Angiotensin II Has Multiple Profibrotic Effects in Human Cardiac Fibroblasts

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Background—Angiotensin II (Ang II) is implicated in cardiac remodeling and is increasingly recognized for its profibrotic activity.

Methods and Results—Because little is known about the direct cellular effects of Ang II on human cardiac fibroblasts, we isolated fibroblasts from ventricles of explanted human hearts and added Ang II (100 nmol/L) to determine its role in growth, extracellular matrix accumulation, and adhesion. To assess which Ang II receptor is involved, Ang II was added in the presence of irbesartan (10 μmol/L), a specific AT1 receptor antagonist; PD 123319 (10 μmol/L), a specific AT2 receptor antagonist, or divalinil (100 nmol/L), an AT4 receptor inhibitor. In human ventricles (n=13), message levels of atrial natriuretic peptide and AT1 receptor were inversely correlated, which suggests a decrease in AT1 receptor expression with progressive heart failure. Northern analysis and fluorescence-activated cell sorting demonstrated AT1 receptor in cultured human cardiac fibroblasts. Ang II increased mitogen-activated protein kinase activity and in DNA synthesis (5-fold, \( P<0.01 \)) stimulated a 2-fold increase in transforming growth factor-β1 (\( P<0.05 \)) mRNA levels at 2 hours and a 2-fold increase in laminin (\( P<0.05 \)) and fibronectin (\( P<0.05 \)) mRNA levels at 24 hours. Ang II also enhanced plasminogen activator inhibitor-1 expression, which inhibits metalloproteinases that degrade the extracellular matrix. All of these effects were inhibited by irbesartan but not PD 123319 or divalinil. In addition, Ang II increased cardiac fibroblast attachment to collagens I and III, which was associated with an increase in focal adhesion kinase activity.

Conclusions—Activation of the AT1 receptor in human heart promotes fibrosis. Ang II plays a novel role in stimulation of plasminogen activator inhibitor-1 expression and adhesion of cardiac fibroblasts to collagen. (Circulation. 2000;101:1130-1137.)

Key Words: angiotensin ■ remodeling ■ receptors ■ cell adhesion molecules

Angiotensin II (Ang II) is a critical mediator of cellular changes associated with left ventricular hypertrophy, postmyocardial infarction (post-MI) remodeling, and heart failure. Ang II, both directly and indirectly by stimulating norepinephrine from cardiac nerve endings and endothelin from endothelial cells, promotes rat cardiomyocyte hypertrophy. Abundant Ang II AT1 receptors present on rat cardiac fibroblasts mediate fibroblast proliferation, extracellular matrix (ECM) production, and the secretion of adhesion molecules such as osteopontin. Cardiac fibrosis develops during long-term Ang II infusion in the rat, and in the post-MI rat model, angiotensin-converting enzyme (ACE) inhibitors and AT1 receptor blockade equally attenuated cardiac fibrosis. Progressive cardiac fibrosis impairs cardiomyocyte performance, metabolism, and oxygen delivery and thus contributes to ventricular dysfunction.

Clinical trials support a key role for Ang II in cardiac remodeling. ACE inhibitors prevent globular heart formation, progression of heart failure, and death compared with placebo after MI. Recently, the AT1 receptor blocker losartan was shown to improve mortality rates in elderly patients with heart failure compared with the ACE inhibitor captopril. Despite these observations, little is known about the direct tissue effects of Ang II in human heart.

On the basis of these observations, we hypothesize that Ang II enhances profibrotic processes in human cardiac fibroblasts. In the present investigation we found that Ang II increases human cardiac fibroblast growth, protooncogene expression, mitogen-activated protein kinase (MAPK) activity, and expression of mRNAs encoding for transforming growth factor (TGF)-β, fibronectin, and laminin. Ang II enhanced cardiac fibroblast adhesion to collagen, which was accompanied by an increase in focal adhesion kinase (FAK) activity. Ang II also increased expression of plasminogen activator inhibitor-1 (PAI-1), which could contribute to de-
increased ECM degradation and hence accumulation. Ang II stimulated these effects in rat cardiac fibroblasts; however, unlike rat cardiac fibroblasts, Ang II did not stimulate osteopontin production, did not increase \( \alpha_1, \beta_1, \beta_3 \), or \( \beta_5 \) integrin expression, and did not downregulate AT\(_1\) receptor message in human cardiac fibroblasts. We conclude that Ang II AT\(_1\) receptors mediate multiple profibrotic actions in human cardiac fibroblasts and that AT\(_1\) receptor blockers will be useful to attenuate the development of cardiac fibrosis in humans.

Methods

Cell Harvest and Culture
Cardiac fibroblasts were isolated from right (n=6) and left (n=7) ventricles of explanted hearts from 7 patients (15 to 65 years old). Five had ischemic cardiomyopathy, 1 had dilated cardiomyopathy (idiopathic), and 1 had primary pulmonary hypertension. Three patients were placed on a left ventricular assist device before transplantation. Ventricular samples were minced and incubated with 0.1% trypsin and collagenase (type IV, 300 U/mL, Sigma Chemical Co) in a shaking waterbath at 37°C. Isolated cells were plated after each 10-minute digestion period. After 5 digestion periods, all the isolated cells were resuspended and cultured in DMEM–Ham’s F-12 medium (DMEM/F12) containing 20% FBS.

Isolation and Analysis of RNA
Total RNA was isolated with the use of Trizol reagent (Life Technologies). Northern analysis was performed with CHOB, a constitutively expressed gene, to correct for differences in loading.\(^3,13\)

Treatment of Fibroblasts
Cultured cardiac fibroblasts (70% confluence) were placed in serum-free medium containing insulin (5 \( \mu \)g/mL), transferrin (5 \( \mu \)g/mL), and selenium (5 ng/mL) (ITS; Sigma Chemical Co) for 24 hours before treatment. Ang II (100 nmol/L, Peninsula Laboratories) or TGF-\( \beta_1 \) (10 ng/mL, R&D system) was added in ITS for 0.5 to 48 hours. Ang II was also added with irbesartan (10 \( \mu \)mol/L), a specific AT\(_1\) receptor antagonist, PD123319 (10 \( \mu \)mol/L), a specific AT\(_2\) receptor antagonist, or divalinal (100 nmol/L), an AT\(_4\) receptor blocker.

Measurement of DNA Synthesis
Incorporation of the thymidine analogue BrdU was measured as described.\(^8\) After serum starvation for 24 hours in DMEM/F12 with ITS, cells were stimulated with Ang II or 10% FBS for the next 20 hours. When used, the MAPK pathway inhibitor PD98059 (New England Biolabs, Inc) was added 30 minutes before growth factor stimulation.

Immunohistochemistry
Cells were grown on culture chamber slides and fixed with 4% omnitix (Zymed Laboratories, Inc). Immunohistochemistry was performed with the use of a streptavidin-peroxidase system (AEC Kit, Zymed Laboratories, Inc).\(^14\) Primary antibodies included anti-AT\(_1\) receptor (Santa Cruz Biotecchnology, Inc) (dilution 1:100), anti-fibroblast (DAKO) (dilution 1:100), anti–von Willebrand factor

Figure 2. AT\(_1\) receptor mRNA is present in human cardiac fibroblasts and is not downregulated by Ang II. Cells were treated with Ang II (100 nmol/L) for the times indicated; AT\(_1\) receptor mRNA levels were determined by Northern blotting of 20 \( \mu \)g of total RNA. The autoradiogram depicted is representative of 5 separate experiments.

Figure 3. Human cardiac fibroblasts express AT\(_1\) receptors as measured by a shift in fluorescence activity. These receptors are not downregulated by Ang II. A, Negative control, without antibody; B, untreated human cardiac fibroblasts; C, human cardiac fibroblasts treated with Ang II (100 nmol/L) for 48 hours. The scan is representative of 3 separate experiments.
(DAKO) (dilution 1:50), antidesmin (Sigma) (dilution 1:400), and anti-α-smooth muscle actin (Sigma) (dilution 1:200).

Fluorescence-Activated Cell Sorting
Human cardiac fibroblasts were grown to confluence, kept for 24 hours in serum-free medium, incubated with or without Ang II (100 nmol/L) for 2 days, then incubated with AT1 receptor antibody (1:100). Fluorescence was measured on a FACSCAN flow cytometer (Becton Dickenson).

Western Blotting
Western blotting was performed as previously described with the use of the anti-human AT1 receptor antibody (1:1000).

Cell Attachment
Adhesion assays were performed as described by Liaw et al, with the use of extracellular matrix substrates human collagen I and III, fibronectin, vitronectin, and laminin at 10 μg/mL. Nonspecific binding was blocked with 1% BSA at 37°C for 1 hour.

MAPK Assay
MAPK activity was measured by immunocomplex assay.

FAK Assay
After treatment with Ang II (100 nmol/L), cells were lysed in buffer containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerol-phosphate, 1 mmol/L Na3VO4, 1 μg/mL leupeptin, 1 mmol/L PMSF, and immunoprecipitated with FAK antibody (dilution 1:100, Santa Cruz Biotechnology, Inc) overnight at 4°C. Protein G sepharose was then added to collect the immunoprecipitated complex. Pellets were washed 3 times with lysis buffer, resuspended with cell lystate buffer (25 μL) and 3× SDS sample buffer (75 μL), and boiled for 5 minutes. After SDS-PAGE, proteins were immunoblotted.

Statistical Analysis
Values are expressed as mean±SEM. Group means were compared by use of the 2-tailed Student’s t-test. Linear regression analyses were performed with the use of ANOVA.

Results

Ventricular AT1 Receptor Expression
All ventricles expressed mRNA coding for atrial natriuretic peptide (ANP), whereas 3 of 13 ventricles contained no detectable AT1 receptor mRNA. The ventricular ANP/CHOB mRNA ratio inversely correlated with the AT1 receptor/CHOB mRNA ratio (Figure 1). ANP or AT1 receptor mRNA levels were not different in ventricles in patients receiving versus those not receiving a left ventricular assist device.

Characterization of Nonmyocyte Cultures
Immunohistochemical staining with an antibody raised against a fibroblast-specific antigen, human prolly 4–hydroxylyase, suggested that 97±2% of the cells isolated were fibroblasts, although this antibody also stains positively for endothelial and vascular smooth muscle cells. However, only 3% of the cells stained positively for desmin and von Willebrand factor, which suggests little contamination with smooth muscle or endothelial cells, respectively. Only 5% of the cells stained positively for α-smooth muscle actin, which indicates that most of the cells were fibroblasts and not myofibroblasts in the presence 20% FBS. The addition of Ang II (100 nmol/L) for up to 72 hours did not result in increased staining for either desmin or α-smooth muscle actin (data not shown).

Northern analysis (Figure 2) and FACScan (Figure 3) demonstrated the presence of AT1 receptor mRNA and protein in human cardiac fibroblasts, which was not downregulated by Ang II. However, the morphology of the cells differed between the second and third passages; second-passaged cells were elongated, and most (75±9% in 5 fields) immunostained positively for the AT1 receptor, whereas third-passaged cells were rounder and only 12±2% (P<0.01) of the cells stained positively (Figures 4A and 4B). Nonimmune IgG demonstrated no staining (not shown). Western blotting revealed 2 major protein bands at 46 and 39 kDa, similar to that reported for AT1 receptors in human placenta.

There was a strong positive signal in second-passaged cells, which progressively diminished in third- and fourth-passaged cells (Figure 4C). This difference in protein resulted from a posttranscriptional defect in AT1 receptor production, since there was no detectable difference in AT1 receptor mRNA levels between these passages. Because of the substantial loss of AT1 receptor with cell passage, we used only second-passaged cells in this study. We also found that changing the media alone in the absence of the addition of a growth factor could induce c-fos; therefore Ang II or vehicle was added to cultures 24 hours after a media change.

Ang II Enhances Cardiac Fibroblast Growth
Ang II (100 nmol/L) induced c-fos and early growth response gene-1 (Egr-1) (Figures 5A and 5B and Table 1) and increased BrdU incorporation (Figure 6). Irbesartan completely blocked these growth responses, which were not affected by PD 123319 or divalinil (not shown). Because inhibition of the AT1 receptor could activate the AT1 receptor, which is reported to mediate antiproliferative effects, we added Ang II with irbesartan and PD 123319 together. Inhibition of the AT1 receptor did not attenuate the effect of the AT1 receptor blocker, which suggests that the effect of irbesartan to inhibit growth responses was mediated directly through the AT1 receptor. Another AT1 receptor blocker, losartan, also blocked Ang II-induced proto-oncogene expression (not shown).

Both Ang II (100 nmol/L) and serum enhanced MAPK activity, which was completely inhibited by irbesartan (Figure 7). The MAPK inhibitor PD98059 also completely inhibited Ang II-induced DNA synthesis, which indicates the importance of this pathway in human cardiac fibroblast growth (Figure 6).

Figure 4. A, Immunostaining with anti-AT1 receptor antibody shows strong staining for AT1 receptor in second-passaged human cardiac fibroblast cultures (magnification ×100); 75±9% of the cells (counted in 5 fields) stained positively. B, Immunostaining of third-passage human cardiac fibroblasts with AT1 receptor antibody reveals only faint, diffuse staining; 12±2% of the cells stained positively (P<0.01, compared with A). Note the cells had a rounder shape compared with the spindly shape in A (magnification ×100). C, AT1 receptor protein in human cardiac fibroblasts at different passage by Western blot analysis. The autoradiogram is representative of 3 separate experiments.
Ang II Increases TGF-β1, PAI-1, and Extracellular Matrix mRNAs

Ang II (100 nmol/L) increased TGF-β1 message levels, which peaked at 2 hours (2-fold, P<0.05, Figure 8A). The Ang II effect was inhibited by irbesartan but not PD123319 (Figure 8B). TGF-β1 itself enhanced TGF-β1 message levels. This autoinduction occurred at 2 hours. (Figure 8C).

Both Ang II and TGF-β1 increased in PAI-1 mRNA levels, although the time courses were different (Figures 9A and 9B). Ang II induction of PAI-1 message peaked at 2 hours and declined by 12 hours. The PAI-1 response to TGF-β1 peaked at 2 hours and remained elevated at 48 hours.

Ang II increased both fibronectin and laminin mRNA by nearly 2-fold at 24 to 48 hours (Table 2) and was inhibited by irbesartan but not PD 123319. There was no detectable effect of Ang II on collagen I mRNA levels during this time period.

Ang II Promotes Adhesion

Human cardiac fibroblasts attached to a variety of matrices including collagens I, III, and IV, fibronectin, laminin, and vitronectin (Figure 10A). However, Ang II added at the time of attachment enhanced adhesion only to collagens I and III but not to the other ECM proteins. Adhesion itself was associated with an increase in FAK activity; Ang II enhanced FAK activity further when added in the presence of collagen I or III (Figure 10B) but not in the presence of other matrixes. In addition, blocking antibodies against human β1 integrin inhibited adhesion to collagen I and III as well as FAK activation (data not shown).

Osteopontin is a prominent adhesion molecule that is regulated by Ang II in rat cardiac fibroblasts and in rat and human vascular smooth muscle cells.15 Osteopontin mRNA was present in low levels in human compared with rat cardiac fibroblasts and did not increase in response to Ang II at 2 to 48 hours (data not shown). In addition, message and protein levels of αv, β1, β3, and β5 integrins were readily detectable in human cardiac fibroblasts but did not change in response to Ang II as measured by Northern analysis and FACS (data not shown).

**Discussion**

With the use of Northern analysis, immunohistochemistry, Western analysis, and FACS, the present study demonstrates the presence of AT₁ receptor message and protein in human cardiac fibroblasts. Although the antibody for the human AT₁ receptor is not highly specific, specific AT₁ receptor blockers inhibit several functions that contribute to the fibrotic process confirming the presence of active receptor. These include (1) growth: proto-oncogene induction, increased MAPK activity, and DNA synthesis; (2) fibrosis: stimulation of TGF-β₁, matrix protein expression, and PAI-1; and (3) adhesion of cardiac fibroblasts to collagen I and III, associated with increased FAK activity and mediated by β₁ integrin. These observations support an important role for Ang II in cardiac fibrosis in humans.

AT₁ receptors have been recognized in human heart20–22; however, the cellular localization of these receptors is unclear. With the use of in situ reverse transcription–polymerase chain reaction, one study suggested that human cardiomyocytes express AT₁ receptors.22 Ang II has been shown to bind to human cardiac fibroblasts, but binding was not consistently displaced by AT₁ or AT₂ receptor blockers,23 which leads to confusion as to whether human cardiac fibroblasts express AT₁ receptors. Although we identified AT₁ receptor protein on human cardiac fibroblasts, levels substantially decreased with cell passage, which clarifies previous studies demon-

**TABLE 1. AT₁ Receptor Mediates c-fos and Egr-1 Expression in Human Cardiac Fibroblasts**

<table>
<thead>
<tr>
<th></th>
<th>c-fos</th>
<th>Egr-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=5)</td>
<td>(n=4)</td>
</tr>
<tr>
<td>Control</td>
<td>0.10±0.03</td>
<td>0.48±0.07</td>
</tr>
<tr>
<td>Ang II</td>
<td>0.32±0.09*</td>
<td>0.86±0.13*</td>
</tr>
<tr>
<td>Ang II+IRB</td>
<td>0.12±0.03</td>
<td>0.42±0.12</td>
</tr>
<tr>
<td>Ang II+PD</td>
<td>0.34±0.06*</td>
<td>0.76±0.18*</td>
</tr>
<tr>
<td>Ang II+IRB+PD</td>
<td>0.13±0.05</td>
<td>0.42±0.06</td>
</tr>
</tbody>
</table>

IRB indicates irbesartan; PD, PD 123319.

*P<0.01.
strating difficulty in detecting the presence of AT1 receptors in these cells.23 Furthermore, AT1 receptor message and protein are downregulated in failing versus nonfailing hearts.21,22 We found an inverse correlation between ventricular mRNA levels of AT1 receptor and ANP, a well-accepted marker of ventricular hypertrophy.24 Thus it is likely that cardiac fibroblasts from normal human heart contain increased AT1 receptor levels compared with the end-stage hearts we studied and thus may be even more responsive to growth, matrix production, or adhesive stimulation by Ang II.

Ang II is known to stimulate growth, MAPK activity, TGF-β1 expression, and ECM production in rat cardiac fibroblasts.3–6,25,26 These Ang II actions have not been clearly demonstrated in human cardiac fibroblasts. Our study provides strong evidence that MAPK mediates the growth effects of Ang II in the human system and suggests that TGF-β1 contributes to the Ang II-induced increase in ECM, since TGF-β increases transcription of collagen I and tissue inhibitors of metalloproteinase in human cardiac fibroblasts.27 Ang II also stimulates PAI-1 mRNA, which in addition to tissue inhibitors of metalloproteinase inhibits metalloproteinase activity, thus allowing for the accumulation of ECM.28 In rat and swine models of left ventricular hypertrophy, ventricular PAI-1 mRNA has been shown to be increased,29,30 which was maintained in animals that developed congestive heart failure compared with those that did not.30 However, the cellular source of PAI-1 was not delineated. The present study is the first demonstration that cardiac fibroblasts express PAI-1 mRNA.
Another new finding is that Ang II enhances adhesion of human cardiac fibroblasts to collagens I and III, the major collagens in the heart. Attachment of fibroblasts to collagen is important for wound healing and scar formation. Ang II-induced attachment increased FAK activity above that seen with attachment alone to collagens I and III. FAK is a tyrosine kinase associated with focal adhesions, which play a key role in cell adhesion and motility.11 Our data further suggest β1 integrin is the major receptor involved in human cardiac fibroblast binding to collagen. Thus Ang II appears to stimulate the formation of focal adhesions containing β1 integrin.

Our previous observations demonstrated that Ang II-enhanced collagen gel contraction by rat cardiac fibroblasts could be inhibited by antibodies against osteopontin or β1 or β3 integrins.6 Since Ang II potently stimulated osteopontin in the rat system, we postulated that this acid phosphoprotein was important for Ang II-mediated fibroblast attachment to collagen. In sharp contrast to rat cardiac fibroblasts, human cardiac fibroblasts contain lower levels of osteopontin message that are not regulated by Ang II. In human heart, the myocyte appears to be the major source of osteopontin.14 In addition, we found that Ang II stimulates expression of α5, β1, β3, and β5 mRNA and protein in rat cardiac fibroblasts (unpublished data), whereas it has little effect on these integrins in human cardiac fibroblasts. Thus the rat cardiac fibroblast is not consistently a model for the human cardiac fibroblast. Furthermore, Ang II downregulates AT1 receptor levels in rat cardiac fibroblasts,7 whereas Ang II had no effect to alter either AT1 mRNA or protein in human cells. Our observation appears consistent with that of Urata et al,20 who found that ACE inhibitor treatment made no difference in AT1 receptor levels in failing human heart.

### Table 2. Effect of Ang II on Extracellular Matrix mRNA Expression

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>2 h</th>
<th>6 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin</td>
<td>0.85±0.10</td>
<td>1.13±0.24</td>
<td>1.39±0.34*</td>
<td>1.44±0.32*</td>
<td>1.60±0.24*</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>0.93±0.15</td>
<td>1.16±0.13</td>
<td>1.34±0.18*</td>
<td>1.43±0.25*</td>
<td>1.73±0.39*</td>
</tr>
<tr>
<td>Collagen I</td>
<td>0.95±0.10</td>
<td>0.93±0.21</td>
<td>1.12±0.13</td>
<td>1.20±0.21</td>
<td>1.16±0.09</td>
</tr>
</tbody>
</table>

*P<0.05, n=4.
Our studies suggest that the AT1 receptor is the major functional Ang II receptor mediating the fibrotic effects of Ang II in human heart. The AT2 or the AT1 receptor blocked had no detectable effects, even with the use of the AT2 receptor blocker with AT1 receptor blockade. In addition, we were unable to identify AT1 message by Northern analysis (unpublished data). Our results thus support a potentially important role for AT1 receptor blockade in heart failure and post-MI remodeling in humans.

Acknowledgments
This work was supported by a grant from the American Heart Association. The authors would like to express their appreciation to Jane H. Teran and Lorraine Reyes for expert secretarial assistance. This article is dedicated to the memory of our colleague Dr Yung S. Do.

References
23. Neuss M, Regitz-Zagrosek V, Hildebrandt A, Fleck E. Human cardiac fibroblasts express an angiotensin receptor with unusual binding characteristics which is coupled to cellular proliferation. Biochem Biophys Res Commun. 1994;204:1334–1339.
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Circulation. 2000;101:1130-1137
doi: 10.1161/01.CIR.101.10.1130

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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