Angiotensin II Stimulates the Autocrine Production of Transforming Growth Factor-β1 in Adult Rat Cardiac Fibroblasts

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Introduction

Angiotensin II (Ang II) has been shown to have a multitude of direct and indirect effects on the cardiovascular system. In addition to its short-term roles which include vasoconstriction and chronotropic and inotropic effects on cardiac muscle, Ang II has been implicated in the long-term regulation of growth in the cardiovascular system (Robertson and Nicholls, 1993). A number of studies have suggested a role for Ang II in the development of vascular hypertrophy. In vivo studies have demonstrated that angiotensin converting enzyme (ACE) inhibitors significantly prevented neointimal lesion formation after balloon injury (Powell et al., 1989). In vitro studies have shown that Ang II promoted cellular hypertrophy in normal vascular smooth muscle cells (VSMCs) (Gibbons et al., 1992).

Please address all correspondence to: Francisco J. Villarreal, Department of Medicine (8412) University of California, San Diego, 200 West Arbor Drive, San Diego, California 92103, USA.

Key Words: Hypertrophy; Cardiac interstitial; Growth factors.

A. A. LEE, W. H. DILLMANN, A. D. McCULLOCH AND F. J. VILLARREAL. Angiotensin II Stimulates the Autocrine Production of Transforming Growth Factor-β1 in Adult Rat Cardiac Fibroblasts. Journal of Molecular and Cellular Cardiology (1995) 27, 2347–2357. Angiotensin II (Ang II) has been implicated in the development of cardiac hypertrophy and myocardial fibrosis. While recent in vivo and in vitro studies performed in cultured cardiac myocytes and fibroblasts support this role for Ang II, the mechanisms of Ang II action at the cellular level remain unclear. In the present study, we postulated that Ang II action in adult cardiac fibroblasts may stimulate the autocrine production and release of transforming growth factor-β1 (TGF-β1), a known regulator of cardiac fibroblast and myocyte function. We examined the ability of Ang II to regulate the gene expression, biological activity, and protein production of TGF-β1 in cultured adult rat cardiac fibroblasts. Treatment of fibroblast cultures with Ang II (10⁻⁸M) induced a two-fold increase in TGF-β1 mRNA levels within 4 h that was sustained through 24 h (P<0.01). TGF-β1-like activity in Ang II-treated cultures was significantly increased compared with control as measured by bioassay (P<0.001). Specificity for TGF-β1-like activity was confirmed through its neutralization with a TGF-β1 specific antibody (100 µg/ml). Total concentration of TGF-β1 (latent plus active forms) in conditioned media from Ang II-treated cardiac fibroblasts was also found to be greater than control (P<0.01). These findings suggest that the effects of Ang II in the adult myocardium may be mediated in part by autocrine/paracrine mechanisms, including the production and release of TGF-β1 by cardiac fibroblasts.

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Ang II has also been implicated as a regulator of normal and pathophysiological growth in the heart. Recent in vivo studies have shown that ACE inhibitors attenuate left ventricular hypertrophy in the abdominally banded rat model (Baker et al., 1990) and reduce collagen accumulation in spontaneously hypertensive rats (Brilla et al., 1991). Increased ACE activity has also been associated with altered diastolic properties (Hirsch et al., 1990). These studies suggest that Ang II may play an important role in the regulation of myocardial remodeling by myocytes and fibroblasts. Recent in vitro studies performed in our laboratory (Villarreal et al., 1993) and by others (Schorb et al., 1993; Sadoshima and Izumo, 1993) have characterized the presence of AT1 subtype Ang II receptors on cardiac fibroblasts. In these studies, Ang II was shown to directly stimulate cardiac fibroblast proliferation and protein production through these receptors.

One potential mechanism of action of peptide growth factors is the autocrine/paracrine release of endogenous regulatory factors (Roberts and Sporn, 1990). In VSMCs, the cellular actions of Ang II have been linked to the autocrine release of growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1) (Weber et al., 1994). The release of TGF-β1 has also been implicated in other autocrine/paracrine systems, such as autoinduction by TGF-β1 (Kim et al., 1989) or its stimulation by mechanical stretch (Komuro et al., 1991). TGF-β1 has been shown to have important regulatory effects on both myocyte and fibroblast function in the heart. In recent studies, TGF-β1 has been shown to regulate growth and contractile activity in neonatal cardiac myocytes in culture (MacLellan et al., 1993; Roberts et al., 1992). In rabbit cardiac fibroblasts, TGF-β1 has been shown to be a potential regulator of collagen mRNA levels (Eghbali et al., 1991). In a thoracic-banded rat model of cardiac hypertrophy, we have previously demonstrated that levels of TGF-β1 mRNA preceded increases in mRNA levels for extracellular matrix proteins (Villarreal and Dillmann, 1992), suggesting a possible regulatory role for TGF-β1 in remodeling processes in the myocardium.

The potential mediation of Ang II action in the myocardium via regulatory factors has not been explored. Since we have previously established the presence of Ang II receptors on cardiac fibroblasts, we postulated that Ang II may directly regulate potential paracrine/autocrine growth factors, such as TGF-β1, in these cells. To examine this hypothesis, we studied whether Ang II stimulates increases in the gene expression, biological activity, and total protein levels of TGF-β1 in adult rat cardiac fibroblast cultures.

Material and Methods

Cell culture

Adult rat cardiac fibroblasts were prepared using a modification of a previously described protocol (Eghbali et al., 1991). Briefly, three to four hearts from 7- to 8-week-old male Sprague-Dawley rats (200–250 g) were finely minced and placed together in an enzymatic digestion solution containing collagenase (100 Mandl units/ml) and pancreatin (0.6 mg/ml). Pooled cell suspensions from four to five digestions were centrifuged and resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin, streptomycin, and fungizone; Gibco). The resuspension was plated onto uncoated cell culture dishes for 30 min, which allowed for preferential attachment of fibroblasts to the bottom of the culture dish. Non-adherent and weakly attached cells were removed and the medium was changed. Cells were grown to confluence and subsequently passaged either 1:3 or 1:4 by trypsin/EDTA. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO2/95% air. Studies were conducted on cardiac fibroblasts (passages two through four) that were grown to subconfluence in serum-containing media and then growth arrested for 24 h in serum-free medium before treatment. Conditioned medium (CM) is defined as cell culture medium collected from cultured adult rat cardiac fibroblasts which were either untreated or treated with Ang II (10^-9 M) for 24 h. The TGF-β1 specific neutralizing antibody (Ab) and the non-specific control immunoglobulin (Ig) were used at 100 μg/ml in cell culture (R&D Systems, Minneapolis, MN, USA).

Immunocytochemistry

Fibroblast identification and purity of cultures were verified by immunocytochemical staining as previously described (Villarreal et al., 1993). Monoclonal antibodies directed against sarcomeric (striated muscle) actin (Sigma Chemical Co., St. Louis, MO, USA) and desmin (Amersham Corp., Arlington Heights, IL, USA) were used for positive
identification of cardiac myocytes and smooth muscle cells. A polyclonal antibody against human factor VIII (DAKO Corp., Carpinteria, CA, USA) was used for identification of endothelial cells. Adult cardiac fibroblasts were characterized by negative staining for the factors mentioned above and by positive staining using a polyclonal anti-vimentin antibody (Polysciences Inc., Warrington, PA, USA). Immunocytochemical staining of the cell culture preparations was performed using a Streptavidin alkaline phosphatase-based protocol (Alvaro-Gracia et al., 1990) with Vector Red (Vector Laboratories, Burlingame, CA, USA) as a chromogen. The effects of immunostaining under this method are enhanced by a red reaction that is also visible under fluorescent microscopy with rhodamine and fluorescein lights. Cells were initially fixed in a 1:1 mixture of cold acetone and methanol at 4°C for 15 min and allowed to dry. Blocking was performed, using 10% serum (from the animal source of the secondary antibody) in 0.1% bovine serum albumin/Tris-buffered saline. Primary antibodies were added and incubated at room temperature for 60 min. Negative controls were generated by using preimmune sera of the animal species in which the primary antibody was developed. Biotinylated IgGs as secondary antibodies were subsequently added and incubated for 60 min. After the addition and incubation of alkaline phosphatase Streptavidin for 30 min, an alkaline phosphatase substrate kit was used to develop the reaction. Any endogenous fibroblast phosphatase activity was blocked by inclusion of levamisole in the mixture of developing reactants. Ideal dilution and exposure times were determined for each of the staining protocols used.

**Northern blots**

For RNA analysis by Northern blot, 5 μg of total RNA was electrophoresed on 1% agarose gels and processed according to an established protocol (Villarreal and Dillmann, 1992). RNA was transferred to nylon membranes, which were baked in an 80°C vacuum oven for 90 min. The following cDNAs were used for hybridization to the nylon membranes: a rat cDNA for fibronectin containing 875 bp of the 3’ coding region, a rat collagen-α, (I) of 900 bp for the 3’ non-coding and coding regions, a mouse collagen-α; (III) probe of 500 bp for the coding region, and a rat cDNA probe for TGF-β1 with 985 bp of the coding region (Villarreal and Dillmann, 1992). Hybridization of mRNA to cDNAs for fibronectin and collagens I and III was carried out in 50% formamide, 5 × SSPE, 5 × Denhart’s solution, and 25 μg/ml herring sperm DNA at 42°C for a minimum of 36 h. Hybridization and washing of membrane for TGF-β1 followed the protocol of Church and Gilbert (1984) to avoid cross-hybridization of the cDNA probe to 18S and 28S ribosomal RNAs. All membranes were subsequently exposed to Kodak XAR film (Rochester, NY, USA) at -70°C using intensifying screens. To correct for loading differences, nylon filters were dehybridized and rehybridized with a 29-bp DNA oligomer to 28S ribosomal RNA. Video images of the autoradiographic signals were captured digitally to a Macintosh Centris 650 (Apple Computer, Cupertino, CA, USA) via a Vicon CCD camera with the Nikon MicroNikkor 60 mm lens. All data are expressed as the ratio of mRNA/28S signals as determined by quantitative image analysis using the NIH Image software.

**RNA extraction**

Total cellular RNA was extracted from 100-mm cell culture dishes following a modified procedure of Chomczynski and Sacchi (1987). Cell culture dishes were placed on ice and cell layers were washed twice with 1X phosphate-buffered saline (PBS). Cells were harvested by lysis in a 4 M guanidium isothiocyanate solution. A phenol-chloroform extraction was performed and the aqueous phase was transferred to a new tube after centrifugation at 10,000 g at 4°C for 20 min. Total RNA was precipitated through two consecutive ethanol precipitations separated by an additional phenol-chloroform extraction step. UV spectrophotometry at 260 nm was used to quantify the final RNA yield.

**TGF-β1 bioassay**

Previous studies have established the use of mink lung epithelial cells in a bioassay for assessment of active TGF-β1 (Danielpour et al., 1989). As described in a modified protocol (Gibbons et al., 1992), CCL-64 mink lung epithelial cells (American Type Culture Collection, Rockville, MD, USA) were cultured in Eagle's minimum essential medium supplemented with non-essential amino acids, 10% heat-inactivated PBS, and antibiotics. Cells were incubated at 37°C in 5% CO2/95% air and were harvested for passaging at confluence by trypsin/EDTA. Freshly plated subconfluent cells in 96-well plates were incubated in serum-containing medium for 4 to 5 h, washed with defined serum-free medium, and incubated with TGF-β1 or fibroblast
conditioned medium (CM) in the defined serum-free medium. To determine DNA synthesis, these cells were pulsed with [3H]thymidine (2.3 µCi/ml) at 18 h after treatment and harvested onto glass fiber filters (Whatman Paper) at 24 h. Fibroblast CM was obtained from subconfluent cardiac fibroblast cultures that were growth arrested as described above for 24 h and then treated with or without AngII (10^-9 M) in serum-free medium for an additional 24 h. A standard curve for each experiment was determined by adding increasing concentrations of TGF-β1 to the cultured mink lung epithelial cells. CM was added to the mink lung epithelial cells in dilutions (up to 1:100) to fall within the detectable range of the standard curve. The activity of TGF-β1 was determined by its growth inhibitory effects in the bioassay and the specificity of the response was confirmed by addition of the TGF-β1 specific neutralizing antibody.

Heat activation of TGF-β1

Total levels of TGF-β1 (latent plus active forms) in CM were assessed by the bioassay after conversion of all latent TGF-β1 to the active form by heat treatment (Roberts and Sporn, 1990). Briefly, protein concentration was adjusted in the CM (50 to 100 µg/ml) by adding crystalline bovine serum albumin (Sigma Chemical). CM was placed in siliconized test tubes, heated at 80°C for 8 min to activate latent TGF-β1, and added to the mink lung epithelial cells in serial dilutions for the bioassay. Specificity of TGF-β1-like activity was verified as described above.

Statistical analysis

Student’s t-tests were used to compare changes between two treatment groups. One-way ANOVA followed by Bonferroni’s test was used for comparisons between multiple groups. Data are expressed as mean ± S.E.M. except where noted. In the heat activation studies, the multiple comparison tests were used after reciprocal transformation of data for equal variances (Fry, 1993). Homogeneity of variances for the transformed data was verified by Bartlett’s test. Data from the heat activation studies were expressed as means with 95% confidence limit and S.E.M. (Sokal and Rohlf, 1995). Results were considered to be statistically significant when P ≤ 0.05. Triplicate wells were used in each experiment and each experiment was performed independently a minimum of three times.

Results

Characterization of adult cardiac fibroblast cultures

Immunocytochemical staining performed to characterize cell population purity showed that the adult rat cardiac fibroblast cell cultures were essentially free of other contaminating cell types (>99% purity). Cells in culture at passage one or higher stained positive for vimentin and negative for sarcomeric (muscle) actin, desmin, and factor VIII indicating minimal contamination with cardiac myocytes, smooth muscle cells, or endothelial cells. Figure 1 illustrates a representative example of the immunocytochemical staining observed for vimentin. Adult cardiac fibroblasts grown in serum also displayed typical rat cardiac fibroblast morphology (Bashey et al., 1992) and exhibited growth inhibition upon confluency, a feature that distinguishes them from smooth muscle cells.

Assessment of mRNA levels for TGF-β1

Messenger RNA levels for TGF-β1 were assessed on a Northern blot using a 985 bp fragment of a cDNA obtained from a rat library. Total RNA was extracted and processed from adult cardiac fibroblasts that were grown to subconfluency and then serum-deprived for 24 h before treatment. Under our culture conditions, incubation with Ang II (10^-9 M) for time periods up to 24 h resulted in increases in TGF-β1 mRNA levels that were detectable within 4 h of incubation. Figure 2 illustrates changes in steady-state levels of TGF-β1 mRNA at 0, 2, 4, and 10 h incubation time. TGF-β1 mRNA levels were increased at both the 4 and 10 h time points. After 24 h of treatment, as shown in Figure 3, Ang II-stimulated TGF-β1 mRNA levels increased 1.94-fold and were significantly different compared with control (P<0.01; n=3).

TGF-β1 activity in fibroblast conditioned media

We used the mink lung epithelial cell bioassay to examine whether Ang II treatment affects the amounts of biologically active TGF-β1 present in culture as measured against a standard curve. A representative standard curve for the inhibitory effects of TGF-β1 on DNA synthesis in mink lung epithelial cells is illustrated in Figure 4a. The linear range for TGF-β1 inhibition of DNA synthesis was generally found to be between 0.01 and 0.08 ng/
ml. The amount of TGF-β1 specific antibody (Ab) needed to neutralize the inhibitory effects of exogenously added TGF-β1 (1 ng/ml) was determined to be 100 μg/ml, as shown in a dose response curve in Figure 4b. The complete neutralization by TGF-β1 Ab verifies the specificity of TGF-β1 activity. TGF-β1-like activity in diluted CM (1:100) from Ang II-treated fibroblasts ranged between 0.01 and 0.06 ng/ml (n = 4), whereas no detectable activity was found in similarly diluted control CM. Figure 5a shows data from one representative experiment (P<0.001). Neutralization of TGF-β1-like activity in CM from both conditions is shown in Figure 5b. The specificity of TGF-β1 activity in diluted CM from Ang II-treated cells was confirmed by incubation with 100 μg/ml of TGF-β1 Ab (P = 0.056; n = 3). Control Ig (100 μg/ml), a non-specific immunoglobulin, was added for comparison with the antibody treatment condition.

The potential effects of Ang II-induced TGF-β1 activity on fibronectin and collagens I and III mRNA levels in adult rat cardiac fibroblasts were assessed. CM from Ang II-treated fibroblasts was added to cultures of untreated fibroblasts. After 24 h of incubation with CM, total RNA was extracted, processed, and subsequently analysed by Northern blot. Probing for fibronectin and collagens I and III mRNAs resulted in no significant changes in mRNA levels under treatment with CM from Ang II-treated cells when compared with treatment with control CM (P = n.s. for each probe; n = 4). Similarly, normalized mRNA levels from adult rat cardiac fibroblasts treated with both Ang II and the TGF-β1 neutralizing antibody (100 μg/ml) showed no sig-
significant changes in the mRNA levels for fibronectin, collagen I, or collagen III when compared with mRNA levels from fibroblasts treated with Ang II and control Ig (100 μg/ml).

**Total production of TGF-β1**

We evaluated whether Ang II treatment in adult cardiac fibroblasts changes the total levels of TGF-β1 (latent plus active forms) in CM from adult cardiac fibroblast cultures. Total levels of TGF-β1 were assessed by bioassay after heat activation. Control Ig (100 μg/ml) was added to both treatment groups for comparison to the antibody studies. Data were normalized against mean control values from each individual experiment (Table 1). Heat activation of CM from control and Ang II-treated cultures indicated detectable levels of TGF-β1 in both conditions. Treatment with Ang II CM and control Ig in cardiac fibroblasts (Ang CM + Ig) significantly increased the total concentration of TGF-β1 compared with untreated CM (Con CM + Ig), as demonstrated by the greater inhibition of [3H]thymidine incorporation by Ang II-treated CM ($P<0.01; n=3$). Incubation with TGF-β1 Ab (100 μg/ml) completely neutralized the inhibitory effects in both control and Ang II-treated CM ($P<0.05$ for both Ab conditions compared with respective Ig controls), thus confirming the specificity of TGF-β1 action for both conditions.

**Discussion**

Recent studies have examined the cellular and molecular mechanisms of Ang II action in the heart. Ang II AT$_1$ receptors have been identified and characterized in rat cardiac fibroblasts. Studies by Schorb et al., (1993) and Sadoshima and Izumo (1993) showed that Ang II-induced hyperplasia in neonatal rat cardiac fibroblasts and upregulated second messengers known to be associated with an AT$_1$ receptor-like response. Our studies in adult rat cardiac fibroblasts (Villarreal et al., 1993) indicate that Ang II stimulation of AT$_1$ receptors resulted in an increased gene expression for ECM proteins. These findings, in addition to *in vivo* studies of ACE inhibition, have important implications for the potential role of Ang II in the regulation of cardiac fibroblast function.

Ang II stimulation of AT$_1$ receptors has been
shown to stimulate a number of intracellular signaling pathways. In neonatal cardiac fibroblasts, Ang II has been shown to activate mitogen-activated protein (MAP) kinase by either Ca^{2+} or protein kinase C-dependent pathways (Booz et al., 1994). While such pathways are directly linked to

![Figure 3](image1.png)

Figure 3 (a) Northern blot analysis of total RNA from adult rat cardiac fibroblasts after 24 h of treatment with Ang II. Five micrograms of total RNA were loaded in each lane. Nylon membranes were initially probed with a cDNA for TGF-β1 and subsequently with an oligonucleotide for 28S RNA. "Con" represents total RNA from untreated cells and "Ang" represents total RNA from Ang II-treated cells after 24 h of treatment. Data are from one representative experiment. (b) Quantitation of mRNA/28S ratio for TGF-β1 mRNA levels in untreated and Ang II-treated adult rat cardiac fibroblasts after 24 h of treatment. Ang II significantly increased TGF-β1 mRNA levels (1.94-fold compared with control; *P<0.01; n = 3).

![Figure 4](image2.png)

Figure 4 (a) Growth inhibitory response of mink lung epithelial cells to increasing concentrations of purified TGF-β1 (0.01 to 1 ng/ml). The concentration of active TGF-β1 in diluted Ang II-treated conditioned media (CM) was determined by matching [³H]thymidine incorporation on the standard curve. Data are from one representative experiment. (b) Effect of increasing concentrations of TGF-β1 specific antibody on mink lung epithelial cells treated with TGF-β1. The concentration of TGF-β1 specific antibody sufficient to completely neutralize the inhibitory effects of 1 ng/ml of TGF-β1 was determined to be 100 μg/ml.

Ang II receptor-coupled action, the cellular responses that follow may be indirectly mediated in part through the induction of one or many growth factors. In fact, stimulation of the autocrine release of growth factors in cells may be an important mechanism of action for Ang II. Recent studies have described the mediation of Ang II effects via the release of growth factors such TGF-β1 and platelet-derived growth factor (PDGF) (Weber et al., 1994).

TGF-β1 has been demonstrated to mediate Ang II-derived effects in a number of cell types. In cultured VSMCs, TGF-β1 has been shown to mediate effects of Ang II on cell proliferation (Gibbons et al., 1992; Stouffer and Owens, 1992) whereas in proximal tubular cells, Ang II-induced TGF-β1 has been reported to influence the development of hypertrophy (Wolf et al., 1993). Kagami et al. (1994)
suggested that both Ang II and TGF-β1 may regulate remodeling processes in the heart (Dzau, 1994; MacLellan et al., 1993), the action of Ang II in the myocardium may be mediated at least in part by the autocrine production of TGF-β1.

The aim of the present study was to examine whether Ang II stimulated the production and release of TGF-β1 in cardiac fibroblasts at several regulatory levels. Specifically, we used an adult rat cardiac fibroblast culture model to examine whether Ang II induced changes in TGF-β1 mRNA levels, biological activity, and total TGF-β1 levels. This study demonstrates that treatment with Ang II stimulates increases in TGF-β1 mRNA levels which were sustained for at least 20 h. Ang II-induced changes in TGF-β1 mRNA levels in cardiac fibroblasts were significant, stimulating a two-fold increase at 24 h. A similar significant increase was detected when the same data were normalized relative to the constitutively expressed heat shock protein 70 (data not shown) (Rohrer et al., 1991). Receptor-coupled Ang II signaling mechanisms that may be involved in the induction of TGF-β1 gene expression in the adult rat cardiac fibroblast may include stimulation of phosphoinositide catabolism and increases in intracellular calcium concentrations (Crabos et al., 1994). In VSMCs, Ang II has been shown to induce the expression of transcription factors c-fos and c-jun (Naftilan et al., 1989), which are known to form a complex which binds to an AP-1 site to activate gene transcription (Curran and Franza, 1988). The promoter of the TGF-β1 gene contains functional AP-1 sites, and thus the induction of TGF-β1 gene expression in VSMCs may involve the activation of transcriptional factors which are regulated by Ang II (Gibbons et al., 1992). It is interesting to consider the possibility that such a scheme of interactions in transcriptional control may also operate in adult cardiac fibroblasts.

TGF-β1 is secreted from cells in a biologically inactive latent form and has been shown to be activated in vitro by proteolytic enzymes, extreme pH, or heat (Roberts and Sporn, 1990). Assessment of its presence in culture involves the determination of its biological activity (active form) and the total amount (latent plus active forms). Using the well-characterized mink lung epithelial cell bioassay, we demonstrated that conditioned medium from Ang II-treated cardiac fibroblasts, diluted 1:100 for detection in the linear range of the standard curve, demonstrated measurable biological activity compared to no activity in similarly diluted control CM. While TGF-β1 activity ranged between 0.01 and 0.06 ng/ml in the diluted CM from Ang II-treated cells, the actual concentrations of TGF-β1 in culture recently demonstrated that Ang II stimulation of fibronectin, biglycan, and collagen type I in glomerular mesangial cells is mediated by the autocrine production of TGF-β1. Since recent studies have

Figure 5  (a) Ang II treatment increased TGF-β1 activity in fibroblast conditioned medium (CM). CM from untreated and Ang II-treated cells were added to mink lung epithelial cells at a dilution of 1:100. Growth inhibition by active TGF-β1 was assessed by [3H]thymidine incorporation. Addition of Ang II-treated CM resulted in a significant decrease in DNA synthesis compared with control as measured by [3H]thymidine incorporation (706 ± 112 vs 2200 ± 251 cpm; mean ± s.d.; *P<0.001), indicating a greater amount of inhibitory TGF-β1-like activity. Data are representative of four experiments. (b) TGF-β1 specific antibody completely abolished the inhibitory effect of TGF-β1 in Ang II-treated CM. The specificity of TGF-β1 activity was determined by incubating diluted Ang II-treated CM with either control Ig (100 μg/ml) or anti-TGF-β1 antibody (100 μg/ml) before addition to the mink lung epithelial cells. CM incubated with anti-TGF-β1 antibody resulted in the complete neutralization of TGF-β1 activity, as assessed by [3H]thymidine incorporation (*P = 0.056 vs Ang + Ig; P = n.s. vs Con + Ig; n = 3).
Table 1: Effects of Ang II on total production of TGF-β1 in conditioned media (CM)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[3H]thymidine incorp. % (Con CM + Ig)</th>
<th>95% Confidence (S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang CM + Ig</td>
<td>52.0*</td>
<td>43.8 (2.4)</td>
</tr>
<tr>
<td>Con CM + Ab</td>
<td>258.4†</td>
<td>162.6 (35.8)</td>
</tr>
<tr>
<td>Ang CM + Ab</td>
<td>199.2†</td>
<td>83.0 (64.4)</td>
</tr>
</tbody>
</table>

*Total TGF-β1 production was assessed by bioassay after heat activation. 
†P<0.01 v Con CM + Ig. 
Data are means shown with lower 95% confidence limit (S.E.M. from original scale).

were not directly measurable on the standard curve and may be difficult to estimate from simple extrapolation of data because of the potential interactions with undefined components in the CM (Stouffer and Owens, 1992). The results of the conditioned medium and the TGF-β1 antibody studies suggest that the potential effects of Ang II-induced TGF-β1 on cellular function may be complex and may involve components such as other growth factors, possibly induced by Ang II, and the likely presence of proteases, such as plasminogen activator, and their inhibitors (Sato et al., 1990).

In addition to bioactivity, this study assessed the total protein content of TGF-β1 in heat activated CM. Ang II treatment was shown to significantly increase the total amount of TGF-β1 in culture compared with control. Thus, bioassay results demonstrated that in the adult rat cardiac fibroblast cultures, Ang II regulates TGF-β1 protein at two key regulatory levels, biological activity and total protein released. While Ang II may increase TGF-β1 activity and protein as shown in vitro, the mechanism of TGF-β1 activation in vivo is not well understood. In vivo, secreted latent TGF-β1 may bind to extracellular matrix proteins (Andres et al., 1989) and then become activated at physiologically significant times by factors such as proteases or local pH changes in environment, as in wound healing, through mechanisms which are currently not well understood (Roberts and Sporn, 1990). A recent study using mouse osteoblastic cells demonstrated that these cells can activate matrix-bound endogenous TGF-β1 for the regulation of ECM network formation (Vukicevic et al., 1992), suggesting a role for latent stores of TGF-β1.

In the heart, TGF-β1 is expressed at high levels during both embryonic and adult life (Thompson et al., 1989), affecting both fibroblast and myocyte function. Levels of TGF-β1 mRNA have also been shown to precede mRNA levels of ECM proteins in a banded rat model of cardiac hypertrophy (Villarreal and Dillmann, 1992). In a rat model of myocardial infarction, increases in TGF-β1 have also been reported in the infarcted myocardium during the scanning phase (Qian et al., 1992). TGF-β1 may also exert direct effects on cardiac myocytes in embryonic development and in adult tissue remodeling. Recently, TGF-β1 was shown to play an important role in Ca²⁺ handling mechanisms in neonatal cardiac myocytes (Neylon et al., 1994). Also, TGF-β1 has been shown to regulate contractile activity in cultured cardiac myocytes (Roberts et al., 1992) and to reproduce the fetal program associated with cardiac hypertrophy (Parker et al., 1990). Since cardiac fibroblasts have been noted to stimulate cardiac myocyte hypertrophy through paracrine regulatory factors (Long et al., 1993), it is possible that Ang II-induced TGF-β1 in adult cardiac fibroblasts may also assume a paracrine role in the regulation of cardiac myocyte function.

While Ang II and TGF-β1 may be involved in an autocrine/paracrine mechanism in cardiac fibroblasts, other complex mechanisms may exist in the cardiac cellular environment. In neonatal cardiac myocytes, mechanical stretch was demonstrated to stimulate the autocrine release of Ang II which in turn initiated a hypertrophic response (Sadoshima et al., 1993). Our studies of pressure-overload hypertrophy indirectly suggest that TGF-β1 and the subsequent induction of fibronectin and collagen gene expression may all participate in a sequence of events triggered by mechanical stress (Villarreal and Dillmann, 1992). It is interesting to speculate that in adult cardiac fibroblasts, mechanical stretch may induce a cascade of cellular responses which may involve the release of autocrine growth factors such as Ang II and TGF-β1. The potential complicated interactions between growth factors, including mechanical factors, in the cardiac interstitium remain to be studied.

In summary, Ang II has been implicated in normal and pathophysiological remodeling of cardiac tissue. In this study, we have shown that Ang II stimulates the autocrine production and release of
TGF-β1 by increasing the mRNA levels, biological activity, and total protein levels of TGF-β1 in the adult rat cardiac fibroblasts. Results from this study suggest a potential autocrine/paracrine mechanism whereby Ang II might regulate fibroblast and myocyte function in the heart via the release of endogenous TGF-β1.

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Angiotensin II Induces TGF-β1 in Cardiac Fibroblasts


