

Original Article

Effect of adrenotensin on cell proliferation is mediated by angiotensin II in cultured rat mesangial cells

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Aim: Both adrenomedullin (ADM) and adrenotensin (ADT) are derived from the same propeptide precursor, and both act as circulating hormones and local paracrine mediators with multiple biological activities. Compared with ADM, little is known about how ADT achieves its functions. In the present study, we investigated the effect of ADT on cell proliferation and transforming growth factor- β (TGF- β) secretion in cultured renal mesangial cells (MCs) and determined whether angiotensin II (Ang II) was involved in mediating this process.

Methods: Cell proliferation was measured by bromodeoxyuridine (BrdU) incorporation assay, Ang II levels were assayed using an enzyme immunoassay, and real time PCR was used to measure Ang II type 1 (AT1) receptor, Ang II type 2 (AT2) receptor, angiotensinogen (AGT), renin, angiotensin converting enzyme (ACE) and TGF- β 1 mRNA levels. TGF- β 1 and collagen type IV protein levels in cell media were measured using enzyme-linked immunoassays.

Results: ADT treatment induced cell proliferation in a concentration-dependent manner; it also increased the levels of TGF- β 1 mRNA and protein as well as collagen type IV excretion by cultured MCs. ADT treatment increased renin and AGT mRNAs as well as Ang II protein, but did not affect the ACE mRNA level. ADT up-regulated angiotensin AT1 receptor mRNA, but not that of the AT2 receptor. The angiotensin AT1 receptor antagonist losartan blocked the effects of ADT-induced cell proliferation, TGF- β 1 and collagen type IV synthesis and secretion.

Conclusion: ADT has a stimulating role in cell proliferation in cultured MCs. Increases in the levels of Ang II and the AT1 receptor after ADT treatment mediate the stimulating effects of ADT on cell proliferation and extracellular matrix synthesis and secretion.

Keywords: adrenotensin; angiotensin II; adrenomedullin; angiotensin AT1 receptor; rat mesangial cells

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Introduction

Adrenomedullin (ADM) is a potent cardiovascular-active peptide that has hypotensive, natriuretic and diuretic actions. It inhibits the angiotensin II (Ang II)-induced proliferation and migration effects on renal mesangial cells (MCs) and has an anti-oxidant action on these cells. It is considered to play an important role in the pathophysiology of many disorders, such as hypertension, acute coronary syndromes and renal failure^[1]. ADM is derived from a larger precursor molecule, proadrenomedullin, and proadrenomedullin was recently reported as a useful new marker for the progression of primary nondiabetic kidney disease^[2]. Proadrenomedullin is further cleaved by endogenous peptidases into 4 bioactive pep-

tides: PAMP (proadrenomedullin N-terminal 20 peptide, preproADM22–41), PreproADM45–92, ADM (PreproADM95–146) and ADT (adrenotensin or PreproADM153–185)^[3]. The four peptides play different roles in physiological processes. Compared with ADM, PAMP shows weaker effects on vasodilation and inhibiting vascular smooth muscle cell proliferation^[4]. The actions of ADT are controversial. Gumusel *et al* reported that ADM induces endothelium-dependent vasoconstriction and elevation of blood pressure^[5, 6]. In a rat model of pulmonary hypertension, Cuifen *et al* reported that ADM and ADT have opposite effects on vasoactivity and showed reciprocal inhibition in their release^[7]. In Qi's study, ADT induced an increase in the concentration of medium ir-ADM^[8]. Zhou *et al* reported that ADT has vasoconstriction activity and induces the proliferation of cultured rat vascular smooth muscle cells^[9], and it has been shown to antagonize the stimulatory effect of ADM on endothelial NO generation. However, this

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peptide also stimulates cAMP production in vascular tissue, suggesting that it could have a vasodilating effect under some circumstances^[10].

Many studies have demonstrated that activation of the renal renin-angiotensin system (RAS) and increased local Ang II production are associated with human and experimental kidney diseases^[11]. Accumulating evidence indicates that a local RAS in MCs plays an important role in the progression of glomerular disease^[12]. There is also evidence that ADM inhibits both the production and action of Ang II^[13]. In this study, we aimed to determine the effects of ADT on cultured MCs and whether Ang II is involved in the regulation of these effects.

Materials and methods

Materials and reagents

Adrenotensin and adrenomedullin 20–50 were purchased from Phoenix Pharmaceuticals (Belmont, CA). Losartan was obtained from Cayman Chemical (Michigan, USA). RPMI-1640 medium was obtained from Sigma RBI (USA). M-MLV reverse transcriptase was purchased from Promega Co (WI, USA). SYBR Green reaction mix was purchased from Applied Biosystems (Tokyo, Japan). The RNA extraction kit was purchased from Sangon Co (Shanghai, China). Steroid hormone-free fetal bovine serum (FBS) was purchased from PAA Laboratories GmbH (Austria). The BrdU assay kit was obtained from Roche (Mannheim, Germany). Other reagents used in the experiment were of analytical grade.

Cell culture

The rat mesangial cell line was obtained from the China Center for Type Culture Collection (Wuhan, China). The cells were cultured in RPMI-1640 containing 4500 mg/L glucose, 100 mg/L penicillin, 100 mg/L streptomycin, 2 mg/L glutamine and 10% FBS. The cells were deprived of FBS for 24 h before treatment.

Cell proliferation analysis

A total of 10^3 cells/well were cultured in 96-well plates in 10% FBS-supplemented medium. After 24 h of sub-culturing, the cells were serum starved for 24 h and then treated with adrenotensin, losartan or a combination of losartan and adrenotensin. In co-treatment experiments, losartan was added 30 min prior to adrenotensin. After 48 h, cell proliferation was assessed by bromodeoxyuridine (BrdU) incorporation assays as described previously^[14]. Briefly, 10 μ mol/L BrdU labeling solution was added to the medium. After incubating the cells for an additional 4 h at 37 °C, the cells were fixed, denatured and then incubated in anti-BrdU-POD (peroxidase) antibody for another 90 min at room temperature. At the end of the incubation, the cells were rinsed with PBS 3 times to remove excessive antibody; 100 μ L of substrate solution was then added into each well. After another 30 min incubation at room temperature, the absorbance of the samples was measured on a TECAN Infinite M200 microplate reader (Salzburg-Umgebung, Salzburg, Austria) at 370 nm, while the absor-

bance obtained at 492 nm served as a reference value.

Isolation of total RNA and synthesis of cDNA

Total RNA was isolated from cultured MCs according to the protocol of the RNA extraction kit. The concentration of the isolated RNA was determined by measuring the specific absorbance at 260 nm, and the integrity of the RNA isolated was checked by agarose gel electrophoresis under denaturing conditions. Total RNA (2 μ g) was used for cDNA synthesis in a 25 μ L reaction mixture. An aliquot of 2 μ L of the cDNA was subsequently used for further real-time PCR quantification of target genes, as described below.

Real-time PCR quantification

For quantitative analysis of the mRNAs encoding angiotensinogen, real-time PCR reactions were performed for the angiotensin AT1 and AT2 receptors, angiotensin converting enzyme (ACE), renin and TGF- β 1 with SYBR Green as an intercalating dye. The primer sequences are listed in Table 1.

Table 1. PCR primer pairs and product characteristics.

Target		Oligonucleotide sequence	Tm (°C)	Product size (bp)
AT1	F	5'-CTC AAG CCT GTC TAC GAA AAT GAG-3'	62	204
	R	5'-CTT CTC CAT GGT GGT GAA GAC-3'	61	
AT2	F	5'-ACC TTT TGA ACA TGG TTT G-3'	62	218
	R	5'-GTT TCT CTG GGT CTG TTT GCT C-3'	62	
AGT	F	5'-GCA AAA ATC AGT GCC TTC ACC C-3'	60	128
	R	5'-AAA CAA ACC CAC ACC CCA GGA G-3'	62	
Renin	F	5'-CTG TGC ATA CTG GCT CTC CA-3'	62	181
	R	5'-GGC TTG GCC TAA AAC TAG GG-3'	62	
ACE	F	5'-GTG TTG TGG AAC GAA TAC GC-3'	60	187
	R	5'-CCT TCT TTA TGA TCC GCT TGA-3'	60	
TGF- β 1	F	5'-TGG CGT TAC CTT GGT AAC C-3'	61	277
	R	5'-GGT GTT GAG CCC TTT CCA G-3'	62	
GAPDH	F	5'-CCC TTC ATT GAC CTC AAC TAC ATG-3'	60	216
	R	5'-CTT CTC CAT GGT GGT GAA GAC-3'	60	

F, forward Primer; R, reverse primer; Tm, melting temperature.

The real-time PCR was carried out on an iCycler thermal cycler (Bio-Rad, Hercules, CA). The PCR procedure was performed with the following time programs: predenaturing of the samples at 95 °C for 10 min, followed by 40 cycles of amplification by denaturation at 95 °C for 30 s, annealing for 1 min at 65 °C (for angiotensinogen and ACE), 64 °C (for renin), 62 °C (for AT1, AT2 receptors and TGF- β 1) or 60 °C (for GAPDH) and extension at 72 °C for 1 min. After a final extension at 72 °C for 10 min, the amplified products were subjected to a stepwise increase in temperature from 55 °C to 95 °C to construct dissociation curves.

Quantification was achieved by comparing the number of cycles needed for the fluorescence of the PCR products to

reach a threshold value. The relative amount of each mRNA was normalized to that of the mRNA for the housekeeping gene GAPDH. Each sample was run and analyzed in triplicate. The samples from control cells were used as calibrators with a given value of 1, and the conditioned groups were compared with this calibrator.

Quantification of secreted TGF- β 1 by enzyme-linked immunosorbent assay (ELISA)

At the end of the experiment, the culture mediums were harvested in siliconized tubes on ice. After centrifugation at $1000\times g$ for 10 min ($4\text{ }^{\circ}\text{C}$) to pellet the cell debris, the supernatants were collected and frozen at $-20\text{ }^{\circ}\text{C}$ until they were used for the TGF- β 1 assay by ELISA. The ELISA assay was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, NM, USA). Briefly, the samples were acidified with 1 mol/L HCl to denature the proteins, neutralized with 1.2 mol/L NaOH/0.5 mol/L HEPES and then transferred to microtiter plates coated with monoclonal anti-TGF- β 1. After a 2-h incubation at room temperature, the wells were aspirated and washed 4 times with PBS buffer. Following incubation with HRP-conjugated anti-TGF- β 1 antibody for another 2 h, the substrate for HRP was added to each well and incubated for 30 min, protected from light. Finally, the chromogenic reaction was halted with a stop solution, and the absorbance at 450 nm was measured using a TECAN Infinite M200 microplate reader. TGF- β 1 concentrations were extrapolated from the standard curve obtained from the same plate.

Measurement of Ang II by enzyme immunoassay

Ang II levels in the conditioned supernatants were assayed using a commercially available enzyme immunoassay-based colorimetric kit (Phoenix Pharmaceuticals, Burlingame, CA, USA). The supernatants were collected and passed through a Centricon-10 column with a cutoff of $>10000\text{ Da}$ (Amicon, Beverly, MA). The filtrate was used for Ang II measurements following the manufacturer's protocol. In brief, the samples were added into a secondary antibody-pre-coated immunoplate. Ang II in the sample competitively bound to the primary antibody with biotinylated Ang II; the biotinylated Ang II then interacted with a streptavidin-horseradish peroxidase conjugate that catalyzed the conversion of a substrate solution. The intensity of yellow was directly proportional to the amount of biotinylated Ang II-SA-HRP complex and inversely proportional to the amount of Ang II in the samples. Absorbance was read at 450 nm using a TECAN Infinite M200 microplate reader, and the sample concentrations were determined by extrapolation from a standard curve.

Measurement of collagen type IV by enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISAs, Exocell, Philadelphia, PA) were used to measure collagen type IV. After 48 h of ADT treatment or combination treatment with losartan and ADT, the conditioned medium was removed gently and

centrifuged at maximum speed in a bench-top microcentrifuge for 15 min to remove the cell debris. The supernatant was used for collagen type IV measurement. Samples and collagen type IV standards were run in triplicate. The yield of collagen type IV in each sample was normalized to the total protein amount.

Statistical analysis

Data are expressed as means \pm standard deviation of the mean for the repeats of individual experiments indicated. Comparisons among different treatment groups were performed by one-way analysis of variance followed by the Newman-Keuls test. A *P* value of less than 0.05 was considered statistically different.

Results

ADT stimulates the proliferation of cultured MCs

BrdU incorporation assay demonstrated that treatment with ADT from 1×10^{-10} to 1×10^{-8} mol/L for 48 h exerted a proliferation effect on cultured renal MCs in a concentration-dependent manner. The maximal effect appeared following 1 nmol/L ADT treatment (Figure 1).

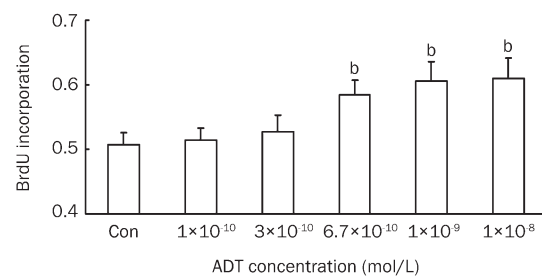


Figure 1. Effect of ADT on MC proliferation. MCs were serum starved for 24 h before ADT application. Cell proliferation was quantified 48 h after ADT treatment using a bromodeoxyuridine (BrdU) incorporation assay. $n=8$. Data are expressed as means \pm SEM. ^b $P<0.05$ vs control.

ADT stimulates angiotensinogen expression in cultured MCs

The results of real-time PCR and an enzyme-linked immunosorbent assay both showed that ADT treatment increased the angiotensinogen mRNA level in MCs and the Ang II level in cultured medium. The ADM receptor antagonist ADM20-50 did not change the effect of ADT. These results suggested that ADT stimulates angiotensinogen synthesis and secretion, and that these effects were not mediated by the ADM receptors in MCs (Figure 2).

ADT increases renin expression, but not the ACE mRNA level

Following a 24-h incubation with 1 nmol/L ADT, renin mRNA expression in MCs was significantly increased. By contrast, the ACE mRNA level tended to be higher than that of the control, but the difference was not statistically significant (Figure 3).

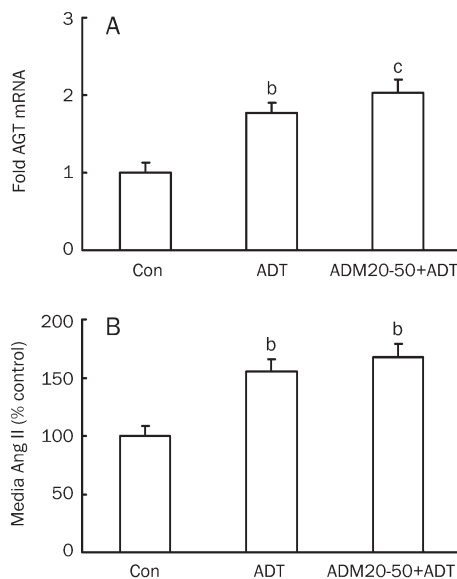


Figure 2. Effects of ADT on AGT mRNA expression and Ang II production by cultured MCs. MCs were cultured in FCS-free RPMI-1640 medium for 24 h and then incubated with ADT (1 nmol/L) with or without the ADM receptor antagonist ADM20-50 for another 24 h. (A) AGT mRNA levels analyzed by quantitative real-time PCR. (B) The Ang II concentration in the culture medium measured by enzyme immunoassay. Data are expressed as means±SEM. ^b*P*<0.05, ^c*P*<0.01 vs control.

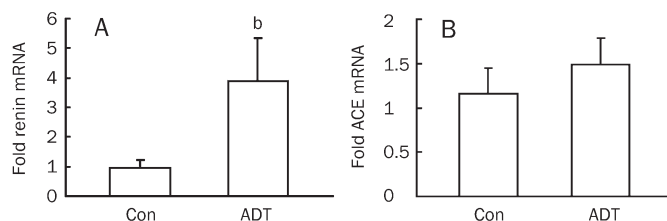


Figure 3. Effects of ADT on renin and ACE mRNA levels in cultured MCs. MCs were cultured in FCS-free RPMI-1640 medium for 24 h and then incubated with ADT (1 nmol/L) for another 24 h. Renin and ACE mRNA levels were measured by real-time PCR. *n*=5. Data are expressed as means±SEM. ^b*P*<0.05 vs control.

ADT increases the mRNA level for the angiotensin AT1 receptor, but not the AT2 receptor

After exposure of MCs to ADT (1 nmol/L) for 24 h, the angiotensin AT1 receptor mRNA level was significantly increased, as shown in Figure 4. By contrast, no obvious effect was observed on the angiotensin AT2 receptor mRNA level. These results suggested that ADT up-regulated AT1 receptor expression, but not AT2 receptor expression, in cultured MCs.

Losartan abolishes the proliferative effect of ADM on MCs

Compared with the control group, MCs incubated in 1 nmol/L ADT for 48 h showed increased cell proliferation. Pretreatment of cells with the angiotensin AT1 receptor antagonist losartan (1 μmol/L) abolished the cell proliferation effect induced by ADT. Losartan alone did not have a significant

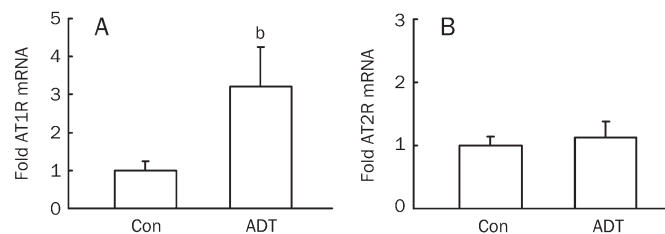


Figure 4. Effects of ADT on the angiotensin AT1 and AT2 receptor mRNA levels in cultured MCs. MCs were cultured in FCS-free RPMI-1640 medium for 24 h and then incubated with ADT (1 nmol/L) for another 24 h. The AT1 (A) and AT2 (B) receptor mRNA levels were measured by real-time PCR. *n*=6. Data are expressed as means±SEM. ^b*P*<0.05 vs control.

effect on cell proliferation (Figure 5A).

Losartan blocks the stimulating effect of ADT on collagen type IV secretion

Following exposure of MCs to ADT (1 nmol/L) for 48 h, the level of collagen type IV in the cultured medium increased significantly. Pre-incubation of cells with losartan (1 μmol/L) abolished ADT's effect on collagen type IV secretion, and losartan alone did not significantly change the collagen type IV level in the cultured medium (Figure 5B).

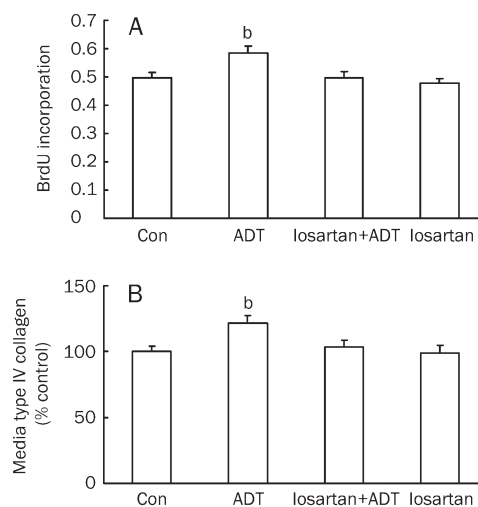


Figure 5. Effects of losartan on ADT-induced cell proliferation and type IV collagen secretion. MCs were cultured in FCS-free RPMI-1640 medium for 24 h and then incubated in 1 nmol/L ADT with or without the AT1 receptor antagonist losartan (1 μmol/L) for another 48 h. (A) Cell proliferation was quantified using a bromodeoxyuridine (BrdU) incorporation assay. *n*=8. ^b*P*<0.05. (B) The type IV collagen protein level was analyzed by ELISA. *n*=6. Data are expressed as means±SEM. ^b*P*<0.05 vs control.

Losartan blocks the stimulating effect of ADT on TGF-β1 synthesis

Following exposure of MCs to ADT (1 nmol/L) for 48 h, TGF-β1 mRNA and protein levels increased significantly. Similar to its affect on collagen type IV secretion, pre-incubation of

cells with losartan (1 $\mu\text{mol/L}$) abolished this effect. Losartan alone did not significantly change the TGF- β 1 mRNA and protein level (Figure 6).

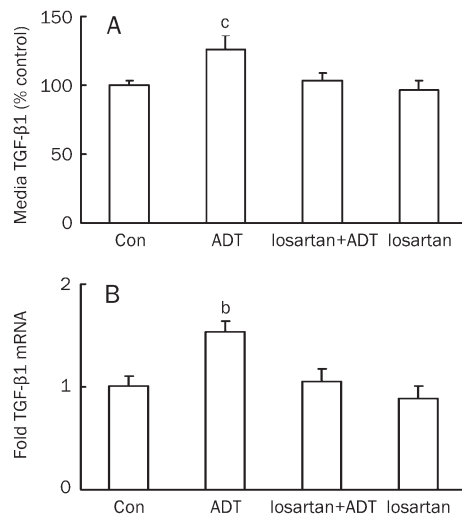


Figure 6. The effects of ADT on TGF- β 1 mRNA and protein levels. MCs were cultured in FCS-free RPMI-1640 medium for 24 h and then incubated in 1 nmol/L ADT with or without the AT1 receptor antagonist losartan (1 $\mu\text{mol/L}$) for another 48 h. (A) TGF- β 1 protein level analyzed by ELISA. (B) TGF- β 1 mRNA level measured by real-time PCR. $n=6$. The results are expressed as means \pm SEM. ^b $P<0.05$, ^c $P<0.01$ vs control.

Discussion

ADM is a potent regulatory peptide that functions as a circulating hormone and local paracrine mediator with multiple biological activities. Intravenous or local intrarenal infusion of ADM increases urine output and urinary sodium excretion. In addition, ADM inhibits connective tissue growth factor expression, extracellular signal-regulated kinase activation and renal fibrosis^[15]. One of the renal targets of ADM is mesangial cells, and accumulating data suggest that there might be a role for ADM in the pathophysiology of MC proliferation and matrix biology^[3]. ADM increases cAMP levels in MCs, leading to their relaxation and an increase in the filtration coefficient, and this effect contributes to an increase in the glomerular filtration rate (GFR). In addition, ADM inhibits angiotensin II-induced migration and proliferation of MCs as well as the generation of reactive oxygen species by these cells. These effects may attenuate the progression of chronic nephropathies^[1].

ADT, which is derived from proadrenomedullin, the common precursor of ADM, is also an active peptide. ADT has been reported to produce contractile responses in cat pulmonary arterial rings in a concentration-dependent manner^[6]. Precontraction of the arterial rings with ADT selectively attenuated the pulmonary vasorelaxant response to ADM. In anesthetized rats, an intravenous bolus injection of ADT increased the mean arterial pressure. In cultured rat vascular smooth muscle cells, 10^{-7} mol/L ADT increased $^3\text{H-TdR}$ incorporation^[9]. However, in the study by Li *et al*, ADT did not

alter the vasorelaxant response to ADM in rat aortic rings, and it had no effect on the vascular tone under either resting or precontracted conditions^[10]. It is uncertain if the results relied on species or the vessels used in the different experiments.

Glomerulosclerosis is a common complication of many chronic kidney diseases and results in chronic renal failure. Previous studies have shown that glomerular MCs play an important role in the pathogenesis of glomerulosclerosis. Previously, there was evidence of a role for ADM in MC biology, but nothing was known about the activity of ADT in MCs. In the present study, we demonstrated that ADT exerts a cell proliferation effect on cultured MCs in a dose-dependent manner, and that ADT stimulates TGF- β 1 expression and secretion.

The receptor involved in signaling the effects of ADT has not yet been identified. ADM20-50 was found to be an antagonist of the ADM receptor in MCs. In the present study, we pretreated cells with ADM20-50 before application of ADT, but it had no effect on AGT mRNA expression or Ang II synthesis induced by ADT, which suggests that ADT might have its own receptor that is independent of the ADM receptor.

The local RAS in MCs actively participates in the progression of glomerular disease. The RAS plays an important role in the regulation of kidney functions and participates in the progression of renal damage through the modulation of cell growth, fibrosis and inflammation^[11]. Ang II, a major effector molecule produced by the RAS, is critical for the development of renal fibrosis through the over-expression of growth factors, such as TGF- β 1 and extracellular matrix (ECM) proteins^[16]. Blockade of Ang II by angiotensin-converting enzyme (ACE) inhibitors or AT1 antagonists is one of the best options to treat renal diseases.

It is well established that glomerular MC proliferation and increased production and accumulation of extracellular matrix proteins are early pathological events in glomerulosclerosis^[17]. Ang II contributes to ECM accumulation^[16]. TGF- β serves as a model profibrogenic cytokine^[18], and it is up-regulated in many fibrotic disorders that are associated with ECM accumulation, including human kidney diseases. Ang II and TGF- β share some responses involved in matrix regulation^[16]. Considerable experimental evidence has revealed that TGF- β is a mediator of Ang II actions. Wolf demonstrated that Ang II stimulates TGF- β synthesis in renal cells^[19]. Ang II treatment of cultured rat MCs has been shown to increase the levels of TGF- β as well as the matrix components fibronectin and collagen. Sánchez-López reported that Ang II participates in renal fibrosis via the up-regulation of growth factors, including TGF- β , and ECM proteins, such as type IV collagen^[11].

To understand the role of the RAS in the ADT-induced effects, we measured Ang II protein levels in culture supernatants and analyzed mRNA expression levels for AGT, the AT1 receptor and the AT2 receptor. The results indicated that ADT increased AGT synthesis, Ang II formation and AT1 receptor expression, but had no effect on AT2 receptor expression. Stimulation of resting MCs with ADT also caused cell proliferation, TGF- β 1 mRNA expression and synthesis and increased type IV collagen levels. The effects were abolished by the

angiotensin AT1 receptor antagonist losartan, which suggests that Ang II is involved in regulating the effects of ADT, as they are mainly mediated by the angiotensin AT1 receptor, not the AT2 receptor.

Besides Ang II and its receptors, we also observed changes in renin and ACE. Our results showed that the renin mRNA level increased significantly after ADT treatment. In RAS, renin is a rate-limiting aspartyl protease enzyme that acts on AGT to form decapeptide Ang I^[20]. Increased AGT and renin induced by ADT may result in an increase in the Ang I concentration. By contrast, the ACE mRNA level did not significantly change after ADT treatment. Therefore, the increase in Ang II level that was observed in the study might mainly be caused by the increase in the ACE substrate concentration. Multiple components in RAS changed after ADT treatment. However, the mechanism by which each of them was influenced by ADT still remains to be investigated.

Our findings are based on cell culture results and do not necessarily reflect the fibrotic tissue conditions in their entirety *in vivo*, where many other factors could also participate in the regulation of the ECM. To understand the exact role of ADT *in vivo*, other specific experiments need to be designed.

In summary, we have provided new evidence that ADT stimulates cell proliferation and TGF- β 1 mRNA expression in cultured rat MCs. In addition, ADT increased renin and AGT mRNA levels and extracellular Ang II protein levels. ADT also increased the expression of angiotensin AT1 receptor mRNA, but not that of AT2 receptor mRNA. Losartan blocked ADT-induced cell proliferation, TGF- β 1 and collagen type IV synthesis, suggesting that ADT plays a stimulating role in MC proliferation and ECM accumulation. These effects might have some clinical significance *in vivo*. The stimulating effect of ADT was mediated by Ang II formation. These observations could help us to gain insight into the mechanisms involved in the regulation of tissue homeostasis and ECM accumulation.

Author contribution

Li-min LU, Tai YAO, Yu HUANG and Hong XUE designed the research; Hong XUE, Ping YUAN and Li ZHOU performed the research; Hong XUE, Ping YUAN, Li ZHOU and Li-min LU analyzed the data; Hong XUE and Li-min LU wrote the paper.

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