Characterization of Angiotensin II Receptors in Cultured Adult Rat Cardiac Fibroblasts

Coupling to Signaling Systems and Gene Expression

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Abstract

Cardiac hypertrophy is largely due to cardiac fibroblast growth and increased synthesis of extracellular matrix. This study has investigated the contribution of the vasoactive hormone, angiotensin II, toward this hypertrophic process. We have demonstrated that cultures of adult rat cardiac fibroblasts express AT_1 but not AT_2 receptors for angiotensin II. The ability of angiotensin II to stimulate phosphoinositide catabolism and to elevate intracellular calcium concentrations in these cells was blocked by losartan, a specific AT_1 receptor antagonist, but not by the AT_2 receptor antagonist CGP 42112. Exposure of adult cardiac fibroblasts to angiotensin II resulted in the induction of several growth-related metabolic events including c-fos protooncogene expression and increased synthesis of DNA, RNA, and protein. Angiotensin II was also found to induce collagen type I, α1 chain transcript expression in cardiac fibroblasts as well as the synthesis and secretion of collagen by these cells. The data demonstrate that angiotensin II, via AT_1 receptors, can stimulate cardiac fibroblast growth and increase collagen synthesis in cardiac tissue. Thus, angiotensin II may contribute toward the development of cardiac hypertrophy in conditions of hypertension that are associated with elevated concentrations of angiotensin II. (*J. Clin. Invest.* 1994. 93:2372-2378.) Key words: AT_1 receptors • collagen synthesis • cardiac hypertrophy • growth

Introduction

Angiotensin II (Ang II),[1] the effector peptide of the renin-angiotensin system, plays an important role in controlling electrolyte balance and blood pressure. Evidence for the existence of a local renin-angiotensin system in the heart includes the intracardiac detection of transcripts for angiotensinogen, renin (1), and Ang I converting enzyme (2), as well as the detection of Ang I, Ang II, and Ang II receptors in neonatal rat cardiac myocytes and fibroblasts (3). Evidence is also accumulating to suggest that Ang II may act as a growth factor for the heart. Ang

II has been shown to elicit a hypertrophic response in neonatal rat cardiac myocytes (4) and a mitogenic response in neonatal rat cardiac fibroblasts (4, 5). In both these cell types, Ang II promotes a rapid induction of many immediate–early genes (c-fos, c-jun, jun B, Egr1, and c-myc) (4). In neonatal rat cardiac myocytes, phospholipase C and protein kinase C appear to be essential second messenger systems for Ang II–induced c-fos expression, whereas Ca^{2+} may play a more permissive role (6). Two distinct Ang II receptor subtypes (AT_1 and AT_2) have been characterized in various tissues (7, for review see reference 8). The growth-related cellular actions of Ang II are mediated by the AT_1 receptor subtype. Binding sites for Ang II have been demonstrated in cardiac tissue and in isolated cardiac myocytes (for review see reference 9), and more recently AT_1 and AT_2 receptor subtypes have been characterized in rabbit or rat ventricular myocardium (10–12). Ang II receptor subtype expression is developmentally regulated, and upregulation of AT_1 receptors occurs in response to hypertrophic changes (11).

The myocardium is composed of cardiac myocytes surrounded by cardiac interstitium and diverse nonmyocyte cells which include fibroblasts, macrophages, vascular smooth muscle, and endothelial cells of blood and lymph-carrying vessels. Nonmyocytes comprise two thirds of the cell population within the myocardium and they are specifically and separately controlled (13). The cardiac interstitium constitutes an elastin network and a complex of fibrillar collagen which is produced by fibroblasts (14, 15). Fibrillar collagen is essential for the alignment of cardiac myocytes during the cardiac cycle and for the transmission of myocyte-generated force to the ventricular chambers, as well as for myofibrillogenesis (16–20). In arterial hypertension, the diastolic properties of the left ventricle are altered before systolic functions, and this alteration is due to both an enhanced wall thickness and a reduced compliance arising from elevated chamber stiffness. Collagen is one of the major determinants of tissue stiffness in normoxic conditions and in the presence of normal pericardium (for review see reference 21). Type I and III collagen gene expression is increased during the early phase of hypertrophy (22), and increases in expression of these genes occur within days of unilateral renal ischemia (23) and are subsequent to elevations of plasma Ang II (24). In chronic pressure overload that is dependent on hormonal control, elevated left ventricular collagen concentrations are associated with myocardial stiffness and hypertrophy (13). In contrast, in aortic regurgitation or intrarenal aortic stenosis, conditions in which Ang II and aldosterone levels are normal, cardiac hypertrophy is not accompanied by elevated collagen concentrations (25, 26). It has been reported that angiotensin converting enzyme inhibitor prevents DNA synthesis in interstitial cells after myocardial infarction and myocardial fibrosis (13, 27). This beneficial effect of angiotensin convert-

1. *Abbreviations used in this paper:* Ang II, angiotensin II; AT, Ang II receptor subtype; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

ing enzyme inhibitors indicates that Ang II may play a major role in the structural remodeling of the cardiac interstitium.

Mitogenic effects of Ang II have been demonstrated in neonatal rat cardiac fibroblasts (4, 5). The response of adult rat cardiac fibroblasts to Ang II has not been studied. This study was therefore undertaken to demonstrate the presence of Ang II receptors in cultured adult rat cardiac fibroblasts, to characterize Ang II receptor subtypes in these cells and their signaling responses, and to assess whether Ang II regulates the production of collagen.

Methods

Preparation and culture of adult rat cardiac fibroblasts. Cardiac fibroblasts were prepared as described by Eghbali et al. (28). Briefly, adult male Sprague-Dawley rats (250 g) were anesthetized (50 mg/kg Nembutal, intraperitoneally), and hearts were excised, minced, and washed in PBS (Gibco AG, Basel, Switzerland) supplemented with antibiotics (penicillin/streptomycin, 100 U/ml/100 mg/ml) (Gibco AG). The tissue was incubated at 35°C in the presence of 0.1% trypsin (Sigma Immunochemicals, St. Louis, MO) and 0.1% collagenase (type CLS; Worthington Biochemical Corp., Freehold, NJ) for 20 min, and disaggregated cells were pelleted by centrifugation at the end of each of several incubation periods. Pelleted cells were resuspended in MEM with Earle's salt (Gibco AG) supplemented with 10% FCS (Gibco AG) and antibiotics (penicillin/streptomycin, 100 U/ml/100 mg/ml) (Gibco AG). Cells were seeded onto a 25-cm^2 flask and incubated in a humidified 5% CO_2/95% air atmosphere at 37°C, and after 2 h unattached cells were discarded, and attached cells (nonmuscle cells, mostly fibroblasts) were cultured further in MEM containing 10% FCS for 2 d. Cells were then rinsed in PBS and harvested by enzymatic disaggregation (in 0.25% trypsin; Gibco AG), centrifugation, and resuspension in MEM containing 10% FCS at a concentration of 10^5 cells/ml. Cells were seeded onto 24- or 6-well culture dishes (Nucon; Gibco AG), and medium changes were made every 3 d. Cell numbers were routinely determined after enzymatic disaggregation (0.25% trypsin) using a Coulter counter (model M4; Instrumenten-Gesellschaft, Basel, Switzerland).

Immunofluorescence cell staining. Cultures were phenotypically characterized by indirect fluorescent immunocytochemistry. Porcine aortic smooth muscle cells and endothelial cells (both kindly provided by Dr. Lüscher, Department of Research, University Hospital, Basel, Switzerland) and neonatal rat cardiac myocytes (29) were used as positive controls for the primary antibodies. The following primary antibodies were tested: a polyclonal antibody against von Willebrand factor (DAKOPATTS, Copenhagen, Denmark) for the detection of endothelial cells, a monoclonal antibody against sarcomeric tropomyosin (Sigma Immunochemicals) for cardiomyocytes, a monoclonal antibody against smooth muscle myosin heavy chain (Sigma Immunochemicals) for vascular smooth muscle cells, and a monoclonal antibody against vimentin (Sigma Immunochemicals). Cells were grown in Lab-Tek tissue culture chamber/slides (Miles Scientific Div., Naperville, IL) containing eight chambers (0.9 × 0.9 cm) and fixed in 4% paraformaldehyde. Cells were permeabilized with 0.5% (wt/vol) Triton X-100 for 10 min. To block nonspecific protein binding, cells were incubated in PBS containing 0.5% (wt/vol) BSA for 20 min. Cells were incubated for 30 min with the first antibody and then for a further 30 min with either Cy3-coupled anti-rabbit IgG or Cy3-coupled anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Slides were washed and incubated for 5 min with Hoechst 33258 reagent (0.5 μg/ml) (Sigma Immunochemicals) for the staining of the nuclei. After washing, preparations were mounted with Fluorsave reagent (Calbiochem-Novabiochem, Lucerne, Switzerland) and observed on a microscope equipped with epilumination and specific filters (560 nm) (Axiophot; Carl Zeiss, Inc., Oberkochen, Germany).

*Determination of Ang II receptors.* Cells were grown (on 6-well dishes) to confluence and then cultured for a further 24 h under serum-free conditions (serum replaced with 0.1% [wt/vol] of BSA). After removal of culture medium, cells were washed twice with 1 ml PBS and then incubated at 25°C in binding medium containing 0.1 nM ^{125}I-Ang II (2,200 Ci/mmol) (ANAWA Laboratories AG, Wangen, Switzerland) and with or without inclusion of different concentrations of unlabeled Ang II or Ang II receptor antagonists (losartan and CGP 42112, specific antagonists for AT$_1$ and AT$_2$ receptor subtypes, respectively; kindly provided by Dr. de Gasparo, Ciba-Geigy AG, Basel, Switzerland). To determine nonspecific binding, parallel wells included 10^{-6} M unlabeled Ang II. The binding medium contained 50 mM Tris, 120 mM NaCl, 4 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 2 mg/ml glucose, 10 mg/ml bacitracin, and 0.25% BSA at pH 7.5, and total assay volume was 0.5 ml. After 90 min at 25°C (equilibrium binding achieved), the supernatant was aspirated, and the cells were washed twice with binding medium. Then, the cells were solubilized in 0.3 M NaOH/1% (wt/vol) SDS. Radioactivity in cell lysates was determined by a gamma counter. Nonspecific binding was < 0.2% of the total radioactivity bound. Binding parameters were determined from competition binding experiments using the iterative nonlinear curve fitting program described by Munson and Rodbard (30).

Measurement of phosphoinositide hydrolysis. Cells (in 24-well dishes) were grown to confluence and prelabeled for 48 h with [^{3}H]-myo-inositol (2 μCi/ml) (ANAWA Laboratories AG) in inositol-free MEM containing 0.1% (wt/vol) BSA. Radiolabeled medium was removed, and cells were washed with 0.5 ml of MEM containing 0.1% (wt/vol) BSA, 10 mM Hepes, pH 7.3, and 15 mM LiCl. A further 0.5 ml of this solution was added to each well, and cells were preincubated for 30 min at room temperature and then for 10 min at 37°C. Thereafter, cells were exposed for 10 min to different concentrations of Ang II. When used, Ang II antagonists were added to cells during the 10-min preincubation at 37°C. Incubations were terminated by addition of 100 μl of 20% TCA, and dishes were maintained for 30 min at room temperature. After the addition of 2 ml of distilled water to each well, samples were applied to columns containing 1 ml anion exchange resin (Dowex AG 1-X8, formate form; Bio-Rad Laboratories, Richmond, CA). Columns were rinsed with water (2 × 6 ml) and 60 mM ammonium formate (1 × 6 ml), and then total inositol phosphates were eluted with 0.1 M formic acid/1 M ammonium formate (1 × 6 ml) and quantitated by liquid scintillation spectrometry as described previously (31, 32).

Measurement of intracellular free calcium. Cells were grown on acid-washed, sterile, 22-mm-diameter glass cover slips (placed in 35-mm culture dishes) in the presence of MEM supplemented with 10% FCS and antibiotics. After removal of culture medium and replacement with physiological salt solution containing 135 mM NaCl, 5 mM KCl, 1.5 mM MgCl$_2$, 2 mM CaCl$_2$, 10 mM Hepes, and 5 mM glucose (pH 7.4), the cells were loaded with 2 μM of fura-2/AM (Molecular Probes, Inc., Eugene, OR) for 20 min at 37°C. Then, the cover slips were washed and mounted in a limited volume chamber on the thermostatic stage (37°C) of an inverted epifluorescence microscope (Diaphot; Nikon, Küsnacht, Switzerland), part of a PhoCal single cell fluorescence analyzer (Joyce Loebl, Gateshead, UK). Substances in prewarmed physiological salt solution were added directly to the chamber. The cells were illuminated with alternating 340 and 380 nm light from a rotating filter wheel (6.25 Hz). Emission was monitored at 510 nm from a single cell within a group of cells, and data were analyzed using the PhoCal software. Calibrations were performed by treating the cells at the end of experiments with 10 μM ionomycin (Calbiochem-Novabiochem) to obtain F_{max}, followed by the addition of 10 μM MnCl$_2$ to obtain F_{min} (33).

Determination of DNA, RNA, and protein synthesis. Cells were grown to subconfluence in 24-well dishes (Nucon; Gibco AG) and then rendered quiescent by serum deprivation for 24 h. Cells were then cultured for a further 24 h in serum-free medium supplemented with either 1 μCi/ml of [^{3}H]thymidine (70 Ci/mmol) (Amersham Corp., Zürich, Switzerland) for DNA synthesis or 1 μCi/ml of [^{3}H]uridine (30 Ci/mmol) (Amersham Corp.) for RNA synthesis, and in the presence or absence of different concentrations of Ang II. Ang II receptor

antagonists were added 20 min before the addition of Ang II. For measurements of protein synthesis, cells were cultured in the presence of Ang II for 48 h (fresh Ang II was added after the first 24 h) and with inclusion of 1 μCi/ml of [^{3}H]leucine (120 Ci/mmol) (Amersham Corp.) for the last 24 h. At the end of the labeling periods (for DNA, RNA, or protein synthesis), radiolabeled medium was aspirated, and the cells were washed twice with PBS and then incubated (2 × 30 min at 4°C) with 10% perchloric acid. Cell precipitates were solubilized in
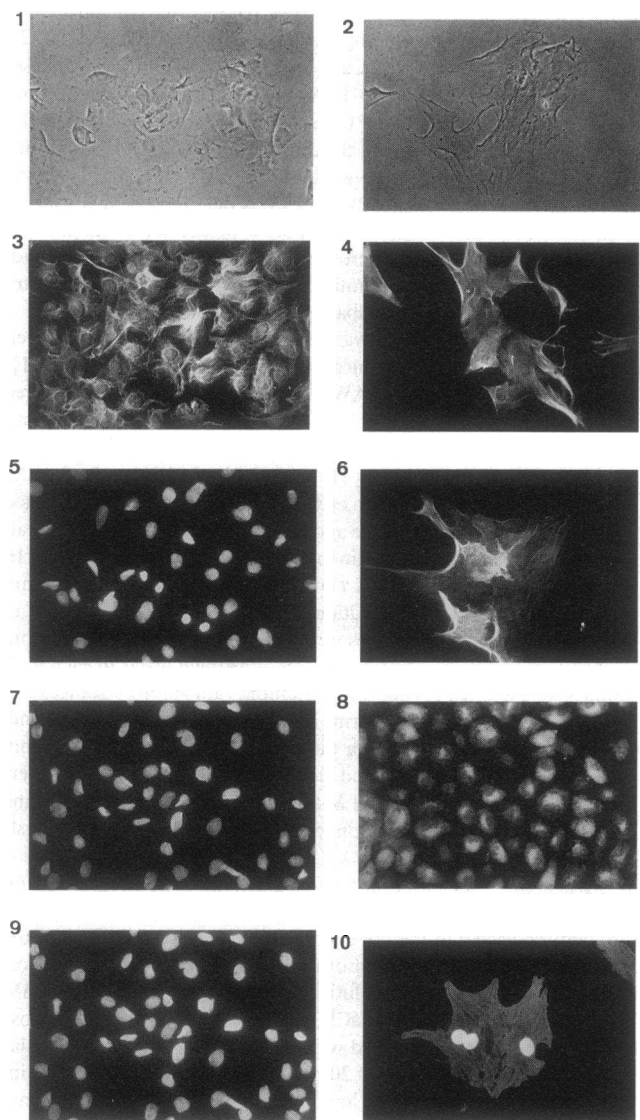

Figure 1. Immunofluorescence characterization of cell cultures. Adult rat cardiac fibroblasts, porcine aortic smooth muscle cells, porcine aortic endothelial cells, and neonatal rat cardiomyocytes were grown in Lab-Tek tissue culture chamber/slides and stained with different antibodies. The nuclei were stained with Hoechst 33258 reagent. All experimental details are given in Methods. (*1* and *2*) Phase-contrast photomicrographs of adult rat cardiac fibroblasts (*1*) and porcine aortic smooth muscle cells (*2*) (×150). (*3–10*) Immunofluorescence photomicrographs of cells (×150). Staining of adult rat cardiac fibroblasts at the first passage with antivimentin antibody (*3*), anti–smooth muscle myosin heavy chain antibody (*5*), anti–von Willebrand factor antibody (*7*), anti–sarcomeric tropomyosin antibody (*9*). Parallel staining of porcine aortic smooth muscle cells with antivimentin antibody (*4*), anti–smooth muscle myosin heavy chain antibody (*6*) was performed. In addition porcine aortic endothelial cells and neonatal rat cardiomyocytes were used as positive controls for von Willebrand factor (*8*) and sarcomeric tropomyosin (*10*), respectively.

0.3 N NaOH/1% SDS for 2 h. Radioactivity was determined by liquid scintillation spectrometry.

Quantitation of collagenous protein synthesis. Fibroblasts were grown to confluence in 100-mm (diameter) petri dishes and rendered quiescent by culture for 24 h in serum-free medium. Then, cells were incubated for 24 h in 10 ml of serum-free medium supplemented with 5 μCi/ml of [^{3}H]proline (90 Ci/mmol) (Amersham Corp.), 25 μg/ml ascorbic acid, and without or with inclusion of 10^{-7} M Ang II. Two 4-ml aliquots of supernatant were taken to which 3 mM $CaCl_2$, 1 mM PMSF, 5 mM N-ethylmaleimide, and 100 U/ml collagenase (type VII; Sigma Immunochemicals) were added to one aliquot and enzyme vehicle (PBS) was added to the second. The samples were incubated for 4 h at 37°C, and then proteins were precipitated with 10% TCA for 30 min at 4°C. After centrifugation, protein pellets were washed with 10% TCA and solubilized in 0.3 N NaOH/1% SDS. Radioactivity was quantitated by liquid scintillation spectrometry. Radioactivity in protein pellets from PBS- and collagenase-treated supernatants represents total and noncollagenous protein, respectively (34, 35).

RNA isolation and Northern blots. The expression of transcripts for c-*fos* and collagen type I, α1 chain was determined using Northern blot analysis. Confluent cultures were serum-deprived for 24 h before stimulation for either 30 min (for c-*fos* mRNA) or 24 h (for collagen type I, α1 chain mRNA). Total RNA was extracted as described by Chomczynski and Sacchi (36). 20 μg of total RNA was fractionated by electrophoresis in 1.2 and 0.8% agarose gels containing 2.2 M formaldehyde for c-*fos* mRNA and collagen type I, α1 chain mRNA, respectively. Gels were blotted onto Hybond nylon membranes (Amersham Corp.) with 20 × SSC as blotting buffer, and the RNA was fixed to the membrane by ultraviolet (UV) irradiation using a UV Stratalinker
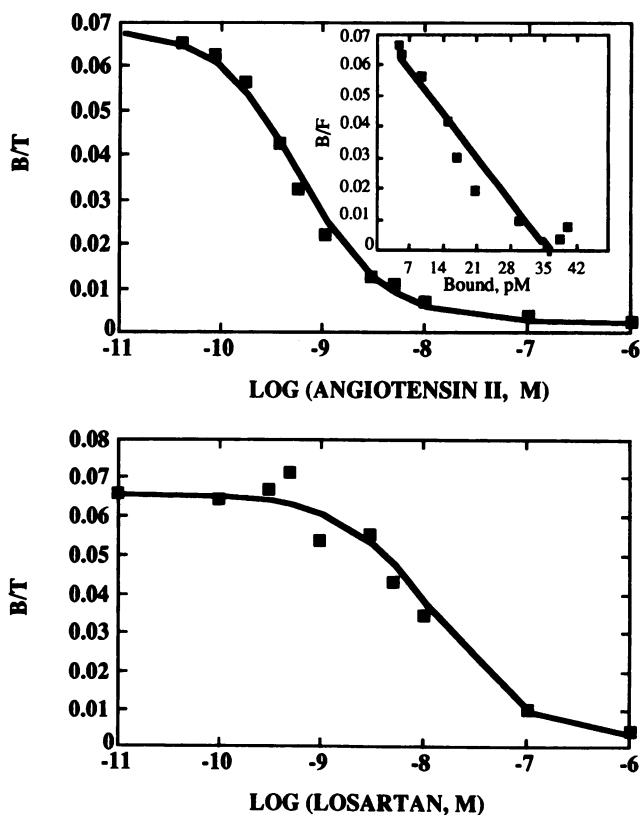
Figure 2. Competition binding of ^{125}I-Ang II. Competition of the binding of ^{125}I-Ang II by Ang II and losartan, a specific Ang II antagonist, was performed as detailed in Methods. The results are expressed as the ratio between specifically bound ^{125}I-Ang II and total ^{125}I-Ang II added; data points represent the mean of three determinations in a representative experiment of three similar experiments. The line was obtained by best-fit analysis, using the Ligand program. Scatchard analysis of the data is shown in the inset.
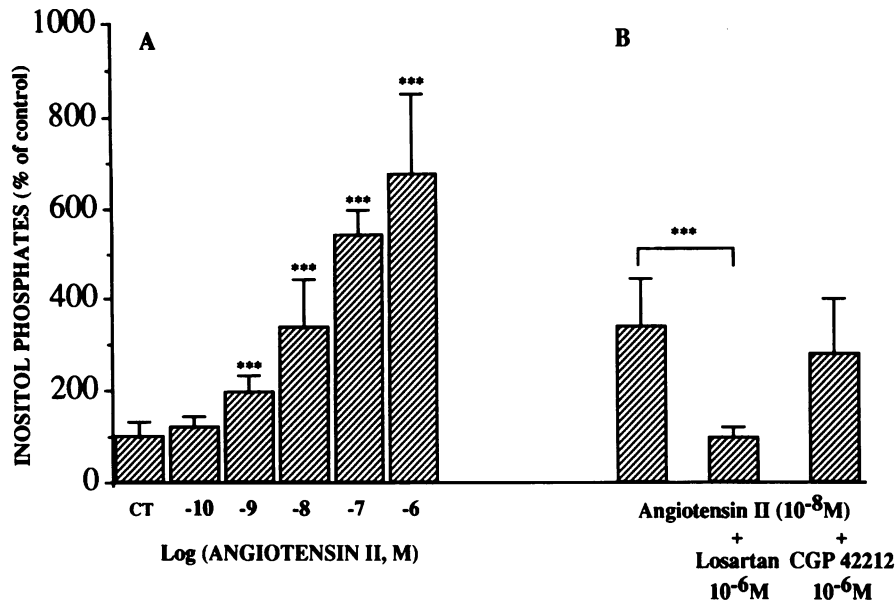
Figure 3. Inositol phosphate production. The dose-dependent accumulation of inositol phosphates in response to Ang II (*A*) and the effect of Ang II receptor subtype antagonists on 10^{-8} M Ang II–stimulated inositol phosphate production (*B*) were studied in [^{3}H]inositol-prelabeled adult rat cardiac fibroblasts as detailed in Methods. Increases in [^{3}H]inositol phosphate content after stimulation with Ang II are expressed as percentages of the mean of that content (arbitrarily taken as 100%) in control samples. Values represent mean±SD of nine determinations in three different cell preparations. Statistical analysis was performed using a nonparametric Mann Whitney U-test. ***$P < 0.0001$ vs control.

(Stratagene GmbH, Heidelberg, Germany). Probes used in this study were a 40-mer oligonucleotide for c-*fos* (British Biotechnology, Cowley, UK), a 1.8-kb cDNA for collagen type I, α1 chain (ATCC 61322; American Type Culture Collection, Rockville, MD), and a 1.2-kb cDNA for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ATCC 57090), which was used as the internal reference. cDNAs were random primed as described previously (37). For c-*fos*, blots were prehybridized at 45°C for 60 min in 50% formamide, 1 M NaCl, 0.1% SDS, 10% dextran sulphate, 7.5% polyethyleneglycol 6000, and 1 mg/ml denatured salmon sperm DNA; blots were then hybridized overnight at 45°C with 40 ng of 3'-end-labeled ([α^{32}P]ATP) c-*fos* oligonucleotide (British Biotechnology). The blots were sequentially washed (each wash for 15 min) with 5 × SSC at 75°C (SSC: 3 M NaCl, 0.3 M Na$_3$ citrate, pH 7.0), 2.5 × SSC at 75°C, and 1 × SSC at room temperature. All blots were rehybridized with GAPDH probes as described previously (37). Blots were exposed to Kodak X-Omat films overnight at −70°C with the use of one intensifying screen. Hybridization signals were densitometrically analyzed using an integrated camera system and the Screen Machine program from Macintosh.

Data analysis. Data are given as mean±SD. Statistical analysis was performed using a nonparametric Mann Whitney U-test. The threshold of significance was taken as $P < 0.05$.

## Results

Characterization of fibroblasts. The differential plastic attachment procedure was selective for fibroblasts. After 2 d in cul-

ture, the cells morphologically resembled spindle-shaped fibroblasts and did not form the cell islands characteristic of endothelial cells. Fig. 1 presents immunocytochemical characteristics of the different cell cultures. In our cell preparations (at first passage) all the cells stained positive for vimentin but were negative for smooth muscle myosin heavy chain (vs positive porcine aortic smooth muscle cells), von Willebrand factor (vs positive porcine aortic endothelial cells), and sarcomeric tropomyosin (vs positive neonatal rat cardiomyocytes).

Determination of Ang II receptors. The competition binding of ^{125}I-Ang II by unlabeled Ang II (Fig. 2) indicates that cardiac fibroblasts express Ang II receptors with a K_d of 4.2±2.6 × 10^{-10} M and a maximum binding capacity of 31.4±11.4 × 10^3 sites/cell (mean±SD, $n = 3$ experiments). Losartan, a specific AT$_1$ receptor antagonist, completely displaced ^{125}I-Ang II, and the IC$_{50}$ was calculated to be 7.9±2.2 × 10^{-9} M (mean±SD, $n = 3$ experiments). The AT$_2$ receptor antagonist, CGP 42112, did not measureably displace bound ^{125}I-Ang II (data not shown). Binding of ^{125}I-Ang II could not be detected after the third passage of adult cardiac fibroblasts (data not shown), and therefore we have used only first passage cultures in all subsequent experiments.

Phosphoinositide catabolism and intracellular calcium mobilization. Ang II dose-dependently increased total inositol phosphate production in cardiac fibroblasts (Fig. 3). The inosi-
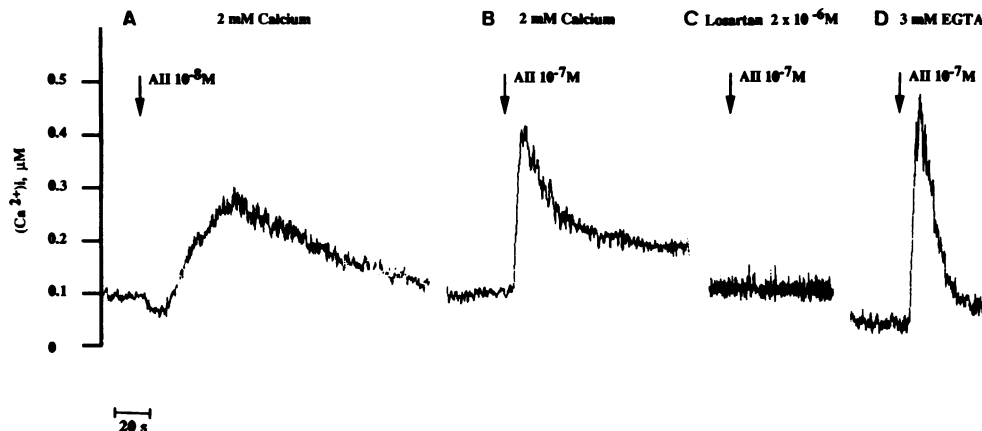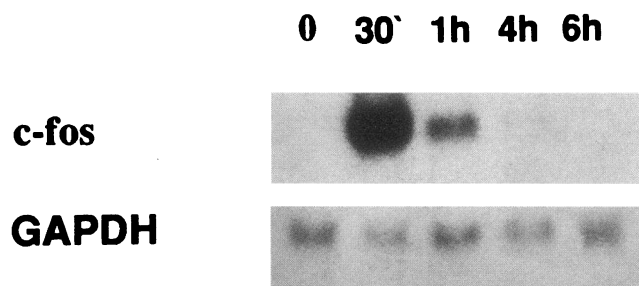

Figure 4. Calcium transient in Ang II–stimulated fibroblasts. Free intracellular calcium concentration was measured after incubation with 10^{-8} M Ang II (*AII*) (*A*), 10^{-7} M AII (*B*), with 10^{-7} M AII after preincubation for 10 min with 2 × 10^{-6} M losartan (*C*), or with 10^{-7} M AII in a calcium-free extracellular medium (calcium was chelated with 3 mM EGTA) (*D*). Experimental details are given in Methods.

tol phosphate accumulation response to 10^{-8} M Ang II ($336\pm106\%$ over control, mean$\pm$SD, $n = 9$) was inhibited completely by 10^{-6} M losartan ($98.5\pm24\%$ over control value, mean$\pm$SD, $n = 9$), but was not affected significantly by 10^{-6} M CGP 42112 ($279\pm118\%$ over control, mean$\pm$SD, $n = 9$). Ang II stimulated an increase in intracellular free calcium concentrations in adult cardiac fibroblasts, the calcium transient being more rapid at higher concentrations of Ang II (Fig. 4). Preincubation of cells with losartan (2×10^{-6} M, 10 min) blocked the Ang II–induced calcium transient (Fig. 4). After chelation of extracellular calcium by EGTA, Ang II still elicited an increase in intracellular calcium concentrations, although the decay of calcium to resting levels was much more rapid and pronounced than in the presence of extracellular calcium (Fig. 4).

Growth-related metabolic events. Since induction of c-*fos* mRNA is one of the earliest transcriptional events associated with growth factor stimulation (38, 39), we studied the effect of Ang II on the expression of this oncogene in cardiac fibroblasts. As shown in Fig. 5, c-*fos* mRNA was expressed maximally after a 30-min stimulation with 10^{-7} M Ang II and returned to basal levels after 4 h. A constitutive expression of c-*fos* could not be detected in control cells. Ang II dose-dependently increased DNA synthesis ([^{3}H]thymidine incorporation), RNA synthesis ([^{3}H]uridine incorporation), and protein synthesis ([^{3}H]leucine incorporation) in adult cardiac fibroblasts (Fig. 6). The maximum levels of incorporation were 2.2-, 4.2-, and 2.6-fold above control for [^{3}H]thymidine, [^{3}H]-uridine, and [^{3}H]leucine, respectively. Ang II (10^{-8} M)-stimulated incorporation of each of these isotopes into adult cardiac fibroblasts was prevented by losartan (10^{-6} M) but not by CGP 42112.

Induction of collagen type I, α1 chain mRNA and synthesis of collagenous protein. We have examined the effect of Ang II on the level of transcripts for collagen type I, α1 chain. Fig. 7 presents Northern blot analysis of RNA from cardiac fibroblasts incubated without or with 10^{-7} M Ang II for 24 h. As described previously (40), two bands (5.7–7 kb), indicated as coll I-a and coll I-b, hybridize with the collagen type I, α1 chain probe used. After Ang II stimulation, transcript levels were increased by 2.1- and 5.7-fold above basal for coll I-a and coll I-b, respectively (Fig. 7). The induction of transcripts for collagen in cardiac fibroblasts was accompanied by their biosynthesis and secretion of collagenous protein. In the presence of 10^{-7} M Ang II, incorporation of collagenase-sensitive [^{3}H]proline

into protein was 4.4-fold greater than in the absence of Ang II. Incorporation of collagenase-resistant [^{3}H]proline in protein was not different between Ang II–stimulated and control cells (Table I).

Discussion

In left ventricular hypertrophy associated with some forms of hypertension, the interstitial space of the ventricle is frequently
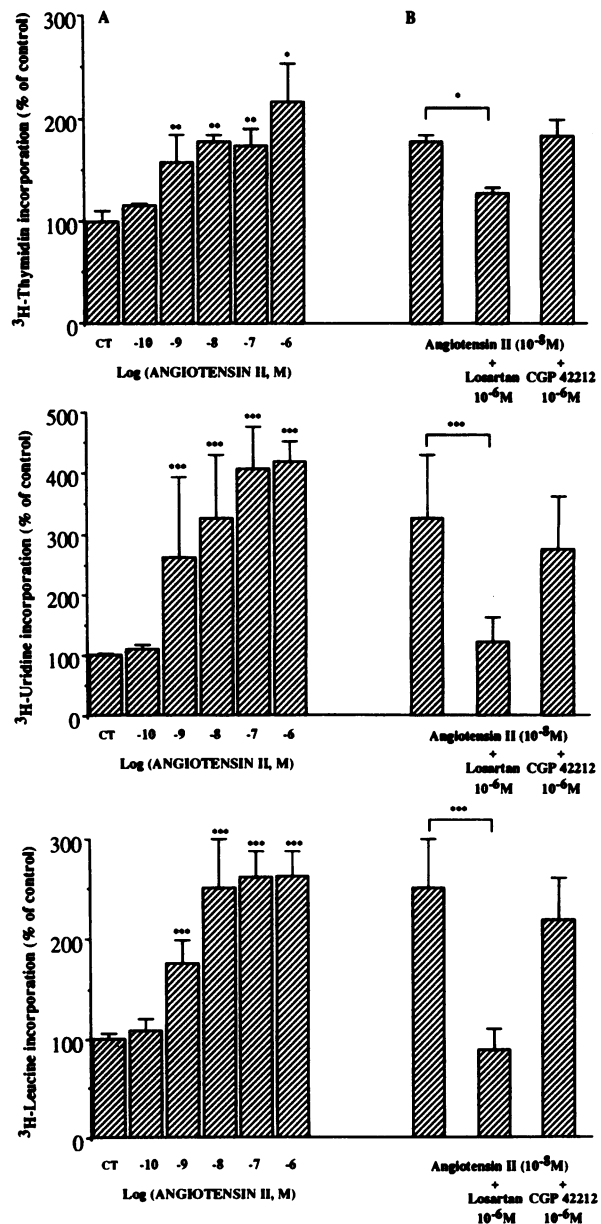
Figure 6. DNA, RNA, and protein synthesis. Incorporation of [^{3}H]-thymidine into DNA, [^{3}H]uridine into RNA, and [^{3}H]leucine into protein synthesis after stimulation of quiescent, cardiac fibroblasts with the indicated doses of Ang II (*A*). The effect of Ang II receptor subtype antagonists on 10^{-8} M Ang II–induced DNA, RNA, or protein synthesis was also measured (*B*). Experimental details are given in Methods. Increases in [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine incorporation are each expressed relative to the mean of [^{3}H]content (100%) in their respective controls. Values represent mean$\pm$SD of 9–12 determinations in three to four different cell preparations. Statistical analysis was performed using a nonparametric Mann Whitney U-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$ vs control.

0 30' 1h 4h 6h

c-fos

GAPDH

Figure 5. c-*fos* mRNA expression. Kinetics of induction of c-*fos* mRNA expression by 10^{-7} M Ang II in quiescent, confluent adult cardiac fibroblasts. c-*fos*-specific transcripts were visualized by hybridization to a specific 3'-end-labeled oligonucleotide, and the blots were rehybridized to a probe for the housekeeping gene GAPDH, to control for the total RNA loaded in each lane. Experimental protocols are described in Methods.
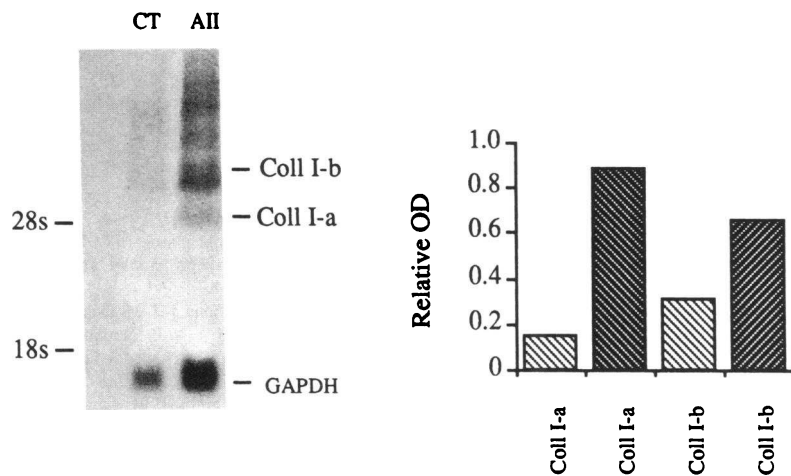
Figure 7. Induction of collagen type I, α1 chain transcripts by Ang II. Quiescent, confluent cultures of cardiac fibroblasts were exposed to Ang II (10^{-7} M) (AII). Northern blot autoradiograph (right panel) shows two transcripts (coll I-a, coll I-b) visualized by hybridization to a random-primed labeled cDNA probe. Striped white bar, control; striped black bar, Ang II. The blot was also hybridized with a probe for a housekeeping gene, GAPDH to control for total RNA loaded per lane. The left panel presents the densitometric analyses of Northern blots. Experimental details are described in Methods. The amount of these transcripts is expressed as relative arbitrary OD units normalized with respect to GAPDH.

the site of an abnormal accumulation of fibrillar collagen. This is probably a result of cardiac fibroblast growth and enhanced collagen synthesis, and Weber and Brilla (13) have proposed that hormones such as aldosterone and Ang II may be involved in this hypertrophic process. To test this hypothesis we have investigated whether adult cardiac fibroblasts express Ang II receptors and whether such receptors are functionally coupled to growth-related cellular processes. Cultures of cardiac fibroblasts from adult rat heart were prepared, and their phenotypic characterization as fibroblasts was confirmed by various criteria including morphology and immunological staining. Indirect fluorescent immunocytochemistry did not reveal a detectable contamination of our first passage cultures with either vascular smooth muscle cells, endothelial cells, or cardiomyocytes.

We have demonstrated that adult rat cardiac fibroblasts express AT_1 receptors, and the affinity of these receptors (K_d of $\approx 4 \times 10^{-10}$ M) is similar to that reported for AT_1 receptors on vascular muscle cells (7), skin fibroblasts (41), neonatal rat cardiac fibroblasts, and cardiomyocytes (5, 42). Skin fibroblasts have been reported to possess both AT_1 and AT_2 receptor subtypes, but the latter were found to decrease during re-

peated passage (41). We have found that repeated passaging of adult cardiac fibroblasts resulted in a complete loss of AT_1 receptor expression. Both AT_1 and AT_2 receptor subtypes have been identified in rabbit or rat ventricular myocardial tissues (10–12). Thus, the lack of AT_2 receptors in isolated adult cardiac fibroblasts (this study) or neonatal rat cardiac fibroblasts (4, 5) may reflect a rapid loss of AT_2 receptors during culture.

We have shown that Ang II induced inositol phospholipid catabolism and an elevation of intracellular free calcium concentration in adult rat cardiac fibroblasts. These effects were inhibited by a specific AT_1 antagonist (losartan), but not by a specific AT_2 competitor (CGP 42112). The observation that Ang II stimulated a rapid calcium transient even in the absence of extracellular calcium suggests that the peptide mobilizes calcium from intracellular calcium pools via inositol triphosphate. Therefore, in keeping with the signaling characteristics of the AT_1 receptor in other cell types (4–8, 10, 41–44), AT_1 receptors in adult rat cardiac fibroblasts are coupled to the activation of phospholipase C.

The induction of c-fos mRNA is one of the earliest transcriptional events associated with growth factor stimulation and has been associated with cell proliferation, cellular differentiation, development, and hypertrophy (38, 39). In addition, c-fos protein is a member of a protein complex (AP-1) that binds specifically to transcriptional control elements, and c-fos has been invoked as a transcriptional regulatory factor (45, 46). As has been described for vascular smooth muscle cells (47, 48) and neonatal rat cardiac myocytes and fibroblasts (4), we found that Ang II induced c-fos expression in adult rat cardiac fibroblasts within 30 min. Furthermore, we have demonstrated that Ang II induces DNA, RNA, and protein synthesis in adult rat cardiac fibroblasts. The ability of Ang II to stimulate such growth-related metabolic events has also been described in neonatal rat cardiac fibroblasts (4, 5). We have also demonstrated that in cultured adult rat cardiac fibroblasts, Ang II induced an increase in collagen type I, α1 chain transcript expression and also stimulated the synthesis and secretion of new collagen. Ang II clearly possesses the potential to stimulate fibroblast growth and increase collagen synthesis in cardiac tissue.

We conclude that Ang II might play an important role in the development of cardiac hypertrophy in those forms of hypertension associated with elevated Ang II concentrations. AT_1 receptor antagonists should be therapeutically useful in reducing collagen synthesis by fibroblasts and thus cardiac hypertrophy.

Table I. [³H]Proline Incorporation into Proteins

| | [³H]Proline incorporation | | |
	Total	Collagenase-resistant	Collagenase-sensitive
		% of control	
Control	99.8±9.1	100.4±3.9	99.9±21
Ang II 10^{-7} M	166.6±32*	92.1±23.3	443.3±65.3*

Quantitation of collagenous protein synthesis. Quiescent cardiac fibroblasts were incubated for 24 h in the presence of [³H]proline (5 μCi/ml) without (control) and with inclusion of 10^{-7} M Ang II. Total and collagenase-resistant [³H]proline-labeled proteins secreted into medium were measured after precipitation of proteins from nonenzyme and collagenase-treated medium aliquots, respectively. [³H]Proline incorporation into collagenous proteins (collagenase-sensitive [³H]proline incorporation) was calculated by subtracting collagenase-resistant [³H]proline from total [³H]proline. All experimental details are described in Methods. Increases in [³H]proline incorporation are expressed relative to the mean of that [³H]proline content (100%) in controls. Values represent mean±SD of six determinations in three different cell preparations. Statistical analysis was performed using a nonparametric Mann Whitney U-test. * $P < 0.01$ vs control.

Acknowledgments

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