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ORIGINAL ARTICLE

Enhanced angiotensin-converting enzyme 2 attenuates angiotensin II-induced collagen production via AT1 receptor–phosphoinositide 3-kinase–Akt pathway

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Abstract Recent reports support a protective role for angiotensin-converting enzyme 2 (ACE2) against glomerular diseases, especially by decreasing of extracellular matrix (ECM) proteins. However, the mechanism regulating this effect appears to be complex and poorly understood. Our aim was to investigate whether or not ACE2 amelioratesthe profibrotic effects of Ang II-mediated, Akt-dependent pathways in the mouse mesangial cell line, MES-13.Gene transfer of ACE2 suppressed Ang II-activated Akt-phosphorylation, accompanied by a decreased level of collagen type I in cells. In addition, Ang II-induced collagen type I synthesis in MES-13s by activating the Ang II/AT-1R–PI3K pathway. This transactivation was dependent on cAMP/Epac but not on PKA. TGF-βRI played a pivotal role in this signaling pathway inducing collagen deposition effects which could be reversed by ACE2 gene transfer in MES-13 cells. The results revealed that gene transfer of ACE2 regulated Ang II-mediated AT1R–TGFβRI–PI3K–Akt signaling and involved the synthesis of collagen. The beneficial effect of ACE2 overexpression appeared to result mainly from blocking phosphorylation of Akt in mesangial cells, suggesting that the ACE2 gene might be a novel therapeutic target for glomerular diseases.

Keywords Angiotensin-converting enzyme 2 (ACE2) · Angiotensin II · Phosphoinositide 3-kinase · Akt · Collagen synthesis

Abbreviations

ACE2	Angiotensin-converting enzyme 2
Ang II	Angiotensin II
Ang (1–7)	Angiotensin-(1–7)
AT1R	Angiotensin type 1 receptor
PI3K	Phosphatidylinositol 3-kinase
RAS	Renin–angiotensin system
GPCR	G protein-coupled receptor
PI3K	Phosphoinositide 3-kinase
RT	Reverse transcription
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
2,5-DOA	2,5-Dideoxyadenosine
AC	Adenylate cyclase
TGFβRI	Transforming growth factor-β receptor, type 1
PKA	Protein kinase A
Epac	Exchange protein directly activated by cAMP-1
AP1	activator protein 1

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Introduction

Activation of the renin–angiotensin system (RAS) plays an important role in the development of experimental and clinical glomerular diseases [1–3]. Increased synthesis and/or uptake of angiotensin II (Ang II) contributes to the synthesis of extracellular matrix (ECM) components, including collagens, which is a hallmark in these

diseases [4, 5]. However, the recent discovery of a classic angiotensin-converting enzyme (ACE) homologue, angiotensin-converting enzyme 2 (ACE2), has revised our understanding of Ang peptide processing. ACE2 functions as a carboxypeptidase, cleaving a single residue from Ang I, generating Ang1–9 [6, 7] and a single residue from vasoconstrictor Ang II to generate vasodilator Ang1–7 [8]. $Ace2^{-/y}Ins2^{WT/C96Y}$ mice exhibit an acceleration of mesangial matrix and glomerular basement membrane injuries which can be reversed by Ang II receptor blockade [9]. The glomerular mesangial matrix injury caused by ACE2 inhibition primarily leads to impaired Ang II degradation and decreased Ang 1–7 formation. Taken together, these studies strongly suggest that ACE2 plays a physiological and/or pathological role against Ang II in glomerular diseases.

Transforming growth factor-β is a potent inducer of ECM synthesis. The aberrant expression of its receptors has long been considered a primary mediator of organ sclerosis and fibrosis [10]. Numerous studies have demonstrated that blockade of TGF-βRI signaling ameliorates the sclerosis response in various experimental models [11–15]. Moreover, a specific role of Ang II stimulating the expression of TGF-βRI was demonstrated in glomerular sclerosis [16]. It is also known that Ang II induces the expression of TGF-βRI and ACE2 antagonizes many Ang II-mediated cell signaling. However, the cellular mechanisms by which ACE2 exerts its physiologic effects have not been fully elucidated. These findings promoted us to explore the possibility that ACE2 may antagonize Ang II-mediated TGF-βRI involved cell signaling concerning collagen synthesis in glomerular diseases. Here, we report that ACE2 gene transfer significantly prevented collagen synthesis stimulated by Ang II. Ang II binding to AT-1R triggered activation of AC-cAMP-dependent PI3K–Akt pathway and TGFβRI, then led to transactivation of the PI3K/Akt signaling pathway resulted in an increase in type I collagen synthesis in MES-13 cells.

Results

The cloning, transfection, and expression of recombinant ACE2 in cells

The full-length cDNA of the ACE2 gene was successfully amplified and inserted into the pcDNA3.1(−) vector. The positive recombinant transformants were analyzed by PCR amplification with the use of ACE2 gene-specific primers in the vector (Fig. 1a). RT-PCR and western blot analyses showed that the ACE2 mRNA and protein were overexpressed in ACE2-transfected cells compared with the controls (Figs. 1b, c).

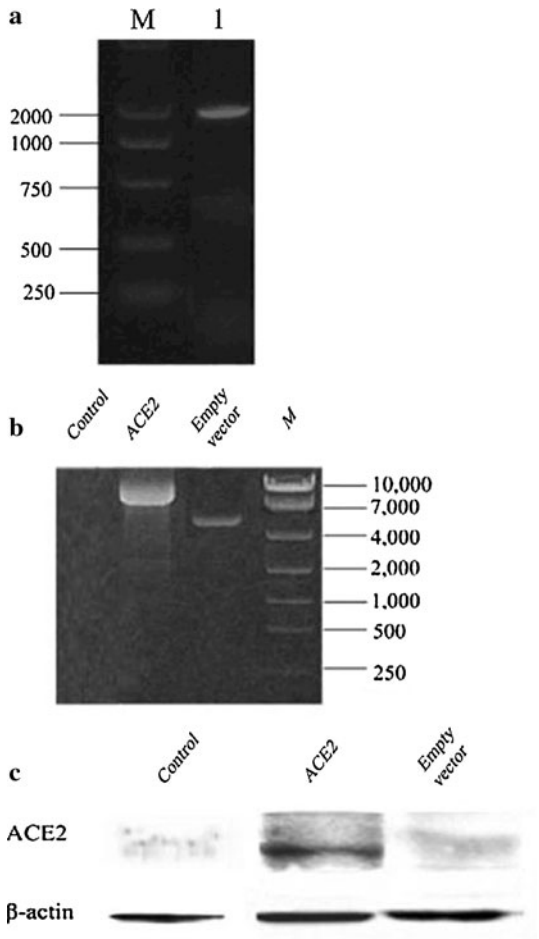

Fig. 1 The identification and expression of recombinant ACE2 in MES-13 mesangial cells. **a** Randomly chosen pACE2 recombinants were analyzed by PCR amplification using ACE2 primers (product size 2001 bp). M and 1 indicates DNA marker and ACE2 PCR product, respectively. **b** The mRNA expression of ACE2 was determined by RT-PCR in cells treated with ACE2, vehicle, or empty vector. **c** The protein expression of ACE2 was determined by western blotting with antibodies against ACE2 (90 kDa) in cells pretreated with ACE2, vehicle, or empty vector. *ACE2* angiotensin-converting enzyme 2

ACE2 transfer inhibits Ang I-stimulated type 1 collagen mRNA synthesis

The cause of many glomerular diseases is an increase in the synthesis of ECM components, including collagens. Ang II has been shown to increase the synthesis of collagen. We first examined the subtypes of collagen affected by Ang II treatment. Quiescent MES-13 cells were treated with Ang II under serum-free condition for various durations (30, 60, 90, 120, 240, and 360 min), and the transcriptional levels of three subtypes of collagen (Coll-1α1, Coll-1α2, and Coll-4α1) were determined by RT-PCR. Before treatment, MES-13 cells expressed only trace amounts of collagen genes. Ang II induced a significant increase in Coll-1α1

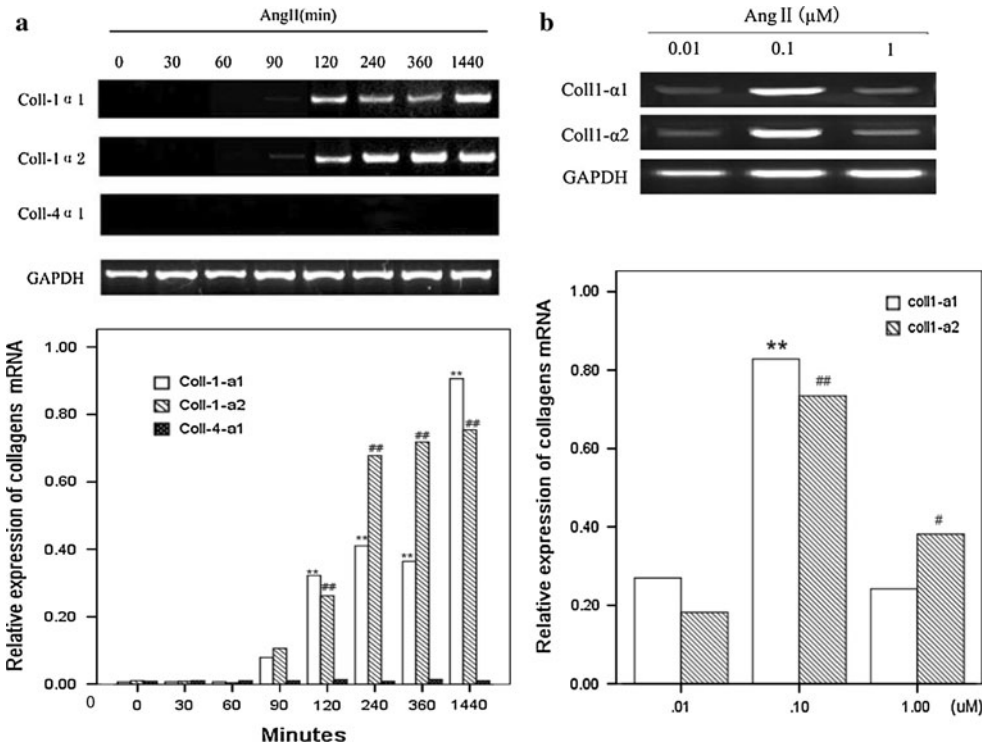

Fig. 2 Ang II induces type I collagen synthesis in MES-13 mesangial cells. **a** Cells were treated with saline vehicle or Ang II (100 nM) for the indicated durations in serum-free condition. RT-PCR was used to detect the mRNA of the three subtypes of collagen, and reversely transcript the stable internal standard segment GAPDH at the same time, then the ratio of two area light degree (IA) was used as the relative expression of mRNA of the three subtypes of collagen. Data were normalized with GAPDH levels and represent means ± SE of percent change in mRNA levels relative to that before Ang II treatment as control. $^{**}P < 0.01$ vs. Coll-1α1 mRNA level before Ang II treatment, $^{\#\#}P < 0.01$ vs. Coll-1α2 mRNA level before Ang II treatment. **b** Cells were treated with saline vehicle or Ang II of differing concentrations (0.01, 0.1, 1 μM) for 24 h in serum-free condition. RT-PCR for type I collagen and GAPDH transcripts was performed using total RNA from cell lysate. The ratio of two area light degree (IA) was used as the relative expression of mRNA of the two subtypes of collagen. Levels of Coll-1α1, Coll-1α2 and GAPDH were determined as described. $^{**}P < 0.01$ vs. Coll-1α1 mRNA level before Ang II treatment, $^{\#\#}P < 0.01$ vs. Coll-1α2 mRNA level before Ang II treatment, $^{\#}P < 0.05$ vs. Coll-1α2 mRNA level before Ang II treatment. *Ang II* angiotensin II

and Coll-1α2 levels 90 min after initiation of treatment, and the effect was still evident after 6 h (Fig. 2a). In contrast, Coll-4α1 transcription was not affected by Ang II.

To further determine the most effective in vitro dosage of Ang II in the experiment, quiescent MES-13 cells were treated with different concentrations of Ang II (10 nM, 100 nM, and 1 μM) for 24 h as previously described [17]. As illustrated by Fig. 2b, Coll-1α1 mRNA synthesis was most strongly stimulated by 100 nM Ang II, and this dose was used in all subsequent experiments.

ACE2 transfer inhibits Ang II-stimulated type 1 collagen protein synthesis

As mentioned above, Ang II was assumed to increase the synthesis of the type I collagen. western blotting results verified a significant increase in the protein levels of pro-collagen type I in Ang II-treated cells, whereas the effect was completely abrogated by ACE2 gene transfer (Fig. 3).

ACE2 transfer interrupts the type I collagen synthesis mediated by Ang II-AT1R

To explore if the Ang II effect is mediated by AT-1R, quiescent MES-13 cells were treated with vehicle, Ang II, or Ang II in combination with losartan, an AT-1R-specific antagonist. The synthesis of the type I collagen protein was completely abrogated by co-treatment with losartan (Fig. 3). These results indicated an AT-1R-mediated increase in collagen synthesis.

The TGFβRI/PI3K–Akt-dependent pathway is initiated by Ang II

To verify if TGFβRI, PI3K, and Akt are downstream signaling molecules induced by Ang II, MES-13 cells were treated with Ang II for 1, 2, 5, or 15 min, and phosphorylation of Akt was examined by western blotting. There was a significant increase in Akt-phosphorylation

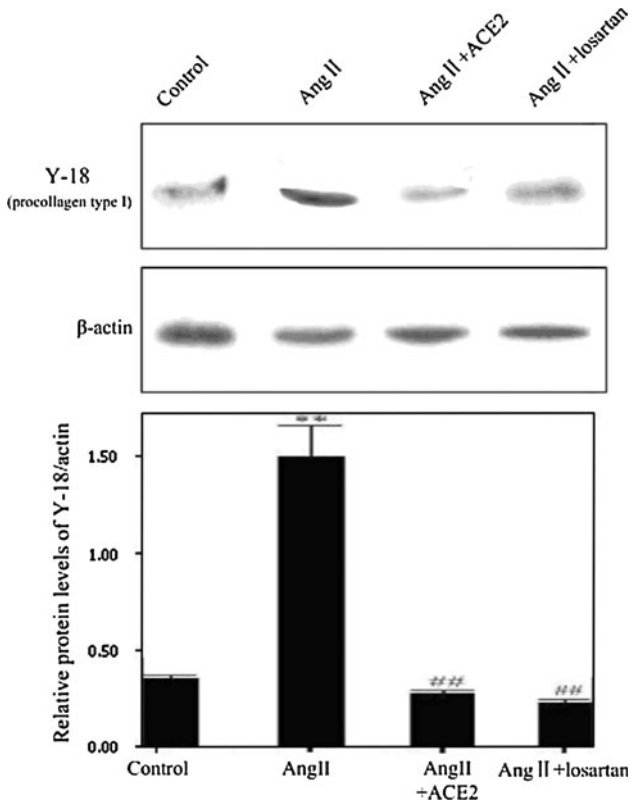

Fig. 3 ACE2 gene transfer inhibits type I collagen synthesis mediated by Ang II-AT1R. MES13 cells with ACE2 gene transfer or not were treated with vehicle, Ang II (0.1 μM) alone, or Ang II in combination with losartan (1 μM, an AT-1R-specific antagonist) for 24 h. Cell lysates were subjected to Western blotting using anti-procollagen type I antibody. The *bar graphs* show the densitometric scanning results from three individual experiments. Data were normalized with *β*-actin levels and represent means ± SE of percent change in protein levels relative to that of vehicle control. $^{**}P < 0.01$ vs. vehicle control, $^{\#\#}P < 0.01$ vs. Ang II alone. *AT-1R* type 1 angiotensin II receptor

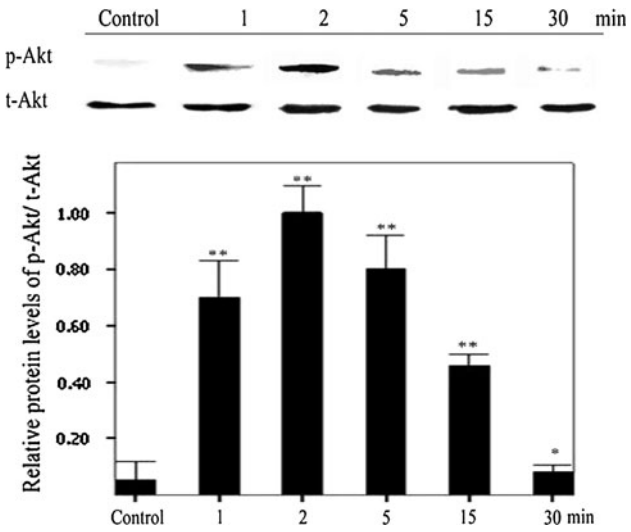

Fig. 4 Ang II enhances phosphorylation of Akt in MES-13 mesangial cells. Cells were treated with vehicle or Ang II (0.1 μM) for the indicated durations. Western blotting was performed on cell lysates (30 μg/lane) with antibodies against phospho-Akt (Thr-308) and total-Akt. The *bar graphs* show the densitometric scanning results from three individual experiments. Data were normalized with *β*-actin levels and represent means ± SE of percent change in protein levels relative to that of vehicle control. $^{**}P < 0.01$ vs. vehicle control, $^{*}P < 0.05$ vs. vehicle control

(threonine 308) 1 min after Ang II treatment. The peak appears at 2 min and then declines (Fig. 4). As shown in Fig. 5, the outcome of Ang II-induced upregulation of phospho-Akt was altered by AT1R-inhibitor losartan, a selective TGF*β*RI inhibitor (SB431542, 10 μM) or wortmannin, a PI3K inhibitor. The results indicate that acute Ang II stimulation affects the activation of TGF*β*RI-PI3K-Akt signaling pathway in MES-13cells.

The AC-cAMP/PKA-dependent pathway stimulated by Ang II regulates the phosphorylation of Akt

To further investigate whether or not an AC-cAMP–PKA-dependent signaling event was responsible for Ang II/AT-1R-mediated activation of Akt, MES-13 cells were treated with vehicle, Ang II alone, or Ang II in combination with 2,5-DOA (100 μM) or H89 (10 μM) for 1 h. The phosphorylation of Akt was examined by western blotting. The phospho-Akt was fully abrogated by 2,5-DOA but not H89 (Fig. 6). The results revealed an AC-cAMP-mediated, PKA-independent pathway was involved in the activation of downstream molecules.

ACE2 transfer interrupts the type I collagen synthesis mediated by Ang II-AT1R/AC-cAMP/TGF*β*RI/PI3K–Akt pathway

We have shown that TGF*β*RI–PI3K–AC/cAMP–Akt pathways are initiated by Ang II. To further detect if Akt, PI3K, AC, and TGF*β*RI inhibitors can downregulate the synthesis of the typeIcollagen, cells were treated with vehicle, Ang II (100 nM) alone, Ang II in combination with Akt inhibitor X, SB431542, 2,5-DOA, or wortmannin. The synthesis of the typeIcollagen was fully abrogated by Akt inhibitor X, SB431542, 2,5-DOA, and wortmannin. The western blotting results demonstrated that a TGF*β*RI involved and AC-cAMP dependent PI3K-Akt pathway was critical for AT-1R-mediated induction of collagen synthesis (Fig. 7).

Discussion

Regardless of etiology, most end-stage glomerular diseases are characterized by accumulation of ECM proteins, including collagens, in mesangium and other areas in

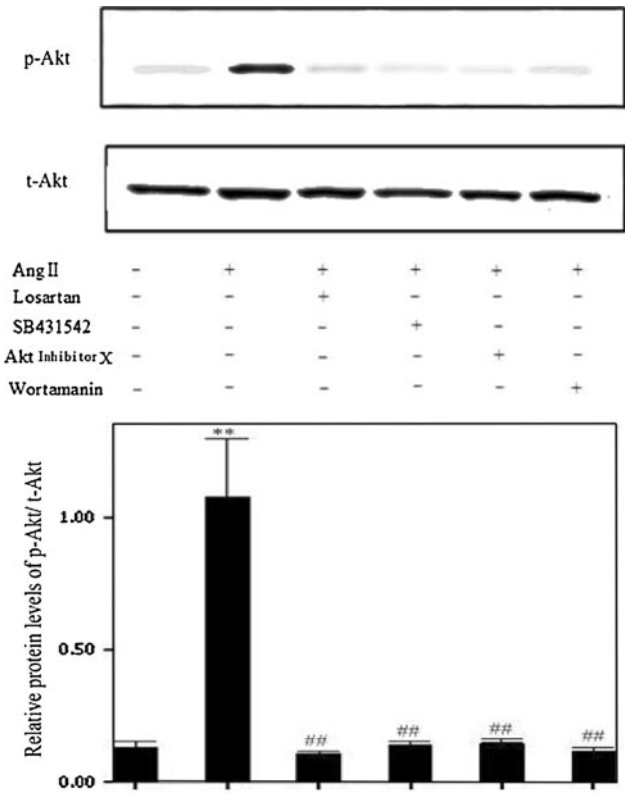

Fig. 5 ACE2 transfer inhibits phosphorylation of Akt mediated by the AT1R–PI3K–Akt pathway. Cells were treated for 2 min with vehicle, Ang II (0.1 μM) alone, Ang II (0.1 μM) in combination with losartan (1 μM), SB431542 (10 μM, a TGFβRI inhibitor), Akt inhibitor X (10 μM), or wortmannin (20 nM, a PI3K inhibitor). Inhibitors were added 1 h before starting Ang II treatment. After treatment, levels of phospho-Akt were determined as described. *PI3K* phosphoinositide 3-kinase

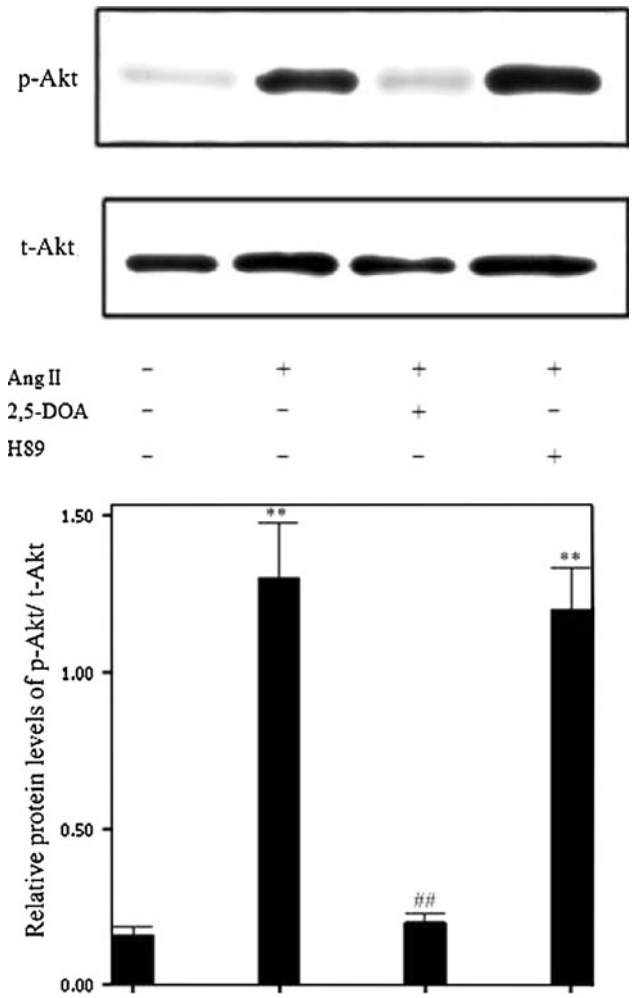

Fig. 6 An AC-cAMP mediated, PKA independent pathway is involved in phosphorylation of Akt. Cells were treated for 2 min with vehicle, Ang II (0.1 μM) alone, Ang II (0.1 μM) in combination with 2,5-DOA (100 μM), or H89 (10 μM). Inhibitors were added 1 h before starting Ang II treatment. After treatment, levels of phospho-Akt were determined as described

glomeruli. The mechanisms underlying this abnormal accumulation of ECM have not been fully elucidated. RAS has been implicated in the development of chronic progressive glomerular diseases, and the precise mechanism of this effect has been a subject to be investigated clinically and experimentally. It has been suggested that Ang II induces glomerular injuries through renal hemodynamic effects and stimulation of growth and ECM production by glomerular cells [18–21]. Several factors such as TGF-β and endothelin-1 have been identified as crucial components in this mechanism, but the detailed signaling mechanisms responsible for the effects remain largely unknown.

Since angiotensin-converting enzyme-2 (ACE2) was identified as a negative regulator of Ang II, there have been many reports concerning its role in several tissues, including the kidney. Recent reports support a protective role for ACE2 against glomerular diseases, especially with regard to the accumulation of ECM proteins [8]. Treatment with hrACE2 increased plasma ACE2 activity and reduced the urinary albumin excretion in Akita Ins2 (WT/C96Y) mice in association with a decreased glomerular mesangial matrix expansion and normalization of increased alpha-smooth muscle actin and collagen III expression [22]. However, the mechanism regulating this effect appears to be complex and poorly understood. In this study, we investigated whether or not ACE2 can blockade the profibrotic effects of Ang II in mesangial cells as well as the potential role of Ang II–PI3K–Akt-dependent pathways in this regulation.

During the last decade, the effect of Ang II on collagen synthesis has been demonstrated in aortic smooth muscle [23], liver [24], lung [25], cardiomyocytes [26], bone cells [27], ureteric bud cells [28], pancreatic stellate cells [29], proximal tubular cells [30], and mesangial cells [31, 32]. In this study, our results reveal the signaling events that

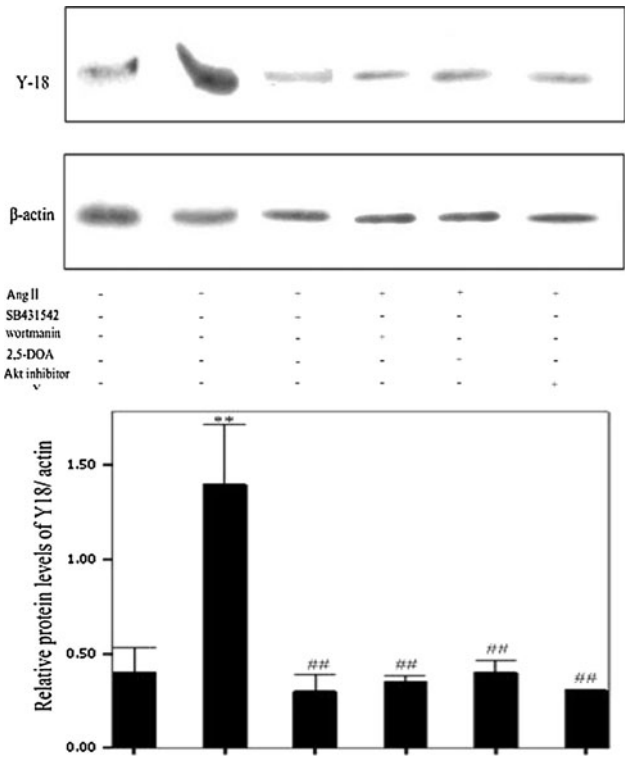

Fig. 7 ACE2 transfer inhibits type I collagen synthesis mediated by the AT1R-PI3K-Akt pathway. MES13 cells were treated with vehicle, Ang II (0.1 μM) alone, Ang II in combination with SB431542 (10 μM), wortmannin (20 nM), 2,5-DOA (100 μM), or Akt inhibitor X (10 μM) for 24 h. Levels of procollagen type I and β-actin were determined as described

mediate the effect of Ang II on collagen synthesis in MES-13 cells. We demonstrate at least four noteworthy results. First, unlike other published reports [8, 33, 34], Ang II induced the mRNA synthesis of neither type III nor type IV, but type I collagens in mesangial cells. Second, Ang II, via AT-1R, increased collagen synthesis through transactivation of the PI3K signaling pathway. Third, the transactivation of PI3K from Ang II/AT-1R was $G_{\alpha 5}$-cAMP-dependent but PKA-independent involving TGFβRI. Finally, ACE2 gene transfer significantly prevented collagen synthesis stimulated by Ang II. Based on these data, we propose ACE2 can intercept the Ang II-induced collagen synthesis in MES-13 cells. Ang II binding to AT-1R triggered sequential activation of adenylyl cyclase and TGFβRI and led to transactivation of the PI3K signaling pathway. Activation of the downstream signaling molecule Akt resulted in an increase in type I collagen synthesis in MES-13 cells.

Most of the known effects of Ang II, e.g., vasoconstriction, stimulation of aldosterone and vasopressin synthesis/release, promotion of salt and water retention, stimulation of cell proliferation/migration, and ECM formation are mediated by activation of the AT1 receptors [34–37]. The AT1 receptor belongs to the G protein-coupled receptor (GPCR) superfamily and is primarily coupled through pertussis toxin-insensitive G proteins. AT-1R is widely expressed in cardiovascular, renal, neuronal, endocrine, and other target cells. Our results suggest an AT-1R dependent activity of Ang II might upregulate the synthesis of collagen type I in mesangial cells.

Ang II binding to the AT1 receptor activates PI3K/Akt signaling [38]. PI3K in the mesangial context has been reported to induce proliferation [39], prevent apoptosis [40, 41], and enhance collagen expression [42]. Our results from this study provide direct evidence for an Ang II-induced, PI3K/Akt-activated upregulation of type I collagen.

In response to binding of GPCR to its ligand Ang II, the heterotrimeric G protein complex separates into two subunits, Gα and Gβ. Receptor-activated $G_{\alpha 5}$ protein activates adenylyl cyclase in all known cases [43]. It was originally believed that most cAMP effects were mediated by the activation of PKA; however, recent studies have shown that PKA are not the only intracellular receptors involved in cAMP signaling in eukaryotes [44–46]. In this study, we demonstrated that inhibition of adenylyl cyclase by 2,5-DOA abolished Ang II-induced PI3K activity, whereas H89, a PKA-specific inhibitor, showed no significant effects. These results suggested that Ang II-induced PI3K–Akt activation in MES-13 cell was mediated by cAMP and did not require the involvement of PKA.

The TGF-β type I receptor is a transmembrane protein kinase that associates with the type II receptor to generate diverse heteromeric serine–threonine kinase complexes with diverse signaling capacities, including the PI3K/Akt pathway, that are associated with the accumulation of ECM, including collagens [47, 48]. Ang II stimulates protein expression of TGF-β receptor type II, but not that of type I, in proximal tubular cells with AP1 sites as a necessary prerequisite for Ang II-induced transcriptional activity. Whereas in vascular smooth muscle cells, Ang II increases TGF-β receptor I mRNA rather than type II. It has previously been demonstrated that Ang II-dependent regulation of TGF-β receptors is at least partially independent of endogenous TGF-β. Our observation indicates that a TGFβRI involved and AC-cAMP-dependent PI3K–Akt pathway is critical for AT-1R-mediated induction of collagen synthesis in mesangial cells.

Recent work has demonstrated that ACE2 may prevent collagen deposition in vitro and vivo. ACE2 overexpression attenuates collagen production, and this attenuation is probably mediated by a reduction in Ang II, an increase in Ang 1–7 and decreased expression of TGF-β [4, 32]. In this study, we constructed a recombinant plasmid encompassing the ACE2 gene and successfully transfected it into cultured mesangial cells. Our results showed gene transfer

of ACE2 significantly prevented type I collagen expression stimulated by Ang II via blockade of Akt-phosphorylation. We speculate that ACE2 expression might represent a novel paradigm for in vivo therapy of glomerular diseases.

In summary, this study defines a regulatory role for the ACE2 gene in the Ang II/AT-1R–PI3K/Akt pathway and a protective effect of ACE2 against collagen deposition in mesangial cells. Although other components of this pathway remain to be identified, our findings nonetheless provide important information for a largely unknown mechanism of RAS-mediated pathogenesis in glomerular diseases. Furthermore, we identified the ACE2 gene as a potential therapeutic target for renal diseases, such as diabetic nephropathy. Elucidating further details in this signaling pathway should allow us to better understand the regulatory roles of ACE2 in pathophysiological homeostasis and promote an ACE2 treatment for glomerular diseases.

Materials and methods

Materials

Ang II was purchased from Sigma Chemical Company (St. Louis, MO, USA). MES-13 mouse mesangial cell line was purchased from the Chinese Academy of Science type culture collection (Shanghai, China). 2,5-DOA, Wortmannin, Akt inhibitor X, H-89, SB431542, antibody against Pro-Coll-1α2 (Y-18), phospho-Akt, total-Akt, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Beverly, MA, USA), respectively. Losartan was obtained from Merck (Wilmington, DE, USA). Trizol and the pcDNA3.1(−) vector were obtained from Invitrogen (Carlsbad, CA, USA).

Cloning of ACE2 gene

Gene-specific primers were designed on the basis of published human ACE2 sequence (GenBank no. AF241254) with the primers (sense: 5′-CAC CAT GTC AAG CTC TTC CTG-3′; antisense: 5′-AAA GGA GGT CTG AAC ATC ATC AGT G-3′). These primers were used to amplify the full-length cDNA from a human fetal heart cDNA library (Gibco, Carlsbad, CA, USA). This was accomplished using a two-step PCR protocol using PfxUltima DNA polymerase (Invitrogen, Carlsbad, CA, USA).

Transfection of recombinant ACE2 gene in MES-13 cells

The purified PCR product with a full length of 2401 bp was ligated into the pcDNA3.1(−) vector (Invitrogen). The positive recombinant transformants were randomly picked and then verified by PCR amplification and DNA sequencing. Stable transfection of the constructs in MES-13 mouse mesangial cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were selected with G418 (Merck, Darmstadt, Germany) at a final concentration of 200 μg ml^{-1}.

Cell culture

MES-13 cells were grown in a 3:1 mixture of Dulbecco's modified Eagle's and Ham's F-12 media (Invitrogen) supplemented with 5% (v/v) fetal bovine serum containing 50 units ml^{-1} penicillin G and 50 g ml^{-1} streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were first grown up to 70% confluency and synchronized overnight in serum-free medium prior to treatment. For RT-PCR, the quiescent cells were treated with ANG II for various concentrations or durations. In some experiments, cells were pretreated with selective inhibitors for indicated durations before Ang II treatment. For western blotting using anti-procollagen type I antibody, MES-13 cells were stimulated with vehicle, Ang II (100 nM) alone, Ang II in combination with losartan (1 μM, an AT-1R-specific antagonist), or Ang II in combination with other antagonists including wortmannin (20 nM, a PI3K inhibitor), Akt inhibitor X (10 μM), 2,5-DOA (100 μM, an AC inhibitor), H89 (10 μM, a PKA inhibitor), or SB431542 (10 μM, a TGFβRI inhibitor) for 24 h. The antagonists were added 1 h before Ang II treatment. Cell lysates were subjected to western blotting using anti-phospho-Akt and anti-total-Akt.

RT-PCR

Total RNA was isolated from MES-13 cells using Quizol reagent (Qiagen,Valencia, CA). The primers (Table 1) were designed with the use of the software program Primer Express (PE, Applied Biosystems). Reverse transcription was done using a one-step RT-PCR kit at the suggested conditions (Qiagen). PCR products were separated on 1% agarose gels and visualized by ethidium bromide techniques.

Western blotting

Lysates from cultured MES-13 cells were subjected to western blot analysis as described previously [49]. Protein concentration was determined using the BCA Protein Array Kit (Pierce, Rockford, IL, USA). Protein samples were separated by 8–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane (Hybond ECL, Amersham, Piscataway, NJ, USA), and then blocked with 1% Blot-Qualified BSA (Promega) for 90 min. The membranes were incubated overnight at 4°C with primary antibodies against

Table 1 Primers used for RT-PCR reactions in cells extracts

	Genbank accession no.	Sense	Anti-sense	Amplicon sizes (bp)
Coll-1α1	NW_047337	5′-CCT GGT AAC ACT GGT CCT CCT G-3′	5′-ATC TTC ACC CTT GCC ACC TG-3′	189
Coll-1α2	NW_047688	5′-GAC ATT GGT GGT GCT GAC-3′	5′-GGA AAC GGG AAA GAG AAA G-3′	272
Coll-4α1	NW_047477	5′-TGG CAA AGT GGA GAT TGT TG-3′	5′-CTT CTG GGT GGC AGT GAT G-3′	280
GAPDH	NW_001084685	5′-AGA TTG CAA ATT ACA TAT TCT A-3′	5′-AGA AAG CAT TTA CCT GAA CTT GGT-3′	206

procollagen type I, phospho-Akt, total-Akt, and β-actin (1:250 ~ 1:1000) and then incubated with alkaline phosphatase-labeled secondary antibody (Biotechnology, Santa Cruz, CA, USA; 1:4000) for 60 min at room temperature. The positive bands corresponding to aim proteins were developed with the Western Blue stabilized substrate for alkaline phosphatase (ProtoBlot II AP System, Promega).

Statistical analysis

Statistical significance of the difference among groups was analyzed by the paired Student's *t* test or one-way analysis of variance followed by Newman–Keuls test. All the statistical analysis was done using Statistica Version 5.0 (StatSoft, Tulsa, OK, USA). All data were represented as the mean ± SE of three different experiments unless otherwise noted in the figure legends. A probability of $P < 0.05$ was considered to represent a significant difference.

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