Chronic renal hypoxia after acute ischemic injury: effects of L-arginine on hypoxia and secondary damage

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Basile, David P., Deborah L. Donohoe, Kelly Roethe, and David L. Mattson. Chronic renal hypoxia after acute ischemic injury: effects of L-arginine on hypoxia and secondary damage. Am J Physiol Renal Physiol 284: F338–F348, 2003. First published October 1, 2002; 10.1152/ajprenal.00169.2002.—Ischemic acute renal failure (ARF) results in the permanent loss of peritubular capillaries and predisposes the progression of chronic renal failure. The present study was undertaken to determine whether renal hypoxia, which may represent an important mediator in disease progression, is persistently exacerbated after recovery from ARF. Rats were subjected to ischemia-reperfusion injury and allowed to recover for 5 or 20 wk. Immunohistochemistry of the hypoxia-sensitive marker 2-pimonidizole at 5 wk revealed an overall increase in incorporation in the outer medullary region after recovery from ARF compared with sham-operated controls. Unilateral nephrectomy, in combination with ischemia-reperfusion injury resulted in greater 2-pimonidizole staining than that observed in the bilateral injury model. In addition, in the unilateral ischemia-nephrectomy model, proteinuria, interstitial fibrosis, and renal functional loss developed significantly faster than in the bilateral model of ARF when animals were allowed to recover for 20 wk. L-Arginine in the drinking water (~0.5 g/day) increased total renal blood flow ~30%, decreased pimonidizole staining, and attenuated progressive proteinuria and interstitial fibrosis (3). It is proposed that a reduction in the peritubular capillary density after ARF results in a persistent reduction in renal PO2 and that hypoxia may play an important role in progression of chronic renal disease after ARF.

fibrosis; proteinuria; blood flow; acute renal failure

ISCHEMIC INJURY TO THE KIDNEY is a leading cause of acute renal failure (ARF). This clinical syndrome is associated with high mortality rates but is largely reversible if patients survive the initiating insult (17). In the setting of renal transplantation, prolonged ischemic time contributes to delayed graft function, which is characterized by the requirement for dialysis in the immediate posttransplant period (29). Both of these are associated with tubular cell damage and a reduction in glomerular filtration rate (GFR), which are restored through a recovery and repair response (5, 20). The occurrence of delayed graft function immediately after transplant strongly correlates with development of hypertension and allograft nephropathy (18, 29, 31). To date, there is no strong or established correlation with ARF in native kidneys and the development of long-term complications; however, renal function is not always fully restored and the development of chronic renal insufficiency often ensues after ARF (4, 8, 25, 27). These clinical observations argue that reversible renal injury predisposes the kidney to the development of long-term renal complications.

Rodent models of ischemia-reperfusion (I/R) injury have been utilized to study some of the features of both ARF and delayed graft function. In rats, I/R injury results in tubular necrosis and a reduction in GFR; the recovery from this type of injury is well studied and described (5, 20, 21, 35, 39). Recently, we reported that several elements of renal structure and function are irreversibly altered after I/R injury (3). We showed that there was a permanent reduction in peritubular capillary density in rats after recovery from bilateral I/R injury. We also observed that recovery from bilateral I/R injury resulted in a permanent alteration in urinary concentrating ability, increased pressor activity in response to a low dose of ANG II, and the development of progressive proteinuria and interstitial fibrosis (3).

The mechanisms by which chronic renal dysfunction ensues after recovery from ARF is not well explored. The development of hypoxia in the postischemic kidney has the potential to activate a number of profibrotic pathways, including those involving transforming growth factor-β activity, which have the potential to contribute to renal scarring (2, 7, 16, 22, 37). It is possible that the alterations in peritubular capillary density may have profound effects on renal function and the development of chronic renal insufficiency. We hypothesize that alterations in peritubular capillary density exacerbate the extent of renal hypoxia and predispose the development of chronic renal disease.

As an initial step toward addressing this hypothesis, the following study was conducted to investigate whether postischemic recovered kidneys are more hypoxic than those of corresponding sham-operated controls. In addition, we sought to determine whether unilateral nephrectomy (UNX), a treatment that is purported to exacerbate renal hypoxia (7), hastens the development of chronic renal insufficiency. Finally, we...
sought to determine whether L-arginine, which is thought to influence renal blood flow (RBF) (6, 14), affects renal hypoxia and progression of CRF.

METHODS

Animal and surgical procedures. Care of the rats before and during the experimental procedures was conducted in accordance with the policies of the Animal Resource Center, Medical College of Wisconsin, and the Guide for the Care and Use of Laboratory Animals (Washington, DC: National Academy Press, 1996). All protocols had received prior approval by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Male Sprague-Dawley rats (~250 g; Harlan, Madison, WI) were housed in pairs in standard shoebox cages with 12:12-h light-dark cycle (lights on, 0600–1800) and access to water and standard laboratory rat chow (0.8% NaCl, Purina Lab Diets 5001) available ad libitum. Animals were anesthetized with ketamine (100 mg/kg ip) for 10 min followed by administration of pentobarbital sodium (25–50 mg/kg ip). ARF was induced in rats according to surgical procedures previously described (3). Briefly, animals were placed on heated surgical tables and midline incisions were made to expose the kidneys. Blood supply to the kidneys was interrupted by applying microaneurysm clamps for the indicated times. After occlusion, the clamps were removed and reflow was verified visually. In some studies, animals were subjected to right UNX in which the right renal pedicle was ligated with a 2–0 silk suture just before the excision of the right kidney. Immediately after this, the animal was subjected to left I/R injury or sham treatment.

The schema for the basic experimental design is shown in Fig. 1. Short-term studies (i.e., 5 wk) were used to address the effects of I/R injury on hypoxia and RBF (see below). This time point was chosen because it is generally associated with the restoration of total RBF and renal tubular structure and GFR in a bilateral I/R injury model (20). In addition, this time point is thought to in

Fig. 1. Schema of experimental design to determine the effects of L-arginine on renal function postischemic injury. All animals were subjected to sham surgery or 30, 45, or 52 min of ischemia-reperfusion (I/R) injury. The 24-h serum creatinine values were utilized to determine the extent of renal injury. Animals were assigned to treatment groups (tap water alone vs. tap water supplemented with L-arginine) such that the 24-h serum creatinine values were similar in both groups. In long-term studies, renal function was monitored for up to 20 wk. Renal histology was evaluated at 5 and 20 wk postsurgery. In all 20-wk studies, all animals were subjected to right unilateral nephrectomy (UNX) just before I/R injury (i.e., 1-kidney I/R injury (1-K) model).

Studies were also performed allowing animals to recover for 20 wk postsurgery. This protocol was designed to assess the effect of treatments on the development of chronic renal insufficiency. All long-term studies were performed with the 1-K model.

Because of the possibility that L-arginine might affect the initiating ischemic insult, treatment of animals with L-arginine did not commence until 72 h of recovery. Animals were placed into groups that were maintained on tap water (untreated) or tap water supplemented with L-arginine (10 g/l) as described by other investigators (30). Under these conditions, L-arginine from dietary sources accounted for ~200–250 mg/day whereas that from the drinking water comprised ~500 mg/day (i.e., ~50 ml of water drunk/day). The total amino acid intake attributable to the diet was ~4,500–5,000 mg/day.

In these studies, values of serum creatinine at 24 h postreperfusion were utilized to match groups placed into the untreated or L-arginine groups (Fig. 1). L-Arginine was replaced daily, and animals were maintained on this treatment for the duration of the study.

Measurement of renal function. Renal function was measured at 24 h and 1, 2, 4, 8, 12, 16, and 20 wk postsurgery. Blood samples were collected as follows. Rats were placed into a closed anesthesia tank containing halothane until they were relaxed but not unconscious. The animals were placed into a restrainer, and the tip of the tail (1–2 mm) was cut with a sterile steel blade. Blood was collected into heparinized tubes and plasma obtained after centrifugation. Urine collection was for 24 h in metabolic cages (Nalgene). Serum and urine creatinine were determined by using standard assays (creatinine kit 555A, Sigma). Urine volume was determined gravimetrically. Creatinine clearance over 24 h was calculated as (Ucreatinine/V)×Pcreatinine, in which Ucreatinine is urinary creatinine, Pcreatinine is plasma creatinine, and V is flow rate.

Urinary protein excretion was determined with a protein assay kit (Bio-Rad) by using the microassay format for enhanced sensitivity. Urine osmolality was determined with the microosmette osmometer (Precision Systems), which functions on the basis of freezing-point depression.
Analysis of RBF. For RBF measurements, the rats were anesthetized with Inactin (100 mg/kg ip) and placed on a heated surgical table. The trachea was cannulated to facilitate breathing, and catheters were placed in the femoral artery to monitor blood pressure and in the femoral vein for intravenous infusion of isotonic NaCl (1.0 ml 100 g body wt \(^{-1} \cdot h^{-1}\)); in these studies, mean arterial pressure ranged between 95 and 115 mmHg and there was no difference among any treatment groups (data not shown). After a midline abdominal incision, the left renal artery and vein were separated and a 2.0- or 2.5-mm-diameter flow probe was placed on the renal artery for measurement of RBF with an electromagnetic flowmeter (model 501, Carolina Instruments, King, NC). After surgery, a 30-min equilibration period was allowed before steady-state blood flow was measured in the rats during a 30-min period. Mean data, averaged over the 30-min collection period, were collected at 2 Hz by computer using data acquisition software (WinDaq acquisition software, DATAQ Instruments, Akron, OH).

Assessment of renal hypoxia. Renal hypoxia was assessed by using the hypoxia-sensitive marker 2-pimonidizole similar to the approach described previously (40). 2-Pimonidizole and a corresponding mouse monoclonal antibody were obtained from Natural Pharmacia International (Belmont, MA). Ninety minutes before termination, rats were given 2-pimonidizole (60 mg/kg ip). Renal tissue was obtained from rats after measurement of RBF (described above) and also from other rats that were treated identically. Anesthetized animals were opened with a midline incision, and the kidneys were removed quickly, cut longitudinally, and fixed by immersion in 10% buffered formalin (Fisher Scientific). The tissue was then prepared for routine histological examination.

The incorporation of 2-pimonidizole was assessed immunohistochemically from 5-μm paraffin sections by using standard staining procedures. After deparaffinization and rehydration, tissue was prepared as follows: 1) endogenous peroxidase activity was blocked by incubation in 3% H₂O₂; 2) endogenous biotin was blocked with sequential incubations with avidin and biotin (Avidin-Biotin blocking kit, Zymed); and 3) nonspecific sites were blocked by incubation in 0.01 M PBS containing 0.3% Triton X-100, 10% goat serum, and 0.3% BSA. The mouse monoclonal antibody (1:100 dilution) was incubated for 2 h at room temperature; detection was performed by using a streptavidin-biotin immunoperoxidase technique with aminoethylcarbazole as a substrate (Histostain SP, Zymed).

Analysis of microvascular structure. In some studies, renal capillary density was assessed by using Microfil as described previously (3). Microfil was visualized under light microscopy in 20-μm unstained sections with a Nikon Eclipse E400 microscope equipped with a Spot Insight color video camera (Diagnostic Instruments, Sterling Heights, MI). Images were captured online by using Metamorph imaging software (version 4.0, Universal Imaging). At least five random images of the cortex, outer stripe of the outer medulla, and inner stripe of the outer medulla were stored by using a \(\times 20\) objective lens and a field dimension of \(\sim 0.26 \text{ mm}^2\). The images were subsequently analyzed with Metamorph imaging software by a study group member who was blinded to the experimental groups. In the absence of counterstain, the sharp contrast between stained structures and the translucent renal parenchyma facilitated image thresholding by the software program and allowed for computer-generated determination of percent area stained for ECM.

Similarly, α-actin-containing myofibroblasts were identified immunohistochemically by using an antibody from Zymed. Detection of this antibody by using dianaminobenzidine as a substrate also generated images that were easily thresholded in the absence of a counterstain. Similar image analysis techniques were applied; data were expressed as the percent surface area stained with α-actin.

Statistical analysis. Unless otherwise indicated, data for renal function, blood flow, and morphometry were analyzed by one-way ANOVA and a Student-Newman-Keuls post hoc test for significance. These analyses were carried out by using SigmaStat software.

RESULTS

To determine the potential long-term effects of ARF on the development of chronic renal hypoxia, we subjected 2-K rats to bilateral I/R injury for 30, 45, or 52 min and allowed them to recover for 4–5 wk postinjury. These injuries resulted in 24 h postreperfusion values of serum creatinine of 1.0 ± 0.2, 2.2 ± 0.2, and 2.9 ± 0.5 mg/dl, respectively (\(n = 5\)/group). These injuries were less severe than the one we utilized previously to demonstrate the chronic deleterious effect of I/R injury. Nevertheless, these injuries substantially reduce peri-tubular capillary density (3). Within 1 wk, serum creatinine values returned to those observed in sham-operated controls (not shown). At 4–5 wk postinjury, urinary concentrating ability was the only obvious functional alteration observed in these animals; urinary output was 11.7 ± 1.4 ml/day in sham, 19.0 ± 1.2 ml/day in 30-min postischemic animals, 26.9 ± 4.0 ml/min in 45-min postischemic animals, and 21.45 ± 3.2 ml/day in 52-min postischemic animals.

Figure 2 illustrates the extent of the recovery of tubular morphology in the renal outer medulla after 5 wk of recovery from 1-K or 2-K injury. After a 30-min injury, tubular morphology is essentially restored in the postischemic recovered animals; tubules appear fully hypertrophied and redifferentiated and express a periodic acid-Schiff-positive brush border (Fig. 2B). In these kidneys, increased numbers of tubulointerstitial cells are occasionally observed (Fig. 2B, black arrow). Similarly, 5 wk after a 52-min injury, tubular structure in the outer medulla looks essentially normal (Fig. 2C). However, there are several focal areas of these postischemic kidneys in which structure is clearly abnormal; dilated tubules are observed occasionally in the outer medulla (Fig. 2, C–E, *) while atrophic-appearing nephrons (Fig. 2D, white arrow) and dilated tubules are also apparent in the renal cortex (Fig. 2D). Similar structures are often seen after 45 min of I/R injury (data not shown) but are almost never observed after the more mild, 30-min I/R injury. When 5 wk of recov-
ery is allowed after I/R injury in the 1-K model, normal-appearing proximal tubules are observed, but dilated tubules and interstitial cellularity are commonly present (Fig. 2E, * and black arrow, respectively).

The reactive hydroxylamine intermediate that results from the reduction of 2-pimonidizole binds to cellular thiols in the presence of low-tissue Po2 levels (1, 34). The parent compound was administered 90 min before death, and immunohistochemistry was utilized to evaluate incorporation. Staining was absent or mild in kidneys of sham-operated rats; when detectable, 2-pimonidizole immunoreactivity was observed in the outer medulla (Fig. 3A). In contrast, robust 2-pimonidizole staining was observed in the outer medulla of rats after recovery from 30- or 52-min 2-K I/R injury (Fig. 3, C–E). No signal was detectable in postischemic tissue by using the anti-pimonidizole antibody in tissues from rats that did not receive the parent compound (Fig. 3B) or when the primary antibody was replaced with nonimmune IgG (data not shown).

The degree of 2-pimonidizole staining was substantially and consistently more intense and had a wider distribution when the same studies were performed in a 1-K model of 45-min I/R injury (Fig. 3F). Additional studies were performed to determine whether oral L-arginine could affect the extent of renal hypoxia. In both 1-K and 2-K models, the degree of 2-pimonidizole staining was consistently less intense and more diffuse in postischemic animals maintained on L-arginine (Fig. 3, G and H). Indeed, less staining of 2-pimonidizole was observed in the most severely damaged regions of L-arginine-treated rat kidneys (Fig. 3G). These data suggest that kidneys of rats at 5 wk postinjury are more hypoxic than kidneys from sham-operated controls and further suggest that the degree of hypoxia can be manipulated by UNX or administration of oral L-arginine.

The possibility that oral L-arginine manifested its effects on renal hypoxia by influencing RBF was addressed by using electromagnetic flowmetry. RBF was measured ~5 wk after 2-K I/R injury. When expressed on a per kidney basis, total RBF was not different between postischemic and sham-operated controls (sham, 6.5 ± 0.5, vs. I/R, 6.1 ± 0.3 ml/min). However, due to the mild hypertrophy present in postischemic kidneys in this experiment (~10%), there was a modest but significant reduction in RBF when data were expressed on a per gram-tissue basis (Fig. 4). Total RBF was significantly enhanced in both sham-operated and postischemic animals that were maintained on L-arginine compared with the untreated postischemic group (Fig. 4).

We performed an additional study in which vessel density was measured by using Microfil in 2-K animals 5 wk after 45-min I/R injury (Fig. 5, Table 1). Untreated postischemic animals showed a significant decrease in vessel density through the cortex, outer stripe of the outer medulla, and inner stripe of the inner medulla relative to sham-operated controls. L-Arginine had no effect on vessel density at 5 wk postinjury.

The effects of renal mass reduction and oral L-arginine supplementation on the long-term effects of I/R injury were addressed in the 1-K model (45-min ischemia). Serum creatinine values were determined 24 h postinjury, and the animals were stratified on the basis of these values; L-arginine treatment commenced on day 3 (see the schema in Fig. 1). Unilateral ischemic injury in combination with contralateral UNX resulted
in an immediate decrease in renal function. The increase in serum creatinine and decrease in creatinine clearance was evident at 24 h postsurgery (Fig. 6, A and B). Serum creatinine values 24 h postsinschma were similar in the untreated group compared with the group supplemented with L-arginine (Fig. 6A). The recovery of GFR-related measurements was apparent in the first week. In our previous report that used a 2-K model of I/R injury, serum creatinine and creatinine clearance were unchanged after the initial recovery for up to 40 wk postinjury (3). However, in the 1-K model, there was an increase in serum creatinine vs. corresponding sham-operated controls at week 20 (Fig. 6A), whereas creatinine clearance values were significantly decreased at 16 and 20 wk postsurgery (Fig. 6B; week 20 values: sham, 3,904 ± 411 vs. postischemic, 2,901 ± 441 ml/day). Conversely, creatinine clearance values of postischemic animals maintained on L-arginine were not different from those of sham-operated controls (Figs. 6, A and B).

Urine protein excretion increased dramatically after ischemic injury in the untreated compared with the corresponding sham-operated UNX controls (Fig. 7). The development of proteinuria in postischemic animals maintained on L-arginine was significantly repressed vs. levels observed in postischemic animals maintained on tap water (Fig. 7). In addition, protein excretion developed more severely in 1-K postischemic animals than what was observed previously by using our 2-K model (Fig. 7) (3).

Two of twelve animals in the untreated postischemic group were euthanized during the course of the recovery period before the 20-wk time point. These animals had elevated serum creatinine values and a high urine protein content (data not shown); the loss of these most severely affected animals reduced the

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Fig. 3. Immunohistochemical detection of pimonidazole uptake after recovery from I/R injury. Shown are low-magnification micrographs of rat renal cortex (c), outer medulla (om), and inner medulla (im) from rats in which immunohistochemistry was performed to reveal 2-pimonidazole incorporation. Representative staining in a kidney is shown from a 2-K sham-operated rat (A), 2-K 30-min postischemic rat (C), 2-K 52-min postischemic rat (E), and 1-K 45-min postischemic rat (F). G and H: kidneys corresponding to 2-K and 1-K postischemic rats, respectively, treated with l-arginine. B: representation of staining with the 2-pimonidazole antibody of a kidney from a postischemic rat not treated with 2-pimonidazole. Staining patterns are representative of a minimum of 6 samples/group. D: higher magnification of C, demonstrating tubular staining (arrow).
number of individuals in this group and resulted in diminution of the apparent reduction in renal function after the initial recovery period.

Renal ischemia resulted in a large increase in urine flow that resolved partially during the first 4 wk of recovery; however, urine flow remained significantly elevated at all time points during the recovery period (Fig. 8A). The effects of L-arginine on urine flow are shown in Fig. 8, B and C. Animals maintained on L-arginine manifested significantly larger urine output compared with either the untreated sham-operated or untreated postischemic groups. Measurement of urine osmolarity demonstrated the reciprocal relationship; i.e., postischemic animals and animals maintained on L-arginine had more dilute urine compared with their appropriate control groups (data not shown).

Renal structural data are shown in Figs. 9 and 10. Representative cortical sections stained with silver to highlight ECM are shown for rats 20 wk after surgery (Fig. 9, A–C). Basement membrane thickening and tubulointerstitial ECM material can be observed in the postischemic group compared with the sham-operated group (compare Fig. 9B with 9A). There was a significant increase in cortical and outer medullary ECM content compared with the sham-operated group; the increase in ECM staining was attenuated in the postischemic group maintained on L-arginine (Fig. 9D).

The presence of tubulointerstitial scarring characterized by the presence of myofibroblasts was revealed by α-actin immunohistochemistry (Fig. 10). Staining of α-actin in kidneys of sham-operated control rats was present in renal blood vessels and only moderately in the interstitial region (Fig. 10A). In kidneys from untreated postischemic animals, there was a substantial and significant increase in α-actin-containing interstitial cells (Fig. 10B); the presence of these cells was largely attenuated in kidneys from L-arginine-treated animals (Fig. 10, C and D). Taken together, the data suggest that L-arginine attenuates or delays the progression of chronic renal insufficiency after recovery from acute ischemic injury.

Table 1. Effect of L-arginine on capillary density after 45-min I/R injury

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham (n = 6)</th>
<th>45-min I/R Untreated (n = 6)</th>
<th>45-min I/R L-Arginine (n = 5)</th>
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<tr>
<td>Serum creatinine, mg/dl</td>
<td></td>
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<tr>
<td>1 day postinjury</td>
<td>0.45 ± 0.1</td>
<td>2.1 ± 0.2*</td>
<td>2.4 ± 0.2*</td>
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<tr>
<td>7 days postinjury</td>
<td>0.42 ± 0.1</td>
<td>0.71 ± 0.1</td>
<td>0.72 ± 0.2</td>
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<tr>
<td>35 days postinjury</td>
<td>0.41 ± 0.1</td>
<td>0.47 ± 0.1</td>
<td>0.44 ± 0.1</td>
</tr>
<tr>
<td>Renal vascular density at 35 days, % sham control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>100 ± 4</td>
<td>52 ± 7*</td>
<td>63 ± 6*</td>
</tr>
<tr>
<td>OSOM</td>
<td>100 ± 4</td>
<td>53 ± 7*</td>
<td>56 ± 5*</td>
</tr>
<tr>
<td>ISOM</td>
<td>100 ± 5</td>
<td>54 ± 9*</td>
<td>68 ± 5*</td>
</tr>
</tbody>
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Values are means ± SE, vascular density values normalized to values obtained with sham-operated controls. I/R, ischemia-reperfusion; OSOM, outer stripe of outer medulla; ISOM, inner stripe of outer medulla. *P < 0.05 vs. sham-operated control; P < 0.05 vs. untreated acute renal failure by Student’s t-test.
DISCUSSION

Both experimental and clinical studies demonstrate that the recovered postischemic kidney is not normal. The observation that recovery from I/R injury in rats predisposes the development of features indicative of chronic renal failure has now been demonstrated by several different groups of investigators (3, 9, 13, 19, 33). These observations may have clinical implications with regard to the recovery of patients after ARF or delayed graft function.

In an effort to study the potential factors that predispose the development of chronic renal failure after I/R injury, we have examined renal tissue at time points in which renal function, and to a large degree tubular structure, is restored after injury (i.e., 5 wk postinjury). Pagtalunan et al. (33) have performed morphometric analyses of kidneys after recovery from I/R injury. They demonstrated the incomplete recovery of a large population of nephrons and that some tubules lost continuity with their parent glomeruli. We did not perform the same type of elegant nephron reconstruction utilized by these investigators, but results of gross morphology at 5 wk postinjury are consistent with abnormal tubular structure indicative of incomplete recovery. However, on gross examination, it is apparent that there is heterogeneity with regard to

Fig. 6. Renal function postischemia. A: serum creatinine values are shown at 24 h and 1 and 20 wk postinjury. B: creatinine clearance values were determined on the basis of 24-h urine collections. The number of animals decreased after the 5-wk time point during the course of the study. Thus n = 20 and 12 in the postischemic water group before and after the 5-wk time point, respectively. In addition, 2 animals in this group were euthanized before 20 wk (1 each after weeks 8 and 12). Thus n = 10 at the completion of the study. Values are means ± SE, expressed relative to the values obtained in the sham-operated/water-treated group. *P < 0.05, postischemic water group vs. sham-operated control by Student-Newman-Keuls.

Fig. 7. Development of proteinuria postischemic injury. Daily urinary protein excretion was determined at the indicated times from 24-h urine collections. Development of proteinuria in animals after bilateral renal ischemia (○) was originally presented in a previous publication (3). Values are means ± SE for all groups in this study. bw, Body wt. *, a, b: P < 0.05 in UNX postischemic water group vs. corresponding UNX sham-operated control group, UNX sham-operated group on L-arginine, and UNX postischemic group on L-arginine, respectively (by ANOVA and Student-Newman-Keuls test); c, P < 0.05 in UNX postischemic group vs. bilateral postischemic group (by Student’s t-test).

Fig. 8. Urinary output postischemic injury. Urine flow rates are on the basis of 24-h urine collections at the indicated time points. A: urine flow rate after UNX and either sham rats or postischemic rats maintained on tap water. B: urine flow rate of rats after UNX and sham surgery maintained on either tap water or L-arginine. C: urine flow rate of rats after UNX and I/R injury maintained on either water or L-arginine. Values are means ± SE. *P < 0.05, postischemic vs. sham-operated group. a and b, P < 0.05 L-arginine group vs. tap water group in sham and postischemic groups, respectively.
the degree of incomplete regeneration at 5 wk postinjury. The degree and number of persistently dilated tubules appear to be dependent on the severity of the injury as well as renal mass, such that renal structure is more disrupted in 1-K animals.

In an earlier study, we examined vessel density by using Microfil after I/R injury to 2-K animals at 4–8 wk. In contrast to the heterogeneity of dilated, atrophic, or incompletely regenerated nephrons whose presence in the recovered kidney is sporadic and focal, the pattern of vessel reduction revealed by Microfil after I/R injury is homogeneous and was observed after even mild, i.e., 30 min, of 2-K I/R injury (3). In addition, we have not observed any evidence of recovery of vessel density.

Coinciding with a reduction in peritubular capillaries, we also demonstrated a persistent urinary concentrating defect after the apparent recovery from ARF (3). The persistent urinary concentrating defect is consistent with medullary dysfunction and could potentially be attributed to hypoxia (7). Although reduction in peritubular capillary density is the greatest in the inner stripe of the inner medulla, all zones of the kidney manifested a significant decrease in vessel density and the potential for exacerbated hypoxia (3). Our present hypothesis is that exacerbated hypoxia occurs...
secondary to ischemic injury and predisposes the kidney to develop chronic renal disease. The purpose of the present study was to test one particular component of this hypothesis; i.e., whether postischemic recovered kidneys are more hypoxic than kidneys of sham-operated control animals. In addition, we hoped to determine whether manipulation of tissue $PO_2$ might influence the development of chronic renal dysfunction.

Clearly, other studies are required before the hypothesis as a whole can gain wide acceptance. For example, it would be useful to devise a treatment that blocks the reduction in vessel density and measures hypoxia; however, we do not yet know the means by which to accomplish this. Of interest to this issue are studies by Kang et al. (23, 24) in which VEGF reversed the reduction of peritubular capillary density after either cyclosporin A treatment or reduced renal mass; VEGF in these settings ameliorated the progression of chronic renal disease. However, in those studies, hypoxia was not directly addressed.

A number of investigators have suggested that exacerbated medullary hypoxia might trigger the development of chronic disease (2, 7, 16, 22, 37). Hypoxia can trigger a number of profibrotic pathways; e.g., transforming growth factor-$\beta$, collagen, and fibronectin (2, 7, 16, 22, 37). It is also possible that these pathways can become activated in the cortical region as a result of focal hypoxia too small or subtle to be detected with our present methodology. Exacerbated medullary hypoxia might affect cortical structure and function either directly or indirectly. For example, it is thought that scarring in the outer medulla exacerbates local ischemia and the spread of the fibrotic area to encompass even the most superficial cortex (7). In addition, it is also possible that hypoxia in the renal medulla can reduce Na reabsorption in the thick ascending limbs of Henle’s loop and contribute to afferent vasoconstriction and increased ANG II generation at the macula densa. This locally produced ANG II could affect glomerular structure, and development of proteinuria (7). It is important to note that Pagtalunan et al. (32) demonstrated the beneficial effects of blocking the renin-angiotensin system on the secondary development of proteinuria after recovery from I/R injury to a solitary kidney.

Although evidence in favor of hypoxia as a mitigating factor in progression of renal disease after ARF is compelling, other suggestions have been raised. The activation of costimulatory pathways in response to ischemic injury, such as the B7 pathway, has been shown to play a contributory role in the development of renal disease after ischemic injury (9). Moreover, the observation that there is incomplete regeneration of the tubule has led to the suggestion that remnant nephrons can cause the development of scarring while hyperfiltration of surviving nephrons can result in progressive nephron damage (33). Whether hypoxia plays a primary role in the genesis of secondary renal dysfunction after ARF or whether it modifies the rate of disease progression set in motion by some other trigger is presently unclear. In our opinion, costimulatory pathways, hyperfiltration of remnant nephrons, and development of renal hypoxia may all be considered potentially interrelated phenomena.

The results of the present study are consistent with exacerbated renal hypoxia, at least within the medullary zone, after recovery from an I/R insult. These results are on the basis of studies showing enhanced 2-pimonidizole uptake. The pimonidizole technique is becoming utilized with increasing frequency because this compound and corresponding monoclonal antibodies are now available commercially. Reports have demonstrated that the binding of the reactive hydroxylamine intermediate to cellular thiols occurs at $PO_2$ below $\approx 10$ mmHg (1). Studies in tumor models have verified the reliability of this technique by performing simultaneous measurements with oxygen electrodes (34). To date, at least one other study has utilized this technique in kidney in which acute cyclosporin A administration was shown to exacerbate renal hypoxia (40).

Although our results are encouraging, the immunohistochemical method is nonquantitative. Furthermore, reductions in $PO_2$ that remain $>10$ mmHg are undetectable. Moreover, the generation of the thiol-binding intermediate is dependent on reduction via NADH or reduced NADP, which may affect the intensity of the signal (1). Thus the method has several limitations. It is of interest that pimonidizole uptake in the kidney was consistently lower in the inner medulla vs. the outer medulla. The reason the inner medulla demonstrates consistently less staining is unknown at this time but may be related to delivery, transport, or conversion of the parent compound in the deeper region of the medulla.

Nevertheless, our ability to consistently observe differences in the intensity and the area of staining in kidneys from postischemic animals vs. those from sham-operated controls suggests that recovery from I/R injury is associated with exacerbated renal hypoxia, which is likely secondary to the reduction in peritubular capillary density. With this in mind, it is important to mention the early models of oxygen transport proposed by Krogh (26) that suggest that reduced capillary density increases the effective distance of oxygen transport to the tissues and promotes tissue hypoxia (36). Although more detailed models now exist, the basic tenets of Krogh’s model are widely held. In addition, the model also suggests that increased flow through the same number of vessels will decrease hypoxia by increasing convective delivery of oxygen (26, 36). Thus it is possible to reduce hypoxia by increasing flow through a reduced number of vessels.

Two important observations in this study are that 2-pimonidizole uptake is increased and progressive renal disease occurs more rapidly in the 1-K model than in the 2-K model. In this study, we utilized serum creatinine and creatinine-clearance determinations to measure loss of renal function posts ischemia. Whether changes in creatinine clearance truly reflect alterations in GFR are unclear because of the known contribution of tubular secretion of creatinine and how this may be affected by loss of peritubular capillaries.

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Nevertheless, there is a clear enhancement of proteinuria and renal fibrosis, suggesting that progressive renal disease is present. These results are similar to those reported by Cruzado et al. (13), who have also reported an effect of renal mass on the development of secondary complications after I/R injury. Thus it is clear that in a rat model of I/R injury, renal mass affects the rate of progression of secondary renal dysfunction after injury. Because UNX is associated with increased metabolic demand and exacerbated hypoxia (7), our observations are also consistent with a role for chronic hypoxia as a mediator of renal disease progression.

However, it should be emphasized that other potentially deleterious compensatory adaptations of reducing renal mass may predispose the development of chronic renal disease and that there are clear differences in the number of incompletely regenerated nephrons in the 1-K model of I/R injury. It is simplistic but reasonable to suggest that renal dysfunction in patients after delayed graft function is more prevalent than in patients after recovery from ARF in their native kidneys, in part, because of mitigating factors related to compensation for reduced renal mass.

L-Arginine has been utilized by many investigators to attenuate the progression of chronic renal disease induced by a variety of stimuli including cyclosporine, ureteral obstruction, hyperglycemia, and various forms of hypertension (10, 14, 15, 30, 38). However, the mechanism of the protection afforded by L-arginine in these models has not been directly addressed. Infusion of L-arginine either systemically or directly into the renal medullary interstitial space increases RBF in a nitric oxide-dependent fashion (14, 28). Thus it is possible that L-arginine might attenuate deleterious effects of renal pathological stimuli by affecting total and/or medullary RBF and renal hypoxia. In this study, oral L-arginine increased RBF and partially attenuated the uptake of 2-pimonidazole. This effect of L-arginine on RBF and hypoxia is likely the result of vasodilation and appears to be independent of any effect on vascular density (Fig. 5). In addition, oral L-arginine profoundly enhanced urinary output; this observation is consistent with washout of the medullary tonicity that would be expected with increased medullary blood flow.

The effects of L-arginine on the long-term function of postischemic kidneys was consistent with the protection afforded in other models characterized by interstitial fibrosis (10, 14, 15, 30, 38). L-Arginine-treated animals had less scarring and a reduced rate of proteinuria compared with animals maintained on tap water. Although other potential protective mechanisms might be postulated for L-arginine in this setting, it is tempting to speculate that renal hypoxia may be a central mechanism that contributes to the progression of several disease models that are characterized by interstitial fibrosis and have been shown, experimentally, to be amenable to L-arginine intervention.

In summary, there are a number of alterations in the postischemic kidney that might affect long-term outcome and function. We suggest that chronic renal hypoxia present after recovery from I/R injury is one important parameter that may influence progressive disease. We suggest hypoxia is secondary to the alteration in peritubular capillary density after I/R injury, but evidence for a direct link between these two observations has not yet been made. The possibility that enhanced renal hypoxia postrecovery represents an important element of progressive disease is suggested by studies in which exacerbated hypoxia (via UNX) hastens development of long-term complications while L-arginine attenuates the development of long-term complications.

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