Effects of sodium nitrite on ischemia-reperfusion injury in the rat kidney

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Basireddy, Mahesh, T. Scott Isbell, Xinjun Teng, Rakesh P. Patel, and Anupam Agarwal. Effects of sodium nitrite on ischemia-reperfusion injury in the rat kidney. Am J Physiol Renal Physiol 290: F779–F786, 2006. First published November 8, 2005; doi:10.1152/ajprenal.00334.2005.—Reactive oxygen and nitrogen species play a key role in the pathophysiology of renal ischemia-reperfusion (I/R) injury. Recent studies have shown that nitrite (NO2−) serves as an endogenous source of nitric oxide (NO), particularly in the presence of hypoxia and acidosis. Nanomolar concentrations of NO2− reduce injury following I/R in the liver and heart in vivo. The purpose of this study was to evaluate the role of NO2− in renal I/R injury. Male Sprague-Dawley rats underwent a unilateral nephrectomy followed by 45 min of ischemia of the contralateral kidney or sham surgery under isoflurane anesthesia. Animals received normal saline, sodium NO2−, or sodium nitrate (NO3−; 1.2 nmol/g body wt ip) at 22.5 min after induction of ischemia or 15 min before ischemia. A separate set of animals received saline, NO2−, or NO3− (0.12, 1.2, or 12 nmol/g body wt iv) 45 min before ischemia. Serum creatinine and blood urea nitrogen were increased following I/R injury but were not significantly different among treatment groups at 24 and 48 h after acute renal injury. Interestingly, NO3− administration appeared to worsen renal injury. Histological scoring for loss of brush border, tubular necrosis, and red blood cell extravasation showed no significant differences among the treatment groups. The results indicate that, contrary to the protective effects of NO2− in I/R injury of the liver and heart, NO2− does not provide protection in renal I/R injury and suggest a unique metabolism of NO2− in the kidney.

nitric oxide; nitrate; acute renal injury; tubular necrosis; hypoxia

ACUTE RENAL FAILURE (ARF) remains a major cause of morbidity and mortality in hospitalized patients (45). In addition, renal dysfunction develops in 5% of all general surgical patients and complicates the course of recovery in 15–25% of critically ill patients (34). Current therapeutic options are limited to supportive measures and renal replacement therapy, necessitating the need for the development of more viable therapies for ARF.

Ischemia-reperfusion (I/R) injury is the predominant underlying cause of ARF (26). Reactive oxygen and nitrogen species play a key role in mediating cell damage during I/R injury (35, 36). Nitric oxide (NO) and NO-derived products have heterogeneous effects in ARF depending on the location of production, duration of action, and the presence of reactive oxygen intermediates (12, 14). NO derived from the inducible isoform of nitric oxide synthase (iNOS) worsens injury in both in vitro and in vivo models of ARF (27, 28, 36, 37, 50). On the other hand, NO derived from the constitutive endothelial isoform of NOS (eNOS) is protective in ARF, because eNOS-deficient mice exhibit increased susceptibility to renal injury (48) compared with iNOS-deficient mice (27). Recent studies have shown that during ischemia or hypoxia, NOS-independent sources of NO play a major role in mediating protective responses (10, 25, 49). One such source is inorganic nitrite (NO2−), which can be produced biologically via oxidation of NO, consumption via the diet, and either directly or indirectly after reduction of nitrate (NO3−) by nitrate reductases present in oral bacteria (1, 2, 30).

NO2− may represent a circulating and tissue storage form of NO. NO2− is reduced to NO during hypoxia and acidosis by the enzymatic action of xanthine oxidoreductase (XOR) and by heme proteins such as deoxyhemoglobin, myoglobin, and tissue heme proteins (3, 5, 9, 16, 25, 29, 32, 33, 46, 47). This has been demonstrated with endogenous tissue and circulating NO2−, the latter of which in the presence of red blood cells has been proposed to mediate systemic blood flow responses to hypoxia (3, 7, 17). Taken together, NO2− may offer a novel and efficacious therapy to replete NO in disorders associated with tissue ischemia. In support of this concept, Duranski et al. (10) recently demonstrated that NO2− can afford tissue protection in vivo in I/R-induced injury of the liver and the heart through mechanisms involving NO formation. Similarly, NO2− was protective in an isolated cardiac I/R injury model (49), in hypoxia-induced pulmonary vasoconstriction (18), and in a hemorrhagic model of stroke (40).

Given the recent evidence that NO2− affords cytoprotection in vivo in I/R injury in the heart and liver (10), its role in I/R injury in the kidney was evaluated in this study. Unlike the protective effects of NO2− in the heart and liver, NO2− did not protect against renal I/R injury.

MATERIALS AND METHODS

Chemicals

Sodium NO3− and sodium NO2− (Sigma, St. Louis, MO) were dissolved in normal saline (NS) and injected at a concentration of 1.2 nmol/g body wt for intraperitoneal or 0.12, 1.2, or 12 nmol/g body wt (equivalent to 30, 300, and 3,000 nmol/250 g body wt) for intravenous administration. An equal volume (0.5 ml) of NS was injected in controls.

Animals

Sprague-Dawley rats, aged 8–12 wk and weighing ~250–300 g, were purchased from Harlan (Indianapolis, IN). Animals were fed a standard diet and allowed free access to water. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

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Renal I/R Injury Model

Rats were anesthetized using 2.5% isoflurane. Under aseptic precautions, a right nephrectomy was performed via a right loin incision. A similar incision was made in the left loin, and the left renal pedicle was exposed, secured, and clamped with an atraumatic clamp for 45 min according to the specified protocol (see below). During this period, the kidney was kept moist and warm using a heat lamp and sterile gauze soaked in warm (37°C) saline. At the end of ischemia, the clamp was removed to allow reperfusion and kidney was restored to the abdominal cavity in its original position. The incision was closed with sutures. Blood samples for serum creatinine and urea nitrogen (BUN) were collected at baseline, 24 h, and 48 h following I/R injury. Test solutions were administered for the three specific protocols outlined in Fig. 1. For protocol 3, an additional group of animals underwent sham surgery in which a right nephrectomy was performed and the left renal pedicle was exposed, but was not clamped. Both sham and I/R animal groups received sterile NS, sodium NO2- (1.2 nmol/g body wt), or sodium NO3- (1.2 nmol/g body wt). For protocol 3, a separate set of animals was also treated with 0.12 or 12 nmol/g body wt of sodium NO2- and sodium NO3-, respectively. Each treatment group consisted of at least five to seven animals.

Protocol 1. Renal ischemia was induced for 45 min and NS, NO2-, or NO3- was administered by intraperitoneal injection after 22.5 min of ischemia. Ischemia was continued for an additional 22.5 min (total ischemia time: 45 min), and then reperfusion was initiated.

Protocol 2. Immediately after the right loin incision, NS, NO2-, or NO3- was administered intraperitoneally followed by a right nephrectomy (Nx) and left renal ischemia (Fig. 1). For protocol 2, the time interval between the administration of test solution and induction of left renal ischemia was kept constant at 15 min.

Protocol 3. NS, NO2-, or NO3- was administered via tail vein injection, and then a right nephrectomy was performed. Blood was drawn for measurement of NO3- at baseline, after 30 min following administration of test solution, and again at 5 min following reperfusion. Left renal ischemia was induced for 45 min, or sham surgery was performed.

Histological Assessment

On day 6 postischemia, the animals were killed under anesthesia and their organs were harvested as detailed below. The left kidneys were removed, cut transversally into four equal sections, immersed in 10% formalin for 24 h, and then transferred into 70% ethanol. Tissues were embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin. Morphological assessment of injury was determined in the cortex and outer medulla by counting the number of tubules exhibiting loss of brush border, necrosis, and areas of red cell extravasation from 10 high-power fields/animal in a blinded fashion.

Laboratory Assays

Serum creatinine and BUN were assayed using a Vet Ace spectrophotometer analyzer (Alfa Wasserman, West Caldwell, NJ). Plasma NO2- levels were assayed as previously described (6). Briefly, blood (0.4 ml) collected at the indicated time points (Fig. 1, protocol 3) was centrifuged and plasma was supplemented immediately (<3 min after blood collection) with N-ethylmaleimide (NEM; 1 mM) and diethylamine pentaacetate (DTPA; 100 μM) in PBS. Stabilized samples were analyzed for NO2- by I3-based reductive chemiluminescence.

Statistical Analysis

All results are expressed as means ± SE. Student’s t-test was used to compare the control vs. treated groups. ANOVA and the Student-Newman-Keuls test were used to compare the mean values for multiple group comparisons. All values were considered significant at P < 0.05.

RESULTS

The effects of NO2- on renal I/R injury were determined following intraperitoneal administration of sodium NO2- (1.2 nmol/g) at 22.5 min before the onset of reperfusion (protocol 1) or 15 min before the onset of ischemia (protocol 2). Assessment of renal function revealed that such administration of NO2- had no effect on the I/R-induced increase in serum creatinine or BUN (Fig. 2, A and B). Serum creatinine and BUN following I/R injury were significantly increased over baseline in the NS, NO2-, and NO3- groups at 24 h (P < 0.05). However, no significant differences were noted between the groups, suggesting that NO2- was not protective in renal I/R-induced injury. Interestingly, NO3- appeared to worsen renal function, although this was not statistically significant.

Because intraperitoneal administration would lead to increased NO2- levels through the portal circulation and the liver, which may, in turn, limit renal availability, we administered NO2- (1.2 nmol/g) intravenously as described in protocol 3. As in protocols 1 and 2, no beneficial effect was observed with intravenous administration of NO2- in the I/R animals (Fig. 2C). Again, NO3- administration led to worsening of renal function, which was significant at 24 h (P < 0.05; Fig. 2C).
Previous studies have shown a bell-shaped dependence on the protective effects of NO$_2$ on the liver and with myocardial I/R injury. Table 1, however, shows that using 10-fold lower or higher NO$_2$ doses (corresponding to 0.12 and 12 nmol/g body wt, respectively) administered intravenously as in protocol 3 showed no significant renal protection. All animals that underwent sham surgery showed a relatively modest increase in serum creatinine and BUN at 24 and 48 h, an expected consequence of unilateral nephrectomy with no significant differences between the NS, NO$_2$, or NO$_3$-treated animals (Table 2).

To verify that NO$_2$ levels were elevated following intravenous administration, circulating NO$_2$ concentrations were measured at baseline, 30 min following infusion (15 min before ischemia), and 5 min following reperfusion or sham surgery. As shown in Fig. 3, plasma NO$_2$ levels increased significantly at 30 min following intravenous administration of NO$_2$ (1.2 nmol/g) in the animals undergoing I/R injury. The plasma levels of NO$_2$ after administration of 0.12 and 12 nmol/g of NO$_2$ increased by 0.05 ± 0.02 and 4.5 ± μM, respectively, at 30 min after injection. Similar temporal changes in plasma NO$_2$ were observed in the sham group of animals receiving NS, NO$_2$, or NO$_3$ (data not shown).

We also performed histological analysis of kidneys to assess the severity of structural injury among the treatment groups. No significant differences were observed in the number of tubules exhibiting loss of brush border, necrosis, and red cell extravasation in the NS, NO$_2$, or NO$_3$ treatment groups in all three protocols. Figure 4 shows the renal histology and the results of the scoring for renal injury for protocol 3 (1.2 nmol/g). Similar results were seen in the kidneys in protocols 1 and 2 as well as with the 0.12 or 12 nmol/g dose of NO$_2$ for protocol 3 (data not shown). There was no evidence of renal injury in the animals undergoing sham surgery. These findings demonstrate that under these conditions, NO$_2$ treatment does not provide protection with respect to both functional and structural indexes of renal injury.

![Fig. 2. Effects of NS, NO$_2$, or NO$_3$ on serum creatinine and BUN at 24 and 48 h after ischemia-reperfusion (I/R) in protocol 1 (A), protocol 2 (B), and protocol 3 (NO$_2$, 1.2 nmol/g, C). *P < 0.05 vs. baseline values at 0 h. #P < 0.05 vs. NS and NO$_2$ at 24 h; n = 5–7 animals/treatment group.](http://ajprenal.physiology.org/)

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Several factors support the application of NO$_2^-$ as a NO donor for treatment of ischemia-related disorders, which include 1) the fact that NO$_2^-$ is a naturally occurring anion that is formed endogenously, 2) NO$_2^-$ is relatively safe being tolerated even at high concentrations as evidenced by FDA approval for its use as the active ingredient in cyanide antidote kits, 3) NO$_2^-$ is relatively inexpensive, and 4) specificity of NO$_2^-$ to deliver NO during ischemia. These factors and the promising results of NO$_2^-$ in hepatic and cardiac I/R injury prompted us to evaluate its efficacy in renal I/R injury.

The results of the present study demonstrated that the administration of NO$_2^-$ did not provide protection in the rat model of I/R-induced renal injury, in contrast to its beneficial effects in cardiac and hepatic I/R injury (10). Doses of 0.12, 1.2, and 12 nmol/g body wt of NO$_2^-$, resulting in a 2, 20, and 200 µM final circulating concentrations, respectively (assuming rat blood volume of ~6 ml/100 g), were used in these experiments based on previous studies in mouse hepatic and cardiac I/R injury (10). In protocol 1, NO$_2^-$ was administered intraperitoneally midway through 45 min of renal ischemia. Because there was no benefit in terms of renal I/R injury, we administered NO$_2^-$ 15 min before renal ischemia to provide adequate time for the NO$_2^-$ to perfuse the kidney as in protocol 2. Recently, Okamoto et al. (38) demonstrated that maximal NO derived from NO$_2^-$ was observed after 40 min of renal ischemia; however, the functional effects of such NO generation on renal injury were not determined in this study. Hence, we tested the effects of intravenous administration of NO$_2^-$ 45 min before ischemia as in protocol 3. Despite a significant increase in circulating NO$_2^-$ 15 min before the onset ischemia (Fig. 3), NO$_2^-$ did not protect against renal I/R injury. Surprisingly, with NO$_3^-$ (1.2 nmol/g), which was used as a control for NO$_2^-$, a trend toward worsened functional renal I/R injury was observed for reasons that remain unclear at this time. Previous studies have suggested that NO$_3^-$ alters transport of sodium and chloride in the distal nephron (21) and may also adversely affect renal function (53).

Recent studies have highlighted the potential for NO$_2^-$ to serve as a biological source of NO in the vascular compartment during hypoxia (11, 30). This concept has led to studies demonstrating that NO$_2^-$ can protect or reverse tissue injury during ischemia, particularly in settings characterized by loss of NO bioavailability. These include hypoxia-induced pulmonary vasoconstriction (18), hemorrhagic stroke (40), as well as hepatic and cardiac I/R injury (10). The concentrations of NO$_3^-$ that have led to protection in these models were relatively low (20 µM) and consistent with regulation of NO-dependent signaling, and provided the rationale for the dose of NO$_2^-$ used in the present study. Several studies have shown that NO$_2^-$ can modulate blood flow under physiological conditions and can be converted to other nitrosating intermediates during hypoxia (3, 5) and acidic conditions leading to beneficial effects on gastric mucosal and intestinal function (1). These findings have spurred much recent interest in exploring the therapeutic potential of NO$_2^-$.

However, factors that might mitigate the use of NO$_2^-$ as a therapeutic agent are its potential for promoting inflammatory tissue injury via production of NO-derived reactive species (e.g., nitrogen dioxide, peroxynitrite) (44, 47) and the lack of information on its effects in other models of I/R injury.

Physiological concentrations of NO$_2^-$ range from 0.3 to 0.5 µM in plasma and from 1 to 20 µM in tissues (2, 3, 8, 22). In the presence of hypoxia or acidosis or following conditions that lead to activation of XOR, NO$_2^-$ can serve as a donor for NO, the functional effects of which are dependent on several factors including the concentration, site of release, and duration of
Fig. 4. A: effect of NS, NO$_2^-$, or NO$_3^-$ on renal histology on day 6 postischemia (protocol 3). Hematoxylin and eosin staining of kidney sections from sham (top) and I/R (bottom) animal groups receiving NS or 1.2 nmol/g body wt of NO$_2^-$ or NO$_3^-$ is shown. Note the changes in the renal tubules in the outer medullary and inner cortical regions (*). B: scoring for structural renal injury. Each bar graph represents total no. of tubules showing loss of brush border (BB loss) and necrosis and red blood cell (RBC) extravasation from 10 high-power fields as described in MATERIALS AND METHODS.
action. Although the precise mechanism of how NO\textsubscript{2} confers tissue protection in the heart and liver is still unclear, a direct role for NO has been implicated (10). In the kidney, NO plays a major role in the regulation of renal hemodynamics under both physiological and pathophysiological conditions (13, 14, 23). Transient spikelike generation of NO at concentrations of 10–100 nmol/l, similar to NO release following eNOS activation, causes guanylate cyclase-dependent vasorelaxation, scavenging of reactive oxygen species, and antiapoptotic effects (13). Higher sustained generation of NO at concentrations >300 nmol/l, as seen with iNOS activation, is proapoptotic and leads to increased lipid peroxidation (13). Given the previous findings that NO is produced from NO\textsubscript{2} during renal I/R (38), our results suggest that this NO\textsubscript{2}-derived NO is not associated with renal protection.

Another source of NO production from NO\textsubscript{2} is the activity of XOR (12, 25, 49). It is generally thought that XOR activity is damaging to tissue due to its ability to increase the production of reactive oxygen species. The NO produced from the XOR-NO\textsubscript{2} interactions has been shown to both protect (49) and contribute (46) to I/R injury in the isolated heart. In renal I/R injury, the role of XOR is more controversial. Studies have demonstrated an increased or unchanged XOR activity following renal I/R, and the use of XOR inhibitors has led to conflicting results (15, 20, 39, 51, 52).

In the setting of renal I/R, NO-based therapies have demonstrated both protective and harmful effects. Table 3 summarizes the results of several studies that have reported the effects of NO donors, l-arginine supplementation, and pharmacological and genetic inhibition of NOS in renal I/R injury. The administration of NO donors such as sodium nitroprusside, molsidomine, and FK-409 is beneficial in renal I/R injury (19, 24, 31, 42). l-Arginine supplementation and NOS inhibitors have shown varying results (4, 24, 37, 41, 43, 50). Inhibition of iNOS using antisense oligonucleotides is beneficial in renal I/R injury (37). Similarly, iNOS-deficient mice are protected in renal I/R injury, whereas eNOS-deficient mice exhibit worsening of renal function in a model of endotoxin-induced ARF (27, 28, 48). Understanding the mechanisms that regulate the pleiotropic effects of NO in the kidney is therefore critical before therapeutic modalities that target NO metabolism can be successfully implemented.

The present data also suggest organ-specific effects of NO\textsubscript{2} in I/R injury, the reasons for which are not entirely clear but may be related to several factors. First, enough NO\textsubscript{2} might not have reached the kidney, as it derives its only blood supply from the renal artery, which, if clamped, would prevent achievement of adequate tissue levels of NO\textsubscript{2}. In the liver and the heart, direct exposure of NO\textsubscript{2} to the organ surface was protective and resulted in elevations in NO\textsubscript{2} in plasma (10). However, the degree of absorption from the kidney surface might not be adequate due to a thick capsule. To obviate this concern, NO\textsubscript{2} was injected intravenously before ischemia, which resulted in elevated concentrations of plasma NO\textsubscript{2}. However, this did not preserve renal function. Second, there may be organ specificity and sensitivity to NO\textsubscript{2} metabolism and NO production. Finally, species differences may account for our results that were derived from experiments in the rat, whereas previous studies reporting on the beneficial effects of NO\textsubscript{2} in I/R in vivo have been performed in mice.

In summary, the results of this study suggest that NO\textsubscript{2} therapy does not protect in renal I/R injury. Although initial studies have demonstrated the therapeutic efficacy and potential for NO\textsubscript{2} in I/R injury in the liver and heart as well as in other ischemia-related disorders, the lack of a beneficial effect in the kidney underscores the importance for further investigation, specifically in the context of organ-specific effects of NO\textsubscript{2}.

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l-NAMe, N\textsuperscript{\textdagger}-nitro-l-arginine methyl ester; l-NMMA, N\textsuperscript{\textdagger\textdagger}-monomethyl-l-arginine; iNOS and eNOS, inducible and endothelial nitric oxide synthase, respectively.
GRANTS
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