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Angiotensin Type 2 Receptor Antagonism Confers Renal Protection in a Rat Model of Progressive Renal Injury

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Abstract. The role of the angiotensin type 2 (AT_2) receptor in the pathogenesis of progressive renal injury has not been previously elucidated. The renal expression of the AT_1 and AT_2 receptors in subtotally nephrectomized rats (STNx) and the effects of AT_2 receptor blockade on renal injury were explored. Reduced renal expression of the AT_1 but not the AT_2 receptor was observed in STNx by reverse transcription–PCR, by *in vitro* autoradiography, and by immunohistochemical staining. The STNx rats were randomly assigned to AT_1 receptor antagonist valsartan, AT_2 receptor antagonist PD123319, or the combination of both for 4 wk. Increased proteinuria in STNx rats was reduced by PD123319 but to a lesser degree when compared with valsartan. Reduced gene and protein expression of the slit diaphragm protein nephrin was prevented by either valsartan or PD123319. Expression of osteopontin, proliferating cell nuclear antigen, and monocyte/macrophage infiltration was increased in STNx rats and was reduced by both AT_1 and AT_2 receptor antagonists. These effects of AT_2 receptor antagonism were observed in the presence of increased BP in STNx rats. These findings suggest that blockade of the AT_2 receptor alone confers a degree of renal protection; in particular, it seems that the combination of the AT_1 and AT_2 receptor antagonists may confer additive renal effects than either receptor antagonist as monotherapy.

Clinical and experimental studies have suggested that angiotensin II plays an important role in the pathophysiology of various kidney diseases. Most studies have evaluated the effects of blockade of the renin-angiotensin system (RAS) in conferring renal protection using either an angiotensin-converting enzyme (ACE) inhibitor or an angiotensin type 1 (AT_1) receptor antagonist (1,2). It has been considered by most investigators that the AT_1 receptor is the major receptor mediating the actions of angiotensin II, whereas the AT_2 receptor has no or a minimal role in renal pathology, particularly in adulthood. However, several studies have demonstrated the presence of the AT_2 receptor not only in fetal tissue (3) but also in adult kidney (4,5). Furthermore, the AT_2 receptor has been identified to have a range of effects related to kidney disease, including the regulation of the chemokine RANTES (6) and the matrix protein osteopontin (4), and mediates effects of the vascular kinin system (7), nitric oxide release (8), and prostaglandin E_2 production (9). Therefore, the AT_2 receptor could have effects on a range of pathophysiologic processes implicated in progressive renal injury.

Recent studies in the heart have highlighted the controversy relating to the role of the AT_2 receptor in various diseases. For example, the AT_2 receptor has been reported to mediate an inhibitory effect on coronary arterial remodeling (10). By contrast, a recent study in the heart suggested that this receptor subtype has trophic effects (11). Indeed, whether the AT_2 receptor is protective, neutral, or injurious in terms of end-organ protection, particularly in pathophysiologic states, has not been clarified (12). Specifically with respect to the kidney, whether the AT_2 receptor plays a role in the pathogenesis of progressive renal injury is largely unknown. The aims of the present study were first to explore the status of angiotensin II receptor subtypes in the remnant kidney, a classical nonimmunologically mediated form of renal injury that is responsive to interruption of the RAS (1,13), and second to assess the effects of blockade of the AT_2 receptor with the selective nonpeptide antagonist PD123319 alone or in combination with the AT_1 receptor antagonist valsartan on functional, structural, cellular, and molecular aspects of renal injury. On the basis of postulated effects of the AT_2 receptor, macrophage accumulation, cellular proliferation, and osteopontin expression were specifically evaluated. In addition, because recent studies have implicated a slit pore protein, nephrin, in the development of

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proteinuria in a number of renal diseases (14–16), we also assessed nephrin gene and protein expression in this study.

Materials and Methods

Protocol 1: Assessment of AT_1 and AT_2 Receptor Expression in the Remnant Kidney

Rat Model. Adult male Sprague-Dawley (SD) rats were subjected to subtotal nephrectomy or sham operation at the age of 8 wk ($n = 6$ per group). Subtotal nephrectomy (STNx) was performed by right nephrectomy, followed by infarction of approximately two thirds of the left kidney with selective ligation of all but one extrarenal branch of the left renal artery (17). Anesthesia was achieved by intraperitoneal injection of pentobarbitone sodium (60 mg/kg body wt; Boehringer Ingelheim, Artarmon, NSW, Australia). Animals were caged in groups of two and given access to food and water *ad libitum*. At the end of the experiment, animals were anesthetized by intraperitoneal injection of pentobarbitone sodium (60 mg/kg body wt). A midline incision of the abdomen was made, and the kidney was removed and frozen for extraction of RNA for subsequent assessment of gene expression, *in vitro* binding studies, and immunohistochemistry. The protocols for animal experimentation and the handling of animals were in accordance with the principles set forth by the National Health and Medical Research Council and the Animal Welfare Committee of the Austin and Repatriation Medical Center.

Reverse Transcription–PCR. Three micrograms of total RNA extracted from each kidney was used to synthesize cDNA with the Superscript First Strand synthesis system for reverse transcription–PCR (RT-PCR; Life Technologies BRL, Grand Island, NY). AT_1 and AT_2 receptor gene expression was analyzed by real-time quantitative RT-PCR performed with the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700; Perkin-Elmer, Foster City, CA) (18). Fluorescence for each cycle was quantitatively analyzed by an ABI Prism 7700 Sequence Detection System (Perkin-Elmer, PE Biosystems, Foster City, CA). To control for variation in the amount of DNA available for PCR in the different samples, gene expression of the target sequence was normalized in relation to the expression of an endogenous control, 18S ribosomal RNA (rRNA) (18S rRNA TaqMan Control Reagent kit; ABI Prism 7700). Primers and Taqman probes for AT_1 and AT_2 receptors and the endogenous reference 18S rRNA were constructed with the help of Primer Express (ABI Prism 7700). For amplification of the AT_1 receptor cDNA, the forward primer was 5'CGGCCTTCGGATAACATGA3', and the reverse primer was 5'CCTGTCACTCCACCTCAAAACA 3'. The probe specific for AT_1 receptor was FAM-5'–CTCATCGGCCAAAAAGCCTGCGT-3'-TAMRA; FAM = 6-carboxyfluorescein, TAMRA (quencher) = 6-carboxy-tetramethylrhodamine. For the AT_2 receptor cDNA, the forward primer was 5' CAATCTGGCTGTGGCTGACTT 3' and the reverse primer was 5' TGCACATCACAGGTCCAAAGA 3'. The probe specific to AT_2 receptor was FAM-5' CAACCCTTCCTCTCTGGGCAACCTATTACTCTTATA-3'-TAMRA. The amplification was performed with the following time course: 50°C for 2 min and 95°C for 10 min and 40 cycles of 94°C for 20 s and 60°C for 1 min. Each sample was tested in triplicate. Results were expressed as relative to control kidneys, which were arbitrarily assigned a value of 1.

AT_1 and AT_2 Receptor Binding by Autoradiography. Binding densities of AT_1 and AT_2 receptors were determined in the kidneys of control and STNx rats (4). In brief, the kidneys were removed and frozen in liquid nitrogen–cooled isopentane and stored at −20°C. Twenty-micron sections were cut on a cryostat at −20°C, then dehydrated overnight under reduced pressure at 4°C before *in vitro* binding studies were performed. The sections were preincubated for 15 min in 10 mM sodium phosphate buffer (pH 7.4) followed by 1 h of incubation in a fresh volume of the same buffer containing the AT_2 receptor-selective radioligand ^{125}I-CGP42112B (15 pmol/L) (4), 0.1% bovine serum albumin, and 0.3 mM bacitracin in the absence (total binding) or the presence (nonspecific binding) of nonradiolabeled CGP42112B (10^{-6} mol/L).

Binding for the AT_1 receptor was assessed using an analogue of angiotensin II, ^{125}I-Sar^1, Ile^8 angiotensin II (19). Sections were preincubated for 15 min in 10 mM sodium phosphate buffer (pH 7.4) followed by 1 h of incubation in a fresh volume of the same buffer containing ^{125}I-Sar^1, Ile^8 angiotensin II (approximately 90 pmol/L), 0.1% bovine serum albumin, and 0.3 mM bacitracin. Specific AT_1 receptor binding was determined in the presence of the AT_2 receptor antagonist PD123319 (10^{-6} mol/L; Parke-Davis, Ann Arbor, MI), whereas nonspecific binding was determined in parallel incubations in the presence of both 10^{-6} mol/L PD123319 and 10^{-6} mol/L valsartan (Novartis Pharma AG, Basel, Switzerland) (20).

After incubation with either ^{125}I-CGP42112B or ^{125}I-Sar^1, Ile^8 angiotensin II, the sections were washed four times for 1 min each in ice-cold buffer and air dried at room temperature. Slides were exposed to Kodak BioMax x-ray film at room temperature for 5 d. After exposure, the films were processed and the optical densities were quantified by a microcomputer imaging device (MCID Imaging system, St. Catherines, Ontario, Canada) connected to an IBM Pentium computer (21). The computer program using the radioactivity standards constructed a calibration curve of optical density *versus* radioactivity density. Specific binding densities were calculated as the difference between total and nonspecific binding densities.

AT_1 and AT_2 Receptor Expression by Immunohistochemistry. Immunohistochemical staining of the AT_2 receptor was performed using a specific polyclonal antiserum to this receptor (22). This polyclonal antiserum was raised in the rabbit against a synthetic peptide sequence derived from the AT_2 receptor, and its specificity has been previously determined (23). Immunohistochemical staining of the AT_1 receptor was performed using a specific rabbit polyclonal antibody to this receptor subtype (sc 1173;, Santa Cruz Biotechnology, Santa Cruz, CA). In brief, 20-μ frozen kidney sections were cut on a cryostat at −20°C. Frozen sections were fixed with cold acetone, and endogenous peroxidase was inactivated using 0.1% hydrogen peroxide in phosphate-buffered saline. The sections were incubated with protein-blocking agent, and endogenous nonspecific binding for biotin/avidin was blocked using a biotin/avidin blocking kit (Vector Laboratories, Burlingame, CA). Kidney sections were incubated overnight (16 h) at 4°C after 1 h of incubation at room temperature with polyclonal antiserum to either the AT_1 receptor or the AT_2 receptor (both 1:50 dilution). Biotinylated horse anti-rabbit Ig (Vector Laboratories) was used as a second antibody, followed by horseradish peroxidase-conjugated streptavidin. Peroxidase activity was identified by reaction with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co., St. Louis, MO) substrate.

Protocol 2: Effect of AT_2 Receptor Blockade Alone or in Combination with the AT_1 Receptor Antagonist

Experimental Protocol. STNx ($n = 50$) was performed as described in protocol 1. The STNx animals were randomly allocated to one of the following groups: (*1*) no treatment (STNx, $n = 13$); (*2*) AT_1 receptor antagonist valsartan at a dose of 30 mg/kg/d by gavage (STNx+valsartan, $n = 15$); (*3*) AT_2 receptor antagonist PD123319 at a dose of 5 mg/kg/d delivered by subcutaneous osmotic minipump (Alzet model 2ML4, Alza Corporation, Palo Alto, CA) implanted

immediately after STNx (STNx+PD123319, $n = 16$); or (*4*) The combination of valsartan and PD123319 (STNx+valsartan+PD123319, $n = 6$). The doses of valsartan and PD123319 were chosen according to a previous study showing effective blockade of these receptor subtypes at these doses, as assessed by *in vitro* binding (4). Sham-operated rats were used as control ($n = 12$).

Systolic BP (SBP) was measured weekly by indirect tail-cuff plethysmography. Animals were housed weekly in metabolic cages for collection of 24-h urine samples and measurement of urinary protein excretion using the Coomassie Brilliant Blue method. Before the rats were killed after 4 wk of treatment, GFR was measured by using the ^{99m}Tc-DTPA method (24). Plasma urea and creatinine concentrations were measured by autoanalyzer (Beckman Instruments, Palo Alto, CA) at the conclusion of the experiment. At the end of the experiment, animals were anesthetized and the kidneys were removed, weighed, and fixed in 10% formalin for subsequent pathologic examination and *in situ* hybridization and frozen for RT-PCR as well as immunostaining with nephrin antibody.

Kidney Histopathology. Assessment of glomerulosclerosis and tubulointerstitial injury was performed by semiquantitative methods as described previously (17,25). In brief, kidney sections were stained with hematoxylin and eosin and observed under light microscope in a masked manner at a magnification of ×400. All glomeruli in each kidney section were graded according to the severity of the glomerular damage: 0, normal; 1, slight glomerular damage, the mesangial matrix and/or hyalinosis with focal adhesion, involving <25% of the glomerulus; 2, sclerosis of 25% to 50%; 3, sclerosis of 50% to 75%; 4, sclerosis of >75% of the glomerulus. The tubulointerstitial area in the cortex was observed and graded as follows: 0, normal; 1, the area of interstitial inflammation and fibrosis, tubular atrophy, and dilation with cast formation involving <25% of the field; 2, lesion area between 25% and 50% of the field; and 3, lesions involving >50% of the field. Indices of glomerular damage or tubulointerstitial injury were calculated by averaging the grades assigned to all glomeruli or tubular fields.

In Situ Hybridization for Osteopontin and Nephrin. *In situ* hybridization studies were performed using techniques as reported pre-

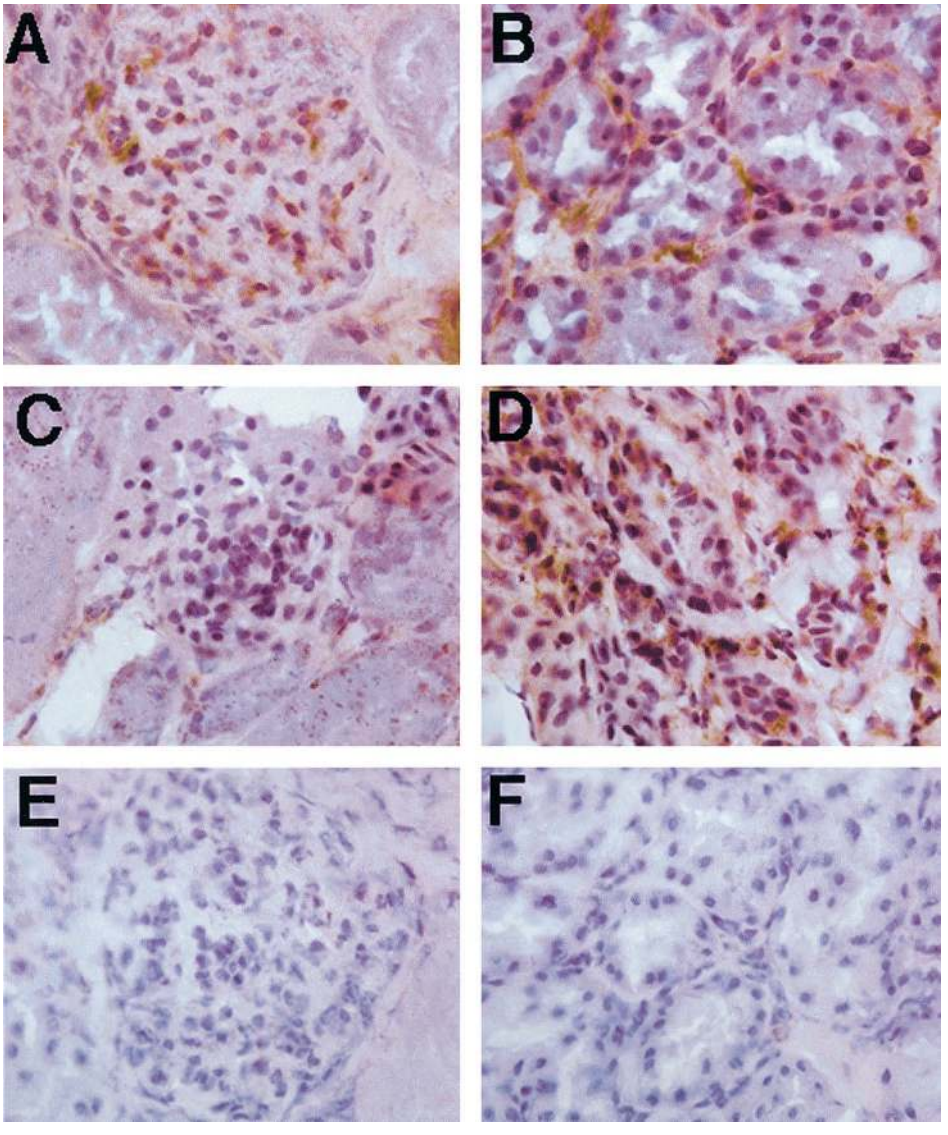

Figure 1. Representative photomicrographs of immunohistochemical staining for the AT_1 receptor in frozen section in control rats (A, B) and subtotally nephrectomized (STNx) rats (C, D). (E and F) Negative staining. (A, C, and E are the glomerulus; B, D, and F are the tubulointerstitium). Magnification, ×1000.

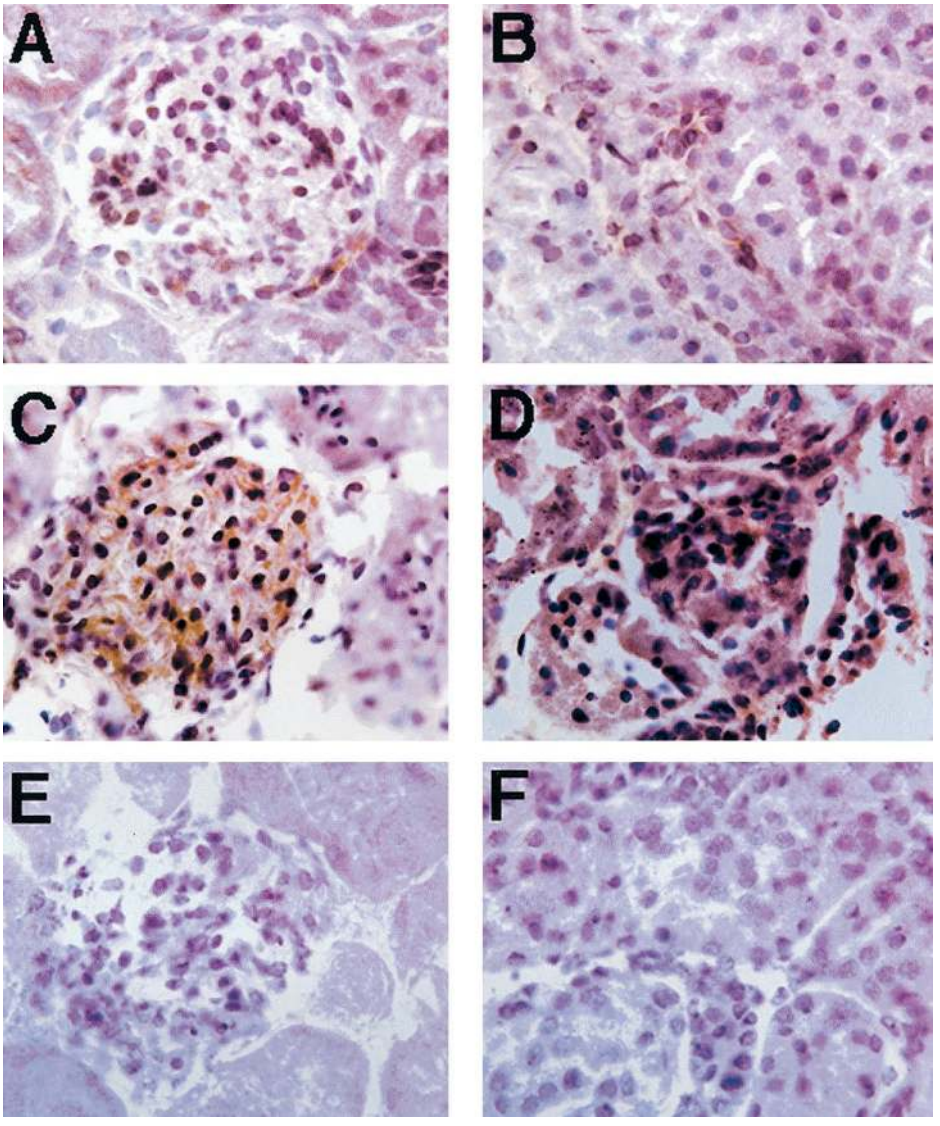

Figure 2. Representative photomicrographs of immunohistochemical staining for the AT_2 receptor in frozen section in control (A, B) and STNx rats (C, D). (E and F) Negative staining. (A, C, and E are the glomerulus; B, D, and F are the tubulointerstitium). Magnification, ×1000.

Table 1. Body weight, kidney weight, and 24-h urinary volume

Group	*n*	Body Weight (g)			Kidney Weight (g)	Kidney/BW (g/g*100)	Urinary Volume (ml/24)
		week 0	week 4	weight gain			
Control	12	252 ± 4	380 ± 5	127 ± 8	1.5 ± 0.1	4.0 ± 0.2	20 ± 1
STNx	13	231 ± 3	292 ± 20*	60 ± 21*	1.6 ± 0.1	5.9 ± 0.4*	39 ± 4*
STNx + Valsartan	15	241 ± 4	331 ± 5*	91 ± 4*	1.7 ± 0.1	5.3 ± 0.2*	36 ± 3*
STNx + PD123319	16	241 ± 3	301 ± 18*	61 ± 18*	1.6 ± 0.1	5.8 ± 0.5*	40 ± 2*
STNx + Valsartan + PD123319	6	234 ± 5	363 ± 7†	128 ± 6†	1.8 ± 0.1	5.6 ± 0.2*	45 ± 5*

* $P < 0.05$ *versus* control.
† $P < 0.05$ *versus* STNx.

viously (26) and outlined below. Antisense riboprobes for rat nephrin were generated as described previously (14). Osteopontin RNA probes were generated from the rat smooth muscle osteopontin cDNA (a gift from Dr. Richard Johnson, Department of Nephrology, University of Washington, Seattle, WA, and currently Chief, Renal Section, Baylor College of Medicine, Houston, TX) (27). ^{35}S-labeled RNA probes for

nephrin or osteopontin were prepared with transcription kits (Promega, Madison, WI). Purified riboprobe length was adjusted to approximately 150 bases by alkaline hydrolysis. Four-micron sections were cut onto slides precoated with 3-aminopropyltriethoxysilane and baked overnight at 37°C. Tissue sections were dewaxed and rehydrated in graded ethanol and milliQ water, equilibrated in P buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA) and incubated in 125 μg/ml Pronase E in P buffer for 10 min at 37°C. Sections were then washed twice in 0.1 M sodium phosphate buffer (pH 7.2), postfixed in 4% paraformaldehyde for 10 min, washed twice in 0.1 M sodium phosphate buffer, then rinsed in milliQ water, dehydrated in 70% ethanol, and air dried. Hybridization buffer containing 2×10^4 cpm/μl riboprobe in 300 mM NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM Na_2HPO4, 5 mM EDTA (pH 8.0), 50% deionized formamide, 20 mg/ml yeast RNA, 10% wt/vol dextran sulfate, and 100 mM dithiothreitol was heated to 85°C for 5 min. Twenty-five microliters of this solution was then added to each section and incubated at 60°C overnight in a 50% formamide humidified incubator. As controls for nonspecific signal, sections were incubated with sense riboprobe. Slides were washed in 2× standard saline citrate containing 50% formamide and then prewarmed to 50°C to remove coverslips. Sections were then washed in the above solution for 1 h shaking at 55°C, rinsed three more times in RNAse buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 8.0], 0.5 M NaCl) and then incubated with RNAse A (150 μg/ml) for 1 h at 37°C. Sections were later washed in 2× standard saline citrate for 45 min at 55°C, dehydrated in graded ethanol, air-dried, and exposed to BioMaxMR autoradiographic film for 3 to 5 d. Slides were then dipped in Amersham nuclear emulsion (Ilford, Mobberley, Cheshire, UK), stored in a light-free box at 4°C for 21 to 28 d, brought to room temperature, then immersed in Kodak D19 developer, washed in acetic acid, and fixed in Ilford Hypan before staining with hematoxylin and eosin.

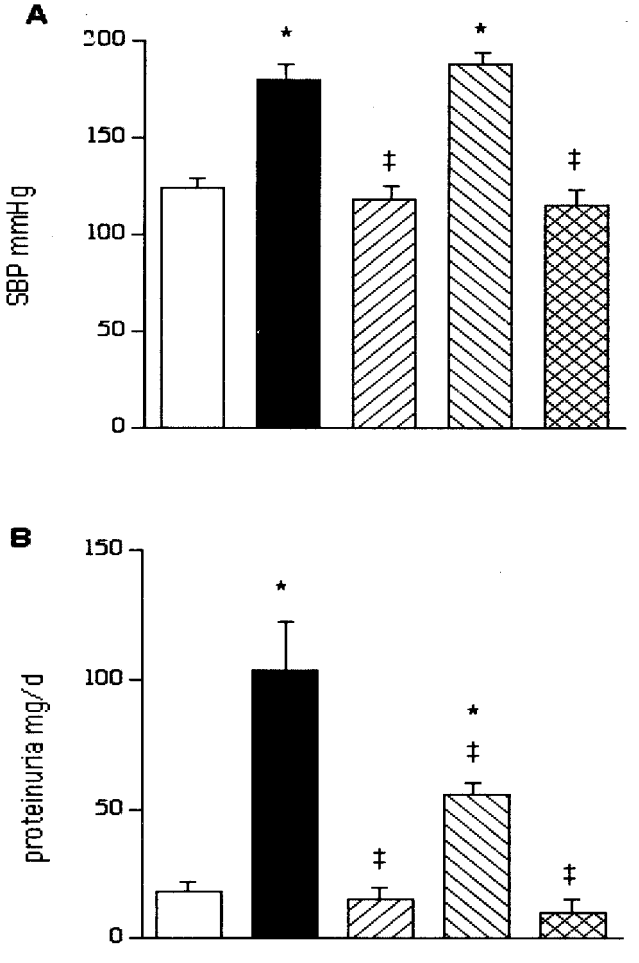

Figure 3. Mean systolic BP (A) and proteinuria (B) in control, STNx, STNx+valsartan, STNx+PD123319, and STNx+valsartan+PD123319 rats. Data are shown as mean ± SEM. *$P < 0.05$ *versus* control; ‡$P < 0.05$ *versus* STNx. □, control; ■, STNx; ▨, STNx+valsartan; ▧, STNx+PD123319; ▩, STNx+valsartan+PD123319.

RT-PCR for Nephrin Gene Expression. Gene expression for nephrin was also assessed by RT-PCR as described previously (15). In brief, the probe specific to nephrin was FAM-5'–CAACCCTTC-CTCTCTGGGCAACCTATTACTCTTATA-3';-TAMRA; FAM = 6-carboxyfluorescein, TAMRA (quencher) = 6-carboxy-tetramethylrhodamine.

Immunohistochemistry. Immunohistochemical staining of nephrin was performed as described previously according to a modified method using a specific antibody to 5-1-6 antigen, which is identical to rat nephrin (14). In brief, 20-μ frozen kidney sections were incubated for 1 h at room temperature with monoclonal 5-1-6 antibody. Biotinylated horse anti-mouse Ig (Vector Laboratories) was used as a second antibody, followed by horseradish peroxidase-conjugated streptavidin. Peroxidase activity was identified by reaction with DAB.

Immunohistochemistry for osteopontin, proliferating cell nuclear antigen (PCNA), and ED-1 was performed with paraffin-embedded sections as described previously (20,28). In brief, sections were labeled with a monoclonal antibody to PCNA (PC-10; DAKO A/S, Copenhagen, Denmark), the monocyte/macrophage antigen (ED-1; Serotec, Oxford, UK), or osteopontin (a gift from Dr. Richard Johnson) (27). Biotinylated Ig was used as a second antibody, followed by horseradish peroxidase-conjugated streptavidin. Detection was accomplished by reaction with DAB.

Histomorphometery. Quantification of nephrin immunostaining was performed by calculation of the proportion of area occupied by the brown staining in all glomeruli per section using the Imaging Analysis System (AIS, Imaging Research, St. Catherines, Ontario, Canada) associated with a videocamera and computer as described previously (15,29). Quantification of osteopontin immunostaining was performed by assessment of the proportion of area of positive staining in tubules in the cortex areas per section. The positive cell numbers stained with PCNA or ED-1 were counted manually in the 30 renal cortical areas (nonischemic, nonscar area) including the glomeruli at a magnification of ×400. The PCNA- or ED-1–positive cells were expressed as number per field.

Table 2. GFR and plasma concentration of urea and creatinine

Group	GFR (ml/min)	Plasma Urea (mmol/L)	Plasma Creatinine (μmol/L)
Control	3.7 ± 0.1	7.0 ± 0.1	33 ± 3
STNx	1.3 ± 0.2*	23.3 ± 2.8*	68 ± 7*
STNx + Valsartan	1.3 ± 0.1*	17.6 ± 1.8*†	62 ± 3*
STNx + PD123319	1.2 ± 0.1*	18.3 ± 1.1*†	56 ± 2*†
STNx + Valsartan + PD123319	1.2 ± 0.1*	17.8 ± 1.2*†	61 ± 2*

* $P < 0.05$ *versus* control.
† $P < 0.05$ *versus* STNx.

Statistical Analyses

Data were analyzed by analysis of variance using Statview SE (Brainpower, Calabasas, CA) on a Macintosh Computer (Cupertino, CA). Comparisons of group means were performed by Fisher's least significant difference method. Data are shown as mean ± SEM, and $P < 0.05$ was viewed as statistically significant unless otherwise specified.

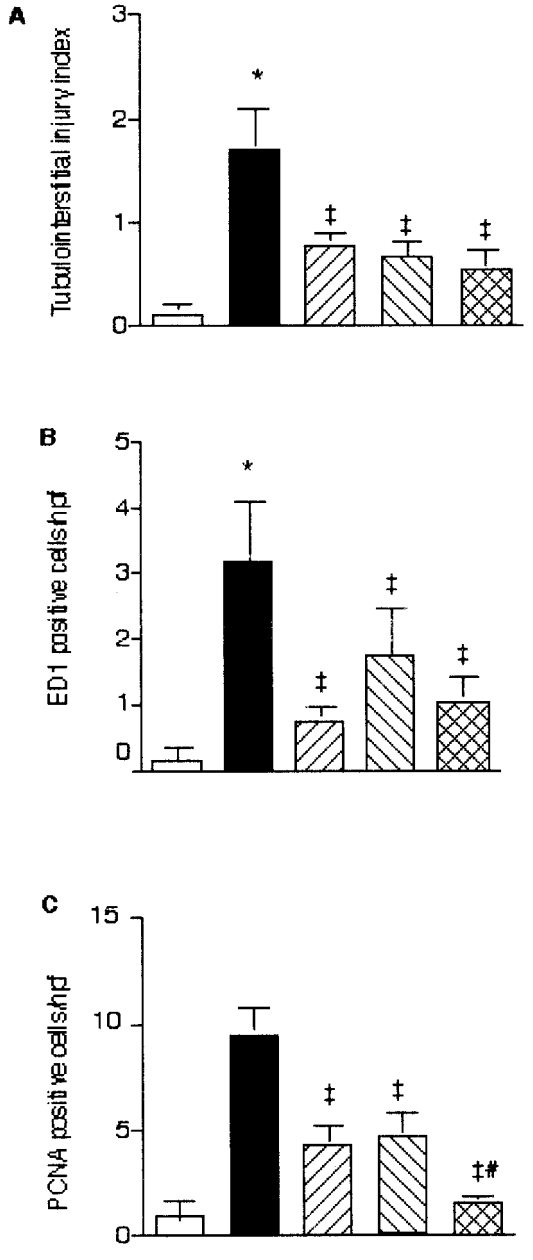

Figure 4. Tubulointerstitial injury index (A), monocyte/macrophage infiltration (B), and proliferating cell nuclear antigen (PCNA; C). All of these parameters were assessed in the nonischemic, nonscar cortex area. Data are shown as mean ± SEM. *$P < 0.05$ *versus* control; ‡$P < 0.05$ *versus* STNx; #$P < 0.05$ *versus* STNx+valsartan and STNx+PD123319. □, control; ■, STNx; ▨, STNx+valsartan; ▧, STNx+PD123319; ▩, STNx+valsartan+PD123319.

Results

Protocol: AT_1 and AT_2 Receptor Expression in the Remnant Kidney

AT_1 Receptor Expression in the Remnant Kidney. Gene expression of the AT_1 receptor was modestly reduced in the remnant kidney (control 1.0 ± 0.1 *versus* STNx 0.73 ± 0.1; $P < 0.05$, *t* test). Binding density for the AT_1 receptor was reduced in the remnant kidney when compared with control kidney as measured by *in vitro* autoradiography with ^{125}I-Sar^1, Ile^8 angiotensin II (control 59 ± 5 dpm/mm^2 *versus* STNx 39 ± 2 dpm/mm^2; $P < 0.01$, *t* -test). Immunostaining using the AT_1 receptor antibody revealed a similar pattern of reduced AT_1 receptor protein in the glomeruli but increased staining in the tubulointerstitium in the remnant kidney (Figure 1).

AT_2 Receptor Expression in the Remnant Kidney. Gene expression of the AT_2 receptor in the kidney was increased in the STNx (control 1.0 ± 0.1 *versus* STNx 2.4 ± 0.2; $P < 0.01$, *t* test). In contrast, binding density for AT_2 receptor was similar in the two groups as assessed by *in vitro* autoradiography with ^{125}I-CGP 42112B (control 15 ± 2 dpm/mm^2 *versus* STNx 17 ± 5 dpm/mm^2; $P > 0.05$, *t* test). Immunostaining for the AT_2 receptor revealed no global difference in the AT_2 receptor protein between remnant and control kidneys. However, the AT_2 receptor protein was identified specifically in the injured tubules after renal mass reduction (Figure 2).

Protocol 2: Effect of AT_2 Receptor Blockade Alone or in Combination with AT_1 Receptor Antagonist

Body Weight, Kidney Weight, and Urine Volume. Untreated STNx rats gained 60 g of weight over 4 wk, which was approximately half of the weight gain of control animals during the same period (Table 1). Similar reduced body weight was observed in PD123319-treated rats, whereas valsartan-treated rats gained 50% more weight than PD123319 or untreated STNx rats. The STNx rats treated with combination of valsartan and PD123319 gained similar weight to control rats (Table 1).

The remnant kidney weight was similar to that of control rats, but the kidney/body weight ratio was increased by 50% in untreated STNx compared with control rats (Table 1). The kidney weight and kidney/body weight ratios were not significantly altered by any of the treatments. Urinary volume nearly doubled in STNx rats compared with control rats but was not influenced by any treatment (Table 1).

BP. SBP increased significantly after STNx (Figure 3A). This rise was prevented by valsartan administered alone or in combination with PD123319. Treatment with PD123319 alone did not affect the increase in SBP, and there was no significant difference in the antihypertensive effect of valsartan when PD123319 was added.

Renal Function. Urinary protein excretion increased approximately fivefold in untreated STNx rats (Figure 3B). Proteinuria was completely prevented in valsartan-treated rats, and the addition of PD123319 did not reduce proteinuria further. PD123319 alone also significantly reduced proteinuria, but its effect was only approximately 50% of that observed with valsartan monotherapy.

Plasma urea and creatinine concentrations were increased in STNx rats compared with control animals (Table 2). Treatment with either valsartan or PD123319 alone and in combination had similar effects in reducing plasma urea concentration. GFR was markedly reduced by 70% in STNx rats but was not significantly influenced by any of the treatments.

Kidney Histopathology. STNx rats had increased glomerulosclerosis compared with control rats (GSI, control 0.39 ± 0.2 *versus* STNx 0.78 ± 0.2; $P < 0.05$). Treatment with valsartan alone or in combination reduced the glomerulosclerotic indices (valsartan, 0.58 ± 0.1; combination, 0.48 ± 0.09; $P < 0.05$, respectively). PD123319 treatment did not affect the glomerulosclerotic indices (0.77 ± 0.22).

Severe tubulointerstitial injury followed renal mass reduction, and this was significantly attenuated to a similar extent by all treatments (Figure 4A). Similarly, increased monocyte/macrophage infiltration observed in the STNx rats was reduced to similar levels by all treatments (Figures 4B and 5). PCNA in the renal tubules was markedly increased in the STNx rats (Figures 4C and 6). Treatment with either valsartan or PD1233319 reduced the number of PCNA-positive renal tubular cells. The combination of valsartan and PD1233319 was

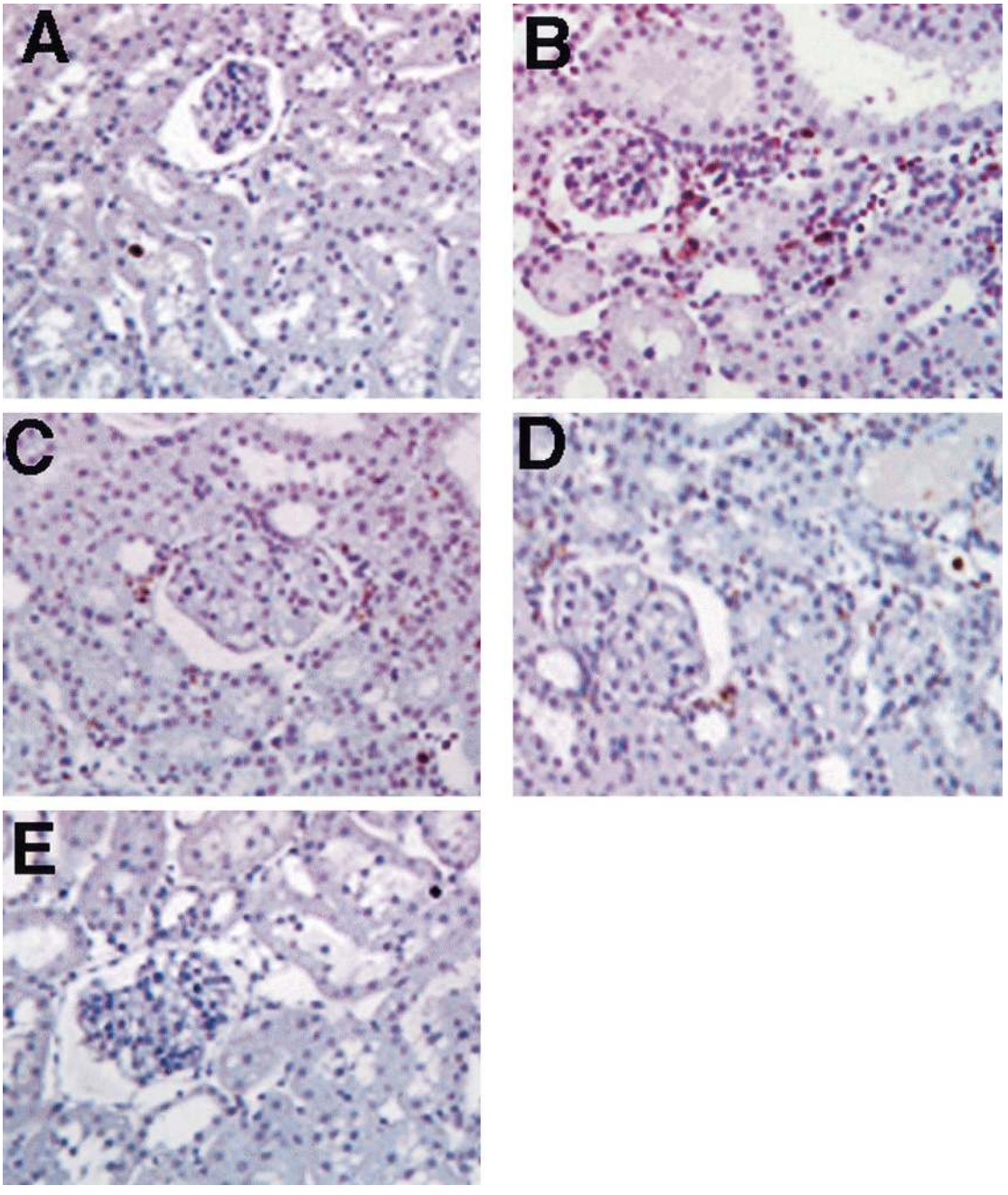

Figure 5. Representative photomicrographs of ED-1 immunostaining in control (A), STNx (B), STNx+valsartan (C), STNx+PD123319 (D), and STNx+valsartan+PD123319 (E). Magnification, ×200.

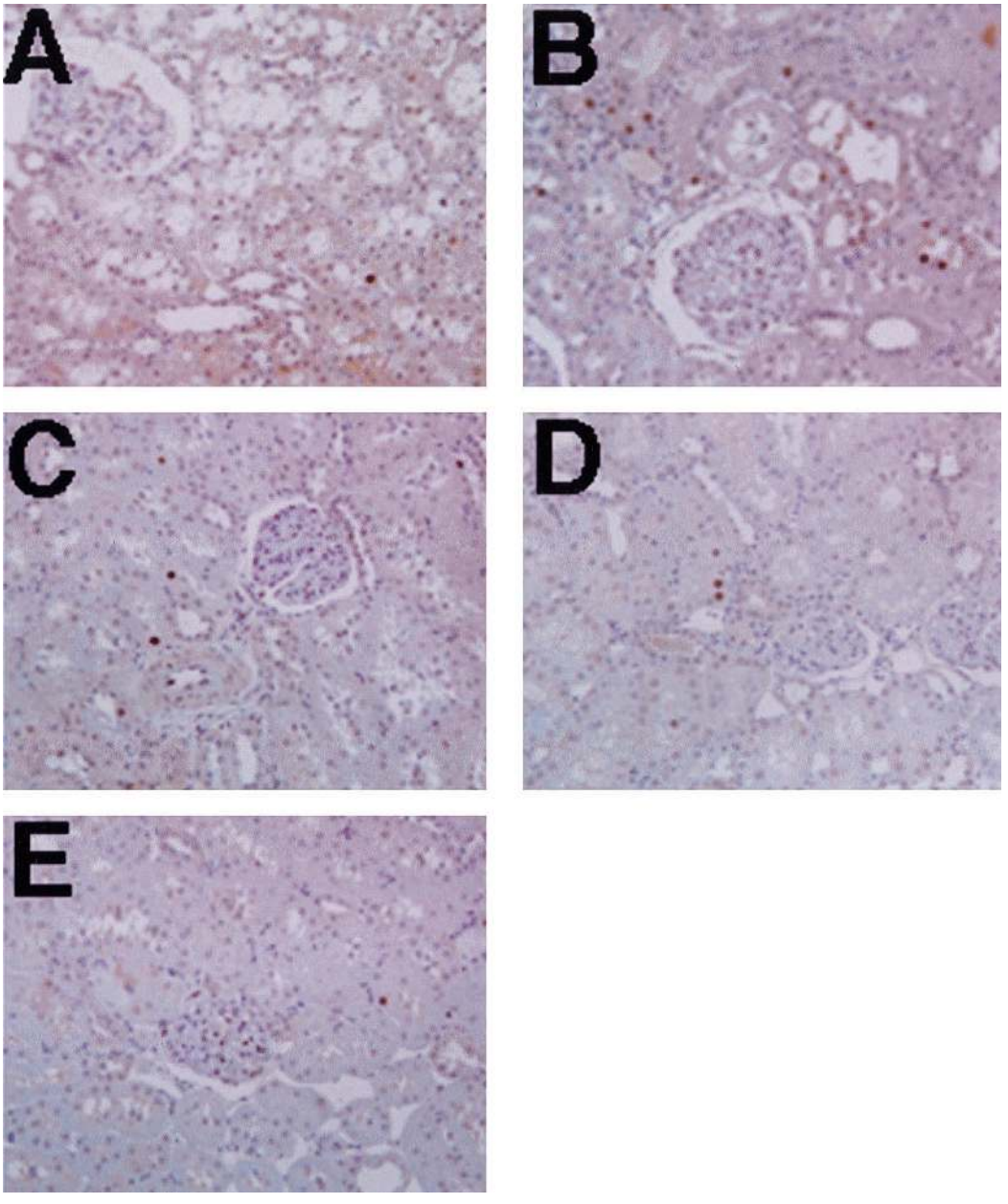

Figure 6. Representative photomicrographs of PCNA immunostaining in control (A), STNx (B), STNx+valsartan (C), STNx+PD123319 (D), and STNx+valsartan+PD123319 (E). Magnification, ×200.

associated with an additional reduction in PCNA-positive cells than seen with either monotherapy.

Gene and Protein Expression of Nephrin. Nephrin gene expression, as assessed by RT-PCR, was reduced in STNx compared with control rats (control 1.0 ± 0.1 *versus* STNx 0.7 ± 0.1; $P < 0.05$). Nephrin gene and protein expression was localized to the glomeruli in control and STNx rats as assessed by *in situ* hybridization and immunohistochemistry (Figures 7 and 8). Nephrin protein expression, as assessed by immunostaining, was reduced in the glomeruli of the remnant kidney when compared with control rats (control 14.3 ± 1% *versus* STNx 6.4 ± 1%; $P < 0.05$). Reduced nephrin gene expression after STNx was attenuated by treatment with valsartan (1.0 ± 0.1; $P < 0.05$ *versus* STNx) or PD123319 (1.6 ± 0.2; $P < 0.05$ *versus* STNx). The combination of valsartan and PD123319 was associated with a marked increase in nephrin mRNA levels when compared with control rats (2.6 ± 0.6; $P < 0.05$). All treatments produced a similar effect on nephrin protein expression as assessed by immunohistochemistry with no evidence of reduced nephrin protein expression in those treated when compared with untreated STNx rats (valsartan, 18.5 ± 1.2%, PD123319, 17.4 ± 1.2%, valsartan+PD123319, 21 ± 2%). In the scarred glomeruli after STNx, nephrin expression was increased as assessed by both *in situ* hybridization (Figure 7C) and immunohistochemistry (Figure 8F), but this was not influenced by any of the treatments.

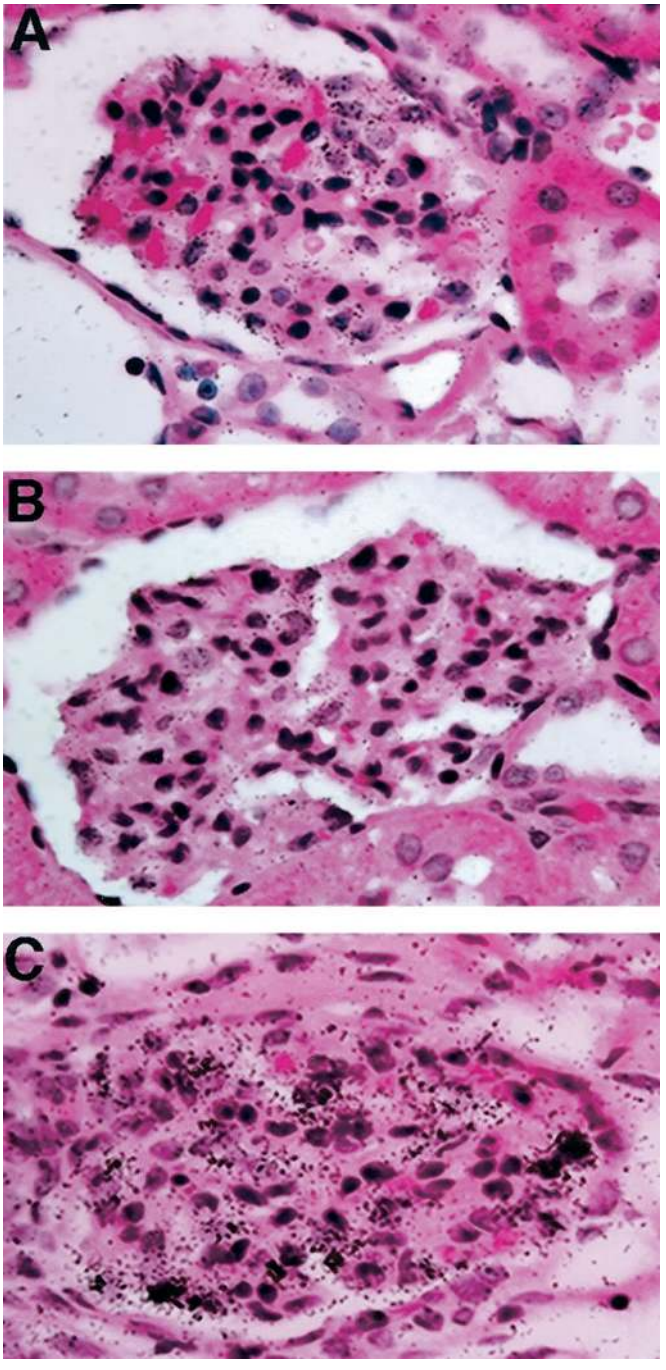

Figure 7. Representative photomicrograph of nephrin gene expression as assessed by *in situ* hybridization in glomeruli from control (A) and STNx rats (B) and in scar area of STNx rats (C). Magnification, ×400.

Gene and Protein Expression of Osteopontin. Osteopontin gene expression was localized primarily to the medulla in control rats (Figure 9A). In STNx rats, there was at least a threefold increase in osteopontin gene expression in the renal cortex as assessed by *in situ* hybridization (Figures 9 and 11A). Both AT_1 and AT_2 receptor antagonists reduced renal cortical osteopontin mRNA levels. The combination was associated with an additional reduction in osteopontin gene expression. A similar pattern was observed with respect to osteopontin protein expression as assessed by immunohistochemistry (Figures 10 and 11B). In STNx rats, there was a marked increase in osteopontin immunostaining, particular in tubules (Figures 10 and 11). This tubular staining was reduced by all treatments, including the AT_2 receptor antagonist, either as monotherapy or in combination with the AT_1 receptor antagonist. In the scarred tubules, osteopontin expression was increased as assessed by both *in situ* hybridization (Figure 9F) and immunohistochemistry (Figure 10F), but this was not influenced by any of the treatments.

Discussion

This study clearly demonstrates that administration of the AT_2 receptor antagonist PD123319 is associated with beneficial effects on a range of renal functional and structural parameters. Moreover, the combination of both AT_1 and AT_2 receptor antagonists may provide additional renal protection than either antagonist alone because a range of molecular and cellular mediators of renal injury was significantly reduced by this combination. This includes effects on expression of various proteins, including osteopontin and nephrin, and tubular cell proliferation, which occur after STNx.

In the present study, we reported the status of AT_1 and AT_2 receptor expression in the kidney after STNx. With the use of a number of complementary techniques to assess gene and protein expression and status of the receptor at the level of ligand binding, reduced AT_1 receptor expression was detected in this model. There seems to be a significant increase in AT_2 receptor gene expression in this model. Global radioligand binding assessment of the AT_2 receptor revealed no overall change in the remnant kidney, although this does not exclude local changes in receptor activation particularly at sites of injury. Indeed, immunohistochemical staining confirmed evidence of the AT_2 receptor protein in the remnant kidney primarily at sites of tubulointerstitial injury. With reduced AT_1 receptor expression and binding density, there seems to be an imbalance in the relative proportion of AT_1 and AT_2 receptors in this model. Changes of AT_1 and AT_2 receptors in the present study are similar to those reported in other models of renal injury (4,30). Renal AT_1 but not AT_2 receptors are downregulated in angiotensin II–induced hypertension and in renal hypertension caused by clipping renal arteries as assessed by immunohistochemistry (30). Reduced AT_1 but not AT_2 receptor binding was also reported in the kidney after angiotensin II infusion as assessed by specific radioligand binding (4). It is important to appreciate that assessment of receptor status *per se* in an experimental model does not predict the response of that model to interruption of that receptor subtype. Indeed, despite STNx animals having reduced AT_1 receptor expression, this model is very sensitive to the renoprotective effects of AT_1 receptor antagonists (17,28). Nevertheless, the identification and characterization of the AT_2 receptor in this model provided the rationale to investigate specific blockade of this receptor subtype.

A hallmark of renal injury is excessive urinary excretion of protein (proteinuria). The mechanisms of proteinuria or albu-

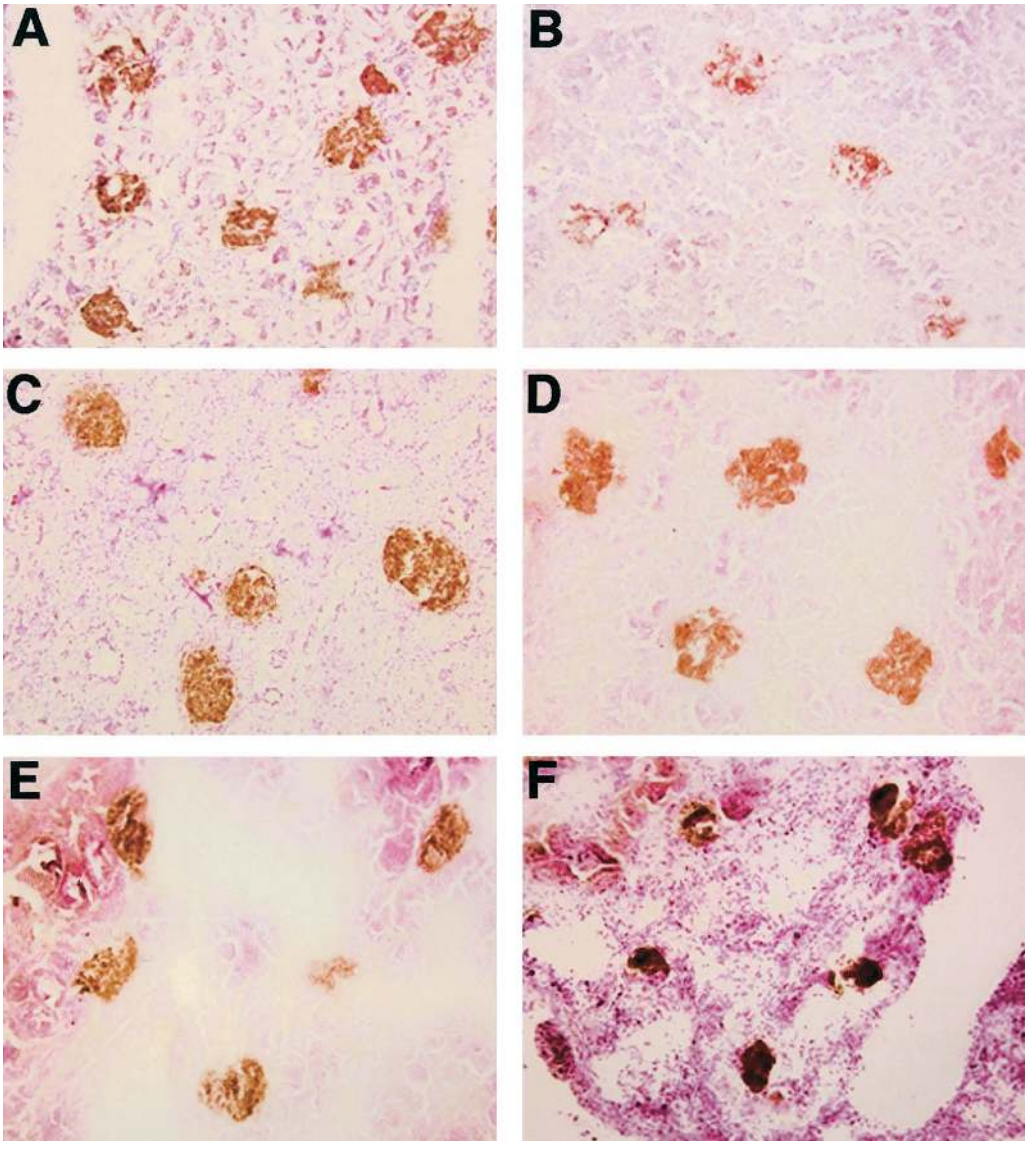

Figure 8. Representative photomicrographs of nephrin immunostaining in control (A), STNx (B), STNx+valsartan (C), STNx+PD123319 (D), and STNx+valsartan+PD123319 (E) and in the scar of untreated STNx (F). Magnification, ×200.

minuria continue to be investigated extensively. Recent studies have suggested that the human gene NPHS protein, nephrin, a cytoskeletal protein, localizes to the slit pore of podocytes in the glomerulus, playing a pivotal role in the pathogenesis of proteinuria (14,31). Several investigators have postulated a link between a deficiency in renal nephrin expression and proteinuria on the basis of a number of recent findings. First, mutation within the nephrin NPHSI gene has been reported to be responsible for the massive proteinuria observed in patients with congenital nephrotic syndrome of the Finnish type (32). Second, injection of an antibody to this protein has been shown to promote the development of proteinuria (14). Third, the association between the development of proteinuria and the deficiency in nephrin expression has been reported in various models of experimental renal injury, including puromycin aminonucleoside nephropathy (33), Passive Heymann nephritis (16), and diabetic nephropathy (15). In the present study, reduced gene and protein expression of nephrin expression in the renally ablated rats was associated with increased proteinuria. This is consistent with previous findings that indicate that there is a link between reduced nephrin expression and development of proteinuria (albuminuria) in the proteinuric renal disease.

The possible links between the RAS and the regulation of nephrin expression have been suggested by recent studies (15,16). Administration of an ACE inhibitor or an AT_1 receptor antagonist attenuated the deficiency in renal nephrin expression in association with reduced proteinuria in two different models of progressive renal injury, Passive Heymann nephritis and diabetic nephropathy (15,16). These findings are consistent with those of our present study that demonstrated that AT_1 receptor antagonism attenuated reduced nephrin expression in the remnant kidney. Furthermore, the AT_2 receptor antagonist PD123319 also attenuated the deficiency of nephrin expression

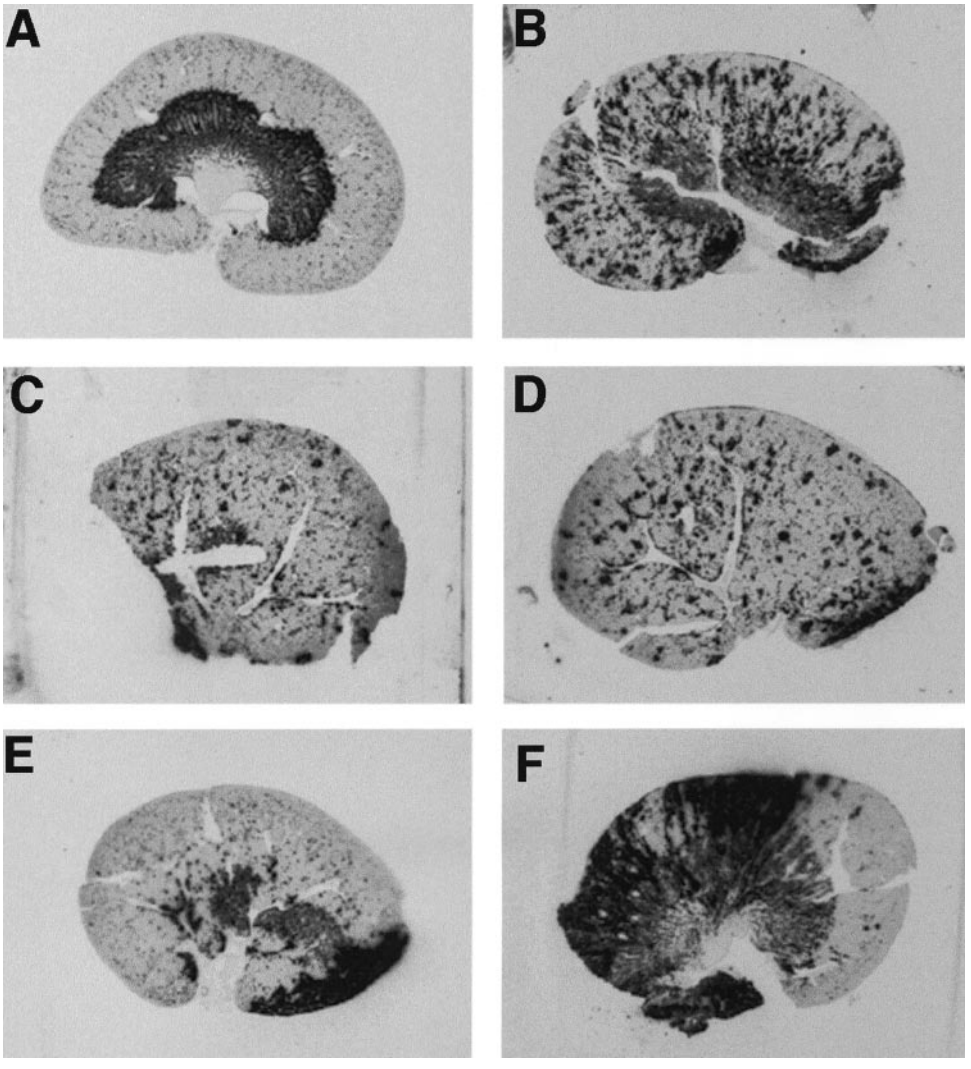

Figure 9. Representative photomicrographs of osteopontin gene expression by *in situ* hybridization in control (A), STNx (B), STNx+valsartan (C), STNx+PD123319 (D), and STNx+valsartan+PD123319 rats (E) and in the scar of untreated STNx (F).

in the context of less proteinuria in these STNx rats. These findings could be interpreted as suggesting that at least part of the antiproteinuric effects of the RAS blockers, including the AT_1 and AT_2 receptor antagonists, is related to attenuation of nephrin expression. However, one cannot exclude that the changes in nephrin expression are secondary to improved renal ultrastructure in response to these agents that block the RAS. Indeed, it has been shown that agents that block the RAS influence slit pore structure in various disease states, including diabetes (34).

There is increasing evidence to suggest that increased urinary protein excretion not only is an indicator of renal injury but also may contribute to tubulointerstitial injury. In the normal setting, protein is filtrated from glomerulus and is reabsorbed by proximal tubule cells, where they are degraded by lysosomes. Accumulation of protein in proximal tubules may induce lysosome rupture and phenotypic changes, which will release or activate local vasoconstrictor or chemotactic proteins leading to inflammation (35). Blockade of angiotensin II by an ACE inhibitor or an AT_1 receptor antagonist has been shown to reduce proteinuria in this model (1,2,4). This antiproteinuric effect has been reproduced in many human proteinuric renal diseases (36,37). A major finding in the present study was that the STNx rats treated with the AT_2 receptor antagonist as well as the AT_1 receptor antagonist had less proteinuria than untreated rats. It is possible that the antiproteinuric effects of AT_2 receptor antagonist as has been previously observed with AT_1 receptor antagonist lead to reduced tubular exposure to proteins, thereby providing and/or attenuating tubulointerstitial injury.

The finding of the AT_2 receptor antagonist PD123319 on proteinuria in the present study is in contrast with a previous study, which showed a lack of beneficial effects after the administration of the AT_2 receptor antagonist PD123319 (38). This previous study involved PD123319 being given via drinking water (38). Therefore, the discrepancy between two studies may be due to mode of administration of PD1233319 because other investigators have reported beneficial and positive find-

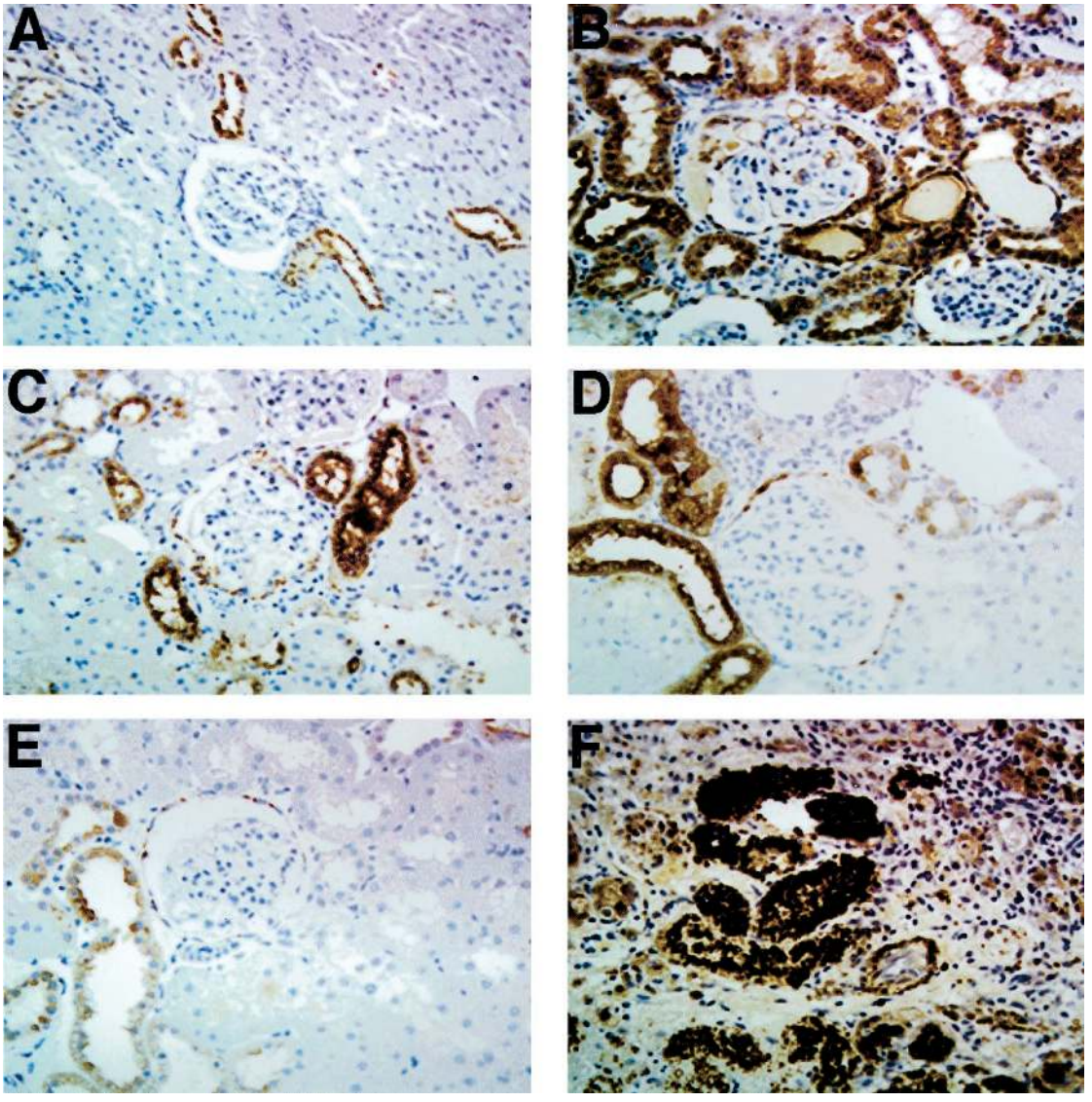

Figure 10. Representative photomicrographs of osteopontin staining in control (A), STNx (B), STNx+valsartan (C), STNx+PD123319 (D), and STNx+valsartan+PD123319 (E) and in scar area of STNx rats (F). Magnification, ×200.

ings with respect to PD123319 in the kidney and the vasculature when the compound was administered by continuous osmotic minipumps (4,20,39). Indeed, administration of PD123319 by continuous osmotic minipumps, as used in the present study, has been shown previously by our group to be an effective approach to block the AT_2 receptor, specifically in the kidney and the vasculature (4,18).

Matrix protein accumulation is a pivotal pathologic process that leads to progressive renal injury, including fibrosis. It has been shown that osteopontin is increased in various models of renal injury (40) and that the expression of osteopontin is modulated directly by angiotensin II (27,41). Our previous study in angiotensin II–infused rats showed that both AT_1 and AT_2 receptors modulate osteopontin expression (4). In the present study, we confirmed that there was increased osteopontin gene and protein expression in this model of progressive renal injury as previously reported (42). Furthermore, blockade of either the AT_1 or AT_2 receptor reduced osteopontin accumulation in the proximal tubules, albeit not to control levels. Importantly, combined AT_1 and AT_2 receptor blockade was superior to monotherapies with osteopontin mRNA levels in this group being similar to those observed in control rats.

Monocyte/macrophage infiltration is considered to play an important role in the development of inflammatory changes in the tubulointerstitium. Monocytes/macrophages are attracted to sites of injury by chemokines such as osteopontin (40). In addition, accumulation of monocytes/macrophages further releases inflammatory chemokines and cytokines, potentially leading to a vicious cycle perpetuating the pathways responsible for progressive tubulointerstitial injury. It has been shown that angiotensin II infusion induces inflammatory changes in renal tubules and the interstitium (27). In the present study, accumulation of monocytes/macrophages was noted in the remnant kidney after STNx, and this was prevented not only by

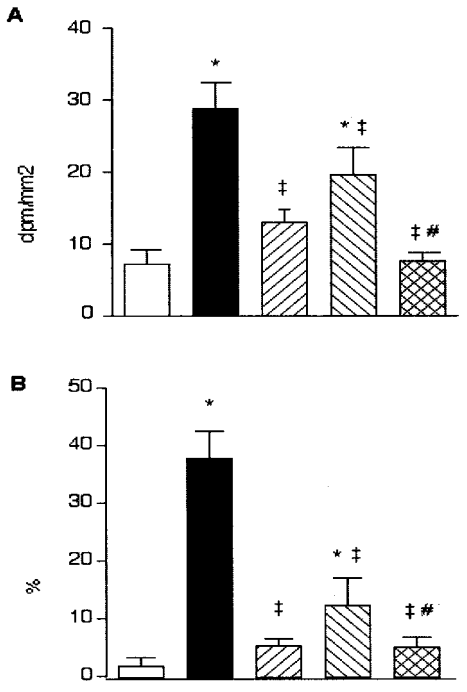

Figure 11. Gene expression of osteopontin assessed by *in situ* hybridization (A) and protein expression assessed by immunohistochemistry (B). Data are shown as mean ± SEM. $*P < 0.01$ *versus* control; $‡P < 0.05$ *versus* STNx; $\#P < 0.05$ *versus* STNx+PD123319. □, control; ■, STNx; ▨, STNx+valsartan; ▧, STNx+PD123319; ▩, STNx+valsartan+PD123319.

AT_1 but also by AT_2 receptor blockade. A previous study showed that angiotensin II stimulates the expression of the chemokine RANTES, which promotes monocyte/macrophage infiltration in the kidney (6). This action of angiotensin II was mediated by the AT_2 receptor (6). Therefore, the finding of this and previous studies linking the AT_2 receptor to expression of osteopontin and RANTES, both proteins involved in macrophage recruitment, provides a potential pathway by which AT_2 receptor blockade could reduce various aspects of tubulointerstitial damage.

Renal remodeling after renal mass reduction involves hypertrophy, increased proliferation, and extracellular matrix production (43). Cellular proliferation plays an important role in renal remodeling and is considered a compensatory mechanism in response to the loss of renal mass (43). The effect of the AT_2 receptor on cell proliferation remains controversial. Increasing evidence from both *in vitro* and *in vivo* studies suggests that the conventional view that the AT_2 receptor is antiproliferative (44–46) may not be correct in a range of pathophysiologic contexts (12). For example, several studies that explored the AT_2 receptor in vascular proliferation suggested that the AT_2 receptor is proliferative (20,39,47). The present study indicates that both AT_1 and AT_2 receptor antagonists reduce tubular cell proliferation, a phenomenon that we observed in the kidney from angiotensin II–infused rats (4). The additive effects of PD123319 to the AT_1 receptor antagonist on PCNA staining further suggest the specific role of the AT_2 receptor in preventing the proliferative response in the kidney in this context.

The findings of the present study further challenge the previous view that only the AT_1 receptor is important in mediating the actions of angiotensin II. Increasing evidence is accumulating for a role of the AT_2 receptor in modulating the pathogenesis of proteinuria, cellular proliferation, and matrix protein accumulation, which ultimately lead to the classical functional and structural hallmarks of progressive renal injury. Because the present study has identified potential renoprotective actions of AT_2 receptor blockade, this strategy may have therapeutic benefits that need to be evaluated in the context of the currently available approaches to interrupt the RAS, such as ACE inhibitors and AT_1 receptor antagonists. ACE inhibitors and AT_1 receptor antagonists have disparate effects on the availability of angiotensin II to interact at the AT_2 receptor. Previously, Klahr *et al.* (48) showed a difference between an ACE inhibitor and an AT_1 receptor antagonist in reducing monocyte/macrophage infiltration in obstructive nephropathy that could partially relate to the ability of ACE inhibition to reduce availability of angiotensin II for the AT_2 receptor. However, this difference between ACE inhibitors and AT_1 receptor antagonists has not been a uniform finding (49) and may partly relate to differences in renal models that have been evaluated. It remains to be determined whether these disparate effects of AT_1 receptor antagonists and ACE inhibitors are of clinical relevance and whether there is ultimately a role for specific AT_2 receptor antagonism. The findings from the present study need to be evaluated in the setting of increasing evidence of crosstalk between AT_1 and AT_2 receptors in mediating the actions of angiotensin II (50–52). Furthermore, whether the results of this crosstalk lead to an overall neutralization of the effects of the two receptor subtypes or these receptors may act in a more synergistic or additive manner in terms of their renal effects has not been defined.

In the present study, although AT_2 receptor blockade was shown to influence a range of molecular and cellular processes implicated in progressive renal injury as well as reducing proteinuria, no clear-cut effects on GFR or glomerulosclerosis were observed. It remains to be determined whether these early changes observed with AT_2 receptor blockade ultimately lead to preserved renal function and structure.

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