Renal ischemic injury results in permanent damage to peritubular capillaries and influences long-term function

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Basile, David P., Deborah Donohoe, Kelly Roethe, and Jeffrey L. Osborn. Renal ischemic injury results in permanent damage to peritubular capillaries and influences long-term function. Am J Physiol Renal Physiol 281: F887–F899, 2001. First published August 9, 2001; 10.1152/ajprenal.00050.2001.—Acute episodes of severe renal ischemia result in acute renal failure (ARF). These episodes are followed by a characteristic recovery and repair response, whereby tubular morphology and renal function appear completely restored within ~1 mo. However, the chronic effects of such an injury have not been well studied. Male rats were subjected to 60-min bilateral ischemia followed by reperfusion, yielding a characteristic injury. Postischemic animals manifested severe diuresis, peaking at 1 wk postinjury (volume: >45 ml/day, ARF vs. 18 ml/day, sham; P < 0.05). Urine flow subsequently declined but remained significantly elevated vs. sham animals for a 40-wk period. The prolonged alteration in urinary concentrating ability was attributable, in part, to a diminished capacity to generate a hypertonic medullary interstitium. By wk 16, proteinuria developed in the post-ARF group and progressed for the duration of the study. Histological examination revealed essentially normal tubular morphology at 4 and 8 wk postinjury but the development of tubulointerstitial fibrosis at 40 wk. Transforming growth factor (TGF)-β1 expression was elevated at 40 wk, but not at 4 and 8 wk postinjury. Microfil analysis revealed an ∼30–50% reduction in peritubular capillary density in the inner stripe of the outer medulla at 4, 8, and 40 wk in post-ARF groups vs. sham animals. In addition, post-ARF rats manifested a significant pressor response to a low dose of ANG II (15 ng·kg⁻¹·min⁻¹). We hypothesize that severe ischemic injury results in a permanent alteration of renal capillary density, contributing to a urinary concentrating defect and the predisposition toward the development of renal fibrosis.

acute renal failure; regeneration; chronic renal failure; fibrosis; proteinuria; diuresis

ISCHEMIC RENAL INJURY IS a leading cause of acute renal failure (ARF). Despite a high mortality of ∼50%, most surviving patients are thought to recover full renal function (6, 17, 22, 23, 45). The restoration of renal function after episodes of ARF is attributable largely to the ability of renal tissue to recover from sublethal or lethal cellular damage (6, 17, 22, 23, 45). However, ARF may result in incomplete recovery of renal function and/or the development of chronic and progressive renal disorders. (1, 17, 39, 41). Furthermore, delayed graft function (DGF), which manifests many characteristics of ARF, is a strong predictor of long-term graft loss (18, 21, 29, 33). The aforementioned results suggest that acute renal injury predisposes the kidney to the development of future complications.

Acute ischemic injury primarily results in proximal tubular damage. Regeneration of the proximal tubule occurs through a coordinated series of events that includes cellular proliferation, migration, and subsequent hypertrophy of a new population of proximal tubule cells. In a typical model of ischemic ARF (i.e., 60-min ischemia in the rat), the glomerular filtration rate (GFR) returns to baseline values within ~1 wk postinjury, but complete restoration of proximal tubular morphology may take up to 4 wk or more (6, 22, 23, 45). Several studies have indicated that mitogenic and hypertrophic growth factors mediate tubular regeneration after acute renal injury (4, 6, 22, 23, 45). Separate from the well-characterized proximal tubular abnormalities, ischemia causes several other derangements in renal function. There is a transient decrease in total renal blood flow that appears to exacerbate the extent of renal injury (6, 46), and renal vascular reactivity is altered for up to 1 wk postinjury (11, 12). Sodium transport in the thick ascending limb is compromised, and urinary concentrating ability is diminished (2, 40).

Although much attention has been focused on events occurring during the ischemic event or in the early recovery phase, the long-term effects of ischemic injury have received little study. Inasmuch as clinical data suggest that acute renal injury has the potential to result in future renal compromise, we set out to perform long-term studies of renal function and structure in rats after their apparent complete recovery from ARF. Our results contained herein suggest that there are permanent alterations in renal structure and function after ischemia-reperfusion injury in rats that are associated with the development of features indicative of chronic renal disease.

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METHODS

Animal and surgical procedures. Care of 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 National Institutes of Health guidelines for the care and use of laboratory animals. All protocols had received prior approval from 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 a 12:12-h light-dark cycle (lights on 0600–1800) and access to water and standard laboratory rat chow (0.8% NaCl, Purina) 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. 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 around the renal arteries. After 60 min of occlusion, the clamps were removed, and reflow was verified visually. Sham-operated control animals received the same treatment, except for the occlusion of the renal arteries.

In the first study (study 1), renal ischemia was performed in 45 animals and sham-operated control surgery in an additional 25. To assess the degree of renal injury, serum creatinine values were determined 24 h post surgery. To ensure that all animals in the study had received comparable and sufficient injury, five rats with 24-h postsurgery creatinine levels of <2.0 mg/dl were excluded from further analysis. In addition, three animals died within 3 days of surgery. In the remaining 57 animals, 24-h serum creatinine values ranged between 2.1 and 4.2 mg/dl, with a mean of 3.4 mg/dl. Of these, only one animal died before the scheduled time of euthanasia (36 wk postsurgery). In the second study (study 2), in which the effects of dehydration stress were determined, 10 postischemic rats with a mean 24-h creatinine value of 2.7 ± 0.3 mg/dl and a range of 2.0–3.5 mg/dl were included. An additional study (study 3) comprised sham-operated rats as well as rats subjected to 30 or 45 min of bilateral renal ischemia. Of the 18 rats used in this study, all survived for the 4-wk study period.

Measurement of renal function. Parameters of renal function were measured at 24 h and 1, 2, 4, 6, 8, 10, 12, 14, 16, 20, 24, 28, 32, 36, and 40 wk postsurgery. Tail blood samples (0.5 ml) were collected under light halothane anesthesia into heparinized tubes and plasma obtained after centrifugation. Urine was collected for 24 h in metabolic cages (Nalgene). Serum and urine creatinine were determined using standard assays (Sigma creatinine kit 555A). Urine volume was determined gravimetrically. Creatinine clearance over 24 h was calculated as $\text{U}_{\text{creatinine}} \times V / \text{P}_{\text{creatinine}}$, where $\text{U}_{\text{creatinine}}$ and $\text{P}_{\text{creatinine}}$ are urinary and plasma creatinine, respectively, and $V$ is flow rate.

Urinary protein excretion was determined with a Bio-Rad protein assay kit (catalog no. 500–0006), using the microassay format based on the manufacturer's recommendation for enhanced sensitivity. The assay was scaled down for use on microtiter plates, with a final volume of 200 µl/sample. Uroinary albumin excretion was determined with the use of the albumin blue 580 fluorescence method, as previously described (27).

Urine osmolarity was determined with the µ-Osmette osmometer (Precision Systems), which functions on the basis of freezing-point depression. Tissue osmotic content was determined in renal papillae that were microdissected from kidneys of rats at termination of the study. Microdissected papillae were frozen in liquid N₂ and stored at −70°C. Papillae were thoroughly homogenized in 250 µl of deionized water, and membranes were separated by centrifugation. Tissue osmotic content was determined from values obtained from the resulting supernatant by the osmometer. Urine sodium excretion was determined by flame photometry (Instrumentation Laboratories, Lexington MA).

Blood pressure measurements. Values for baseline blood pressure and sensitivity to ANG II were determined in a subpopulation of animals that were killed at 4 or 8 wk postsurgery. After a final urine collection, animals were anesthetized with ketamine HCl (60 mg/kg ip), xylazine (6 mg/kg ip), and acepromazine maleate (0.9 mg/kg ip). Chronic indwelling catheters, constructed as described previously (20), were inserted into the femoral artery and vein and advanced ~5 cm so that the tips were in the aorta and the vena cava but remained distal to the renal vessels. Catheters were exteriorized at the scapula and placed inside a stainless steel cannula that was secured to the rat with a specialized jacket. The opposite end of the spring was attached to a three-channel microswivel (Alice King Medical Arts), which provided the animals with free and unrestrained movement. Rats were housed individually in metabolic pens, and the microswivels were attached to a holder above the pen. The arterial catheter was filled with 1,000 U/ml heparin in sterile saline to prevent clotting. The venous catheter was connected to a continuous-infusion pump, through which 5% dextrose in water (no sodium) supplemented with chloramphenicol sodium succinate (1 mg/ml) was infused at a rate of 8 ml/day.

Mean arterial pressure (MAP) was measured on the first day after recovery from the instrumentation surgery from the arterial catheter for 3 h (0900–1200) with a pressure transducer connected to an eight-channel amplifier and a pressure display unit (Dept. of Physiology, Medical College of Wisconsin). The amplified analog signal was converted to a digitized signal (Significat model RTS-132) and analyzed on-line with a sampling frequency of 100 Hz (Significat Data Acquisition Software, version 2.4). MAP was averaged over 60-s intervals, and the final MAP was calculated as the mean of ~180 data points during the 3-h recording period. Immediately after the first 3-h recording period, the venous infusate was supplemented with ANG II (Sigma, St. Louis, MO) such that the infusion rate was 15 ng·kg⁻¹·min⁻¹, which we had determined to be near the threshold for pressor activity under these conditions (data not shown). The following day, MAP was determined for 3 h as before. ANG II was removed from the infusate, and the animals were prepared for euthanasia the following morning.

Analysis of renal structure and capillary density. At termination, animals were prepared for euthanasia by administration of ketamine HCl (60 mg/kg), xylazine (6 mg/kg), and acepromazine maleate (0.9 mg/kg). The animals were placed on a heated surgical table, and the right renal kidney was tied off and quickly removed. One-half of the kidney was saved for biochemical analysis (see below) by snap-freezing in liquid N₂. The remaining half was fixed in Bouin’s solution and prepared for routine histological examination, including periodic acid-Schiff and silver staining.

For analysis of the renal microvasculature, Microfil infu- sion was employed in a fashion similar to that described previously by Lennon et al. (30). A Tygon catheter (outer diameter 0.090 in.) connected to a 16-g needle was inserted into the aorta distal to the renal arteries, and loose 2-0 ligatures were placed around the left renal pedicle and the aorta proximal to the renal arteries. Approximately 3 ml of
heparinized saline were infused through the catheter, and
the animal was allowed to stabilize for ~30 min. Microfil
(Flow Tech, Carver MA) was prepared by combining MV-130
red with MV diluent at a 1:2 ratio and 10% vol/vol catalyst
(final volume 5.5 ml/kidney). After the renal vein was cut, the
proximal aortic ligature was tightened to isolate renal circu-
lation. Microfil was infused with a filling pressure held
manually between 100 and 140 mmHg as determined by a pres-
sure transducer connected to the system through a side arm.
The renal pedicle ligature was tightened, and the kidney was
removed and placed in 5% formalin for several days. The
kidney was cleared with successive changes in graded alcohol
and methyl salicylate according the manufacturer’s instruc-
tions. To aid in visualization and analysis, the kidney was
embedded in paraffin and sectioned at a 20-µm thickness.

For quantification of renal interstitial fibrosis or vascular
density, sections were visualized using an Olympus BH2
microscope equipped with a Sony 3CCD DXC960MD color
video camera. The images were captured on-line using Meta-
morph imaging software (Version 4.0, Universal Imaging).
For all image-analysis studies (vascular density, interstitial
volume, and extracellular matrix), at least five random im-
ages were stored from the cortex, outer stripe of the outer
medulla, and inner stripe of the outer medulla, and at least
three images were stored from the inner medulla using a ×10
objective with a field dimension of ~0.48 mm². All images
were stored and subsequently analyzed by a study group
member who was blinded to the experimental groups. In
these studies, two different, but complementary, types of
analysis were performed. For studies determining microvas-
cular density, the sharp contrast between the opaque-filled
vessels and adjacent translucent renal parenchyma facili-
tated image thresholding by using the software program and
allowed for computer-generated determination of percent
area occupied by Microfil. An additional method based on
previously published techniques (44) was used, whereby ref-
ersence gridlines (12 × 12) were overlaid by a 10
array of dots using Meta-
morph software. Of the 100 dots, the number that lay directly
over interstitial space, but not over tubules, tubule luminas,
glomeruli, and arterioles, were determined. Data are ex-
pressed as percent area above threshold in a fashion similar
to that used in Microfil studies.

mRNA expression studies. Total RNA was isolated from
frozen renal tissue using the Ultraspec RNA isolation solu-
tion (Biotex, Houston, TX) according to the manufacturer’s
instructions. Renal transforming growth factor (TGF)-β1
mRNA was determined using an RNase protection assay
according to the procedure previously described (4). After
hybridization and RNase digestion, the resulting protected
fragments were separated on a sequencing gel and analyzed
using a phosphorimager (Molecular Dynamics, Sunnyvale,
CA). To aid in comparisons among multiple gels, all data are
expressed relative to the mean value obtained in three sam-
ple s of RNA from 4-wk sham-operated control rats; these
samples were included in every assay as an internal stan-
dard. Statistical analysis was performed using Student’s
t-test for unpaired sample means. P < 0.05 for two-tailed
analysis was considered significant.

Aquaporin-2 (AQP2) mRNA was measured by an RNase
protection assay using a probe generously provided by Drs.
Baozhi Yaun and Allen W. Cowley, Jr. (Dept. of Physiology,
Medical College of Wisconsin). A 296-bp PCR product was
cloned into pCR2.1-TOPO (Invitrogen), and the sequence was
verified to be identical to that of rat AQP2 (Yaun B and
Cowley AW Jr., unpublished observations). The riboprobe
template was generated by PCR of the subcloned fragment
using the following primers: forward, 5’- AGCCGCCAGA-
AGTCGGAGCA-3’; and reverse, 5’-GGATCCATTTAGGT-
GACACTATAGAACAGCCACATAGAAGGCAGCT-3’. The
underlined region refers to the bacterial promoter sequence
of SP6 polymerase used to generate the antisense riboprobe
from the purified product of the PCR amplification.

RESULTS

Effects of ischemic injury on long-term renal func-
tion. To determine the potential long-term effects of
ARF on renal structure and function, we subjected rats
to 60 min of bilateral ischemia-reperfusion injury and
allowed them to recover from surgery for 4, 8, or 40 wk
(study 1). Renal functional data are shown in Fig. 1. Serum
creatinine values rose to 3.4 ± 0.4 mg/dl within
24 h after surgery, and the estimated creatinine clear-
cence based on 24-h urine collection between post-
surgery days 1 and 2 decreased to ~12% of sham-operated
control values (sham: 2.058 ± 0.376 ml/day vs. ARF:
248 ± 55 ml/day). By 7 days, serum creatinine had
decreased to values similar to those in sham-operated
controls, and creatinine clearance values had returned
to 87% of control values and remained significantly

![Fig. 1. Renal function after bilateral ischemia/reperfusion injury. A: serum
creatinine values. B: ]C creatinine [as %sham-operated controls (solid line)].
NS, not significant. As animals were killed during the course of the exper-
iment, the number remaining in the study decreased. Therefore, n = 37, 24,
and 12 for time points up to and including 4, 8, and 40 wk, respectively, in
the post-acute renal failure (ARF) group and n = 28, 18, and 8 in the sham-
operated control group. Values are means ± SE. *P < 0.05 by Student’s
1-tailed t-test.](https://ajprenal.org/ajpur/281/12/269/h2001269f1.jpg)
different from controls. There was no difference in the serum creatinine levels or creatinine clearance at any time point between 2 and 40 wk postsurgery.

Despite the recovery of filtration function in the postischemic animals, other elements of renal function appear to remain altered after ischemia-reperfusion injury. Figure 2 illustrates the urinary concentrating defect seen in postischemic rats. At 1 wk postschemia, there is an $\sim 2.5$-fold increase in urinary output (45.2 ± 2.2 vs. 18.3 ± 1.4 ml/day, Fig. 2B) and a corresponding decrease in urinary osmolarity (Fig. 2A). The values for both urinary output and urinary osmolarity initially shifted back toward values in sham-operated controls in the first 4–6 wk postsurgery. However, these trends did not continue after the first 4–6 wk, such that urinary output and osmolarity remained significantly different in the two groups for the duration of the study.

The inability to retain sodium is a well-described feature after postischemic ARF. Therefore, we measured sodium excretion in these animals to determine the potential relationship between sodium excretion and urinary output. Total sodium excretion peaked at 1 wk postinjury and gradually decreased up to week 4 postsurgery (Fig. 2C). By week 8, total sodium excretion was similar in both sham-operated and postischemic animals. Fractional excretion of sodium ($\%F_{\text{ENa}}$) peaked at 24–48 h, remained elevated in postschemic rats by 2 wk postinjury, and returned to control values by 4 wk (Fig. 2D).

Effect of dehydration on urinary concentrating ability in postischemic animals. To investigate further the abnormality in urinary flow in the postischemic rat, an additional study (study 2) was performed to determine the renal response to dehydration stress after apparent recovery from ischemic injury. Consistent with the results of study 1, urine flow rates were significantly higher at 1 wk postinjury and gradually trended toward but did not reach control values. At week 4 postsurgery, animals were randomized into either water-deprived or water-replete groups, and three successive 12-h urine samples were obtained. In water-replete groups, the difference in urine flow rates and in urine osmolarity persisted in all three collection periods (Fig. 3B). In response to water deprivation, urine flow decreased and urine osmolarity increased in both sham-operated and postischemic animals (Fig. 3A and B). The difference in urine osmolarity between control and postischemic animals persisted for each of the three 12-h collection periods (Fig. 3B). It appeared that the rate of increase in urine osmolarity for each of the

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Fig. 2. Alterations of urinary concentrating ability and Na handling after renal injury. Analysis of urinary Na and water excretion are shown. At the indicated times, rats were placed in metabolic cages for 24-h urine collection. A and B: 24-h urine volume and osmolalities, respectively. C: 24-h total Na excretion. D: %fractional Na excretion ($F_{\text{ENa}}$) was calculated on the basis of $C_{\text{creatinine}}$. Values are means ± SE. *$P < 0.05$ by Student’s 1-tailed $t$-test.
successive collection periods was similar in both groups (Fig. 3B).

We sought to determine whether the alteration in urinary concentrating ability was related to the magnitude of the osmotic gradient generated in the renal papilla. Papillary osmotic content in hydrated postischemic animals is significantly reduced compared with corresponding hydrated, sham-operated controls (Fig. 3C). Papillary osmotic content significantly increased after dehydration stress in both groups but remained different between postischemic animals and sham-operated controls. The data indicate that ischemic injury chronically compromises the ability to generate a concentrated medullary interstitium.

Finally, we also measured the renal expression of AQP2 mRNA after this protocol. In hydrated groups, there was no difference in the expression of AQP2 mRNA in postischemic, recovered animals vs. levels observed in sham-operated, hydrated rats (Fig. 4). AQP2 mRNA was significantly enhanced in kidneys of animals after 36 h of dehydration (sham: 1.6-fold; ischemic: 1.8-fold ischemic; \( P < 0.05 \) vs. comparable hydrated controls) (Fig. 4). Taken together, these data suggest that the vasopressin-AQP2 axis is intact in the postischemic rat.

Development of proteinuria in postischemic recovered rats. As animals in study 1 progressed through the postischemic period, other abnormalities became man-
ifest. During the first 14 wk postinjury, protein excretion was low in both sham-operated and posts ischemic animals (Fig. 5A). Detectable increases in protein excretion began by week 16 postsurgery in both groups of animals. Proteinuria rose consistently with each subsequent sampling, and the degree of proteinuria was significantly higher in posts ischemic animals than in sham-operated rats between weeks 16 and 40 postsurgery. Consistent with these observations, urinary albumin revealed a qualitatively similar relationship and comprised between 30 and 40% of urinary protein (Fig. 5B).

Development of renal fibrosis in posts ischemic recovered rat kidney. Renal histology was assessed in kidneys at 4, 8, and 40 wk postinjury (Fig. 6). At 4 and 8 wk postinjury, there was ample evidence of tubular repair. Despite this, it was not uncommon to identify a small proportion of tubules with abnormal morphology (Fig. 6B, thin black arrow). At 40 wk postinjury, renal morphology was profoundly abnormal. In the cortex, we observed several instances of glomerulosclerosis, glomerular atrophy (Fig. 6E, arrows), and glomerular hypertrophy (Fig. 6F, arrow). In the outer medulla, tubulointerstitial fibrosis was prominent (Fig. 6H). These changes were not prominent in the 40-wk sham-operated control group (Fig. 6G).

The data in Table 1 represent quantitative assessments of scores corresponding to tubulointerstitial space in the kidneys of posts ischemic and sham-operated animals. At 40 wk postinjury, there was a significant increase in the tubulointerstitial space in posts ischemic kidneys vs. kidneys from sham-operated animals. This relationship was not observed at 8 wk postsurgery. However, at 4 wk, the measured value of interstitial space was elevated in the post-ARF group vs. sham-operated rats; visual examination revealed that this was largely due to hypercellularity in the interstitium (see Fig. 6B, thick black arrow).

Figure 7, A–D, are silver-stained sections of renal tissue from sham-operated and posts ischemic rats. Corresponding extracellular matrix-area scores derived from silver-stained tissues are shown in Table 2. Scores are significantly greater in posts ischemic animals vs. corresponding sham-operated controls at all time points, and it appeared that the fibrosis was progressive. In addition to the area occupied by silver staining, an increase in the intensity of silver staining can be appreciated in the 40 wk post-ARF samples vs. age-matched controls and vs. posts ischemic animals at earlier time points (Fig. 7).

TGF-β1 expression post-ischemic injury. The mRNA for the profibrotic growth factor TGF-β1 was measured in kidney of rats at various times after renal injury. Figure 8 demonstrates there is an approximately four-fold increase in TGF-β1 mRNA expression in the early posts ischemic period (i.e., 3 days) but that TGF-β1 mRNA returns to basal levels at 4 and 8 wk postinjury. When measured at 40 wk postinjury, TGF-β1 mRNA was elevated in both sham-operated and posts ischemic animals but was significantly elevated in the posts ischemic group compared with sham-operated rats. These results demonstrate that TGF-β1 expression posts ischemia is not persistent but does increase sec ondarily to the initial recovery phase of this injury.

Analysis of renal microvascular structure. To determine whether the above results could be attributable to alterations in the renal blood supply after renal injury, Microfil was infused through the renal circulation to visualize microvascular structures. Figure 9A demonstrates the filling pattern seen in a representative sham-operated control rat kidney under low-power stereomicroscopy. In these kidneys, the increased vascularity in the inner stripe of the outer medulla is prominent, indicative of the peritubular capillary "puffs" in this region. Representative filling patterns for animals at 4 and 8 wk postinjury are shown in Fig. 9, B and C, and demonstrate a reduction in overall vascularity. Vascular density was determined by measuring the total surface area of a region occupied by Microfil (%thresholded area, Fig. 10A) or by determining the number of vessel intersections across an arbitrary grid (Fig. 10B). Acute ischemic injury resulted in a significant reduction in vessel density within 4 wk of the insult. Although all regions of the kidney were affected, the most dramatic reduction in vessel density was observed in the inner stripe of the outer medulla; the reductions in vessel density were persistent up to 40 wk postinjury.

To address whether the severity of ischemic injury affects damage to the renal vasculature and renal function, we subjected rats to lesser degrees of ischemia (study 3), i.e., 30 and 45 min. Serum creatinine values 24 h postsurgery were 1.0 ± 0.2 and 2.2 ± 0.2 mg/dl in each of these groups, respectively (Table 3).
Table 1. Interstitial volume scores of kidney regions after recovery from ARF

<table>
<thead>
<tr>
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<th>4 Wk</th>
<th>8 Wk</th>
<th>40 Wk</th>
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<tr>
<td></td>
<td>Cortex</td>
<td>OSOM</td>
<td>ISOM</td>
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<tr>
<td>Sham</td>
<td>16.6±2.6</td>
<td>26.0±2.8</td>
<td>38.12±4.2</td>
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<tr>
<td>ARF</td>
<td>28.7±3.5*</td>
<td>41.6±7.0*</td>
<td>43.1±5.7</td>
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Values are mean dots/field ± SE, representing dots that overlaid interstitial space, from an arbitrary 10 × 10 array superimposed on random images. n, No. of rats; ARF, acute renal failure; sham, sham-operated rats; OSOM, outer stripe of the outer medulla; ISOM, inner stripe of the outer medulla. *P < 0.05 vs. sham-operated control for given kidney region.

Fig. 6. Renal structure after recovery from ischemic ARF. Shown are cross-sections through rat renal cortex and outer medulla stained with pericid acid-Schiff. A–C: sections through the outer medulla of a sham-operated rat kidney (A) 4 (B) and 8 wk (C) after ischemic ARF. Note that at 4 wk some tubules appear unrepaired (B, thin black arrow) and that interstitial hypercellularity was frequently observed (B, thick black arrow). In C, tubular structures appear normal, except for tubular hypercellularity (white arrowheads). D–H: sections through rat renal cortex and outer medulla at 40 wk postsurgery. D: cortex from sham-operated rat. E and F: 2 strikingly different glomeruli observed in 40-wk posts ischemic animals. In E, glomerular atrophy (thin black arrows) and nearby cellular infiltrate (thick black arrow) are observed. In contrast, in F glomerular hypertrophy was also observed at 40 wk postinjury. Tubular interstitial scarring is evident in outer medulla at 40 wk (H, white arrow) but not appreciably in 40-wk sham-operated rats (G). Bar, 50 μm.
The rats recovered uneventfully from ARF. However, at 4 wk the diuresis was unresolved in both postischemic groups and was greater in the 45- vs. the 30-min group. Microfil analysis revealed that renal capillary density 1 mo after either 30 or 45 min of ischemia-reperfusion injury was significantly reduced compared with the corresponding sham-operated controls (Table 3). The degree of capillary destruction appeared similar in the cortex and the outer stripe of the outer medulla. However, capillary density in the inner stripe of the outer medulla and the inner medulla showed a trend toward a greater reduction in the 45- vs. the 30-min group.

Enhanced pressor response in postischemic animals.
Reductions in peritubular capillary density and/or reductions in renal medullary blood flow have been shown to predispose the development of elevated arterial pressures. To address this possibility, we instrumented animals that recovered from 60 min of bilateral ischemia (from study 1) with chronic indwelling catheters just before scheduled euthanasia at 4 and 8 wk postsurgery. In the subgroup of animals tested, there was no difference in the MAP between sham-operated animals and postischemic animals 4 or 8 wk postsurgery. When these animals were administered a threshold dose of ANG II (15 ng·kg⁻¹·min⁻¹), MAP significantly increased in both groups of postischemic animals (Fig. 11). Conversely, sham-operated control animals did not elicit a response to ANG II at this dose. The data suggest that animals are hypersensitive to pressor stimulation after recovery from ischemic acute renal failure.

**DISCUSSION**

The long-term effects of ARF have been largely unexplored. Herein we report, using a rat model of ischemic ARF, that several manifestations of renal function and structure are permanently altered after injury. In addition, kidneys of animals that have undergone a renal repair response appear to be predisposed to the development of chronic renal dysfunction. In addition, at least four other separate reports using animal models have recently been published demonstrating

Table 2. Extracellular matrix scores of kidney regions after recovery from ARF

<table>
<thead>
<tr>
<th></th>
<th>Cortex</th>
<th>OSOM</th>
<th>ISOM</th>
<th>n</th>
<th>Cortex</th>
<th>OSOM</th>
<th>ISOM</th>
<th>n</th>
<th>Cortex</th>
<th>OSOM</th>
<th>ISOM</th>
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<tr>
<td>Sham</td>
<td>3.3 ± 0.4</td>
<td>3.6 ± 0.7</td>
<td>3.5 ± 0.7</td>
<td>6</td>
<td>8.0 ± 1.6</td>
<td>4.3 ± 1.1</td>
<td>2.6 ± 0.5</td>
<td>10</td>
<td>11.9 ± 1.7</td>
<td>3.4 ± 0.2</td>
<td>1.3 ± 0.5</td>
<td>9</td>
</tr>
<tr>
<td>ARF</td>
<td>4.4 ± 0.5</td>
<td>9.3 ± 0.7*</td>
<td>6.3 ± 0.6*</td>
<td>6</td>
<td>10.3 ± 1.8*</td>
<td>11.1 ± 1.7*</td>
<td>9.0 ± 2.9*</td>
<td>14</td>
<td>23.8 ± 2.0*</td>
<td>11.4 ± 0.9*</td>
<td>8.5 ± 0.9*</td>
<td>12</td>
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Values are means ± SE derived from % thresholded areas from Jones-stained kidneys. n, No. of rats. *P < 0.05 vs. sham-operated control for given kidney region.
chronic deleterious effects of ischemic injury on long-term renal function and structure in rats (14, 19, 37, 38).

In a patient population, the long-term effects of ARF are not clear and are somewhat controversial. The issue is complicated by the diverse etiology of ARF and a paucity of long-term follow-up studies. The general ill-health of most patients suffering from ARF also makes long-term follow-up studies difficult. In surviving patients, renal function is thought to return, in part due to an endogenous renal repair response. However, this view is simplistic, and the complete return of renal function may never be appreciated in a significant proportion of patients. For example, studies by Briggs et al. (8) (4- to 75-mo follow-up), Lewers et al. (31) (2- to 15-yr follow-up), Bonomini et al. (5) (1- and 5-yr follow-up), and Kjellstrand et al. (28) (<1-yr follow-up) report that between 35 and 71% of patients surviving acute tubular necrosis had incomplete renal function as assessed by measurement of creatinine clearance or serum creatinine (5, 8, 28, 31). A common feature of two of these studies was the persistent lack of urinary concentrating ability (8, 31). In addition, Bonomini et al. (5) reported that a small percentage of patients manifested a loss of GFR between follow-up years 1 and 5. Similarly, a small percentage of patients in the report by Lewers et al. (31) showed a progressive loss of function over time. Despite the presence of residual or declining renal function, most patients in these follow-up studies were not symptomatic for renal disease.

Many of the features reported in these studies resemble the observations that we have made in postischemic rats and highlight the need to understand the long-term implications of renal injury and of therapies geared toward facilitating recovery after ARF. Although chronic renal failure may ensue after ARF in only a small number of cases, it is possible that our observations may also relate to DGF after renal transplant. DGF is defined as a poor graft function, with a requirement for dialysis in the immediate posttransplant period; ischemic acute tubular necrosis is the most common etiology of DGF (34). The influence of posts ischemic acute tubular necrosis on long-term graft function is controversial, as some studies do not demonstrate any correlation with DGF and graft survival (e.g., Ref. 9). Other more recent studies do demonstrate that DGF is an independent risk factor for graft survival and the development of posttransplant hypertension (18, 34, 36). Thus after the resolution of ischemic ARF in rats, the predisposition to secondary chronic renal injury and hypersensitivity to pressor stimuli may be of clinical relevance.

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Fig. 8. Secondary increase in transforming growth factor (TGF)-β1 mRNA after recovery from ischemic ARF. Total RNA from whole kidney was analyzed for the expression of TGF-β1 mRNA by RNase protection assay. The signal intensity of TGF-β1 mRNA was determined on a phosphorimager and divided by the signal obtained for the housekeeping gene cyclophilin. The data were obtained from multiple gels; results were normalized to the signal obtained in the 4-wk sham-operated group, which were run on every gel as an internal standard. Values are means ± SE. *P < 0.05 vs. sham-operated control. #P < 0.05 sham-operated at 40 wk vs. sham-operated control at 4 wk postsurgery (by Student’s 1-tailed t-test).

Fig. 9. Gross renal morphology and capillary filling in normal and postischemic kidneys. Representative stereoscopic views of 20-μm Microfil-infused kidney section. Shown are Microfil-infused kidneys from a sham-operated rat (A) at 4 (B) and 8 wk (C) post-ischemic injury. In this stereoscopic view, Microfil appears as bright yellow against a dark background. A reduction in Microfil-infused structures in recovered postischemic kidneys is evident. c, cortex; os, outer stripe of the outer medulla; is, inner stripe of the outer medulla; im, inner medulla. Magnification is shown.
In the present study, the return of GFR and proximal tubular morphology occurred in a time frame that is similar to that in previous reports on this model (6, 22, 23, 45). However, we were struck not only by the robust diuresis postinjury but also by the fact that diuresis was never completely resolved. To our knowledge, this is the first report suggesting that urinary concentrating ability is permanently impaired in rats after ischemic ARF. The early and more robust phase of diuresis is likely the result of multiple factors. One factor is osmotic diuresis due to profound sodium loss. Sodium excretion returns to control values within 2–4 wk. We suggest that this is related to the repair of the proximal tubule, but it may also be related to the restoration of distal nephron function. Regardless, sodium excretion cannot explain the sustained diuresis that was maintained throughout the study.

A previous report by Fernandez-Llama et al. (15) demonstrated decreased expression of aquaporins in the kidney in the immediate posts ischemic period. However, it appears unlikely that a sustained alteration in aquaporins can account for the sustained diuresis reported here. First, in their study, AQP2 expression returned to control levels by 7 days postinjury (15). Second, we show that posts ischemic animals (4 wk postsurgery) responded to dehydration in the same fashion as did sham-operated animals but with an altered set point at which baseline urine concentration is determined. Therefore, vasopressin-mediated mechanisms appear intact. Finally, AQP2 mRNA levels, which are under the influence of vasopression during dehydration stress, are similar between posts ischemic and sham-operated animals at 4 wk. Moreover, AQP2 mRNA levels are enhanced similarly in response to dehydration in both groups. Taken together, disruption of the arginine vasopressin-AQP2 axis is not implicated in the sustained diuresis posts ischemic injury.

There are reports of decreased renal medullary tonicity after injury due to either ischemia- or cisplatin-induced ARF (2, 40). In this study, renal medullary tonicity was decreased even as late as 4 wk in posts ischemic kidneys. On the basis of these results, we suggest that the primary mechanism by which urinary output is permanently altered is a decrease in the baseline level of medullary tonicity after ischemia; this decrease in tonicity alters the set point at which steady-state urine concentration is determined.

Fig. 10. Image analysis of Microfil-infused kidneys using computerized image analysis. Microfil-infused kidneys were subjected to quantification using computerized image-analysis tools. In A, a 12 × 12 grid overlaid an image as described in METHODS, and the number of vessel-grid intersections was determined. In B, vessel density was determined as % total surface area in the field. In the cortex, the area covered by glomeruli was excluded from analysis. For clarity, the bars representing sham-operated controls are pooled values from 4-, 8-, and 40-wk groups. For statistical analysis, only the values for sham-operated rats from time-matched groups were used. No differences were observed among these groups (not shown). Values are means ± SE. Cx, cortex; OSOM and ISOM, outer stripe and inner stripe of outer medulla, respectively; IM, inner medulla. *P < 0.05 vs. sham-operated control (Student’s 1-tailed t-test).

Table 3. Effect of variations on ischemic time vs. renal function and vascular structure

<table>
<thead>
<tr>
<th>Serum creatinine, mg/dl</th>
<th>Sham (n = 6)</th>
<th>30-Min I/R (n = 6)</th>
<th>45-Min I/R (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Day postinjury</td>
<td>0.45 ± 0.1</td>
<td>1.0 ± 0.2*</td>
<td>2.2 ± 0.2*</td>
</tr>
<tr>
<td>7 Days postinjury</td>
<td>0.42 ± 0.1</td>
<td>0.44 ± 0.1</td>
<td>0.68 ± 0.2</td>
</tr>
<tr>
<td>28 Days postinjury</td>
<td>0.42 ± 0.1</td>
<td>0.46 ± 0.1</td>
<td>0.57 ± 0.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Urine flow rate, ml/day</th>
<th>7 Days postinjury</th>
<th>14 Days postinjury</th>
<th>28 Days postinjury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>12.1 ± 1.1</td>
<td>19.1 ± 2.2*</td>
<td>29.0 ± 3.3*</td>
</tr>
<tr>
<td>OSOM</td>
<td>14.9 ± 1.3</td>
<td>17.5 ± 1.5*</td>
<td>25.6 ± 4.0*</td>
</tr>
<tr>
<td>ISOM</td>
<td>11.7 ± 1.4</td>
<td>19.0 ± 1.2*</td>
<td>26.9 ± 4.0*</td>
</tr>
</tbody>
</table>

Renal vascular density at 28 days, % sham control

| Cortex                  | 100 ± 5           | 53 ± 6*           | 57 ± 5*            |
| OSOM                    | 100 ± 4           | 59 ± 6*           | 45 ± 4*            |
| ISOM                    | 100 ± 5           | 74 ± 12*          | 60 ± 5*            |
| IM                      | 100 ± 5           | 44 ± 8*           | 35 ± 5*            |

Values are means ± SE, n, No. of rats; IM, inner medulla. *P < 0.05 vs. sham-operated control.
groups, but not until week 16. The proteinuria is likely an effect of aging, and ischemic ARF exacerbates this process. It is possible that the proteinuria is a secondary result of compensatory activity of surviving nephrons after the development of interstitial fibrosis. Therefore, our hypothesis suggests that there is an increase in single-nephron GFR of surviving nephrons whereas total GFR is held constant as long as the functional reserve is adequate. It is of interest that animals with only one kidney manifest secondary increases in creatinine after ischemic injury (14; Basile DP, unpublished observation) an effect likely related, in part, to a decreased functional reserve.

TGF-β1 is a major profibrotic factor in the kidney, and we have previously reported that its expression is enhanced after renal ischemic injury (4) However, consistent with our earlier reports (3, 4), TGF-β1 expression postischemic injury is transient and returns to basal values by 4 wk postinjury. Thus we wish to emphasize that the enhanced TGF-β1 expression observed chronically in this model is triggered by signals secondary to the initial insult and are independent of the initial response to injury.

Our observations concerning the development tubulointerstitial fibrosis and altered medullary tonicity suggest that renal blood flow is permanently altered after ischemic injury. The long-term consequences of ischemic injury on the renal vasculature remain incompletely understood. The available evidence, using electromagnetic flowmeters and ⁸⁶Rb extraction techniques, suggests that renal blood flow returns to normal (sham-operated) values between 7 and 28 days postischemia with no apparent alteration in regional distribution of flow by 4 wk (12, 26). Despite quantitative precision, the aforementioned studies lacked fine spatial resolution. Therefore, we employed Microfil to visualize renal capillary filling at times when total renal blood flow is thought to have returned to normal. This technique revealed a reduction in the number of microvessels in most regions of the kidney, most prominently in the inner stripe of the outer medulla at 4, 8, and 40 wk postinjury. Therefore, although damage to the tubules is reversible, damage to the microvessels in this model is permanent.

We suggest that the loss of renal microvessels exacerbates renal hypoxia and that this predisposes the kidney to alterations in urinary concentrating ability. Disruption in blood flow could explain the diminished medullary tonicity either by reducing the driving force of the Na-K ATPase in the thick ascending limb or by disturbing the normal countercurrent exchange of urea that occurs between the vasa recta capillaries and the inner medullary collecting duct.

In addition, we suggest that the loss of microvessels could also explain, in part, the gradual build-up of extracellular matrix that contributes to the development of renal interstitial fibrosis. Recent studies have correlated a loss of peritubular capillaries and the development of tubulointerstitial fibrosis (10). An emerging view is that chronic hypoxia may develop in several forms of renal disease and thereby trigger the expression of TGF-β1 and other profibrotic molecules that contribute to the development of tubulointerstitial fibrosis (3, 7, 16, 25, 43). Hypoxia may regulate TGF-β1 expression by affecting transcription factor activity or by modulating the activation of TGF-β1 from its latent form (3). Whether renal ischemia-reperfusion injury does, in fact, chronically exacerbate renal hypoxia remains to be determined.

Finally, we suggest that reductions in peritubular capillary density may contribute to the hypersensitivity of pressor stimuli. Johnson and colleagues (24, 25, 32) have shown that acute insults to the kidney affect the integrity of peritubular capillaries and predispose animals to salt-sensitive hypertension. It is now becoming evident that reductions in medullary blood flow play a predominant role in the control of arterial blood pressure (13). For example, microinfusion of N⁵-nitro-L-arginine methyl ester exclusively into the renal medulla of conscious Sprague-Dawley rats decreased medullary blood flow and simultaneously increased pressor sensitivity to low-dose intravenous infusions of
ANG II (42). Similarly, in our study we measured the response to a fixed low dose of ANG II and showed that postischemic animals manifested a significant pressor response. The mechanism for this increased pressor activity is not yet completely understood. We suggest that permanent alterations in medullary blood flow brought about by ischemic injury may predispose these animals to the genesis of hypertension through other stimuli.

In conclusion, we have demonstrated that many aspects of renal function and structure are restored after severe ischemic injury, whereas several other aspects of the kidney are permanently altered. We hypothesize that the underlying abnormalities in renal microvascular structure account for many of the long-term effects on renal structure and function after renal injury. These results may have implications for patients recovering from severe cases of ARF or the demise of renal allografts after DGF.

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