Nitric Oxide Modulates Vascular Disease in the Remnant Kidney Model

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A loss of the microvascular endothelium occurs in the remnant kidney model of renal disease and may play an important role in progression (Kang et al, J Am Soc Nephrol, 12:1434, 2001). Given that nitric oxide (NO) is a potent endothelial cell survival factor, we hypothesized that stimulating (with L-arginine) or blocking (with nitro-L-arginine methyl ester, (L-NAME)) NO synthesis could modulate the integrity of the microvasculature and hence affect progression of renal disease. Rats underwent 5/6 nephrectomy (RK) and then were randomized at 4 weeks to receive vehicle, L-NAME, or L-arginine for 4 weeks. Systolic blood pressure and renal function was measured, and tissues were collected at 8 weeks for histological and molecular analyses. The effect of modulation of NO on vascular endothelial growth factor (VEGF) expression in rat aortic vascular smooth muscle cells (SMC) and mouse medullary thick ascending limb tubular epithelial cells (mTAL) was also studied. Inhibition of NO with L-NAME was associated with more rapid progression compared to RK alone, with worse blood pressure, proteinuria, renal function, glomerulosclerosis, and tubulointerstitial fibrosis. The injury was also associated with more glomerular and peritubular capillary endothelial cell loss in association with an impaired endothelial proliferative response. Interestingly, the preglomerular endothelium remained intact or was occasionally hyperplastic, and this was associated with a pronounced proliferation of the vascular SMCs with *de novo* expression of VEGF. Cell culture studies confirmed a divergent effect of NO inhibition on VEGF expression, with inhibition of VEGF synthesis in mTAL cells and stimulation of VEGF in vascular SMC. In contrast to the effects of NO inhibition, stimulation of NO with L-arginine had minimal effects in this rat model of progressive renal disease. These studies confirm that blockade of NO synthesis accelerates progression of renal disease in the remnant kidney model, and support the hypothesis that one of the pathogenic mechanisms may involve accelerated

capillary loss and impaired angiogenesis of the renal microvasculature. Interestingly, inhibition of NO synthesis did not lead to a loss of the preglomerular endothelium, which may relate to the effect of NO blockade to stimulate VEGF synthesis in the adjacent vascular smooth muscle cell. (Am J Pathol 2002, 161:239–248)

Recent studies suggest a key role for the microvasculature in progressive renal disease. In the classic remnant kidney model, a progressive loss of glomerular and peritubular (PTC) capillaries has been documented.^{1–4} The capillary loss may lead to chronic ischemia of the tissues that stimulate scarring.⁵ Indeed, the glomerular and peritubular capillary loss correlates directly with the severity of glomerulosclerosis and interstitial fibrosis.³ Furthermore, stimulation of endothelial cell proliferation with vascular endothelial growth factor (VEGF) reduces the capillary loss and slows progression.⁴

Recently an important role for nitric oxide (NO) has been shown in progressive renal disease.^{6–9} Specifically, Fujihara et al⁶ reported that inhibition of nitric oxide (NO) accelerates renal progression in the remnant kidney model. The mechanism responsible for the worsening of renal disease by NO inhibition is not known, but may relate to the increased systemic and glomerular blood pressure in these rats.⁶ The increase in blood pressure observed with NO inhibition results from both loss of NO and increased angiotensin II and endothelin-1,^{10,11} and is likely to have as a primary target the vascular endothelium. In addition to pressure-related vascular injury, NO is a major trophic and survival factor for endothelium, and a decrease in local NO could also potentiate endothelial cell loss by both increasing endothelial cell apoptosis and by decreasing endothelial cell proliferation and repair.^{12–14} Indeed, we have recently documented a critical role for NO in mediating endothelial cell integrity in a rat model of thrombotic microangiopathy.¹⁵

Given the importance of the endothelium in progressive renal disease and the critical role of NO in maintaining its integrity, we hypothesized that blockade of NO

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synthesis in the remnant kidney model would be associated with more severe endothelial cell loss and that this would correlate with both the deterioration in renal function and with the severity of the renal injury. We now report that inhibition of NO synthesis markedly accelerates progression in association with impairment of the angiogenic response and loss of the capillary endothelium which is greater than expected for the increase in systolic blood pressure, suggesting the important role of NO in maintaining renal microvasculature. We also found a marked difference in the response of the preglomerular arterial (macrovascular) endothelium when compared to the microvascular endothelium. In contrast to the loss of the capillary endothelium that occurs with blockade of NO synthesis in the remnant kidney model, the preglomerular arterial endothelium remained preserved, possibly due to a pronounced proliferation of adjacent vascular smooth muscle cells with local expression of VEGF.

Materials and Methods

Experimental Protocol

All animal procedures were approved by the Animal Care Committee of the University of Washington. Male Sprague-Dawley rats (weight 200–240 g) underwent baseline measurement of blood pressure and renal function, and underwent a remnant kidney (RK) operation. The RK operation was performed by a right subcapsular nephrectomy and surgical resection of the upper and lower thirds of the left kidney. Four weeks after the RK operation, blood pressure and renal function were measured, and the animals were divided into three groups. Group 1 ($n = 6$) received no specific therapy; group 2 ($n = 6$) received L-arginine (1 g/dl in the drinking water, Sigma Chemical, St Louis, MO); and group 3 ($n = 7$) received L-NAME (5 mg/dl in the drinking water, Sigma Chemical), all for 4 weeks. These three groups were also compared with a historic group of control RK rats ($n = 6$) that had systolic blood pressure equivalent to those present in the L-NAME-treated RK rats.^{3,4}

Animals were fed a standard laboratory diet and water *ad libitum*, and the amount of daily water and diet intake was measured. Mean daily drug intake in RK+L-NAME and RK+L-arginine group was 6.6 ± 1.4 mg/kg/day and 1.23 ± 0.24 g/kg/day, respectively. This resulted in a significant reduction in urinary nitrate/nitrite excretion measured in RK+L-NAME rats by the modified Griess reaction⁴ (74 ± 28 vs. 135 ± 75 pmol/day, RK+L-NAME vs. RK alone, $P < 0.05$) whereas no significant changes were noted in the RK+L-arginine group (210 ± 105 pmol/day).

Renal Morphological Assessment

Tissue was fixed in methyl Carnoy's solution, paraffin-embedded, sectioned (4- μ m), and stained with periodic acid Schiff (PAS) reagent or by indirect immunoperoxidase as reported previously.¹⁶ Endothelial cells were

detected with monoclonals JG-12 (gift of D. Kerjaschki, University of Vienna, Austria) and RECA-1 (Serotec, Indianapolis, IN); VEGF with rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA); α -smooth muscle actin with mouse monoclonal α -SM-1 (Sigma, St. Louis, MO), collagen type III with goat anti-human antibody (Southern Biotechnology, Birmingham, AL), osteopontin with goat anti-osteopontin antibody 199 (gift from C. Giachelli, University of Washington, Seattle, WA); and monocyte-macrophages with mouse monoclonal antibody ED-1 (Serotec, Indianapolis, IN). Controls included omitting the primary antibody and substitution of the primary antibody with pre-immune rabbit or mouse serum.

To examine whether there is any evidence of endothelial or smooth muscle proliferation, double immunostaining was performed with α -smooth muscle cell actin and an antibody to the proliferating cell nuclear antigen (PCNA) (PC 10, Cappel, Aurora, OH). Tissue sections were first incubated with PCNA antibody overnight at 4°C, followed sequentially by biotinylated horse anti-mouse IgG serum, peroxidase-conjugated avidin D, and color development with diaminobenzidine (DAB) with nickel chloride. After incubation in 3% H₂O₂ for 8 minutes to eliminate any remaining peroxidase activity, sections were incubated with primary antibody for α -smooth muscle cell actin for 3 hours at room temperature, followed by biotinylated horse anti-mouse IgG for 30 minutes at room temperature. After incubation in alkaline phosphatase streptavidin (Vector, Burlingame, CA), color was developed using AP-RED substrate kit (Zymed, San Francisco, CA).

All quantification was performed blinded. The numbers of endothelial and smooth muscle cells were counted in the afferent arteriole, interlobular artery, and arcuate artery. Vessels which were not sectioned transversally, providing an asymmetrical wall, were excluded from the present study. To assess smooth muscle cell hypertrophy and/or hyperplasia, the cross-sectional wall area and thickness of each artery was measured by computer image analysis (Optimas 6.2, Media Cybernetics, Silver Spring, MD). Arteries and arterioles were identified by their anatomical location and branching pattern from neighboring vessels, and shape of endothelial and smooth muscle cells described elsewhere.¹⁷ Considering the continuous tapering course of interlobular artery, we evaluated the interlobular artery at the same level (proximal one-third from corticomedullary junction) of individual kidneys. Afferent arterioles were carefully distinguished from efferent arterioles by their general characteristics, including the presence of thin and continuous endothelial cells with a thicker smooth muscle wall than the efferent arteriole.¹⁷

The number of glomerular capillary loops, glomerular capillary density, peritubular capillary rarefaction index, and density were assessed.^{3,4} The percent glomerulosclerosis and tubulointerstitial fibrosis score^{0–5} were evaluated based on PAS staining previously described.¹⁶ The degree of osteopontin expression and type III collagen deposition was determined by computer image analysis of complete sagittal sections.¹⁶

Table 1. Comparison of Renal Injury in L-Arginine and L-NAME-Treated RK Rats

Injury	RK (n = 6)	RK + L-arginine (n = 6)	RK + L-NAME (n = 5)
Glomerulosclerosis (%)	11.1 ± 1.8	10.0 ± 3.4	34.2 ± 14.9*
Tubulointerstitial fibrosis score (0–5)	2.0 ± 0.24	1.70 ± 0.23	2.61 ± 0.51*
Cortical osteopontin expression (%)	9.1 ± 2.2	7.8 ± 3.8	13.4 ± 5.1*
Cortical collagen III deposition (%)	15.1 ± 3.8	13.0 ± 2.3	32.4 ± 5.8*
Glomerular macrophage infiltration (/glomerulus)	5.4 ± 1.7	5.0 ± 3.2	7.7 ± 2.0*
Tubulointerstitial macrophage infiltration (/mm ²)	150.3 ± 32.7	137.5 ± 59.8	197.8 ± 25.1*

Data are expressed as means ± SD.

**p* < 0.05 vs. RK or RK + L-arginine.

In Vitro Studies

Murine thick ascending limb cells (mTAL) (gift of K. Madson, University of Florida, Gainesville, FL) and rat vascular smooth muscle cells (SMC) (American Type Culture Collection (CRL-2018)) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), gentamicin (G418, 50 mg/ml) and L-glutamine. After cells were grown to 70% confluency in multi-well plates (Becton Dickinson, Franklin Lakes, NJ), the culture medium was changed into serum-free media for 24 hours before each experiment. After synchronization of cell growth, cells were washed three times with Hanks' Balanced Salt Solution (HBSS) and exposed to L-NAME (2 mmol/L) (Sigma, St. Louis, MO) for 1, 4, 8, and 24 hours. All experiments were also conducted under hypoxic condition to simulate *in vivo* condition since the normal pO_2 in the renal medulla is 20–30 mmHg¹⁸ and the microvascular loss in the RK model can result in tissue hypoxia and/or ischemia. To achieve hypoxia, cells with or without L-NAME were incubated in a GasPak anaerobic culture pouch (BBL Microbiology Sys-

tems, Kansas City, MO), resulting in partial pressure of oxygen (PaO_2) of 25–30 mmHg for hypoxic conditions compared to 120–130 mmHg in normoxic conditions in the culture medium.³ These conditions did not result in any loss of cell viability as measured by lactate dehydrogenase (LDH) assay (Sigma, St. Louis, MO).

To quantify the level of VEGF protein secretion under the different conditions, VEGF protein was measured in cell culture supernatants using a commercial murine ELISA kit for VEGF (R & D Systems, Minneapolis, MN) which is sensitive to 3 pg/ml. Interassay coefficient of variation is <7% and the intra-assay coefficient of variation is <5% among the standards. All data for VEGF production detected by ELISA are expressed as pg/10⁵ cells.

Generation of Mouse and Rat VEGF Riboprobes for RNase Protection Assay (RPA)

Mouse VEGF Probe

An anti-sense primer (GCGAATTCTATCTCGTCGT CGGGGTACTC, *Bam*HI site is incorporated at the 5' end with three extra nucleotides to guarantee restriction enzyme digestion) and T7 primer (sense primer) were used for polymerase chain reaction (PCR) with a plasmid DNA containing mouse VEGF as a template (the contained VEGF cDNA region is from bp 1 to 290 of NM_009505). The PCR fragment of mouse VEGF was inserted into the pCR-II-TOPO with the TA cloning kit (Invitrogen, CA). After linearization with *Hind*III, an anti-sense riboprobe was synthesized with T7 RNA polymerase in the presence of [³²P]-labeled UTP.

Rat VEGF Probe

The rat VEGF probe (1–327 of a EST clone, AA850734) was generated by PCR and subcloned into pGEM4 Z with 3' end toward T7 promoter. After linearization with an appropriate restriction enzyme, an anti-sense riboprobe was synthesized with T7 polymerase in the presence of [³²P]-labeled UTP.

RNA Isolation and RPA

Following incubation with L-NAME under normoxic and hypoxic conditions, total RNA was prepared from the

Figure 1. Changes in blood pressure, proteinuria, and renal function in the RK, RK+L-arginine and RK+L-NAME rats. During L-NAME administration, systolic blood pressure, urinary protein excretion and renal functions expressed as blood urea nitrogen (BUN) and creatinine were significantly higher in RK+L-NAME rats (●) compared to RK (◇) or RK+L-arginine (○) rats.

Table 2. Changes in Microvasculature in L-Arginine and L-NAME-Treated RK Rats

Vasculature	RK	RK + L-arginine	RK + L-NAME
Glomerular capillary			
Total glomerular capillary number (/cross-section)	33.7 ± 3.5	32.1 ± 5.5	24.5 ± 5.0*
Glomerular capillary density (/0.01 mm ²) [†]	14.5 ± 4.5	14.8 ± 3.7	11.0 ± 5.2*
Proliferating endothelial cell (/glomerulus)	0.04 ± 0.01	0.04 ± 0.01	0.02 ± 0.01*
Peritubular capillary (PTC)			
% positive area of PTC (/100 tubules)	0.74 ± 0.21	0.80 ± 0.35	0.52 ± 0.25*
PTC rarefaction index (%)	13.2 ± 3.4	12.2 ± 4.8	20.7 ± 3.3*
Proliferating endothelial cell (/mm ²)	0.96 ± 0.17	0.99 ± 0.23	0.71 ± 0.29*

Data are expressed as means ± SD.

**p* < 0.05 vs. RK or RK + L-arginine.

[†]Total glomerular capillary number/glomerular tuft area.

mTAL cell and rat vascular SMC monolayers using the RNeasy96 total RNA isolation protocol manufactured by Qiagen (Valencia, CA). After determination of RNA purity and concentration by spectrophotometry, 2 µg of RNA samples were hybridized for 30 minutes at 90°C with a mixture of [³²P]-UTP labeled riboprobe and the house-keeping gene (L32) (1 × 10⁵ cpm for each probe), and RPA was performed as described previously using a RPA kit¹⁹ (Torrey Pines Biolabs, Houston, TX) according to the manufacturer's instruction. The protected hybridized RNA was denatured at 85°C and electrophoresed on

10% polyacrylamide gels. The gels were transferred to 3 mol/L Whatman filter paper, dried, and exposed to Kodak X-O mat film overnight at -70°C.

Statistical Analysis

All data are presented as mean ± standard deviation (SD). Differences in the various parameters between groups were evaluated by unpaired comparisons for non-parametric data. Differences in parameters at each time point after RK surgery were compared by paired *t*-test. The relation between variables was assessed by Pearson correlation analysis. Significance was defined as a *P* value <0.05.

Results

Consistent with a previous study,⁶ blockade of NO synthesis in the remnant kidney model was associated with accelerated progression of renal disease (Table 1, Figure 1). L-NAME-treated rats showed higher systolic blood pressure, more proteinuria, worse renal function, and more severe glomerulosclerosis. Interstitial fibrosis was also greater in L-NAME-treated rats, and this was associated with significantly more tubular osteopontin expression, greater macrophage infiltration, and more collagen type III deposition (Table 1). Interestingly, there was no difference in the severity of renal injury between RK control and RK+L-arginine rats (Table 1, Figure 1). Two rats in the RK+L-NAME group died spontaneously between the second and third week of L-NAME administration whereas there was no mortality in the control RK and RK+L-arginine group.

Microvascular Changes Associated with Blockade of NO Synthesis

L-NAME treatment was associated with more severe endothelial cell loss than that observed with RK alone, with a greater loss of glomerular capillary loops and more peritubular capillary rarefaction (Table 2). L-NAME-treated rats also had significantly less capillary endothelial cell proliferation.

The number of glomerular capillary loops and the PTC rarefaction index significantly correlated with the % glo-

Figure 2. Correlation of glomerular and peritubular capillary losses with renal damage. There was a significant inverse correlation between the number of glomerular capillary loop and the development of glomerulosclerosis (A). Peritubular capillary loss was also correlated with tubulointerstitial fibrosis (B), suggesting that renal microvascular loss was associated with renal scarring.

Figure 3. Morphology of preglomerular vessels in the RK and RK+L-NAME rats. In RK+L-NAME rats, the number of smooth muscle cell was increased (C, interlobular artery; $\times 200$, PAS) with characteristic medial wall thickening and increase in extracellular matrix compared to normal rat (A, $\times 200$, PAS) and RK rats (B, $\times 200$, PAS). Representative interlobular arteries in each group were selected at the same level (within the inner one-third of renal cortex from the corticomedullary junction).

merulosclerosis and tubulointerstitial fibrosis score, respectively (Figure 2).

Given the potential role of systemic blood pressure on microvascular capillary loss, we also compared the RK+L-NAME rats with a historical RK group^{3,4} that were examined at 8 to 12 weeks after renal mass reduction and had similar systolic blood pressure (178.3 ± 21.8 vs. 175.7 ± 45.9 mmHg, RK+L-NAME vs. historical RK control, $P = \text{NS}$). When compared to the L-NAME-treated RK rats, the historical RK rats had more preserved glomerular capillary loops (34.1 ± 4.9 vs. 24.5 ± 5.0 /glomerular cross-section, $P < 0.05$) and less PTC rarefaction (15.2 ± 2.5 vs. $20.7 \pm 3.3\%$, $P < 0.05$). There was no significant correlation of systolic blood pressure with the numbers of glomerular and peritubular capillaries, suggesting that blocking NO synthesis accelerates the loss of renal microvasculature in the kidneys independent of an increase in systemic blood pressure.

Effects on the Preglomerular (Arterial) Vasculature

While endothelial cell loss characterized the glomerular and postglomerular vasculature, the endothelium in the preglomerular vessels (afferent arteriole and more prox-

imal vessels) in the RK rats was preserved in association with a significant increase in the vascular smooth muscle cell layer (Figure 3). There was no significant difference in endothelial cell number in afferent arteriole, interlobular or arcuate artery between RK vs. RK+L-NAME rats (Table 3). However, the number of proliferating endothelial cells was higher in arterioles and arteries of RK rats administered L-NAME (Table 4) in contrast to less endothelial cell proliferation of both glomerular and peritubular capillaries in RK+L-NAME rats. In occasional vessels, nests of proliferating endothelial cells were apparent, which rarely resulted in an obliterative arteriopathy (Figure 4, F).

Remnant kidney rats administered L-NAME also showed more prominent vascular injury with greater medial wall thickness, higher numbers of smooth muscle cells, and more SMC proliferation when compared to RK alone or RK+L-arginine groups (Figures 3 and 4, Tables 3 and 4). Periarterial adventitial fibrosis was also prominent in RK+L-NAME rats in association with more cell infiltration (Figure 5).

We originally hypothesized that the SMC proliferation and wall thickening in L-NAME-treated rats might be due to the higher blood pressure in these animals, and hence might be more likely to have pressure-related vascular injury. However, the increase in SMC number of preglomer-

Table 3. Changes in Macrovasculature in L-Arginine and L-NAME-Treated RK Rats

Vasculature	RK	RK + L-arginine	RK + L-NAME
Afferent arteriole			
Number of SMC (/cross-section)	3.59 ± 0.34	3.04 ± 0.30	$6.0 \pm 0.32^*$
Number of EC (/cross-section)	2.51 ± 0.28	2.71 ± 0.17	3.0 ± 0.31
Wall area (μm^2)	241.7 ± 20.34	232.4 ± 17.9	$275.9 \pm 21.9^*$
Interlobular artery			
Number of SMC (/cross-section)	7.01 ± 3.13	7.40 ± 2.41	$12.7 \pm 3.04^*$
Number of EC (/cross-section)	4.55 ± 0.80	3.9 ± 0.61	5.0 ± 1.2
Wall area (μm^2)	670.8 ± 211.3	654.9 ± 232.0	$1275.2 \pm 260.9^*$
Arcuate artery			
Number of SMC (/cross-section)	17.0 ± 3.16	17.5 ± 2.19	$25.9 \pm 3.17^*$
Number of EC (/cross-section)	12.3 ± 2.47	10.3 ± 2.14	13.1 ± 5.08
Wall area (μm^2)	3217 ± 647.5	3004 ± 1275.3	$6274 \pm 846.3^*$

Data are expressed as means $\pm$ SD.
 Abbreviations: SMC, smooth muscle cell; EC, endothelial cell.
 $^*p < 0.05$ vs. RK or RK + L-arginine.

Table 4. Smooth Muscle Cell and Endothelial Cell Proliferation in Preglomerular Vessels

Vessel	SMC proliferation (cell/each vessel)			Endothelial cell proliferation (cell/each vessel)		
	RK	RK + L-arginine	RK + L-NAME	RK	RK + L-arginine	RK + L-NAME
Afferent arteriole	0.031 ± 0.02	0.035 ± 0.01	0.260 ± 0.05*	0.110 ± 0.09	0.105 ± 0.07	0.370 ± 0.14*
Interlobular artery	0.25 ± 0.02	0.24 ± 0.01	0.57 ± 0.15*	0.15 ± 0.04	0.17 ± 0.02	0.295 ± 0.18*
Arcuate artery	0.16 ± 0.10	0.19 ± 0.06	0.53 ± 0.01*	0.34 ± 0.11	0.32 ± 0.10	0.62 ± 0.05*

Data are expressed as means ± SD.

* $p < 0.05$ vs. RK or RK + L-arginine.

merular arteries as well as medial wall thickness observed in L-NAME-treated RK rats were significantly greater than that observed in RK rats with comparable blood pressure (Figure 6), suggesting the possibility of blood pressure-independent SMC proliferation induced by blocking of NOS.

Differential Effects of NO Blockade on VEGF Expression

Consistent with our prior study,³ we documented a loss in VEGF expression in both podocytes and in tubular epithelial cells in the RK model, but there was no significant difference among groups in the percent positive area of VEGF expression by immunostaining as assessed by computer image analysis (4.2 ± 1.8 vs. 4.3 ± 1.5 vs. $4.1 \pm 1.2\%$, RK vs. RK+L-arginine vs. RK+L-NAME) or Western blotting (data not shown). However, in L-NAME-treated RK rats, arteriolar SMC showed *de novo* staining of VEGF, which contrasted with the uniformly negative

staining for VEGF in smooth muscle cells of control or L-arginine-treated RK rats (Figure 7, A and B).

Since we observed *de novo* SMC VEGF staining in the L-NAME-treated RK rats whereas comparable or decreased VEGF expression was observed in renal tubular cell in this group, we hypothesized that VEGF expression regulated by NO synthase inhibition might be different in renal tubular cells and SMC. We therefore examined the effect of inhibiting NO synthesis (with L-NAME) on the expression of VEGF in cultured mTAL cells and rat vascular SMC.

In mTAL cells, L-NAME inhibited VEGF mRNA expression, whereas, in rat vascular SMC, L-NAME increased VEGF expression under hypoxic but not normoxic conditions (Figure 7, C and D) consistent with the *in vivo* finding which showed *de novo* expression of VEGF in SMC of RK+L-NAME rats. The VEGF protein levels in the supernatant of cultured cells as measured by ELISA were consistent with the changes in VEGF mRNA expression (Figure 7, E and F). L-NAME treatment of rat vascular

Figure 4. Proliferation of smooth muscle cell (SMC) and endothelial cell (EC) of arteries in the RK (A,C) and RK+L-NAME (B,D,E,F) rats. Compared to RK rats (A and C, respectively, $\times 400$), the number of proliferating smooth muscle cells is increased in both preglomerular arteries (B, interlobular artery, $\times 200$) and afferent arterioles (D, $\times 400$) in RK+L-NAME rats. Some afferent arteriole of RK+L-NAME rats show medial wall thickening with SMC proliferation (E, $\times 400$), suggesting both hyperplasia and hypertrophy developed in arterioles. In some arteries, nests of proliferating (PCNA+) endothelial cells are present (F, $\times 400$).

Figure 5. Perivascular fibrosis in the RK and RK+L-NAME rats. In RK+L-NAME rats, there is more perivascular adventitial cell fibrosis (**B**, $\times 200$, interlobar artery, collagen type III immunolabeling; **C**, $\times 200$, PAS) compared to RK rats (**A**, $\times 200$, interlobar artery, collagen type III immunolabeling).

SMC resulted in a 2.1-fold increase in VEGF protein secretion under hypoxic conditions whereas L-NAME decreased VEGF protein levels in mTAL cells.

Discussion

The remnant kidney model is widely considered to be the classic model of progressive renal disease. A previous study has reported that blockade of NO production in this model is associated with increased systemic and glomerular pressure and a more rapid course of renal scarring.⁶ The increased systemic and glomerular pressure is thought to mediate renal progression by initiating injury to the endothelium,¹ but until recently few studies have addressed the role of the endothelium in progression of renal disease. Recently, both our group^{3,16} and that of Yamanaka's^{2,20,21} have demonstrated that there is a loss of both glomerular and peritubular capillary endothelium in experimental models of progressive renal disease, and we have found that administration of the angiogenic growth factor, vascular endothelial growth factor (VEGF), can slow progression by maintaining capillary number.⁴ Studies by Adalbert Bohle²² have similarly implicated a critical role for the peritubular capillaries in the progressive interstitial fibrosis that characterizes human renal disease.

Figure 6. Blood pressure-independent effect of L-NAME on SMC proliferation. The number of SMC of interlobular arteries in RK+L-NAME rats was significantly higher compared to the RK rats with comparable blood pressure.

In this study we examined the changes in endothelial cells that occur in the remnant kidney model in which NO synthesis was stimulated or blocked. Our hypothesis was that endothelial cell injury would be more severe in those rats with NOS blockade due to higher systemic and glomerular pressure leading to greater vascular injury, which could be protected by the stimulation of NO synthesis. More importantly, we also hypothesized that a lack of NO itself might lead to endothelial cell loss, as NO is an endothelial cell survival factor and is important in angiogenesis.¹²⁻¹⁴ A lack of NO may also accelerate endothelial loss by blocking VEGF action, as NO is required for VEGF-dependent endothelial cell proliferation.^{12,13} In a recent study, we documented that blocking NO synthesis markedly increases endothelial cell death and worsens renal disease in a model of thrombotic microangiopathy.¹⁴

Consistent with the study by Fujihara et al,⁶ we found that blockade of NO synthesis with L-NAME in the remnant kidney model resulted in higher systemic blood pressure, greater proteinuria, worse renal function, and more severe glomerulosclerosis. We also documented worse interstitial fibrosis with greater tubular osteopontin expression, macrophage infiltration, and type III collagen deposition.

Our first new finding was that blockade of NO synthesis was associated with significantly greater glomerular and peritubular capillary endothelial cell loss in association with a reduced endothelial proliferative response. The loss of the glomerular capillary endothelium may predispose to glomerular capillary loop collapse and glomerulosclerosis,^{2,17} whereas a loss of the peritubular capillary endothelium could impair oxygen delivery to the tubules which would accentuate ischemic injury and stimulate fibrosis.⁵ Consistent with these findings, we found a close correlation between the microvascular endothelial cell loss and the degree of glomerulosclerosis ($r^2 = 0.79$, $P < 0.05$) and tubulointerstitial fibrosis ($r^2 = 0.78$, $P < 0.05$). These studies are consistent with a critical role for the microvasculature in progressive renal disease.

The loss of the endothelium could be secondary to pressure-mediated injury. However, when a historical control group of remnant kidney rats^{3,4} were compared to L-NAME-treated RK rats with similar blood pressures

Figure 7. Expressions of VEGF mRNA and protein *in vitro* and *in vivo*. In RK+L-NAME rats, VEGF could be documented in the arterial walls (**B**, $\times 100$) whereas minimal VEGF staining was present in the vessels of RK rats (**A**, $\times 100$). *In vitro*, L-NAME (NA, 2 mmol/L) decreased VEGF mRNA expression in mTAL cells under hypoxic conditions (**C**, $n = 4$; 8 hours) compared to controls (C) whereas L-NAME increased hypoxia-induced VEGF mRNA expression in SMC (**D**, $n = 4$). L-NAME exposure for 24 hours also resulted in a decrease in VEGF protein secretion in mTAL cells under hypoxic conditions (**E**, $n = 6$) whereas L-NAME increased hypoxia-induced VEGF secretion in SMC (**F**, $n = 6$).

(178.3 $\pm$ 21.8 vs. 175.7 $\pm$ 45.9 mmHg), there was significantly greater glomerular and peritubular capillary loss in the L-NAME-treated rats, suggesting the presence of some blood pressure-independent microvascular injury by NOS blockade. This would be consistent with an additional direct effect of NO as an endothelial cell survival and trophic factor.^{12–15} An antiangiogenic effect of NOS inhibition has been reported in tumor tissue.¹⁴ Ziche et al²³ also observed that VEGF-induced angiogenesis was blocked by systemic administration of L-NAME using a rabbit corneal pocket assay.

An interesting contrast was the observation that the preglomerular arterial endothelium was preserved or hyperplastic in L-NAME-treated remnant kidney rats. The preglomerular arterial vessels also showed an increase in the number of total and proliferating smooth muscle cells and an increase in medial wall thickness, a finding consistent with vascular changes associated with hypertension²⁴ and which could also be a consequence of the loss of NO which normally has an inhibitory effect on SMC growth.²⁵ The vascular changes are also consistent with previous studies that have documented medial wall thickening with lipid accumulation and inflammatory changes in the preglomerular vasculature of L-NAME-treated animals.^{26,27} Interestingly, these changes in preglomerular vessels were also not solely a consequence of an elevation in systemic blood pressure (Figure 6) as evidenced by more prominent vascular changes (SMC hyperplasia and medial wall thickening) in preglomerular vessels of L-NAME-treated RK rats when compared to RK rats with similar systemic blood pressure. Similarly, the development of the arteriopathy induced by L-NAME administration has been shown to be independent of blood pressure in normal Wistar rats.²⁸

To better understand the potential mechanisms for the differential response of the preglomerular and microvascular endothelium, we studied the expression and regulation of VEGF, which we have previously found has a key role in maintaining microvascular endothelial cell integrity in the remnant kidney model.^{3,4} We found that inhibition of NO synthesis blocked VEGF expression in tubules (mTAL cells) under hypoxic conditions, whereas VEGF was up-regulated in hypoxic smooth muscle cells under similar conditions. Consistent with these *in vitro* findings, we found up-regulated expression of VEGF in the media of preglomerular vessels in L-NAME-treated rats with remnant kidneys whereas arteries in control remnant kidney rats showed minimal expression. It is unclear if this up-regulated VEGF in the media is responsible for maintaining the preglomerular arterial endothelium, however, as numerous studies suggest that the angiogenic effects of VEGF depend on an intact NO system.^{13,23} Nevertheless, it seems likely that growth factors expressed by the arterial smooth muscle cells may have a role in maintaining survival of the adjacent endothelium²⁹ whereas decreased VEGF expression in renal tubular cells by NOS blockade may contribute to PTC loss via reducing endothelial cell survival signals. This differential regulation of NO inhibition on VEGF expression in tubular cells *versus* vascular SMC is similar to that which has recently been reported with angiotensin II.^{30,31} Thus, angiotensin II

stimulates VEGF in vascular SMCs but inhibits VEGF in mTAL cells. These studies underscore the importance of considering the different intrarenal compartments when considering the progression of renal disease.

We were not able to confirm the previous report that L-arginine slows progression in the RK model. This may relate to difference in timing and dose of the L-arginine. The level of urinary nitrite/nitrate was higher in the RK+L-arginine group compared to RK rats, but it was not statistically significant. A possible explanation for an inability of L-arginine to provide benefit in our RK model may be related to chronic renal failure *per se*, as NOS is profoundly decreased at 4 weeks after renal mass reduction,³² and therefore providing substrate may not be effective in this specific model of progressive renal disease. This finding is consistent with a human study by DeNicola et al,³³ who was unable to show enhanced NO generation in response to L-arginine in patients with chronic renal disease. They also did not observe any improvement in proteinuria, renal function, or blood pressure after L-arginine treatment. It is possible that starting the L-arginine administration earlier (before the significant decrease in NOS) or treatment with an NO donor rather than with the physiological NO precursor⁹ might be a more effective way to prevent progressive renal disease.

Inhibition of NO synthesis has been known to exacerbate renal disease in the RK model by hemodynamic changes. Our study supports the hypothesis that blood pressure-independent vascular changes induced by NOS blockade may be an additional mechanism for progression of renal disease. Inhibition of NO synthesis was associated with significant loss of the microvasculature and with an impairment in local angiogenesis. NO blockade also was associated with more severe preglomerular vascular disease. Both of these effects could provide a mechanism for inducing renal ischemia with the stimulation of renal scarring. Furthermore, both effects could not be attributed to the higher blood pressure in the rats in which NO synthesis was blocked. Thus, these studies suggest that the maintenance of adequate NO may be an additional mechanism for the preservation of the renal vasculature in progressive renal disease.

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