Effect of AT₂ receptor blockade on the pathogenesis of renal fibrosis

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Morrissey, Jeremiah J., and Saulo Klahr. Effect of AT₂ receptor blockade on the pathogenesis of renal fibrosis. Am. J. Physiol. 276 (Renal Physiol. 45): F39–F45, 1999.—Cellular and molecular events contributing to tubulointerstitial fibrosis of the kidney during obstructive nephropathy are driven in large part through increased angiotensin II levels in the obstructed kidney. Angiotensin converting enzyme inhibition or AT₁ receptor antagonism have been shown to ameliorate the fibrosis of the kidney due to obstruction of the ureter. In this investigation, we determine the effects of the AT₂ receptor antagonist PD-123319 on pathophysiologcal events within the kidneys of rats with unilateral ureteral obstruction. Treatment with PD-123319 was found to exacerbate the increase in interstitial volume and collagen IV matrix score of the ureteral obstructed kidney. Monocyte/macrophage infiltration of the injured kidney was no different between treated and untreated animals. The AT₂ receptor antagonist did, however, inhibit apoptosis of tubular cells, α-smooth muscle actin expression within the interstitium, and p53 expression in the ureteral obstructed kidney. These results suggest that angiotensin II operating through the AT₂ receptor exerts an antifibrotic effect on the kidney during obstructive nephropathy in opposition to the profibrotic effects of angiotensin II operating through the AT₁ receptor.

angiotensin II; tubulointerstitial fibrosis; α-smooth muscle actin; monocytes/macrophages; apoptosis

INTERSTITIAL FIBROSIS is a common pathological feature associated with clinical (17, 24, 30) and experimental (4, 24) renal diseases of different etiology. Unilateral ureteral obstruction (UUO) is a well-characterized model of progressive tubulointerstitial fibrosis (9, 11, 21, 32). The pathophysiology of obstructive nephropathy is initiated and maintained in large part by increased levels of intrarenal angiotensin II (8, 11, 27). The autocrine, paracrine, and endocrine actions of angiotensin II contribute to the progression of renal disease in many experimental models, as evidenced by the beneficial effect of angiotensin converting enzyme (ACE) inhibitors on the course of the disease (3, 12, 13, 23).

Angiotensin II alters cell function by signaling through two distinct cell surface receptors designated AT₁ and AT₂. Several recent studies have shown that many of the pathophysiological alterations of renal diseases in general (reviewed inRefs. 5 and 7) and obstructive nephropathy in particular (8) are ameliorated by treating experimental animals with AT₁ receptor antagonists. The AT₁ receptor is relatively abundant in adult kidney, whereas the AT₂ receptor is the predominant receptor present in the fetal and early neonatal kidney (1, 10, 25, 31). Although the AT₁ receptor is the predominant receptor type for angiotensin II actions in the adult kidney, the AT₂ receptor impacts renal function by controlling pressure natriuresis (15) and decreasing cGMP production in sodium-depleted rats (33). Furthermore, in addition to regulating organo-genesis in fetal organs (1, 10, 25, 31), the AT₂ receptor appears to regulate cell proliferation in experimental models of tissue injury (14, 22) or cells in culture (6, 34, 36). During obstructive nephropathy, interstitial fibroblasts proliferate and contribute, in part, to the increased interstitial volume seen in this setting (8, 9, 20, 21). Also during obstructive nephropathy, there is cell loss through apoptosis (35) and an increase in the expression of p53 (2, 19), which regulates apoptosis and other aspects of cell proliferation (29). To examine the role of the AT₂ receptor in obstructive nephropathy, we administered the compound PD-123319, an inhibitor of the AT₂ receptor, to rats with UUO. The influence of this pharmacological treatment on cellular and molecular parameters of tubulointerstitial fibrosis due to obstructive nephropathy was then assessed.

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METHODS

Animal preparation. Female Sprague-Dawley rats weighing 180–210 g were used (8, 9, 11, 20). All animals underwent UUO under halothane anesthesia; the left ureter was ligated with 4-0 silk at two points and then severed between the ligatures to prevent retrograde urinary tract infection. Animals were fed Purina rat chow ad libitum along with tap water (n = 5 animals) or water containing 12.5 mg/l PD-123319 (n = 5) (Research Biochemicals International, Natick, MA). This dose was used by other investigators in rats, with significant functional consequences on natriuresis (15). Animals were killed under pentobarbital anesthesia 5 days after UUO. It has been shown in rats (8, 9, 11, 12, 19, 20, 27, 35) that by 5 days of chronic ureteral obstruction, inflammatory and noninflammatory processes develop in a quantifiable and reproducible fashion in the renal parenchyma. The kidneys were perfused in situ with ice-cold Hanks’ balanced salt solution (HBSS) until free of blood, then quickly removed. A 2-mm coronal section of each kidney was placed into the fixative Histochoice (Amresco, Solon, OH). The cortex of the remainder of the kidney was rapidly dissected on a cold glass plate, and the total RNA was quickly extracted with Ultraprep RNA (Biotecx Laboratories, Houston, TX).

In other groups, animals were killed after 1, 3, or 5 days of UUO or after treatment with enalapril (200 mg/l) or SC-51316 (20 mg/l) in the drinking water for the 5 days of UUO as previously described (8, 11, 12). The total RNA of the cortex of

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both kidneys of these rats was then prepared as described above.

Morphometric analysis of interstitial volume, collagen IV, or α-smooth muscle actin matrix score and monocyte/macrophage quantitation. Both the contralateral and ureteral obstructed kidneys from the same rat were embedded in one paraffin block. For all morphological evaluations, the observer was naive to the treatment (or lack thereof) of the animals prior to death and embedding of the coronal slices of kidney. Sections were deparaffinized with AmeriClear (Baxter Health Care, Deerfield, IL) and rehydrated into HBSS.

A standard point count method adapted in our laboratory (8, 9, 11, 20) was used to quantitate the volume of the renal interstitium (Vvint). Under high magnification (>400), consecutive nonoverlapping fields were photographed from each section of renal cortex. A grid containing 117 points (13 × 9) was placed on each 5 × 3½-inch photograph, and a total of 5 or 10 photographs (585 or 1,170 points) were evaluated for each kidney. The results from the kidneys of five separate animals were then averaged. In general, five photos of the contralateral unobstructed kidney sections were examined, and 10 photos of the ureteral obstructed kidney sections were examined. The points overlaying the tubule basement membrane and interstitial space were counted, whereas points falling within large vessels, Bowman’s capsule, or within tubular and peritubular capillaries were considered outside the interstitium.

The matrix score for collagen type IV or α-smooth muscle actin expression in the renal cortical interstitium was determined as described previously (8, 9, 20). Twenty random, nonoverlapping ×200 fields of sections stained for collagen IV or for α-smooth muscle actin antibody were assigned scores of zero to three. A score of zero was assigned when the histological appearance was indistinguishable from that of a normal kidney processed at the same time. In normal or contralateral kidneys, collagen IV is confined to the basement membrane, whereas α-smooth muscle actin is confined to arterioles. Progressively higher scores were assigned, depending on the degree and amount of interstitium that reacted with antibodies specific for collagen IV or α-smooth muscle actin. The scores of the 20 fields were averaged for the score of that kidney. The scores of kidneys of five separate animals were then averaged.

Monocyte/macrophage infiltration was determined as the number of cells within the cortex that stained positively with the monoclonal antibody ED-1. As in the method described previously (8, 9, 20), the number of ED-1-positive cells within three randomly chosen ×200 fields from the same section were counted to arrive at the number of cells from that kidney. The number of ED-1-positive cells in the kidneys of five separate animals was calculated.

Quantitation of apoptosis. Paraffin sections of the kidneys with an obstructed ureter of rats that were untreated or treated with the AT2 receptor antagonist were deparaffinized and rehydrated in sterile phosphate-buffered saline. A kit from Boehringer Mannheim (Indianapolis, IN), which was based upon terminal deoxynucleotidyl transferase incorporation of fluorescein-dUTP and subsequent alkaline phosphatase-linked detection, was employed to visualize the nuclei of cells undergoing apoptosis. Sections were lightly counterstained with Mayers hematoxylin (Sigma Chemical, St. Louis, MO). The number of alkaline phosphatase-positive nuclei was counted in five nonoverlapping ×200 microscopic fields of kidney cortex and averaged for the value of one kidney. The number of kidneys of five separate animals were then averaged.

Quantitation of AT2 receptor and p53 mRNA. Oligo(dT)-primed cDNA was prepared from 4 µg of total RNA as described (19). The cDNA was used to amplify for the AT2 receptor mRNA or for p53 mRNA using primers specific for the rat sequences. For the AT2 receptor, primers (accession number D16840) were 5′-GAG TCC GCA TTT AAC TGC-3′ (sense) and 5′-CCA CTG AGC ATA TTT CTG AGG-3′ (antisense) to yield a 534-bp product, whereas for p53 (accession numbers LO703 and LO7181), the primers were 5′-CTG AGG TCG GCT CCG ACT ATA CCA TCT TCC-3′ (sense) from exon 6 and 5′-CTG ATT CGT CTC TCG GAA CAT CTC GAA GGC-3′ (antisense) from exon 9, yielding a 360-bp product. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, primers from the rat gene were used as follows: 5′-AA TCG ATC CTG CAC CAC CAA-3′ (sense) and 5′-CTA GCC ATA TTC ATT GCA ATC-3′ (antisense); this yielded a 515-bp product. The sequence of each PCR product was verified by cloning into the vector pCR11 (Invitrogen, San Diego, CA) and dideoxy chain termination sequencing. The RT-PCR products were quantitated by densitometry of photographs of ethidium bromide-stained agarose gels as described previously (8, 9, 11, 19, 20). The number of PCR cycles was optimized to measure the amount of mRNA in the linear range. The amount of AT2 or p53 mRNA was factored by the amount of mRNA of GAPDH. The GAPDH mRNA was not influenced by the treatment with the AT2 receptor antagonist, since the densitometry signal for GAPDH mRNA was the same, based upon the amount of total mRNA, regardless of treatment.

Statistical analysis. Data reported are the means ± SD of five separate animals. ANOVA was employed to determine statistical significance of the measured values (P ≤ 0.05).

RESULTS

AT2 mRNA levels. Although the amount of AT2 mRNA in the kidney precipitously declined during development (1, 10, 25, 31), there is a finite and demonstrable level of this mRNA in the kidney cortex of the adult rat (Fig. 1). There is a progressive decrease in the amount of the AT2 mRNA in both the ureteral obstructed kidney, to 3 ± 2% of normal, and the contralateral unobstructed kidney, to 1 ± 2% of normal, by 5 days of UUO. The decrease in AT2 mRNA was blunted by treating the animals with the ACE inhibitor enalapril for the duration of UUO (Fig. 1A). By 5 days of UUO the AT2 mRNA level was decreased to 76 ± 9% of normal in the kidney with an obstructed ureter and to 78 ± 5% in the contralateral kidney with continuous enalapril treatment of the animals. Treatment of the animals with the AT2 receptor antagonist PD-123319 similarly blunted the decrease in the AT2 receptor mRNA that occurs in both the contralateral and UUO kidneys of animals after 5 days of UUO without treatment (Fig. 1B). The amount of GAPDH mRNA was not changed by UUO or ACE inhibitor treatment or by specific receptor antagonist (Fig. 1). In the continuous presence of the AT2 receptor antagonist, the level of AT2 receptor mRNA was reduced to 53 ± 10% of normal in the obstructed kidney and 41 ± 13% in the contralateral kidney. Interestingly, treatment of rats with UUO with a specific AT1 receptor antagonist (SC-51316) was not found to prevent loss of the AT2 receptor mRNA due to UUO (not shown). There were four to five animals in each group in the studies employing enalapril and the receptor antagonists. The retention of AT2 receptor mRNA due to enalapril or PD-123319 treatment in each sample is significantly different from the results.
in samples from animals in the untreated state (P < 0.01 each).

Interstitial volume. During ureteral obstruction, there was an increase in interstitial volume (Table 1). This is reflective of what we (8, 9, 20, 25) and others (21, 32) have reported previously. The interstitial volume increased in untreated rats from an average of 9.5 ± 0.3% in the contralateral unobstructed kidney to 37 ± 1.9% in the UUO kidneys. In rats receiving the AT2 receptor antagonist there was no change in the interstitial volume of the contralateral unobstructed kidney (Table 1). There was, however, a significant (29%, P < 0.001) increase in the interstitial volume of the kidneys with an obstructed ureter, to 44.9 ± 2.0%, in rats given PD-123319. This increase was calculated by subtracting the interstitial volume of the contralateral kidney from that of the kidney with an obstructed ureter in treated and untreated rats and comparing those differences.

In a previous review of renal scarring (12), we suggested that histological observations indicated no semiquantitative effect of AT2 receptor inhibition on interstitial volume (12). The present detailed and quantitative point count method, however, uncovered a 29% increase in interstitial volume.

Interstitial matrix. A component of the increased interstitial volume is an overproduction of extracellular matrix proteins. Collagen type IV is normally confined to the basement membrane surrounding tubules, peritubular capillaries, and the parietal epithelial cells of Bowman's capsule (Fig. 2A). During ureteral obstruction, collagen IV is produced in excess, and this excess contributes to a widened basement membrane and to the expansion of the extracellular matrix surrounding interstitial cells (Fig. 2B). As we have shown previously (8, 9, 20; see also Table 1), the collagen IV matrix score was substantially increased in kidneys with an obstructed ureter compared with the unobstructed contralateral kidney. There was no demonstrable difference between the contralateral kidneys of untreated (Fig. 2A) or treated (Fig. 2C) rats. The ureteral obstructed kidney of rats treated with PD-123319 contained more collagen IV than untreated animals (in Fig. 2, compare B and D). The percentage increase in the collagen IV matrix score (10%) of the obstructed kidney of treated rats above the baseline of the contralateral kidneys was less than the increase in interstitial volume, but was still significant (P < 0.006).

Smooth muscle actin matrix score. Another factor contributing to the increased interstitial volume of the kidney cortex after ureteral obstruction is the proliferation of fibroblasts (8, 9, 20, 21). These fibroblasts subsequently differentiate into myofibroblasts as evidenced by the expression of α-smooth muscle actin (8, 9, 20). The interstitial volume of the cortex of the ureteral obstructed kidneys increased regardless of whether the animals were treated with the AT2 receptor antagonist PD-123319 (Table 1). Surprisingly, at day 5 of ureteral obstruction, the amount of interstitial α-smooth muscle

### Table 1. Effect of AT2 receptor inhibition on histological parameters of renal fibrosis

<table>
<thead>
<tr>
<th></th>
<th>Interstitial Volume, %</th>
<th>Collagen IV Matrix Score</th>
<th>α-Smooth Muscle Actin Matrix Score</th>
<th>Monocyte/Macrophage Infiltration</th>
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<tr>
<td>Untreated animals</td>
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<tr>
<td>Contralateral kidney</td>
<td>9.5 ± 0.3</td>
<td>0.34 ± 0.06</td>
<td>0.26 ± 0.06</td>
<td>6.8 ± 0.8</td>
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<td>Ureteral obstructed kidney</td>
<td>37.0 ± 1.9*</td>
<td>3.24 ± 0.18*</td>
<td>3.08 ± 0.25*</td>
<td>57.4 ± 7.8*</td>
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<tr>
<td>PD-123319-treated animals</td>
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<tr>
<td>Contralateral kidney</td>
<td>9.4 ± 0.4</td>
<td>0.36 ± 0.09</td>
<td>0.32 ± 0.05</td>
<td>8.0 ± 1.0</td>
</tr>
<tr>
<td>Ureteral obstructed kidney</td>
<td>44.9 ± 2.0*†</td>
<td>3.54 ± 0.15*†</td>
<td>1.40 ± 0.32†</td>
<td>59.6 ± 5.6</td>
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Values are means ± SD of 5 separate animals. Monocytes/macrophages were determined as the number of ED-1 positive cells per ×200 microscopic field of kidney cortex. *P < 0.001, ureteral obstructed kidney vs. contralateral kidney. †P < 0.001, ureteral obstructed kidney, treated, vs. untreated animals. ‡P < 0.006, ureteral obstructed kidney, treated vs. untreated animals.
actin expression was considerably less in the kidney cortex of animals treated with the AT$_2$ receptor antagonist (Fig. 3A) compared with untreated animals (Fig. 3B). The $\alpha$-smooth muscle actin matrix score of the AT$_2$ receptor antagonist-treated animals increased to only 38% that of the untreated animals after subtracting the value of the contralateral kidney in both treated and untreated animals (Table 1). This suggests that inhibition of the AT$_2$ receptor blunts myofibroblast differentiation.

**Monocyte/macrophage infiltration.** Another major contributor to the increased interstitial volume during ureteral obstruction is an infiltration of the kidney by lymphohematopoietic cells (8, 9, 20). There was no significant difference between treated or untreated rats in the number of monocytes/macrophages that infiltrated the kidney with ureteral obstruction (Table 1). Inhibition of the AT$_2$ receptor, therefore, did not prevent the migration of monocytes/macrophages into the renal parenchyma.

**p53 expression.** The increased cellularity of the renal interstitium during ureteral obstruction is more than likely the algebraic sum of fibroblast proliferation and monocyte/macrophage infiltration, balanced through loss of interstitial cells by apoptosis. The p53 protein is involved in apoptosis (29), and we found that p53 mRNA and protein are increased in the kidney with an obstructed ureter (19). We therefore measured p53 mRNA expression in the kidney cortex of rats with UUO that remained untreated or that were treated with the AT$_2$ receptor antagonist PD-123319. As shown in Fig. 4, p53 mRNA was dramatically increased 5 days after ureteral obstruction compared with the contralateral unobstructed kidney of untreated rats. The induction of p53 mRNA in the kidney with an obstructed ureter was significantly blunted when the AT$_2$ receptor was pharmacologically inhibited (Fig. 4). In animals with UUO that remained untreated, there was an $11.2 \pm 3.1$-fold induction of p53 mRNA by 5 days of UUO. This induction was significantly reduced to $2.7 \pm 0.6$-fold ($P < 0.008$) by PD-123319 treatment ($n = 3$). The amount of GAPDH mRNA within the cDNA pool of treated or untreated rats was not different (Fig. 4).
Apoptosis. The above results with respect to p53 mRNA expression suggest that PD-123319 might inhibit apoptosis. This was verified by labeling the ends of DNA strands with fluorescein-dUTP in the presence of terminal transferase. Figure 5 shows that numerous tubule cell nuclei are labeled by this procedure in the kidney of both the PD-123319-treated animal (Fig. 5A) and the untreated animal (Fig. 5B). There are considerably fewer apoptotic nuclei present in the kidney of AT2 receptor antagonist-treated rats (58 ± 12 apoptotic nuclei/0.01 cm²) than in the cortex of untreated rats with an obstructed ureter (127 ± 14 apoptotic nuclei/0.01 cm²; P < 0.001). In the contralateral unobstructed kidney, the number of apoptotic nuclei did not exceed 2 per ×200 field and did not differ between treated and untreated rats (not shown).

**DISCUSSION**

In these studies, we found that the level of AT2 mRNA in the kidney cortex of rats with UUO was decreased in both kidneys. The decrease was blunted by ACE inhibition (Fig. 1A) and by the AT2 receptor inhibitor PD-123319 (Fig. 1B). This suggests that modulation of angiotensin II levels and occupancy of the receptor by an antagonist affect the level of AT2 mRNA. The probable change in receptor levels in the contralateral unobstructed kidney may be related to the increased physiological demand placed on this kidney to excrete waste, salts, and water. The AT2 receptor activity modulates natriuresis (15) and prostanoid metabolism (33) of the normal kidney. Sodium depletion has been found to increase the expression or cause "reexpression" of the AT2 receptor in the kidney of the adult rat (26). Perhaps the need to excrete sodium may cause a decrease in the need to express the AT2 receptor in the contralateral unobstructed kidney in the UUO model.

The overall results of this study suggest that AT2 receptor blockade exacerbates the fibrosis of the tubulo-interstitium in obstructive nephropathy, probably by removal of counterregulatory mechanisms that would oppose the effects of angiotensin II operating through the AT1 receptor. Previous studies from this laboratory (8) indicated that AT1 receptor antagonism during UUO inhibited fibroblast proliferation and the increase in the collagen IV matrix score. Use of an ACE inhibitor is expected to decrease angiotensin II levels, thereby inactivating the functional status of AT1 and AT2 receptors. In the presence of enalapril, we found a decrease in interstitial volume expansion and the collagen IV matrix score (8, 9, 11).

Our attempt to inhibit the AT2 receptor pharmacologically resulted in significant increases in Vv_int, collagen IV matrix scores, and a significant reduction in apoptosis in the kidneys with an obstructed ureter. This is similar to findings of Ichikawa and coworkers (16), who utilized mutant mice in which the AT2 receptor gene was knocked out, in the model of obstructive nephropathy. Thus pharmacological knockout of receptor function in the rat (present study) and gene knockout of the receptor in mice produce similar findings. The present results with PD-123319 are, therefore, attributable to its inhibitory effect on the AT2 receptor per se and probably not to theoretically possible non-receptor-mediated effects. Additionally, in our studies, we found that the reduction in AT2 mRNA, interstitial smooth muscle actin expression, and p53 mRNA induction were each blunted by AT2 receptor inhibition in the kidneys with obstructed ureter. This suggests that AT2 receptor antagonism delays or inhibits fibroblast differentiation into myofibroblasts and delays or inhibits p53-mediated processes in kidney disease. It is thought that smooth muscle actin inhibits migration of fibroblasts (28). Differentiation of the newly proliferated
fibroblasts may not occur until these cells reach their final destination within the tubulointerstitium. A p53-mediated process, the blunting of which would contribute, in part, to the observed increase in $V_{\text{int}}$ is apoptosis. This method of cell loss is operative in the ureteral obstructed kidney (35), and inhibition of this process would lead to increased cellularity of the kidney interstitium. These observations in the kidney are supported by findings in other systems. The AT2 receptor antagonist PD-123319 was found to blunt angiotensin II-mediated apoptosis of cells in culture (18, 34, 36). The AT2 receptor was also found to blunt proliferation of vascular smooth muscle cells (36), coronary endothelial cells (34), and renomedullary interstitial cells (18). Although we have no firm evidence of an antiproliferative effect of the AT2 receptor in these studies, this does remain somewhat of a consideration, in view of the increased cellularity found. This antiproliferative effect of AT2 receptor function is more than likely through the inhibition of the mitogen-activated protein kinases ERK1 and ERK2 (35).

The increase in the collagen IV matrix score is probably multifactorial. An increase in the fibroblast number (caused by the inhibition of apoptotic removal) can account for some of the increased collagen IV found deposited in the cortical interstitium. Another possible factor is that in the presence of the AT2 receptor antagonist there is no counterbalance to the profibrotic and pro-proliferative effect(s) of angiotensin II operating exclusively through the AT1 receptor. We have shown that angiotensin II acting through either the AT1 or the AT2 receptor activates different homodimer and heterodimer combinations of the NF-κB family of transcription factors (12). Perhaps angiotensin II operating solely or more efficiently through the AT1 receptor activates transcription factor combinations that promote fibrosis and proliferation, thereby exacerbating fibrosis of the tubulointerstitium.

We acknowledge the excellent assistance of Linda Duan during these studies. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-09976. Received 23 March 1998; accepted in final form 17 September 1998.

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