Nitrite-Derived Nitric Oxide Protects the Rat Kidney against Ischemia/Reperfusion Injury In Vivo: Role for Xanthine Oxidoreductase

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In normal conditions, nitric oxide (NO) is oxidized to the anion nitrite, but in hypoxia, this nitrite may be reduced back to NO by the nitrite reductase action of deoxygenated hemoglobin, acidic disproportionation, or xanthine oxidoreductase (XOR). Herein, is investigated the effects of topical sodium nitrite administration in a rat model of renal ischemia/reperfusion (I/R) injury. Rats were subjected to 60 min of bilateral renal ischemia and 6 h of reperfusion in the absence or presence of sodium nitrite (30 nmol) administered topically 1 min before reperfusion. Serum creatinine, serum aspartate aminotransferase, creatinine clearance, fractional excretion of Na\(^+\)/H\(^+\), and plasma nitrite/nitrate concentrations were measured. The nitrite-derived NO-generating capacity of renal tissue was determined under acidic and hypoxic conditions by ozone chemiluminescence in homogenates of kidneys that were subjected to sham, ischemia-only, and I/R conditions. Nitrite significantly attenuated renal dysfunction and injury, an effect that was abolished by previous treatment of rats with the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (2.5 μmol intravenously 5 min before ischemia and 50 nmol topically 6 min before reperfusion). Renal tissue homogenates produced significant amounts of NO from nitrite, an effect that was attenuated significantly by the xanthine oxidoreductase inhibitor allopurinol. Taken together, these findings demonstrate that topically administered sodium nitrite protects the rat kidney against I/R injury and dysfunction in vivo via the generation, in part, of xanthine oxidoreductase–catalyzed NO production. These observations suggest that nitrite therapy might prove beneficial in protecting kidney function and integrity during periods of I/R such as those encountered in renal transplantation.


Until recently, the nitrite anion was regarded merely as an inactive metabolite of nitric oxide (NO) oxidation produced under normal physiologic conditions, accounting for approximately 70% of the endogenous nitrite pool (1). However, we (2) and others (3) recently demonstrated that, far from being inactive, nitrite has marked protective effects in ischemia/reperfusion (I/R) injury of both the heart and the liver. Moreover, these studies demonstrate that the beneficial effects of nitrite are related to its reduction to NO, under ischemic conditions.

The generation of NO is attributed conventionally to the enzyme NO synthase (NOS), of which there are three isoforms. Endothelial NOS (eNOS)-derived NO plays an important role in determining and maintaining aspects of normal renal function, for instance proximal tubule sodium reabsorption (4,5), but in elevated concentrations, NO also contributes to renal pathophysiology (6), such as in proximal tubule ischemic injury (4). This dual nature of NO perhaps is an oversimplification, because NO either ameliorates or exacerbates renal injury, depending on the site and the rate of NO production and the chemical fate of NO (7,8). Furthermore, inhibition of the activity of inducible NOS (iNOS) activity or absence of iNOS protein (iNOS knockout mice) reduces renal (proximal tubule) injury that is caused by ischemia (hypoxia), demonstrating that NO from iNOS contributes to renal injury (9–11). Neuronal NOS (nNOS) has been demonstrated to be expressed in the macula densa (12), where NO is an important modifier of the tubuloglomerular feedback response (13).

During ischemia, eNOS activity is compromised as a result of its essential dependence on oxygen and the increasing acidosis (14). However, in ischemic conditions, the endogenous nitrite
pool may serve as an important alternative source of NO in the heart, liver, and kidney by providing NOS-independent NO generation (2,3,15,16). Indeed, this nitrite-derived NO generation increases with increasing hypoxemia and acidosis. Such a source of NO thus may compensate for the diminishing production of NO from L-arginine and iNOS. Three main mechanisms that seem to play a role in the reduction of nitrite to NO have been identified: (1) Acidic reduction of nitrite via disproportionation (16,17), (2) reduction of nitrite by deoxygated hemoglobin (deoxyHb) (18,19), and (3) enzymatic conversion of nitrite by xanthine oxidoreductase (XOR) (2,15,17,20–22).

We hypothesized that the generation of NO from nitrite may be beneficial in maintaining NO balance during I/R of the rat kidney. There is evidence that the systemic administration of nitrite does not reduce renal I/R in the rat (23). However, we investigated whether direct topical application of sodium nitrite to the renal surface in vivo reduces renal dysfunction, injury, and inflammation in a rat model of I/R injury. Having found that topical nitrite reduces I/R injury, we investigated the role of XOR in the conversion of nitrite to NO within the kidney, because XOR is expressed in both the glomeruli and the tubulointerstitium of allograft kidneys (24).

Materials and Methods

Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich Co. Ltd. (Poole, Dorset, UK). All solutions used were prepared using nonpyrogenic saline (0.9% NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, UK). Thiopentone sodium (Intraval sodium) was obtained from Rhone Merieux (Harlow, Essex, UK). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (C-PTIO) was purified from Cayman Chemical Company (Tallinn, Estonia).

Renal I/R Injury In Vivo

A total of 103 male Wistar rats (Charles River Ltd., Margate, UK) that weighed 250 to 360 g were used in this study. Rats received a standard diet and water ad libitum and were cared for in accordance with both the UK Home Office Guidance in the Operation of the Animals (Scientific Procedures) Act 1986 and the Guide for the Care and Use of Laboratory Animals, published by the American Physiologic Society. Rats were surgically anesthetized using sodium thiopentone (Intraval sodium, 120 mg/kg intraperitoneally; Merial Animal Health Ltd., Harlow, Essex, UK) and then subjected to bilateral renal ischemia for 60 min followed by reperfusion for 6 h as described previously (25).

Rats were randomly allocated into seven groups and treated with saline (I/R group) or nitrite administered topically (I/R nitrite group; 60 μmol/L, 2 ml/kg, onto kidneys 1 min before reperfusion) or intravenously (I/R nitrite [intravenous] group; 60 μmol/L, 2 ml/kg, intravenously 1 min before reperfusion). For determination of whether NO was responsible for any effects of nitrite, rats were treated with the NO scavenger C-PTIO (5 mM C-PTIO, 2 ml/kg, intravenously 5 min before renal ischemia and 100 μmol/L C-PTIO, 2 ml/kg, onto kidneys 6 min before reperfusion) in the absence (I/R C-PTIO group) and presence of topical nitrite treatment (I/R nitrite + C-PTIO group). For determination of whether XOR is responsible for the conversion of nitrite to NO, rats were treated with the XOR inhibitor allopurinol (40 mg/kg, 1 ml/kg, intravenously) 8 min before renal ischemia and 8 min before reperfusion, in the absence (I/R allopurinol group) or presence of topical nitrite treatment (I/R nitrite + allopurinol group). The vehicle for allopurinol was NaOH; therefore, an additional group was administered NaOH (0.4 M, 1 ml/kg, intravenously) 8 min before renal ischemia and 8 min before reperfusion (I/R NaOH group). Responses were compared with those that were seen in sham-operated rats that were subjected to all procedures except I/R in the absence (sham group) and presence of topical nitrite treatment (sham nitrite group). The dosages of nitrite and allopurinol used were based on those that were shown previously to provide protection against I/R injury in the rodent (3,26).

Determination of Renal Injury and Dysfunction

At the end of the reperfusion period, 1-ml blood samples were collected via the carotid artery into S/1.3 tubes that contained serum gel (Sarstedt, Germany), after which the heart was removed to terminate the experiment. The samples were centrifuged (6000 × g for 3 min) to separate serum from which biochemical parameters were measured within 24 h (Vetlab Services, Sussex, UK). Serum creatinine concentration was used as an indicator of impaired renal (glomerular) function (27). The rise in the serum level of aspartate aminotransferase (AST) was used as an indicator of reperfusion injury (28). Urine samples were collected during the reperfusion period, and the volume of urine produced was recorded. Urinary creatinine was measured and was used in conjunction with serum creatinine concentration and urine flow to calculate creatinine clearance (CCL) using standard formulas, which was used as an indicator of glomerular function (27). Urinary Na+ was measured at the end of the reperfusion period and used in conjunction with serum Na+ to estimate fractional excretion of Na+ (FENa) using standard formulas and was used as an indicator of tubular dysfunction (29).

Histologic Evaluation

Histology and histologic scoring of renal sections were prepared as described previously and used for the assessment of renal I/R injury (28). Briefly, 100 intersections were examined for each kidney and a score from 0 to 3 was given for each tubular profile that involved an intersection: 0, normal histology; 1, tubular cell swelling, brush border loss, nuclear condensation, with up to one third of tubular profile showing nuclear loss; 2, as for score 1 but greater than one third and less than two thirds of tubular profile shows nuclear loss; and 3, greater than two thirds of tubular profile shows nuclear loss. The total score for each kidney was calculated by addition of all 100 scores with a maximum score of 300.

Immunohistochemical Localization of Nitrotyrosine

Tyrosine nitration was detected in kidney sections by immunohistochemistry, as described previously (10).

Western Blotting for eNOS, iNOS, and nNOS

Longitudinal sections of kidney were homogenized individually in homogenizing buffer (50 mmol/L Tris-HCl; 150 mmol/L NaCl; 1% Triton X-100; 2 mM EDTA; 8 mmol/L EGTA; and 1 μg/ml each of benzamidine, leupeptin, antipain, and aprotinin) using an automated high-throughput homogenizer (Precellys24; Bertin Technologies, Montigny-le-Bretonneux, France). Samples (20 μg) then were subjected to Western blotting for detection of eNOS (rabbit polyclonal anti-eNOS, C-20, 1/5000; Santa Cruz Biotechnology, Santa Cruz, CA), iNOS (purified iNOS rabbit polyclonal antibody, 1/1000; BD Biosciences, Oxford, UK), and nNOS (purified nNOS polyclonal antibody, 1/1000; Cayman Chemicals) as described previously (30).
Measurement of NO Formation from Nitrite by Rat Kidney Homogenates

Kidneys that were removed from sham-operated rats, from rats that were subjected to ischemia only (60 min), and from rats that were subjected to ischemia (60 min) and reperfusion (2 min) were snap-frozen in liquid nitrogen and kept at −80°C until processing. Tissue samples then were homogenized and protein concentration was determined using the Bradford protein assay as described previously (2). Nitrite (100 μmol/L)-derived NO formation by kidney homogenates (500 μg) was determined using ozone chemiluminescence under pH 5.5 and hypoxic conditions (over 2 min) in a 10-ml reaction chamber as described previously (2).

Statistical Analyses

All values described in the text and figures are expressed as means ± SEM for n observations. Each data point represents biochemical measurements, hemodynamic monitoring, plasma nitrite/nitrate concentrations, rates of NO production, and eNOS protein expression that were obtained from up to 12 separate rats. Two-way ANOVA with Bonferroni post hoc test was performed on hemodynamic data, and one-way ANOVA with Dunnett post hoc test was performed on all other data using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com). P < 0.05 was considered to be significant.

Results

Effect of Nitrite on Renal/Glomerular Dysfunction and Injury Caused by I/R in Rats

Rats that underwent renal I/R exhibited a significant increase in serum creatinine and serum AST, attenuation in CCL, decrease in urine flow, and increase in FENa, indicating renal, glomerular, and tubular dysfunction (Figure 1). Treatment of rats with topical nitrite significantly attenuated the deleterious effects of I/R with respect to serum creatinine, serum AST, CCL, and FE\textsubscript{Na+} (Figure 1). Topical nitrite seemed to improve urine flow; however, this did not quite reach significance (Figure 1). In all cases, the effect of topical nitrite was abolished by treatment with the NO scavenger C-PTIO (Figure 1). The administration of C-PTIO in the absence of nitrite had no effect per se on I/R-induced dysfunction (Figure 1). The administration of allopurinol alone to rats that were subjected to I/R had a protective effect per se, significantly (P < 0.05) attenuating the rise in serum creatinine compared with rats that were administered NaOH alone (176.2 ± 8.0 [n = 6] versus 200.3 ± 7.4 [n = 6], respectively). However, the co-administration of allopurinol with topical nitrite resulted in a similar level of serum creatinine as allopurinol alone (175.3 ± 3.8; n = 6), abolishing (P < 0.05) the protective effect of nitrite alone on serum creatinine (152.6 ± 6.4; n = 12).

In contrast to topical nitrite administration, the systemic application of nitrite at an equivalent dosage had no significant effect on any of the parameters assessed (serum creatinine: 185.8 ± 5.2 [n = 12] versus 181.0 ± 7.3 [n = 6]; serum AST: 1907 ± 158 [n = 12] versus 2177 ± 129 [n = 6]; CCL: 0.007 ± 0.002 [n = 12] versus 0.006 ± 0.002 [n = 6]; FE\textsubscript{Na+}: 60.5 ± 10.8 [n = 12] versus 63.7 ± 10.2 [n = 6]) for the I/R group and I/R nitrite (intravenous) group, respectively.

Effects of Nitrite on Histologic Alterations after I/R in Rats

On comparison with the renal histology that was observed in kidneys that were taken from sham-operated rats (Figure 2A), rats that underwent renal I/R demonstrated the recognized features of renal injury (Figure 2B). Specifically, this included degeneration of tubular structure, tubular dilation, swelling and necrosis, luminal congestion, and the presence of eosinophilia. In contrast, renal sections that were obtained from rats that were administered topical nitrite before reperfusion (Figure 2C) demonstrated marked reduction of the histologic features of renal injury on comparison with kidneys that were obtained from rats that were subjected to I/R only (Figure 2B).

On comparison with the histology score that was measured from kidneys that were obtained from sham-operated animals, renal I/R produced a significant increase in histology score (Figure 2D). Administration of topical nitrite significantly reduced the histology score when compared with that obtained from rats that were subjected to renal I/R only (Figure 2D), indicating a reduction in renal injury.

Effect of Nitrite on Nitrotyrosine Staining after I/R in Rats

When compared with sham-operated rats (Figure 3A), immunohistochemical analysis of kidney sections that were obtained from rats that were subjected to I/R demonstrated a positive staining for nitrotyrosine (Figure 3B). In contrast, renal sections that were obtained from rats that were administered topical nitrite and underwent I/R demonstrated no positive staining for nitrotyrosine (Figure 3C).

Effect of Nitrite on NO Production and NOS Expression Caused by Renal I/R in Rats

When compared with sham-operated rats, the plasma that was obtained from rats that were subjected to I/R demonstrated a significant increase in nitrite/nitrate levels (Figure 4A), suggesting increased NO formation. This increase was absent in the plasma of rats that were subjected to I/R subsequent to topical administration of nitrite (Figure 4A). The effect of topical nitrite treatment was prevented by C-PTIO (Figure 4A).

No detectable iNOS was found in any samples tested; however, I/R injury resulted in a significant increase in eNOS expression, above that seen in sham-operated rats, that was absent in the kidneys of rats that were treated with topical nitrite (Figure 4B). In addition, I/R injury resulted in a significant increase in nNOS expression that was not attenuated with the administration of topical nitrite (Figure 4C).

Rates and Mechanism of Nitrite-Derived NO Production from Rat Kidney Homogenates

The rates of NO that were generated from nitrite by rat kidney homogenates at acidic pH (pH 5.5) under anaerobic conditions were significantly higher than the rates that were produced by rat kidney homogenates in the absence of nitrite (Figure 5A). This finding supports the conclusion that exogenous nitrite can be a source of NO production under hypoxic (acidic) conditions. The nitrite reductase activity of kidneys that had been exposed to 60 min of renal ischemia and 2 min of
reperfusion was significantly greater than that of sham-treated kidneys and greater than that of kidneys that had undergone ischemia only (Figure 5A). Treatment of kidney homogenates with allopurinol attenuated NO production by approximately 71% (Figure 5B). Allopurinol had no effect per se in the absence of tissue (data not shown).

Effect of Nitrite on Mean Arterial BP Caused by I/R in Rats

Overall, there was no significant difference in mean arterial pressure (MAP) between groups at baseline (time = 0 min; Figure 6). Topically administered nitrite produced a significant attenuation in the I/R-mediated rise in MAP 1 h after the commencement of reperfusion (time = 120 min; Figure 6). Administration of C-PTIO to nitrite-treated rats not only reversed this effect of nitrite but also restored the rise in MAP 1 h after the commencement of reperfusion compared with rats that were subjected to I/R only (Figure 6).

Discussion

Nitrite-derived NO protects the heart and the liver against experimental I/R injury. Herein, we demonstrate that topical
but not systemic administration of nitrite protects the kidney against the effects of renal I/R injury. Moreover, we demonstrate that this protection is due specifically to the activity of NO that is generated after the enzymatic reduction of nitrite to NO achieved, in part, via the nitrite reductase activity of XOR.

Topical administration of nitrite attenuated the degree of (1) renal dysfunction (increased plasma creatinine levels), (2) reperfusion injury (increased plasma AST levels), (3) glomerular dysfunction (reduced CCL), and (4) tubular injury (increased FENa). It is interesting that whereas topical nitrite administration was of clear benefit, intravenous administration of nitrite had no effect on the renal dysfunction and injury that were caused by the I/R insult, confirming the recent study of Basireddy et al. (23). Because the concentration of nitrite that was used in this study was the same for both routes (60 μmol/L, identical to that of Basireddy et al.) and the timing of nitrite application at 1 min before reperfusion (i.e., 59 min in to the ischemic event) was the same in both protocols, it is likely that the route of administration underlies the difference in efficacy. However, one still cannot discount that this study and that of Basireddy et al. are two very different models of I/R injury. Basireddy et al. hypothesized that the topical route would be unlikely to be effective in the kidney, because the degree of absorption from the kidney surface may not be adequate as a result of the thick capsule. However, our study clearly demonstrates that the capsule is not a major barrier to the absorption and the efficacy of nitrite. On the contrary, it is likely that the topical route provides a higher local concentration of nitrite to the kidney than systemic administration. When nitrite is administered by a systemic route, it is probable that substantial amounts of nitrite are metabolized to NO by deoxyHb in the circulation before it reaches the kidney. With topical application, it is likely that considerably less metabolism occurs via this route.

Having established that topical administration of nitrite does indeed protect the kidney against I/R injury, we wished to investigate whether this beneficial effect was, indeed, due to formation of NO in vivo. We demonstrate here that the NO scavenger C-PTIO abolished the beneficial effects of nitrite, whereas C-PTIO alone (i.e., in the absence of nitrite) did not aggravate the renal injury and dysfunction that were caused by I/R injury, indicating that, as in the heart (2), NO mediates the beneficial effects of nitrite. This is in accordance with the study of Okamato et al. (15), who, using [(15)N]nitrite, demonstrated nitrite-derived NO generation as evidenced by formation of [(15)N]NO adducts after renal ischemia.

Our findings of a protective effect of nitrite via its provision of NO in I/R are consistent with previous studies demonstrat-
ing beneficial effects of NO in acute renal failure. However, NO also has been associated with damaging effects. The protective effects of NO seem to be associated with eNOS-derived NO, which causes vasodilation and inhibits leukocyte adhesion and platelet aggregation (31), whereas the damaging and proinflammatory effects have been associated with iNOS-derived NO, which forms the peroxynitrite anion from the interaction of NO and superoxide (32). The plasma levels of nitrite (used as an indicator of NO formation) measured at the end of the 6-h reperfusion period indicate I/R-induced elevated NO production as demonstrated previously (10). This elevated NO has been proposed to account, in part, for the proinflammatory processes that occurs within the kidney and contribute to acute renal failure (9) and due in part to the generation of peroxynitrite. The reduction of nitration of proteins after application of nitrite indicates that this treatment results in a reduction in peroxynitrite formation, and it is likely that this, in part, is responsible for the beneficial effects of nitrite treatment. Indeed, this is a surprising finding, because XOR has a large capacity to generate reactive oxygen species (ROS) in I/R, and with the increased NO production from nitrite, an increase in peroxynitrite production would have been expected. However, we hypothesize that the reduction of nitrite to NO at the molybdenum site of XOR will result in competition for electrons, limiting the rate of oxygen reduction at the flavin adenine dinucleotide site of the enzyme. Consequently, superoxide production and, therefore, peroxynitrite would be reduced. Hence, providing XOR with nitrite as an alternative substrate may result in a protective effect through the generation of NO and inhibition of ROS production, rather than a damaging effect, and this indeed is consistent with what we previously demonstrated in the heart (2).

It is interesting that Western blotting of kidney supernatants indicate that the source of this increased NO during I/R is likely to be eNOS and nNOS rather than iNOS. eNOS and nNOS expression was elevated after the full I/R insult, but, surprisingly, no iNOS was detectable. This lack of iNOS expression is at odds with previous publications, and it is unclear why this should be the case but may relate to differences in the severity of the I/R insult. Our data suggest, for the first time, that nNOS has been induced during renal I/R injury. Whether this is a protective mechanism is unknown, and further investigation is required. In a model of cerebral ischemia, it was found that nNOS inhibition improved hippocampal function after ischemia, suggesting a proinflammatory role of nNOS (33). Our observations of elevated eNOS expression are in agreement with Shoskes et al. (34), who demonstrated that during reperfusion, eNOS is upregulated rapidly for 6 h after commencement of reperfusion, after which eNOS is downregulated rapidly on the third day after reperfusion and returns to preischemia levels only on the 21st day. The balance between ROS and NO in the microvasculature promotes an anti-inflammatory phenotype under normal conditions while favoring a proinflammatory phenotype during I/R. Under normal conditions, the balance between ROS and NO favors an anti-inflammatory phenotype, because NO chemistry predominates as a result of the approximately 1000-fold greater production of NO compared with superoxide in vascular endothelial cells (35). During ischemia, eNOS activity becomes suppressed profoundly as a result of its essential dependence on oxygen. It is likely that, rather than directly altering eNOS activity, provision of nitrite and thereby NO under ischemic conditions re-

Figure 3. Effect of nitrite on nitrotyrosine staining that was caused by I/R in rats. Nitrotyrosine staining of renal sections from a sham-operated rat (A), a rat that was subjected to renal I/R only (B), and a rat that was administered nitrite (60 μmol/L, onto kidneys) 1 min before reperfusion and subjected to renal I/R (C). Figures are representative of at least three experiments that were performed on different experimental days (n = 12 for all groups; primary antinitrotyrosine antibody).
places the constitutive protective NO that is provided by eNOS and maintains normal homeostasis and in so doing prevents the proinflammatory processes from being activated as a result of repressed NO production. Therefore, when the balance of NO and ROS is disrupted, this can lead to a proinflammatory phenotype in the microcirculation. In this instance, the production of superoxide relative to NO increases such that ROS-dependent mechanisms predominate and NO-dependent mechanisms are rendered inactive. The precipitous decline in NO bioavailability in the ischemic state may result from a reduction in NO biosynthesis, inactivation of NO by superoxide, or both (35). Indeed, several lines of recent evidence support the hypothesis that inflammatory stimuli, in the early stages of an inflammatory response, upregulate eNOS-derived

Figure 4. Effect of nitrite on nitric oxide (NO) production and NO synthase (NOS) expression caused by I/R in rats. (A) Plasma nitrite/nitrate concentrations were measured as an indicator of NO levels at the end of the 6-h reperfusion period subsequent to sham operation (sham; \( n = 12 \)) or renal I/R (I/R; \( n = 12 \)). Rats were administered nitrite (60 \( \mu \text{mol/L} \), onto kidneys) 1 min before reperfusion (I/R nitrite; \( n = 12 \)) and C-PTIO (5 mM, 2 ml/kg, intravenously) 5 min before renal ischemia and (100 \( \mu \text{mol/L} \), 2 ml/kg, onto kidneys) 6 min before reperfusion (I/R nitrite + C-PTIO; \( n = 9 \)). An additional group of rats received nitrite (60 \( \mu \text{mol/L} \), onto kidneys) 1 min before sham reperfusion (sham nitrite; \( n = 8 \)). (B) Western blot analysis of endothelial NOS (eNOS) and \( \alpha \)-actin expression in the rat kidney from three different experiments that were performed on different experimental days. (C) Western blot analysis of neuronal NOS (nNOS) and \( \alpha \)-actin expression in the rat kidney from three to five different experiments that were performed on different experimental days. Data are means ± SEM for \( n \) observations. *\( P < 0.05 \) versus I/R; **\( P < 0.05 \) versus I/R nitrite + C-PTIO.
NO that consequently exerts proinflammatory actions, including the induction of iNOS (30,36).

Having shown that an enhanced formation of NO from nitrite accounts for the beneficial effects that were seen in this study, we tried to address the underlying mechanism(s). Clearly, in the presence of a low pH of approximately 5.5, which has been reported to occur in the kidney during I/R (37), rat kidney homogenates that are subjected to 60 min of ischemia and 2 min of reperfusion convert nitrite to NO (as determined by chemiluminescence) above that achieved as a result of simple acidic disproportionation. Simple chemical acidification/reduction accounted for approximately 50% of the total nitrite reductase activity that was measured using the chemiluminescence technique in the incubation experiments, indicating that 50% of the response could be attributed to tissue nitrite reductase activity that was measured using the chemiluminescence technique in the incubation experiments, indicating that 50% of the response could be attributed to tissue

Figure 5. Rates of nitrite-derived NO production from rat kidney homogenates. (A) Rates of NO production at pH 5.5 under anaerobic conditions from kidney homogenates (300 μg of protein) in the absence (sham-nitrite; n = 4) and presence of nitrite (sham+nitrite [n = 6]; ischemia+nitrite [60 min of ischemia only; n = 4]; I/R+nitrite [60 min of ischemia and 2 min of reperfusion; n = 4]). (B) Rates of NO production at pH 5.5 under anaerobic conditions in the presence of kidney homogenates (300 μg of protein) 1 min before reperfusion (I/R+nitrite; n = 5) in the absence (I/R+nitrite; n = 6) and the presence of allopurinol (I/R+nitrite+allo; n = 6). Data are means ± SEM for n observations. *P < 0.05 versus sham+nitrite; **P < 0.05 versus I/R+nitrite.

Figure 6. Effect of topically administered nitrite on mean arterial pressure (MAP) caused by I/R in rats. MAP was measured during and subsequent to sham operation (sham; n = 12) or renal I/R (I/R; n = 14). Rats were administered nitrite (60 μmol/L, onto kidneys) 1 min before reperfusion (I/R nitrite; n = 12) and C-PTIO (5 mmol/L, intravenously) 5 min before renal ischemia and (100 μmol/L, 2 ml/kg, onto kidneys) 6 min before reperfusion (I/R nitrite + C-PTIO; n = 9). A separate group of rats received C-PTIO only, same dosages and times as described above (I/R C-PTIO; n = 11). An additional group of rats received nitrite (60 μmol/L, onto kidneys) 1 min before sham reperfusion (sham nitrite; n = 8). Data are means ± SEM for n observations. *P < 0.05 I/R versus I/R nitrite; **P < 0.05 I/R nitrite versus I/R nitrite + C-PTIO.
tion of nitrite to NO results in a protective, rather than a damaging, effect.

A third mechanism that has been shown to be responsible for nitrite-derived NO formation, which cannot be excluded, is nitrite reduction by deoxyHb (19,42). In vitro red blood cell studies show that under hypoxic conditions, deoxyHb within the red blood cell reacts with nitrite to produce methemoglobin (Hb[III]), and this then reduces nitrite to a labile intermediate Hb(III)NO, which eventually is converted to Hb(II)NO (19). This mechanism also was evident in humans: Cosby et al. (42) demonstrated that nitrite was reduced to NO by deoxyHb and caused dosage-dependent vasodilation. This possibly may be one of the mechanisms for the protective effect of topically administered nitrite against I/R injury in the rat kidney. It is likely that both this pathway and the XOR pathway have a role to play and that their contribution depends largely on the prevailing environmental conditions (O₂ levels and pH). Indeed, Okamoto et al. (15) showed important nitrite reductase roles for both Hb and XOR in vivo in the kidney after the intravenous administration of nitrite.

We also found a significant rise in systemic MAP at 1 h after reperfusion in rats that were subjected to I/R when compared with sham-operated rats. This might be the result of the activation of the renin-angiotensin system (RAS), a major endocrine regulatory system of cardiovascular homeostasis, which is involved in reperfusion injury of the kidney (43). Renal function and pathology are improved markedly by angiotensin-converting enzyme (ACE) inhibitors such as captopril (44), saralasin (45), and enalapril (46) in several models of renal ischemia. ACE inhibitors are thought to protect against renal I/R by attenuating renal vascular resistance, thereby increasing oxygen delivery and consumption (46–48). In addition, inhibition of type I angiotensin II receptors causes reduced renal vascular resistance and increased synthesis of prostacyclin and NO (49). This would be in agreement with other evidence that suggests that NO that is generated from NOS can counteract the RAS by inhibiting renin release, ACE, or angiotensin II (50,51). Therefore, NO that is generated from nitrite may play a similar role to that just described; however, further studies would need to be carried out to confirm this.

Conclusion

Our observations demonstrate for the first time that topically administered nitrite protects the rat kidney against I/R injury. This effect of nitrite is due to formation of NO, as it was abolished by an NO scavenger. The conversion of nitrite to NO depends on low pH and the enzymatic activity of XOR, but we cannot exclude the possibility that other mechanisms contribute to the observed conversion. These findings do not support the concept of organ-specific effects of nitrite in I/R injury or a unique metabolism in the kidney; rather, they suggest similar protective effects of nitrite in the kidney to those of the heart and the liver, with similar metabolism, but highlight the importance of the route of administration. On the basis of these findings, we propose that transplant solutions that contain nitrite (or other molecules that release NO in an acidic environment) will protect the kidney and other organs against I/R injury and may have beneficial effects in organ transplantation.

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Disclosures

None.

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