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Angiotensin II stimulates cardiac myocyte hypertrophy via paracrine release of TGF-β_1 and endothelin-1 from fibroblasts

Mary O. Gray[a], Carlin S. Long[a], Judith E. Kalinyak[b], Hong-Tai Li[a], Joel S. Karliner[a,*]

[a]*Cardiology Section, San Francisco Veterans Affairs Medical Center, Department of Medicine, Cardiovascular Research Institute, University of California, 4150 Clement Street, San Francisco, CA 94121, USA*

[b]*Department of Medicine, Endocrinology Division, San Francisco General Hospital, University of California, San Francisco, CA 94143, USA*

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Abstract

Objective: We sought to determine whether angiotensin II (Ang II) promotes hypertrophy of cardiac myocytes directly or via paracrine mechanisms mediated by cardiac fibroblasts. **Methods:** We studied neonatal rat cardiac myocytes and fibroblasts in culture as a model system. Paracrine effects of Ang II were identified using conditioned medium and co-culture experiments. **Results:** Ang II type 1 (AT_1) receptors responsible for myocyte growth localized to fibroblasts in radioligand binding, emulsion autoradiography, Western analysis, and immunofluorescence staining experiments. The bulk of AT_1 receptor binding in myocyte cultures (1343±472 sites/cell) was to Ang II receptors on contaminating fibroblasts (9747±2126 sites/cell). Ang II induced significant paracrine trophic effects on myocytes in conditioned medium (40% increase in protein synthesis over control) and co-culture (4-fold increase over control) experiments. TGF-β_1 and endothelin-1 were paracrine mediators of hypertrophy in neutralization experiments. **Conclusions:** Ang II stimulates cardiac myocyte hypertrophy via paracrine release of TGF-β_1 and endothelin-1 from cardiac fibroblasts in a neonatal rat cell culture model.

Keywords: Angiotensin; Cell communication; Cell culture; Cytokines; Endothelins; Growth factors; Myocytes; Rat; Receptors

1. Introduction

Angiotensin II (Ang II) receptors have long been known to exist in heart tissue and have been identified on isolated cardiac cells maintained in culture [1]. Recognition of a localized renin–angiotensin system in heart capable of synthesizing Ang II and of responding to its effects [2,3] has led to intense investigation of this network in cardiac growth and development. However, the mechanism of action of Ang II is incompletely understood, and further insights into its effects may have important therapeutic implications. In clinical trials, blockade of this system in patients with acute myocardial infarction or congestive heart failure has led to increased longevity [4,5]

Ang II promotes hypertrophy of cultured rat cardiac myocytes by activation of the Ang II type 1 (AT_1) receptor [6]. Binding of Ang II to the AT_1 receptor stimulates multiple signal transduction pathways involved in hypertrophy such as tyrosine kinases, mitogen-activated protein (MAP) kinases, and 90-kD S6 kinase [7]; 70-kD S6 kinase [8]; c-Jun NH_2-terminal kinase [9]; and extracellular signal-regulated protein kinases (ERKs) and Raf-1 kinase [10]. Activation of these pathways induces transcription of immediate-early genes (c-*fos*, c-*jun*, *jun B*, *Egr-1*, and c-*myc*) and the re-expression of cardiac fetal gene products such as skeletal α-actin and atrial natriuretic factor [7]. Ang II-mediated increases in protein synthesis and induction of the fetal isogene program in the absence of increased DNA synthesis [6] result in growth of cultured myocytes resembling that in whole heart models of hypertrophy [11,12]. Overexpression of the AT_1 receptor transgene in mouse myocardium produces a lethal phenotype associated with atrial but not ventricular myocyte hyperplasia and heart block [13].

The AT_1 receptors responsible for Ang II-stimulated

*Corresponding author. Tel.: +1 (415) 750 2112; Fax: +1 (415) 750 6950.

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cardiac hypertrophy have been thought to reside on the myocytes themselves [14,15]. However, recent studies raise questions regarding the validity of this assumption. First, both intact myocardium and isolated cardiac cells maintained in culture exist as co-populations of myocytes and nonmyocyte cells, predominantly fibroblasts [16]. Abundant Ang II receptors have been identified on neonatal [17] and adult [18] rat cardiac fibroblasts. Binding of Ang II to the fibroblast AT_1 receptor initiates proliferative growth [19] and increases net collagen production [17,18]. Furthermore, activation of the AT_1 receptor increases transforming growth factor-β_1 (TGF-β_1) mRNA levels and bioactivity in neonatal [20] and adult [21] rat cardiac fibroblast culture models. We and others have previously shown that TGF-β_1 acts as a potent stimulant of myocyte growth and fetal contractile protein gene transcription [16], suggesting one potential paracrine mechanism for Ang II-mediated cardiac myocyte hypertrophy. Second, when efforts are made to strictly limit fibroblast contamination of cardiac myocyte preparations, the putative direct effects of Ang II on myocytes are also diminished. For example, Ang II has long been known to modulate myocardial contractility and heart rate [22,23]. However, when Lefroy et al. examined myocytes isolated from rat, guinea pig, and human myocardium rather than muscle strips or whole heart preparations, they found that Ang II no longer influenced contraction amplitude or velocity whereas responses to isoproterenol and increased extracellular calcium were preserved [24]. They concluded that in these preparations active intermediate substances released by nonmyocyte cells were minimized and that there was no evidence for a direct effect of Ang II on cardiac myocyte contraction. Finally, direct receptor identification techniques such as microscopic autoradiography reveal localization of AT_1 receptors predominantly to fibroblasts in isolated rat cardiac cells maintained in culture under basal conditions [25]. Fibroblast AT_1 receptor binding assessed by autoradiography of tissue sections of intact myocardium is further increased in pathological settings such as myocardial infarction and co-localizes with enhanced collagen deposition [26,27]. These data support the hypothesis that AT_1 receptors reside primarily on fibroblasts in heart tissue and that effects of Ang II on myocytes occur via paracrine mechanisms mediated by fibroblasts.

In the present study we addressed the issue of whether Ang II promotes hypertrophy of cardiac myocytes directly or via paracrine mechanisms mediated by fibroblasts using four approaches. First, we determined the relative abundance of AT_1 receptors on neonatal rat cardiac myocytes and fibroblasts by radioligand binding, emulsion autoradiography, Western analysis, and immunofluorescence staining. Second, we assessed the trophic effect on myocytes of factors released by Ang II-stimulated fibroblasts in conditioned medium and co-culture experiments. Finally, we used neutralizing antibodies against TGF-β_1 and antagonists of endothelin-1, another potent myocyte growth factor [28], to identify specific paracrine roles for these two peptides in Ang II-mediated hypertrophy.

2. Methods

2.1. Cell culture model

This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Primary cardiac myocyte cultures were prepared as previously described [29]. Ventricles from day-old Sprague-Dawley rats (Simonsen) were minced and subjected to trypsinization and mechanical dissociation. Suspensions were preplated to reduce the number of fibroblasts then replated into 100 mm plastic culture dishes (Fisher) for radioligand binding studies, for Northern and Western analyses, and for determination of total cell protein content. Myocytes were plated into 2-well glass chamber slides (Nunc) for emulsion autoradiography and immunofluorescence staining and into 6-well culture plates (Fisher) for protein labeling experiments. Myocytes attached at a final density of 150–200 cells/mm^2 after overnight incubation. Cultures were maintained in minimal essential medium (MEM) with Hanks salt solution (UCSF Cell Culture Facility) supplemented with 5% bovine calf serum (Hyclone) and 0.1 mM bromodeoxyuridine (BrdU) to prevent fibroblast proliferation [29]. For autoradiography, immunofluorescence staining, and Northern and Western analyses, myocytes were fed with defined medium consisting of MEM supplemented with 1.5 μM vitamin B_{12}, 50 U/ml penicillin, 10 μg/ml insulin, and 10 μg/ml transferrin for 24 to 48 h prior to harvest. For determination of total protein content and for protein labeling experiments, myocytes were maintained in defined medium beginning on culture day 1. Preparations contained 90–95% cardiac myocytes as determined by their appearance under phase contrast microscopy and by immunocytochemical staining with antibodies against sarcomeric myosin (MF-20), smooth muscle actin (Sigma), and factor VIII (Sigma). In addition, we performed flow cytometry studies using MF-20 antibody to demonstrate that 90% of cells in primary myocyte preparations were cardiac myocytes [30].

Cardiac fibroblasts obtained during the preplating step of the myocyte isolation procedure were maintained in serum-supplemented medium, allowed to proliferate, then trypsinized and passaged once at 1:3 dilution. Culture medium was supplemented with 0.1 mM BrdU as cells reached subconfluence but could still be counted under phase-contrast microscopy. For emulsion autoradiography, immunofluorescence staining, Northern and Western analyses, and protein labeling experiments, fibroblasts were fed with defined medium for 24 to 48 h prior to use.

Preparations contained >95% cardiac fibroblasts as determined by morphological appearance under phase-contrast microscopy and by routine immunocytochemical staining with antibodies against sarcomeric myosin (MF-20), smooth muscle actin (Sigma), and factor VIII (Sigma).

For co-culture experiments, primary cardiac fibroblasts were harvested on culture day 1 in defined medium containing 0.1 mM BrdU and seeded into 6-well plates of cardiac myocytes at a 1:4 ratio of fibroblasts to myocytes. Cells were incubated with experimental agents or with vehicle for 72 h. Cell numbers were constant over time. Separate culture dishes of standard fibroblast and myocyte preparations were used as concurrent controls.

2.2. Growth measurement

Cardiac myocyte growth was measured by two methods as previously described. First, asymptotic labeling with [^{14}C]phenylalanine (Amersham) was used to quantitate new cell protein synthesis [31]. Cultured cardiac myocytes were incubated with [^{14}C]phenylalanine (0.1 μCi/ml) and either experimental agents or vehicle for 24 to 72 h. At the end of each experiment cellular protein, defined as trichloroacetic acid (TCA)-insoluble and sodium dodecyl sulfate (SDS)-soluble material, was taken for liquid scintillation counting. Second, total protein was measured using a detergent compatible system (Bio-Rad) based on the spectrophotometric assay of Bradford [32]. Cells were washed with phosphate buffered saline (PBS) and dissolved in 0.1% SDS at 37°C. Triplicate aliquots were assayed using bovine serum albumin (BSA) dissolved in 0.1% SDS as standard.

2.3. Radioligand binding

Saturation binding studies were performed according to a general protocol previously described in our laboratory [33]. Myocytes and fibroblasts were counted, incubated with cold lysing solution (1mM Tris-HCl pH 7.5 and 2 mM EGTA), removed from plates by scraping, and centrifuged at 100,000 *g* for 30 min at 4°C. Pellets were resuspended in SET buffer (0.25 M sucrose, 1 mM EDTA, 5 mM Tris-HCl) and stored at −70°C. For binding studies, thawed pellets were resuspended in assay buffer (50 mM Tris-HCl pH 7.4, 120 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM bacitracin, and 0.25% BSA) and sonicated. Membrane preparations were incubated in triplicate with varying concentrations (100–900 pM) of [^{125}I]Sar^1, Ile^8-angiotensin II (Peptide Radioiodination Center, Washington State University) in a total volume of 100 μl containing 70,000–100,000 cells at room temperature for 60 min. Preliminary experiments indicated that the reaction reached equilibrium by this time point. Radioactivity was determined in a gamma counter at a counting efficiency of 73%. Nonspecific binding was determined in the presence of 10 μM Sar^1-angiotensin II. Specific binding (total binding minus non-specific binding) ranged between 60% and 90%. The maximum number of binding sites (B_{max}) and equilibrium binding constant (K_d) were determined by least-squares linear regression analysis using the method of Scatchard [34]. Preliminary experiments showed that less than 2% of binding sites were retained in the supernatant from a 100,000 *g* pellet. Displacement binding experiments using [^{125}I]Sar^1, Ile^8-angiotensin II and increasing concentrations (10^{-10} to 10^{-5}M) of the nonpeptide antagonists losartan (Ang II type 1 receptor selective) and PD123319 (Ang II type 2 receptor selective) were performed to determine Ang II receptor subtype distribution using a protocol identical to that described above.

2.4. Emulsion autoradiography

Emulsion autoradiography studies were performed as previously described [35]. Cardiac myocytes cultured on glass chamber slides were incubated with 250 pM [^{125}I]Sar^1, Ile^8-angiotensin II in binding buffer (0.75 mM KH_2PO_4, 2.8 mM Na_2HPO_4 pH 7.3, 140 mM NaCl, 4 mM KCl, 4 mM $MgSO_4$, 0.9 mM $CaCl_2$, 0.3 mM bacitracin, and 0.1% BSA) for 90 min at 18°C. Additional myocytes were incubated concurrently with 1 μM Sar^1-angiotensin II to determine non-specific binding or with 10 μM losartan or 10 μM PD123319 to identify Ang II receptor subtypes. Slides were fixed with paraformaldehyde vapor, coated with photographic emulsion (Kodak NTB2), and exposed in the dark at 4°C for 2–4 weeks. Slides were then developed (Kodak D-19), counterstained with hematoxylin and eosin, and viewed with both bright- and dark-field optics using a Nikon Optiphot microscope at 40X magnification.

2.5. Northern analysis

Myocytes and fibroblasts were lysed with guanidinium isothiocyanate (Fluka), and total RNA was isolated by pelleting the sheared cellular lysates through cesium chloride gradients. Samples were fractionated by electrophoresis in a 1% agarose gel containing 0.6 M formaldehyde and transferred to nylon membranes (Schleicher and Schuell). For measurement of steady-state levels of mRNA for the AT_1 receptor, Northern blots were hybridized overnight at 60°C with an AT_1 receptor cDNA probe labeled with ^{32}P-dCTP (DuPont NEN) by random priming (Ambion). Autoradiograms were obtained by exposing the hybridized blot to Kodak X-OMAT film at −70°C for 72 h. To confirm RNA loading and integrity, membranes were stripped and rehybridized with a probe to 28S ribosomal RNA.

2.6. Western analysis

Myocytes and fibroblasts were washed with PBS, lysed

in cold homogenization buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.25 M sucrose, and 20 μg/ml each phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, leupeptin, and aprotinin), and centrifuged at 100,000 *g* for 30 min at 4°C. Pellets were resuspended in homogenization buffer, subjected to 12% SDS–PAGE, and transferred to nitrocellulose sheets (Schleicher and Schuell). AT_1 receptor protein levels were determined by incubating blots with anti-AT_1 antibody (Santa Cruz Biotechnology catalog #sc-579), an affinity-purified rabbit polyclonal antibody raised against amino acids 306–359 of the human AT_1 receptor [36,37]. Immunoreactive bands were detected with ^{125}I-protein A (ICN). For TGF-β_1 experiments, cultured cardiac fibroblasts in fresh defined medium were incubated with 100 nM Ang II for 6, 12, and 24 h. Cells were washed with PBS, lysed in homogenization buffer, and centrifuged at 100,000 *g* for 30 min at 4°C. Concentrated fibroblast soluble fractions were subjected to 12% SDS-PAGE under non-reducing conditions followed by transfer to nitrocellulose sheets. TGF-β_1 protein levels were determined by incubating blots with anti-TGF-β_1 antibody (R&D Systems catalog #MAB240), an affinity-purified monoclonal antibody produced by a murine hybridoma elicited from a mouse immunized with purified, CHO cell-derived, recombinant human latent TGF-β_1. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham).

2.7. Immunofluorescence staining

Myocytes and fibroblasts were washed with PBS, fixed with methanol and acetone, and blocked with 1% normal goat serum (Cappel). Cells were incubated with anti-AT_1 antibody at 1:100 dilution for 4 h at room temperature in a humidified chamber. Cells were incubated with FITC-conjugated goat anti-rabbit IgG antibody (Cappel) diluted 1:1000, mounted with glass coverslips, and viewed with a Zeiss IM35 microscope using a 40X water immersion objective. Images were recorded on Kodak TMAX 400 film with exposure time of 1 second. All photomicrographs were processed by an automated, commercial developer without additional adjustment.

2.8. Conditioned medium experiments

Passage 1 fibroblasts were allowed to proliferate to subconfluence then fed with defined medium supplemented with 0.1 mM BrdU for 24 h. Following a second defined medium change, cells were incubated with either 100 nM Ang II or with vehicle for 24 h. The resultant fibroblast conditioned medium was mixed 1:1 with fresh defined medium and 0.1 μCi/ml [^{14}C]phenylalanine to constitute myocyte labeling medium. Multiwell plates of myocytes maintained from culture day 1 in defined medium were incubated for 72 h with myocyte labeling medium in the presence of losartan to block the action of any residual Ang II. The relative growth-promoting effect of conditioned medium from fibroblasts incubated in the presence or absence of Ang II was determined by aspirating labeling medium, washing myocytes with PBS, and measuring [^{14}C]phenylalanine incorporation into TCA-insoluble, SDS-soluble myocyte protein by liquid scintillation counting. Preliminary experiments showed no augmentation of the growth-promoting effect of conditioned medium by either acid or heat pretreatment. Blocking experiments were carried out by pre-incubating myocyte labeling medium with 10 μg/ml pan-specific TGF-β neutralizing antibody (R&D Systems catalog #AB-100-NA), an affinity-purified rabbit polyclonal antibody raised against a mixture of recombinant human TGF-β_1, porcine TGF-$\beta_{1.2}$, porcine TGF-β_2, and recombinant amphibian TGF-β_5.

2.9. Reagents

AT_1 receptor cDNA was a gift from Dr. T. Murphy of Emory University [38]. Losartan (DuP 753) was a gift from DuPont Merck (Wilmington, DE). PD 123319 and PD 142893 were gifts from Parke-Davis (Ann Arbor, MI). BQ 123 and BQ 788 were gifts from Hoffman-LaRoche (Basel, Switzerland). Porcine TGF-β_1 was purchased from R&D Systems (Minneapolis, MN). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

2.10. Statistical analysis

All data are expressed as mean±SEM. Numerical data were compared using Student's *t* test for paired observations between two groups and by ANOVA followed by the Dunnett *t* test when more than two groups were analyzed. A P value of <0.05 was considered significant.

3. Results

3.1. Ang II stimulates cardiac myocyte hypertrophy

We determined the effect of Sar^1-angiotensin II, hereafter referred to as Ang II, on cultured neonatal rat cardiac myocytes using [^{14}C]phenylalanine incorporation as a marker of protein synthesis as previously described in our laboratory [31]. The hypertrophic response was concentration-dependent (Fig. 1A) with a maximal increase in [^{14}C]phenylalanine incorporation of 35±6% above basal at 100 nM Ang II ($n=6$, $P<0.05$). The response was completely inhibited by the AT_1 receptor antagonist losartan, was unaffected by the AT_2 receptor antagonist PD 123319 (Fig. 1B), and led to a corresponding increase in total myocyte protein content of 21±5% above basal (526±94 vs. 435±91 mg protein per dish, $n=8$, $P<0.05$). Therefore, Ang II-stimulated myocyte protein synthesis is mediated by activation of AT_1 receptors in this model.

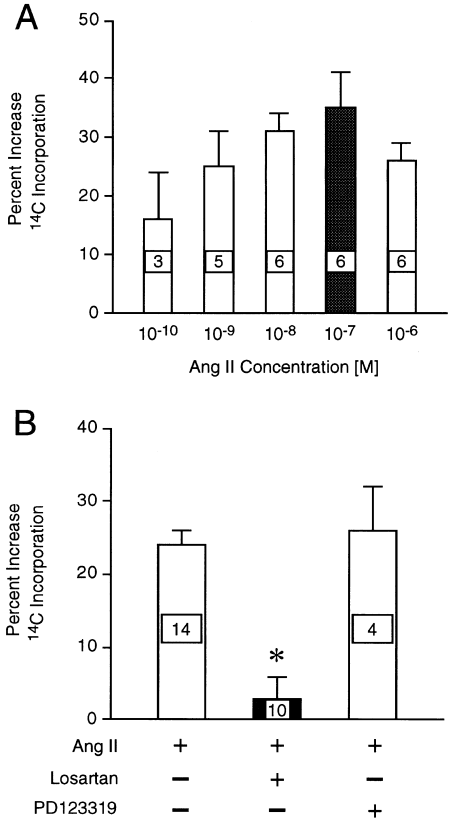

Fig. 1. Bar graphs showing Ang II stimulation of myocyte protein synthesis by activation of the AT_1 receptors. Data shown are percent increases in [^{14}C]phenylalanine incorporation above basal, expressed as mean±SEM. The number in each bar is the number of experiments per condition, each performed using a separate culture preparation **(A)** Myocytes were incubated with the indicated concentration of Ang II for 72 h. Growth response was maximal at 100 nM Ang II. **(B)** Myocytes were incubated with 100 nM Ang II for 72 h and vehicle (−), 10 μM losartan, or 10 μM PD 123319 as indicated. Ang II-stimulated protein synthesis (24±2% above basal) was inhibited by the AT_1 receptor antagonist losartan (3±3% above basal) and unaffected by the AT_2 receptor antagonist PD 123319 (19±8% above basal). *$P<0.05$ versus vehicle or PD 123319.

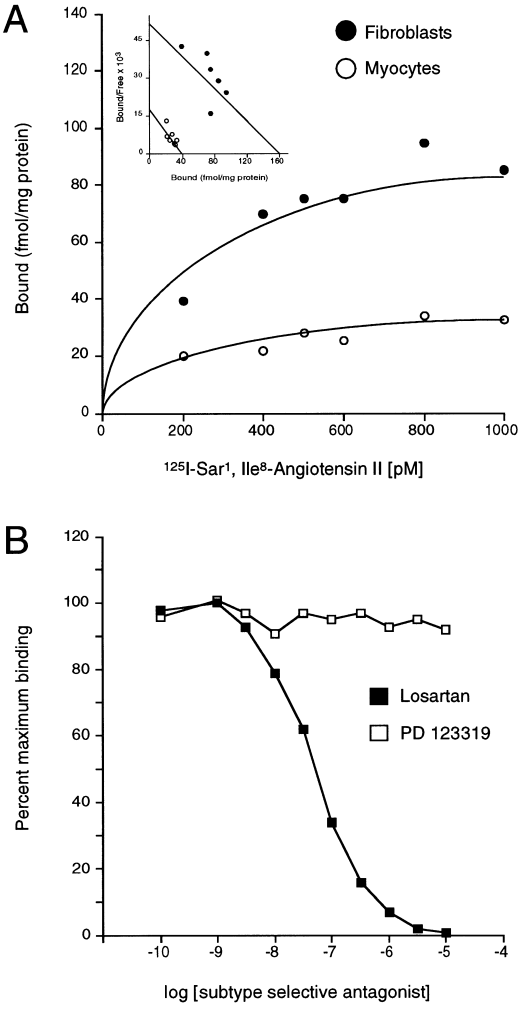

Fig. 2. Binding curves of [^{125}I]Sar^1, Ile^8-angiotensin II in membrane preparations showing localization of Ang II receptors to cardiac fibroblasts and predominance of the AT_1 subtype. (**A**) Representative saturation binding curves with Scatchard transformations (inset) using fibroblast (closed circles) and myocyte (open circles) membranes from the same heart preparation. In four independent experiments, the maximal number of binding sites (B_{max}) was 4.6 times greater in fibroblasts than in myocytes. **(B)** Representative displacement binding experiments in which fibroblast membranes were incubated with 450 pM [^{125}I]Sar^1, Ile^8-angiotensin II and increasing concentrations of losartan (closed squares) and PD 123319 (open squares). Data are expressed as percent of maximum binding of radioligand in the absence of antagonist. Results are representative of three independent experiments.

3.2. Direct identification of Ang II receptors by radioligand binding

We localized Ang II receptors to specific cell types in our model in concurrent saturation radioligand binding studies using fibroblasts and myocytes isolated from the same heart preparation and maintained under identical culture conditions (Fig. 2A). We found that the maximum number of binding sites (B_{max}) for the antagonist radioligand [^{125}I]Sar^1, Ile^8-angiotensin II was 4.6 times greater in fibroblast membranes than in myocyte samples (105±27 vs. 23±10 fmol/mg protein, $n=4$, $P<0.05$). There was no difference in K_d between the two cell types (264±62 pM for fibroblasts versus 370±156 pM for myocytes). Furthermore, because fibroblasts and myocytes were counted prior to harvest, we could also calculate the number of Ang II binding sites per cell. Ang II receptors were 7.3 times more abundant on fibroblasts than on myocytes (9747±2126 versus 1343±472 sites/cell, $n=4$, $P<0.05$) and almost exclusively of the AT_1 subtype as determined in subsequent displacement binding experiments (Fig. 2B). Because neonatal rat cardiac myocyte preparations are actually co-cultures containing 5–10% fibroblasts, the bulk of binding attributed to myocytes is the result of radioligand binding to Ang II receptors on contaminating fibroblasts.

We used emulsion autoradiography with concurrent cytochemical staining to localize binding of the same

radioligand to intact cells in primary myocyte preparations. Intense, specific binding was observed only in the minority of cells exhibiting the morphologic characteristics of cardiac fibroblasts (Fig. 3A). Co-incubations with losartan (Fig. 3B) and PD 123319 (Fig. 3C) confirmed the almost exclusive presence of the AT_1 subtype. No specific binding to cardiac myocytes was noted. Therefore, in this neonatal cell culture model, binding of radioligand to AT_1 receptors is saturable, specific, and limited almost exclusively to cardiac fibroblasts.

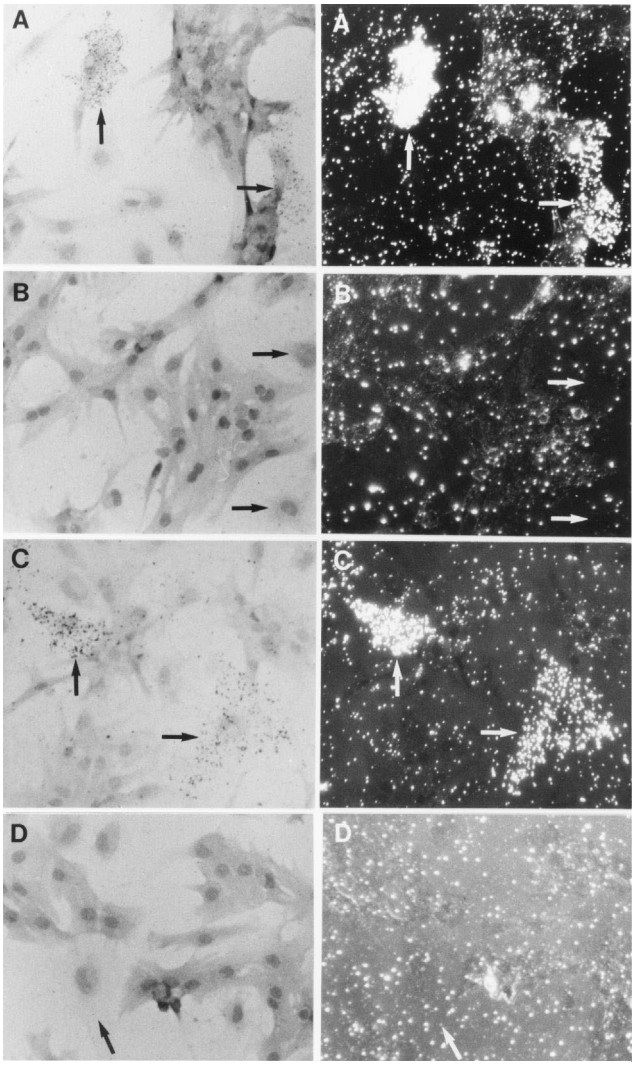

Fig. 3. Localization of AT_1 receptors to cardiac fibroblasts by emulsion autoradiography. Primary myocyte preparations were incubated with 250 pM [^{125}I]Sar^1, Ile^8-angiotensin II followed by emulsion autoradiography and counterstaining with hematoxylin and eosin. Left panels: brightfield views. Right panels: darkfield views. **(A)** In the absence of competitors, intense binding of radioligand was limited to the minority of cells (white arrows, right panel) exhibiting the characteristic flattened cytoplasm and nuclear morphology of cardiac fibroblasts (black arrows, left panel). **(B)** Co-incubation with losartan reduced radioligand binding to minimal background levels. Arrows indicate typical fibroblast without overlying silver grains. **(C)** Co-incubation with PD 123319 had no effect on radioligand binding. Arrows indicate typical fibroblast with overlying silver grains. **(D)** Co-incubation with unlabeled Ang II reduced binding to minimal background levels. Results are representative of three independent experiments.

3.3. Direct immunological detection of Ang II receptors

We localized Ang II receptors to specific cell types in concurrent Western analyses using fibroblasts and myocytes isolated from the same heart preparation and maintained under identical culture conditions. We found that AT_1 receptor protein signal in fibroblast particulate fractions appeared as an intense band at the expected molecular weight of 67 kD (Fig. 4A *left*). Almost no AT_1 receptor signal was detected in myocyte fractions. We

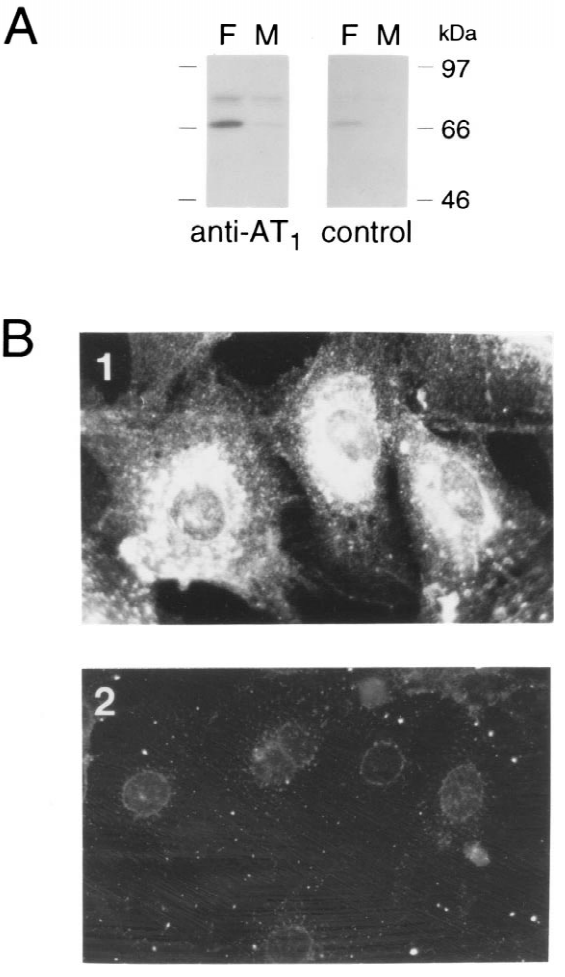

Fig. 4. Localization of AT_1 receptors to cardiac fibroblasts using immunological detection methods. **(A)** Western blot analysis of fibroblasts (F) and myocytes (M) from the same heart preparation. Particulate fractions (50 μg per lane) were subjected to 12% SDS–PAGE and transferred to nitrocellulose. Blots were incubated with anti-AT_1 receptor antibody (left panel) or with control rabbit serum (right panel) followed by detection with [^{125}I]protein A. **(B)** Immunofluorescence staining of cardiac fibroblasts (upper panel) and myocytes (lower panel) was performed by incubating fixed cells with anti-AT_1 antibody followed by FITC-conjugated goat anti-rabbit IgG antibody. Intense staining of fibroblasts and minimal staining of myocytes were observed. Results are representative of three independent experiments.

localized AT_1 receptor protein in intact fibroblasts and myocytes by immunofluorescence staining. Intense staining of fibroblasts was observed (Fig. 4B, panel 1). In contrast, staining of cardiac myocytes was minimal (Fig. 4B, panel 2). Therefore, in this model AT_1 receptors localize to cardiac fibroblasts using immunological detection methods.

3.4. Northern analysis of AT_1 receptor mRNA levels

In a recent study Asano et al. used RT-PCR techniques to identify AT_1 receptor mRNA in human heart tissue but did not localize expression to individual cell types [39]. We used Northern analysis to compare steady-state levels of mRNA for the AT_1 receptor in fibroblasts and myocytes isolated from the same heart preparation and maintained under identical culture conditions. Others have reported 10-fold greater AT_1 receptor mRNA signal in total RNA extracted from neonatal rat cardiac fibroblasts compared with myocytes [17]. Using a ^{32}P-labeled AT_1 receptor cDNA probe [38], we found that AT_1 receptor mRNA signal intensity in fibroblast total RNA was twice that in cardiac myocyte samples at four levels of RNA loading (Fig. 5). Although our results do not exclude the presence of substantial amounts of AT_1 receptor mRNA in cardiac myocytes, the data summarized in Fig. 2 through 4 suggest that AT_1 receptor mRNA in myocytes is not transcribed into receptor protein.

3.5. Paracrine regulation of myocyte growth in conditioned medium experiments

We previously reported that the TGF-β family of peptide growth factors stimulated myocyte hypertrophy and fetal contractile protein gene transcription in our cardiac cell culture model [16]. Furthermore, TGF-β mRNA localized primarily to cardiac fibroblasts with predominance of the TGF-β_1 and TGF-β_3 isoforms. Sharma et al. reported upregulation of TGF-β_1 mRNA by Northern analysis in neonatal rat cardiac fibroblasts incubated with Ang II for 1 h that reached a plateau at 16 h [40]. Fisher and Absher reported upregulation of TGF-β_1 and TGF-β_2 mRNA expression and enhanced TGF-β secretion from fibroblasts after 48 h of incubation with Ang II [20]. In the present study we found that incubation of cardiac fibroblasts with Ang II for 6 h increased expression of the 25 kD TGF-β_1 homodimer by several fold, an effect that persisted for at least 24 h (Fig. 6). TGF-β_1 expression in myocyte fractions could not be documented by Western analysis nor could TGF-β_3 expression in either cell type using two different isoform-specific anti-TGF-β_3 antibodies (not shown).

We used conditioned medium experiments to test the hypothesis that Ang II promotes cardiac myocyte hypertrophy via paracrine mechanisms mediated by fibroblasts. We found that Ang II augmented the growth-

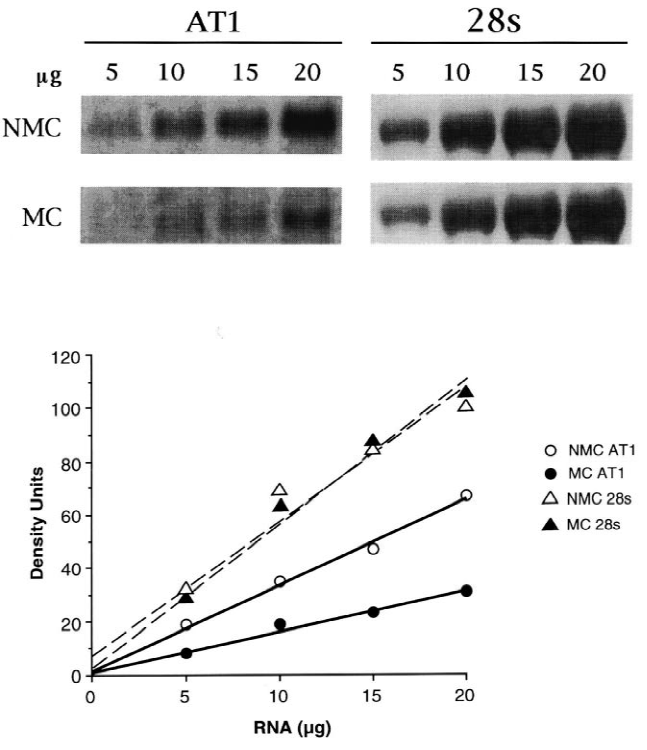

Fig. 5. Northern analysis comparing AT_1 receptor mRNA in fibroblasts and myocytes. (Upper panel) Increasing amounts of total RNA from fibroblasts (NMC) and myocytes (MC) were hybridized with a ^{32}P-labeled AT_1 receptor cDNA probe (AT_1). Blots were stripped and rehybridized with a 28S ribosomal RNA probe (28S). **(Lower panel)** AT_1 receptor mRNA and 28S ribosomal RNA signal intensities were quantitated by scanning densitometry and expressed as arbitrary density units. AT_1 receptor signal intensity in fibroblasts was twice that present in myocytes at each level of total RNA loading. Results are representative of three independent experiments.

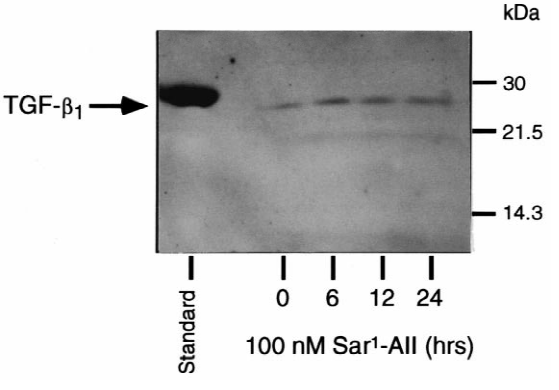

Fig. 6. Western analysis of Ang II-induced TGF-β_1 expression in cardiac fibroblasts. Fibroblasts were incubated with vehicle (0) or Ang II for 6, 12, or 24 h prior to harvest. Concentrated fibroblast soluble fractions (50 µg per lane) were subjected to 12% SDS–PAGE under non-reducing conditions and transferred to nitrocellulose. Blots were incubated with anti-TGF-β_1 antibody followed by detection with enhanced chemiluminescence. Porcine TGF-β_1 was used as a positive control (Standard). Ang II increased expression of the 25 kD TGF-β_1 homodimer, an effect that persisted for at least 24 h.

promoting effect of fibroblast conditioned medium on myocytes by 40±5% above control (n=5, P<0.05) when fibroblasts were incubated with 100 nM Ang II for 24 h (Fig. 7A). Augmentation was inhibited by losartan and unchanged by PD 123319 (not shown). Furthermore, augmentation was inhibited by pre-incubation of conditioned medium with TGF-β neutralizing antibody prior to transfer to myocyte cultures (Fig. 7A). Addition of TGF-β neutralizing antibody directly to myocyte cultures had no effect on basal [^{14}C]phenylalanine incorporation (5092±693 vs. 5400±670 cpm per dish, n=4). However, direct addition of TGF-β antibody to myocyte cultures inhibited Ang II-stimulated protein synthesis (Fig. 7B). Taken together, these data suggest that the effects of Ang II on cardiac myocyte growth are mediated in part by the paracrine production of TGF-β_1 following activation of fibroblast AT_1 receptors.

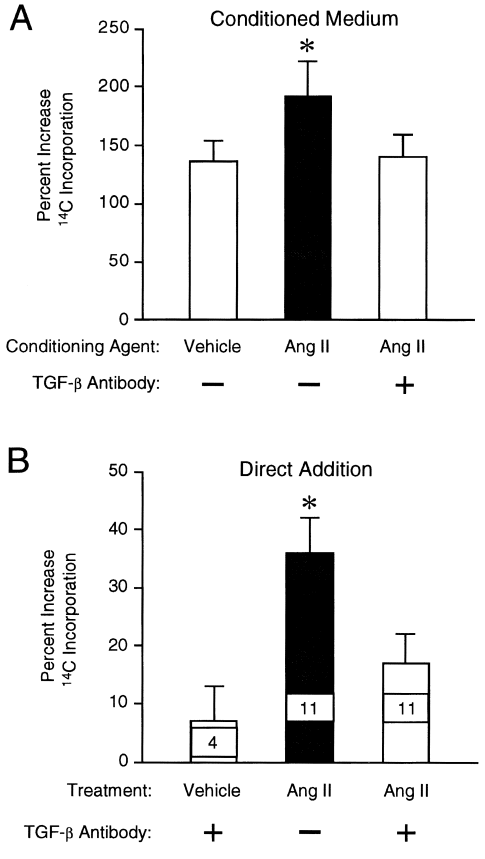

Fig. 7. Bar graphs showing paracrine regulation of cardiac myocyte growth by TGF-β. Data shown are percent increases in [^{14}C]phenylalanine incorporation above basal, expressed as mean ± SEM. **(A)** The growth-promoting effect on myocytes of conditioned medium from fibroblasts incubated with Ang II for 24 h was 40±5% greater than control conditioned medium (Vehicle). The enhanced trophic effect was inhibited by pre-incubation of conditioned medium with TGF-β neutralizing antibody (+) prior to transfer to myocytes. *P<0.05 versus Vehicle or Ang II+Antibody. **(B)** Direct addition of TGF-β neutralizing antibody (+) to myocyte cultures had no effect on basal [^{14}C]phenylalanine incorporation. However, Ang II-induced protein synthesis was inhibited by 57±6% in the presence of TGF-β neutralizing antibody. *P<0.05 versus Antibody alone or Ang II+Antibody. The number in each bar is the number of experiments per condition, each performed using a separate culture preparation.

3.6. Paracrine regulation of myocyte growth in co-culture experiments

We reasoned that if Ang II promotes hypertrophy of cardiac myocytes via paracrine mechanisms mediated by fibroblasts, addition of fibroblasts to myocyte cultures should enhance Ang II-stimulated protein synthesis. In preliminary experiments we found that Ang II had no effect on [^{14}C]phenylalanine incorporation by cardiac fibroblasts cultured in the presence of 0.1 mM BrdU as described in Materials and Methods (7527±2475 vs. 7760±2526 cpm per dish, n=9). However, when additional fibroblasts were co-cultured in a 1:4 ratio with myocytes (Fig. 8A), Ang II-stimulated increases in [^{14}C]phenylalanine incorporation were four times greater than those of standard myocyte preparations in which contaminating fibroblasts are present in 1:10 to 1:20 ratio with myocytes. Furthermore, TGF-β neutralizing antibody inhibited Ang II-stimulated increases in [^{14}C]phenylalanine incorporation by 60% (n=4, P<0.05), again suggesting that the hypertrophic effect of Ang II was mediated in part by TGF-β in this co-culture model.

Endothelin-1 (ET-1) is another peptide growth factor that has been shown to stimulate cardiac myocyte hypertrophy and fetal contractile protein gene transcription [28]. ET-1 was first proposed as a paracrine factor in Ang II-stimulated myocyte hypertrophy by Ito et al. who found that Ang II-induced protein synthesis could be inhibited by selective antagonists of the ET_A receptor subtype [41]. More recently, Ponicke et al. reported that addition of non-myocyte cells to highly purified myocyte cultures restored the ability of Ang II to increase [^{3}H]phenylalanine uptake and that the response could also be inhibited by ET_A receptor antagonists [42]. In the present study, direct addition of 1 nM ET-1 to standard myocyte preparations (Fig. 8B) increased [^{14}C]phenylalanine incorporation by 79±10% above basal (n=3, P<0.05). The response was inhibited by 60% in the presence of the non-selective ET_A/ET_B receptor antagonist PD 142893 and to a similar degree by the ET_A receptor selective antagonist BQ 123 (Fig. 8B). The ET_B receptor selective antagonist BQ 788 had no significant effect on ET-1 stimulation of myocyte protein synthesis. Furthermore, PD 142893 inhibited Ang II-stimulated increases in [^{14}C]phenylalanine incorporation in co-culture preparations (Fig. 8A) by 54% (n=4, P< 0.05). These data suggest that effects of Ang II on cardiac myocyte growth are also mediated in part by the paracrine production of endothelin-1 following activation of fibroblast AT_1 receptors. In three additional experiments (Fig. 8A), Ang II-stimulated myocyte protein synthesis was further inhibited in the combined presence of PD 142893 and TGF-β neutralizing antibody (21±11% above basal).

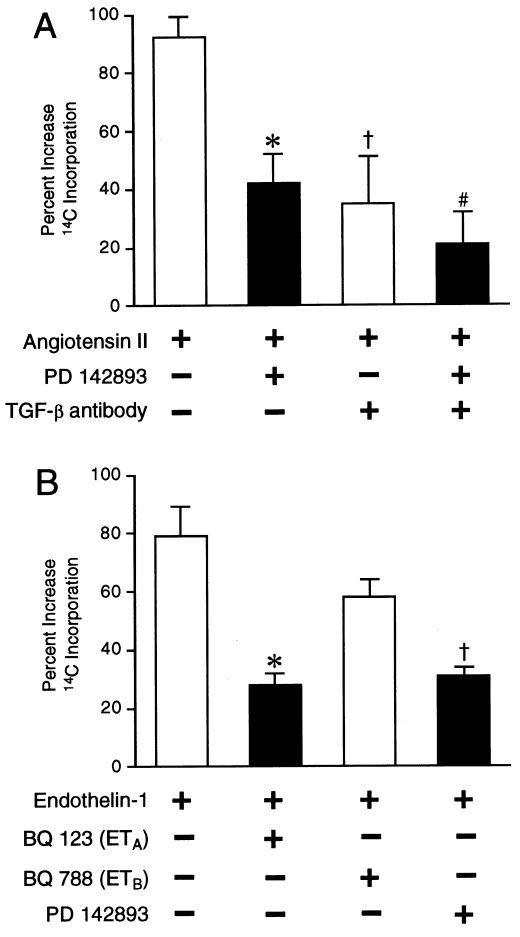

Fig. 8. Bar graphs showing paracrine regulation of myocyte growth by endothelin-1. Data shown are percent increases in [^{14}C]phenylalanine incorporation above basal, expressed as mean±SEM. **(A)** When fibroblasts were co-cultured in a 1:4 ratio with myocytes in 4 independent experiments, Ang II induced a 92±7% increase in [^{14}C]phenylalanine incorporation above basal ($P<0.05$). In contrast, in experiments using the standard myocyte preparation (Figure 1B), Ang II induced a 24±2% increase in protein synthesis above basal. Blocking experiments were conducted using 10 μg/ml TGF-β neutralizing antibody and 1 μM PD 142893. *$P<0.05$ versus Ang II alone. †$P<0.05$ versus Ang II alone. #$P<0.05$ versus Ang II alone. **(B)** Endothelin-1 (1 nM) increased [^{14}C]phenylalanine incorporation by 79±10% above basal in 3 independent experiments using standard myocyte preparations. Inhibitor studies were conducted using 1 μM BQ 123, BQ 788, or PD 142893. *$P<0.05$ versus ET-1 alone. †$P<0.05$ versus ET-1 alone.

4. Discussion

Ang II has long been known to promote cardiac hypertrophy [43]. More recently its role in ventricular remodeling has been recognized [44] and has led to the widespread use of angiotensin-converting enzyme inhibitors [4] and AT_1 receptor antagonists [5] in patients with myocardial infarction and congestive heart failure. In the present study we examined the trophic effects of Ang II and the localization of the AT_1 receptors responsible for growth in isolated neonatal rat cardiac myocytes and fibroblasts maintained in culture. These culture models have been used extensively to characterize the role of the renin–angiotensin system and of many putative growth factors in cardiac development [45,46]. Two lines of evidence support the hypothesis that Ang II promotes hypertrophy of cardiac myocytes via paracrine mechanisms mediated by fibroblasts. First, we found that neonatal cardiac myocytes expressed very few Ang II receptors as demonstrated by radioligand binding, emulsion autoradiography, Western analysis, and immunofluorescence staining. The small number of Ang II receptors identified in quiescent cardiac myocyte preparations could be explained in large measure by the presence of contaminating fibroblasts. Second, conditioned medium and co-culture experiments indicated that Ang II induced a significant paracrine trophic effect on myocytes that was mediated by cardiac fibroblasts. More specifically, myocyte protein synthesis induced by Ang II activation of fibroblast AT_1 receptors was inhibited by TGF-β neutralizing antibody and by antagonists of endothelin-1. Therefore, Ang II stimulation of myocyte protein synthesis differs from the effects of norepinephrine, which directly activates myocyte α_1-adrenoceptors, a subtype not expressed by neonatal rat cardiac fibroblasts [47].

There is considerable information on the biochemical pathways activated in rat cardiac myocyte preparations in response to Ang II, and many of these pathways appear to be operative in cardiac fibroblasts as well [48]. Not all studies have found that cardiac myocytes express Ang II receptors. For example, Villarreal et al. identified AT_1 receptors on neonatal and adult rat cardiac fibroblasts using radioligand binding and emulsion autoradiography techniques and expression of AT_1 receptor mRNA by Northern analysis [17]. However, they reported that AT_1 receptor mRNA was 10-fold less abundant in total RNA from neonatal rat cardiac myocytes and undetectable in total RNA from adult rat cardiac myocytes. Using radioligand binding techniques from the same laboratory, Kim et al. found that specific binding of Ang II receptors in neonatal cardiac myocyte preparations was less than 10% of the specific binding obtained in comparable numbers of fibroblasts [25]. Furthermore, emulsion autoradiography studies showed no specific binding of radioligand to intact neonatal cardiac myocytes, supporting their conclusion that Ang II receptors were localized predominantly on cardiac fibroblasts in this culture model.

Our findings in the present study are in agreement with these observations. In additional experiments employing Western blot analysis and immunofluorescence staining we were unable to detect AT_1 receptors on neonatal cardiac myocytes, but these immunological methods identified abundant AT_1 receptor protein in cardiac fibroblast preparations (Fig. 4). Fareh et al. reported minimal specific binding of antagonist radioligand to adult myocyte preparations and were unable to generate saturation and competition binding curves, suggesting at most a very low number of Ang II receptors on adult rat cardiac myocytes

[49]. Physiological concentrations of Ang II had a negligible effect on calcium transients in control adult myocytes in their study, whereas Ang II induced much larger, concentration-dependent increases of intracellular calcium in cardiac fibroblasts. Asano et al. recently reported the identification of both AT_1 and AT_2 receptor subtypes at low density (<8 fmol per mg protein) in normal human myocardium [39]. However, these studies were performed in tissue homogenates in which individual cell types were not distinguishable. Moreover, LeFroy et al. were unable to find any significant effect of Ang II on the contraction characteristics of myocytes isolated from rat, guinea pig, or human hearts using video microscopy with a computerized edge detection system, whereas responses to isoproterenol and increased extracellular calcium concentrations were preserved [24]. These studies suggest that cardiac myocytes express few functional Ang II receptors.

The apparent discrepancies between these findings and previous reports that attribute Ang II-mediated signal transduction to receptors located on myocytes may result from differences in methods of culturing neonatal rat cardiac cells. As discussed by Long [16], all such myocyte preparations are actually co-cultures of myocytes and fibroblasts. The absolute number of contaminating fibroblasts is directly related to myocyte plating density, and high-density cultures inevitably contain large numbers of fibroblasts despite the light microscopic appearance of a confluent population of contracting myocytes. One consequence of non-homogeneous cardiac cell preparations is that biochemical analyses may be affected either by cross-contamination of cell types or by interactions between myocytes and fibroblasts, particularly in high density cultures [16]. To minimize such confounding factors, we routinely plate our myocyte cultures to achieve a final density of 150–200 cells/mm^2. However, densities reported by others typically range from 1000–1500 cells/mm^2 [10,14], values that also signify large absolute numbers of fibroblasts.

Our co-culture experiments, in which additional fibroblasts were deliberately added to standard sparse myocyte cultures, confirmed the paracrine influence of fibroblasts in Ang II-stimulated hypertrophy initially observed in conditioned medium experiments. These results suggest that both TGFβ_1 and endothelin-1 are released by fibroblasts in response to Ang II and thereby promote cardiac myocyte growth. Kim et al. were unable to demonstrate that TGF-β_1 mediated Ang II-induced hypertrophy of neonatal cardiac myocytes [25], despite reports from the same laboratory that Ang II stimulates production of TGF-β_1 in adult rat cardiac fibroblasts [21]. Orita et al. performed similar co-culture experiments using neonatal myocytes and fibroblasts without identifying the paracrine factors involved [50]. Recently, Ponicke et al. prepared cultures of neonatal rat cardiac myocytes virtually free of contaminating fibroblasts by the continuous use of cytosine-β-D-arabinofuranoside and found that Ang II failed to increase [^{3}H]phenylalanine incorporation. Addition of fibroblasts to myocyte cultures restored the ability of Ang II to induce myocyte protein synthesis [42]. They also reported that a major paracrine factor for Ang II-stimulated myocyte hypertrophy was endothelin-1 released from cardiac fibroblasts. Our data using a co-culture system are consistent with their results. The minimum percentage of fibroblasts necessary to produce a paracrine effect in co-cultures of fibroblasts and myocytes has not been determined. However, as noted by Long in quiescent cardiac myocyte cultures containing 10% non-muscle cells [16], myocyte size progressively increased in proportion to overall cell density and to the absolute number of non-muscle cells present in the preparation.

Our experiments and many of those reported by others were carried out in quiescent, serum-deprived cardiac myocytes and fibroblasts maintained in culture. However, in adult rats after myocardial infarction Lefroy et al. identified regional increases in Ang II receptor density in the infarcted tissue that co-localized with fibroblast infiltration and collagen deposition [27]. Fareh et al. showed that application of supraphysiological concentrations of Ang II induced minimal calcium transients in isolated, control cardiac myocytes and 10-fold larger responses in cardiac fibroblasts [49]. However, under conditions such as volume overload leading to myocyte hypertrophy, both Ang II receptor density on cardiac myocytes and calcium responses were increased. Data such as these suggest factors that regulate Ang II-mediated signal transduction as well as pathological settings in which cardiac myocytes may express functional Ang II receptors.

Although we have not directly studied stretch-induced myocyte hypertrophy, in our cell culture preparation cardiac myocytes beat in the presence of serum or β-adrenergic stimulation [45]. Under the serum-free conditions described in this study, Ang II has little effect on the force or rate of cardiac myocyte contraction. Therefore, the paracrine mechanisms we propose for Ang II stimulation of myocyte protein synthesis are unlikely to be responsible for stretch-induced myocyte enlargement in this model. Prolonged exposure of cardiac cells to Ang II may lead to alterations in the number or function of other receptors (receptor crosstalk). For example, we have reported that 24 to 48 h incubation of myocytes with Ang II downregulates the cardiac myocyte α_{1A}-adrenoceptor and its mRNA but does not influence α_{1B}- or α_{1C}-AR subtypes [51]. Whether this effect is mediated by activation of a very small number of AT_1 receptors on cardiac myocytes or by paracrine mechanisms has not been established.

In summary, we have shown that extravascular Ang II receptors in heart reside largely on cardiac fibroblasts. Following activation of AT_1 receptors by Ang II these cells release at least two paracrine effectors, endothelin-1 and TGF-β_1, that cause cardiac myocyte hypertrophy. Much of the published biochemical data regarding Ang II

signal transduction in cardiac myocytes may be more applicable to cardiac fibroblasts, since it is likely that large numbers of these cells were studied in what were effectively co-cultures of the two cell types. In future studies, it will be important to identify cardiac myocyte plating densities carefully and to use sparse cultures to minimize the number of contaminating fibroblasts. Alternatively, parallel experiments in myocytes and fibroblasts or deliberate co-culture studies may be used to resolve these issues.

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