Dietary arginine supplementation attenuates renal damage after relief of unilateral ureteral obstruction in rats

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Background. Progression of renal injury after relief of unilateral ureteral obstruction (UUO) has been demonstrated. Nitric oxide (NO) may be an effective intervention due to its vasodilatory, antifibrotic, and anti-apoptotic effects. Herein, we used dietary L-arginine (ARG) supplementation in a UUO relief model.

Methods. This study comprised group 1, control (no treatment). All other rats were subject to 3-day UUO, which was then relieved, and the rats maintained for 7 additional days. Group 2, no additional treatment; group 3, L-ARG; group 4, L-NAME, NO synthase inhibitor; group 5, ARG and L-NAME. Urinary NO$_2$/NO$_3$ was quantified. GFR and ERPF were measured at day 10. Interstitial fibrosis and fibroblast expression, macrophage infiltration, tubular apoptosis, and proliferation, NOS expression, and the levels of tissue TGF-$\beta$ were evaluated.

Results. Urinary NO$_2$/NO$_3$ was significantly increased by ARG treatment and decreased by L-NAME. GFR and ERPF measured 7 days following relief were not significantly different in the previously obstructed kidneys (POK) of groups 2 and 3. L-NAME significantly reduced GFR and ERPF. ARG significantly reduced apoptosis, macrophage infiltration, and fibroblast expression in the POK. L-NAME exacerbated the effects on apoptosis and fibroblasts. Fibrosis was minimal in groups 1 through 3, but was significantly increased by L-NAME. ARG did not affect renal NOS expression and tissue TGF-$\beta$1 levels.

Conclusion. Dietary ARG supplementation during UUO relief did not improve ERPF or GFR. However, renal damage, including fibrosis, apoptosis, and macrophage infiltration was significantly improved by ARG treatment. This suggests that increasing NO availability could be beneficial in the setting of UUO relief.

METHODS

Experimental protocol

Five groups were studied (Table 1). Renal function of rats (male Sprague-Dawley; 300 to 400 g) without UUO
was measured as control (group 1, \(N = 8\)). Animals in groups 2 through 5 were subject to left UO, and obstruction was relieved after 3 days of UUO, as described below. In all relieved groups, GFR and ERPF in both kidneys were measured 7 days after relief. Group 2 (\(N = 8\)) rats had no further treatment. Rats in group 3 (\(N = 7\)) were treated with 1\% L-arginine in their drinking water for 25 days (starting 15 days prior to UUO and continuing through 3 days of UUO and 7 days of relief), at which time renal function was measured. Rats in group 4 (\(N = 6\)) were treated with NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME) for 12 days (daily intraperitoneally injection, starting 2 days prior to UUO and continuing through 3 days of UUO and 7 days of relief), at which time renal function was measured. Group 5 (\(N = 6\)) was treated with L-arginine and L-NAME (as above), and then renal function was measured. All protocols were approved by the institutional animal care committee.

### Renal function measurement

Renal function was measured as previously described [16]. A 24 g × 19 mm angiocatheter was secured in the right jugular vein. A bolus of lactated Ringer’s (LR) solution was slowly injected intravenously in the amount of 1\% body weight (BW), and bolus of 1\% parahippurate (PAH) and 1.5\% inulin in LR was injected in the amount of 0.2\% BW. A solution of 1\% PAH and 1.5\% inulin in LR was infused at 10 L/min/100g BW, and was maintained for at least 60 minutes. A midline abdominal incision was made, followed by a small transverse incision in the ureter. PE10 tubing was inserted into the right and left ureters of control rats, whereas PE50 tubing was inserted into the left ureter of the obstructed kidney due to its dilatation. Ten minutes after catheter insertion, urine collections were started. Twenty-minute urine collections were made from each kidney 3 times, and 450 \(\ell\) of arterial blood was collected at the midpoint of each collection. This volume was replaced by the infusions so the rat was not volume depleted. After measurement of renal function, both kidneys were harvested for analysis. Following renal function measurements, a tube was inserted into the middle ureter, and was ligated with 3–0 silk. Saline solution (0.9\%) was introduced into the ureter from a level of approximately 18 inches using gravity, and the patency of the anastomosis was confirmed. Then, both kidneys were harvested for histologic and protein analyses.

### Kidney weight

At harvest, each kidney was decapsulated, washed with saline, bisected coronally, blotted dry on gauze, and weighed. The whole kidney weight was expressed as a percentage of BW determined at the time rats were euthanized.

### Determination of ERPF (using para-hippurate) and GFR (using inulin)

Urine and plasma concentrations of parahippurate and inulin were detected using standard chemical methods as previously described [16, 19].

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**Table 1. Percent kidney weight 7 days following relief of UUO**

<table>
<thead>
<tr>
<th>Group # (N)</th>
<th>Treatment</th>
<th>Right kidney (contralateral) (% BW)</th>
<th>Left kidney (previously obstructed) (% BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (8)</td>
<td>Untreated control</td>
<td>0.36 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.52 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 (8)</td>
<td>3-day UUO + 7-day relief</td>
<td>0.50 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.45 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3 (7)</td>
<td>3-day UUO + 7-day relief + arginine</td>
<td>0.46 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.47 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 (6)</td>
<td>3-day UUO + 7-day relief + L-NAME</td>
<td>0.48 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.47 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5 (6)</td>
<td>3-day UUO + 7-day relief + arginine + L-NAME</td>
<td>0.49 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.46 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Kidney weight, control right kidney in group 1.

<sup>b</sup>\(P < 0.05\), compared to control kidney.
Para-aminohippurate was detected colorimetrically at 550 nm and inulin at 595 nm. Clearances were calculated using standard formulas. For each rat, the values obtained for 3 clearance periods were averaged. Values were normalized to BW.

**Urinary NO$_{2}$/NO$_{3}$ and cyclic GMP**

To assess the effects of arginine and L-NAME, a 24-hour urine sample was collected in metabolic cages 1 day prior to UUO in groups 2 through 5. Twenty-four-hour urine was collected in a reservoir containing 2-propanol and samples were frozen at $-80^\circ$C until assay. Urinary NO$_{2}$/NO$_{3}$ levels were measured by the Griess reaction after copperized cadmium treatment, as previously described [16]. Urinary cyclic GMP was measured by enzyme-linked immunosorbent assay (ELISA) (Assay Designs, Inc., Ann Arbor, MI, USA). The results were normalized to BW.

**Determination of tissue TGF-β1 level**

Samples of frozen tissue were assayed for TGF-β1 using ELISA as previously described (20; Quantikine; R&D Systems, Minneapolis, MN, USA). Analysis was completed using dual wavelength readings. Standard curve and all samples were run in duplicate.

**Assessment of renal morphology and interstitial fibrosis**

After renal function measurement, kidney sections were fixed in 10% neutral buffered formalin and embedded in paraffin. Five-micrometer sections were stained with Masson’s trichrome, and were examined by a board-certified pathologist (S.V.S.) in a blinded fashion. Proximal tubules were identified by their plump lining cells containing eosinophilic granular cytoplasm and prominent luminal brush borders. Distal tubules are lined with low cuboidal cells containing clear to amphophilic cytoplasm and central nuclei with absence of brush borders. The distinction between distal tubules and collecting ducts was made on the basis of location in the renal parenchyma, and the size and frequency of occurrence in that location. In the outer and inner medulla, which are depicted in most of the figures, segments of the distal tubules are much narrower than collecting ducts. The presence of interstitial fibrosis was assessed in slides stained with Masson’s trichrome and validated by measurements of interstitial volume, using a point-counting method as previously described [21].

**Immunohistochemical analysis**

Immunohistochemical analyses for iNOS, eNOS, macrophage, and PCNA were performed as previously described [15, 16]. Macrophages and renal tubules (for PCNA) were counted in 10 high-power fields ($\times$400) by 2 different independent investigators in a blinded fashion. To quantitate apoptotic cells, the TUNEL assay was performed as previously described [15, 20]. TUNEL-positive renal tubules were quantitated as above. We also analyzed tissue for the presence of fibroblasts using immunohistochemistry. The antibody utilized was Dako # S100-A4 (Dako Corp., Carpinteria, CA, USA). The S100-A4 antigen is also known as FSP-1, and has been found to be expressed by fibroblasts, but not epithelial cells [22, 23]. Antigen was retrieved by incubating cells with pronase K for 20 minutes 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.

**Western blotting analysis**

Western blot analyses for iNOS and eNOS were performed using standard techniques as previously described [15, 16]. iNOS and eNOS were detected at 130 kb and 133 kb, respectively. Relative intensity was expressed as percent control intensity. Five samples were analyzed per group.

**Statistical analysis**

All results are expressed as the mean ± SE, and were analyzed for significance by the one-way analysis of variance (ANOVA) and multiple comparison tests.

**RESULTS**

**Kidney weight**

Kidney weights were measured at the time of euthanasia (Table 1). The relative kidney weight in normal kidney was 0.36 ± 0.01% BW. In all other groups, both kidney weights were significantly higher than normal kidney ($P < 0.05$). Kidney weights were not significantly different between POK and contralateral kidney (CK) in any groups.

**The effect of L-arginine and L-NAME on urinary NO$_{2}$/NO$_{3}$ and cyclic GMP**

To verify the effect of L-arginine and L-NAME treatment on NO production, urinary NO$_{2}$/NO$_{3}$, and cyclic GMP were measured 1 day prior to UUO. Urinary NO$_{2}$/NO$_{3}$ was significantly higher in arginine-treated rats (group 3, 2053 ± 172 nmol/24hr/100g) than group 2 (1111 ± 107 nmol/24hr/100g; Fig. 1A), confirming increased NO production. L-NAME treatment significantly blunted the
Fig. 1. The effect of L-arginine and L-NAME treatment on urinary NO$_2$/NO$_3$ and cyclic GMP. A 24-hour urine specimen was collected one day prior to UUO, and NO$_2$/NO$_3$ and cGMP measured as in Methods. (A) Urinary NO$_2$/NO$_3$ was significantly higher in the arginine-treated group 3 as compared to control group 2, and this was blunted by L-NAME (group 4). (B) Urinary cGMP was significantly higher in the arginine-treated group 3 as compared to control group 2, and this was blunted by L-NAME (group 4). *P < 0.05, compared to group 2. ∞ P < 0.05 compared to indicated group.

Table 2. GFR in normal rats and rats which underwent relief of UUO

<table>
<thead>
<tr>
<th>Group</th>
<th>GFR (CK) mL/min/100g</th>
<th>GFR (POK) a mL/min/100g</th>
<th>Total GFR mL/min/100g</th>
<th>%GFR (POK) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>0.30 ± 0.02</td>
<td>0.31 ± 0.02</td>
<td>0.61 ± 0.04</td>
<td>50.3 ± 1.50</td>
</tr>
<tr>
<td>Group 2</td>
<td>0.43 ± 0.04b</td>
<td>0.24 ± 0.03</td>
<td>0.67 ± 0.06</td>
<td>36.1 ± 3.0b</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.39 ± 0.05</td>
<td>0.26 ± 0.03</td>
<td>0.64 ± 0.07</td>
<td>40.2 ± 1.9b</td>
</tr>
<tr>
<td>Group 4</td>
<td>0.33 ± 0.11</td>
<td>0.11 ± 0.01b,c</td>
<td>0.44 ± 0.03b,c</td>
<td>25.5 ± 3.1b,c</td>
</tr>
<tr>
<td>Group 5</td>
<td>0.29 ± 0.03c</td>
<td>0.11 ± 0.03b,c</td>
<td>0.40 ± 0.06b,c</td>
<td>25.5 ± 4.0b</td>
</tr>
</tbody>
</table>

aUO and relief were performed in the left kidney in groups 2 through 5.
bP < 0.05, compared to group 1.
cP < 0.05, compared to groups 2 and 3.

Renal function after relief of UUO

In group 1, GFR was 0.30 ± 0.02 mL/min/100g in the right kidney (Table 2), with no significant differences between left and right kidneys. In groups 2 through 5, GFRs were significantly different between POK and CK (P < 0.05). In the POK, GFRs in groups 2 (0.24 ± 0.03 mL/min/100g) and 3 (0.26 ± 0.03 mL/min/100g) were not significantly different from group 1. In contrast, GFRs in the POK of groups 4 and 5 were significantly lower than group 1. L-NAME treatment significantly decreased GFRs in groups 4 and 5 compared to groups 2 and 3. In CK, GFR in group 2 was significantly higher than group 1. GFR of CK in groups 3 through 5 was not significantly different from group 1.

We also examined total GFR as the sum of the GFRs in both left and right kidneys (Table 2). Total GFR in groups 2 and 3 was not significantly different from that in group 1. L-NAME treatment significantly decreased total GFR in groups 4 and 5. We calculated the percent GFR in the POK. In groups 2 through 5, the percent GFR in POK was significantly lower than group 1 (50.33 ± 1.51% of total). Percent GFR in the arginine-treated group was not significantly different from group 2. L-NAME treatment, with or without concomitant arginine treatment, significantly decreased total GFR compared to groups 2 and 3 (P < 0.05).

In group 1, ERPF was 1.02 ± 0.11 mL/min/100g in the right kidney and 1.01 ± 0.14 mL/min/100g in the left kidney (Fig. 2). In groups 2 and 3, ERPF in POK was not significantly different from group 1. In contrast, L-NAME
treatment decreased ERPF in both kidneys in groups 4 and 5 compared to group 1.

Renal morphology and interstitial fibrosis

Interstitial fibrosis and renal morphology were assessed by examination of Masson’s trichrome slides. Mild dilatation of distal and collecting tubules was seen in all POK. Inflammation (leukocytes infiltration), mainly in peripelvic area, was also noted. In L-NAME–treated rats, damaged tubules surrounded by interstitial fibrosis were seen in some cortical area. CKs in all groups had no remarkable change in their architectures or evidence of inflammation. In CK, there was no significant fibrosis in any group. In POK, mild blue staining was seen in some animals in group 2 (Fig. 3A), and less blue staining was seen in group 3 (Fig. 3B). Significantly increased blue staining was seen in group 4 compared to group 2 (Fig. 3C) and group 5 (not shown). We evaluated blue-stained collagen deposition. In the POK, mild blue staining was seen in some animals in group 2 (4.80 ± 1.21%, Fig. 4A), and less blue staining was seen in group 3 (2.42 ± 1.21%, Fig. 4B). Increased blue staining was seen in group 4 (12.88 ± 2.93%, \( P < 0.05 \), compared to group 2, Fig. 5C) and group 5 (6.45 ± 1.68%).

In situ TUNEL

POK in groups 2 through 5 showed significantly higher tubular apoptosis than control normal kidneys (0.93 ± 0.16 nuclei per high-power field; nuclei/HPF; Fig. 4A). TUNEL\(^+\) cells were localized to dilated distal and collecting tubules. Arginine treatment significantly lowered apoptosis in the POK in group 3 (Fig. 4C).

Fig. 2. The effect of L-arginine on the recoverability of ERPF following the relief of 3-day UUO. ERPF was measured in both POK and CK in all groups by PAH clearance as in Methods. ERPF was not significantly different in groups 2 and 3, but was significantly decreased by L-NAME treatment (groups 4 and 5). ∗ \( P < 0.05 \), compared to group 1. †† \( P < 0.05 \), compared to group 2, † \( P < 0.05 \), compared to group 3.

Fig. 3. Trichrome staining of POK. Interstitial fibrosis was visualized by Masson’s trichrome staining, and interstitial volume determined by a point counting method (see text for numbers). Mild dilatation of collecting ducts and dilated tubules was seen in all released groups. Renal medulla is shown for all groups. (A) In POK, mild blue staining was seen in some animals in group 2. (B) Less blue staining was seen in group 3 (arginine treatment). (C) Increased blue staining was seen in group 4 (L-NAME treatment). Abbreviations (this and all following figures): DT, distal tubule; CD, collecting duct.
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Fig. 4. Apoptosis in kidney following relief of UUO. Apoptosis was measured by the TUNEL assay as in Methods. (A) Graphic representation of apoptosis in all groups demonstrating decreases in apoptosis following arginine treatment (group 3) and increases following L-NAME treatment (group 4). Renal medulla is shown for all groups. (B–D) In situ TUNEL. (B) POK, group 2; (C) POK, group 3; (D) POK, group 4. *P < 0.05 compared to group 1; ∞ P < 0.05 compared to corresponding CLK. # P < 0.05 compared to POK of group 2.

compared to group 2 (Fig. 4B). L-NAME treatment alone (Fig. 4D) resulted in significantly more apoptosis (17.8 ± 3.7 cells/HPF) than all other groups, and blunted the effect of L-arginine when both were administered together. Apoptosis in POKs in groups 2 through 5 was significantly higher than in their CKs (P < 0.05), and there was no significant difference among CKs, which were not different from control.

Tissue TGF-β1 level

Tissue TGF-β1 levels in POK of groups 2 through 5 were all significantly higher than group 1 (Fig. 5). Tissue TGF-β1 levels in CK of groups 2 through 4 were significantly higher than control kidney. Although TGF-β1 levels tended to be higher in POK, there was no significant difference between POK and CK in all groups. Neither L-arginine nor L-NAME affected TGF-β1 in the POK or CLK.

Macrophage infiltration in the kidney

In groups 2 through 5, the numbers of macrophages in the POK were significantly higher than in control kidney (Fig. 6A, P < 0.05). In the POK, arginine treatment (9.22 ± 0.56 macrophages/HPF, Fig. 6C) significantly reduced the number of macrophages compared to group 2 (27.43 ± 4.01 macrophages/HPF, Fig. 6B; P < 0.05). L-NAME treatment had no significant effect on macrophage infiltration compared to group 2 (32.75 ± 2.42 macrophages/HPF, Fig. 6D), and did not blunt the effect of L-arginine alone.
Fibroblast expression in the kidney

There was a small number of interstitial fibroblasts present in control kidneys (5.4 ± 0.2/HPF; Fig. 7A and B). All POK in groups 2 and 5 had significantly higher fibroblast expression compared to group 1. L-arginine caused a small but significant decrease in fibroblast expression in group 3 (Fig. 7D; 13.6 ± 0.9 FSP+ cells/HPF vs. 17.6 ± 0.8 FSP+ cells/HPF) compared to group 2 (Fig. 7C). In contrast, L-NAME treatment alone significantly increased fibroblast expression (Fig. 7A and E), and was able to reverse the effect of L-arginine. CLK fibroblast expression was significantly lower than POK expression in all groups, and was not significantly different from control.

Detection of PCNA expression

To determine the effect of L-arginine on renal tubular proliferation after relief of UUO, immunostaining of PCNA was performed (Fig. 8A). Both POK and CK in groups 2 and 4, and POK in group 5, showed significantly higher tubular proliferation than group 1 (P < 0.05; Fig. 8B). L-arginine treatment significantly reduced the numbers of PCNA-positive cells in both kidneys (4.70 ± 0.36 cells/HPF in CK and 6.25 ± 1.07 cells/HPF in POK; Fig. 8C) when compared to group 2 (10.52 ± 0.83 positive cells/HPF in CK and 15.53 ± 1.98 cells/HPF in POK; P < 0.05). L-NAME alone did not affect the number of PCNA-positive cells when compared to group 2.

Immunostaining of iNOS and eNOS

To evaluate the effect of L-arginine on the expression of iNOS and eNOS, and to determine their localization, immunostaining for iNOS and eNOS was performed. iNOS was weakly expressed in renal tubular cells in control normal kidney. Relatively higher staining was seen in cortex (not shown). In POK, clear staining was seen in proximal tubules and distal tubules (Fig. 9A). Glomeruli had weak staining. Tubular cells in medulla were also stained. In CK, staining similar to that seen in the POK was noted (not shown). In the arginine-treated group (Fig. 9B), no significant difference in localization or expression was seen compared to group 2. eNOS staining was also performed. In control kidney, eNOS was weakly expressed in tubular cells in both cortex and medulla (not shown). In POK, clear staining was seen in proximal tubules and distal tubules (Fig. 9C). Slightly less staining was seen in collecting duct. Glomeruli had some staining. In CK, staining was also seen in the same location. As with iNOS, no significant difference was found between groups 2 and 3 (Fig. 9D).

Western analysis for iNOS and eNOS

To confirm the immunohistochemical results, Western blot analyses were performed in groups 2 and 3. Intensity was expressed as an arbitrary unit, with CK of group 2 set arbitrarily at 100%; comparisons are shown in Table 3. Representative Western blots are shown in Figure 10. iNOS expression (Fig. 10A) was not different between CK and POK in groups 2 and 3. There was no significant difference in iNOS expression between groups 2 and 3 in both CK and POK. Similarly, eNOS expression (Fig. 10B) was not different between CK and POK in both groups. There was no significant difference in eNOS expression between groups 2 and 3 in both kidneys. These results suggested that L-arginine treatment did not affect the levels of NOS expression.

DISCUSSION

The obstructed kidney is characterized by progressive renal fibrosis and damage, and a decline in both renal blood flow (RBF) and GFR. Relief of obstruction is characterized by a variable recovery in renal function, with less known about changes in renal damage. In the present experiments, we relieved a 3-day UUO and examined rats at 7 days. When L-arginine was present during the recovery phase, there was a diminution in the many of the markers of renal damage, without effects on renal function. In contrast, in the presence of the NOS inhibitor, L-NAME, there were severe decreases in renal function, as well as increases in renal damage. These results suggest that NO is important in maintaining renal function following relief of UUO, and that providing additional NOS substrate may be beneficial for improving renal damage in the post-relief period.

The recovery of the kidney after relief of UUO has been studied by several investigators [3–6]. In most cases, the studies have involved an examination of either renal function or renal damage, but not both. However, since...
several studies have demonstrated that GFR can be correlated with interstitial fibrosis [24, 25], it is important to examine not only how relief of UUO affects GFR and RBF, but also if interstitial fibrosis and other markers of renal damage progress. Previous studies from our laboratory [19] demonstrated that, after relief of 3-day UUO, recovery of renal function is complete by 14 days after relief, whereas renal damage progressed through 28 days of recovery. These results are similar to those of Chevalier et al in a neonatal rat model. At 28 days after relief of UUO, interstitial collagen, α-smooth muscle actin, and TGF-β1 were dramatically increased in the POK compared to sham-operated rats [6]. After 28 days of relief, GFR in the POK was not significantly different from baseline GFR. At 1 year, however, following relief of UUO, interstitial collagen, macrophage infiltration, and α-smooth muscle actin immunostaining were significantly increased in both POK and CLK, and GFR in the POK was at a level of only 20% of sham-operated rats [7]. Thus, relief of UUO may provide protection of renal function in the short term, but the fibrotic mechanisms set in motion by obstruction appear to lead to eventual long-term renal dysfunction.

NO has been shown to be protective in both acute and chronic UUO models, with effects on both renal function and renal damage. Although L-arginine has not previously been used during the relief of UUO, its restorative effect on renal function have been reported in acute UUO [10, 11]. In addition, the NO donor, SNAP, also attenuated renal blood flow in a 24-hour UUO model [9]. Gene therapy with iNOS has also been shown to be
Fig. 7. Fibroblast expression in kidney following relief of UUO. Fibroblasts were localized immunohistochemically using the S100A4 antibody as described in Methods. (A) Graphic representation of fibroblast expression in all groups demonstrating decreases in fibroblasts following arginine treatment (group 3) and increases following L-NAME treatment (group 4). (B–E) Immunohistochemistry. Renal medulla is shown for all groups. (B) Control kidney, group 1; (C) POK, group 2; (D) POK, group 3; (E) POK, group 4. *P < 0.05 compared to group 1 and to corresponding CLK. #P < 0.05 compared to POK of groups 2, 4, and 5.
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Fig. 8. PCNA expression in kidney following relief of UUO. PCNA was localized immunohistochemically as described in Methods. (A) Graphic representation of PCNA expression in all groups demonstrating decreases in PCNA expression following arginine treatment (group 3), and which was blunted by L-NAME treatment (group 5). (B and C) Immunohistochemistry. Renal medulla is shown. (B) POK, group 2; (C) POK, group 3. NS, not statistically significant. *P < 0.05, compared to group 1; **P < 0.05, compared to group 2.

beneficial in acute UUO [12]. In experiments examining relief of 1-, 2-, and 3-week UUO in rats, an angiotensin-converting enzyme (ACE) inhibitor showed a restorative effect on renal function [4]. Although not measured (or even known about) in that study, NO production has been shown to be augmented by use of an ACE inhibitor in a 5-day UUO model [13]. Thus, the restorative effect of the ACE inhibitor in the relief experiments cited above may be in part due to NO production. NO has also been shown to be protective against renal damage in UUO, with both antifibrotic and anti-apoptotic effects. Morrissey et al [13] demonstrated that L-arginine treatment significantly decreased interstitial fibrosis. In our previous studies using iNOS knockout mice, we found both interstitial fibrosis and apoptosis were increased. In vitro studies examining stretch-induced apoptosis in renal epithelial cells demonstrated a protective effect of L-arginine or SNAP, and enhanced apoptosis with NOS inhibitors [15, 26].

In the present experiments, we used L-ARG, L-NAME, or L-ARG + L-NAME in a model of relief of 3-day UUO. Measurement of urinary NO metabolites demonstrated that ARG alone increased urinary NO. When L-NAME was given, there was a significant decrease in NO that was not completely reversed by concomitant administration of L-ARG. Although ERPF and GFR were lower in group 2 than group 1, they were not statistically significantly different; therefore, it was difficult to discern an effect of L-ARG alone. Nevertheless, there was a striking effect of L-NAME, which severely decreased both ERPF and GFR. These results suggest that NO is involved in maintaining ERPF and GFR during the 7-day recovery period. Providing substrate, along with L-NAME, was not able to reverse the effects of the NOS inhibitor. It is possible that there was insufficient L-ARG provided to compete with L-NAME, or that the L-ARG was not accessible to sites at which it could be metabolized to NO, and additionally, to improve renal function.

Renal damage in UUO is characterized by interstitial fibrotic changes, tubular apoptosis, and cellular changes, including macrophage infiltration and the presence of interstitial fibroblasts. We examined the effect of arginine and L-NAME treatment on each of these parameters compared to relief of UUO with no other treatment. In the present study, minimal fibrosis was seen in rats in which a 3-day UUO was relieved, and the rats followed for an additional 7 days. Therefore, it would be difficult to note an improvement with L-arginine treatment. However, the blocking of NO production by L-NAME significantly increased interstitial fibrosis in this model. These results are in agreement with Morrissey et al [13], who demonstrated that L-arginine treatment significantly decreased interstitial fibrosis in UUO, and who showed that L-NAME blocked the beneficial effects of an ACE inhibitor by decreasing NO synthesis.
In the control 7-day relieved POK, there was a significant increase in apoptotic tubular cells. This increase was attenuated with arginine treatment, and exacerbated with L-NAME treatment, which also blunted the effect of L-arginine. Although both proapoptotic and anti-apoptotic effects of NO have been demonstrated [27], these results demonstrate that NO is anti-apoptotic, not only during obstruction, but during relief, as well. The specific target of NO in this model has not been determined, but some involvement of caspase-3 in epithelial tubular cell apoptosis has been demonstrated [28].

In addition, macrophage infiltration, one of the markers of interstitial damage, was significantly decreased by L-arginine in this study. A similar result was previously seen when L-arginine was used in UUO [13] and a bilateral UO model [27]. NO may decrease the number of macrophages in the kidney through direct effects on apoptosis, or through effects on macrophage recruitment. Thus, NO has been shown to cause apoptosis in macrophages [30]. Furthermore, NO has also been shown to regulate RANTES, a protein involved in the recruitment of macrophages. In wound healing, it has been shown that NO down-regulates expression of RANTES in keratinocytes, leading to decreased macrophage infiltration [32]. In an animal model of endotoxemia, arginine administration decreased glomerular RANTES and, conversely, L-NAME increased both RANTES and glomerular macrophage infiltration [32]. Surprisingly, L-NAME treatment alone did not increase the number of macrophages in the kidney, perhaps suggesting differences in localization of arginine and L-NAME, which could influence their effect on macrophage infiltration.

The number of spindle-shaped cells expressing S100A4 increased in the relieved POK. It is known that FSP-1 is
Table 3. Western blot for iNOS and eNOS in control group and arginine-treated group

<table>
<thead>
<tr>
<th>Group 2–CK</th>
<th>Group 2–POK</th>
<th>Group 3–CK</th>
<th>Group 3–POK</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS expression</td>
<td>8795 ± 1347</td>
<td>8995 ± 1286</td>
<td>8357 ± 1020</td>
</tr>
<tr>
<td>(100%)</td>
<td>(102.3 ± 14.6%)</td>
<td>(95.0 ± 11.6%)</td>
<td>(99.8 ± 14.6%)</td>
</tr>
<tr>
<td>eNOS expression</td>
<td>10,093 ± 1155</td>
<td>9862 ± 1572</td>
<td>9625 ± 872</td>
</tr>
<tr>
<td>(100%)</td>
<td>(97.7 ± 15.6%)</td>
<td>(95.4 ± 8.6%)</td>
<td>(96.6 ± 8.6%)</td>
</tr>
</tbody>
</table>

All percentages were expressed as % of control.

expressed in interstitial fibroblasts, and that FSP is identical to S100-A4 [22]. Thus, we have identified the spindle-shaped interstitial cells immunolocalized by S100-A4 as fibroblasts. These fibroblasts may arise by the process of epithelial mesenchymal transition [33, 34]. It has been suggested that tubular cells may undergo EMT in an attempt to escape apoptosis, induced possibly by TGF-β, which is increased in UUO. EMT is accompanied by a breakdown of the basement membrane, allowing “escape” of the now spindle-shaped epithelial cells into the interstitium. In the present study, the number of fibroblasts significantly increased in the POK of the 7-day relief group compared to control. L-arginine treatment decreased and L-NAME treatment increased the number of fibroblasts in the POK. NO has been shown to cause apoptosis in several fibroblastic cell lines, thus increasing NO may account for the decreased number of fibroblasts. Alternatively, NO could affect EMT directly. However, there is a virtual absence of literature on the involvement of NO in EMT. In an animal model of Peyronie’s disease, a penile fibrotic disease in which TGF-β is injected into the tunica albuginea of the penis, there is an activation of fibroblast to myofibroblasts. Although fibroblast activation is not the same as EMT, activated fibroblasts have also been implicated in UUO and fibrosis. In the Peyronie’s model, inhibition of NOS increased myofibroblast abundance and collagen synthesis [35]. Thus, the absence of NO exacerbated fibroblast activation, similar to results found here using L-NAME.

TGF-β1 has been reported to play an important role in the cellular and molecular changes characteristic of UUO. We, and others, have shown increases on TGF-β in UUO [20, 36]. Although TGF-β1 is increased in the relief model of UUO and presumably involved in tubular apoptosis, fibroblast expression, and fibrosis, L-arginine treatment did not alter tissue TGF-β1 levels. This result was unexpected. In iNOS knockout mice, we have previously demonstrated an increase in renal TGF-β1 levels. Furthermore, several models of fibrosis induced by NOS inhibition are characterized by increases in renal TGF-β1, e.g., in 5-day UUO model of rat, L-arginine treatment did not affect mRNA expression of TGF-β1 in obstructed kidney [13]. Our in vitro data support this finding. Under stretched condition, NRK-52E, rat kidney epithelial cells, up-regulated TGF-β production. However, L-arginine and L-NAME treatment did not affect TGF-β1 expression [39]. That NO attenuated renal damage, and L-NAME exacerbated it, without effects on TGF-β suggests several possibilities. One is that other cytokines, besides TGF-β, are involved. Possible candidates include angiotensin II or endothelin. A second possibility is that there were localized effects on TGF-β that were not noted when we assayed for TGF-β in the whole kidney.
The obstructed kidney is characterized by both tubular atrophy and tubular (and interstitial) proliferation. In the present experiments, the number of PCNA-positive cells was increased in the POK after relief of UUO. Arginine treatment decreased tubular proliferation, and L-NAME blunted its effects. These results are similar to that seen in iNOS knockout mice, which demonstrated an antiproliferative effect of NO [15]. However, other studies with renoprotective agents, including TGF-β antibody [20], anti-inflammatory agent [40], and COX-2 inhibitor [41] have noted increased tubular proliferation when other parameters of renal damage are attenuated. There may be multiple factors involved in both tubular proliferation and apoptosis whose balance is affected by various treatments, and therefore, divergent effects may be seen.

We also examined the changes in the CK in this model. For the most part, the CK shows much less damage than the POK, and was little affected by either L-arginine or L-NAME. However, TGF-β was increased in the CK in all groups compared to control kidney. Chevalier et al also demonstrated prominent renal damage in CK 1 year following relief of UUO and, thus, increased levels of TGF-β1 in CK might be involved in this process [7]. GFR was significantly higher in the CK of arginine-treated rats compared to control rats. This demonstrates the compensatory change in the CK when the other kidney is obstructed. In contrast, GFR in CK of arginine-treated rats was not significantly different from control. The lesser change in the CK of arginine-treated rats might reflect less damage in the POK following arginine treatment. Similarly, the CK shows increased proliferation, which may reflect a compensatory change in the CK. As in the POK, L-NAME treatment significantly decreased ERPF and GFR in CK compared to rats without L-NAME treatment, suggesting that NO might be needed for this compensatory change.

CONCLUSION

The recovery of renal function after relief of obstruction is an important clinical problem. Surgical relief of obstruction is carried out, and recovery of renal function is followed as the marker of recovery. However, pharmacologic agents that affect interstitial fibrosis may be beneficial adjuncts to this process. There have been very few studies examining this. When bone morphogenetic protein-7 (BMP-7) was administered concomitant with relief of UUO, both GFR and interstitial volume changes were improved compared to relief alone [42]. In the present study, we determined that L-arginine administration decreased tubular apoptosis, macrophage infiltration, interstitial fibrosis, and fibroblast expression in the previously obstructed kidney. In this study, arginine was administered prior to UUO. In the clinical setting, any intervention would be done following the diagnosis or discovery of obstruction. Thus, studies will need to be carried out in which arginine is administered at the time of reversal of UUO. Furthermore, long-term follow-up of reversal needs to be carried out to determine the long-term effects of reversal on renal function and damage, as well as to assess the effects of L-arginine on this process.

ACKNOWLEDGMENTS

This study was supported in part by grant of the National Institutes of Health (R01-DK53555).

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REFERENCES


