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

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RUNNING HEAD: Acute injury influences long-term renal structure function
SUMMARY

Acute episodes of severe renal ischemia result in acute renal failure (ARF). This episode is followed by a characteristic recovery and repair response in which tubular morphology and renal function appear completely restored within approximately 1 month. 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. Post-ischemic animals manifested severe diuresis peaking at 1 week post-injury (V>45 ml/day, ARF vs. 18 ml/day, sham; P <0.05). Urine flow subsequently declined but remained significantly elevated vs. shams for the 40 week period. The prolonged alteration in urinary concentrating ability was attributed in part to a diminished capacity to generate a hypertonic medullary interstitium. By week 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 wks post-injury but the development of tubulointerstitial fibrosis at 40 wks. TGF-ß1 expression was elevated at 40 weeks, but not at 4 and 8 weeks post-injury. Microfil analysis revealed an approximate 30-50% reduction in the peritubular capillary density in the inner stripe of the outer medulla at 4, 8 and 40 wks of post ARF groups vs. shams. In addition, post-ARF rats manifested a significant pressor response to a low-dose of Ang II (15 ng/kg/min). We hypothesize that severe ischemic injury results in a permanent alteration in renal capillary density contributing to a urinary concentrating defect and predisposing the development of renal fibrosis.

Abbreviations used in this manuscript:

DGF, Delayed Graft function. GFR, Glomerular filtration rate; MAP, mean arterial pressure; ECM, extracellular matrix; TAL thick ascending limb of Henle.
Introduction

Ischemic renal injury is a leading cause of acute renal failure. Despite a high mortality of approximately 50%, most surviving patients are thought to recover full renal function (6, 17, 22, 23, 45). The restoration of renal function following episodes of acute renal failure is attributed largely to the ability of renal tissue to recover from sublethal or lethal cellular damage (6, 17, 22, 23, 45). However, acute renal failure 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 acute renal failure, is a strong predictor of long-term graft loss (18, 21, 29, 33). These reports suggest that acute renal injury predisposes the kidney toward the development 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), GFR returns to baseline values within approximately 1 week post-injury but complete restoration of proximal tubular morphology may take up to 4 weeks 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 one week post-injury (11, 12). Sodium transport in the thick ascending limb is compromised and urinary concentrating ability is diminished (2, 40).
While 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 has received little study. Inasmuch as clinical data suggests that acute renal injury has the potential to result in future renal compromise, we set out to perform long-term studies on renal function and structure in rats following the apparent complete recovery from acute renal failure. Our results contained herein suggest that there are permanent alterations in renal structure and function following ischemia/reperfusion injury in rats that are associated with the development of features indicative of chronic renal disease.

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 National Institutes of Health guidelines for the care and use of laboratory animals. All protocols had received prior approval by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Male Sprague-Dawley rats (Harlan, Madison, WI; approx 250 g) were housed in pairs in standard shoe-box cages with 12 hour light cycle (lights on 0600-1800) and access to water and standard laboratory rat chow (0.8% NaCl, Purina) available ad libitum. Animals were anaesthetized with ketamine (i.p., 100 mg/kg) for 10 minutes followed by administration of pentobarbital (i.p., 25-50 mg/kg). Acute renal failure 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 micro-aneurysm 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 the occlusion of the renal arteries.

In the first study (Study 1), renal ischemia was performed on 45 animals and sham-operated control surgery on an additional 25 animals. To assess the degree of renal injury, serum creatinine values were determined 24 hours post-surgery. To ensure that all animals in the study had received comparable and sufficient injury, 5 rats with 24-hour post surgery creatinine levels of less than 2.0 mg/dl were excluded from further analysis. In addition, 3 animals died within 3 days of surgery. Of the remaining 37 animals, 24 hour serum creatinine values ranged between 2.1 and 4.2 mg/dl with a mean of 3.4 ± 0.4 mg/dl. Of these, only 1 animal perished prior to the time of scheduled sacrifice (at 36 weeks post-surgery). In the second study (Study 2), in which the effects of dehydration stress were determined, 10 post-ischemic rats with a mean 24-hour creatinine of 2.7 ± 0.3 mg/dl and a range of 2.0 to 3.5 mg/dl were included in the study. An additional study (Study 3), contained 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 week study period.

**Measurement of renal function:**

Renal functional parameters were measured at 24 hours, 1, 2, 4, 6, 8, 10, 12, 14, 16, 20, 24, 28, 32, 36 and 40 weeks post surgery. Tail blood samples (0.5 ml) were collected under light halothane anesthesia into heparinized tubes and plasma obtained following centrifugation. Urine collection was for 24 hours 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 hours was calculated using \( \frac{U_{\text{creatinine}} \times V}{P_{\text{creatinine}}} \).

Urinary protein excretion was determined with the BioRad protein assay kit (catalog 500-0006) using the microassay format based on the manufacturers recommendation for enhanced sensitivity. The assay was scaled down for use on microtiter plates with a final volume of 200 µl/sample. Urinary albumin excretion was determined using the albumin 580 blue method as previously described (27).

Urine osmolarity was determined with the µosmette 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 nitrogen and stored at -70°C. Papillae were thoroughly homogenized in 250 µl deionized water and membranes 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 angiotensin II were determined in a sub-population of animals that were sacrificed at 4 weeks and 8 weeks post-surgery. Following a final urine collection, animals were anesthetized with ketamine HCl (60 mg/kg), xylazine (6 mg/kg), and acepromazine maleate (0.9 mg/kg) by i.p. injection. Chronic indwelling catheters, constructed as described previously (20), were inserted into the femoral artery and vein and advanced approximately 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 spring that was secured onto the rat with a specialized jacket. The opposite end of the spring was attached to a 3-channel microswivel (Alice King Medical Arts Inc) which provided free and unrestrained movement of the animal. Rats were housed individually in metabolic pens and the microswivels were attached to a holder above the pen. The arterial catheter was filled with 1000U/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.

MAP was measured on the first day following recovery from the instrumentation surgery from the arterial catheter for 3 hours (0900-1200h) with a pressure transducer connected to an 8-channel amplifier and a pressure display unit (Department 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-second intervals and final MAP was calculated as the mean of approximately 180 data points during the 3 hour-recording period. Immediately following the first 3-hour recording period, the venous infusate was supplemented with angiotensin II (Sigma, St Louis, MO) such that the infusion rate was 15 ng/kg/min 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 hours as before. Angiotensin II was removed from the infusate and the animals were prepared for sacrifice the following morning.

Analysis of renal structure and capillary density:

At termination, animals were prepared for sacrifice by administering 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 removed quickly. One half of this kidney was saved for biochemical analysis (see below) by snap freezing in liquid nitrogen. The remaining half was fixed in Bouin's solution and prepared for routine histological examination including PAS and silver staining.

For analysis of the renal microvasculature, microfil infusion was employed in a fashion similar to that described previously by Lennon et al (30). A tygon catheter (OD 0.090") 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 was infused through the catheter and the animal was allowed to stabilize for approximately 30 min. Microfil (Flow Tech Inc, Carver MA) was prepared by combining MV-130 red with MV diluent at a 1:2 ratio and 10% v/v catalyst (final volume 5.5 ml/kidney). After cutting the renal vein, the proximal aortic ligature was tightened to isolate the renal circulation. Microfil was infused with a filling pressure held manually between 100-140 mm Hg as determined by a pressure transducer connected to the system through a side arm. The renal pedicle ligature was tightened and kidney removed and placed in 5% formalin for several days. The kidney was cleared with successive changes in graded alcohol and methyl salicylate according to the manufacturers instructions. To aid in visualization and analysis, the kidney was embedded in paraffin and sectioned at 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 Metamorph imaging software (Version
For all image analysis studies, (vascular density, interstitial volume and extracellular matrix), at least 5 random images were stored for cortex, outer stripe of the outer medulla, inner stripe of the outer medulla and at least 3 images stored from the inner medulla using a 10X objective with a field dimension of approximately 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 microvascular density, the sharp contrast between the opaque-filled vessels, and adjacent translucent renal parenchyma facilitated image thresholding by 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 reference grid-lines (12 X 12) were overlain onto images. A blinded observer manually counted the number of intersections between a microfilled structure and a reference gridline.

For calculations of interstitial volume, we modified the technique recently described by Morrissey et al. (35). PAS-stained kidney sections were obtained from all groups and overlain 10 X 10 array of dots using Metamorph software. Of the 100 dots, the number which lied directly over interstitial space, but not tubules, tubule luminae, glomeruli and arterioles were determined. Data are expressed as the number of dots/field.

Extracellular matrix content was calculated on basis of data generated from silver-stained sections. The black staining of basement membrane components using this technique allowed for facilitated image thresholding. Data are expressed 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 solution (Biotecx, Houston, TX) according to the manufacturer's instructions. Renal TGF-β1 mRNA was determined using ribonuclease protection assay according to the procedure previously described (4). Following 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 between multiple gels, all data are expressed relative to the mean value obtained in 3 samples of RNA from 4 week sham-operated control rats; these samples were included in every assay as an internal standard. Statistical analysis was performed using the Student's t-test for unpaired sample means; P < 0.05 for 2-tailed analysis was considered significant.

Aquaporin-2 mRNA was measured by ribonuclease protection using a probe generously provided by Drs Baozhi Yaun and Allen W. Cowley Jr. (Department 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 aquaporin-2 (Drs. Yaun and Cowley, unpublished data). The riboprobe template was generated by PCR of the subcloned fragment using the following primers; forward 5'-AGCGCGCAGAAGTCGGAGCA-3'; reverse 5'-GGATCCATTTAGGTGACACTATAGAACAGCCACATAGAAGGCAGCT-3'. The underlined region refers to the bacterial promoter sequence of SP6 polymerase used to generate antisense riboprobe from the purified product of the PCR amplification.

**RESULTS**

*Effects of Ischemic injury on long-term renal function*
To determine the potential long-term effects of acute renal failure on renal structure and function, we subjected rats to 60 min bilateral ischemia/reperfusion injury and allowed them to recover from surgery for 4, 8 or 40 weeks (Study 1). Renal functional data are shown in Figure 1. Serum creatinine values rose to 3.4 ± 0.4 mg/dl within 24 hours following surgery and the estimated creatinine clearance based on a 24-hour urine collection between day 1 and day 2 post-surgery decreased to approximately 12% of sham-operated control values (2058 ± 378 ml/day, sham vs. 248 ± 55 ml/day, ARF). By 7 days, serum creatinine had decreased to values similar to sham-operated controls and creatinine clearance values had returned to 87% of control values and remained significantly different from controls. There was no difference in the serum creatinine levels or creatinine clearance at any time point between 2 and 40 weeks post-surgery.

Despite the recovery of filtration function in the post-ischemic animals, other elements of renal function appear to remain altered following ischemia/reperfusion injury. Figure 2 illustrates the urinary concentrating defect seen in post-ischemic rats. At 1 week post-ischemia, there is an approximate 2.5 fold-increase in urinary output (45.2 ± 2.2 ml/day vs. 18.3 ± 1.4 ml/day, Figure 2B) and a corresponding decrease in urinary osmolarity (Figure 2A). The values for both urinary output and urinary osmolarity initially moved back toward values in sham-operated controls in the first 4-6 weeks post-surgery. However, these trends did not continue after the first 4-6 weeks 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 following post-ischemic acute renal failure. Therefore we measured sodium excretion in these animals to determine the potential relationship between Na excretion and urinary output. Total sodium excretion peaked at
1 week post-injury and gradually decreased up to week 4 post-ischemia (Figure 2C). By week 8, total sodium excretion was similar in both sham-operated and post-ischemic animals. Fractional excretion of Na (%FENa) peaked at 24-48 hours, remained elevated in post-ischemic rats by 2 weeks post-injury and returned to control values by 4 weeks (Figure 2D).

*Effect of dehydration on urinary concentrating ability in post-ischemic animals*

To investigate further the abnormality in urinary flow in the post-ischemic 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 the first study, urine flow rates were significantly higher at one week post injury and gradually trended toward but did not reach control values. At week 4 post-surgery, animals were randomized into either water-deprived or water replete groups and 3 successive 12-hour urine samples were obtained. In water replete groups, the difference in urine flow rates and in urine osmolarity persisted in all 3 collection periods (Figure 3B). In response to water deprivation, urine flow decreased and urine osmolarity increased in both sham-operated and post-ischemic animals (Figure 3A and 3B). The difference in urine osmolarity between control and post-ischemic animals persisted for each of the three 12-hour collection periods (Figure 3B). It appeared that the rate of increase in urine osmolarity for each of the successive collection periods was similar in both groups (Figure 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 post-ischemic animals is significantly reduced when compared with corresponding hydrated sham-operated controls (Figure 3C). Papillary osmotic content significantly increased
following dehydration stress in both groups, but remained different between post-ischemic 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 aquaporin-2 (AQ2) mRNA following this protocol. In hydrated groups, there was no difference in the expression of AQ2 mRNA in post-ischemic recovered animals vs. levels observed in sham-operated hydrated rats (Figure 4). AQ2 mRNA was significantly enhanced in kidneys of animals following 36 hours of dehydration (1.6 fold, sham; 1.8 fold ischemic; P < 0.05 vs. comparable hydrated controls) (Figure 4). Taken together, these data suggest that the vasopressin/AQ2 axis is intact in the post-ischemic rat.

**Development of proteinuria in post-ischemic recovered rats**

As animals in Study 1 progressed through the post-ischemic period, other abnormalities became manifest. During the first 14 weeks post-injury, protein excretion was low in both sham-operated and post-ischemic animals (Figure 5A). Detectable increases in protein excretion began by week 16 post-surgery in both groups of animals. Proteinuria rose consistently with each subsequent sampling and the degree of proteinuria was significantly higher in post-ischemic animals than in shams between week 16 and week 40 post-surgery. Consistent with these observations, urinary albumin revealed a qualitatively similar relationship and comprised between 30-40% of urinary protein (Figure 5B).

**Development of renal fibrosis in post-ischemic recovered rat kidney**

Renal histology was assessed in kidneys at 4, 8 and 40 weeks post-injury (Figure 6). At 4
and 8 weeks post-injury, there was ample evidence of tubular repair. Despite this, it was not uncommon to identify a small proportion of tubules with abnormal morphology (Figure 6B, small arrow). At 40 weeks post-injury renal morphology was profoundly abnormal. In the cortex, we observed several instances of glomerulosclerosis, glomerular atrophy (Figure 6E, arrow) and glomerular hypertrophy (Figure 6F, arrow). In the outer medulla, tubulointerstitial fibrosis was prominent (Figure 6H). These changes were not prominent in the 40-week sham-operated control group (Figure 6E).

Data in Table 1 represent quantitative assessments of scores corresponding to tubulointerstitial space in the kidneys of post-ischemic and sham-operated animals. At 40 weeks post-injury, there is a significant increase in the tubulointerstitial space in post-ischemic kidneys vs. kidneys from sham-operated animals. This relationship was not observed at 8 weeks post surgery. However at 4 weeks, the measured value of interstitial space was elevated in the post-ARF group vs. shams; visual examination revealed that this was largely due to hypercellularity in the interstitium (see Figure 6B, thick arrow).

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

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

Analysis of renal microvascular structure

To determine if the above results could be attributable to alterations in the renal blood supply following 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 weeks post-injury are shown in Figures 9B and 9C, 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, Figure 10A) or by determining the number of vessel intersections across an arbitrary grid (Figure 10B). Acute ischemic injury resulted in a significant reduction in vessel density within 4 weeks of the insult. While all regions of the kidney were
affected, the most dramatic reduction in vessel density was observed in the ISOM; the reductions in vessel density were persistent up to 40 weeks post-injury.

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 minutes and 45 minutes. Serum creatinine values 24 hours post-surgery were 1.0 ± 0.2 and 2.2 ± 0.2 mg/dl, in each of these groups respectively (Table 3). The rats recovered uneventfully from ARF. However, at 4 weeks, the diuresis was unresolved in both post-ischemic groups and was greater in the 45 min vs. the 30 min group. Microfil analysis revealed that renal capillary density 1 month following either 30 or 45 min of ischemia/reperfusion injury was significantly reduced when compared with the corresponding sham-operated controls (Table 3). The degree of capillary destruction appeared similar in the cortex and the OSOM. However, capillary density in the ISOM and IM showed a trend toward a greater reduction in the 45 min group vs. 30 min group.

*Enhanced pressor response in post-ischemic animals*

Reductions in peritubular capillary density and/or reductions in renal medullary blood flow have been shown 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 prior to scheduled sacrifice at 4 and 8 weeks post-injury. In the subgroup of animals tested, there was no difference in the MAP between sham-operated animals and post-ischemic animals 4 or 8 weeks post-surgery. When these animals were administered a threshold dose of Ang II (15 ng/kg/min), MAP significantly increased in both groups of post-ischemic animals. 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 following recovery from ischemic acute renal failure.

DISCUSSION

The long-term effects of acute renal failure have been largely unexplored. We report here, using a rat model of ischemic ARF, that several manifestations of renal function and structure are permanently altered following 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 4 other separate reports using animal models have recently been published demonstrating 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 due, in part, to an endogenous renal repair response. However, this view is simplistic and complete return of renal function may never be appreciated in a significant proportion of patients. For example, studies by Briggs et al (4-75 month follow-up), Lewers et al. (2-15 years follow up), Bonomini et al. (1 and 5 year follow up) and Kjellstrand et al (<1 year 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 2 of these studies was the persistent lack of urinary concentrating ability (8, 31). In addition, Bonomini et al., reported that a small percentage of patients manifested a loss of GFR between the year 1 and year 5 follow up (5). Similarly, a small percentage of patients in the
report by Lewers et al., showed a progressive loss of function over time (31). 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 post-ischemic rats and highlight the need to understand the long-term implications of renal injury and of therapies geared toward facilitating recovery following ARF. While CRF may ensue following ARF in only a small in number of cases, it is possible that our observations may also relate to delayed graft function (DGF) following renal transplant. DGF is defined as poor graft function with a requirement for dialysis in the immediate post-transplant period; ischemic ATN is the most common etiology of DGF (34). The influence of post-ischemic ATN on long-term graft function is controversial as some studies do not demonstrate any correlation with DGF and graft survival e.g. (9). Other, more recent studies do demonstrate that DGF is an independent risk factor for graft survival and the development of post-transplant hypertension (18, 34, 36). Thus, following the resolution of ischemic ARF in rats, the predisposition of secondary chronic renal injury and hypersensitivity to pressor stimuli may be of clinical relevance.

In the current study, the return of GFR and proximal tubular morphology occurred in a time frame that is similar to previous reports in this model (6, 22, 23, 45). However, we were struck not only by the robust diuresis post-injury but also 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 following 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. Na excretion returns to control values within 2-4 weeks.
We suggest this is related to the repair of the proximal tubule but may also be related to the restoration of distal nephron function. Regardless, Na excretion cannot explain the sustained diuresis that is maintained throughout the study.

A previous report by Fernandez-Llama et al., demonstrated decreased expression of aquaporins in kidney in the immediate post-ischemic period (15). However, it appears unlikely that a sustained alteration in aquaporins can account for the sustained diuresis reported here. Firstly, in their study, AQ-2 expression returned to control levels by 7 days post-injury (15). Secondly, we show that post-ischemic animals (4 weeks post-surgery) responded to dehydration in the same fashion as did shams but with an altered set-point at which baseline urine concentration is determined. Therefore, vasopressin-mediated mechanisms appear intact. Finally, AQ-2 mRNA levels, which are under the influence of vasopression during dehydration stress, are similar between post-ischemic and sham-operated animals at 4 weeks. Moreover, AQ-2 mRNA levels are enhanced similarly in response to dehydration in both groups. Taken together, disruption of the AVP-AQ-2 axis is not implicated in the sustained diuresis post-ischemic injury.

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

Besides alterations in urine flow, we observed manifestations of chronic renal disease
such as the development of progressive tubulointerstitial fibrosis. Proteinuria was also apparent and progressive in both 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 following the development of interstitial fibrosis. Therefore, our hypothesis suggests that there is an increase in single nephron GFR of surviving nephrons while total GFR is held constant as long as functional reserve is adequate. It is of interest that animals with only 1 kidney manifest secondary increases in creatinine following ischemic injury (14) (and our unpublished observations), an effect likely related, in part, to decreased functional reserve.

TGF-β is a major profibrotic factor in the kidney and we have previously reported that its expression is enhanced following renal ischemic injury (4) However, consistent with our earlier report (3, 4), TGF-β expression post-ischemic injury is transient and returns to basal values by 4 weeks post-injury. Thus, we wish to emphasize that the enhanced TGF-β 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 suggested that renal blood flow is permanently altered following ischemic injury. The long-term consequences of ischemic injury on the renal vasculature remains incompletely understood. The available evidence using electromagnetic flowmeters and $^{86}$Rb extraction techniques suggests that renal blood flow returns to normal (sham-operated) values between 7 and 28 days post-ischemia with no apparent alteration in regional distribution of flow by 4 weeks (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 but most prominently in the inner stripe of the outer medulla at 4, 8 and 40 weeks post-injury. Therefore, while 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 TAL 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-ß1 and other pro-fibrotic molecules that contribute to the development of tubulointerstitial fibrosis (3, 7, 16, 25, 43). Hypoxia may regulate TGF-ß expression by affecting transcription factor activity or by modulating the activation of TGF-ß 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 his colleagues have shown that acute insults to the kidney affect the integrity of peritubular capillaries and predispose animals to salt-sensitive
hypertension (24, 25, 32). 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, micro-infusion of L-NAME 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 angiotensin II (42). Similarly, in our study we measured the response to a fixed low-dose of Ang II and showed that post-ischemic 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 following severe ischemic injury, while 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 following renal injury. These results may have implications for patients recovering from severe cases of ARF or the demise of renal allografts after delayed graft function.
ACKNOWLEDGMENTS

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References


significance of acute renal failure after renal transplantation in patients treated with cyclosporin.


Figure 1. Renal function following bilateral ischemia/reperfusion injury. A) Serum creatinine values are shown. B) $C_{\text{creatinine}}$ is shown as % of sham-operated controls (Straight-line). As animals were sacrificed during the course of the experiment the number of animals remaining in the study decreased. Therefore N= 37, 24 and 12 for time points up to and including 4, 8 and 40 weeks respectively in the post-ARF group; N= 28, 18 and 8 in the sham-operated control group. Data are mean " SEM; * indicates P < 0.05 by Student one-tailed t-test.

Figure 2. Alterations of urinary concentrating ability and Na handling following renal injury. Analysis of urinary sodium and water excretion are shown. At the indicated times, rats were placed in metabolic cages for 24 hour urine collection. (A) 24-hour urine volume and (B) osmolalities are shown. (C) 24 hour total Na$^+$ excretion; (D) % FENa was calculated on the basis of $C_{\text{creatinine}}$. Data are mean " SEM; * indicates P < 0.05 by Student one-tailed t-test.

Figure 3. Urinary concentrating ability and papillary tonicity in response to dehydration during recovery from acute renal failure. Ischemic acute renal failure was induced and urine flow rate (A) and urine osmolarity (B) was determined at the indicated times in the post-recovery period. After 28 days, animals were subjected to dehydration stress (bar) and urine collected in three 12-hour intervals. Panel C; tissue osmotic content was determined in renal papillae that were microdissected at the termination of the study. Values represent mean " SEM. *, P < 0.05
ARF-hydrated animals vs sham-operated hydrated animals; a, P < 0.05 sham-operated dehydrated vs. sham-operated water replete; b, P < 0.05 sham-operated dehydrated vs. post-ischemic dehydrated; c, P < 0.05 post-ischemic dehydrated vs. post-ischemic water replete (by Student’s one-tailed t-test).

**Figure 4. Effect of dehydration stress and ischemic injury on the expression of aquaporin-2 mRNA levels in kidney.** Total RNA from whole kidney was analyzed for the expression of aquaporin-2 (AQ-2) mRNA by ribonuclease protection assay. A representative gel showing the protected fragment corresponding to rat AQ-2 mRNA and 28S RNA. Note the increased intensity of the signal obtained from both post-ischemic and sham-operated groups in response to water deprivation.

**Figure 5. Development of proteinuria in rats following recovery from post-ischemic acute renal failure.** Protein values were determined on the basis of 24-hour urine collections using standard assays. (A) Total daily protein excretion, (B) Total daily Albumin excretion. Values represent mean ± SE. *, P < 0.05 vs sham-operated control by Student’s one-tailed t-test.

**Figure 6. Renal structure following recovery from ischemic acute renal failure.** Shown are cross-sections through rat renal cortex and outer medulla stained with periodic acid Schiff (PAS). A-C represent sections through the outer medulla of a sham-operated rat kidney (A), 4 weeks (B) and 8 weeks (C) following ischemic ARF. Note that at 4 weeks some tubules appear unrepaird (thin black arrow) and that interstitial hypercellularity was frequently observed (thick black
arrow). In C, tubular structures appear normal with the exception of tubular hypercellularity (small white arrow). Sections through rat renal cortex and outer medulla at 40 weeks post surgery are shown in D-H. A sham-operated cortex is shown in D. E and F show two strikingly different glomeruli observed in 40 week post-ischemic animals. In E, glomerular atrophy is observed (thin black arrows) and nearby cellular infiltrate (black arrow) (E). In contrast, glomerular hypertrophy was also observed at 40 weeks post injury (F). Tubular interstitial scarring is evident in outer medulla at 40 weeks (H, white arrow) but not appreciably in 40 weeks shams (G). White bar in H is 50 µm.

**Figure 7. Silver stain of extracellular matrix from sham-operated and post-ischemic rats.**

Shown are cross-sections through rat outer medullae stained with the Jones silver stain method. Renal outer medulla from a sham-operated rat (A) and a post-ischemic rat (B) 4 weeks post surgery. C and D represent outer medullae from sham and post-ischemic rats at 40 weeks post-surgery. Note significant interstitial staining in the post-ischemic kidney. White bar in D is 50 µm.

**Figure 8. Secondary increase in TGF-β1 mRNA following recovery from ischemic acute renal failure.**

Total RNA from whole kidney was analyzed for the expression of TGF-β1 mRNA by ribonuclease 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
week sham-operated group, which were run on every gel as an internal standard. Values represent mean ± SE. *, P < 0.05 vs sham-operated control; #, P < 0.05 sham-operated at 40 weeks vs. sham-operated control at 4 weeks post-surgery (by Student's one-tailed t-test).

Figure 9. Gross renal morphology and capillary filling in normal and post-ischemic kidneys

Representative stereoscopic views of 20 µm microfilled kidney section. Shown are microfilled kidneys from a sham-operated rat (A) at 4 weeks (B) and 8 weeks (C) post-ischemic injury. In this stereoscopic view, microfil appears as bright yellow against a dark background. A reduction in microfilled structures in recovered post-ischemic 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.

Figure 10. Image analysis of microfilled kidneys using computerized image analysis.

Microfilled kidneys were subjected to quantification using computerized image analysis tools. In panel A, a 12 X 12 grid was overlaid onto an image as described in methods and the number of vessel-grid intersections determined. In panel B, vessel density was determined as % of total surface area in the field. In the cortex, the area covered by glomeruli were excluded from analysis. For clarity, the bars representing sham-operated controls are pooled values from 4 week, 8 week and 40 week groups. For statistical analysis, only the values for shams from time-matched groups were used; no differences were observed between these groups (not shown). Values represent mean ± SE. *, P < 0.05 vs sham-operated control (by Student's one-tailed t-test).
Figure 11. Post-ischemic rats manifest an enhanced pressor response to low-dose angiotensin II. (A) Baseline measurements of MAP in conscious rats were determined for 3 hours on day-1 post-catheter implantation. (B) Immediately after recording, rats were subjected to infusion of angiotensin II (15 ng/kg/min for 24 hours, in Na-free D5W) and MAP was again recorded during the final 3 hours of infusion on day 2. Values for each animal were determined in at least 180 one-minute averages. Values represent mean ± SEM. *, P < 0.05 vs sham-operated control by Student's one-tailed paired t-test comparing pressures obtained in the same animal pre- and post infusion.
Table 1. Interstitial volume scores of kidney regions following recovery from ARF

<table>
<thead>
<tr>
<th></th>
<th>4 weeks</th>
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<th></th>
<th>8 weeks</th>
<th></th>
<th></th>
<th>40 weeks</th>
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<tr>
<td></td>
<td>Cx</td>
<td>OS</td>
<td>IS</td>
<td>N</td>
<td>Cx</td>
<td>OS</td>
<td>IS</td>
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<tr>
<td>Sham</td>
<td>16.6±2.6</td>
<td>26.04±2.8</td>
<td>38.12±4.2</td>
<td>6</td>
<td>19.2±1.3</td>
<td>23±2.2</td>
<td>41.7±2.4</td>
</tr>
<tr>
<td>ARF</td>
<td>28.7±3.5*</td>
<td>41.6±7.0*</td>
<td>43.1±5.7</td>
<td>6</td>
<td>20.3±1.9</td>
<td>26.3±3.9</td>
<td>41.2±1.9</td>
</tr>
</tbody>
</table>

Values represent dots that overlie interstitial space from an arbitrary 10 X 10 array superimposed on random images. Cx, cortex; OS, outer stripe of the outer medulla; IS, inner stripe of the outer medulla. Data are mean dots/field ± SEM; * P < 0.05 vs sham-operated control for given kidney region.
Table 2. Extracellular matrix scores of kidney regions following recovery from ARF

<table>
<thead>
<tr>
<th></th>
<th>4 weeks</th>
<th></th>
<th>8 weeks</th>
<th></th>
<th>40 weeks</th>
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<tr>
<td></td>
<td>Cx</td>
<td>OS</td>
<td>IS</td>
<td>N</td>
<td>Cx</td>
<td>OS</td>
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<tr>
<td>Sham</td>
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<td>3.6±0.7</td>
<td>3.5±0.7</td>
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<td>8.0±1.6</td>
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<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>
</tr>
</tbody>
</table>

Values are derived from % thresholded area from Jones-stained kidneys and are mean ± SEM; * P < 0.05 vs sham-operated control for given kidney region.
Table III Effect of variations on ischemic time vs renal function and vascular structure

<table>
<thead>
<tr>
<th>Group</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>Serum Creatinine (mg/dl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day post-injury</td>
<td>0.45±0.1</td>
<td>1.0±0.2*</td>
<td>2.2±0.2*</td>
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<tr>
<td>7 days post-injury</td>
<td>0.42±0.1</td>
<td>0.44±0.1</td>
<td>0.69±0.2</td>
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<tr>
<td>28 days post-injury</td>
<td>0.42±0.1</td>
<td>0.46±0.1</td>
<td>0.57±0.1</td>
</tr>
<tr>
<td>Urine Flow rate (ml/day)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>7 days post-injury</td>
<td>12.1±1.1</td>
<td>19.1±2.2*</td>
<td>29.0±3.3*</td>
</tr>
<tr>
<td>14 days post-injury</td>
<td>14.9±1.3</td>
<td>17.5±1.5*</td>
<td>25.6±4.0*</td>
</tr>
<tr>
<td>28 days post-injury</td>
<td>11.7±1.4</td>
<td>19.0±1.2*</td>
<td>26.9±4.0*</td>
</tr>
<tr>
<td>Renal Vascular Density at 28 days (% of sham control)</td>
<td>Cortex</td>
<td>OSOM</td>
<td>ISOM</td>
</tr>
<tr>
<td></td>
<td>100±5</td>
<td>100±4</td>
<td>100±5</td>
</tr>
<tr>
<td></td>
<td>53±6*</td>
<td>59±6*</td>
<td>74±12*</td>
</tr>
<tr>
<td></td>
<td>57±5*</td>
<td>45±4*</td>
<td>60±5*</td>
</tr>
</tbody>
</table>

OSOM, outer stripe of outer medulla; ISOM, inner stripe of outer medulla; IM, inner medulla. * P < 0.05 vs sham-operated control.
Figure 1 Basile et al.
Figure 2, Basile et al.
Figure 3 Basile et al.
Figure 4, Basile et al
Figure 5, Basile et al.
Figure 6, Basile et al.
Figure 7, Basile et al
Figure 8, Basile et al.
Figure 9 Basile et al.
Figure 10, Basile et al

**Microvessel Density** (Number of grid intersections/field)

- Sham, N=18
- ARF 4 weeks, N=6
- ARF 8 weeks N=6
- ARF 40 weeks N=8

**Vascular Perfusion Index** (% Thresholded area)

- Sham, N=18
- ARF 4 weeks, N=6
- ARF 8 weeks N=6
- ARF 40 weeks N=8
Figure 11, Basile et al.