Evidence for Peroxynitrite Formation in Renal Ischemia-Reperfusion Injury: Studies with the Inducible Nitric Oxide Synthase Inhibitor L-N6-(1-Iminoethyl)lysine

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ABSTRACT

Reactive oxygen species are suggested to participate in ischemia-reperfusion (I-R) injury. However, induction of inducible nitric oxide synthase (iNOS) and production of high levels of nitric oxide (NO) also contribute to this injury. NO can combine with superoxide to form the potent oxidant peroxynitrite (ONOO−). NO and ONOO− were investigated in a rat model of renal I-R injury using the selective iNOS inhibitor L-N6-(1-iminoethyl)lysine (L-NIL). Sprague-Dawley rats were subjected to 40 min of bilateral renal ischemia followed by 6 h of reperfusion with or without L-NIL administration. Control animals received a sham surgery and had plasma creatinine values of 0.4 ± 0.1 mg/dl. I-R surgery significantly increased plasma creatinine levels to 1.9 ± 0.3 mg/dl (P < .05) and caused renal cortical necrosis. L-NIL administration (3 mg/kg) in animals subjected to I-R significantly decreased plasma creatinine levels to 1.2 ± 0.10 mg/dl (P < .05 compared with I-R) and reduced tubular damage. ONOO− formation was evaluated by detecting 3-nitrotyrosine-protein adducts, a stable biomarker of ONOO− formation. Immunohistochemistry and HPLC revealed that the kidneys from I-R animals had increased levels of 3-nitrotyrosine-protein adducts compared with control animals. L-NIL-treated rats (3 mg/kg) subjected to I-R showed decreased levels of 3-nitrotyrosine-protein adducts. These results support the hypothesis that iNOS-generated NO mediates damage in I-R injury possibly through ONOO− formation.

The pathophysiology of acute renal failure (ARF) is complex and not well understood (Star, 1998). Numerous models of ARF suggest that oxygen-derived reactive species are important in renal ischemia-reperfusion (I-R) injury (Ueda et al., 1995), but the nature of the mediators is still controversial. Treatment with oxygen radical scavengers, antioxidants, and iron chelators such as superoxide dismutase, dimethylthiourea, allopurinol, and deferoxamine are protective in some models, and suggest a role for the hydroxyl radical formation (Paller et al., 1984; Paller and Hedlunk, 1988). However, these compounds are not protective in all models of I-R injury (Gamelin and Zager, 1988), and direct evidence for the generation of hydroxyl radical is absent (Zager et al., 1992). Furthermore, these inhibitors have another property in common. They all directly scavenge or inhibit the formation of peroxynitrite (ONOO−), a highly toxic species derived from nitric oxide (NO) and superoxide (Denicola et al., 1995; Whiteman and Halliwell, 1997). Thus, the protective effects seen with these inhibitors may be due in part to their ability to inhibit ONOO− formation.

NO is an important signaling molecule produced by nitric oxide synthase (NOS) (Gross and Wolin, 1995). Constitutive NOS isoforms, endothelial and neuronal, are found in the kidney in the vasculature and the macula densa, respectively (Mundel et al., 1994; Noiri et al., 1996). Treatment with an antisense DNA construct that prevents expression of iNOS protects renal function 24 h after I-R injury in rats (Noiri et al., 1996), and iNOS knockout mice are partially protected against renal I-R injury (Ling et al., 1999). This suggests that NO, generated by iNOS, contributes to I-R injury. NO and superoxide anions react spontaneously to form ONOO−. This potent and versatile oxidant can react with lipids, proteins, and DNA (Pryor and Squadrino, 1995). These reactions can explain many of the cytotoxic actions of NO. Because both superoxide and NO contribute to renal I-R injury, we rationalized that ONOO− may be formed during renal I-R. The goals of this study were to determine whether ONOO− is formed during I-R injury and whether pharmacological inhibitions of NOS would reduce renal damage.

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ABBREVIATIONS: ARF, acute renal failure; I-R, ischemia-reperfusion; ONOO−, peroxynitrite; NO, nitric oxide; NOS, nitric oxide synthase; NOS, inducible nitric oxide synthase; L-NIL, L-N6-(1-iminoethyl)lysine; PAS, periodic acid-Schiff.
bition of iNOS reduces ONOO⁻ formation and protects renal function.

Materials and Methods

I-R Surgery. All animals were housed and sacrificed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. I-R surgery was performed on male Sprague-Dawley rats (225–250 g). Rats were placed on a warming pad and anesthetized with pentobarbital sodium (50 mg/kg). Using aseptic technique, bilateral flank incisions were made to expose the kidneys, and both renal pedicles were isolated and occluded for 40 min with microvascular clamps. After clamp release, incisions were closed with skin staples. The rats were allowed to awake on the warming pad and were returned to clean cages with free access to food and water.

Experimental Design. iNOS was inhibited using the selective inhibitor L-N⁵-(1-iminoethyl)lysine (L-NIL; Alexis Biochemicals, San Diego, CA) (Moore et al., 1994). Four treatment groups (n = 5–6 rats/group) were used in this study. The sham group (1) received sham surgery (incisions were made to expose the kidneys, but the renal pedicle was not clamped). The I-R group (2) was subjected to 40 min of bilateral renal ischemia. The L-NIL + I-R group (3) was subjected to I-R and was administered two injections of L-NIL (3 mg/kg i.p.). The first dose was given 30 min before surgery, and the second was given at the time of clamp release (40 min later). The L-NIL control group (4) received a sham operation and L-NIL injections at times equivalent to the L-NIL + I-R treatment group. The sham group and the I-R group received vehicle (0.9% NaCl) on an equivalent schedule.

Pathology Injury Score. Renal tissue injury was assessed in tissue sections stained using the periodic acid-Schiff (PAS) reaction. Sections were scored in a blinded, semiquantitative manner (Walker, 1994). The numerical scores indicate the following: 0, normal structure; 1, areas of tubular epithelial cell swelling, vacuolar degeneration, necrosis, and desquamation involving less than 25% of cortical tubules; 2, similar changes involving greater than 25% but less than 50% of cortical tubules; 3, similar changes involving greater than 50% but less than 75% of cortical tubules; 4, similar changes involving greater than 75% of cortical tubules; and 5, complete cortical necrosis.

Immunohistochemistry. Paraffin-embedded tissue sections (3 μm) were cleared in xylene, rehydrated, and washed in PBS. Slides were incubated in methanol with 1% H₂O₂ to block endogenous peroxidase activity. Nonspecific protein binding was blocked by incubation with 10% goat serum in PBS. Rabbit anti-nitrotyrosine antibody (1:100 dilution; Upstate Biotechnology, Lake Placid, NY) was incubated with the sections for 1 h at room temperature. Primary antibody was blocked with 3-nitrotyrosine (10 μg/ml) for 1 h at room temperature. The anti-nitrotyrosine antibody was detected using the Vectastain Elite peroxidase ABC kit and 3,3’-diaminobenzidine (Vector Laboratories, Inc., Burlingame, CA). A brown precipitate forms where the anti-nitrotyrosine antibody binds the tissue section. Gilf’s hematoxylin was used as a counterstain. As a negative control, the antigenic binding site of the anti-nitrotyrosine antibody was blocked with 3-nitrotyrosine (10 mM) for 1 h at room temperature.

3-Nitrotyrosine HPLC. Protein-incorporated and free 3-nitrotyrosine and tyrosine kidney tissue concentrations were determined by Coularray-HPLC electrochemical detection method with slight modifications (Imam and Ali, 2000). Frozen tissue was sonicated (5% w/v) in 10 mM sodium acetate, pH 6.5. The homogenates were centrifuged at 14,000g for 10 min at 4°C. The supernatant was collected and treated with 5 mg/ml pronase for 18 to 20 h at 50°C. Enzymatic digests were then treated with an equal volume of 40% trichloroacetic acid and were centrifuged at 14,000g for 10 min at 4°C. Supernatants were passed through a 0.2-μm polyvinylidene fluoride filter before injection into an ESA (Cambridge, MA) CoulArray-HPLC equipped with eight electrochemical channels using platinum electrodes in line and set to increasing specified potentials [channel (potential): 1 (180 mV); 2 (240 mV); 3 (350 mV); 4 (500 mV); 5 (550 mV); 6 (690 mV); 7 (875 mV); and 8 (900 mV)]. The analytical column was a TSK-GEL ODS 80-TM reversed phase column with a column size of 4.6 mm × 25.0 cm (TosoHaas, Montgomeryville, PA). The mobile phase was 50 mM NaOAc/5% methanol (v/v), pH 4.8. HPLC was performed under isocratic conditions. 3-Nitrotyrosine and tyrosine were quantified relative to known standards. 3-Nitrotyrosine values were represented as 3-nitrotyrosine molecules per 100 tyrosine molecules.

Plasma NO₂⁻ + NO₃⁻ Concentration. NO₂⁻ + NO₃⁻ concentration was determined in the plasma using a colorimetric nonenzymatic NO assay kit (Oxford Biomedical Research, Oxford, MI). Plasma samples (50 μl) were diluted in H₂O₂, and ZnSO₄ was added (final concentration = 1.5%) to precipitate protein. Samples were incubated 15 min at room temperature and were centrifuged at 16,000g for 5 min to separate particulate matter. The supernatant was collected and added to a microcentrifuge tube containing six to seven cadmium beads. The samples were mixed on a tube shaker overnight. The following day, samples were centrifuged and the resulting supernatant was tested for NO₂⁻ using the Griess reagent. Griess reagent consisted of equal volumes of 1% sulfanilamide in 2.5% H₃PO₄ and 0.1% N-(1-naphthyl)-ethylenediamine in H₂O. The samples were diluted in H₂O and mixed with an equal volume of the Griess reagent. Plates were incubated 5 min at room temperature, and the absorbance was read at 550 nm. Results were compared against a standard curve of NaNO₂ and the concentration (μM) of NO₂⁻ was determined for each sample.

Data Analysis. Data are reported as mean ± S.E. Each n represents one rat. All data were analyzed by a one-way ANOVA followed by the Student-Newman-Keuls test unless otherwise indicated. P < .05 was considered statistically significant.

Results

An initial study was performed using 3 mg/kg L-NIL to inhibit iNOS during I-R. The I-R group had significantly elevated plasma creatinine concentration (1.9 ± 0.3 mg/dl) compared with the sham group (0.4 ± 0.1 mg/dl, P < .05) (Fig. 1). The L-NIL + I-R treatment group showed signi-
cantly decreased plasma creatinine values (1.2 ± 0.2 mg/dl, \( P < .05 \)) compared with the I-R group. The L-NIL control group had creatinine levels (0.4 ± 0.1 mg/dl) that were not different from sham.

Histological sections were examined for morphological changes (Fig. 2). Kidneys from sham animals showed the normal structure of healthy tubules with abundant luminal brush-border membranes. Kidneys from the I-R group showed extensive tubular damage. Kidneys from the L-NIL + I-R group had an intermediate level of damage. Brush-border membranes were disrupted in many tubules, but the damage was less severe than that found in the I-R group. The L-NIL control group was similar to the sham group, and did not show structural damage (photograph not shown). Tissue sections were graded in a blinded manner for injury on a 5-point scale (Fig. 3). These results support the renal function data that L-NIL also provided partial protection against morphological damage.

Formation of 3-nitrotyrosine-protein adducts is a reliable biomarker of ONOO\(^-\) formation, and specific immunohistochemical assays have been developed (Beckman et al., 1994; Kaur and Halliwell, 1994). Immunohistochemical detection of 3-nitrotyrosine-protein adducts was used as a marker of ONOO\(^-\) formation in the kidney. Tissue sections were probed with a polyclonal anti-nitrotyrosine antibody and detected with a peroxidase secondary system. Representative photographs are presented in Fig. 4. The sham group displayed very low levels of diffuse staining. The I-R group showed intense staining in tubules. The L-NIL + I-R group showed an intermediate level of staining, indicating L-NIL treatment decreased the relative levels of ONOO\(^-\) formed. The antigenic binding specificity of the anti-nitrotyrosine antibody was confirmed by blocking the antigen-binding site with 3-nitrotyrosine before addition to the tissue section. 3-Nitrotyrosine-protein adducts were quantified in kidney homogenates, and the results are shown in Fig. 5. These data support the results from immunohistochemical staining. There are low, but detectable levels of 3-nitrotyrosine-adducts in the sham group. In the I-R group 3-nitrotyrosine-protein adducts were significantly increased compared with the sham group (\( P < .05 \)). Levels in the L-NIL + I-R group were not different from the sham group.

Because the dose of L-NIL used did not provide complete protection, additional doses were tested in this model. The data in Fig. 6 show plasma creatinine values for all doses of L-NIL tested. A protocol consisting of two doses of 1 mg/kg L-NIL did not reduce plasma creatinine values after I-R (1.94 ± 0.2 mg/dl). A protocol with 10-mg/kg dose of L-NIL also did not reduce plasma creatinine values (1.73 ± 0.2 mg/dl). The I-R group without L-NIL administration had creatinine levels of 1.8 ± 0.1 mg/dl, and the sham had values of 0.33 ± 0.02 mg/dl. The plasma creatinine values for all doses of the L-NIL sham groups were not significantly different from the sham group (data not shown).

Plasma NO\(_2^-\) + NO\(_3^-\) concentration was measured as a marker of NOS activity in animals administered L-NIL (\( n = 3 \) for plasma NO\(_2^-\) + NO\(_3^-\) concentration determinations).
Sham animals had a plasma NO$_2^-$ + NO$_3^-$ concentration of 20 ± 3.6 μM. The NO$_2^-$ + NO$_3^-$ concentration in the 3 mg/kg L-NIL sham group and 10 mg/kg L-NIL sham group was 19 ± 4.4 and 10 ± 1.2 μM, respectively (P = .05 for sham group versus 10 mg/kg L-NIL sham control with a two-tailed t test). Plasma NO$_2^-$ + NO$_3^-$ concentration in the I-R group (28 ± 4.3 μM) was not different compared with the sham group.

Discussion

We found the presence of 3-nitrotyrosine-protein adducts, a marker of ONOO$^-$ generation, in renal tubules of I-R injured kidneys. Treatment with the iNOS inhibitor L-NIL (3 mg/kg) improved renal function and decreased apparent ONOO$^-$ formation. These data support the hypothesis that
ONOO\textsuperscript{−} formation occurs during I-R and that pharmacological inhibition of iNOS can reduce ONOO\textsuperscript{−} formation and preserve renal function.

Several lines of evidence support the notion that excess NO production occurs during I-R and contributes to renal injury in the rat. In vitro studies using isolated rat proximal tubules demonstrate that 15 min of hypoxia and 35 min of reoxygenation cause cell death. This cell death is prevented using the nonselective NOS inhibitor N-nitro-L-arginine methyl ester and is enhanced by exogenous L-arginine, the substrate for NOS (Yu et al., 1994). In vivo, antisense DNA directed against iNOS prevents renal injury at 24 h (Noiri et al., 1996). Finally, NOS activity is significantly increased in rats during the first 24 h of reperfusion after 60 min of ischemia (Shoskes et al., 1997).

Nonselective NOS inhibitors that inhibit constitutive NOS worsen renal I-R injury (Chintala et al., 1993; Noiri et al., 1996). This is caused presumably by inhibiting constitutive endothelial NOS in the renal vasculature, which reduces blood flow to the kidney. The effects of selective iNOS inhibitors have never been reported in this I-R model of renal injury. L-NIL is described as a selective inhibitor of iNOS in vitro (Moore et al., 1994). It has at least a 5-fold selectivity for iNOS in vivo (Faraci et al., 1996), and has been used to evaluate the role of iNOS in a number of studies (Connor et al., 1995; Schwartz et al., 1997). A 3-mg/kg dose of L-NIL decreases lipopolysaccharide-mediated renal injury in rats (Schwartz et al., 1997; Zhang et al., 2000). Although the 3-mg/kg treatment with L-NIL reduced injury in our study, it is important to note that 10 mg/kg did not. This result may reflect a loss of isoform selectivity of L-NIL at higher doses. The apparent decrease in plasma NO\textsubscript{2} + NO\textsubscript{3} concentration after the 10-mg/kg dose of L-NIL in sham animals supports the notion that higher doses of L-NIL may inhibit basal NO formation. Because the pharmacokinetics of L-NIL is unknown, dosing schedules will need to be optimized to fully evaluate the usefulness of this drug.

ONOO\textsuperscript{−} formation has been detected in several models of oxidant-mediated injury. Studies have found 3-nitrotyrosine-protein adducts in myocardial tissue after I-R injury in vivo (Liu et al., 1997). 3-Nitrotyrosine residues are also found in myocardial samples from patients with myocarditis or sepsis (Kooy et al., 1997) and in lung tissue from patients with acute lung injury (Kooy et al., 1995). In lipopolysaccharide-treated animals, 3-nitrotyrosine-protein adducts in the kidney are associated with the development of oxidant stress (Zhang et al., 2000). Toxic insults have also been shown to cause ONOO\textsuperscript{−} formation. 3-Nitrotyrosine-protein adducts are found in rats lungs in response to asbestos inhalation (Tanaka et al., 1998). Carbon monoxide exposure generates ONOO\textsuperscript{−} in vascular endothelial cells (Thom et al., 1997), and toxic doses of acetaminophen cause 3-nitrotyrosine-protein adducts in livers of mice (Hinson et al., 1998).

Although it has been suspected for some time that superoxide and other reactive oxygen species are important in the development of renal I-R injury in rats (Paller et al., 1984; Paller and Hedlunk, 1988), the nature of the reactive species is controversial (Gamelin and Zager, 1988; Zager et al., 1992). Because preventing induction of iNOS is protective (Noiri et al., 1996; Ling et al., 1999), NO must be involved as well. The rate constant for the reaction of superoxide dismutase with superoxide is approximately $2 \times 10^9 \text{M}^{-1} \cdot \text{s}^{-1}$. However, the rate constant for the reaction of NO with superoxide is 3-fold higher ($6.7 \times 10^9 \text{M}^{-1} \cdot \text{s}^{-1}$) (Crow and Beckman, 1996). Thus, the formation of ONOO\textsuperscript{−} is favored in conditions where both NO and superoxide are formed. The appearance of ONOO\textsuperscript{−} in renal I-R indicates a period of cogeneration of NO and superoxide. In the mouse 3-nitrotyrosine-protein adducts are found in the outer stripe of the kidney medulla at 24 h after I-R (Chiao et al., 1997), and our studies found 3-nitrotyrosine-protein adducts localized to the tubular epithelium at an even earlier time, 6 h. In vitro studies have also suggested that ONOO\textsuperscript{−} is generated in isolated proximal tubules by hypoxia-reoxygenation injury as early as 30 min after reoxygenation (Paller, 1998). In vitro studies have shown that ONOO\textsuperscript{−} generation in tubular epithelium may impair the adhesion of tubular epithelium to the basement membrane and this may contribute to tubular obstruction during ARF (Wangsiriapisan et al., 1999).

In summary, 3-nitrotyrosine-protein adducts were detected in renal tubules after I-R injury. Selective inhibition of iNOS by L-NIL decreased injury, improved renal function, and decreased apparent ONOO\textsuperscript{−} formation. Although this study did not address the role of ONOO\textsuperscript{−} in I-R directly, it is tempting to speculate that an early interaction between NO and superoxide generates ONOO\textsuperscript{−} and that this reactive nitrogen species participates in the development of injury. Thus, reactive nitrogen species should be considered potential therapeutic targets in the prevention and treatment of renal I-R injury.

References
Imam S and Ali S (2000) Selenium, an antioxidant, attenuates methamphetamine-induced dopaminergic toxicity, in at ASPET Journals on November 13, 2017 jpet.aspetjournals.org Downloaded from


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