Renal damage progresses despite improvement of renal function after relief of unilateral ureteral obstruction in adult rats

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Ito, Keichi, Jie Chen, Maher El Chaar, Joshua M. Stern, Surya V. Seshan, Jonathan J. Khodadadian, Ingrid Richardson, Michael J. Hyman, E. Darracott Vaughan, Jr., Dix P. Poppas, and Diane Felsen. Renal damage progresses despite improvement of renal function after relief of unilateral ureteral obstruction in adult rats. Am J Physiol Renal Physiol 287: F1283–F1293, 2004. First published August 24, 2004; doi:10.1152/ajprenal.00441.2003.—Progression of renal damage after relief of unilateral ureteral obstruction (UUO) has been demonstrated, especially in neonatal rats. We evaluated renal function and renal damage after relief of 3-day UUO in five groups of adult rats: group 1, no treatment; group 2, 3-day UUO; groups 3-5, 3-day UUO followed by relief; group 3, 7-day relief; group 4, 14-day relief; and group 5, 28-day relief. Glomerular filtration rate (GFR), renal blood flow (RBF), tissue transforming growth factor-β (TGF-β), interstitial fibrosis and fibroblast expression, tubular apoptosis, macrophage infiltration, expression of nitric oxide synthases (NOS), and urinary nitrate/nitrite (NO2/NO3) were evaluated. RBF and GFR were decreased to <10% of baseline by 3 days of UUO. GFR and RBF in a previously obstructed kidney (POK) returned to baseline by 14 days after relief. Both tissue TGF-β-1 and interstitial fibrosis were significantly higher in POK of groups 3-5 compared with groups 1 and 2. In group 5, the numbers of infiltrating macrophages, fibroblasts, and apoptotic tubular cells were higher in POK compared with group 1. Urinary NO2/NO3 was significantly higher than baseline from 3 to 27 days after relief of UUO. Expression of NOS isoforms was increased in tubules. As interstitial fibrosis contributes to decreased renal function, these results suggest that the acute recovery in function may be compromised in the long term by the progressive renal fibrosis which was found. Furthermore, pharmacological intervention at the time of relief of UUO, targeted to fibrotic processes, may contribute to long-term recovery of renal function.

Sprague-Dawley rats; interstitial fibrosis; apoptosis; transforming growth factor-β; FSP; nitric oxide

UNILATERAL URETERAL OBSTRUCTION (UUO) is a common clinical finding (17). Prominent changes in UUO include decreases in renal function and increased fibrosis, tubular apoptosis, and cellular proliferation. After UUO, both renal blood flow (RBF) and glomerular filtration rate (GFR) decrease, and the decreases are permanent without intervention. If UUO is relieved within a certain period, both RBF and GFR increase. However, the recoverability of renal function in the previously obstructed kidney (POK) depends on the duration of UUO and the species being examined (1, 14, 27, 43).

It has been shown in a variety of chronic renal diseases that the presence of interstitial fibrosis is a major determinant of glomeruli (5, 6, 36). Thus, despite the presence of seemingly normal glomeruli, significant decreases in GFR have been found. Interstitial fibrosis may contribute to decreased GFR by a variety of mechanisms including tubular atrophy, tubular ischemia, and obliteration of postglomerular peritubular capillaries (12).

Many factors have been implicated in fibrosis in UUO. Two significant mediators are transforming growth factor-β (TGF-β) and nitric oxide (NO) (2, 7). TGF-β is a profibrotic and proapoptotic mediator in UUO (29). Increases in TGF-β have been noted in both neonates (9) and adult kidneys (8) after relief of UUO. In contrast, NO, a multifunctional mediator synthesized from L-arginine by NO synthase (NOS) (33), has been shown to be antiapoptotic (29) and antiinflammatory in UUO (21, 32), in addition to modifying renal hemodynamics (11, 13, 19, 37). Other studies suggest a reciprocal relationship between NO and TGF-β.

Because interstitial fibrosis is implicated in renal functional loss, it is possible that recovery of renal function after relief of UUO is at least partially dependent on changes in the interstitium. Therefore, in the present study, we examined the recoverability of both GFR and RBF over a period of 28 days following relief of 3 days of UUO. In addition to examining renal function, we also examined renal tissue for the presence of fibrosis and fibroblasts, tubular apoptosis, macrophage infiltration, and expression of both TGF-β and NO. Our goal was to determine if changes in renal function and renal histopathology were convergent or divergent.

MATERIALS AND METHODS

Experimental protocol. Male Sprague-Dawley rats (300 to 400 g) were kept at constant temperature and lighting conditions and fed standard rat chow. All animal treatment adhered to approved institutional guidelines. Determination of renal function, measurement of urinary NO2/NO3, tissue TGF-β, immunohistochemical analyses, and Western blot analyses were performed as described below.

In vivo UUO and relief of UUO. Left UUO was performed as previously described (29). To ensure sufficient length of ureter for the ureteroneocystotomy, the lower left ureter was ligated using 3–0 silk at two places close to the ureterovesical junction.

Three-day UUO was reversed using microsurgical methods, described briefly here. Rats were anesthetized intraperitoneally and positioned supine under two heating lamps. A midline abdominal incision was made, and under ×8 magnification, the dilated left ureter was identified and dissected free of surrounding tissues. An 18-gauge angiocatheter was introduced through the midanterior bladder wall.

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and exited through the left lateral bladder wall. The ureter was transected −1 cm above the level of obstruction, and the open end was cannulated with the angiocatheter. With the use of a 10−0 nylon suture, a ureteral bladder anastomosis was effected in an interrupted fashion. The catheter was then removed and the bladder-puncture defect was repaired with a 7−0 prolene suture. The abdominal incision was closed in two layers including muscle and skin.

**Renal function measurement**. Renal function was measured as detailed below and described previously (22). RBF was calculated using PAH clearance; GFR was calculated using inulin clearance, both according to standard formulas. Urine and plasma concentrations of PAH and inulin were detected using standard chemical methods (15, 18). PAH was detected colorimetrically at 550 nm using p-dimethylaminocinnamaldehyde, and inulin at 595 nm using anthrone. For each rat, the values obtained for three clearance periods were averaged. Values were normalized to body weight (body weights shown in Table 3).

Rats were anesthetized, and a 24-gauge × 19-mm angiocatheter was secured in the right jugular vein. A bolus of lactated Ringer (LR) solution was slowly injected intravenously in the amount of 1% body wt and a bolus of 1% PAH and 1.5% inulin in LR was injected in the amount of 0.2% body wt. A solution of 1% PAH and 1.5% inulin in LR was infused at 10 μl·min^−1·100 g body wt^−1 and was maintained for at least 60 min to allow for equilibration before urine and blood collections. A midline abdominal incision was made, the ureters were identified, and a small transverse incision was made in the ureter. PE-10 tubing was inserted into the right and left ureters of control rats, whereas PE-50 tubing was inserted into the left ureter of the POK due to its dilatation. Ten minutes after catheter insertion, urine collections were started. Twenty-minute urine collections were made from each kidney three times, and 450 μl of arterial blood were collected at the midpoint of each collection. The volume loss due to removal of blood for renal function measurements was compensated for by infusion of the same amount of saline. Renal function values were averaged over the three measurements.

To document that patency of the anastomosis had been maintained throughout the period following relief of UUO, an antegrade study was carried out at the conclusion of the measurement of renal function. A tube was inserted into the midureter and was ligated by 3–0 silk; 0.9% saline solution or ink was introduced into the ureter from a level of ~18 inches using gravity, and patency of the anastomosis was confirmed by examining the entry into/efflux from the bladder. Only rats with patent anastomoses were used in these studies. After measurement of renal function, both kidneys were harvested for analysis.

RBF and GFR were measured in both left and right kidneys of group 1 rats (group 1). In groups 3−5, RBF and GFR were measured at 7, 14, and 28 days postrelief of UUO, respectively. In group 2, RBF and GFR were measured at 1 day following UUO, because it was not possible to measure RBF and GFR at 3 days following UUO (too little urine flow). However, previous data demonstrate that RBF and GFR at 3 days of UUO are either comparable to or decreased from that measured 1 day after UUO (25); the present results obtained at 24 h of UUO document a significant decrease in renal function, consistent with previous findings (1, 25, 42). For tissue analysis in group 2, tissues were harvested from rats subjected to UUO for 3 days. Analyses of renal function and tissue were made using n = 8 rats in groups 1, 4, and 5 and n = 7 rats in groups 2 and 3.

**Urinary NO_2/NO_3**. A 24-h urine sample was collected from rats in group 5 placed in metabolic cages at the following times: 1 day before UUO, and 3, 7, 14, 21, and 27 days after relief of UUO. The urine was collected in a reservoir containing 2-propanol and samples were frozen at −80°C until assay. Urinary NO_2/NO_3 levels were measured by the Griess reaction after copperized cadmium treatment. The results were normalized to body weight, as the weight of the animals changed during the study (see Table 3).

**Assessment of renal morphology and interstitial fibrosis**. After renal function measurement, sections of previously obstructed and contralateral kidneys were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections 5-μm thick were stained with hematoxylin and eosin and Masson’s trichrome for light microscopic analysis. Slides were examined by a board-certified pathologist (S.V.S.). Histological studies were performed in a blinded fashion. Renal morphology including tubular dilatation and leukocyte infiltration was assessed. The general presence of interstitial fibrosis was assessed in slides stained with Masson’s trichrome. In addition, interstitial fibrosis was assessed by measurements of interstitial volume, using a point-counting method as previously described (45).

**Determination of tissue TGF-β1**. Kidney tissues were stored at −80°C until assay. All samples were assayed using ELISA, following manufacturer’s instructions (R&D Systems, Minneapolis, MN). In brief, to activate TGF-β1 in tissue, the samples were homogenized with 1 N HCl and neutralized with 1.2 N NaOH/0.5 M HEPES. Samples were analyzed using a microplate reader set to 450 nm with correction set at 550 nm. Standard curve and all samples were run in duplicate.

**Immunohistochemical analysis**. Immunohistochemical analyses for inducible and endothelial NOS (iNOS and eNOS) and macrophages were performed. Paraffin-embedded tissue sections were made as described above. Immunohistochemical analyses were performed as previously described (28, 30). In brief, the concentrations of primary antibodies were 1:100 for iNOS (monoclonal, Santa Cruz Biotechnolog), 1:200 for eNOS (polyclonal, Santa Cruz Biotechnology), and 1:75 for macrophage (monoclonal, ED1 macrophage, Serotec). Biotinylated rabbit antimouse secondary antibody (Vector Laboratories, Burlingame, CA) was used for iNOS staining, and biotinylated goat antirabbit secondary antibody (Vector Laboratories) was used for eNOS staining. Horseradish peroxidase (HRP)-linked rabbit antimouse secondary antibody (Dako) was used for macrophage detection. Avidin-biotin peroxidase complex (Vector Laboratories) was used for iNOS and eNOS detection. Sections were developed with diaminobenzidine (DAB). Sections were then counterstained with hematoxylin. Positive macrophages were counted in 10 high-power fields (×400) by two independent investigators in a blinded fashion and an average was obtained.

We also analyzed tissue for the presence of fibroblasts using immunohistochemistry. The antibody used was DAKO #S100-A4. The S100-A4 antigen is also known as FSP-1 and has been found to be expressed by fibroblasts, but not epithelial cells (35, 41). Antigen was retrieved by incubating cells with protease K for 20 min in an oven. Slides were then processed as is carried out routinely for immunoperoxidase. The primary antibody was used at a dilution of 1:100. Ten high-power fields were counted in each slide and averaged together. Staining for S100-A4 was found in spindle-shaped interstitial cells and also in cells that were round and were identified as inflammatory cells by the pathologist. Only spindle-shaped cells were included in the counts. Samples incubated without primary antibody exhibited no staining.

**In situ detection of DNA strand breaks**. To quantitate nuclei with fragmented DNA, the TUNEL assay was performed with in situ TUNEL kit (Intergen, Purchase, NY) as previously described (28, 30). Briefly, after deparaffinization and quenching endogenous hydrogen peroxide, to retrieve antigen, sections were treated with 1 mg/ml proteinase K for 15 min. The sections were rinsed with PBS and incubated with deoxynucleotidyl transferase (TdT) with digoxigenin-dNTP for 1 h. The reaction was stopped with terminating buffer. Slides were then washed with PBS, and peroxidase-conjugated antidigoxigenin antibody was applied for 30 min. After being washed with PBS, slides were developed with DAB and counterstained with 10% hematoxylin. Positive cells in renal tubules were quantitated in the same fashion as macrophages.

**Western blotting analysis**. Western blot analyses for iNOS and eNOS were performed in rats in all groups. After the measurement of...
renal function, kidney samples were harvested and stored in −80°C until use. Appropriate amount of kidney samples was homogenized in lysis buffer and centrifuged at 13,000 rpm for 30 min. The supernatant was separated, and the protein concentration was determined with the Bradford protein assay (Bio-Rad, Hercules, CA). For the Western blot analysis, equal amounts of protein were loaded. Western blot analysis was then performed as previously described (28, 30). In brief, the concentrations of primary antibodies were 1: 5,000 for iNOS (polyclonal, Transduction Laboratories, Lexington, KY) and 1:3,000 for eNOS (polyclonal, Santa Cruz Biotechnology). The concentration of HRP-conjugated secondary antibody was 1:5,000. The intensity of each band was quantified by National Institutes of Health image. iNOS and eNOS were detected at 130 and 133 kb, respectively. Relative intensity was expressed as percent control intensity (or an arbitrary unit).

Statistical analysis. All results are expressed as means ± SE and were analyzed for significance by one-way ANOVA and multiple comparison tests.

RESULTS

Recoverability of renal function after relief of 3-day UUO.

To determine the recovery of renal function and markers of renal damage, five groups were studied, as shown in Table 1. Group 1, rats without UUO, were used for control (baseline) measurements. Rats in group 2 were subject to UUO for 3 days, with no relief of UUO. Rats in groups 3-5 were subject to left UUO; obstruction was relieved at 3 days by reimplanting the ureter in the bladder (ureteroneocystotomy), as described below. Rats were studied at the following times after relief of UUO: group 3, 7 days; group 4, 14 days; group 5, 28 days.

In group 1, GFR was 0.30 ± 0.02 ml·min⁻¹·100 g⁻¹ in the right kidney, which was not significantly different from 0.31 ± 0.03 ml·min⁻¹·100 g⁻¹ in the left kidney. GFR of the obstructed group 2 kidney was 0.02 ± 0.01 ml·min⁻¹·100 g⁻¹, a decrease greater than 90%. By day 7 after relief of UUO, GFR in the POK had improved and was not significantly different from baseline (Table 1). The improvement was maintained throughout the 28 days. GFR of contralateral (right) kidneys (CLK) in groups 3, 4, and 5 was higher than group 1 (Table 1). Because we measured the GFR in individual kidneys, we were able to calculate the percent of GFR which was accomplished by each kidney. In group 1, the kidneys each maintained 50% of the GFR. In group 2, the obstructed kidney maintained only 5% of the total GFR. At 7 and 14 days of relief, the percentage of GFR in the POK was significantly lower than group 1 (Table 1). There was no significant difference between group 1 and group 5 in the percentage of GFR in the POK. However, the total GFR in group 5 was significantly higher than group 1.

Table 1. Experimental treatments and GFR measurements

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GFR Right Kidney, ml/min · 100 g⁻¹</th>
<th>GFR Left Kidney, ml/min · 100 g⁻¹</th>
<th>Total GFR</th>
<th>% GFR Left Kidney</th>
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<tr>
<td>Group 1 None</td>
<td>0.30 ± 0.02</td>
<td>0.31 ± 0.03</td>
<td>0.61 ± 0.04</td>
<td>50.3 ± 1.5</td>
</tr>
<tr>
<td>Group 2 3-day UUO</td>
<td>0.39 ± 0.01</td>
<td>0.02 ± 0.01†</td>
<td>0.41 ± 0.01</td>
<td>5.1 ± 2.8*</td>
</tr>
<tr>
<td>Group 3 3-day UUO + 7-day Relief</td>
<td>0.42 ± 0.03*</td>
<td>0.22 ± 0.03†</td>
<td>0.64 ± 0.05</td>
<td>33.8 ± 3.1*</td>
</tr>
<tr>
<td>Group 4 3-day UUO + 14-day Relief</td>
<td>0.39 ± 0.02*</td>
<td>0.28 ± 0.02†</td>
<td>0.67 ± 0.04</td>
<td>42.2 ± 1.1*</td>
</tr>
<tr>
<td>Group 5 3-day UUO + 28 day Relief</td>
<td>0.43 ± 0.04*</td>
<td>0.40 ± 0.04</td>
<td>0.83 ± 0.08*</td>
<td>48.0 ± 1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. GFR, glomerular filtration rate; UUO, unilateral ureteral obstruction. *P < 0.05 compared with group 1. †P < 0.05 compared with contralateral side.

RBF in group 1 was 1.02 ± 0.11 ml·min⁻¹·100 g⁻¹ in the right kidney, which was not significantly different from 0.10 ± 0.14 ml·min⁻¹·100 g⁻¹ in the left. RBF of the obstructed group 2 kidney decreased by greater than 90% (Table 2). By day 7 after relief of UUO, RBF in the POK had improved, and by day 14 was not significantly different from baseline. By 28 days of relief, RBF of the POK was significantly higher than untreated control (P < 0.05). In groups 4 and 5, RBF of the CLK was significantly higher than group l RBF. Furthermore, total RBF after 14 and 28 days of relief was significantly higher than group 1. At 7 and 14 days of relief, the percentage of RBF in the POK was significantly lower than control group; however, there was no significant difference between control group and group 5.

Renal morphology and interstitial fibrosis. Kidneys of group 1 demonstrated no evidence of pathology. In group 2, there was dilatation of collecting ducts and distal tubules. In all groups in which obstruction was relieved, there was a persistence of dilatation of collecting ducts and distal tubules and inflammation (leukocyte infiltration), mainly in the peripelvic area, which gradually increased throughout the 28-day period. Tubular atrophy was seen mainly in group 5. CLK in all groups had no remarkable change in their architecture and demonstrated no inflammation. The glomeruli were preserved with normal architecture in all groups.

In group 2 obstructed kidneys, almost no fibrosis was seen (interstitial volume = 0.64 ± 0.2%). In the POK, mild blue staining was seen in two of five animals in group 3 (4.80 ± 1.21%; Fig. 1A). Increased blue staining was seen in almost all group 4 kidneys (10.35 ± 1.02%, P < 0.05, compared with group 3) and group 5 kidneys (24.56 ± 3.53%, P < 0.05, compared with group 4; Fig. 1B). When the CLK was examined, there was minimal fibrosis in group 3 (0.60 ± 0.39%) and some slight fibrotic changes in groups 4 (1.19 ± 0.73%) and 5 (3.62 ± 0.71%). Kidneys in group 5 had significantly more fibrosis.
28 days after relief (group 5; compared with higher at all time periods after relief of unilateral ureteral obstruction (UUO) followed by another rise by 28 days (Fig. 3). There was a significant decrease by 14 days of relief, which was maintained at 29.2 days postrelief, the number of macrophages in the POK was 1.7 cells/high-power field (HPF) from 4.1 cells/HPF (Fig. 3, A and B). The number of macrophages in POK was significantly higher than that in the CLK in all groups.

In group 1 kidneys, there were almost no apoptotic cells (0.93 ± 0.16 nuclei/HPF). At 3 days of UUO, there was a significant increase in apoptotic cells (5.5 ± 1.6 nuclei/HPF). The level of apoptotic cells in the POK was maintained throughout the 28-day period following relief of UUO (Fig. 4, A, B, and C). The POK showed significantly higher tubular apoptosis than CLK in all groups, and CLK in the relief groups showed no significant difference in tubular apoptosis compared with control kidneys.

Expression of FSP-1. We also examined kidneys for the presence of interstitial fibroblasts, by staining for FSP-1. In group 1 kidneys, there were very few FSP+ cells (5.4 ± 0.2 cells/HPF; Fig. 5, A and B). After 3 days of UUO, there was a significant increase in FSP+ cells in the obstructed kidney (18.5 ± 0.8; Fig. 5C). The number of FSP+ cells remained stable following relief of UUO (7-day postrelief shown in Fig. 5D; 14- and 28-day data in Fig. 5A).

Urinary NO2/NO3 excretion. A 24-h urine sample was collected in group 5 before and at various times after relief of UUO. Urinary NO2/NO3 excretion was significantly higher at all time points examined following the relief of UUO, compared with those amounts in preoperative urine (Table 3). Results are presented as micromoles of NO excreted per 24 hours; the findings are the same if the results are normalized to body weight, not shown. Urinary NO2/NO3 was highest 7 days after the relief of UUO and remained significantly elevated throughout the period examined.

Evaluation of iNOS and eNOS. Because urinary NO2/NO3 was increased in group 5, we were interested in examining renal expression of NOS by both immunostaining and Western blot. iNOS (Fig. 6A) was weakly expressed in renal tubular cells in group 1 kidney. Relatively higher staining was seen in cortex. In 3-day UUO (group 2), increased iNOS staining in tubular cells was seen in both medulla and cortex. Especially proximal and distal tubules in cortex were strongly stained with iNOS (Fig. 6B). In the POK (groups 3, 4, and 5), positive staining was seen in proximal tubules, convoluted distal tubules, and thick ascending limbs (Fig. 6C). Less staining was
seen in other distal tubules and collecting ducts. Glomeruli had minimal staining. In the CLK, staining was also increased, similarly to that seen in the POK (not shown).

In group 1, eNOS (Fig. 6D) was expressed weakly in tubular cells in both cortex and medulla. In 3-day UUO (group 2), increased eNOS staining in tubular cells was seen in both medulla and cortex, similarly to iNOS staining (Fig. 6E). In the POK, increased staining was evident in both proximal tubules and distal tubules (Fig. 6F). Less staining was seen in the collecting duct. Glomeruli had a slight increase in eNOS staining. In the CLK, positive staining was also seen in the same location.

iNOS was also examined by Western blot analysis. The 3-day UUO showed a small increase in iNOS compared with the control (Table 4 and Fig. 7A). Expression was increased in both CLK and POK in all relieved groups compared with groups 1 and 2 (Table 4 and Fig. 7B). There was no significant difference in these increases between CLK and POK in all groups.

The level of eNOS expression was also examined by Western blot (Fig. 7C). eNOS expression was significantly increased in group 2 obstructed kidney; both CLK and POK in all groups after relief of UUO compared with groups 1 and 2 (Table 4 and Fig. 7B). There was no significant difference in these increases between CLK and POK in groups 3 and 4 ($P < 0.05$). The expression of eNOS in POK of group 5 was significantly higher than that in its CLK ($P < 0.05$). These data validated the result of immunostaining for iNOS and eNOS.

**DISCUSSION**

The recovery of renal function after relief of obstruction is an important clinical problem. It has been shown that recovery is dependent on several factors including the duration of the obstruction, its location, whether it is partial or complete, and the presence of intercurrent infection. In this study, we examined both the recovery of renal function, measured as GFR and RBF, and also measured several markers of renal damage. This study demonstrated that renal function returned to normal within 14 days after relief of 3 days of complete UUO in adult rats. However, renal damage continued after the relief of UUO. Interstitial fibrosis and tubular apoptosis increased after relief of UUO, along with an associated increase in tissue TGF-$\beta_1$, a profibrotic and proapoptotic cytokine. NO and its synthetic enzyme NOS were increased after the relief of UUO. These results demonstrate that even short-term obstruction is followed by sustained renal damage and suggest that the complete recovery of renal function noted by 14 days may be compromised in the long term by renal fibrotic and apoptotic changes.

Both RBF and GFR of the POK returned to control levels by 14 days after relief of 3-day UUO. These results are similar to previous studies that demonstrated recovery of renal function after relief of 24-h UUO in rats (1). Relief of longer-term UUO, with variable recovery times, has not led to complete recovery of renal function in UUO (14, 27). Recovery of GFR or RBF at 1 mo after relief of UUO may not predict long-term recovery. Furthermore, recovery of total GFR may not indicate
complete recovery from renal injury. After relief of 24-h UUO, 15% of superficial nephrons were not filtering at 14 days, and the remainder exhibited increased single nephron GFR (1). Hyperfiltration of single nephrons can lead to renal damage. Future studies will examine the long-term results of relief of 3 days of UUO to determine if the immediate recovery of renal function is sustained.

We examined several indicators of renal damage after the release of UUO. We found a gradual increase in the amount of trichrome staining, indicating interstitial fibrosis, which was strongest 28 days after relief of UUO. The tissue concentration of TGF-β1 was increased in the POK after relief of 3 days of UUO. Tubular apoptosis was still evident 28 days after the relief of UUO. An increase in macrophages was also found postrelief of UUO. Furthermore, the increase in interstitial fibroblasts noted at 3 days of UUO was also sustained. Thus several different markers of renal damage were significantly increased or maintained at levels higher than control following relief of UUO. There was also evidence of progressive histopathological changes in the CLK, although these were not as severe as in the POK. In the CLK, tissue TGF-β was significantly increased after relief of UUO, and there was a slight but significant increase in interstitial volume at 28 days. It is not clear why TGF-β is increased in the CLK. However, we previously showed that renal tubules are a source of TGF-β in the kidney (45).

Our unpublished data show increased tubular proliferation in the CLK, and these cells may account for the increased TGF-β. In contrast to the POK, tubular apoptosis, fibroblasts, and macrophage numbers were not different in the CLK compared with controls. Thus, despite similar increases in TGF-β, the contralateral kidney is less affected than the previously obstructed kidney. In this study, we examined only whole kidney levels of TGF-β. It is possible that there are differences in the regional expression of TGF-β between POK and CLK that would differentially affect the response of the kidneys. Even if there is similar expression of TGF-β in both kidneys, the POK, having been previously injured, may be more susceptible to the effects of TGF-β. Or, alternatively, other factors in addition to TGF-β may be responsible for the increased damage in the POK more than in the CLK.

Urinary NO metabolites increased after relief of UUO compared with baseline. Western blot and immunohistochemistry confirmed increased expression of both iNOS and eNOS in the POK and CLK, suggesting increased renal synthesis of NO. Previous studies have implicated NO in the changes in RBF in the obstructed kidney following UUO (13, 37). As NO is a known vasodilator (33), it may also contribute to the recovery of RBF after relief of UUO in the POK. Furthermore, we and others demonstrated that NO
appears to be protective in UUO. Both interstitial fibrosis and apoptosis are increased in mice with a targeted deletion of iNOS (21, 30). Arginine feeding was shown to decrease fibrosis, and angiotensin-converting enzyme inhibitors that increase NO are also antifibrotic (23, 32). As we demonstrated increased urinary NO at 28 days postrelief, it is possible that the increased NO results from an attempt by the kidney to oppose the action of TGF-β. This rise in NO would thus be beneficial to both renal function, as shown above, and to offset the deleterious effects of TGF-β. Alternatively, other mediators may be involved.

Fig. 5. Number of FSP+ cells in the POK was higher at 7, 14, and 28 days postrelief than baseline. The level of FSP+ cells in the POK was maintained throughout the 28-day period following relief (A). *P < 0.05 compared with baseline. #P < 0.05 compared with corresponding CLK. Representative immunohistochemistry for FSP (B: control; C: 3-day UUO; D: 7 days of relief). A few selected cells are shown with arrows.
The increased expressions of iNOS and eNOS in the CLK suggest that it may contribute to the functional changes in the CLK. In our study, both GFR and RBF in CLK were increased after relief of UUO. Similar findings were reported previously. In dogs, the CLK exhibited compensatory functional changes after relief of UUO (43). Similarly in rats, renal function in the CLK was increased either 7 days (14) or 3 mo (27) after relief of UUO. We demonstrated herein that the CLK maintained a significantly higher percentage of the RBF and GFR at 7 and 14 days postrelief. The findings of increased expression of NOS in the CLK suggest that NO may contribute to this.

In a neonatal rat model, Chevalier et al. (9) demonstrated progression of interstitial fibrosis after relief of UUO. Twenty-eight days after relief of UUO, interstitial collagen, α-smooth muscle actin, and TGF-β1 were dramatically increased in the POK compared with sham-operated rats (10). Consistent with the present report, GFR after 28 days of relief was not significantly different from baseline GFR. At 1 yr following relief, interstitial collagen, macrophage infiltration, and α-smooth muscle actin immunostaining were significantly increased in both POK and CLK (10). GFR in the POK was at a level of only 20% of sham-operated rats. The neonatal rat is born with an immature kidney (39), and thus it might have been anticipated that an insult such as obstruction would lead to long-term damage, even if reversed. The data shown here demonstrate that even in an adult kidney, 3 days of UUO are sufficient to set off a chain of events that will lead to irreversible renal damage.

### Table 3. Body weight and urinary NO excretion in group 5 rats

<table>
<thead>
<tr>
<th>Experimental Day</th>
<th>Body Weight, g</th>
<th>Urinary NO, μmol/24 h</th>
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<tbody>
<tr>
<td>Pre-Op</td>
<td>354±19</td>
<td>2.51±0.31</td>
</tr>
<tr>
<td>Post-UUO day 3</td>
<td>331±16</td>
<td>6.87±1.03</td>
</tr>
<tr>
<td>Post-UUO day 7</td>
<td>347±15</td>
<td>8.39±0.71</td>
</tr>
<tr>
<td>Post-UUO day 14</td>
<td>395±12</td>
<td>6.32±0.89</td>
</tr>
<tr>
<td>Post-UUO day 21</td>
<td>425±14</td>
<td>7.07±1.52</td>
</tr>
<tr>
<td>Post-UUO day 27</td>
<td>457±14</td>
<td>7.51±1.95</td>
</tr>
</tbody>
</table>

Values are means ± SE. NO, nitric oxide.

Fig. 6. Inducible nitric oxide synthase (iNOS) immunostaining (A, B, and C). iNOS expression in tubular cells was increased after 3 days of UUO (B) compared with baseline (A). Increased expression of iNOS remained after the relief (C: group 3). D, E, and F: endothelial (e)NOS immunostaining. eNOS expression in tubular cells was increased after 3 days of UUO (E) compared with baseline (D). Increased expression of iNOS remained after the relief (F: group 3) similar to iNOS staining.
over time. Our results are similar to those reported by Morrissey et al. (31) who used a 3-day UUO followed by a 7-day relief model. In that study, interstitial volume and interstitial collagen IV deposition were increased in the relieved POK compared with control. In contrast to both of these studies, Koo et al. (24) reported a modest improvement in the POK at 10 days after relief of a 10-day UUO (0.26 ± 0.04 volume units) compared with an unrelieved 10-day UUO (0.32 ± 0.05). It is possible that with a longer time after relief, there would be a progression of fibrosis.

Interstitial fibrosis is a complex process that involves synthesis and degradation of extracellular matrix proteins, cellular infiltration, epithelial mesenchymal transformation, and tubular apoptosis and atrophy (12, 34, 40). The end result of interstitial fibrosis is decreased GFR, which may result from several factors. Tubular atrophy can result in atubular glomeruli; these glomeruli, which are detached from their corresponding proximal tubules, are unable to filter properly (26). The accumulation of extracellular matrix can isolate tubules from their oxygen supply and may also result in obliteration of peritubular capillaries, which can also result in decreased GFR (3, 4, 38). Given the importance of interstitial fibrosis, it is not unexpected that a large number of studies have examined interventions targeted to numerous cytokines designed to ameliorate interstitial fibrosis (12, 17). However, almost none have examined renal function in those animals. Because the ultimate goal of these interventions is preservation of renal function, it is surprising that there has been so little study of renal function in fibrotic models. In the study by Morrissey et al. (31) cited above, RBF was not significantly different from control after 7-day relief of 3-day UUO, whereas in the present study, we noted recovery by 14 days. GFR was still decreased compared with control. When bone morphogenetic protein-7 (BMP-7) was administered concomitant with relief of UUO, both GFR

Table 4. Quantification of Western blot analysis results in control rats and following relief of UUO

<table>
<thead>
<tr>
<th>Group 1</th>
<th>CK</th>
<th>POK</th>
<th>Group 2</th>
<th>CK</th>
<th>POK</th>
<th>Group 3</th>
<th>CK</th>
<th>POK</th>
<th>Group 4</th>
<th>CK</th>
<th>POK</th>
<th>Group 5</th>
<th>CK</th>
<th>POK</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>4.57±0.95(100%)</td>
<td>4.811±0.82 (105±10%)</td>
<td>4.842±0.233 (106±5%)</td>
<td>6.943±1.017* (182±35%)</td>
<td>6.719±0.652 (227±94%)</td>
<td>5.307±0.709 (155±44%)</td>
<td>5.992±0.962 (160±36%)</td>
<td>7.778±0.922* (206±38%)</td>
<td>8.928±0.832* (254±64%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>4.682±1.187 (100%)</td>
<td>6.515±0.407* (139±9%)</td>
<td>6.212±0.863* (133±18%)</td>
<td>7.587±0.866* (332±169%)</td>
<td>7.951±0.616* (372±213%)</td>
<td>5.951±0.667 (287±169%)</td>
<td>6.202±0.669* (241±106%)</td>
<td>7.275±0.848* (293±135%)</td>
<td>9.296±0.510* (433±238%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. POK, previously obstructed kidney; iNOS and eNOS, inducible and endothelial nitric oxide synthase; CK, contralateral kidney. *P < 0.05 compared with control kidney. †P < 0.05 compared with contralateral side.

Fig. 7. Western blot analysis for iNOS and eNOS in kidney. A: iNOS Western blot demonstrating control and 3-day obstruction, obstructed (obstr), and contralateral (unobstr) kidneys, including iNOS standard. B: representative Western blots for iNOS and eNOS for groups 1, 3, 4, and 5. C: eNOS Western blot demonstrating control and 3-day obstruction.
and interstitial volume changes were improved compared with relief alone.

Thus, in the present study, we examined both renal function and renal histopathology concomitantly in UUO, a model of rapidly progressing fibrosis. We chose to study surgical reversal of obstruction to mimic the clinical situation. The success of surgical reversal is monitored by following renal function. However, our results suggest that although RBF and GFR appear to recover in the short term, that the renal fibrosis set in motion by obstruction actually progresses. This suggests that in the long term, renal function may again decrease as the fibrosis progresses further. This also suggests that concomitant treatment with an antifibrotic agent may be an appropriate treatment when obstruction is reversed, as noted above using BMP-7. Preliminary studies in our laboratory using an antibody to TGF-β showed an amelioration of the fibrotic process in postrelief UUO, validating the effectiveness of this strategy.

GRANTS

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REFERENCES


