*Original Article*

# Angiotensin II–Stimulated Collagen Synthesis in Aortic Adventitial Fibroblasts Is Mediated by Connective Tissue Growth Factor

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**Angiotensin II (Ang II), a potent mediator of vascular remodeling, can stimulate the synthesis of extracellular matrix in vascular cells. Recent studies indicate that connective tissue growth factor (CTGF) is involved in collagen synthesis. There is also increasing evidence that adventitial fibroblasts (AFs) are actively involved in vascular remodeling. However, whether collagen synthesis by AFs is mediated by CTGF, or whether it is relevant to Ang II, has not been studied. The present study was conducted to determine whether CTGF is expressed in AFs, and if so, whether the CTGF produced by AFs participates in collagen synthesis. The AFs were isolated from thoracic aorta of Wistar-Kyoto rats (WKY). The expression of CTGF was measured by Western blot or real-time PCR. Collagen synthesis was assessed by [$^3$H]proline incorporation. Our results suggested that CTGF was expressed in AFs and secreted into medium. Ang II increased CTGF mRNA and protein expression in a time- and dose-dependent manner, with the maximal protein increase occurring at 24 h with an Ang II dose of $10^{-7}$ mol/L, and this increase was inhibited by the Ang II receptor type 1 ($AT_1$-R) antagonist losartan, but not by the Ang II receptor type 2 ($AT_2$-R) antagonist PD123319. Ang II dose-dependently stimulated the incorporation of [$^3$H]proline into cultured AFs, and this effect was inhibited by a CTGF antisense oligodeoxynucleotide. Overexpression of CTGF by pcDNA3.1(+)/CTGF increased [$^3$H]proline incorporation in cultured AFs. The results demonstrated that, in cultured AFs, Ang II increased CTGF production *via* $AT_1$-R, which could be mediators of collagen synthesis by Ang II. This finding suggests that CTGF might be a novel target for antifibrotic therapy in vascular diseases. (*Hypertens Res* 2008; 31: 1233–1240)**

***Key Words*: connective tissue growth factor, angiotensin II, vascular adventitial fibroblasts, collagen synthesis**

## Introduction

The blood vessel wall is composed of three tunicae: the intima, media and adventitia. The role of the intima and media on vascular functions and their implication for cardiovascular diseases has been extensively studied. The influence of the adventitia on vascular functions was recently recognized (*1*). The structural changes in the adventitial layer, termed adventitial remodeling, have been observed in vascu-

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lar disease settings, such as hypertension (*2*), atherosclerosis (*3*), restenosis after balloon angioplasty (*4*), arteritis (*5*), and thoracic aortic dissection (*6*). Interestingly, adventitia remodeling has been shown to proceed to intima or media remodeling (*7*).

Adventitial remodeling is characterized by increases in adventitia thickness and the number of fibroblasts, phenotypic change of adventitial fibroblasts (AFs) to myofibroblasts, and an increase in extracellular matrix (ECM) protein deposition. ECM overexpression is involved in vascular and heart hypertrophy, chronic renal disease and heart failure. Collagen is one of the most abundant ECM components in the vessel wall. Fibroblasts are the main cell type capable of synthesizing collagen, and thus are responsible for the fibrosis in various tissues and organs. Our previous study revealed that the adventitia is the main deposition site of collagen in the aortic wall (*8*). An increase in collagen accumulation in the adventitia might alter the mechanical properties of the vessels.

Angiotensin II (Ang II) participates in the pathogenesis of cardiovascular and renal diseases through the regulation of cell growth, inflammation and fibrosis. In cultured AFs, Ang II stimulates the proliferation (*9*) and migration of AFs (*10*), and myofibroblast formation (*11*). It is well known that Ang II stimulates collagen production in various fibroblasts, including cardiac fibroblasts. However, little is know about the role of Ang II in ECM production in vascular AFs and the underlying mechanisms.

Connective tissue growth factor (CTGF) plays an important role in stimulating ECM production in fibroblasts. Although increasing evidence implicates CTGF in the pathogenesis of fibrotic diseases, the potential link between Ang II–induced collagen synthesis and CTGF expression in adventitial remodeling has not yet been investigated.

## Methods

### Materials

Dulbecco's modified Eagle's medium (DMEM) and newborn calf serum (NCS) were purchased from Gibco (Gaithersburg, USA). Cell culture materials were from Corning Costar (Corning, USA). Ang II was obtained from Sigma-Aldrich (St. Louis, USA). Polyclonal antibody against CTGF was purchased from Santa Cruz Biotechnology (Santa Cruz, USA), and [$^3$H]proline was purchased from Amersham (Buckinghamshire, UK). Lipofectamine 2000 was purchased from Invitrogen (San Diego, USA). Losartan was a gift from the Merck pharmaceutical company.

### Cell Culture

The methods used for the isolation and culture of AFs from thoracic aorta of 6–8 week old male Wistar-Kyoto rats (WKY) have been previously described (*12*). Cells were grown in DMEM supplemented with 10% heat-inactivated FCS, 1% L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The subconfluent cells were made quiescent by incubation in serum-free DMEM for 48 h before stimulation. These cells were characterized by positive staining for vimantin, and negative staining for α-smooth muscle (α-SM)-actin as described previously (*13*).

### Real-Time Quantitative PCR

Total RNA was extracted from culture cells in TRIzol (Invitrogen) according to the manufacturer's instructions. The absolute number of CTGF mRNA molecules was measured using real-time RT-PCR amplification. The primers for CTGF were designed using Primer Premiere software (Premiere Biosoft, Palo Alto, USA). The real-time measurements were performed with a Rotor-Gene 2000 Real-Time Cycler instrument (Corbett Research, Mortlake, Australia). Samples were analyzed in duplicate. The CTGF gene was amplified with the following primers: forward, 5′-cctgacccaactatgatgc-3′; reverse, 5′-cccttactccctggcttt-3′. Quantitative RT-PCR was performed in 20 μL reactions consisting of 1× PCR buffer, 3.5 mmol/L $MgCl_2$, 20 pmol/L primers, 1 μL of RT product and 1× SYBR Green I nucleic acid gel stain (Molecular Probes, Eugene, USA). The amplification protocol used was as follows: an initial 3 min denaturation at 95°C, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 74°C for 45 s. Concentrations of samples were calculated by using Rotor-Gene 2000 software (Corbett Research). Results were expressed as fold increases.

### Western Blot Analysis

Cells were scraped and lysed as described previously (*11*). The supernatant of cells was concentrated in a centrifugal filter device (Amicon Ultra-15; Millipore, Bradford, USA) at 4,000× *g* for 20 min at 4°C. Protein concentrations were determined by the Bradford method. Proteins (20 μg) were then transferred to nitrocellulose membranes and blocked with 5% non-fat milk/TBST (Tris-buffered saline/Tween 20) for 1 h at room temperature. Membranes were incubated with primary antibodies directed against CTGF (1:1,000), and β-actin (1:5,000), in 5% milk/TBST at 4°C overnight. After washing three times with TBST, the membranes were incubated with horseradish peroxidase–conjugated antibody (1:2,000) for 1 h at room temperature. Western blots were developed using ECL (Roche, Manheim, Germany) and quantified by scanning densitometry.

### Collagen Synthesis Analysis

Incorporation of [$^3$H]proline into cells was evaluated to examine collagen synthesis. After the stimulation of cells with Ang II, [$^3$H]proline (1 μCi/mL) was added to each well for the last 6 h of culture.Then, the cells were rinsed twice with cold PBS and incubated with cold 10% trichloroacetic acid at 4°C for

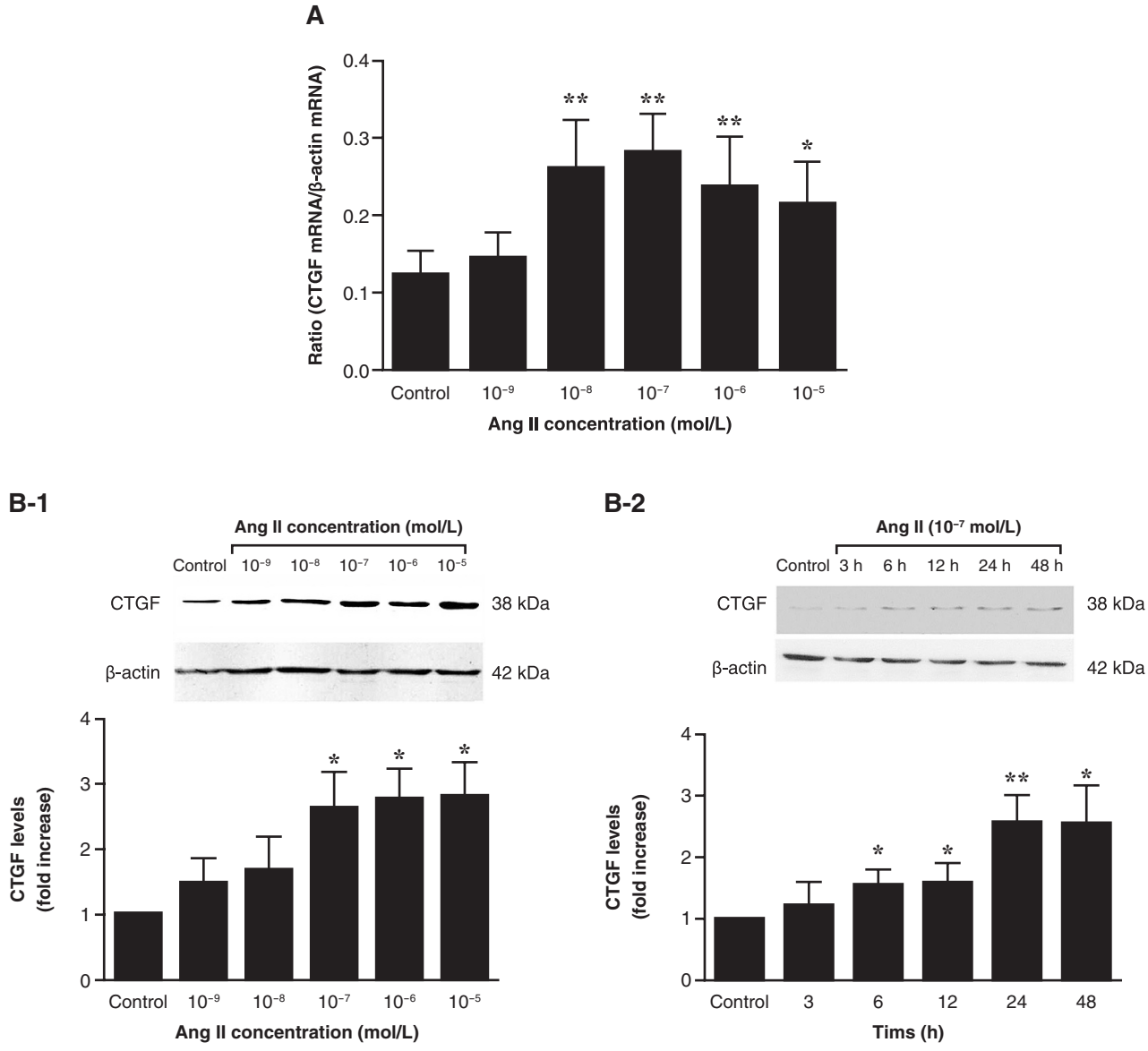


**Fig. 1.** *Ang II increased CTGF expression in AFs. A: Ang II increased CTGF mRNA expression in AFs. CTGF mRNA levels were measured by real-time quantitative RT-PCR, as described in the Methods section. AFs were treated with Ang II at concentrations of $10^{-9}$–$10^{-5}$ mol/L for 24 h. Data are the mean±SD of 3 separate experiments performed in duplicate. *$p < 0.05$ vs. the control. **$p < 0.01$ vs. the control. B: Ang II increased CTGF protein expression in AFs. B-1: After serum starvation for 48 h, AFs were treated with increasing concentrations of Ang II ($10^{-9}$–$10^{-5}$ mol/L) for 24 h. B-2: As a pretreatment, AFs were treated with $10^{-7}$ mol/L Ang II for 3 h, 6 h, 12 h, 24 h, or 48 h. Total proteins were extracted from AFs and probed with specific CTGF polyclonal antibody. Each lane contained 20 μg total protein. β-Actin was used as an internal loading control. Data are the mean±SD of 3 separate experiments. *$p < 0.05$ vs. the control. **$p < 0.01$ vs. the control.*

30 min. The precipitates were then washed twice with deionized water and solubilized in 0.3 mol/L NaOH–0.1% SDS at 37°C for 2 h. The radioactivity of aliquots of trichloroacetic acid–insoluble material was determined using a liquid scintillation counter. The data were normalized to the cell number.

## Construction of CTGF Expression Plasmid and Transient Transfection

CTGF expression plasmid was constructed as described previously (*14*). CTGF cDNA was cloned and amplified by RT-PCR with the following primers: forward, 5′-atatggtaccaccat gctcgcctccgtc-3′; reverse, 5′-acgttctagattacgccatgtctccata-3′.

The purified products were subcloned into pcDNA3.1(+) vector (Invitrogen). Cells were transfected with the CTGF expression vectors using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's specifications. Briefly, 0.4 μg of plasmid DNA and 2 μL Lipofectamine Plus reagent were mixed, and then 2 μg Lipofectamine Plus reagent was added. After transfection for 3 h, the cells were cultured in normal complete medium for another 24 h. The infectivity of vectors was evaluated by the percentage of green light–emitting cells under a fluorescent microscope (Zeiss, Jena, Germany).

### Antisense CTGF Oligodeoxynucleotide Transfection

The antisense oligodeoxynucleotide (ODN) for CTGF was synthesized based on 17 base phosphorothioate ODN with the sequence 5′-acggaggcgagcatggt-3′. The following sense and mismatch ODNs were used as controls: 5′-accatgctcgcctccgt-3′ and 5′-atagaggcgggcacggt-3′, respectively. Fibroblasts were incubated with 50 nmol/L ODN and Lipofectamine Plus (Invitrogen) for 6 h. Cells were washed prior to the addition of medium containing $10^{-7}$ mol/L Ang II. The transfection efficiency varied between 35% and 45% by analysis of fluorescent cells. The transfected cells were subjected to [$^3$H]proline incorporation analysis as described above.

### Statistical Analyses

Data are presented as the mean±SD. Statistical analysis was carried out by Student's *t*-test. For multiple groups ANOVA was performed. Differences were considered to be significant at $p<0.05$.

## Results

### Ang II Increases CTGF Expression in Adventitial Fibroblasts

Cultured AFs were treated with increasing concentrations of Ang II ($10^{-9}$–$10^{-5}$ mol/L) for 24 h, and CTGF mRNA expression was determined by RT-PCR. Ang II dose-dependently upregulated CTGF mRNA expression. The maximal response of Ang II was found at $10^{-7}$ mol/L (Fig. 1A).

We examined whether Ang II regulates CTGF protein production by Western blot analysis. When AFs were stimulated with Ang II ($10^{-9}$–$10^{-5}$ mol/L) for 24 h. Ang II increased CTGF protein production in a dose-dependent manner, and the response was maximal at $10^{-7}$ mol/L Ang II (Fig. 1B-1). The time-course effect of Ang II is shown in Fig. 1B-2. When cells were treated with Ang II ($10^{-7}$ mol/L) for increasing amounts of time (3–48 h), Ang II up-regulated CTGF protein production, and the effect reached a peak at 24 h and remain elevated up to 48 h. Our data suggest that cultured AFs were able to express CTGF under the basal unstimulated condition, and CTGF expression was upregulated by Ang II.

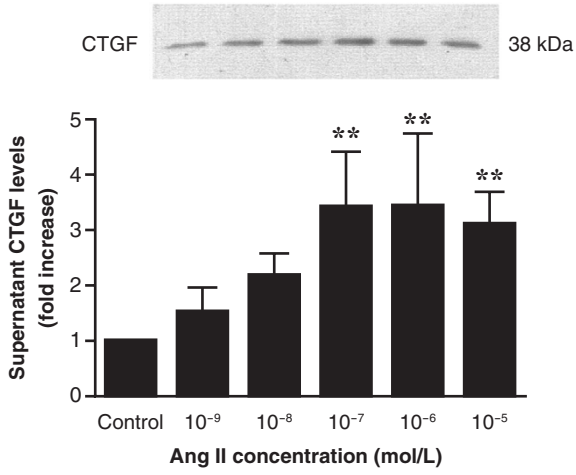


**Fig. 2.** *CTGF secretion from fibroblasts into medium. After serum starvation for 48 h, AFs were treated with increasing concentrations of Ang II ($10^{-9}$–$10^{-5}$ mol/L) for 24 h, and the conditioned medium was collected and concentrated by ultracentrifuge. By Western blotting as described in the Methods and relatively even protein loadings were verified by Ponceau S stain. Data are the mean±SD of 3 separate experiments.* **$p<0.01$ vs. the control.*

### Ang II Increases Soluble CTGF Production in Adventitial Fibroblasts

We next examined whether Ang II regulates soluble CTGF production by Western blot analysis. AFs were cultured in serum-free DMEM for 24 h. The conditioned medium was collected and concentrated before CTGF measurement. CTGF protein was detected in the conditioned basal medium and dose-dependently increased by Ang II ($10^{-9}$–$10^{-5}$ mol/L) (Fig. 2). These data suggest that CTGF protein was secreted by cultured fibroblasts into the conditioned medium.

### Ang II Induces CTGF Expression through Ang II Receptor Type 1 in Adventitial Fibroblasts

Ang II–induced CTGF protein expression was abrogated when fibroblasts were pretreated with the Ang II receptor type 1 ($AT_1$-R) antagonist losartan ($10^{-5}$ mol/L). In contrast, the Ang II receptor type 2 ($AT_2$-R) antagonist PD123319 ($10^{-4}$ mol/L) had no such effect, suggesting that Ang II–induced CTGF upregulation was mediated through $AT_1$-Rs (Fig. 3).

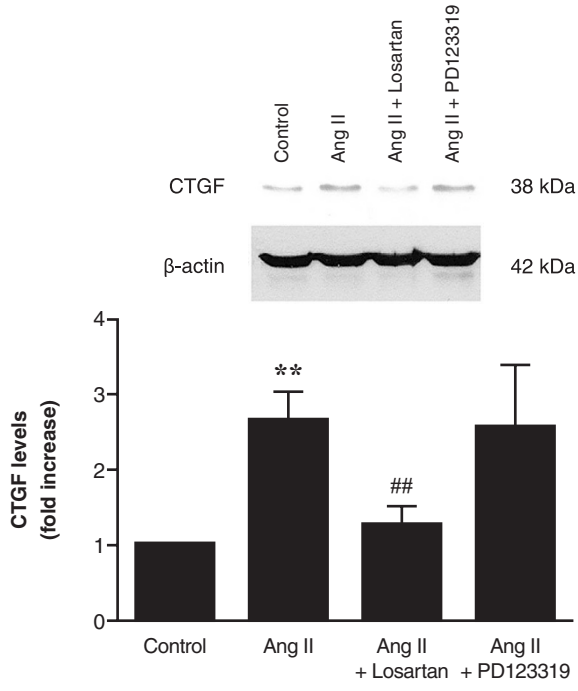


**Fig. 3.** *Ang II–induced CTGF expression via $AT_1$ receptors in AFs. After serum starvation for 48 h, AFs were pretreated for 60 min with the $AT_1$ antagonist losartan ($10^{-5}$ mol/L), or $AT_2$ antagonist PD123319 ($10^{-4}$ mol/L), then cells were stimulated with Ang II ($10^{-7}$ mol/L) for an additional 24 h. CTGF was quantified by Western blot analysis. Each lane contained 20 μg total protein. β-Actin was used as an internal loading control. Data are the mean±SD of 3 separate experiments. $^{**}p<0.01$ vs. the control, $^{\#\#}p<0.01$ vs. the Ang II group.*

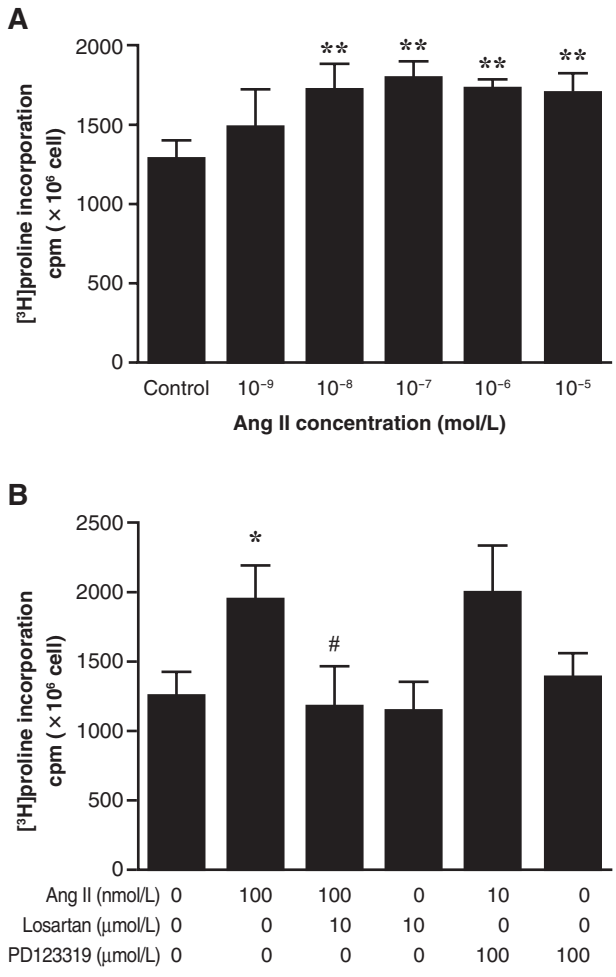


**Fig. 4.** *Ang II–stimulated [$^3$H]proline incorporation via $AT_1$ receptor in AFs. A: Ang II, $10^{-9}$ mol/L to $10^{-5}$ mol/L, increased [$^3$H]proline incorporation in AFs, and the maximal effect of Ang II was at a dose of $10^{-7}$ mol/L. [$^3$H]Proline incorporation was normalized to the cell number and the values in the panels are the mean±SD of 3 separate experiments performed in triplicate. $^{**}p<0.01$ vs. the control. B: $10^{-7}$ mol/L Ang II increased collagen synthesis in AFs, which was selectively inhibited by $10^{-5}$ mol/L losartan, but not by $10^{-4}$ mol/L PD123319.*

## Ang II Induces Collagen Synthesis through $AT_1$-R in Adventitial Fibroblasts

[$^3$H]proline incorporation was used to study the effects of Ang II on collagen synthesis. In cultured AFs, Ang II ($10^{-9}$–$10^{-5}$ mol/L) caused a dose-dependent increase in [$^3$H]proline incorporation. Maximal stimulation of [$^3$H]proline incorporation was observed at $10^{-7}$ mol/L Ang II (Fig. 4A).

Consistent with the results in CTGF expression assays, we found that the $AT_1$-R also mediated Ang II–induced collagen synthesis. The $AT_1$-R antagonist losartan ($10^{-5}$ mol/L) inhibited the Ang II–induced [$^3$H]proline incorporation, but the $AT_2$-R antagonist PD123319 ($10^{-4}$ mol/L) had no effect. Losartan and PD123319, when administered alone without Ang II, had no effect on [$^3$H]proline incorporation (Fig. 4B). These data suggest that Ang II stimulated collagen synthesis in AFs and the effect was mediated through $AT_1$-R.

## Role of CTGF on Collagen Synthesis in Adventitial Fibroblasts

We increased CTGF expression with a CTGF overexpression plasmid in AFs. Overexpression of CTGF increased CTGF protein expression (Fig. 5A) and [$^3$H]proline incorporation in cultured AFs (Fig. 5B). Then, we blocked CTGF expression with CTGF antisense oligonucleotides. Incubation of cells with CTGF antisense oligonucleotides reduced CTGF protein expression (Fig. 6A) and diminished Ang II–induced [$^3$H]proline incorporation. A CTGF mismatched oligonucleotide had no effect on Ang II–induced collagen synthesis (Fig. 6B).

## Discussion

Vascular fibrosis is one of the major changes in cardiovascu-

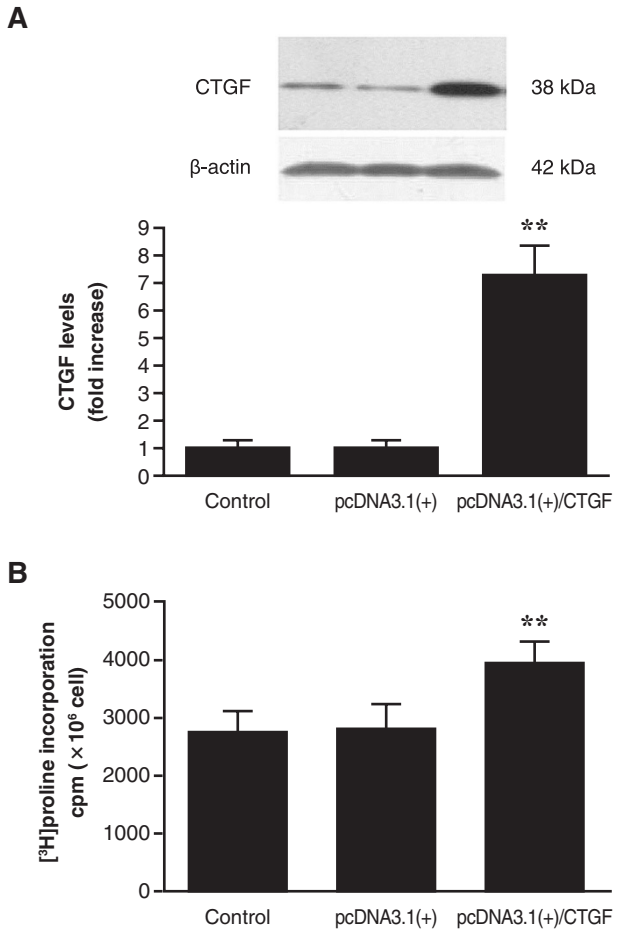


**Fig. 5.** *CTGF overexpression increased CTGF protein expression and [$^3$H]proline incorporation in AFs. A: CTGF protein expression in AFs after transfection of pcDNA3.1(+)/CTGF. The pcDNA3.1(+)/CTGF and control vectors were transfected in AF for 24 h, and then total proteins were extracted and probed with specific CTGF polyclonal antibody. Each lane contained 20 μg total protein and β-actin was used as an internal loading control. **$p < 0.01$. B: AFs were transfected as previously described. [$^3$H]proline incorporation was normalized to the cell number and the values in panels are the mean±SD of 3 separate experiments performed in triplicate. **$p < 0.01$ vs. the control.*

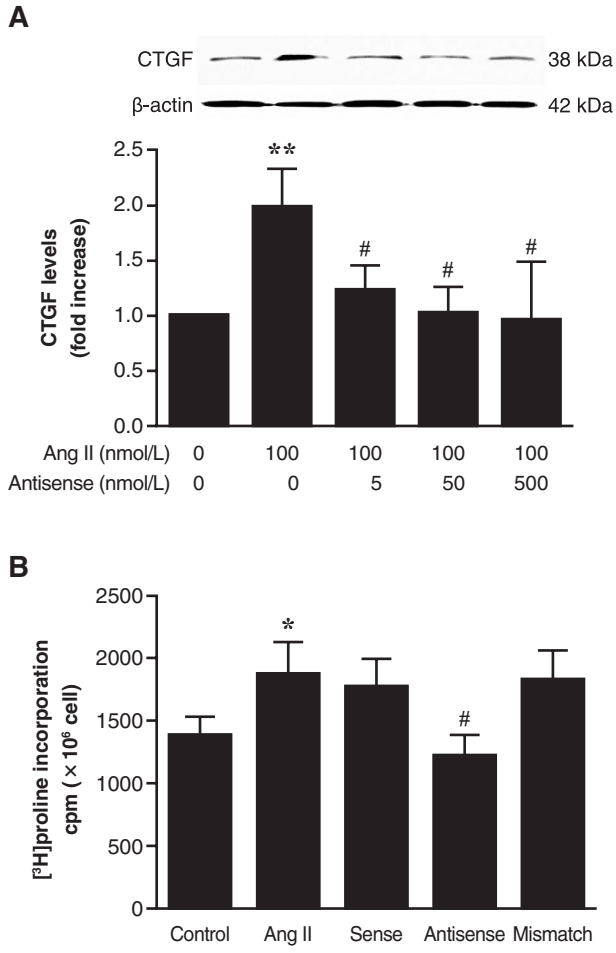


**Fig. 6.** *CTGF antisense ODN inhibited CTGF protein expression and [$^3$H]proline incorporation in AFs. A: CTGF antisense ODN (5 nmol/L, 50 nmol/L, 500 nmol/L) dose-dependently inhibited CTGF expression induced by $10^{-7}$ mol/L Ang II in AFs. *$p < 0.05$ vs. the control. $^{\#}p < 0.05$ vs. the Ang II–treated group. B: Effects of CTGF sense ODN (50 nmol/L), antisense ODN (50 nmol/L), and mismatch ODN (50 nmol/L) on [$^3$H]proline incorporation induced by $10^{-7}$ mol/L Ang II in AFs. *$p < 0.05$ vs. the control. $^{\#}p < 0.05$ vs. the Ang II–treated group. The quantification of CTGF expression and [$^3$H]proline incorporation were expressed as fold increases. Data are the mean±SD of 3 separate experiments.*

lar diseases. There is increasing evidence that Ang II participates in the fibrotic process during vascular damage. In vascular smooth muscle cells (VSMCs), it has been established that CTGF is a mediator of the profibrogenic effect of Ang II (*15*). Recently, increasing attention has been given to the role of the AFs, which play an important role in vascular remodeling (*1–7*, *16*). We previously showed that the adventitia is the main deposition site of collagen in the aortic wall (*8*). However, the mechanisms regulating Ang II–induced fibrosis in AFs have not yet been studied. The present study was the first to demonstrate that CTGF is a mediator of Ang II–induced collagen production *via* $AT_1$-Rs in vascular fibroblasts.

Our study showed that growth-arrested AFs expressed a low level of CTGF, and Ang II increased the CTGF gene expression and protein production. Moreover, a CTGF antisense ODN diminished, while a CTGF overexpression plasmid increased Ang II–induced collagen production. Our data clearly demonstrated that CTGF was involved in vascular collagen production induced by Ang II in AFs. We observed that

in cultured AFs, Ang II–induced CTGF and collagen production were inhibited by the Ang II type 1 ($AT_1$) antagonist losartan. These results indicated that Ang II *via* $AT_1$ stimulated collagen synthesis through CTGF upregulation. In agreement with our findings, CTGF has been shown to be a mediator of Ang II–induced myocardial remodeling in cardiac fibroblasts (*17*) and of Ang II–induced fibronectin synthesis in the kidneys (*18*). In both cell types, these effects of Ang II are mediated though $AT_1$-R (*17*, *18*). Although we did not determine the expression of collagen by Western blotting in this study, we demonstrated that Ang II stimulated the expression of pro-collagen I mRNA in cultured aortic fibroblasts by RT-PCR measurement (data not shown).

Transforming growth factor-β (TGFβ) is a potent profibrogenic factor and a stimulator of CTGF gene expression (*19*). In many cells, TGFβ-induced ECM production is mediated by CTGF (*20*, *21*). Blockade of TGFβ by a neutralizing antibody against TGFβ has been shown to diminish Ang II–induced CTGF production in VSMCs (*15*) and in renal cells (*22*), suggesting that endogenous TGFβ synthesis is involved, at least in part, in the CTGF production caused by Ang II. However, several studies have shown that Ang II–induced CTGF production was independent of TGFβ (*23*), indicating that Ang II–induced CTGF production might be either TGFβ-dependent or TGFβ–independent. Our preliminary study showed that neutralizing antibody against TGFβ1 (10 μg/mL) partially blocked the CTGF protein expression induced by $10^{-7}$ mol/L Ang II (data not shown), suggesting that Ang II–induced CTGF expression might be partially induced by indirect effects of Ang II–induced production of TGFβ1 in AFs.

We have previously reported that Ang II induces AF transition to myofibroblasts (*11*). Myofibroblasts demonstrate a marked increase in proliferative and synthetic activities, including ECM synthesis. Our previous study demonstrated that Ang II induced myofibroblast differentiation *via* a pathway that involves NADPH oxidase generation of reactive oxygen species (ROS) (*11*). ROS mediates many Ang II effects, including $AT_1$-mediated CTGF production (*15*). In addition to ROS, calcineurin (*23*), endothelin-1 (*24*), protein kinase C (*15*) and Smad (*25*) have been shown to be mediators of Ang II–induced CTGF production. Future studies are needed to determine the role of these factors on CTGF production in AFs. Blockade of CTGF overexpression might be a potential therapeutic target in the treatment of Ang II–induced vessel wall damage.

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