Expression of Functional Angiotensin-Converting Enzyme and AT$_1$ Receptors in Cultured Human Cardiac Fibroblasts

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**Background**—Angiotensin II (Ang II) has been implicated in the development of cardiac fibrosis. The aims of the present study were to examine expression and activity of ACE and of angiotensin receptors in human cardiac fibroblasts cultured from dilated cardiomyopathic and ischemic hearts. The effects of Ang II on fibroblasts were also investigated.

**Methods and Results**—Human cardiac fibroblasts were cultured from ventricular and atrial myocardium and characterized immunohistochemically. Expression of ACE and the angiotensin AT$_1$ receptor was demonstrated in cardiac fibroblasts by reverse transcriptase–polymerase chain reaction and radioligand binding. Functional ACE activity, measured by radiolabeled substrate conversion assay, was detected in both ventricular (V$_{max}$ · K$_m$ \textsuperscript{-1} · mg$^{-1}$, 0.031 ± 0.010; n = 13) and atrial (0.034 ± 0.012; n = 6) fibroblasts. Fibroblast ACE activity was increased after 48 hours of treatment with basic fibroblast growth factor, dexamethasone, and phorbol ester. Ang II did not affect DNA synthesis but stimulated [3 H]proline incorporation in cardiac fibroblasts (20.0 ± 4.0% increase above control by 10 μmol/L; P < 0.05, n = 7), which was abolished by losartan 10 μmol/L but not PD123319 1 μmol/L. Ang II also stimulated a rise in intracellular calcium (basal, 56 ±1 nmol/L; Ang II, 355 ± 24 nmol/L) via the AT$_1$ receptor, as shown by complete inhibition with losartan.

**Conclusions**—We have demonstrated expression and activity of ACE and AT$_1$ receptor in cultured human cardiac fibroblasts. In addition, cardiac fibroblasts respond to Ang II with AT$_1$ receptor–mediated collagen synthesis. The presence of local ACE and AT$_1$ receptors in human fibroblasts suggests their involvement in the development of cardiac fibrosis. (Circulation. 1998;98:2553-2559.)

**Key Words:** angiotensin receptors, enzymes, collagen, cells

Myocardial fibrosis is a pathological feature associated with cardiac hypertrophy, myocyte necrosis, and hypertension. Components of the renin-angiotensin system, including ACE and angiotensin II (Ang II), have been implicated in the development of cardiac fibrosis. Significantly, numerous studies have provided evidence for the presence of local ACE, and expression of ACE has been demonstrated in both rat and human cardiac tissues. Furthermore, the cardiac renin-angiotensin system has been shown to be functional in humans, with ACE being the predominant pathway for the local generation of Ang II in human cardiac tissues. The microvascular endothelium is considered to be the main site of ACE expression in both rat and human hearts, although other cardiac cells may express the enzyme. ACE binding and immunostaining, for example, have been localized to interstitial cells at sites of myocardial infarction (MI) in the rat, and ACE expression has been demonstrated in isolated rat cardiac fibroblasts. In contrast, immunohistochemical studies on human tissues have provided conflicting information concerning the cellular localization of ACE immunoreactivity after MI. We have shown that interstitial cells in the failing human heart display ACE binding, albeit at a relatively low level compared with the vascular endothelium. However, the expression of ACE in human cardiac fibroblasts is still uncertain. In addition, little is known about the regulation of fibroblast ACE activity, and possible regulatory factors include glucocorticoids and basic fibroblast growth factor (bFGF), both of which have been demonstrated to enhance ACE activity in other cell types.

Several studies have suggested that Ang II is a trophic agent with the ability to influence myocardial fibrosis and modulate cardiac fibroblast growth and collagen synthesis. The effects of Ang II are mediated through specific receptors, AT$_1$ and AT$_2$, being the main subtypes. Both receptors occur in rat and human myocardium, whereas the AT$_1$ subtype predominates in isolated adult rat cardiac fibroblasts. The expression of angiotensin receptors in cultured human cardiac fibroblasts and the effects of Ang II...
on cell growth and collagen metabolism remain to be established.

In the present study, we have determined the expression and activity of ACE in isolated ventricular and atrial fibroblasts, studied the effects of Ang II on cardiac fibroblast collagen synthesis and proliferation, and characterized the receptor subtype mediating such responses.

### Methods

#### Materials

Human Ang II, platelet-derived growth factor AB (PDGF-AB), bFGF, dexamethasone, phosphor myristate acetate (PMA), DMEM, HBSS, FCS, PBS, L-glutamine, penicillin/streptomycin, HEPES, EDTA, SDS, BSA, trichloroacetic acid, and collagenase II were purchased from Sigma Chemical Co. 125 I-labeled (1-sarcosine,8-iso-leucine)angiotensin II ([125 I]-[S 1 ,I 8 ]Ang II, 2200 Ci/mmol) was purchased from NEN Life Science Products. A 125 I-labeled tyrosyl derivative of lisinopril ([N-(s)-1-carboxy-3-phenylpropyl]-l-lysyl-tyrosyl-l-proline, [35 S]-351A) was iodinated (2000 Ci/mmol) as previously described. Losartan (DuP 753) and PD123319 were gifts from DuPont Merck, Wilmington, Del, and Parke-Davis, Ann Arbor, Mich, respectively. Tissue culture plastics (Falcon) were from Marathon Laboratory Supplies. [Methyl-3 H]thymidine 64 Ci/mmol and [3,4- 3 H]benzoyl-phenylalanine-alanine-proline ([3 H]BPAP, 400 μCi/mL) was synthesized and radiolabeled in our laboratory. Liquid and tolune scintillation cocktails were from Canberra Packard.

#### Human Cardiac Fibroblast Culture and Characterization

Samples of left ventricular and atrial myocardium were obtained from recipient hearts (age range, 2 to 59 years, with dilated cardiomyopathy or ischemic heart disease) at the time of cardiac transplantation and stored at 4°C for ≤12 hours in HBSS containing 20 mmol/L HEPES (pH 7.4). Myocardium from the free walls of left ventricles and atria, dissected free of epicardial and valvular regions, was minced fine and incubated in a sterile-filtered dissociation solution containing collagenase II 1000 U/mL for 2 hours under shaking in a water bath at 37°C. After 1 hour, the mixture was passed through a syringe several times to aid cell dissociation and then incubated for another hour. Dispersed cells were filtered (100-μm nylon mesh), washed in fibroblast growth medium, centrifuged (1200 rpm, 5 minutes), resuspended in growth medium, and incubated in 25-cm² plastic flasks in a humidified atmosphere of 5% CO₂ in air. Fibroblast growth medium (DMEM supplemented with L-glutamine 2 mmol/L, penicillin 100 U/mL, streptomycin 100 μg/mL, and 20% FCS) was replaced every 3 days. Fibroblasts were identified by positive immunostaining for fibroblast-specific α-smooth muscle actin, also a feature of cardiac fibroblasts in culture, which may reflect either variation in myocardial scarring between patients or possible phenotypic transformation of some cells in culture. Human endothelial cells, isolated from unused portions of donor aorta and recipient coronary artery as previously described, exhibited the endothelial cobblestone morphology and CD31 immunostaining. Cells of passages 1 through 6 played a-smooth muscle actin immunoreactivity, also a feature of cardiac fibroblasts in culture, which may reflect either variation in myocardial scarring between patients or possible phenotypic transformation of some cells in culture. Human endothelial cells, isolated from unused portions of donor aorta and recipient coronary artery as previously described, exhibited the endothelial cobblestone morphology and CD31 immunostaining. Cells of passages 1 through 6 were used for all experiments.

#### Expression of ACE and the AT₁ Receptor Genes

Expression of ACE and AT₁ receptor was confirmed by reverse transcription–polymerase chain reaction (RT-PCR) analysis. Total RNA was prepared from cell pellets according to the method of Chomczynski and Sacchi and was reverse transcribed into cDNA with random primers (In Vitrogen). Oligonucleotide primers were synthesized according to the nucleotide sequences and genomic DNA sequences (296 bp; lanes 7 through 9) after RT-PCR amplification of transcripts isolated from cultured human atrial (lanes 1 through 3) and ventricular fibroblasts (lanes 4 through 9) and aortic endothelial cells (EC, lane 9). Lane 3 represents a control amplification (no cDNA).

### Figure 1.

Expression of AT₁ receptor and ACE in human cardiac fibroblasts (fibs). Autoradiogram of specific products corresponding to AT₁ receptor (551 and 466 bp; lanes 1 through 6) and ACE cDNA sequences (296 bp; lanes 7 through 9) after RT-PCR amplification of transcripts isolated from cultured human atrial (lanes 1 through 3) and ventricular fibroblasts (lanes 4 through 8) and aortic endothelial cells (EC, lane 9). Lane 3 represents a control amplification (no cDNA).

### Figure 2.

Human cardiac fibroblasts exhibited specific [25 S]-([S 1,I 8 ]Ang II binding sites (0.2 nmol/L, 90 minutes at 37°C) characteristic of AT₁ receptor subtype. Top, Binding was significantly reduced (**P<0.001) in presence of unlabeled ([S 1,I 8 ]Ang II 1 μmol/L and losartan 1 μmol/L but was unaffected by PD123319 1 μmol/L. Values represent mean±SEM of ligand bound (cpm/μg protein, n=4 ventricular cultures). Comparisons between values were made by ANOVA followed by Bonferroni's correction. Bottom, Competitive inhibition of [25 S]-([S 1,I 8 ]Ang II binding to ventricular fibroblasts in presence of increasing concentrations of nonselective [□], ([S 1,I 8 ]Ang II; ○, Ang II), AT₁-selective (□, losartan), and AT₂-selective (●, PD123319) competitors. Points represent mean of 3 determinations, expressed as percentage of maximum binding.
organization of human AT\(_1\) receptor and ACE genes. The AT\(_1\) receptor primer sequences were 5'-GATGGGAGCGGT-GAGCGG-3' (sense) and 5'-TGCCAAAGGCCACGGGTAT-TC-3' (antisense), the PCR amplifier spanning exons 1, 2, 3, 4, and 5 over a region of 755 bp. For human ACE, the sense primer was GACCATGTCCTTCAGCACC-3'.

ACE Activity Assay

Confluent cardiac fibroblasts in 24-well plates were incubated in DMEM containing 0.4% FCS for 48 hours before assay for ACE activity or treatment with either PDGF-AB 15 ng/mL, bFGF 50 ng/mL, dexamethasone 100 nmol/L, or PMA 1 μmol/L for 24 to 48 hours. Before assay, cells were rinsed with PBS and incubated in serum-free DMEM (1 mL/well), and enzyme activity was measured with the tripeptide [\(^{3}\)H]BPAP as substrate, as previously described. 22

Half of all samples were incubated with captopril 1 μmol/L for 1 hour before the start of the reaction (addition of [\(^{3}\)H]BPAP 0.1 μCi/mL), the ACE inhibitor remaining present throughout the reaction. Enzyme activity (U/mg protein) was calculated by the formula V = V_{max} / K_{m} = ln([S]/[I])/t, where [S] and [I] are the initial and final substrate concentrations, respectively, and t is time of incubation. One unit of ACE activity is the V_{max}/K_{m} value equivalent to 1% substrate metabolism in 1 minute under first-order conditions.

Collagen Synthesis and DNA Synthesis Assays

Collagen synthesis was assessed by measurement of the cellular uptake of [\(^{3}\)H]proline. Fibroblasts were seeded into 24-well plates at 3×10^4 cells per well (1 mL/well) in growth medium and incubated overnight. Cells were then incubated in DMEM containing 0.4% FCS for 48 hours before addition of Ang II 1 μmol/L to 10 μmol/L. For experiments using selective antagonists, the cells were incubated with either losartan 10 μmol/L or PD123319 1 μmol/L for 1 hour before addition of Ang II and remained present throughout the experiment. [\(^{3}\)H]Proline was added to each well at a final concentration of 1 μCi/mL, and cells were incubated for 48 hours. After incubation, the supernatant in each well was replaced with ice-cold 10% trichloroacetic acid for 20 minutes at 4°C. The acid-precipitable material was rinsed with deionized water, then solubilized in 0.25 mL of 0.3 mol/L NaOH–0.1% SDS at 37°C for 2 hours. The cell lysate was added to 3 mL of liquid scintillant, and the incorporated radioactivity (cpm) was measured. The same protocol was used for assessing DNA synthesis, except that [methyl-\(^{3}\)H]thymidine incorporation between 20 and 24 hours was measured, in response to Ang II 10 μmol/L to 10 μmol/L and PDGF-AB 15 ng/mL.

Measurement of Intracellular Calcium Concentration

Cardiac fibroblasts on glass coverslips were loaded with the calcium indicator fura-2 AM 1 μmol/L for 30 minutes at room temperature in Krebs-Henseleit buffer containing (in mmol/L) NaCl 120, KCl 4.8, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 25, glucose 25, CaCl\(_2\) 1.3, and HEPES 25 (pH 7.4), and 0.1% BSA. Coverslips were then placed in a temperature-controlled holder (34°C) and mounted onto the stage of an epifluorescence microscope (Zeiss Axiovert 35). Cells were challenged with Ang II 1 μmol/L and monitored visually over time as previously described. 23 When used, losartan 10 μmol/L and PD123319 1 μmol/L were present at all stages of the experiment. The intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) was determined from fluorescence values by the formula described by Grynkiewicz et al. 23

Statistics

Data are presented as mean±SEM. Multiple groups of data underwent a 1-way ANOVA followed by Bonferroni's t test. Student's t test
was used to compare paired observations, and a value of \( P < 0.05 \) was considered significant.

### Results

#### Expression of AT₁ Receptor and ACE mRNA and Radioligand Binding

Specific PCR products corresponding to human AT₁ receptor and ACE cDNA sequences were generated from both atrial and ventricular fibroblasts (Figure 1). Expression of the AT₁ receptor gene displayed alternative splicing of the 5′ untranslated exons, with 2 transcripts being detected, one encoding exons 1 and 5 (466-bp PCR product) and the other exons 1, 2, and 3 (551-bp PCR product).

Atrial and ventricular fibroblasts exhibited specific \([^{125}I]\)(S¹I₈)Ang II binding with characteristics of the AT₁ receptor subtype. Binding was selectively inhibited in the presence of losartan, whereas PD123319 had no apparent effect (Figure 2A). Binding was competitively inhibited by unlabeled \((S¹I₈)\)Ang II and Ang II, as well as by losartan, and nonspecific binding represented <10% of total binding (Figure 2B). Cardiac fibroblasts also exhibited specific binding of the radiolabeled ACE inhibitor \([^{125}I]\)-351A, albeit at a lower level than that displayed by endothelial cells (Figure 3), and this was abolished in the presence of either EDTA (data not shown) or 1 \( \mu \)mol/L lisinopril.

#### ACE Activity of Cardiac Fibroblasts

Ventricular and atrial fibroblasts exhibited functional ACE activity, as determined by cleavage of the radiolabeled substrate \([^{3}H]\)BPAP and its blockade in the presence of captopril 1 \( \mu \)mol/L. (Figure 4). There was no significant difference between the ACE activities (U/mg 21 ± 1/min) of ventricular (0.031 ± 0.010, \( n = 13 \)) and atrial (0.034 ± 0.012, \( n = 6 \)) fibroblasts. However, human aortic and coronary endothelial cells exhibited 6-fold greater ACE activity per milligram total protein (0.213 ± 0.034, \( P < 0.001; \ n = 4 \)). Quiescent fibroblasts exhibited a significant increase in ACE activity after exposure for 48 hours to 100 nmol/L dexamethasone (152.6 ± 5.3%, \( P < 0.01; \ n = 6 \)), 50 ng/mL bFGF (156.8 ± 14.0%, \( P < 0.01; \ n = 4 \)), or 1 \( \mu \)mol/L PMA (170.0 ± 20.0%, \( P < 0.05; \ n = 3 \)). Increased ACE activity was also detected at 24 hours, albeit at a lower level (data not shown), but no change was detected after treatment with 15 ng/mL PDGF-AB.

#### Effect of Ang II on Collagen and DNA Synthesis and Intracellular Calcium Concentration

Cardiac fibroblasts responded to Ang II 1 nmol/L to 10 \( \mu \)mol/L with a concentration-dependent increase in \([^{3}H]\)proline incorporation (Figure 5A). A maximum 20±4% increase in \([^{3}H]\)proline incorporation was achieved by 10 \( \mu \)mol/L Ang II (\( P < 0.05 \), data from 7 experiments pooled together), and this was abolished by preincubation with losartan 10 \( \mu \)mol/L but not PD123319 1 \( \mu \)mol/L (Figure 5B). The response to Ang II was essentially the same in the 33 different cell cultures isolated from explanted human hearts, irrespective of the varying levels of scarring. Ang II 10

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**Figure 5.** Effect of Ang II on collagen synthesis in human cardiac fibroblasts. Top, Ang II 1 nmol/L to 10 \( \mu \)mol/L stimulated an increase in \([^{3}H]\)proline incorporation in ventricular fibroblasts. Values represent mean ± SEM \([^{3}H]\)proline incorporation (0 to 48 hours) in quadruplicate treatments from a representative of 7 experiments with similar results; *\( P < 0.05 \) vs control. Similar results were obtained in 5 experiments on atrial fibroblasts. Bottom, Ang II-induced collagen synthesis was selectively inhibited by 1 hour of preincubation with losartan 10 \( \mu \)mol/L but not PD123319 1 \( \mu \)mol/L. Bars represent mean ± SEM \([^{3}H]\)proline incorporation (0 to 48 hours) in quadruplicate treatments from a representative of 4 experiments with similar results; **\( P < 0.001 \) vs control and vs all others unmarked. Similar results were obtained in experiments on atrial fibroblasts.
nmol/L to 10 μmol/L exhibited no effect on [methyl-^3\text{H}]thymidine incorporation at 24 hours, whereas PDGF-AB caused a 7-fold increase (data not shown). Neither was any stimulation of DNA synthesis observed between 18 and 42 hours, which was measured to account for possible delayed mitogenesis.

Human cardiac fibroblasts responded to Ang II with a rapid increase in [Ca^{2+}], (basal, 56±1 nmol/L to Ang II, 355±24 nmol/L; \( P<0.001 \); \( n=66 \) cells) (Figure 6). The peak response occurred \( \approx 16 \) seconds after stimulation, returning to baseline levels thereafter (Figure 6B). The Ang II–induced calcium increases were completely inhibited by pretreatment with...
Ang II induced a net stimulation of collagen synthesis, this being in agreement with the results of previous studies on isolated human \( \text{AT}_2 \) and rat cardiac fibroblasts.\(^{16,18} \) The Ang II–induced collagen synthesis and intracellular calcium transients in human cardiac fibroblasts were both shown to occur via the \( \text{AT}_1 \) receptor, the expression of which was confirmed by RT-PCR analysis and radioligand binding. The \( \text{AT}_2 \) receptor antagonist PD123319 had no apparent effect on either \( ^{125}\text{I}-(\text{S},\text{P})\text{Ang II} \) binding, collagen synthesis, or intracellular calcium transients, which suggests the absence of \( \text{AT}_2 \) receptors. These results contrast with those obtained by ourselves and others in tissue sections and myocardial membrane preparations, indicating the presence of both receptor subtypes in human myocardium and the predominance of the \( \text{AT}_2 \) subtype in regions of fibrosis.\(^{11,22,35,36} \) This apparent discrepancy between in vivo and in vitro findings probably reflects changes in the proportion of Ang II receptors, particularly downregulation of \( \text{AT}_1 \) receptors, after isolation of cells and in response to culture conditions.\(^{16,17,37,38} \) Thus far, \( \text{AT}_1 \) receptor expression has not been demonstrated in human primary cell cultures, and the relative instability of \( \text{AT}_2 \) receptor expression in isolated cells represents a significant limitation of in vitro investigations. Knowledge about the function of the \( \text{AT}_1 \) receptor is limited to studies using animal models and isolated rodent cells, in which the receptor mediates effects such as inhibition of collagen synthesis\(^{39} \) and DNA synthesis,\(^{40} \) thereby opposing responses mediated by the \( \text{AT}_2 \) subtype. Recently, however, blockade of the \( \text{AT}_2 \) receptor has been shown to inhibit DNA synthesis in interstitial cells after MI in the rat.\(^{31} \) Therefore, although the present findings implicate the \( \text{AT}_1 \) receptor in human cardiac fibrosis, the potential involvement of the \( \text{AT}_2 \) receptor in vivo cannot be excluded and requires further investigation.

In conclusion, the present results indicate that cardiac fibroblasts represent a site for the local generation and action of Ang II in the human heart and therefore may contribute to the development of cardiac fibrosis. This highlights the potential of ACE inhibition and \( \text{AT}_1 \) receptor antagonism in strategies for prevention as well as possibly regression of fibrosis.

**Acknowledgments**

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**References**


**Discussion**

We have demonstrated expression of both ACE and the \( \text{AT}_1 \) receptor subtype in cultured human cardiac fibroblasts and have shown that the \( \text{AT}_1 \) receptor subtype mediates Ang II–induced increases in collagen synthesis and intracellular calcium in these cells. Together, these findings implicate ACE and Ang II in the development of myocardial fibrosis, independent of changes in hemodynamics.

The demonstration of ACE expression and activity in human cardiac fibroblasts supports the findings of several recent studies. In the rat heart, ACE expression has been detected at sites of fibrosis after Ang II infusion\(^{14} \) and after MI,\(^{16} \) as well as in myofibroblasts isolated from cardiac scar tissue.\(^{23} \) Expression of ACE has also been detected in human cardiac tissues\(^{33,36} \) and a correlation found between the levels of ACE and fibrillar collagen type I mRNA.\(^{30} \) More specifically, increased ACE activity has been found in aneurysmal left ventricular tissue\(^{31} \) and ACE immunoreactivity localized to myocytes as well as other cell types, including fibroblasts, adjacent to scar tissue\(^{11} \) in patients after MI. In the present study, no differences were detected between the level of ACE activity in fibroblasts cultured from failing (dilated cardiomyopathy or ischemic heart disease) and normal donor hearts.

ACE and potential autocrine control of cardiac fibroblasts. The positive effect of PMA may be attributed to either short-term activation or long-term downregulation of protein kinase C (PKC) in cardiac fibroblasts. Together, these results provide evidence for the dynamic regulation of ACE activity and potential autocrine control of cardiac fibroblasts.

Losartan 10 µmol/L, whereas no inhibition was observed with PD123319 1 µmol/L (Figure 6A and 6C).

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