Angiotensin II increases fibronectin and collagen I through the β-catenin-dependent signaling in mouse collecting duct cells

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ANGIOTENSIN II (ANG II) plays a key role in the development and progression of chronic kidney disease (CKD) (27). It has been shown that increased levels of ANG II and renin in renal tubules after subtotal nephrectomy are pathogenically linked to the development of tubulointerstitial injury (10). In particular, alterations in the ANG type 1 (AT1) receptor, angiotensin-converting enzyme 2 (ACE-2), and the newly described (pro)renin receptor precede the development of renal fibrosis (41). Evidence from in vivo studies has shown that AT1 receptor antagonists ameliorate renal tubulointerstitial fibrosis caused by unilateral ureteral obstruction (17). Further, in vitro studies indicate that ANG II activates renal cells to produce profibrotic factors and extracellular matrix (ECM) proteins (37, 48, 51). These profibrotic factors lead to tubulointerstitial injury and glomerulosclerosis due to excessive accumulation and deposition of ECM components (3, 49), which are the final manifestations of CKD and renal failure (24).

METHODS

Cell line culture and treatments. M-1 cortical collecting duct cells (American Type Culture Collection; CRL-2038) were grown in DMEM-F12 media supplemented with 10% FBS, 5 μM dexamethasone, 1× insulin-transferrin-selenium, and 100 U/ml penicillin/streptomycin in a humid atmosphere of 5% CO2-95% room air at 37°C. Cells were then treated with ANG II (10^{-7} M) for 16 h. An additional
group of cells was treated with either an inhibitor of the Wnt signaling pathway (pyrvinium pamoate; 10−9 M) or an AT1 receptor (candesartan; 10−7 M) blocker. The dose of pyrvinium pamoate was based on previous studies showing its effectiveness at nanomolar concentrations in reducing β-catenin phosphorylation and proliferation in tumor cells (22, 28, 38, 45). Additionally, it has been shown that pyrvinium pamoate selectively potentiates the casein kinase alpha activity with an EC50 of 10 nM (45).

**Immunofluorescence in M-1 cells.** Subconfluent M-1 cells (50−60%) cultured in chamber slides (Nalge Nunc, Rochester, NY) were fixed in cold methanol for 20 min, blocked with PBS-Tween (0.1%) plus BSA (3%) for 1 h, and stained with rabbit anti-β-actin (Cat. no. sc-130657; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilutions, and detected with Alexa Fluor 488 conjugated to anti-rabbit IgG (Invitrogen, Carlsbad, CA) at 1:1,000 dilution. Samples were counterstained with 4', 6-diamidino-2-phenylindole (Invitrogen). Negative controls were obtained by omission of the specific primary antibody.

**Measurements of fibronectin, collagen I, cyclin D1, and c-myc mRNAs by qRT-PCR.** Quantitative real-time RT-PCR (qRT-PCR) was performed using the TaqMan PCR system. Total RNA (20 ng) was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). The following primers and probes were used to amplify the genes: 

- **Fibronectin**: 5′-TCAGTGGCTTTCCCTAC-3′ (sense), 5′-GGTAGGTGGTGG-GAATGA-3′ (antisense), and 5′-FAM-AGCATGCACA-CTGTC-3′ (fluorogenic probe);
- **Collagen I**: 5′-TCAGTGGCTTTCCCTAC-3′ (sense), 5′-GTGAATGAGTTGCAG-3′ (antisense), and 5′-FAM-AGCATGCACA-CTGTC-3′ (fluorogenic probe);
- **cyclin D1**: 5′-CATTGAACTGCTTC-3′ (sense), 5′-GAATGAGTTGCAG-3′ (antisense), and 5′-FAM-AGCATGCACA-CTGTC-3′ (fluorogenic probe);
- **c-myc**: 5′-TCAGTGGCTTTCCCTAC-3′ (sense), 5′-GTGAATGAGTTGCAG-3′ (antisense), and 5′-FAM-AGCATGCACA-CTGTC-3′ (fluorogenic probe);
- **β-catenin**: 5′-TCAGTGGCTTTCCCTAC-3′ (sense), 5′-GTGAATGAGTTGCAG-3′ (antisense), and 5′-FAM-AGCATGCACA-CTGTC-3′ (fluorogenic probe).

Data were normalized against β-actin mRNA levels using primer and probe sequences as previously described (11).

**Protein expression of α-smooth muscle actin, fibronectin, collagen I, cyclin D1, c-myc, and β-catenin.** Protein expression levels were quantified after immunoblotting using a 1:1,000 dilution of the following specific antibodies: 

- α-smooth muscle actin (α-SMA; Cat. no. sc-7882, sc-53142 Santa Cruz Biotechnology), fibronectin, collagen I (Cat. no. sc-6458, sc-4500363; Sigma, St. Louis, MO), activated β-catenin (anti-ABC, Cat. no. sc-130657, sc-717, sc-788, and sc-130657, respectively; Santa Cruz Biotechnology). 

Primary antibodies were followed by incubation with either donkey anti-rabbit or anti-mouse IgG IRDye 800 CW (Li-cor Biosciences, Lincoln, NE) at a 1:30,000 dilution. Densitometric analyses were performed by normalization against β-actin.

**Statistics analysis.** Each experiment constituted an average of five to six independent observations (each well represented an independent observation). Experiments were performed in at least three different cell passages. Cells were used until passages 10−12. Differences between groups were assessed by one-way ANOVA followed by Tukey’s test using the GraphPad Prism software v 5.0 (GraphPad Software, San Diego, CA). P < 0.05 was considered statistically
significant. The results shown in plots and texts are expressed as means ± SD.

RESULTS

ANG II induces myofibroblast-like phenotype and α-SMA protein levels in M-1 cells. To evaluate whether ANG II treatment induces epithelial-mesenchymal transition, M-1 cells were treated with ANG II (10^-7 M). After 48 h, cells were fixed in methanol and staining with β-actin antibody to visualize the cell shape using immunofluorescence. Figure 1A shows three representative fields demonstrating that the number of myofibroblast-like cells was augmented after 48 h of ANG II treatment. As a marker of myofibroblast phenotype induction, we quantified the protein levels of α-SMA by Western blot. Figure 1B shows that protein abundance of α-SMA was upregulated after 24 h of ANG II treatment (fold change: 2.12 ± 0.32 vs. control: 1.00 ± 0.23, P < 0.05). Pretreatment with losartan (10^-6 M) prevented the ANG II-dependent induction of myofibroblast-like phenotype and α-SMA protein levels (fold change: 1.11 ± 0.42 vs. control: 1.00 ± 0.23, P = 0.212).

ANG II induces the expression of fibronectin and collagen I via AT1 receptor in M-1 collecting duct cells. Figure 2A shows that ANG II (10^-7 M) treatment in M-1 cells induced significant increases in mRNA levels of fibronectin (fold change: 1.55 ± 0.21 vs. control: 0.94 ± 0.11, P < 0.05) and collagen I (fold change: 1.46 ± 0.20 vs. control: 0.87 ± 0.17, P < 0.05). Figure 2B displays similar results in protein levels (fibronectin: 1.53 ± 0.16 vs. control: 0.95 ± 0.09, P < 0.05; collagen I: 2.18 ± 0.23 vs. control: 1.06 ± 0.08 fold change, P < 0.05). Candesartan (10^-7 M), an AT1 receptor blocker, prevented the ANG II-mediated induction of fibronectin (mRNA: 0.91 ± 0.14, protein: 0.84 ± 0.23, P = NS) and collagen I (mRNA: 1.09 ± 0.14, protein: 1.07 ± 0.16, P = NS).

ANG II increases the expression of β-catenin target genes in M-1 cells. Cyclin D1 and c-myc are β-catenin target genes. ANG II augmented the mRNA and protein levels of cyclin D1 and c-myc (fold change cyclin D1: 1.86 ± 0.48 and 1.65 ± 0.11; c-myc: 1.63 ± 0.04 and 2.11 ± 0.36, respectively, P < 0.05). In the presence of candesartan, the effects of ANG II on cyclin D1 and c-myc were completely abolished (Fig. 3, A and B). We next evaluated whether ANG II treatment was able to increase β-catenin expression, which would explain the induction of cyclin D1 and c-myc. As shown in Fig. 4, ANG II increased β-catenin protein levels after 4 h, with the highest induction at 16 h (fold change: 2.61 ± 0.15 vs. 1.10 ± 0.21, P < 0.001). This induction was still significantly higher after 24 h (fold change: 1.61 ± 0.20 vs. 1.01 ± 0.21, P < 0.05).

Angiotensin II induces the expression of collagen I and fibronectin via activation of the β-catenin signaling in M-1 cells. To determine whether ANG II interacts with the β-catenin signaling pathway to induce the expression of profibrotic genes, we treated M-1 cells with ANG II in the presence of pyrvinium pamoate, which targets β-catenin by activating CK-1α (38). As shown in Fig. 5, A and B, ANG II-mediated upregulation of collagen I and cyclin D1 and c-myc mRNA levels were prevented by pyrvinium pamoate. In a new set of experiments, we evaluated the effect of pyrvinium pamoate on the ANG II-induced expression of β-catenin. As shown in Fig. 6, ANG II treatment induced β-catenin protein level changes (fold change: 2.21 ± 0.43 vs. 1.01 ± 0.06, P < 0.05). Along with the ANG II-induced upregulation of...
β-catenin, ANG II also promoted the phosphorylation of ser-9-GSK-3β (fold change p-ser-9-GSK-3β/GSK-3β ratio: 3.56 ± 0.47, P < 0.05). Previous treatment with pyrvinium pamoate suppressed the ANG II-mediated upregulation of β-catenin (fold change: 1.23 ± 0.19, P = nonsignificant vs. control) and p-ser-9-GSK-3β/GSK-3β ratio (fold change: 1.58 ± 0.48, P = nonsignificant). Treatment with pyrvinium pamoate in control cells showed nonsignificant changes in the p-ser-9-GSK-3β/GSK-3β ratio; however, we observed a slight, but significant reduction in β-catenin expression levels (fold change: 0.78 ± 0.22, P < 0.05; Fig. 5). Phosphorylated β-catenin levels were reduced by ANG II treatment, whereas pyrvinium pamoate prevented this effect (Fig. 6).

**DISCUSSION**

In the present study, we demonstrate that in mouse collecting duct cells, ANG II via AT1 receptor induces the expression of fibronectin and collagen I as well as cyclin D1 and c-myc, which are both target genes of the β-catenin signaling pathway. Our results indicate that the activation of the AT1 receptor induces fibrotic factors in the collecting duct cells through the stimulation of β-catenin signaling.

Angiotensin II is the major fibrogenic factor in the kidney and most of its effects are related to cell cycle dysregulation (51). Systemic chronic infusion of ANG II induces the overexpression of fibronectin and collagen I deposition in the kidney (52). Some of the ANG II-dependent mechanisms involved in the development of CKD involve oxidative stress, intrarenal RAS overactivation, and high blood pressure (11, 21, 46). Additionally, other factors may contribute to the stimulation of fibrosis, independent of ANG II (15, 16). The sustained formation of peroxynitrite in rats fed a high-salt diet may induce sulfhydryl oxidation, protein nitration, and lipid peroxidation, which all contribute to kidney injury (8), as reflected by the glomerular expansion and tubulointerstitial fibrosis. We reported that an excessively high-salt diet, by itself, leads to a marked increase in peroxynitrite formation and predisposes the kidney to greater tubulointerstitial injury when associated with chronic ANG II infusions (23). High salt also independently stimulates TGF-β1 (47) and other intracellular signaling pathways leading to an enhanced response to ANG II. In fact, we showed in rats that physiological intrarenal RAS activation in response to a chronic low-salt diet, despite absence of major renal tissue injury, augmented renal tubulointerstitial fibrosis (42).

Although most of the profibrotic effects of ANG II have been reported in mesangial cells, podocytes, and proximal tubule epithelial cells (1, 50), a direct link between ANG II and
Angiotensin II (Ang II) stimulates profibrotic genes via \(\beta\)-catenin pathway. Collecting duct cells can undergo epithelial-mesenchymal transition via stimulation of insulin-like growth factor (IGF)-1 by TGF-\(\beta\) (18). Immunohistochemical studies of renal biopsies from adult patients with a variety of underlying renal diseases associated with interstitial fibrosis demonstrate the expression of mesenchymal proteins such as \(\alpha\)-SMA and vimentin in tubular epithelial cells, including those of collecting ducts, adjacent to regions of interstitial fibrosis and tubular basement membrane disruption (19, 36). The mechanism by which collecting duct cells undergo extracellular matrix transformation is complex and may involve IGF-induced Akt and GSK-3\(\beta\) phosphorylation associated with early disruption of E-cadherin-\(\beta\)-catenin membrane colocalization with the translocation of E-cadherin to endosomes and \(\beta\)-catenin to the nucleus (18).

\(\beta\)-Catenin is expressed along the nephron and plays a role in kidney disease (20, 30). However, there is little evidence about its role in collecting duct cells. Recent studies have suggested that lithium decreases renal medullary GSK-3\(\beta\) activity (35) and is associated with nephrotoxicity (33), and changes in cellular composition in the distal nephron (4, 5). Kidneys from lithium-treated rats show upregulation of p-ser-9GSK-3\(\beta\) and proliferating cell nuclear antigen in the cortex and medulla. Lithium is an unspecific activator of the Wnt pathway (34). Despite its effects on GSK3-\(\beta\), lithium also inhibits other enzymes like the inositol monophosphatase, inositol polyphosphate 1-phosphatase, fructose 1,6-bisphosphatase, bisphosphate nucleotidase, adenylate cyclase, and PKC, among others (34).

Because the dysregulation of the evolutionarily conserved Wnt/\(\beta\)-catenin signaling system has been involved in the development of fibrotic processes (13, 18), we further examined whether Ang II induces profibrotic genes via \(\beta\)-catenin sig-

![Fig. 4. Time course of \(\beta\)-catenin expression levels in the M-1 cells incubated with Ang II. Ang II increased \(\beta\)-catenin at 4, 8, and 16 h. \(*P < 0.05, **P < 0.01\) vs. control (n = 6).](http://ajprenal.physiology.org/)

![Fig. 5. \(\beta\)-Catenin signaling inhibition prevented the Ang II-mediated upregulation of profibrotic genes and \(\beta\)-catenin target genes. Pyrvinium pamoate (Pyr) prevented the stimulation of both fibronectin and collagen I (A) and the Wnt target genes: cyclin D1 and c-myc (B). \(*P < 0.05\) vs. control, n = 6.](http://ajprenal.physiology.org/)
naling pathway activation. We tested the expression of cyclin D1 and c-myc, both classic target genes of this pathway. Our data agree with previous in vitro studies showing that ANG II upregulates cyclin D1 expression in Chinese hamster ovary cells expressing AT1 receptor (12). Moreover, Diep et al. (6) showed that in ANG II-infused rats there was an increased cyclin D1 expression in blood vessels. Additionally, in rat vascular smooth muscle cells, ANG II induced the expression of c-myc (29). Diez et al. (7) showed that inhibition of ACE reduced c-myc levels in smooth muscle cells in spontaneous hypertensive rats. Although the binding of ANG II to the AT1 receptor exerts most of the pathophysiological effects of ANG II by promoting cell proliferation and inflammation (26), our data indicate that AT1 receptor-dependent activation of the β-catenin signaling pathway also plays an important role on the stimulation of profibrotic factors in collecting duct cells.

Free β-catenin is phosphorylated by GSK-3β and is rapidly targeted to proteosomal degradation (32). However, different stimuli can inhibit GSK-3β via phosphorylation at the Ser9 site leading to β-catenin stabilization (32). AT1 receptor is widely expressed along the nephron (14) and its activation leads to PKC activation and calcium accumulation in collecting duct cells (11). It has been suggested that β-catenin stabilization can be induced by the activation of PKC with phorbol esters in T cells (25). Therefore, we examined whether the effect of ANG II on β-catenin signaling trans-activates transcription factors of the TCP/LEF family for the activation of gene expression (43). Our data suggest that the direct AT1 receptor-dependent activation of β-catenin signaling stimulates β-catenin downstream target genes in M-1 cells since candesartan prevented this effect. The inhibition of β-catenin signaling by pyrvinium pamoate decreases protein and mRNA levels of profibrotic genes and β-catenin target genes induced by ANG II. Notably, the expression of cyclin D1 and c-myc, both widely known β-catenin target genes implicated in regulating cell proliferation, was correlated with β-catenin stabilization. Our results indicated that ANG II treatment increases Ser9-GSK-3β phosphorylation leading to the stabilization of β-catenin. Interestingly, ANG II also induced the upregulation of total β-catenin and decreased its phosphorylation. We confirmed our observations demonstrating that after 48 h, ANG II treatment induces a mesenchymal phenotype demonstrating that the M-1 cells can undergo epithelial mesenchymal transition as previously suggested (18).

In conclusion, this study demonstrates that ANG II increases β-catenin protein levels in a time-dependent manner, whereas cotreatment with pyrvinium pamoate decreases both β-catenin and p-ser9-GSK-3β. Our findings support the concept that in collecting duct cells, ANG II has a stimulatory effect on the β-catenin signaling pathway to induce the expression of fibrotic factors. Our data also indicate that AT1 receptor activation is necessary for induction of the profibrotic factors, fibronectin and collagen I, through a mechanism involving β-catenin stabilization in collecting duct cells. These results suggest a role for AT1 receptor in the development of fibrosis in tubulop epithelial cells of the collecting duct.

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An Inspector calls: cerrar la p... de la penna... a la computadora...


