

## Full-length article

## All-*trans* retinoic acid inhibited angiotensin II-induced increase in cell growth and collagen secretion of neonatal cardiac fibroblasts<sup>1</sup>

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### Key words

cardiac fibroblast; angiotensin II; all-*trans* retinoic acid; collagen secretion; transforming growth factor- $\beta_1$ <sup>1</sup> Project supported by the National Natural Science Foundation of China (No 39970241).<sup>4</sup> Correspondence to Prof Tai YAO.

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### Abstract

**Aim:** To determine whether all-*trans* retinoic acid (*at*RA) acts to modulate angiotensin II (Ang II)-induced cardiac fibroblast cell growth and collagen secretion. **Methods:** Cultured neonatal rat cardiac fibroblasts (CF) were used in the experiment. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to detect cell growth of the CF; and immunocytochemistry and Western blotting were used to measure the production and secretion of collagen and the expression of transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) by the CF. **Results:** *at*RA ( $1 \times 10^{-7}$  to  $1 \times 10^{-5}$  mol/L) inhibited the Ang II-induced increase in cell growth of CF ( $P < 0.05$ ). Ang II stimulated the secretion of collagen types I and III by the CF. This effect was blocked by AT<sub>1</sub> receptor antagonist losartan ( $1 \times 10^{-6}$  mol/L), but not by AT<sub>2</sub> receptor antagonist PD123319 (up to  $1 \times 10^{-6}$  mol/L). Exposure of CF to *at*RA ( $1 \times 10^{-5}$  mol/L) attenuated the Ang II-induced increase in the secretion of collagen types I and III ( $P < 0.05$ ). *at*RA ( $1 \times 10^{-5}$  mol/L) also blocked the Ang II-induced increase in the expression of TGF- $\beta_1$ . **Conclusion:** *at*RA inhibits the Ang II-induced increase in cell growth and collagen secretion of neonatal rat CF. The effect of *at*RA is possibly mediated by lowering the TGF- $\beta_1$  level. These observations support the notion that *at*RA is a potential candidate for the prevention and therapy of cardiac remodeling.

### Introduction

Increased hemodynamic workload is well known to alter the structure and muscle content of the heart<sup>[1,2]</sup>. These responses, as component parts of cardiac remodeling, are thought to be an adaptive mechanism to decrease cardiac wall stress and maintain heart function. However, cardiac remodeling also results in increased myocardial stiffness and ventricular diastolic dysfunction, potentially leading to chronic heart failure. This is an important cause of morbidity and mortality associated with cardiovascular disease.

Cardiac remodeling is the composite result of many factors, including mechanical stretching, ischemia, and the effects of hormones and cytokines. During cardiac remodeling, cardiac fibroblasts (CF) undergo significant changes: they proliferate and produce an increased amount

of extracellular matrix, including fibronectin, laminin and collagen types I and III. In addition, they may assume a myofibroblast character and express  $\alpha$ -actin, and thus contain myofilaments<sup>[3–7]</sup>. These changes may lead to fibrous material deposition, alterations of the highly organized extracellular matrix and interconnections between cardiomyocytes and their neighboring cells, thus impairing the systolic and diastolic functions of the heart.

Angiotensin II (Ang II) is a major final effector molecule of the renin-angiotensin system. Ang II may act as a mitogen for the proliferation of some types of cells. It has also been implicated as an important factor that contributes to the pathogenesis of atherosclerosis, hypertension, and cardiac remodeling<sup>[4,5,8–10]</sup>. Ang II is also known to stimulate cell proliferation and secretion of types I and III in CF, and activate the expression of some growth factors, such as the

transforming growth factor- $\beta_1$  (TGF- $\beta_1$ )<sup>[6]</sup>, which in turn modulates the cell's response to Ang II. Therefore, Ang II plays an important role in cardiac fibrosis. In this context, drugs that decrease or antagonize the effect of Ang II may be beneficial for alleviating cardiac remodeling and heart failure.

All-*trans* retinoic acid (*atRA*), a derivative of vitamin A, influences cellular differentiation in organ development<sup>[11,12]</sup>, and inhibits proliferation of a variety of cell types<sup>[12–14]</sup>. WANG *et al* determined that *atRA* inhibited Ang II-induced cardiomyocyte hypertrophy<sup>[15]</sup>, and LÜ *et al* demonstrated that chronic treatment with *atRA* prevented medial thickening of intramyocardial and intrarenal arteries, and ventricular fibrosis during the development of hypertension in spontaneously hypertensive rats<sup>[16]</sup>. There is evidence that retinoids reduce the expression of platelet-derived growth factor, inducible nitric oxide synthase, interleukin-6, and endothelin<sup>[12,13,17]</sup>, thus opposing the action of Ang II. The effects of *atRA* on the expression and function of TGF- $\beta_1$  as well as extracellular matrix proteins are cell specific<sup>[18,19]</sup>. It has been reported that *atRA* blocks the stimulatory action of Ang II on TGF- $\beta_1$  mRNA in vascular smooth muscle cells<sup>[20]</sup>. Therefore, it is interesting to ascertain whether *atRA* has similar effects on CF. The purpose of the present study was to determine whether *atRA* alleviated the Ang II-induced increase in cell growth and collagen secretion in cultured CF from neonatal rats.

## Materials and methods

**CF cell culture** CF were isolated from 1-day-old Sprague-Dawley rat pups with 0.1% trypsin. To select pure cardiac fibroblasts, dissociated cells were preplated in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Rockville, MD, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37 °C with 5% CO<sub>2</sub> for 1 h. The nonmyocytes attached readily to the bottom of the culture dish. Cells adhered to the culture dishes during the preplating procedure were resuspended with culture medium and then allowed to reattach for 30 min at 37 °C. After this procedure was repeated twice, the cells were distributed into culture dishes and incubated in the culture medium containing 10% fetal bovine serum. In the third passage, the cells were mainly CF. Cells from the third or fourth passages were used for experiments.

**Cell growth assay** Subconfluent CF were seeded into 96-well plates at a density of 4000 cells per well and cultured for 12 h in the presence of 10% fetal bovine serum (Gibco). Subsequently, cells were washed twice with serum-free medium and growth was arrested in serum-free medium for 24 h.

Then the quiescent cells were treated with Ang II and/or *atRA* for 24 h. More Ang II was added after 12 h to compensate for a decrease due to degradation by endogenous angiotensinase. Control cultures contained dimethyl sulfoxide (Me<sub>2</sub>SO; Sigma, St Louis, MO, USA). After incubation of the cultures for 24 h at 37 °C, 10–20  $\mu$ L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) was added per well. The culture plate was preplated in a cabinet at 37 °C with 5% CO<sub>2</sub> for 4 h, then the old medium was removed and 150  $\mu$ L Me<sub>2</sub>SO was added per well. The culture plate was shaken for 15 min. Absorbance at 490 nm was considered to be directly proportional to the number of cells in the wells and was therefore used as a measure of cell growth.

**Immunocytochemistry analysis** CF cultured on 12-mm glass coverslips were serum-starved for 24 h and then cultured for 48 h in media alone (control) or treated with Ang II and/or *atRA*. CF were then fixed in 10% buffered formalin for 10–30 min, washed twice with distilled water, and immersed in 30% H<sub>2</sub>O<sub>2</sub>/carbinol for 30 min at room temperature. After that, the cells were washed 3 times with distilled water, incubated with 5% bovine serum albumin (BSA; Boster, Wuhan, China) for 20 min, then with collagen type I or collagen type III antibodies (1:50) (Boster) for 12 h at 4 °C. After 3 washes with phosphate-buffered saline (PBS) and incubation with horseradish peroxidase-conjugated goat anti-mouse antibody (1:2000; Boster) for 20 min at 37 °C, CF were washed again with PBS, then incubated with streptavidin-biotin-peroxidase complex (SABC, Boster) for 20 min. After being washed for 20 min with PBS by using a DAB-kit (Boster), CF were mounted on cover slides for microscopic imaging.

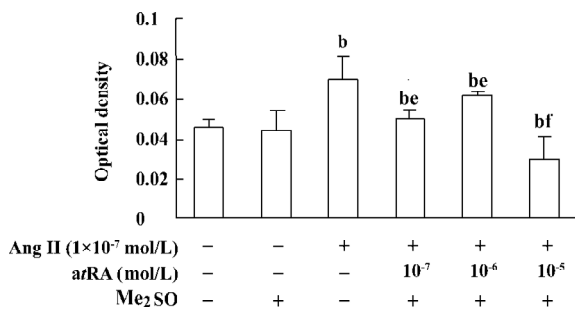
**Western blot analysis** The protein concentrations were determined by using a BCA-100 protein quantitative analysis kit (Shenergy Biocolors). An equal amount of protein from the medium was subjected to 5%–12% gradient sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Amersham Pharmacia). After electrophoretic separation, proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Amersham Pharmacia), which was then incubated overnight at 4 °C with the primary antibody (1:500; Santa Cruz, Santa Cruz, USA). Antibody binding was detected by counter-staining with horseradish peroxidase-conjugated antibodies (1:2000; CalBiochem, USA) and visualized using an ECL-detection kit (Pierce Biotechnology, Rockford, IL, USA). The efficiency of transfer was confirmed by staining the membrane with Ponceau S. The relative intensity of immunoreactive bands on the exposed film was quantified by using a computer-assisted densitometry program (Smart View; Life Science Research Products and

System Engineering).

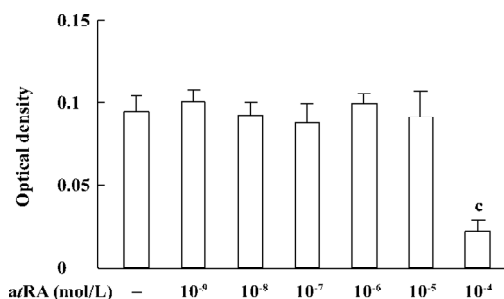
**Statistical analysis** Data are presented as mean±SD. Statistical significance was evaluated by one-way ANOVA followed by the Turkey *post hoc* test (SPSS software). Significance was accepted at  $P<0.05$ . Each experiment was repeated 3 times to verify the reproducibility of the results.

**Results**

**azRA attenuated the Ang II-induced increase in cell growth of CF** Exposure of neonatal rat CF for 24 h to Ang II ( $1\times 10^{-9}$  to  $1\times 10^{-6}$  mol/L) induced a significant increase in cell growth, therefore Ang II at a concentration of  $1\times 10^{-7}$  mol/L was used in the subsequent experiments. The Ang II-induced increase in cell growth was attenuated by azRA ( $1\times 10^{-7}$  to  $1\times 10^{-5}$  mol/L). When CF were co-treated with azRA ( $1\times 10^{-5}$  mol/L) and Ang II for 24 h, the cell growth level was even below that of the control (Figure 1). azRA alone at concentrations of  $1\times 10^{-9}$  to  $1\times 10^{-5}$  mol/L produced no effect on the cell growth of CF, but caused a significant decrease at a higher concentration ( $1\times 10^{-4}$  mol/L; Figure 2).

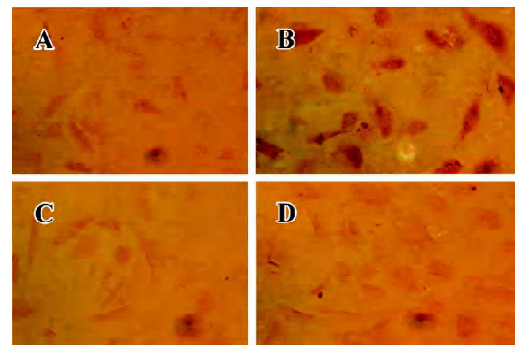


**Figure 1.** Effects of azRA ( $1\times 10^{-7}$ ,  $1\times 10^{-6}$ , and  $1\times 10^{-5}$  mol/L) on Ang II-induced increases in cell growth of cultured neonatal rat CF. Cell growth was measured by using the MTT assay, and optical density was expressed in arbitrary units.  $n=3$ . Mean±SD. <sup>b</sup> $P<0.05$  vs control. <sup>e</sup> $P<0.05$ , <sup>f</sup> $P<0.01$  vs Ang II alone-treated group.



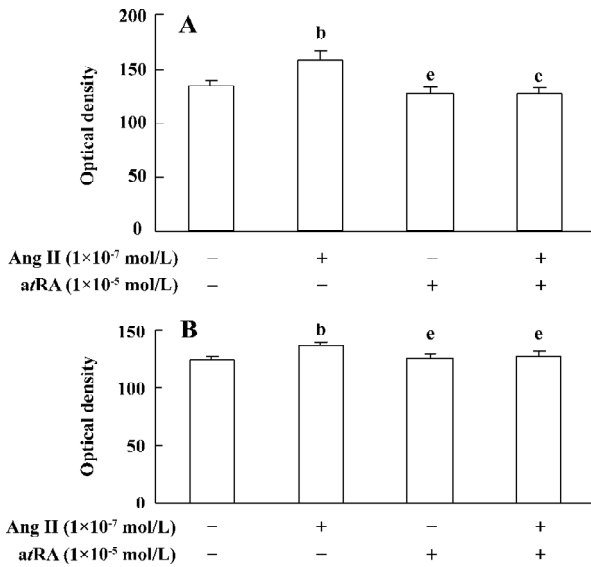
**Figure 2.** Effect of azRA alone on cell growth of cultured neonatal rat CF.  $n=3$ . Mean±SD. <sup>c</sup> $P<0.01$  vs control.

**azRA attenuated the Ang II-induced increase in intracellular collagen types I and III in CF** Immunocytochemical staining was used to detect intracellular collagen types I and III. In the control neonatal rat CF, only sparsely distributed collagen type III staining was observed (Figure 3A). Exposure to Ang II ( $1\times 10^{-7}$  mol/L) for 48 h resulted in an accumulation of collagen type III in CF (Figure 3B). azRA ( $1\times 10^{-5}$  mol/L) treatment reduced the Ang II-induced accumulation of collagen type III (Figure 3D). azRA by itself ( $1\times 10^{-5}$  mol/L) caused no apparent change in the quantity of collagen type III (Figure 3C). The results of image analysis are shown in Figure 4A. Similarly, the Ang II-induced increase in the content of collagen type I in the CF was also abolished by azRA (Figure 4B).

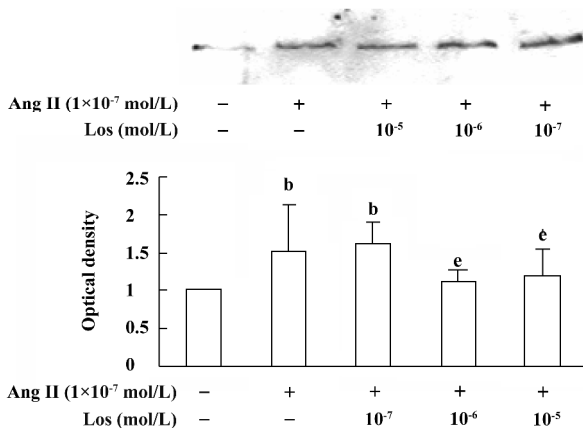


**Figure 3.** Immunocytochemical staining of collagen type III in neonatal rat CF. (A) Control; (B) CF treated with Ang II ( $1\times 10^{-7}$  mol/L) alone; (C) CF treated with azRA ( $1\times 10^{-5}$  mol/L) alone; (D) CF treated with Ang II ( $1\times 10^{-7}$  mol/L) and azRA ( $1\times 10^{-5}$  mol/L).

**azRA attenuated the Ang II-induced increase in collagen secretion of CF** Western blot analysis was used to measure collagen secretion by the neonatal rat CF into the culture medium. Figure 5 shows that an increase in collagen type III in the culture medium was detected in Ang II ( $1\times 10^{-7}$  mol/L)-treated cultures and that this response was blocked by angiotensin AT<sub>1</sub> receptor antagonist losartan ( $1\times 10^{-6}$  and  $1\times 10^{-5}$  mol/L). However, this effect of Ang II was not affected by angiotensin AT<sub>2</sub> receptor antagonist PD123319 (up to  $1\times 10^{-6}$  mol/L; Figure 6). azRA ( $1\times 10^{-5}$  mol/L) alone slightly reduced the basal secretion of collagen types III and I, and completely abolished the Ang II-induced stimulation of collagen secretion (Figure 7A, 7B). When the CF were co-treated with azRA and Ang II, the collagen levels in the culture medium were significantly lower than those of the controls (Figure 7). We also observed that when PD123319 ( $1\times 10^{-5}$  mol/L) was added to the culture medium, application of azRA ( $1\times 10^{-6}$  or  $1\times 10^{-5}$  mol/L) together with Ang II ( $1\times 10^{-7}$  mol/L) again



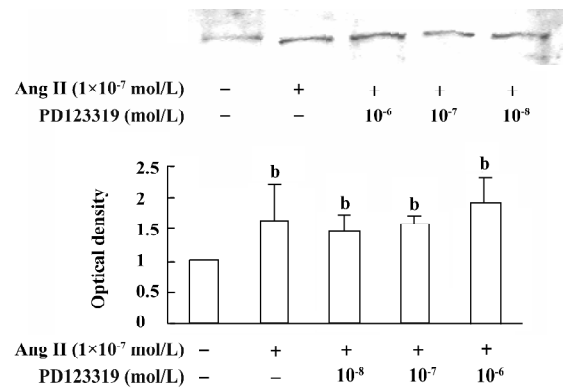
**Figure 4.** Image analysis of immunocytochemical staining of intracellular collagen type III (A) and collagen type I (B) in neonatal rat CF. *n*=3. Mean±SD. <sup>b</sup>*P*<0.05 vs control. <sup>c</sup>*P*<0.05 vs Ang II alone-treated groups.



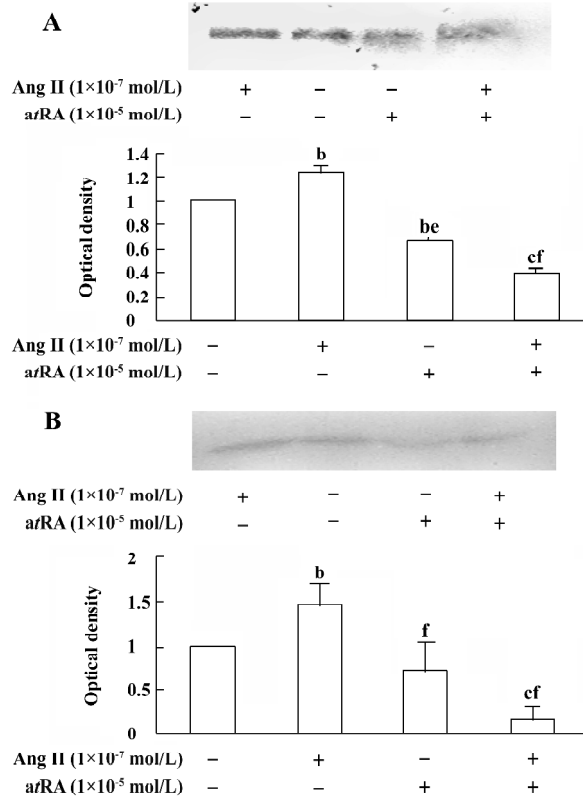
**Figure 5.** Effect of losartan on Ang II-induced secretion of collagen type III by neonatal rat CF. *n*=3. Mean±SD. <sup>b</sup>*P*<0.05 vs control. <sup>c</sup>*P*<0.05 vs Ang II alone-treated group.

significantly reduced the secretion of collagen type III into the medium (Figure 8).

**a<sub>1</sub>RA attenuated the Ang II-induced increase in TGF-β<sub>1</sub> expression in CF** Western blot analysis was used to measure the expression of TGF-β<sub>1</sub> in CF. After 48 h of exposure to Ang II ( $1 \times 10^{-7}$  mol/L), the expression of TGF-β<sub>1</sub> in the CF was significantly increased compared with the controls. a<sub>1</sub>RA ( $1 \times 10^{-5}$  mol/L) alone caused no obvious change in the basal level of TGF-β<sub>1</sub> in the CF, but it completely abolished the Ang II-induced stimulation of TGF-β<sub>1</sub>, the TGF-β<sub>1</sub> content



**Figure 6.** Effect of PD123319 on Ang II-induced secretion of collagen type III by neonatal rat CF. *n*=3. Mean±SD. <sup>b</sup>*P*<0.05 vs control.

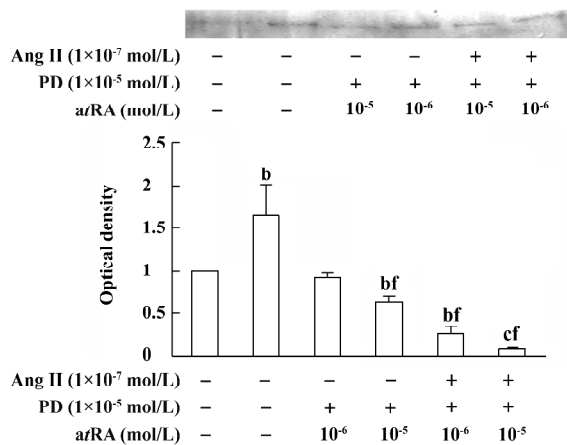


**Figure 7.** Effect of a<sub>1</sub>RA on the Ang II-induced increase in collagen type III (A) and collagen type I (B) secretion by neonatal rat CF. *n*=3. Mean±SD. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs control. <sup>e</sup>*P*<0.05, <sup>f</sup>*P*<0.01 vs Ang II alone-treated group.

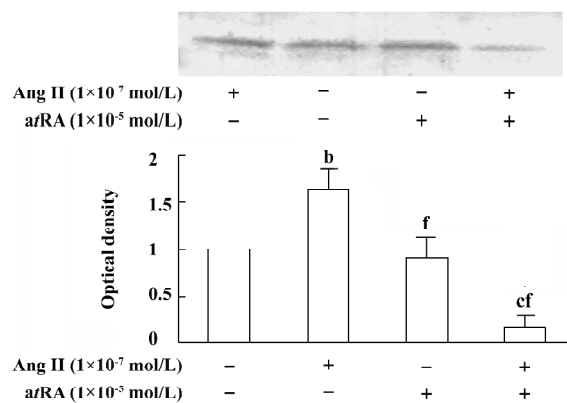
was significantly lower than that of the control (Figure 9).

**Discussion**

Our observations demonstrate that a<sub>1</sub>RA inhibits the



**Figure 8.** Effect of arRA on the Ang II-induced increase in collagen type III secretion in PD12319-treated neonatal rat CF.  $n=3$ . Mean $\pm$ SD. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs control. <sup>f</sup> $P<0.01$  vs Ang II alone-treated group.



**Figure 9.** Effect of arRA on the Ang II-induced increase in TGF- $\beta_1$  expression in neonatal rat CF.  $n=3$ . Mean $\pm$ SD. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs control. <sup>f</sup> $P<0.01$  vs Ang II alone-treated group.

stimulatory effect of Ang II on cultured neonatal rat CF, which is consistent with the results previously achieved in our laboratory by measuring the myocyte protein content<sup>[15]</sup>. In the present study, we also found that arRA (up to  $1 \times 10^{-5}$  mol/L) by itself generally produced no apparent effect on CF cell growth, but a high concentration of arRA ( $1 \times 10^{-4}$  mol/L) caused a significant decrease, possibly indicating toxicity. Therefore, arRA at concentrations of  $1 \times 10^{-5}$  and  $1 \times 10^{-6}$  mol/L were used in the present study to observe its effect on the actions of Ang II.

The induction of growth and proliferation of a variety of cell types by Ang II has been intensively studied. The cell response to Ang II depends on the interactions between pro- and anti-proliferative factors, and it is well known that

Ang II stimulates the expression of a number of pro-proliferative autocrine factors. Ang II has been demonstrated to be closely associated with the production of extracellular matrix. An accumulation of fibrillar collagen in the interstitial space of the hypertrophied heart is thought to be responsible for abnormal ventricular wall stiffness and for impaired cardiac pumping capacity<sup>[21,22]</sup>. It has been shown that Ang II increases cardiac fibroblast-mediated collagen synthesis and mRNA levels of collagen types I and III<sup>[23]</sup>. The immunocytochemistry results in the present study showed that 48 h exposure of neonatal rat CF to Ang II resulted in an increase in the collagen types I and III content. Because collagen is secreted into the extracellular matrix as soon as it is synthesized, we measured the collagen content in the culture medium, and our results from Western blotting showed that the secretion of collagen types I and III was stimulated by Ang II. We also demonstrated that the stimulatory action of Ang II was completely blocked by the angiotensin AT<sub>1</sub>, but not AT<sub>2</sub>, receptor antagonist, indicating that the Ang II-induced increase in collagen secretion was mediated mainly by activating angiotensin AT<sub>1</sub> receptors.

It has been documented that arRA promotes differentiation and fibrinolysis, but also inhibits cell proliferation, migration, thrombosis, angiogenesis, platelet aggregation, and inflammation<sup>[24-27]</sup>. In line with the finding that arRA attenuated the effect of Ang II on cell growth, we also found that arRA inhibited the Ang II-induced increase in secretion of collagen types I and III by the cultured neonatal rat CF.

TGF- $\beta_1$  is an important growth factor involved in the development of myocardial fibrosis. Low expression levels of TGF- $\beta_1$  and collagen types I and III mRNA are seen in the normal rat heart; however, expression of TGF- $\beta_1$  and collagen types I and III is markedly increased in the infarcted heart. The increase in TGF- $\beta_1$  mRNA precedes the increases in collagen mRNA levels. In *in vitro* studies, TGF- $\beta_1$  has been found to induce an increase in collagen production and secretion, and enhance the mRNA levels of collagen types I and III in rat CF<sup>[28]</sup>. Increased myocardial TGF- $\beta_1$  and collagen mRNA are found in myocardial fibrosis induced by Ang II infusion<sup>[28]</sup>. There is also evidence indicating that TGF- $\beta_1$  inhibits the production and secretion of collagenase (MMP-1), leading to the accumulation of collagen in the extracellular matrix<sup>[29]</sup>. Our present results have revealed that Ang II produces a significant activation of TGF- $\beta_1$  in cultured neonatal rat CF, and that this effect of Ang II is abolished by arRA. Combined administration of arRA and Ang II caused a significant decrease in TGF- $\beta_1$  below the basal level.

The mechanism underlying the effect of arRA on Ang II-induced activation of CF remains to be further clarified. In

the present study, we did not measure the expression levels of AT<sub>1</sub> and AT<sub>2</sub> receptors in *at*RA-treated CF; however, there is evidence that *at*RA downregulates AT<sub>1</sub> receptor levels in vascular smooth muscle cells<sup>[20]</sup>. This might be also true in CF. Because the Ang II-induced activation of CF is mediated via the AT<sub>1</sub> receptor, it is possible that *at*RA inhibits the effect of Ang II on CF via downregulation of the AT<sub>1</sub> receptor. An interesting observation in the present study is that combined treatment with *at*RA and Ang II resulted in a significant decrease in the secretion of collagen and the expression of TGF-β<sub>1</sub> below their respective basal levels. It is known that activation of angiotensin AT<sub>2</sub> receptor produces effects that are opposite to those of AT<sub>1</sub> receptor activation, so a possible explanation is that when cells are treated with both *at*RA and Ang II, the AT<sub>1</sub> receptor-mediated effect is blocked or attenuated by *at*RA, whereas the AT<sub>2</sub> receptor-mediated effect becomes apparent, leading to decreases in TGF-β<sub>1</sub> expression and collagen secretion. However, in the present study we found that when AT<sub>2</sub> receptor antagonist PD123319 was administered together with *at*RA and Ang II, the secretion of collagen type III was as low as that without PD123319 administration. We therefore suggest that the inhibition of collagen secretion upon combined treatment of the CF with *at*RA and Ang II is not mediated by activation of AT<sub>2</sub> receptors. Combined treatment with *at*RA and Ang II probably results in activation of some other signals or signaling pathways, leading to a decrease in the secretion of collagen. Because TGF-β<sub>1</sub> is known to inhibit the expression of collagenase, and in the present study we observed that the expression of TGF-β<sub>1</sub> was significantly decreased when CF were co-treated with *at*RA and Ang II, we therefore hypothesize that combined administration of *at*RA and Ang II results in a decrease in the expression of TGF-β<sub>1</sub> via some unknown pathway and, in turn, a low level of TGF-β<sub>1</sub> leads to an increase in the activity of collagenase and thus decreases the collagen content. Our data are similar to those reported by Haxsen *et al*<sup>[20]</sup>, who noted that combined administration of *at*RA (1 μmol/L) and Ang II (1 μmol/L) in a culture of vascular smooth muscle cells resulted in a significant decrease in the level of TGF-β<sub>1</sub> mRNA. They suggested that the abrogation of Ang II-dependent TGF-β<sub>1</sub> induction by *at*RA in vascular smooth muscle cells was possibly mediated via the activator protein-1 (AP-1)<sup>[20]</sup>.

In summary, our observations provide further evidence that *at*RA inhibits Ang II-induced increases in cell growth and secretion of collagen types I and III in cultured neonatal rat CF. We hypothesize that this inhibition of collagen secretion might be related to the decrease of TGF-β<sub>1</sub> expression in CF. These data support the notion that *at*RA exerts a

beneficial effect on cardiac structural impairment during the process of cardiac remodeling. The details of the mechanism by which *at*RA acts remain to be further elucidated.

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