirmed using three different specific iNOS antibodies for immunoblot analysis, as well as gel loadings of up to 450 µg of protein per lane in an attempt to detect low abundance of iNOS. The inability to detect iNOS in mouse renal cortex and outer medulla indicates that there is a low abundance or absence of iNOS protein induction in mouse kidney during 48 h of reperfusion after ischemia. The sensitivity of our antibody for iNOS protein was confirmed by a positive control. In wild-type mice treated with LPS, there was an induction of iNOS protein detected at 12 h after LPS injection, reaching a maximum at 24 h. There is only one study that detected iNOS protein during ischemia in mouse kidney (4). In this study, a non-commercially available rabbit polyclonal antibody to the NH2-terminal peptide of mouse iNOS was used. An increase in iNOS protein was only found in the outer medulla and not in the cortex or whole kidney, after ischemia and 24 h of reperfusion. There is no ready explanation for the difference between this result and the present study.

In a further attempt to implicate NO in the ischemia-reperfusion injury, we also examined whether peroxynitrite was generated from the reaction between NO and superoxide during ischemia-reperfusion in the wild-type mice. Protein extracts were immunoblotted for nitrotyrosine as a footprint for peroxynitrite formation. Again, there was no significant increase in tyrosine nitration after ischemia-reperfusion as detected by either a monoclonal or polyclonal antibody. We could therefore find no evidence that peroxynitrite contributed in any substantial way to the injury induced by ischemia-reperfusion in the wild-type mice.

The absence of either iNOS induction or an increase of tyrosine nitration suggested that the renal protection against ischemia-reperfusion injury in iNOS knockout mice was not likely due solely to the deletion of iNOS itself; rather, compensatory mechanism(s), possibly resulting from the depletion of the iNOS gene, may have contributed to the renal protection in these mice. We speculated that expression of a putatively cytoprotective agent such as a HSP may be altered in the iNOS knockout mice.

There is evidence that the heat shock response is a primitive defense mechanism in cells exposed to toxic insults such as heat, ischemia-reperfusion, heavy metals, or amino acid analogs (12, 19, 27). HSPs represent a family of peptides that have been highly conserved through evolution and appear to provide protection to injured cells. Studies have revealed that HSPs, in particular the HSP-70 family, not only confer thermotolerance but protect against ultraviolet light-induced injury, oxygen radical toxicity, acute lung injury, myocardial ischemia, and renal ischemia-reperfusion injury (12, 19, 27, 28). In the HSP-70 group, HSP-73 is a constitutive isoform and HSP-72 an inducible one. HSP-73 participates in intracellular protein targeting, processing, and transport and thereby plays an essential role in the maintenance of cell function. In response to a wide range of stresses, HSP-72 is rapidly synthesized and acts as a molecular chaperone (5, 27). This isoform binds to denatured or unfolded proteins and subsequently renatures or refolds the damaged proteins. Induction of HSPs in the kidneys of animals after renal ischemia-reperfusion is well characterized (1, 25, 28). Therefore, we next examined whether an upregulation of a HSP could account for the resistance to ischemia-reperfusion that we observed in the iNOS knockout mice.

As expected from previous reports, we found increased expression of HSP-72 in the renal cortex of wild-type mice subjected to ischemia-reperfusion. Surprisingly, however, we found that HSP-72 expression in the renal cortex of the iNOS knockout mice was dramatically increased under basal conditions. In support of the involvement of upregulation of HSP-72 in the protection afforded the iNOS knockout mice was our finding that renal function was protected against ischemia-reperfusion injury by the pretreatment of wild-type mice with ketamine, a potent inducer of HSPs (2, 23). These results are therefore compatible with the
upregulation of HSP-72 contributing, at least in part, to the renal protection in iNOS knockout mice.

In this regard, prior induction of HSP-70 has been shown to precondition cells against injury in cultured myocytes exposed to energy depletion (26). Our results are consistent with several reports in which HSPs are shown to play an important role in the protection of renal function against ischemia-reperfusion in experimental models (1, 25, 28). Chatson et al. (3) also reported that heat shock protected renal function with an enhanced survival rate against ischemia-reperfusion in rats. The protective effects were associated with an upregulation of HSP-72 in the kidneys (3). Besides the role of HSP as a molecular chaperone, the induction of HSP has been shown to inhibit the synthesis of cytokines and acute phase reactants (6), which are implicated in the pathogenesis of ischemic ARF (4, 24). It has also recently been suggested that there is a close relationship between HSP-70 and iNOS at the gene level (6, 7, 26). In this regard, we did not find overexpression of HSP-72 in the kidneys of either endothelial NOS or nNOS knockout mice (data not shown), implying that there may be a specific link between upregulation of HSP-72 and iNOS gene deletion.

In summary, iNOS knockout mice were shown to be protected functionally and histologically from ischemia-reperfusion injury. This protection was not associated with prevention of induction of iNOS protein expression during ischemia-reperfusion. However, increased expression of HSP-72 was observed under basal conditions in the iNOS knockout mice and may serve as a protective mechanism against ischemia-reperfusion injury in these animals.

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