Expression of Functional Angiotensin-Converting Enzyme and AT₁ 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 AT₁ 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 (\(V_{\text{max}} \cdot K_{\text{m}}^{-1} \cdot mg^{-1} \cdot 0.031 \pm 0.010; n=13\)) and atrial (\(0.034 \pm 0.012; n=6\)) 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 \([3 \text{H}]\)proline incorporation in cardiac fibroblasts (20.0±4.0% increase above control by 10 \(\mu\)mol/L; \(P<0.05, n=7\)), which was abolished by losartan 10 \(\mu\)mol/L but not PD123319 1 \(\mu\)mol/L. Ang II also stimulated a rise in intracellular calcium (basal, 56±1 nmol/L; Ang II, 355±24 nmol/L) via the AT₁ receptor, as shown by complete inhibition with losartan.

Conclusions—We have demonstrated expression and activity of ACE and AT₁ receptor in cultured human cardiac fibroblasts. In addition, cardiac fibroblasts respond to Ang II with AT₁ receptor–mediated collagen synthesis. The presence of local ACE and AT₁ 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 a local ACE activity, 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, 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 and 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, AT₁ and AT₂ being the major subtypes. Both receptors occur in rat and human myocardium, whereas the AT₁ 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 human 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, phenol 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-EDTA, SDS, BSA, trichloroacetic acid, and collagenase II were purchased from NEN Life Science Products. A 125 I-labeled tyrosyl L-proline 52 Ci/mmol were from ICN Biomedicals, and [3,4-3 H] L-proline 52 Ci/mmol were from ICN Biomedicals, and [3H]benzoyl-phenylalanine-alanine-proline ([3H]BPAP, 400 μCi/mL) was synthesized and radiolabeled in our laboratory. Liquid and toluene 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 120 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 antigen, α-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 5 μg was reverse transcribed into cDNA with random primers (In Vitrogen). Oligonucleotide primers were synthesized according to the nucleotide sequences and genomic
organization of human AT₁ receptor and ACE genes. The AT₁ receptor primer sequences were 5'-GATGGGACCGCT-GGACCCG-3' (sense) and 5'-TGCAAAAGGGCAACCGTGATC-3' (antisense), the PCR amplimer spanning exons 1, 2, 3, 4, and 9 over a region of 755 bp. For human ACE, the sense primer was as previously described.11

Collagen Synthesis and DNA Synthesis Assays
Collagen synthesis was assessed by measurement of the cellular uptake of [3H]proline. Fibroblasts were seeded into 24-well plates at 3×10⁴ 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. [3H]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 mol/L NaOH–0.1% SDS for 2 hours. The cell material was rinsed with deionized water, then solubilized in 0.25 mol/L NaOH–0.1% SDS 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-3H]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, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, glucose 25, CaCl₂ 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.25 When used, losartan 10 μmol/L and PD123319 1 μmol/L were present at all stages of the experiment. The intracellular calcium concentration ([Ca²⁺]) was determined from fluorescence values by the formula described by Grynkiewicz et al.25

Statistics
Data are presented as mean±SEM. Multiple groups of data underwent a 1-way ANOVA followed by Bonferroni t test. Student’s t test of DMEM containing either 0.2 nmol/L [3H]-(S¹,I⁸)Ang II or 0.3 nmol/L [3H]-351A, with 0.1% BSA for 90 minutes at 37°C. After a washing with ice-cold DMEM, cells were solubilized in 0.2 mol/L NaOH and 0.1% SDS for 30 minutes, and ligand present in the lysate was measured with a gamma counter. Nonspecific [3H]-(S¹,I⁸)Ang II and [3H]-351A binding was defined as that obtained in the presence of 1 μmol/L unlabeled (S¹,I⁸)Ang II and either 1 μmol/L EDTA or 1 μmol/L lisinopril, respectively. [3H]-(S¹,I⁸)Ang II binding was characterized by inhibition studies with increasing concentrations (1 pmol/L to 1 μmol/L) of nonselective [Ang II, (S¹,I⁸)Ang II], AT₁-selective (losartan), or AT₁-selective (PD123319) competitors.

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. After assay, cells were rinsed with PBS and incubated in serum-free DMEM (1 mL/well), and enzyme activity was measured with the tripeptide [3H]BPAP as substrate, as previously described.27 Half of all samples were incubated with captopril 1 μmol/L for 1 hour before the start of the reaction (addition of [3H]BPAP 0.1 μCi/mL), the ACE inhibitor remaining present throughout the reaction. Enzyme activity (U/mg protein) was calculated by the formula V = ln([S₀]/[S])/t, where [S₀] and [S] are the initial and final substrate concentrations, respectively, and t is time of incubation. One unit of ACE activity is the V₅₀/Kₚ value equivalent to 1% substrate metabolism in 1 minute under first-order conditions.
was used to compare paired observations, and a value of $P<0.05$ was considered significant.

**Results**

**Expression of AT$_1$ Receptor and ACE mRNA and Radioligand Binding**

Specific PCR products corresponding to human AT$_1$ receptor and ACE cDNA sequences were generated from both atrial and ventricular fibroblasts (Figure 1). Expression of the AT$_1$ 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 [125I]-(S1,I8)Ang II binding with characteristics of the AT$_1$ 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 (S1,I8)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 [125I]-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 μmol/L lisinopril.

**ACE Activity of Cardiac Fibroblasts**

Ventricular and atrial fibroblasts exhibited functional ACE activity, as determined by cleavage of the radiolabeled substrate [3H]BPAP and its blockade in the presence of captopril 1 μmol/L (Figure 4). There was no significant difference between the ACE activities (U mg$^{-1}$ min$^{-1}$ ± SEM) 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 μmol/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 μmol/L with a concentration-dependent increase in [3H]proline incorporation (Figure 5A). A maximum 20±4% increase in [3H]proline incorporation was achieved by 10 μmol/L Ang II ($P<0.05$, data from 7 experiments pooled together), and this was abolished by preincubation with losartan 10 μmol/L but not PD123319 1 μmol/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
nmol/L to 10 μmol/L exhibited no effect on [methyl-3H]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 [Ca2+]i, (basal, 56±1 nmol/L to Ang II, 355±24 nmol/L; P<0.001; n=66 cells) (Figure 6). The peak response occurred ≈16 seconds after stimulation, returning to baseline levels thereafter (Figure 6B). The Ang II–induced calcium increases were completely inhibited by pretreatment with

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**Figure 6.** Effect of Ang II on [Ca2+]i in human cardiac fibroblasts. Top, Fluorescent fura 2 imaging of Ang II–triggered intracellular calcium transients in ventricular fibroblasts over time. Images shown are 4 snapshots taken at 0, 16, 32, and 72 seconds after addition of Ang II 1 μmol/L alone (A through D) and in presence of losartan 10 μmol/L (E through H) or PD123319 1 μmol/L (I through L). On pseudocolor scale, dark blue (low pixel intensity) represents low [Ca2+]i and red represents high [Ca2+]i. Bottom left, Time course of Ang II stimulation of [Ca2+]i in human cardiac fibroblasts. Points represent mean±SEM [Ca2+]i (nmol/L) in ventricular (n=66) and atrial (n=55) fibroblasts at each time point (seconds). Bottom right, Losartan but not PD123319 abolishes Ang II–induced calcium transients in cardiac fibroblasts. Bars represent mean±SEM of maximal response to Ang II (n=number of cells from 4 separate cultures). Bars shown are basal [Ca2+]i (n=130) and Ang II (1 μmol/L)-stimulated [Ca2+]i in absence (n=66) and presence (n=88) of losartan 10 μmol/L and PD123319 1 μmol/L (n=58). ***P<0.001 vs control and vs Ang II+losartan.
Ang II induced a net stimulation of collagen synthesis, this being in agreement with the results of previous studies on isolated human\textsuperscript{11} and rat cardiac fibroblasts.\textsuperscript{16,18} The Ang II–induced collagen synthesis and intracellular calcium transients in human cardiac fibroblasts were both shown to occur via the AT\textsubscript{1} receptor, the expression of which was confirmed by RT-PCR analysis and radioligand binding. The AT\textsubscript{2} receptor antagonist PD123319 had no apparent effect on either \textsuperscript{125}I—(S,\textsuperscript{3}P)Ang II binding, collagen synthesis, or intracellular calcium transients, which suggests the absence of AT\textsubscript{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 AT\textsubscript{2} subtype in regions of fibrosis.\textsuperscript{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 AT\textsubscript{2} receptors, after isolation of cells and in response to culture conditions.\textsuperscript{16,17,37,38} Thus far, AT\textsubscript{2} receptor expression has not been demonstrated in human primary cell cultures, and the relative instability of AT\textsubscript{2} receptor expression in isolated cells represents a significant limitation of in vitro investigations. Knowledge about the function of the AT\textsubscript{2} receptor is limited to studies using animal models and isolated rodent cells, in which the receptor mediates effects such as inhibition of collagen synthesis\textsuperscript{39} and DNA synthesis,\textsuperscript{40} thereby opposing responses mediated by the AT\textsubscript{1} subtype. Recently, however, blockade of the AT\textsubscript{2} receptor has been shown to inhibit DNA synthesis in interstitial cells after MI in the rat.\textsuperscript{31} Therefore, although the present findings implicate the AT\textsubscript{1} receptor in human cardiac fibrosis, the potential involvement of the AT\textsubscript{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 AT\textsubscript{1} receptor antagonism in strategies for prevention as well as possibly regression of fibrosis.

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References

34. Lindner V, Reidi MA. Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. Proc Nail Acad Sci U S A. 1991;88:3739–3743.
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