Angiotensin type 2 receptor actions contribute to angiotensin type 1 receptor blocker effects on kidney fibrosis

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Naito T, Ma L-J, Yang H, Zuo Y, Tang Y, Han JY, Kon V, Fogo AB. Angiotensin type 2 receptor actions contribute to angiotensin type 1 receptor blocker effects on kidney fibrosis. Am J Physiol Renal Physiol 298: F683–F691, 2010. First published December 30, 2009; doi:10.1152/ajprenal.00503.2009.—Angiotensin type 1 (AT1) receptor blocker (ARB) ameliorates progression of chronic kidney disease. Whether this protection is due solely to blockade of AT1, or whether diversion of angiotensin II from the AT1 to the available AT2 receptor, thus potentially enhancing AT2 receptor effects, is not known. We therefore investigated the role of AT2 receptor in ARB-induced treatment effects in chronic kidney disease. Adult rats underwent 5/6 nephrectomy. Glomerulosclerosis was assessed by renal biopsy 8 wk later, and rats were divided into four groups with equivalent glomerulosclerosis: no further treatment, ARB, AT2 receptor antagonist, or combination. By week 12 after nephrectomy, systolic blood pressure was decreased in all treatment groups, but proteinuria was decreased only with ARB. Glomerulosclerosis increased significantly in AT2 receptor antagonist vs. ARB. Kidney cortical collagen content was decreased in ARB, but increased in untreated 5/6 nephrectomy, AT2 receptor antagonist, and combined groups. Glomerular cell proliferation increased in both untreated 5/6 nephrectomy and AT2 receptor antagonist vs. ARB, and phospho-Erk2 was increased by AT2 receptor antagonist. Plasminogen activator inhibitor-1 mRNA and protein were increased at 12 wk by AT2 receptor antagonist in contrast to decrease with ARB. Podocyte injury is a key component of glomerulosclerosis. We therefore assessed effects of AT1 vs. AT2 blockade on podocytes and interaction with plasminogen activator inhibitor-1. Cultured wild-type podocytes, but not plasminogen activator inhibitor-1 knockout, responded to angiotensin II with increased collagen, an effect that was completely blocked by ARB with lesser effect of AT2 receptor antagonist. We conclude that the beneficial effects on glomerular injury achieved with ARB are contributed to not only by blockade of the AT1 receptor, but also by increasing angiotensin effects transduced through the AT2 receptor.

podocyte; plasminogen activator inhibitor-1

ANGIOTENSIN II (ANG II) acts at two major receptors, type 1 (AT1) and type 2 (AT2). Classic ANG II effects are mediated through AT1, including vasoconstriction, sodium reabsorption, sympathetic nerve stimulation, aldosterone secretion, and inhibition of renin synthesis (46). ANG II also induces cell growth, oxidative stress, inflammation, and matrix accumulation through upregulation of molecules such as transforming growth factor-β (TGF-β) and plasminogen activator inhibitor-1 (PAI-1) (20). Human and experimental chronic kidney diseases (CKD) are characterized by progressive renal fibrosis. Inhibition of angiotensin actions, whether by angiotensin-converting enzyme inhibitor (ACEI) or AT1 receptor blocker (ARB), has been effective in ameliorating progression of CKD beyond effects on blood pressure (36). These beneficial effects have largely been linked to AT1 action blockade. However, beneficial effects of ARB could also be contributed to by increased levels of unbound ANG II after AT1 blockade being diverted to and activating the AT2 receptor. We sought to determine whether AT2 actions could play a role in these beneficial effects of ARB in a model of CKD widely studied and with relevance to human CKD, namely the renal ablation model (8).

Pivotal studies from this model paved the way for understanding of the beneficial effects of ACEI in lowering glomerular hemodynamic stresses, and ameliorating or even reversing pathogenic events beyond hemodynamics, including direct effects to decrease matrix accumulation, and dampen mesangial, endothelial, and podocyte injuries (20, 21). Of note, these actions of the ARB and ACEI to ameliorate progressive kidney disease have been observed in human CKD even in patients classically regarded as having a low renin state, such as African-American patients with arterionephrosclerosis (51). These findings highlight the importance of local activation and interactions of components of the renin-angiotensin system, rather than systolic blood pressure (SBP) or peripheral renal levels, in mediating effects on sclerosis (51).

The role of the AT2 receptor in renal injury has been less defined than that of the AT1 receptor. The AT2 receptor counteracts the vasoconstrictor effect of the AT1 receptor (11, 26, 47), inhibits cellular proliferation, and promotes apoptosis (10, 14, 27, 35, 38). However, whether these actions are also active in vivo in CKD is controversial. AT2 receptor transgenic mice had less sclerosis than wild-type (WT) mice after 5/6 nephrectomy (Nx), a model of CKD. Furthermore, specific overexpression of a novel molecule that specifically mediates some AT2 receptor actions, namely the ANG II type 2 receptor-interacting protein 1 (ATIP1), resulted in protection of mice against vascular injury (22). Correspondingly, sclerosis was worse in AT2−/− mice (5, 25), and interstitial fibrosis was also worsened with AT2 receptor antagonism (40). On the other hand, AT2 receptor blockade was renoprotective when given in the early phase of 5/6 Nx in rats (9). The balance of AT1 vs. AT2 activation also modulates NF-κB and thus may modify net injury (17). In this study, we therefore investigated the role of the AT2 in the beneficial effects induced by ANG type 1 receptor blocker on remodeling of existing CKD in the widely studied renal ablation model, which importantly has relevance for progression of human CKD (3, 4, 7).

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (200–250 g; Charles River, Nashville, TN) were studied. All protocols were approved by the Institutional Animal Care and Use Committee according to the National...
Institutes of Health guidelines. Rats were housed under normal conditions with a 12:12-h light-dark cycle at 21°C with 40% humidity and 12 air exchanges per hour, standard rat powdered diet (Purina Rodent meal 5001, 23.4% protein, 4.5% fat, 6% fiber, 0.4% sodium; Tusculum Feed Center, Nashville, TN), and water ad libitum. Rats underwent 5/6 Nx by right Nx and ligation of two or three branches of the left renal artery (36). This model is characterized by development of early sclerosis and proteinuria by week 4, which become established by week 8, with subsequent more rapid development of increasingly severe sclerosis, proteinuria, and hypertension (36). The removed right kidney was used as normal baseline tissue. Rats were allocated to treatment groups at 8 wk after 5/6 Nx to achieve equal average starting point of sclerosis assessed by open renal biopsy, and equal average levels of SBP and proteinuria before treatment were started. Rats were then treated as specified below for 4 more wk till death at week 12 after 5/6 Nx. The control 5/6 Nx group received no treatment (untreated control 5/6 Nx, n = 6). The ARB antagonist group (ARB, n = 6) received 80 mg/l drinking water losartan, a dose fourfold over minimum antihypertensive doses that we previously showed can induce regression of sclerosis from week 8 to week 12 in this model in some rats (36). The AT1R antagonist group (AT2RA, n = 6) received PD123319, 15 mg·kg−1·day−1 delivered sc by osmotic minipump (Alzet model 2ML4; Alza, Palo Alto, CA). PD123319 is a nonpeptide highly potent and selective AT2 receptor antagonist (gift of Pfizer) and is made in a trifluoroacetate salt formula (MW 576.4 vs. parent compound MW 508.6) which can be dissolved in normal saline. The dose chosen was based on previous studies in cardiovascular remodeling and kidney fibrosis, using 5–10 mg·kg−1·day−1 of parent compound, equivalent to 15 mg·kg−1·day−1 of the trifluoroacetate compound (9, 30). The combination group (combined, n = 6) received both losartan and PD123319 as above. Normal kidney tissue removed at time of 5/6 Nx was used as normal control for some tissue assessments.

SBP was measured using conscious tail-cuff plethysmography in prewarmed trained rats at ambient temperature of 29°C. Animals were placed in metabolic cages for collection of 24-h urine samples. Urine protein excretion was measured by Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Serum creatinine was measured at 12 wk by Vitros CREA slides (Johnson & Johnson Clinical Diagnostics, Rochester, NY). Creatinine clearance was calculated from 24-h urine samples at 12 wk.

Glomerular injury. The severity of sclerosis in each glomerulus was graded from 0 to 4+ as follows: 0 = no lesion, 1+ = sclerosis of <25%, 2+ = sclerosis of 25–50%, 3+ = sclerosis of 50–75%, 4+ = sclerosis of >75% of the glomerulus, respectively (36). A whole kidney average sclerosis index for each rat was obtained by averaging scores from all glomeruli on PAS-stained sections. All sections were examined without knowledge of the treatment protocol. Change of sclerosis from all glomeruli on each section. Apoptosis in cortical tubules of kidneys was semiquantitatively scored from 0 to 4: 0 = no positivity, 1+ = positivity of tubular nuclei <25%, 2+ = 25–50%, 3+ = 50–75%, 4+ = >75% in 10 nonoverlapping ×200 fields, and average score was calculated for each section. All sections were examined without knowledge of the treatment protocol.

In situ hybridization.35S-labeled sense and antisense riboprobes for PAI-1 were prepared as previously described (41). Negative control in situ hybridization was done with sense probes and showed no signal. PAI-1 mRNA was assessed by image analysis using SCION Image Beta 4.03 (Scion, Frederick, MD). Briefly, at least eight nonoverlapping random ×200 dark fields in the cortex were assessed for positive signals calculated as percent area of field occupied by PAI-1 mRNA signal, and average was calculated for each section. All sections were examined without knowledge of the treatment protocol.

Gene expression studies. Total RNA was isolated from kidney cortex using the RNeasy mini kit according to the manufacturer’s instructions (Qiagen). RNA was reverse transcribed using the TaqMan reverse transcription kit (Applied Biosystems). Real-time PCR amplifications were performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems). Following inventornted TaqMan assays, IDs were used: Rn01435427_m1 for rat AT1R, Rn02132799_s1 for PD123319, and Hs00999991_s1 for 18s ribosome. 18s RNA was used as internal control. The relative levels of mRNA expression for these genes were obtained as previously described (32).

Western blot analysis. Tissue protein extracts were analyzed with rabbit anti-rat PAI-1 antibody (American Diagnostica) and rabbit anti-phi-hospho-p44/42 MAP kinase (Erk1 and Erk2) antibody (Cell Signaling Technology, Beverly, MA) as primary antibodies with appropriate secondary antibody and visualized using an ECL Western Blotting Detection Reagents (Amersham Biosciences). Membranes were reprobed with mouse anti-β-actin (Sigma) and mouse anti-MAP kinase 2 (Erk2; Upstate, Lake Placid, NY) as loading controls and expression was semiquantitatively assessed by SCION Image Beta 4.03 (Scion) as a densitometric ratio relative to each loading control.

Collagen content. Renal cortex was isolated and frozen, and collagen content was calculated from the concentration of hydroxyproline, measured as their phenylisothiocyanate derivatives by reverse-phase high-performance liquid chromatography (RP-HPLC) (35). Briefly, tissue homogenates were hydrolyzed in vacuo in constant boiling HCl (110°C, 16 h) and were dried and taken up in 1 ml of redrying solution [ethanol/water/(C2H5)3N/phenylisothiocyanate, 7:1:1:1] were added, redried under nitrogen. Twenty microliter of derivatization reagent [ethanol/water/(C2H5)3N/phenylisothiocyanato-cyanate, 7:1:1:1] were added, and the reaction was allowed to proceed at room temperature for 20 min. The sample was then lyophilized during centrifugation to remove the reagents. Samples were either frozen at this stage or diluted to 1 ml with buffer (5% acetonitrile in 5 mmol/l disodium phosphate, pH 7.4). A solution containing amino acid standards was dried and coupled in the same manner. The coupling efficiency was >99% under these conditions. Collagen content was expressed as percent of total protein.

Podocyte culture. Podocyte injury is crucial for progression of glomerulosclerosis. We therefore studied the effects of AT1 vs. AT2 blockade on injury induced by ANG II in cultured podocytes. Our in vivo studies pointed to a key role of PAI-1 in glomerulosclerosis. We therefore also investigated the role of PAI-1 and interaction with AT1 vs. AT2 in podocyte injury. To achieve these goals, we cultured podocytes from WT and PAI-1−/− mouse kidneys as previously described (34). Podocyte identity was confirmed by WT-1 and synaptopodin staining (34). WT and PAI-1−/− podocytes were preincubated for 24 h with ARB (10−5 M) or AT1-R (10−5 M) or both or left untreated and then exposed to ANG II (10−7 M) for 48 h in the presence of ARB, AT1-R, or both, and compared with control cells without ANG II or additional treatment. Doses were based on a previous study in cultured cells (23, 31). Collagenα1/IV accumulation in the supernatant was chosen as a marker of profibrotic injury to the podocyte and was assessed by ELISA. Activation of the Erk pathway was assessed in WT podocytes. WT podocytes were serum-
starved (control) or serum-starved with additional treatment for 15 min with ANG II with added ARB, AT2RA, or combination in doses as above as indicated.

Statistical analysis. Data are expressed as means \( \pm \) SE. Differences between groups were examined for statistical significance using one-way ANOVA or Mann-Whitney U-test. \( P \) values <0.05 were considered significant.

RESULTS

SBP and renal function. Body weight was not different among the groups at any time (Table 1). SBP tail-cuff plethysmograph, 24-h proteinuria, and serum creatinine were by study design equivalent among groups before treatments began 8 wk after 5/6 Nx, a time point of established early sclerosis in this model. SBP at 12 wk was decreased only numerically in AT2RA alone and significantly in ARB and combined groups vs. the untreated 5/6 Nx group (untreated 5/6 Nx 206.6 \pm 3.5, ARB 157.1 \pm 12.8, AT2RA 180.3 \pm 12.9 mmHg, combined 164.3 \pm 4.8, untreated 5/6 Nx vs. ARB \( P < 0.05 \), untreated 5/6 Nx vs. combined \( P < 0.05 \); Table 1). Proteinuria at 12 wk was decreased vs. the untreated 5/6 Nx group in ARB and combined groups but not in AT2RA-treated rats. Proteinuria was decreased by ARB, and in contrast remained high in untreated 5/6 Nx rats and in AT2RA-treated rats (untreated 5/6 Nx 463.2 \pm 39.1, ARB 199.3 \pm 48.9, AT2RA 386.5 \pm 34.8, combined 242.1 \pm 79.5 mg/day, untreated 5/6 Nx vs. ARB \( P < 0.01 \), untreated 5/6 Nx vs. combined \( P < 0.05 \), ARB vs. AT2RA \( P < 0.05 \); Table 1). Serum creatinine and creatinine clearance after 4 wk of treatment were not significantly different between groups, mirroring our previous experience in this model and that of others (see Fig. 3; CCR untreated 5/6 Nx 0.8 \pm 0.2, ARB 0.8 \pm 0.3, AT2RA 0.6 \pm 0.1, combined 0.6 \pm 0.2 ml/min) (1).

These relatively insensitive measures of glomerular filtration rate (GFR) did not reflect the improved proteinuria or the structural improvement with ARB, likely reflecting both the marked decrease in GFR resulting from the removal of a large portion of nephron mass before initiating treatment and the relatively short period of treatment.

Kidney scarring. By study design, glomerulosclerosis was similar in 8-wk biopsies in all groups (sclerosis index at 8 wk untreated 5/6 Nx 0.92 \pm 0.16, ARB 0.86 \pm 0.17, AT2RA 0.79 \pm 0.10, combined 0.78 \pm 0.10). At 12 wk after 5/6 Nx, glomerulosclerosis, tubular atrophy, dilatation, cast formation, and interstitial fibrosis were far more advanced compared with biopsy in untreated 5/6 Nx and also in rats treated only with AT2RA (Fig. 1A). Normal baseline kidneys removed at time of 5/6 Nx showed no glomerulosclerosis, interstitial fibrosis, or tubular atrophy. Average sclerosis increase was significantly less in ARB-treated rats, with regression in some rats (percent change in sclerosis from biopsy to autopsy; untreated 5/6 Nx 93.4 \pm 17.8, ARB 50.6 \pm 18.3, AT2RA 121.8 \pm 25.8, combined 57.3 \pm 26.6%; ARB vs. AT2RA \( P < 0.05 \); Fig. 1B).

Kidney cortex collagen content, assessed by HPLC (37), was dramatically increased compared with normal kidney tissue removed at time of 5/6Nx in untreated 5/6 Nx, AT2RA, and combined groups (Fig. 2), and nearly normalized in rats treated with ARB (normal kidney 1.7 \pm 0.3, untreated 5/6 Nx 18.7 \pm 3.8, ARB 2.5 \pm 0.7, AT2RA 14.9 \pm 6.6, combined 16.3 \pm 2.0%, normal vs. untreated 5/6 Nx and combined \( P < 0.01 \), normal vs. AT2RA \( P < 0.05 \), ARB vs. AT2RA \( P < 0.05 \), ARB vs. combined and untreated 5/6 Nx \( P < 0.01 \)).

We next studied whether AT1a vs. AT1b receptor expression shifted with interventions. Compared with untreated 5/6 Nx at 12 wk, expression of AT1a mRNA in kidney cortex increased 9.6 \pm 5.1-fold in ARB-treated rats, 5.0 \pm 2.7-fold in AT2RA-treated rats, and 6.8 \pm 2.3-fold in combined group (\( P = 0.06 \) ARB vs. untreated 5/6 Nx). AT1b mRNA expression in kidney cortex also increased 3.7 \pm 0.5-, 2.5 \pm 0.4-, and 17.2 \pm 11.0-fold, respectively, in ARB, AT2RA, and combined group, compared with untreated 5/6 Nx.

Cellular proliferation and apoptosis. Immunostaining for PCNA was negative in the normal rat glomerulus in kidneys compared with untreated 5/6 Nx vs. AT2RA and combined groups (Fig. 2), and nearly normalized in rats treated with ARB (normal kidney 1.7 \pm 0.3, untreated 5/6 Nx 27.1 \pm 2.5%, combined 16.3 \pm 2.0%, normal vs. untreated 5/6 Nx and combined \( P < 0.01 \), normal vs. AT2RA \( P < 0.05 \), ARB vs. AT2RA \( P < 0.05 \), ARB vs. combined and untreated 5/6 Nx \( P < 0.01 \)).

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<th>Table 1. Systemic and renal parameters</th>
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<td><strong>Week</strong></td>
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<td><strong>Serum creatinine, mg/dl</strong></td>
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Values are means \( \pm \) SE. ARB, angiotensin type 1 receptor blocker; CONT 5/6 Nx, control 5/6 nephrectomy. *\( P < 0.05 \) vs. CONT.
Glomerular PCNA-positive cells included mesangial area and endothelial cells, and rare cells consistent with podocytes. Tubular cells were frequently positive for PCNA in untreated 5/6 Nx and AT2RA, with rare tubular positivity in ARB and combined groups. PCNA-positive interstitial cells were occasionally present in untreated 5/6 Nx and AT2RA groups, and were very rare in ARB and combined groups. Apoptosis as assessed by TUNEL-positive cells was present mostly in tubular epithelial cells, with few positive cells in glomeruli with no difference among groups (glomerulus: untreated 5/6 Nx 0.4 ± 0.2, ARB 0.3 ± 0.1, AT2RA 0.5 ± 0.1, combined 0.3 ± 0.2/glomerulus; cortex: untreated 5/6 Nx 2.1 ± 0.2, ARB 2.0 ± 0.1, AT2RA 2.2 ± 0.2, combined 2.2 ± 0.2). Thus, the balance of proliferation vs. apoptosis was shifted in untreated and AT2RA-treated glomeruli.

Erk 1/2 expression. We next evaluated the activation of Erk1/2, a downstream signaling molecule for the angiotensin system. In normal kidney cortex, phosphorylated Erk was present at low levels. Phospho-Erk2 expression was upregulated by AT2RA vs. ARB or combined (normal kidney 0.39 ± 0.01, untreated 5/6 Nx 0.78 ± 0.10, ARB 0.56 ± 0.04, AT2RA 0.86 ± 0.07, combined 0.61 ± 0.10; normal vs. untreated 5/6 Nx and AT2RA P < 0.01, ARB vs. AT2RA P < 0.05, AT2RA vs. combined P < 0.05; Fig. 3).

Fig. 2. Collagen content, assessed by HPLC of lysates from renal cortex. Compared with normal kidney, collagen content increased significantly in control untreated 5/6 Nx and AT2RA, with significant decrease in ARB but not combined group. **P < 0.01, normal vs. control untreated 5/6 Nx, normal vs. combined, control untreated 5/6 Nx vs. AT2RA, AT2RA vs. combined. *P < 0.05, normal vs. AT2RA, ARB vs. AT2RA; n = 4 for normal; n = 5–6 for all other groups.

Fig. 3. Representative Western blots of phospho-Erk 2 protein in the renal cortex of rats at 12 wk after 5/6 Nx (A). The expression of phospho-Erk 2 protein is presented as phospho-Erk 2/total Erk 2 ratio (B). **P < 0.01, normal vs. control untreated 5/6 Nx, normal vs. AT2RA. *P < 0.05, AT2RA vs. ARB, AT2RA vs. combined; n = 3 for normal; n = 5–6 for all other groups.
PAI-1 and plasmin expression. In normal kidney, PAI-1 mRNA by in situ hybridization showed diffuse low-level tubular and no glomerular expression (data not shown). PAI-1 mRNA expression was augmented in untreated 5/6 Nx and AT2RA at 12 wk and was localized mainly in sclerotic glomeruli, mesangial areas, tubules, interstitial cells, and occasionally in glomerular endothelial cells, podocytes as identified by anatomical location, and small artery endothelial and vascular smooth muscle cells. Image analysis showed that PAI-1 mRNA was significantly increased in untreated 5/6 Nx, and even further increased in AT2RA, compared with significant decrease in ARB and combined group (untreated 5/6 Nx 9.79 ± 1.00, ARB 4.36 ± 0.54, AT2RA 17.24 ± 1.62, combined 7.00 ± 1.69%; untreated 5/6 Nx vs. ARB P < 0.05, AT2RA vs. untreated 5/6 Nx, ARB or combined P < 0.01; Fig. 4).

PAI-1 protein expression paralleled PAI-1 mRNA. PAI-1 immunostaining was mainly increased in areas of sclerosis and fibrosis with less staining in ARB and combined vs. untreated 5/6 Nx or AT2RA (Fig. 5). Western blotting verified that cortical PAI-1 protein expression was increased in untreated 5/6 Nx and AT2RA compared with ARB, with intermediate level in combined (untreated 5/6 Nx 0.70 ± 0.03, ARB 0.56 ± 0.04, AT2RA 0.73 ± 0.03, combined 0.64 ± 0.03; untreated 5/6 Nx vs. ARB P < 0.01, ARB vs. AT2RA P < 0.01; Fig. 6).

Plasmin activity of kidney cortex at 12 wk after 5/6 Nx showed a trend for decrease in AT2RA and combined groups even below the slightly decreased level seen in untreated 5/6 Nx, whereas ARB-treated rats had normalized plasmin levels (normal kidney 0.16 ± 0.05, untreated 5/6 Nx 0.12 ± 0.02, ARB 0.16 ± 0.08, AT2RA 0.08 ± 0.02, combined 0.06 ± 0.01) (28, 36).

Effects of AT1 vs. AT2 receptor blocker on podocytes. The podocyte is a key responder and modulator of development of glomerulosclerosis. We therefore examined podocyte-specific responses to ANG II, evaluating whether this response was influenced by AT1 or AT2 receptor blocker. We further assessed whether PAI-1 contributed to any differential response by studying WT and PAI-1−/− podocytes. We used mouse primary podocyte cultures as rat knockout for PAI-1 is not available. In WT, but not PAI-1−/− podocytes, ANG II significantly increased collagen synthesis measured by ELISA compared with control (control 192.8 ± 19.1 vs. ANG II 557.3 ± 158.8 ng/ml, P < 0.05). ARB completely blocked this response in WT podocytes, with only numerical decrease with AT2RA or combination (ANG II + ARB 188.7 ± 36.7, ANG II + AT2RA 423.3 ± 98.5, ANG II + combination
346.7 ± 186.3 ng/ml, ANG II vs. ARB \( P < 0.05 \), ANG II vs. AT\(_2\)RA or combination \( P = \text{NS} \); Fig. 7). Although phosphorylation of Erk was increased by ANG II, ARB and AT\(_2\)RA were equally effective in blunting this response in WT podocytes (p-Erk/β-actin ratio: control 0.70 ± 0.13, ANG II 1.60 ± 0.09, ANG II + ARB 0.89 ± 0.01, AT\(_2\)RA 0.97 ± 0.18, combination 0.94 ± 0.09; Fig. 8). These studies support that the podocyte’s profibrotic response to ANG II is largely AT\(_1\) mediated and is dependent on the presence of PAI-1.

**DISCUSSION**

The pathophysiological role of the AT\(_2\) receptor in CKD has not been defined. Renoprotection linked to the AT\(_2\) receptor has been variably reported even in the same model, 5/6 Nx (9, 25, 50). These results could, in part, reflect different time points of...
intervention and observation. Thus, the AT2 receptor was linked to injurious effects at 4 wk after 5/6 Nx (9). In contrast, later or earlier time points suggested beneficial effects of the AT2 receptor on sclerosis (19). Species differences in mice vs. rats could also underlie some of the conflicting reports. We chose to study the rat model of 5/6 Nx with ligation of renal artery branches, as results from this model previously showed relevance to insights and treatment of human CKD. Effects of AT2R may differ in other models with less hypertension and other pathogenic mechanisms. Next, we decided to study the role of the AT2 receptor in existing glomerulosclerosis, as this delayed timing of intervention is most relevant to human CKD where treatment is initiated after the disease is clinically evident. Our studies show that in the setting of such delayed intervention, not only is blockade of the AT2 receptor of no delayed timing of intervention is most relevant to human CKD where treatment is initiated after the disease is clinically evident. Our studies show that in the setting of such delayed intervention, not only is blockade of the AT2 receptor of no benefit, it prevents the beneficial effects of ARB on sclerosis and proteinuria when added to this treatment. Of note, these beneficial effects on renal damage are not paralleled by significant decrease in serum creatinine. We and others previously observed this apparent discordance in the 5/6 Nx model, which likely reflects the short duration of treatment, the relative insensitivity of serum creatinine as a measure of GFR in rodents and humans, and the loss of renal function following the initial surgical removal of 5/6 of renal mass that leaves fewer nephrons with the potential to measurably improve function even in the face of less sclerosis (1, 36, 42).

Next, we investigated the mechanisms whereby the AT2 receptor might modulate kidney fibrosis in this model. In vitro studies demonstrated that AT2 receptor-mediated signal induces not only downregulation of cellular proliferation but also upregulation of apoptosis by inhibition of cell signaling Erk1/2 and PI3K, and Akt activation through activation of tyrosine phosphatases (14, 27). However, whether the AT2 receptor has anti-proliferative effects in vivo is controversial (9, 10, 17). Glomerular PCNA positivity was reduced by AT2 receptor antagonism in a rat injury model of ANG II infusion (43). In contrast, our study showed that glomerular PCNA-positive cells remained remarkably high in AT2 receptor antagonist-treated rats, similar to untreated control remnant kidney rats. Moreover, phosphorylated Erk2 remained significantly upregulated in AT2 receptor antagonist-treated rats, similar to the 5/6 Nx-untreated rats. Our in vitro data show that podocytes injured by ANG II also have increased phosphorylated Erk2, but ARB and AT2RA and combination dampened this response. These data indicate that effects of AT2RA to decrease Erk2 activation in vivo are mediated by actions on cells other than podocytes. Erk-MAP kinase signaling induces cellular proliferation via transcriptional factors such as AP-1 (16), and inhibits apoptosis through Bcl-2 phosphorylation, and AT2 modifies these molecules in vitro (18, 27, 44). In vivo in the protein overload model of tubulointerstitial injury, increased apoptosis in proximal tubules was linked with upregulation of AT2 and altered Erk and Bcl-2 (49). In our model of glomerulosclerosis, we did not demonstrate changes in TUNEL staining with ARB or AT2RA; however, this assessment does not exclude possible effects on cell death. Our data indicate that the AT2 receptor might downregulate glomerular cellular proliferation in this setting by inactivation of Erk signaling.

ANG II is one key factor in the development of renal fibrosis (19, 20). Our data show that AT2 receptor antagonism only numerically decreased SBP, and thus we cannot rule out that blood pressure effects could contribute to our observations. However, in the combined group with ARB and AT2RA, blood pressure was even significantly decreased; yet, the added AT2R antagonism prevented the beneficial effects observed with ARB alone. Thus, these data point to additional effects of modulating the AT2 receptor beyond or in addition to any effect related to modest changes in blood pressure.

The rodent has two subtypes of the AT1 receptor, the major AT1A receptor, and the less abundant AT1B receptor. The latter has recently been suggested to contribute to increased inflammation and glomerular injury (13). ARB blocks both receptors. Our mRNA expression data suggest the possibility that AT2RA could not only block the potential beneficial effects of AT2 receptor, but also result in upregulated AT1B receptor, which might also contribute to worsened injury with AT2RA alone. Whether receptor protein and signal transduction of AT1B are affected by AT2 receptor modulation and possible interactions awaits further study.

ANG II has manifold profibrotic effects in addition to induction of hypertension: it induces extracellular matrix (ECM) accumulation directly and indirectly by inducing TGF-ß, and inhibitors of ECM turnover, such as PAI-1, induced by AT1 effects (15, 21, 41). Effects of AT2 on the ECM are less well-delineated. Our results show that PAI-1 mRNA expression was increased by AT2 receptor antagonism even beyond untreated 5/6 Nx levels, in contrast to the marked decrease in PAI-1 induced by ARB. Furthermore, addition of AT2RA to ARB resulted in higher PAI-1 expression by in situ hybridization compared with ARB alone. Western blots confirmed high levels of PAI-1 in untreated 5/6 Nx and AT2RA-treated rats, and decreased PAI-1 with ARB, with numerically intermediate level with combined therapy. These results support that the AT2 receptor might suppress progression of glomerulosclerosis and renal scarring through downregulation of PAI-1 in the remnant kidney model, and conversely, that inhibition of AT2 action increases PAI-1, thus promoting fibrosis.

The Erk pathway could induce PAI-1 through transactivation of downstream transcriptional factors such as hypoxia-inducible factor-1, Elk-1, and AP-1 (12, 24, 48). We speculate that AT2 receptor antagonism augments PAI-1 expression through upregulation of Erk phosphorylation in the 5/6 Nx kidney. Our in vitro studies of podocytes showed that AT1 vs. AT2 effects on ANG II-induced collagen were not linked to altered Erk activation. Possible newly identified mediators of AT2 signaling, such as ATIP1, might be involved in fibrosis responses (22). However, PAI-1 expression might also be modulated by AT2-dependent mechanisms through, e.g., inhibition of bradykinin/NO/cGMP pathway (6). Future in vitro studies may elucidate in greater detail the relationship between the AT2 receptor and PAI-1.

We also showed that renal collagen was significantly decreased by ARB, with even greater benefit of ARB on cortical collagen content, a measure largely reflecting tubulointerstitial fibrosis, than on glomerulosclerosis. Furthermore, added AT2 receptor antagonism in particular dampened ARB benefits on collagen content, supporting key effects of AT1/AT2 balance on the tubulointerstitium in fibrotic states. Effects of the AT2 receptor on fibrosis have been controversial both in vitro and in vivo (2, 9, 29, 30, 33, 35, 39, 40, 45). AT2 receptor stimulation in vitro increased collagen synthesis in vascular smooth muscle and mesangial cells via a Gxa1-mediated mech-
anism, while essentially opposite effects were observed in fibroblasts (39). In vivo studies of transgenic mice with up-regulation of AT1P1 showed protection against neointimal response in the injured femoral artery (22). These results suggest that AT2 receptor-mediated signals might have heterogeneous effects on different cell types.

In summary, our results indicate that the beneficial effects of ARB on renal injury are complex and involve both blockade of deleterious AT1 actions and actions of the AT2 receptor.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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