A Role for Uric Acid in the Progression of Renal Disease

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Abstract. Hyperuricemia is associated with renal disease, but it is usually considered a marker of renal dysfunction rather than a risk factor for progression. Recent studies have reported that mild hyperuricemia in normal rats induced by the uricase inhibitor, oxonic acid (OA), results in hypertension, intrarenal vascular disease, and renal injury. This led to the hypothesis that uric acid may contribute to progressive renal disease. To examine the effect of hyperuricemia on renal disease progression, rats were fed 2% OA for 6 wk after 5/6 remnant kidney (RK) surgery with or without the xanthine oxidase inhibitor, allopurinol, or the uricosuric agent, benziodarone. Renal function and histologic studies were performed at 6 wk. Given observations that uric acid induces vascular disease, the effect of uric acid on vascular smooth muscle cells in culture was also examined. RK rats developed transient hyperuricemia (2.7 mg/dl at week 2), but then levels returned to baseline by week 6 (1.4 mg/dl). In contrast, RK+OA rats developed higher and more persistent hyperuricemia (6 wk, 3.2 mg/dl). Hyperuricemic rats demonstrated higher BP, greater proteinuria, and higher serum creatinine than RK rats. Hyperuricemic RK rats had more renal hypertrophy and greater glomerulosclerosis (24.2 ± 2.5 *versus* $17.5 \pm 3.4\%$; $P < 0.05$) and interstitial fibrosis (1.89 ± 0.45 *versus* 1.52 ± 0.47; $P < 0.05$). Hyperuricemic rats developed vascular disease consisting of thickening of the preglomerular arteries with smooth muscle cell proliferation; these changes were significantly more severe than a historical RK group with similar BP. Allopurinol significantly reduced uric acid levels and blocked the renal functional and histologic changes. Benziodarone reduced uric acid levels less effectively and only partially improved BP and renal function, with minimal effect on the vascular changes. To better understand the mechanism for the vascular disease, the expression of COX-2 and renin were examined. Hyperuricemic rats showed increased renal renin and COX-2 expression, the latter especially in preglomerular arterial vessels. In *in vitro* studies, cultured vascular smooth muscle cells incubated with uric acid also generated COX-2 with time-dependent proliferation, which was prevented by either a COX-2 or TXA-2 receptor inhibitor. Hyperuricemia accelerates renal progression in the RK model via a mechanism linked to high systemic BP and COX-2–mediated, thromboxane-induced vascular disease. These studies provide direct evidence that uric acid may be a true mediator of renal disease and progression.

Hyperuricemia has long been associated with renal disease. Approximately 20 to 60% of patients with gout have mild or moderate renal dysfunction (1); before the availability of uric acid lowering agents, as many as 10 to 25% of patients with gout developed end-stage renal disease (2). The histologic lesion termed "gouty nephropathy" consists of glomerulosclerosis, interstitial fibrosis, and renal arteriolosclerosis, often with focal interstitial urate crystal deposition (2,3). These histologic findings have been observed in autopsies of 79 to 99% of patients with gout (3).

Despite the association of gout with renal disease, controversy exists as to whether uric acid has an etiologic role (4–6). First, it has been difficult to ascribe the generalized renal injury in gout to the deposition of urate crystals, for they are often only focally present. Second, many patients with gout have hypertension or are elderly, and the renal lesions might simply reflect hypertensive or aging-associated renal damage (1). Third, results of the studies are mixed as to whether lowering uric acid will slow renal progression in patients with gout (7,8). The inability to resolve this issue has emphasized the need for additional studies (6).

To investigate the role of uric acid in renal disease, we recently developed a model of hyperuricemia in rats (9). Most mammals have a low serum uric acid due to the presence of uricase; in humans and the Great Apes, the uricase gene was mutated and rendered nonfunctional. We therefore induced hyperuricemia in rats by providing low doses of oxonic acid, which is a uricase inhibitor. Unlike previous models of uricase

inhibition, which result in massive uricosuria with intrarenal crystal deposition and obstructive renal disease, this model resulted in mild hyperuricemia without intrarenal crystal deposition. Nevertheless, subtle interstitial renal injury developed, and this was associated with activation of the renin angiotensin system (RAS) and the development of hypertension (9). The hyperuricemic animals also developed an afferent arteriolopathy that occurred independently of changes in BP (10). The vascular injury was mediated in part by direct effects of uric acid to induce vascular smooth muscle cell (VSMC) proliferation and also by activation of the RAS (10). Whereas hyperuricemia induced both renal injury and vascular disease in normal rats, hyperuricemia was also shown to accelerate cyclosporine-induced vascular injury and interstitial renal disease (11). The exacerbation of cyclosporine nephrotoxicity by uric acid was also associated with increased activation of the RAS and blockade of specific nitric oxide pathways (11).

The observation that mild hyperuricemia activates the RAS and causes renal disease via a crystal independent pathway raised the hypothesis that hyperuricemia may be a general risk factor for renal progression. Interestingly, hyperuricemia is a common feature of renal disease of all etiologies; as GFR falls the serum uric acid increases due to reduced renal excretion. The hyperuricemia in renal failure patients is generally mild, due to a compensatory increase in fractional excretion, reduced production, and increased excretion of uric acid via nonrenal (gastrointestinal) routes (12). Because the hyperuricemia is often mild, gout is rare in patients with end-stage renal disease and the increase in uric acid is often considered innocuous. However, the possibility that the hyperuricemia could be contributing to renal progression has not been tested. To test this hypothesis, we examined the role of uric acid in the classic model of renal progression induced by 5/6 nephrectomy (remnant kidney model). Additionally, given the fact that renal cortical COX-2 expression is increased after subtotal renal ablation (13,14) and COX-2 is a major regulator of renin production, we also investigated changes in COX-2 by hyperuricemia.

Materials and Methods

Experimental Protocol

All animal procedures were approved by the Animal Care Committees of the University of Washington and Baylor College of Medicine. Male Sprague-Dawley rats (190 to 200 g; Simonsen Laboratories, Gilroy, CA) underwent a remnant kidney (RK) operation after baseline measurement of BP and renal function. The RK operation was performed by a right subcapsular nephrectomy and surgical resection of the upper and lower thirds of the left kidney in total of 17 rats. After randomization by the percent remnant kidney weight removed [(right kidney weight − weight of upper and lower poles of left kidney)/right kidney weight × 100], the animals were divided into four groups. Group 1 ($n = 4$) received normal-salt (NS) diet (0.27% NaCl) (RK); group 2 ($n = 5$) received NS diet containing 2% oxonic acid (OA, hepatic uricase inhibitor; Sigma, St. Louis, MO) (RK+OA); group 3 ($n = 4$) received 2% OA NS diet and allopurinol (xanthine oxidase inhibitor, 13 mg/dl in the drinking water; Schein Pharmaceutical, Florham Park, NJ) (RK+OA+AP); and group 4 ($n = 4$) received 2% OA NS diet and benziodarone (uricosuric agent, 13

mg/dl in the drinking water; Sanofi, Barcelona, Spain) (RK+OA+BZ). 2% OA could be given without apparent toxicity to rats according to our previous studies (9–11), and there was no mortality during the study period. The doses of AP and BZ were determined on the basis of the uric acid-lowering effect in previous studies (9,10). The mean daily intakes of allopurinol and benziodarone were 20.4 ± 5.3 mg/kg per d and 21.3 ± 6.2 mg/kg per d in RK+OA+AP and RK+OA+BZ groups, respectively. At 6 wk, rats were sacrificed for histologic evaluation.

Systolic BP, Uric Acid, Proteinuria, and Renal Function

Systolic arterial BP was monitored by a tail cuff sphygmomanometer using an automated system with a photoelectric sensor (IITC; Life Sciences, Woodland Hills, CA) that has been shown to closely correlate with intraarterial measurement of BP (15). All rats were preconditioned for this machine at least two times before the actual measurement of BP. Serum uric acid concentration was determined by a carbonate phosphotungstate method and uric acid standard (Sigma, St. Louis, MO) (16). Urinary protein excretion (24 h) were measured using the sulfosalicylic acid method, and blood urea nitrogen and creatinine were determined colorimetrically utilizing a commercial kit (Sigma Diagnostics, St. Louis, MO).

Renal Morphology and Immunohistochemistry

Tissue for light microscopy and immunoperoxidase staining was fixed in Methyl Carnoy's solution and embedded in paraffin. Four-micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. Indirect immunoperoxidase staining of 4-μm sections was performed as described previously (17), with specific monoclonal and polyclonal antibodies directed to the following antigens: α-smooth muscle actin (α-SMA) with mouse monoclonal (α-SM-1; Sigma, St. Louis, MO), renin with monocloncal mouse anti-human antibody (Sanofi Recherche, Motpellier, France); collagen type III with goat anti-human antibody (Southern Biotechnology, Birmingham, AL); COX-2 with rabbit anti-mouse polyclonal antibody (Cayman, Ann Arbor, MI); monocyte-macrophages with mouse monoclonal antibody ED-1 (Serotec, Indianapolis, IN). Controls included omitting the primary antibody and substitution of the primary antibody with preimmune 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% H_2O_2 for 8 min to eliminate any remaining peroxidase activity, sections were incubated with primary antibody for α-smooth muscle cell actin for 3 h at room temperature followed by biotinylated horse anti-mouse IgG for 30 min at room temperature. After incubation to alkaline phosphatase Streptavidin (Vector, Burlingame, CA), color was developed using AP-RED substrate kit (Zymed, San Francisco, CA).

Quantification of Morphologic Data

All analyses were performed blinded. The numbers of endothelial and smooth muscle cells were counted in the afferent arteriole, interlobular artery, and arcuate artery. All cross-sectioned arterioles and arteries available in each section were evaluated. Vessels that were not

sectioned transversally, providing an asymmetrical wall, were excluded from the present analysis. 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 Springs, MD). Arteries and arterioles were identified by their anatomic location and branching pattern from neighboring vessels and shape of endothelial and smooth muscle cells described elsewhere (18). Considering the continuous tapering course of interlobular artery, we evaluated the interlobular artery at the same level (proximal 1/3 from corticomedullary junction) of individual kidneys. Afferent arterioles were carefully distinguished from efferent arteriole by their general characteristics, including the presence of thin and continuous endothelial cells with a thicker smooth muscle wall than the efferent arteriole.

Renin expression was quantified by the number of glomeruli with positive staining for juxtaglomerular renin using a minimum 40 glomeruli in each biopsy, a method which has previously been shown to correlate with changes in tissue renin content (19). The expression of cortical COX-2 was measured as the percent positive area of COX-2 immunostaining in renal cortex using computer image analyzer. The percent glomerulosclerosis and tubulointerstitial fibrosis score (0 to 5) were evaluated on the basis of PAS staining as described previously (17).

Generation of Rat COX-1 and COX-2 Riboprobe for RNase Protection Assay (RPA)

A fragment of COX-1 cDNA produced by *BstX1* (from bp 1350 to bp 1690), 340 bp, was blunted and subcloned into the *Smad*I site of pGEM4Z. A 241-bp fragment of rat COX-2 cDNA produced by *Bam*HI and *Eco*RI (from bp 409 to bp 650) was subcloned in pGEM4Z (20).

RPA for COX-1 and COX-2 mRNA

Following incubation of VSMC with uric acid for 4, 8, and 24 h, total RNA was prepared from the VSMC 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 min at 90°C with a mixture of ^{32}P-UTP–labeled riboprobe and the housekeeping gene (L32) (1×10^5 cpm for each probe), and RPA was performed as described previously using a RPA kit (21) (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 3M Whatman filter paper, dried, and exposed to Kodak X-O mat film overnight at −70°C.

In Vitro Effect of Uric Acid and Selective COX-2 Inhibitor on Smooth Muscle Cell Proliferation

Rat vascular smooth muscle cells (RVSMC) were purchased from American Type Culture Collection (CRL-2018) and cultured in DMEM supplemented with 10% FBS, gentamicin (G418, 50 mg/ml), and L-glutamine. After cells were grown to 70% confluence in multiwell plates (Beckton Dickinson, Franklin Lakes, NJ), the culture medium was changed into serum-free media for 24 to 48 h before each experiment. After synchronization of cell growth, cells were washed three times with HBSS and exposed to uric acid (3 mg/dl) (Sigma, St. Louis, MO) for 6, 24, 48, and 72 h. At each time point, the number of cells was counted by hemocytometer and Coulter Counter. Alternatively, cell number was determined using CellTiter Proliferation Assay (Promega, Madison, WI). Cell proliferation was also assessed by

measuring ^{3}H-thymidine uptake (22). The effect of a selective COX-2 inhibitor (NS398 [10 μM/L]; Cayman Chemical, Ann Arbor, MI) and thromboxane A2 (TXA-2) receptor antagonist (SQ-29548 [2.5 μM/ml]; Biomol, Plymouth Meeting, PA) on uric acid-induced SMC proliferation was also evaluated. At the concentrations of all reagents used there was no evidence for cytotoxicity as documented by trypan blue staining and LDH release.

Statistical Analysis

All data are presented as mean $\pm$ SD. Differences in the various parameters between groups were evaluated by two-way ANOVA followed by correction for multiple comparison. Differences in parameters at each time point after RK surgery and drug administration were compared by paired t test. The relation between variables was assessed by Pearson correlation analysis. Significance was defined as $P < 0.05$.

Results

Hyperuricemia Induces Hypertension and Renal Hypertrophy in the Remnant Kidney Model

Baseline uric acid levels averaged 1.1 mg/dl in all four groups (Figure 1). In RK rats, a transient increase in uric acid was observed (2 wk, 2.7 ± 0.6 mg/dl; $P < 0.05$), but levels
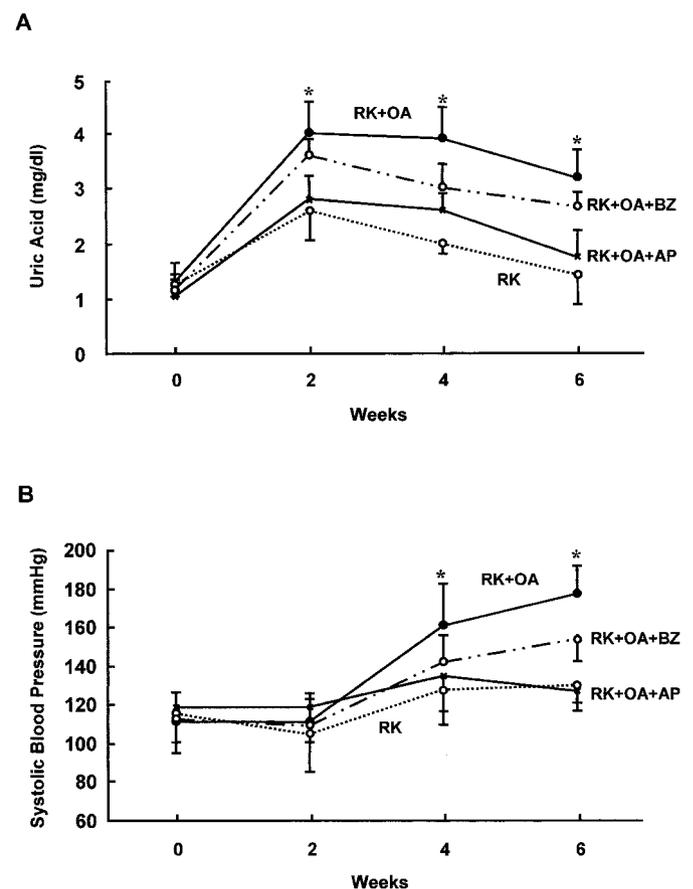
Figure 1. Changes in serum uric acid (A) and BP (B) in the remnant kidney (RK; ···○···), remnant kidney + oxonic acid (RK+OA; ●), remnant kidney + oxonic acid + allopurinol (RK+OA+AP; −×−), and remnant kidney + oxonic acid + benziodarone (RK+OA+BZ; ··−○−··) rats. *$P < 0.05$ *versus* RK and RK+OA+AP.

returned to baseline by week 6 (1.4 ± 0.5 mg/dl). In contrast, RK+OA rats developed more severe and persistent hyperuricemia (2 wk, 4.0 ± 0.6 mg/dl; 6 wk, 3.2 ± 0.6 mg/dl; $P < 0.01$ versus RK alone at 2 and 6 wk).

This rise in serum uric acid in RK+OA rats was followed by an elevation of BP at 4 wk compared with RK rats alone (Figure 1B). Allopurinol, a xanthine oxidase inhibitor, significantly decreased the level of serum uric acid and prevented the development of hypertension. Benziodarone (a uricosuric agent) only partially reduced uric acid and had a lesser effect on BP (Figure 1).

During the study period, the mean daily intake of food was similar among groups (RK versus RK+OA versus RK+OA+AP versus RK+OA+BZ, 24 ± 0.4 versus 21 ± 0.5 versus 22 ± 0.6 versus 22 ± 0.5 g/kg). Despite comparable body weight gain, remnant kidney weight (RKW) and RKW/ body weight (BW) at 6 wk were significantly higher in RK+OA rats, suggesting more severe renal hypertrophy (Table 1). The percent increase in RKW/BW in RK+OA rats was 1.6-fold compared with RK rats (RK+OA versus RK, 380.4 ± 37.3 versus 241.9 ± 20.5%; $P < 0.05$). The renal hypertrophy induced by oxonic acid was significantly prevented by the administration of allopurinol or benziodarone (Table 1).

Hyperuricemia Accelerates Renal Progression in the Remnant Kidney Model

Baseline levels of serum creatinine were comparable in four groups (RK versus RK+OA versus RK+OA+AP versus RK+OA+BZ, 0.72 ± 0.01 versus 0.69 ± 0.04 versus 0.75 ± 0.05 versus 0.70 ± 0.03 mg/dl). At 6 wk of OA administration, RK+OA rats displayed worse renal function with high serum creatinine compared with the RK alone group (1.44 ± 0.08 versus 1.23 ± 0.13 mg/dl, RK+OA versus RK; $P < 0.05$). Administration of allopurinol or benziodarone prevented the increase in serum creatinine (RK+OA+AP, 1.12 ± 0.15 mg/ dl; RK+OA+BZ, 1.23 ± 0.15 mg/dl; $P < 0.05$ versus RK+OA, respectively).

After renal ablation, RK rats showed a progressive increase in urinary protein excretion. The administration of OA in RK rats resulted in greater proteinuria at 4 and 6 wk (Figure 2) compared with the RK rats. Both allopurinol and benziodarone
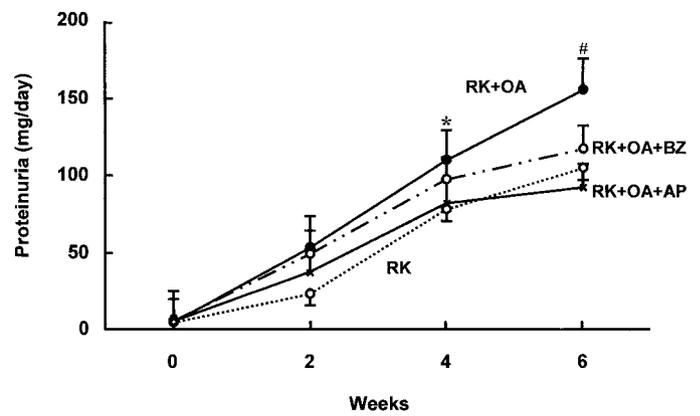

Figure 2. Changes in proteinuria in the RK (···○···), RK+OA (-●-), RK+OA+AP (-×-), and RK+OA+BZ (··-○-··) rats. *$P < 0.05$ versus RK and RK+OA+AP; #$P < 0.05$ versus RK, RK+OA+AP and RK+OA+BZ.

prevented the increase in proteinuria induced by OA (Figure 2). Allopurinol was especially effective and reduced proteinuria to the same level as that observed in the RK model alone.

RK rats displayed classic evidence of progressive renal disease with focal glomerulosclerosis and interstitial fibrosis. RK+OA rats showed similar histologic features; in particular, there was no evidence for tubular obstruction from crystals. Glomerulosclerosis was more common in RK+OA rats compared with RK rats (24.2 ± 2.5 versus 17.5 ± 3.4%; $P < 0.05$) (Figure 3). Interstitial fibrosis was also greater in RK+OA rats compared with RK alone (1.89 ± 0.45 versus 1.52 ± 0.47; $P < 0.05$) (Figure 3). Allopurinol prevented both the increase in glomerulosclerosis and interstitial fibrosis. In contrast, the uricosuric agent, benziodarone, was able to prevent the increase in glomerulosclerosis but not the increase in interstitial fibrosis in RK+OA rats (Figure 3).

Hyperuricemia Induces Severe Preglomerular Vascular Disease in RK Rats

The preglomerular arterial vessels (arcuate artery, interlobular artery, and afferent arteriole) of RK+OA rats showed dramatic differences compared with control RK rats (Figure 4,

Table 1. Changes in body weight (BW) and remnant kidney weight (RKW) at 6 wk[a]

	RK (n = 4)	RK+OA (n = 5)	RK+OA+AP (n = 4)	RK+OA+BZ (n = 4)
Baseline BW (g)	202.3 ± 13.0	206.1 ± 1.3	192 ± 13.1	195.6 ± 6.0
Final BW[b] (g)	358.4 ± 35.0	336.6 ± 23.2	338.1 ± 18.4	345.9 ± 23.2
Final RKW[b] (g)	1.31 ± 0.05	1.47 ± 0.11[e]	1.29 ± 0.28	1.30 ± 0.28
Final RKW/BW[b] (g/kg)	3.7 ± 0.2	4.2 ± 0.4[e]	3.8 ± 0.6	3.7 ± 0.6
Increase in RKW (%)[c]	241.9 ± 20.5	380.4 ± 37.3[e]	256.8 ± 35.7	236.2 ± 28.4

[a] OA, oxonic acid; AP, allopurinol; BZ, benziodarone. Data are expressed as mean ± SD.
[b] BW and RKW after 6 wk of RK operation.
[c] (RKW/BW at 6 wk)/(Baseline RKW[c]/BW) × 100.
[d] Baseline RKW = Baseline right kidney weight − (weight of upper and lower poles of left kidney).
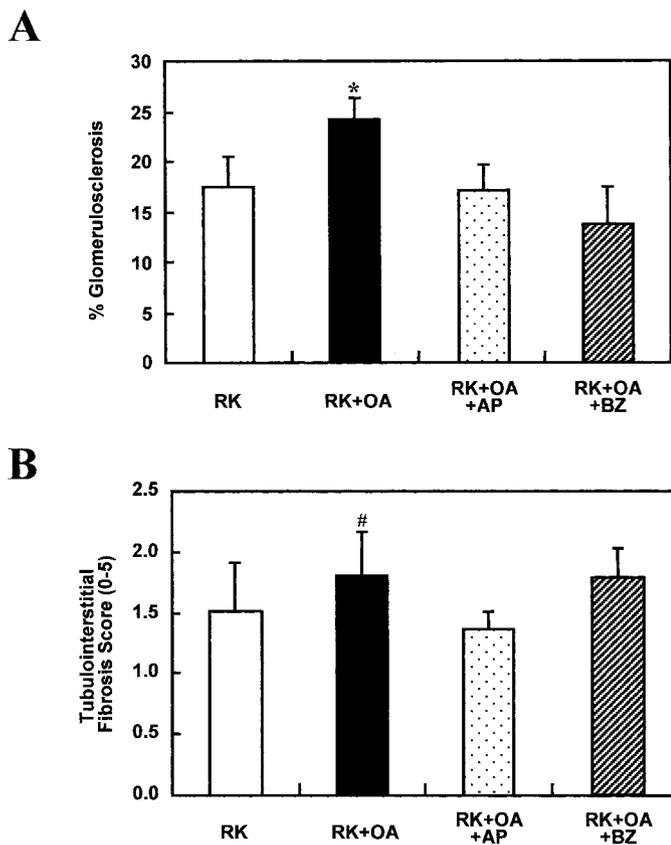[e] $P < 0.05$ versus RK, RK+OA+AP, and RK+OA+BZ.

A

B

Figure 3. Comparison of renal scarring. (A) glomerulosclerosis; (B) tubulointerstitial fibrosis in the RK, RK+OA, RK+OA+AP, and RK+OA+BZ rats. $*P < 0.05$ *versus* RK, RK+OA+AP, and RK+OA+BZ; $^{\#}P < 0.05$ *versus* RK and RK+OA+AP.

Figure 4. Morphology of preglomerular vessels in the RK and RK+OA rats. Compared with RK rats (A, ×400, PAS; D, ×400, PCNA+α-SMA double-staining), there was marked wall thickening with smooth muscle cell (SMC) proliferation in RK+OA rats (B, ×400, PAS; E, ×400, PCNA+α-SMA double-staining). Occasional arteries in RK+OA rats showed an increase in SMC number, migration of SMC into intima consistent with an obliterative arteriopathy (C, ×400, PAS). Perivascular adventitial fibrosis was also prominent in RK+OA rats (F, ×400, Collagen III immunostaining).

A through C). RK+OA rats showed a marked increase in vessel wall thickness as well as an increase in smooth muscle cell number (Figure 4B). In some RK+OA rats, the arterial vessels showed changes consistent with an obliterative arteriopathy (Figure 4C). Double staining of PCNA and α-SMA documented the presence of numerous proliferating SMC in the RK+OA group compared with control RK rats (Figure 4, D and E).

Periarterial adventitial fibrosis was also prominent in RK+OA rats (Figure 4F) in association with more cell infiltration. Quantification of the proliferating smooth muscle cell number (PCNA/α-SMC+cells) and medial wall thickness of the various preglomerular vessels is shown in Table 2. Another characteristic finding in preglomerular vessels was the infiltration of inflammatory macrophages assessed by ED-1$^+$ cells, especially in subendothelial and adventitial areas. There were also some ED-1$^+$ cells in media.

Allopurinol significantly prevented the SMC proliferation and wall thickening of afferent arteriole and interlobular artery in RK-OA rats (Table 2). In contrast, benziodarone did not show any effect on SMC proliferation. Interestingly, the number of endothelial cells in the interlobular and arcuate arteries was also increased with OA administration (Table 2) and was not affected by the administration of allopurinol or benziodarone.
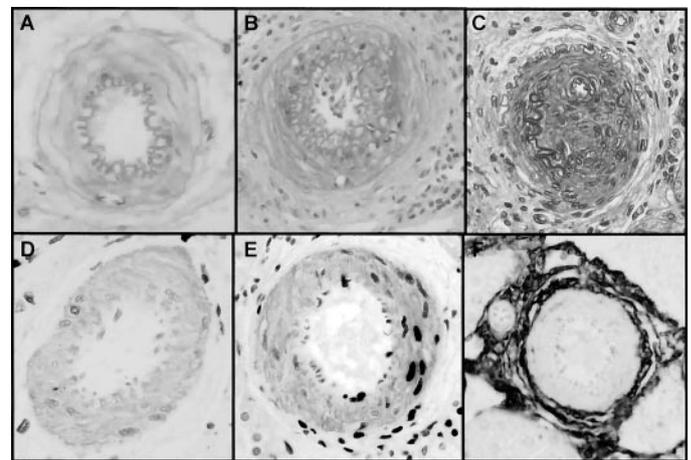
Role of BP in the Vascular Changes Induced by Hyperuricemia

Because the RK+OA rats had higher systolic BP compared with the control RK rats (Figure 1), we hypothesized that the vascular injury observed in the RK+OA rats might be attributed to vascular injury induced by the higher pressure. To differentiate the effect of hyperuricemia-induced elevation in BP from hyperuricemia itself on SMC proliferation, we evaluated the changes in the preglomerular vessels in a historic RK control group with comparable BP to RK+OA rats ($n = 6$; RK+OA *versus* historic RK with high BP, 177.8 ± 20.1 *versus* 175.7 ± 45.8 mmHg; $P = $ NS) and body weights (336.6 ± 23.2 *versus* 362.5 ± 55.4 g; $P = $ NS) (17,23). As shown in Figure 5, the number of SMC of preglomerular arteries in RK+OA rats was higher compared with the RK rats with comparable BP. This suggests that the increase in BP observed in RK+OA rats does not account for the differences in vascular injury observed.

Hyperuricemia Activates the RAS and COX-2 Systems in the RK Model

We have previously reported that hyperuricemia increases juxtaglomerular renin expression in normal rats and in rats treated with cyclosporine (9–11). We have also shown that preglomerular arteriopathy in hyperuricemic rats can be prevented by treatment with either ACE inhibitors or angiotensin II type 1 receptor (AT1) blockers (10). We therefore investigated whether renin was increased by hyperuricemia in the RK model. Consistent with the other studies, we found increased renin expression in RK+OA rats *versus* RK alone (RK+OA *versus* RK, 39.2 ± 7.39 *versus* $31.2 \pm 4.48\%$ of glomeruli with juxtaglomerular renin staining; $P < 0.05$). Both allopurinol

Table 2. Changes in macrovasculature in RK and OA-treated RK rats[a]

	RK	RK+OA	RK+OA+AP	RK+OA+BZ
Afferent Arteriole				
number of SMC (per cross-section)	4.0 ± 0.4	6.7 ± 1.6[b]	4.0 ± 0.5	6.2 ± 0.8
number of EC (per cross-section)	2.8 ± 0.7	3.1 ± 0.6	3.1 ± 0.2	3.3 ± 0.4
wall area (μm^2)	231.3 ± 21.8	269.5 ± 13.8[b]	215.4 ± 10.5	257.3 ± 31.9
Interlobular Artery				
number of SMC (per cross-section)	8.1 ± 1.0	13.2 ± 2.2[b]	9.8 ± 1.1	12.6 ± 4.2
number of EC (per cross-section)	6.7 ± 0.6	7.9 ± 0.7[c]	8.3 ± 0.4	8.4 ± 1.9
wall area (μm^2)	659.3 ± 203.2	1090.0 ± 224.5[b]	670.4 ± 253.0	983.7 ± 310.8
Arcuate Artery				
number of SMC (per cross-section)	17.8 ± 1.7	30.8 ± 7.7[c]	25.4 ± 1.0	27.8 ± 7.8
number of EC (per cross-section)	9.5 ± 1.1	14.5 ± 2.4[c]	13.9 ± 1.2	15.5 ± 2.6
wall area (μm^2)	3058 ± 847.9	5737.6 ± 3206.9[b]	3110.2 ± 1532.9	5935.1 ± 2916.6

[a] Data are expressed as mean ± SD. SMC, smooth muscle cell, EC, endothelial cell.
[b] $P < 0.05$ *versus* RK and RK+OA+AP.
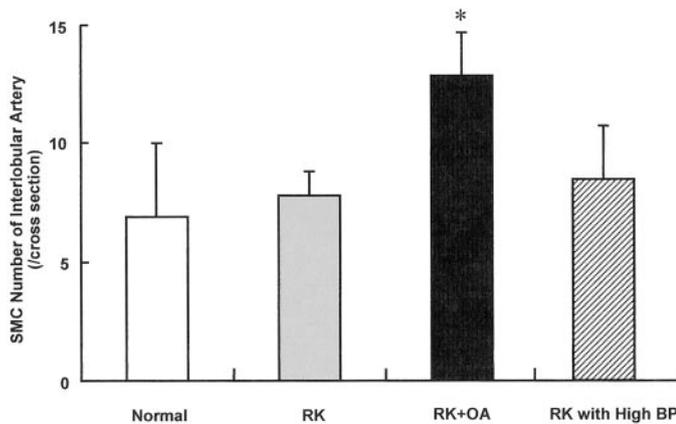[c] $P < 0.05$ *versus* RK.

Figure 5. BP-independent effect of hyperuricemia on SMC proliferation. The number of SMC of interlobular arteries in RK+OA rats was significantly higher compared with the RK rats with comparable BP. *$P < 0.05$ *versus* normal, RK, and RK with high BP.

Figure 6. Correlation of serum uric acid with renin expression in renal cortex.

(20.2 ± 7.93%) and benziodarone (27.8 ± 8.35%) significantly reduced renal renin expression induced by OA ($P < 0.005$). There was a strong correlation between renin content of individual rats with the serum uric acid ($r^2 = 0.52$; $P < 0.05$) (Figure 6).

A major regulator of renin expression is cyclooxygenase 2 (COX-2) (13). We therefore examined the expression of COX-2 in this model. In normal rat cortex, COX-2 immunoreactivity is present almost exclusively in macula densa cells (14). Consistent with the studies by Wang *et al.* (14) in the RK model, COX-2 expression was increased in RK rats compared with normal rats (0.10 ± 0.01 *versus* 0.24 ± 0.05%; $P < 0.05$), especially in the tubular cells of the cortical thick ascending limb. RK+OA rats displayed even more COX-2 expression that involved not only the cortical thick ascending limb but also smooth muscle cells in afferent arterioles and interlobular arteries (Figure 7).
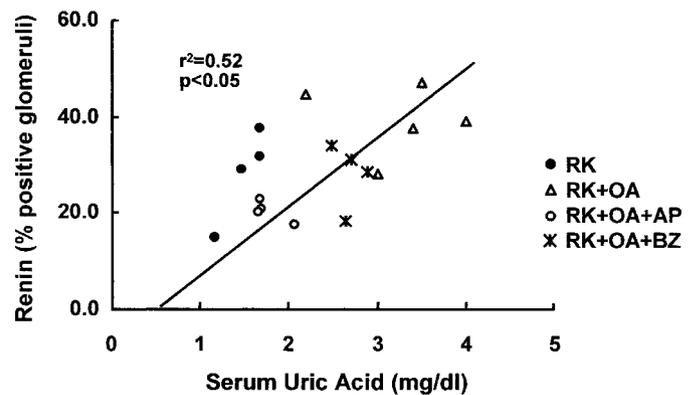
The increase in COX-2 expression was quantified by computer image analysis of the cortex. RK+OA rats showed significantly greater COX-2 expression than RK rats alone (0.24 ± 0.05 *versus* 0.70 ± 0.36%; $P < 0.05$), and both allopurinol (0.27 ± 0.18%) and benziodarone (0.48 ± 0.26%) could prevent the increase in COX-2 expression. Within individual rats, the uric acid levels correlated directly with the degree of COX-2 expression (Figure 8A). In turn, the COX-2 expression also correlated with the increase in juxtaglomerular renin (Figure 8B). These studies demonstrate that both the COX-2 and RAS are induced with hyperuricemia in the RK model.

Uric Acid Stimulates VSMC Proliferation via the COX-2 Pathway

The observation that uric acid induced vascular disease independently of BP suggested a potential direct effect of uric acid on smooth muscle cells. Consistent with *in vivo* finding, stimulation with uric acid (3 mg/dl; Sigma, St. Louis, MO)
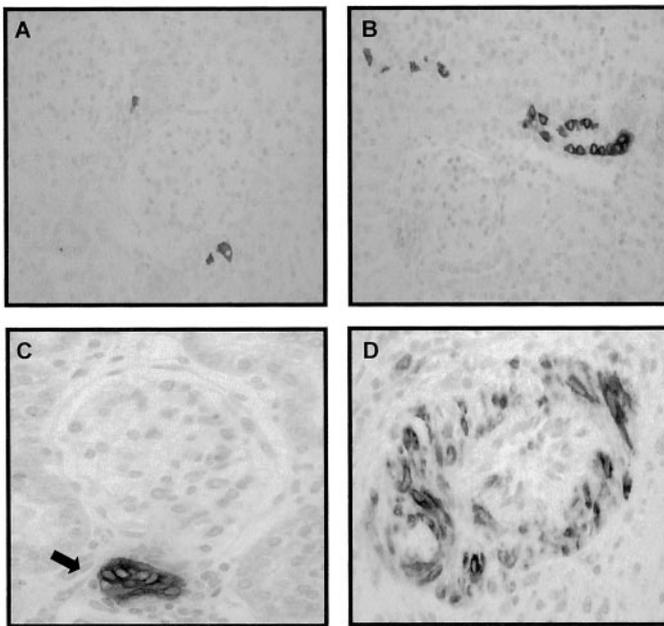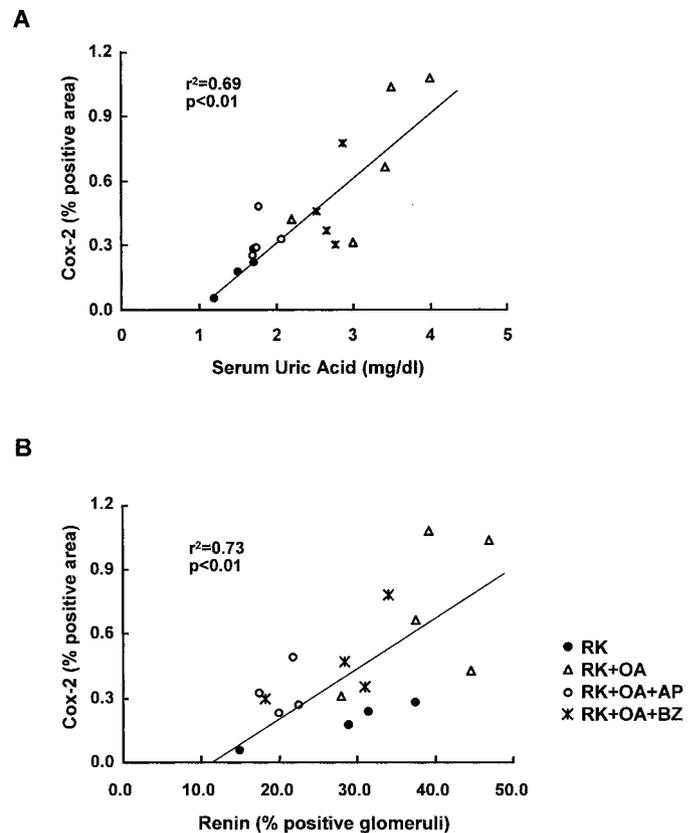
Figure 7. Expression of COX-2 in the RK and RK+OA rats. In normal rats and RK rats, COX-2 immunoreactivity is present almost exclusively in macula densa cells (A, RK rats, ×200), whereas RK+OA rats displayed more COX-2 expression that involved not only the tubular cells of cortical thick ascending limb but also smooth muscle cells in afferent arterioles (C, ×400, arrow) and interlobular arteries (D, ×400).

Figure 8. Correlation of COX-2 expression with serum uric acid (A) and renin expression (B).

resulted in time-dependent increase in rat aortic smooth muscle cell (VSMC) number (Figure 9A).

We observed *de novo* expression of COX-2 in the vascular smooth muscle wall of the RK+OA rats; we therefore hypothesized that COX-2 expression induced by uric acid may play an important role in the development of arteriopathy observed in the RK+OA rats. Consistent with this observation, we found that uric acid could induce a marked increase in COX-2 mRNA in cultured VSMC (Figure 9B), whereas comparable COX-1 mRNA expression was noted. To determine the role of COX-2 in the SMC proliferation, we examined the effect of a selective COX-2 inhibitor (NS398 [10 μM/L]: Cayman Chemical, Ann Arbor, MI) on SMC number. NS398 significantly inhibited uric acid–induced SMC proliferation (Figure 9C), suggesting the possible role of prostaglandins generated by COX-2 in uric acid–mediated SMC proliferation. Importantly, NS398 did not affect cell number in the absence of uric acid stimulation.

Previous studies have reported that angiotensin II-induced VSMC proliferation is mediated by COX-2–generated thromboxane (TX-A2) (24). TX-A2 was therefore hypothesized to be a likely mediator of the proliferative response of VSMC to uric acid. VSMC were incubated with uric acid (3 mg/dl) in the absence or presence of the selective TXA2 antagonist (SQ-29548 [2.5 μM/ml]; Biomol, Plymouth Meeting, PA). SQ-29548 significantly reduced uric acid-induced SMC proliferation.

Discussion

Hyperuricemia is common in patients with renal disease, but it has almost never been considered a risk factor for progression (25). However, two studies have found that hyperuricemia is an independent risk factor for progression in IgA nephropathy (26,27). Furthermore, in a recent study of 6400 subjects with normal renal function, a serum uric acid of >8.0 mg/dl, when compared with a serum uric acid level of <5.0 mg/dl, was associated with a 2.9-fold increased risk for developing renal insufficiency within 2 yr in men and a 10.0-fold increased risk in women (28). This increased relative risk was independent of age, body mass index, systolic BP, total cholesterol, serum albumin, glucose, smoking, alcohol use, exercise habits, proteinuria, and hematuria (28). Indeed, an elevated uric acid was more predictive for the development of renal insufficiency than proteinuria (28). We therefore investigated the effects and the action mechanism of hyperuricemia on the progression of renal disease in the classic RK model in rats.

Serum uric acid increased in RK rats but was greater in rats administered 2% OA (the uricase inhibitor). The serum uric acid level peaked at 2 wk in both RK and RK+OA rats and then gradually decreased over the ensuing 4 wk. The fall in uric acid in the RK and RK+OA rats may reflect enhanced extrarenal (mainly enteric) excretion and depressed production of uric acid (reduced xanthine oxidase activity) in chronic renal failure as reported by Vaziri *et al.* (12).
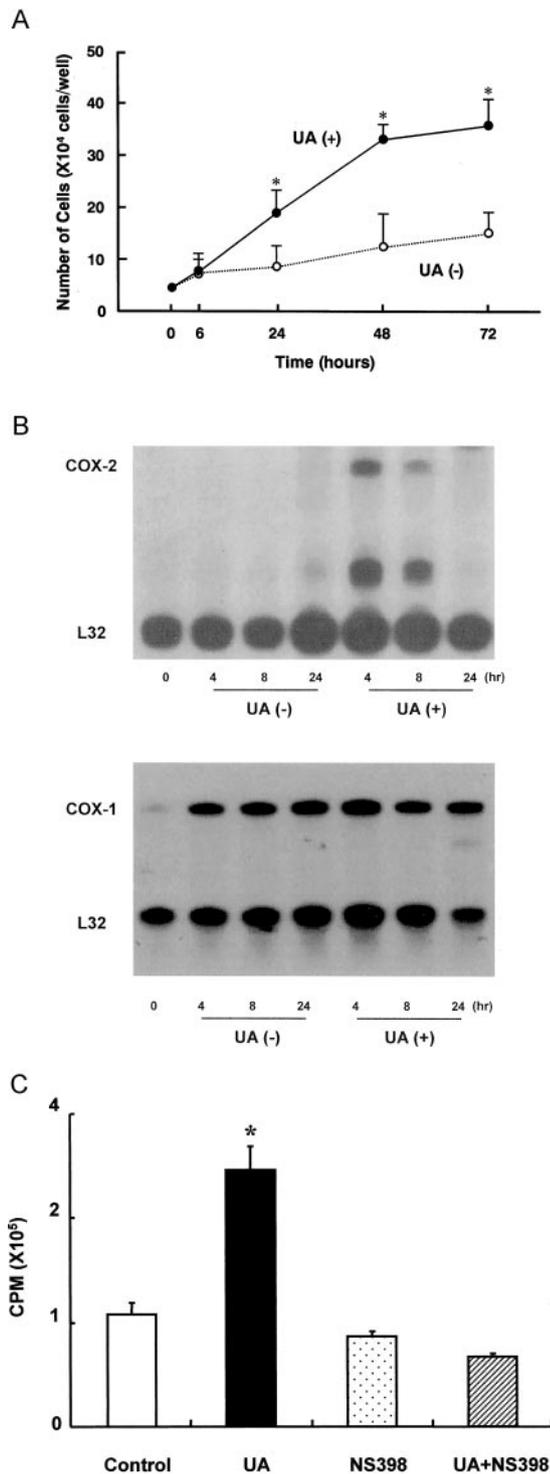
Figure 9. Uric acid–induced VSMC proliferation via COX-2 pathway. Stimulation with uric acid (3 mg/dl, ●) resulted in time-dependent increase in rat aortic smooth muscle cell (VSMC) number (A, $n = 6$) compared with control media without uric acid (○). A marked increase in COX-2 mRNA expression was observed in cultured VSMC at 4 and 8 h after stimulation with uric acid (B, $n = 3$) in contrast to no change in COX-1 mRNA expression. Selective COX-2 inhibitor (NS398, 10 μM/L) significantly inhibited uric acid-induced SMC proliferation, whereas NS398 did not affect ^{3}H-thymidine uptake in the absence of UA stimulation (C, $n = 6$ per group). *$P < 0.01$ *versus* other groups.

There were many new findings in this study. The major novel finding was that modest hyperuricemia markedly exacerbated renal progression. Specifically, hyperuricemic RK+OA rats showed more renal hypertrophy, hypertension, proteinuria, impaired renal function, and greater glomerulosclerosis and interstitial fibrosis. The renal injury did not appear to be mediated by urate crystal deposition, as there were no features of tubular obstruction consistent with intrarenal urate crystal deposition.

One of the mechanisms by which uric acid may have exacerbated the renal injury may be activation of the RAS, which has been shown to be an important mediator of progression in renal disease, not only by its hemodynamic effects to increase systemic and glomerular pressure, but also by its direct fibrogenic effect on renal and vascular cells. We have previously reported that the administration of OA to normal rats increases juxtaglomerular renin expression and that treatment with enalapril can control BP, improve the arteriolopathy, and prevent the renal injury in this model (9,10). We also found that OA treatment in a rat model of cyclosporine nephropathy results in hyperuricemia, increased renin expression, and accelerated progression (11). Saito *et al.* (29) have also reported that serum uric acid levels in humans also correlate with plasma renin activity. Although we did not measure plasma renin activity, we did document an increase in renal renin expression in hyperuricemic rats that correlated with serum uric acid. Furthermore, prevention of the hyperuricemia in OA-treated rats with allopurinol, and to a lesser extent with benziodarone, resulted in lower renin levels in oxonic acid-treated rats in concert with less renal injury.

A second new finding in the study was the observation that hyperuricemic RK rats developed severe preglomerular vasculopathy. Although thickening of the afferent arterioles had been noted in previous studies of hyperuricemic rats with normal renal function (10), hyperuricemia was associated with a severe vasculopathy in the RK model. The preglomerular vessels showed thickening with an increase in SMC number and macrophage infiltration in subendothelial, medial, and adventitial areas. These vascular changes, which occasionally resulted in an obliterative arteriopathy, could potentiate the renal injury by causing ischemia to the postglomerular circulation (30). The reduction in lumen could also provide a stimulus for the increased renin expression observed (31) and might also contribute to the development of the marked hypertension in these rats (32).

The mechanism for the development of the vascular disease was studied. We have previously reported (10), as has Rao *et al.* (33), that uric acid may directly stimulate VSMC proliferation. In this study, we identified a new mechanism involving COX-2. Specifically, we found that rat aortic VSMC showed *de novo* expression of COX-2 mRNA after incubation with uric acid. Incubation of the SMC with either a COX-2 inhibitor or with a TX-A2 receptor inhibitor could prevent the proliferation in response to uric acid. COX-2 was also shown to be expressed *de novo* in the preglomerular vessels of RK+OA rats, and its expression correlated both with the uric acid levels and with the degree of SMC proliferation. These studies thus

suggest a critical role for uric acid-mediated, COX-2–generated thromboxane in VSMC proliferation in this model. Interestingly, COX-2 has also been recently shown to be important in the mechanism by which angiotensin II stimulates VSMC proliferation (24).

In addition to COX-2, it is likely that angiotensin II is also contributing to the vasculopathy. As mentioned, renin was also increased in this model and correlated with the COX-2 content. It is unclear if the increased renin reflects direct effects of uric acid on the juxtaglomerular cells or an indirect effect via stimulation of COX-2 in the macula densa and arterioles (13) or an indirect effect related to the development of vascular disease with reduced renal perfusion. Regardless, the generation of angiotensin II may result in VSMC proliferation and hypertrophy (24) as well as inflammatory cell infiltration (34). We have previously reported that the vasculopathy in normal rats with OA-induced hyperuricemia can be largely prevented by blocking the RAS, and we have also found that uric acid–mediated VSMC proliferation can be partially inhibited by blocking the AT1 receptor (10). It is thus likely that both angiotensin II and COX-2 are involved in the vascular proliferation and inflammation observed in our model. *In vivo* studies evaluating the effect of inhibition of the RAS or COX-2 in this model of progressive renal disease with hyperuricemia would provide a more direct proof of the importance of these systems.

Importantly, the changes in preglomerular vessels observed in our RK+OA rats were not solely a consequence of an elevation in systemic BP (Figure 5), as evidenced by more prominent vascular changes in preglomerular vessels of hyperuricemic RK rats when compared with historic RK rats with comparable systemic BP but significantly lower uric acid level. This finding suggests uric acid–induced vasculopathy is independent of elevated systemic BP. Additionally, although increased COX-2 expression was reported in arteries from hypertensive rats (35), increased cortical expression of COX-2 induced by renal ablation or angiotensin II infusion has been shown to be BP-independent (36).

An interesting observation was that allopurinol was markedly effective at lowering uric acid and preventing OA-induced renal dysfunction, proteinuria, hypertension, vascular disease, renal hypertrophy, and renal scarring. The benefit of allopurinol correlated with its ability to lower uric acid. However, it is possible that some of the benefit may also relate to the known ability of allopurinol to prevent oxidant formation. Benziodarone, a uricosuric agent, was less effective at lowering uric acid and only partially reduced the increase in renin expression, and this may account for its partial benefit on the disease process. Interestingly, it was more effective at blocking the glomerular changes (proteinuria and glomerulosclerosis) than the vascular or interstitial changes. This may reflect that the glomerular changes may be mediated more by a threshold phenomenon related to the uric acid level. Another possibility is that the interstitial injury was not blocked as effectively due to the effects of increased urinary uric acid on tubular cells that occurred as a consequence of the uricosuric action of benziodarone.

In summary, we have identified uric acid as a new and potentially important mediator of renal progression in the remnant kidney model of progressive renal disease. Hyperuricemia increased systemic BP, proteinuria, renal dysfunction, and progressive renal scarring. Hyperuricemia also induces vascular disease via a COX-2–dependent pathway. Although one must be cautious in the interpretation of animal models to human disease, these studies provide a mechanism to explain recent epidemiologic data showing that uric acid is an independent risk factor for renal progression (26–28). Furthermore, our studies suggest hyperuricemia may be one of the key and previously unknown mechanisms for the activation of the renin angiotensin and COX-2 systems in progressive renal disease. Studies are needed to determine if early treatment of hyperuricemia can slow progressive renal disease in humans.

Acknowledgments

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