Angiotensin II Is Mitogenic in Neonatal Rat Cardiac Fibroblasts


Angiotensin II has been reported to be a hormonal stimulus of cardiac growth, a response that may involve myocyte hypertrophy as well as growth of nonmyocytes. This study was designed to determine whether neonatal rat cardiac fibroblasts have an angiotensin II receptor that is coupled with hypertrophic and/or proliferative growth. Competitive radioligand binding studies showed that cardiac fibroblasts have a single class of high-affinity (IC₅₀, 1.0 nM) angiotensin II binding sites (Bₘₐₓ, 778 fmol/mg protein) that are sensitive to the competitive nonpeptide AT₁ receptor antagonist losartan (IC₅₀, 13 nM). Other angiotensin peptides competed for [¹²⁵I]angiotensin II binding in the following rank order: angiotensin II > angiotensin III > angiotensin I > [des-Asp¹-des-Arg⁸]angiotensin II. A nonhydrolyzable analogue of guanosine triphosphate increased the dissociation rate of bound [¹²⁵I]angiotensin II and decreased hormone binding to the receptor at equilibrium. The angiotensin II receptor was coupled with increases in intracellular calcium. Incorporation of precursors into protein, DNA, and RNA in response to angiotensin II was determined. In serum-deprived cultures, a 24-hour exposure to 1 μM [Sar³]angiotensin II increased rates of phenylalanine, thymidine, and uridine incorporation by 58%, 103%, and 118%, respectively. These increases were blocked by the noncompetitive AT₁ receptor antagonist EXP3174. After 48 hours, [Sar³]angiotensin II increased total protein and DNA of cardiac fibroblasts by 23% and 15%, respectively, with no change in the protein/DNA ratio. [Sar³]Angiotensin II increased cell number by 138% after a 24-hour exposure, without affecting cell area. In summary, cardiac fibroblasts have G protein–linked AT₁ receptors that are coupled with proliferative growth. These results suggest that angiotensin II–induced cardiac hypertrophy is, in part, secondary to stimulated increases in nonmyocyte cellular growth. (Circulation Research 1993;72:1245–1254)

KEY WORDS • angiotensin II • hormone receptors • guanine nucleotide binding protein • cardiac fibroblasts • cell growth • cardiac hypertrophy

Angiotensin (Ang) II exerts a number of direct and indirect actions on the heart.¹ Multiple indirect actions of Ang II are mediated through its influence on cardiovascular regulatory sites in the brain, modulation of sympathetic nerve activity, and stimulation of aldosterone synthesis and release from the adrenal gland. Direct cardiovascular actions of Ang II include chronotropic and inotropic effects on cardiac muscle, effects on cardiac metabolism, and vasoconstriction of blood vessels.¹ In addition, clinical studies demonstrating the efficiency of angiotensin converting enzyme (ACE) inhibitors in the treatment of heart failure,² myocardial ischemia,³,⁴ and hypertension⁵,⁶ suggest that Ang II directly promotes pathological cell growth that participates in remodeling of the failing heart. Several animal studies have implicated Ang II in cardiac hypertrophy associated with hypertension; for instance, in a rat model of “pressure-overload” cardiac hypertrophy, treatment with an ACE inhibitor prevented the increase in left ventricular mass with no effect on afterload,⁷ suggesting that the growth effects of Ang II are direct. Moreover, chronic infusion of Ang II into rats increased left ventricular mass, even when the pressor activity of Ang II was blocked or a subpressor dose of Ang II was used.⁸ Recent experiments using cultured embryonic chick cardiomyocytes lend further support to the hypothesis that Ang II can directly produce cellular hypertrophy.⁹,¹⁰

It has not been established whether Ang II also has a direct growth effect on nonmyocyte cells of the heart, although recent studies suggest that cardiac fibroblasts are a target for Ang II; for instance, treatment with the ACE inhibitor captopril prevented myocardial fibrosis in a rat model with renovascular hypertension¹¹ and with induced myocardial infarction.¹² In the latter study,
collagen accumulation and DNA synthesis were prevented by captopril treatment, indicating that Ang II may play a major role in the structural remodeling of the cardiac interstitium by exerting a proliferative effect on fibroblasts. The process of interstitial remodeling may underlie the clinical observation that left ventricular hypertrophy represents the major risk factor associated with the subsequent appearance of symptomatic heart failure. However, in vivo studies implicating Ang II in this process are complicated by the possibility that the effects of Ang II are indirect, that the actions of ACE inhibitors are due solely to a reduction in cardiac afterload, or that ACE inhibitors have additional actions unrelated to the formation of Ang II. To examine some of these shortcomings, we studied the effects of Ang II on cardiac fibroblasts in culture. Our goals were 1) to determine whether cardiac fibroblasts have a membrane receptor for Ang II and 2) to determine whether Ang II stimulates growth of cultured cardiac fibroblasts.

Materials and Methods

Tissue Culture and Media

 Cultures of ventricular fibroblasts were prepared from hearts of 0–3-day-old Sprague-Dawley rat pups. Unless otherwise noted, the preparative steps were performed at 37°C. Ventricles from 80 rats were minced and added to 80 ml CaCl₂- and Mg²⁺-free Hanks’ buffered salt solution (HBSS) in a water-jacketed tissue stirrer (Wheaton Industries, Millville, N.J.). After 20 minutes, six sequential digestions were performed using 80 ml HBSS with 2.5 units/ml bovine trypsin, 2.75 units/ml α-chymotrypsin, 0.5 units/ml elastase, and 25 mM HEPES, pH 6.4. Dissociated cells and debris from the first two digestions (16 and 18 minutes, respectively) were discarded. Supernatants from each of the remaining four digestions (20 minutes each) were added to tubes containing 10 ml plating medium (Dulbecco’s modified Eagle’s medium: medium 199 [4:1], 5% horse serum, 5% fetal calf serum, 34 μg/ml ampicillin, and 3 μg/ml gentamicin) and centrifuged at 200g for 4 minutes (22°C). Cells were resuspended in 5.0 ml plating medium and placed in a humidified incubator (95% air–5% CO₂) for 90 minutes in 25-cm² polystyrene tissue-culture flasks (Corning Glass Inc., Corning, N.Y.) to allow for the selective adhesion of fibroblasts. Newly cultured fibroblasts were maintained in plating medium for 48 hours. The medium was then switched to supplemented minimal essential medium (MEM-SS2) with 10% newborn calf serum. Minimal essential medium (MEM) was supplemented with (per liter) 68 units insulin, 10 mg transferrin, 0.55 g methylocellulose, 1 mg sodium pyruvate, 1.1 mg phosphatidylcholine, 2.2 mg cholesterol, 3.5 μg hydrocortisone, 0.29 g l-glutamine, 2.2 g sodium bicarbonate, 1% (vol/vol) MEM essential amino acid solution, 1% (vol/vol) MEM nonessential amino acid solution, 1% (vol/vol) MEM vitamin solution, and 1% (vol/vol) antibiotic-antimycotic solution (pH adjusted to 7.4). Eight days after the initial dispersion, fibroblasts were passaged using 0.25% porcine trypsin and seeded at a density of 3–8×10⁴/cm² on 35-mm plates (a 15–22-fold dilution). After 72 hours, the medium was changed to serum-free MEM-SS2, and experiments were performed 1–4 days later. At the time of experiments, cultures were subconfluent.

Immunocytochemistry

Indirect fluorescent immunocytochemistry was used to assess the extent of contamination of cardiac fibroblast cultures with vascular smooth muscle cells (VSMCs). Rat aortic smooth muscle cells, which served as a positive control for the primary antibody, were obtained and passaged as described. Cells were seeded at a density of 3×10⁴/cm² into six-well 35-mm dishes containing 22-cm glass coverslips (thickness, 1; Fisher Scientific Co., Pittsburgh, Pa.) in the presence of MEM-SS2 with 10% newborn calf serum. Immunofluorescent staining was performed 3–6 days later. The culture medium was aspirated, and cells were washed three times with phosphate-buffered saline (PBS, containing [mM] NaH₂PO₄ 12.3, KH₂PO₄ 3.17, and NaCl 123.3, pH 7.4) with 0.5 mM CaCl₂ and 100 mM glycine and fixed for 10 minutes at 22°C with 3.7% formaldehyde–244 mM sodium acetate (pH 7.5). Cells were washed three times with PBS containing CaCl₂ and glycine, permeabilized with 0.05% Triton-X (in PBS with CaCl₂) for 5 minutes, and incubated with blotto (50 mM Tris, 100 mM NaCl, and 5% nonfat dry milk, pH 7.4) for 30 minutes. Cells were incubated for 30 minutes at 37°C with either a monoclonal antibody (1:400 dilution of mouse ascites fluid) against smooth muscle myosin heavy chain (MHC), a monoclonal antibody against desmin (diluted 1:40), or a polyclonal antibody against factor VIII (diluted 1:400). Controls of the MHC antibody were performed using a dilution of ascites fluid in which primary antibody was removed by immunoprecipitation with goat anti-mouse antibody and protein A–agarose beads as previously described. After three washes with blotto, the cells were incubated for 30 minutes at 37°C in the dark with goat anti-mouse immunoglobulin (lg) G fluorescein antisera (diluted 1:160) or goat anti-rabbit IgG fluorescein antisera (diluted 1:64). Coverslips were washed three times with blotto, rinsed with water, and placed cell-side down in mounting medium (90% glycerol and 9.9% PBS) with 0.1% p-phenylenediamine added to reduce photobleaching. Cells were viewed using an Axiovert 35 microscope (Zeiss, Oberkochen, FRG) equipped with phase-contrast and epifluorescent optics. Photobleaching was minimized by taking photographs with ASA 400 Kodak Tmax film under ASA 1,600 conditions (4–16 seconds).

Ang II Binding

Cells on 35-mm plates were washed three times with 1 ml binding buffer, which contained (mM) Tris 50 (pH 7.5), NaCl 120, KCl 4, CaCl₂ 1, MgCl₂ 1, and glucose 11, with 0.01 mg/ml bacitracin and 0.25% crystalline bovine serum albumin. Binding studies were initiated by adding [¹²⁵I]Ang II at a final concentration of 0.02 nM (approximately 0.014 μCi/ml) to each plate. Incubations were performed at 22°C for 60 minutes (unless otherwise noted) and terminated by aspirating the binding buffer and washing the cells three times with ice-cold binding buffer. Bound radioactivity was removed by adding 1.0 ml of 0.25 M NaOH–0.5% sodium dodecyl sulfate (SDS) for 10 minutes and washing with an additional 0.5
ml NaOH-SDS. Radioactivity was measured with an LKB Compugamma (model 1282, Pharmacia LKB Biotechnology Inc., Piscataway, N.J.). Counts were corrected for background radioactivity, and specific binding was determined by subtracting the radioactivity bound in the presence of 10^{-7} M unlabeled Ang II.

To evaluate the effect of guanylylimidodiphosphate (GMP-PNP) on the dissociation of bound [^{125}I]Ang II, membranes were prepared as follows: Cells were scraped at 4°C into 50 mM Tris (pH 7.6) containing 1 mM EDTA and 100 μg/ml phenylmethylsulfonyl fluoride and homogenized with a glass Dounce tissue grinder (Wheaton) with a "B" pestle (clearance, 0.0063–0.0089 cm). The homogenate was centrifuged at 48,000g for 30 minutes (4°C). The pellet was washed once and resuspended in binding buffer. Binding assays were performed as previously described using 0.1 nM [^{125}I]Ang II and a final protein concentration of 500 μg/ml. Ang II binding was linear over the range of 100–1,500 μg/ml protein (data not shown).

**Measurement of Intracellular Calcium**

The fluorescent Ca^{2+} indicator dye fura-2 AM was used to monitor changes in intracellular Ca^{2+} in response to Ang II. Fibroblasts were grown to confluence in 25-cm² culture flasks in MEM-SS2 with 10% newborn calf serum. Two days before the experiments, the cells were serum-starved. Fibroblasts (3–4×10^6 cells) were placed in HBSS and incubated for 1 hour at 37°C in 10 ml HEPES-Krebs-Ringer bicarbonate solution (HKRB, containing [mM] HEPES 20, NaCl 103, KCl 4.8, CaCl₂ 0.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, and glucose 15, pH 7.4). After two washes with HKRB, cells were removed from the flasks with 6 ml of 0.25% trypsin and transferred to HKRB containing soybean trypsin inhibitor (0.6 mg/ml). Cells were centrifuged for 2 minutes at 200g, resuspended in 37°C HKRB, and transferred to 3-ml cuvettes. Fluorescence was measured with a spectrometer (model CM II, SPEX Industries Inc., Edison, N.J.) interfaced with a SPEX DM 3000 controller. Excitation wavelengths were 340 and 380 nm, and emission was monitored at 505 nm. Intracellular calcium was calculated as previously described.

**Measurement of Fibroblast Growth and Proliferation**

Total protein and DNA were assayed as parameters of cell growth and proliferation. Cells were incubated in the presence or absence of [Sar^1]Ang II, a protease-resistant analogue of Ang II. Cells adherent to plates were washed with HBSS and resuspended into 0.5 ml standard saline citrate (150 mM NaCl and 15 mM sodium citrate, pH 7.0) with 0.25% SDS. These plates were washed with an additional 0.5 ml standard saline citrate–SDS. Protein in the cell lysate and wash was assayed by the method of Lowry et al. with bovine serum albumin used as a standard. DNA was assayed fluorometrically, with calf thymus DNA used as a standard.

To determine cell area, fibroblasts were plated at a density of 3×10⁴/cm², which was sufficiently sparse to allow individual cells to be outlined and analyzed. For each experiment, five 35-mm plates were treated for 48 hours with [Sar^1]Ang II or vehicle. For each plate, a single field (1.0×1.5 mm, 30–50 cells) was selected at random (by a blinded observer) and recorded at a magnification of ×250 with an Axiovert 35 phase-contrast microscope (Zeiss) fitted with an NC-70 video camera (Dage-MTI, Inc., Michigan City, Ind.). All cells in the field were outlined, and cell area was determined by computer analysis of the digitized images using Image Master 2000 (Technology Resources Inc., Nashville, Tenn.). To determine cell number, fibroblasts were plated at a density of 4×10³/cm² in 75-mm tissue culture flasks, which was sufficiently sparse to avoid clumps of cells on harvesting. After 24 hours, cells were serum-deprived for 24 hours and then incubated in medium with or without Ang II (1 μM). Cells were washed with HBSS, harvested with 0.25% trypsin, diluted (1:200) with Isoton II (Coulter Corp., Hialeah, Fla.), and counted with a Coulter counter model ZM with an orifice size of 100 μm.

**Measurement of Incorporation of Precursor Into Protein, DNA, and RNA**

Serum-starved fibroblasts were exposed to 1 μM [Sar^1]Ang II for 24 hours. Over the final 3 hours, [¹⁴C]phenylalanine (0.25 μCi/ml), [³H]thymidine (2.5 μCi/ml), or [³²P]uridine (0.2 μCi/ml) was added to the medium. For measuring rates of amino acid incorporation, nonlabeled phenylalanine (0.3 mM) was added to the culture medium to minimize variations in the specific activity of the precursor pool. Two or three plates from three separate dispersions were used for each experimental condition (control, Ang II, antagonist, and Ang II plus antagonist). At the end of incubation, cells were washed with PBS and lysed with 0.2 ml of 0.1 M NaOH–0.1% SDS. Macromolecules in the lysates were precipitated with 0.5 ml ice-cold 10% trichloroacetic acid. Precipitates were collected on GF/C filters (Whatman Inc., Clifton, N.J.) and washed three times with 10% trichloroacetic acid and three times with 70% ethanol. Radioactivity was measured with a Beckman LS3801 liquid scintillation counter. Preliminary studies established that incorporation of the radiolabels into the acid-precipitated cellular fraction was linear for 3 hours. Because cells were not growth-arrested, variability was observed in control incorporation of radiolabels, which ranged (in counts per minute per 35-mm plate) from 578 to 761 for [¹⁴C]phenylalanine, from 10,600 to 25,000 for [³H]thymidine, and from 482 to 902 for [³²P]uridine. Results are expressed as percent mean±SEM increase over control.

**Materials**

Sera, HBSS, culture media, MEM essential amino acid solution, nonessential amino acid solution, vitamin solution, antibiotic-antimycotic solution, and porcine trypsin were obtained from Gibco, Grand Island, N.Y. Ang I, Ang II, Ang III, [Sar^1]Ang II, [Sar^1]Leu^5]Ang II, and Ang-(3-8) (i.e., [des-Asp^1]-des-Arg^9]Ang II) were purchased from either United States Biochemical Corp., Cleveland, Ohio, or Peninsula Laboratories, Belmont, Calif. [Methyl-1^-¹⁴C]thymidine (124 Ci/mmol), [²⁻¹⁴C]uridine (54.1 mCi/mmol), and [¹⁻¹⁴C]phenylalanine (479 mCi/mmol) were from Amersham Corp., Arlington Heights, Ill. [²⁻¹³¹I]Tyr^1]Ang II (2,200 Ci/mmol) was from Du Pont NEN Research Products, Boston. ATP, a-chymotrypsin (type II), soybean trypsin inhibitor, goat anti-mouse IgG fluorescein, goat anti-rabbit
IgG fluorescein, mouse monoclonal antibody against desmin, rabbit polyclonal antibody against factor VIII, and phorbol 12,13-dibutyrate (PDB) were from Sigma Chemical Co., St. Louis, Mo. Platelet-derived growth factor-BB homodimer (PDGF-BB) was purchased from Collaborative Research, Bedford, Mass. Bovine trypsin and elastase were obtained from Worthington Biochemical Corp., Freehold, N.J.; GMP-PNP was from Boehringer Mannheim Corp., Indianapolis, Ind.; and protein A–agarose was from Pierce Chemical Co., Rockford, Ill. The acetoxymethyl (AM) ester of fura-2 was obtained from Molecular Probes, Inc., Eugene, Ore. Losartan (DuP753), EXP3174, and AT1 receptor antagonist PD123177 were kindly supplied by DuPont Merck Pharmaceutical Co., Wilmington, Del. Mouse monoclonal antibody (SF5-A10-B10) against smooth muscle MHC was a generous gift of Dr. G. Owens, University of Virginia, Charlottesville. Other chemicals were of reagent grade quality.

Statistics and Analysis of Binding Curves

Data obtained on the effects of [Sar1]Ang II on total protein and DNA were from experiments performed using cells from a single dispersion (minimum of five plates per condition) and are representative of four separate experiments. One-way analysis of variance was used to analyze each experiment, followed by Bonferroni’s t test. For data obtained from multiple experiments, the significance of differences between means (treated versus control) was determined by the two-tailed Student’s t test for paired analysis. Because of variability in control values among dispersions, results are expressed as percent change over control (nontreated) and are reported as mean ± SEM for n dispersions. Results were considered significant at p < 0.05. The time course of binding, competitive binding curves, and dissociation curves were analyzed by the nonlinear least-squares curve-fitting option of the computer software GRAPHPAD InPlot 4.0 (GraphPAD Software, San Diego, Calif.). Receptor number (Bmax) was determined from competitive binding experiments by 1) the equation Bmax=Bo×IC50/L, where Bo is the amount of specific binding at a specified concentration (L) of radioligand and 2) transformation and Scatchard analysis of the data.

Results

Immunocytochemical Characterization of Cell Cultures

Figure 1A (left) shows a phase-contrast photomicrograph of cardiac fibroblasts that were used in this study. The fibroblasts were mononucleated, bipolar, or multinucleate; which appeared spread out on the dish. The cytosol was translucent, and the cell perimeter was not sharply defined. Rat aortic VSMCs exhibited a similar morphology, with the exception that many were multinucleated (Figure 1A, right). Staining with antibody for smooth muscle–specific MHC demonstrated that VSMCs constituted a small proportion of the cells in culture (Figure 1B, top left). In 14 randomly chosen fields (total, 687 cells; magnification x320), the proportion of VSMCs was 4.68 ± 1.04%. Rat aortic VSMCs served as a positive control for MHC staining (Figure 1B, top right). The specificity of the MHC monoclonal antibody was tested by treating immunoreactive ascites with precipitating antibodies and protein A–agarose. Under these conditions, there was no staining of either fibroblasts (Figure 1B, bottom left) or VSMCs (Figure 1B, bottom right). In addition, it was established that cultures of cardiac fibroblasts prepared by the method of differential plating exhibited little staining (1.22% of 594 cells in 12 fields) with monoclonal antibody against desmin, a cytoskeletal protein of cardiomyocytes (data not shown).22 Staining with antibody for factor VIII was performed to determine contamination of the culture with endothelial cells. In 15 randomly chosen fields, none of 593 investigated cells exhibited staining, indicating that there was no contamination of the fibroblast culture with endothelial cells.

Characterization of Ang II Binding to Fibroblasts

The time course for the binding of 0.02 nM [125I]Ang II to cultured neonatal rat cardiac fibroblasts is shown in Figure 2. Binding of [125I]Ang II reached equilibrium by 45 minutes. Nonsaturable Ang II binding contributed a negligible amount to total binding, decreasing from 21% of total binding at 5 minutes to 6% at 120 minutes. Nondiiodinated Ang II competed for the binding of 0.02 nM [125I]Ang II to fibroblasts with an IC50 of 1.0 nM, indicating that Ang II binds to a high-affinity site in cardiac fibroblasts (Figure 3). No evidence was found for a second affinity state (i.e., Kd of >6 nM) as reported for neonatal rat cardiac myocytes.23 As determined from the competitive binding curves, the maximum binding capacity, Bmax, of the fibroblasts was 778±129 fmol/mg protein (n = 7). Transformation and Scatchard analysis of the data yielded a similar value for Bmax of 865±108 fmol/mg protein (n = 7) (Figure 3, insert).

Competitive binding experiments were performed to define the ligand specificity of the Ang II binding site. As Figure 4 shows, Ang I, Ang II, and Ang III were effective in competing for the binding of 0.02 nM [125I]Ang II. The hexapeptide Ang-(3-8) also competed for binding but only at concentrations greater than 100 nM. Losartan, a competitive nonpeptide AT1 receptor antagonist, was able to completely inhibit binding of [125I]Ang II, whereas the AT2 receptor antagonist PD123177 was ineffective. As Table 1 shows, Ang peptides and losartan competed for binding of [125I]Ang II in the following rank order of potency: Ang II > Ang III > losartan > Ang I > Ang-(3-8).

In other cells, the plasma membrane AT1 class of Ang II receptors has been shown to be coupled with G proteins.24 This possibility was examined in cardiac fibroblasts by testing the ability of the nonhydrolyzable GTP analogue GMP-PNP to accelerate the displacement of bound radiolabeled Ang II by excess nonlabeled Ang II. As Figure 5 shows, GMP-PNP together with nonlabeled Ang II accelerated the displacement of [125I]Ang II. The rate constant for dissociation was increased 120% in the presence of GMP-PNP, from 0.010 to 0.022 min−1.

Effects of Ang II on Intracellular Calcium

Increases in intracellular Ca2+ may be an important step in Ang II–mediated growth. As described for other cell types,4 the AT1 receptor on cardiac fibroblasts was found to be coupled with increases in intracellular Ca2+. [Sar1]Ang II (1 μM) produced an increase in intracellular Ca2+. 

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Ca\textsuperscript{2+}. In six experiments, a mean initial peak transient of 329 nM, from a baseline of 56 nM, was followed by a decrease to a sustained level of 129 nM (Figure 6A). The initial increase and sustained plateau phase may represent the sequential contributions of extracellular and intracellular pools of Ca\textsuperscript{2+} as described for VSMCs and other cell types.\textsuperscript{1} Losartan (10 \textmu M) inhibited the Ang II-stimulated increase in intracellular Ca\textsuperscript{2+}. Losartan did not affect baseline Ca\textsuperscript{2+} or the ATP (10 \textmu M)–stimulated increase in Ca\textsuperscript{2+} (Figure 6B).
Effect of Ang II on Cellular Growth

We next examined whether the Ang II receptor in cardiac fibroblasts is coupled with cellular growth. As Figure 7 shows, a 48-hour treatment of cardiac fibroblasts with 1 nM to 1 μM [Sar']Ang II produced significant increases in both total protein and DNA. No significant effect on protein or DNA was observed with 0.1 nM [Sar']Ang II, whereas maximum effects were seen at 1 nM. The EC_{50} for the observed increases in protein and DNA was between 0.1 and 1.0 nM, which agrees with the finding of a high-affinity binding site for Ang II on these cells. With [Sar']Ang II, no difference was observed in the protein/DNA ratio, indicating that [Sar']Ang II exerts a mitogenic effect on cardiac fibroblasts. A 48-hour exposure to 1 μM [Sar']Ang II increased total protein and DNA by 23.2±2.5% and 14.6±3.2% (n=8), respectively. The [Sar']Ang II-induced increases in protein and DNA were receptor-mediated, because they were blocked by the competitive peptide Ang II receptor antagonist [Sar',Leu']Ang II (10 μM). The nonpeptide competitive AT_{1} receptor antagonist losartan (10 μM) totally blocked the increase in protein and DNA induced by 10 nM [Sar']Ang II (total protein: untreated,
TABLE 1. Displacement of $[^{125}I]$Angiotensin II Binding by Angiotensin Peptides and the Nonpeptide Receptor Antagonist Losartan

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC$_{50}$ (nM)</th>
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<tbody>
<tr>
<td>[Sar$^1$]Ang II</td>
<td>1</td>
</tr>
<tr>
<td>Ang III</td>
<td>8.1</td>
</tr>
<tr>
<td>Ang I</td>
<td>67</td>
</tr>
<tr>
<td>Ang-(3-8)</td>
<td>8,900</td>
</tr>
<tr>
<td>Losartan</td>
<td>13</td>
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Ang, angiotensin; Ang-(3-8), [des-Asp$^1$-des-Arg$^2$]Ang II; losartan, AT$_1$ receptor antagonist. Values were obtained from the competitive binding studies shown in Figure 4.

84.5±13.9 μg; [Sar$^1$]Ang II, 113.2±16.8 μg; [Sar$^1$]Ang II plus losartan, 85.5±14.4 μg; total DNA: untreated, 0.48±0.09 μg; [Sar$^1$]Ang II, 0.63±0.10 μg; [Sar$^1$]Ang II plus losartan, 0.51±0.1 μg; n=3). However, with 1 μM [Sar$^1$]Ang II, losartan (10 μM) did not totally block the increases in protein or DNA; i.e., the increase in protein was blocked by 50.3±8.5%, and the increase in DNA was blocked by 38%, 40%, and 82% (three experiments). [Sar$^1$,Leu$^2$]Ang II alone had no effect on total protein or DNA. In three dispersions, exposure of cells (for 48 hours) to EXP3174 (10 μM) had no effect on either basal total protein or total DNA (total protein: untreated, 76.2±8.9 μg; EXP3174, 74.8±6.9 μg; total DNA: untreated, 0.43±0.1 μg; EXP3174, 0.48±0.11; n=3). Also, a 48-hour exposure to EXP3174 (10 μM) did not significantly alter the effect of PDGF-BB (5 ng/ml) on total protein (92.4±4.9 μg for PDGF-BB versus 88.4±7.5 μg for PDGF-BB and EXP3174) or DNA (0.54±0.12 μg for PDGF-BB versus 0.56±0.13 μg for PDGF and EXP3174; n=3; p=NS).

To determine whether the observed increases in total protein and DNA resulted from an increase in rates of synthesis, the incorporation of precursors into protein and DNA was measured by pulse-labeling cells with $[^{14}$C]phenylalanine and $[^{3}H]$thymidine, respectively. As Table 2 shows, exposure of fibroblasts to 1 μM [Sar$^1$]Ang II for 24 hours resulted in significant increases in rates of incorporation of precursors into protein and DNA. The percent increase in the rate of thymidine incorporation induced by [Sar$^1$]Ang II was comparable to that produced by 1 μM PDB (161±33%, n=6) but less than the increase observed with 5 ng/ml PDGF-BB (397±57%, n=5). RNA synthesis, assessed by $[^{3}H]$uridine incorporation, was increased 118% at 24 hours by [Sar$^1$]Ang II (Table 2). Increased rates of phenylalanine, thymidine, and uridine incorporation were blocked by EXP3174 (Table 2), a highly specific noncompetitive AT$_1$ receptor antagonist.

Because the protein/DNA ratio was unaffected by [Sar$^1$]Ang II, this indicated that the peptide exerted a hyperplastic rather than a hypertrophic effect on cardiac fibroblasts. This hypothesis was examined further by measuring cell area and number. Under conditions in which [Sar$^1$]Ang II increased protein and DNA, no significant increase in cell area was detected; i.e., the percent increase over control in cell area for treated cells was 0.7±9.3% (n=3). However, in response to [Sar$^1$]Ang II, the cell number was increased by 138% from 1.6±0.4×10$^4$ to 3.8±0.4×10$^4$ cells per flask (n=3, p<0.01).

**Figure 5.** Graph showing the effect of guanylylimidodiphosphate (GMP-PNP) on dissociation of bound $[^{125}$I$]$angiotensin II ($[^{125}$I]AII) from membranes of neonatal rat cardiac fibroblasts. $[^{125}$I]AII (0.1 nM) was incubated with the particulate membrane fraction at 22°C for 60 minutes. Nonlabeled AII (10 μM) was added to one set of tubes (△), and AII (10 μM) and GMP-PNP (10 mM) were added to another set (○). Free and bound AII were separated at various times by vacuum filtration. Total binding (■) and nonsaturable binding (○) were also determined. Each point represents the mean of three separate experiments.

**Figure 6.** Panel A: Tracing showing the effect of 1 μM [Sar$^1$]Angiotensin II ([Sar$^1$]AII) on intracellular calcium. Primary cultures of neonatal cardiac fibroblasts were grown to confluence and serum-starved for 2 days. Cells were incubated for 1 hour with the fluorescent dye fura-2 AM and stimulated with 10 μM [Sar$^1$]AII and 10 μM ATP as indicated by arrows. Panel B: Tracing showing that AT$_1$ receptor antagonist losartan (10 μM) inhibited the [Sar$^1$]AII (1 μM)–stimulated increase in intracellular calcium. Losartan had no independent effect on baseline calcium or on the ATP (10 μM)–stimulated increase in calcium.
FIGURE 7. Dose–response curves for [Sar]angiotensin II ([Sar]Ang II)–stimulated increases in DNA and total protein from cultured neonatal rat cardiac fibroblasts. Primary cultures were passaged after 8 days and seeded at 3–8×10^6/cm^2 on 35-mm plates. After 3 days, cells were serum-deprived for 24 hours and exposed to various concentrations (from 0.1 nM to 1 μM) of [Sar]Ang II for 48 hours. Cells were lysed, the plates were scraped, and DNA (●) and protein (○) in the lysate were determined. Each point represents the mean±SEM of six determinations using cells from a single dispersion. The data shown are representative of four separate experiments. Protein and DNA for nontreated cells were 106.6±2.1 and 0.92±0.02 μg per dish (n=6), respectively. *p<0.01 vs. nontreated cells for DNA and protein by analysis of variance and Bonferroni’s t test.

Discussion

Autoradiographic studies have shown that receptors for Ang II are widely distributed in the heart, being present in the myocardium as well as on coronary vessels and sympathetic nerves.20 In addition, plasma membrane receptors for Ang II have been described for cardiomyocytes in culture.9,23 There is also evidence to indicate that receptor-mediated effects of Ang II may arise from locally produced peptide.22,27 Although recent studies suggest that Ang II may contribute to the observed fibrosis and interstitial remodeling of the heart under various pathological conditions,28 only one study23 has examined Ang II binding to cardiac fibroblasts, which are the principal regulatory cells in the remodeling of the interstitium. In that study, it was reported that neonatal rat cardiac fibroblasts showed no binding to [125I]Ang II. However, these experiments were performed 5 days after dispersion, which may have been inadequate for receptor recovery, and the cells were exposed to serum until the time of assay, which may have resulted in receptor downregulation.23 In the present study, we found that neonatal rat cardiac fibroblasts have abundant high-affinity (IC50, 1 nM) binding sites for Ang II. The Bmax for Ang II binding was 750–865 fmol/mg protein, which is greater than that reported for the high-affinity site on neonatal rat cardiomyocytes (Kd, 0.63 nM; Bmax, 318 fmol/mg protein).23

Characterization of ligand specificity of the Ang II binding site of cardiac fibroblasts showed that Ang I, Ang II, and Ang III competed for binding in a rank order of potency similar to that reported for rabbit myocardial membranes16 and rat cardiomyocytes.23 The hexapeptide Ang-(3–8) exhibited a much lower affinity, as recently reported for the AT1 receptor in liver.24 The nonpeptide AT1 receptor antagonist losartan completely inhibited binding of [125I]Ang II, whereas the nonpeptide AT2 receptor antagonist PD123319 was ineffective, indicating that these fibroblasts have only AT1 receptors. In other cells, AT1 receptors have been shown to be coupled with G proteins3,16,24 and with increases in intracellular Ca2+.1 In the present study, a nonhydrolyzable GTP analogue accelerated the dissociation of Ang II from the membrane binding site, indicating that the AT1 receptor of cardiac fibroblasts is coupled with a G protein.

In other cell types, binding of Ang II to the receptor has been shown to generate the second messengers inositol 1,4,5-trisphosphate and diacylglycerol.1 The former is known to release Ca2+ from intracellular stores; the latter activates protein kinase C and calcium-activated protein kinases.1 Calcium mobilization was implicated in Ang II–stimulated increases in tyrosine phosphorylation, an event putatively linked to growth in liver cells.29 Thus, the observed increases in intracellular Ca2+ may be important for Ang II–mediated growth in cardiac fibroblasts.

We demonstrate that the Ang II receptor of cardiac fibroblasts is coupled with a functional response, namely cell growth. [Sar]Ang II increased total protein and DNA of cultured fibroblasts with an EC50 that is within the physiological range of circulating Ang II (0.1–1.0 nM). Ang II exerted a hyperplastic effect, increasing cell number, and had no effect on the protein/DNA ratio or cell area. The percent increase in cell number after a 48-hour exposure to [Sar]Ang II exceeded the increase in DNA, perhaps because more sparsely plated cultures were used for cell number determinations. The responses were mediated by AT1 receptors, since the noncompetitive AT1 receptor antagonist EXP3174 blocked the observed increases in protein, DNA, and RNA synthesis. Losartan, which is commonly used to characterize Ang II receptors as AT1 receptors, blocked the increases in protein and DNA produced by 10 nM [Sar]Ang II. However, the effects of 1 μM [Sar]Ang II on total protein and DNA were incompletely blocked by 10 μM losartan. Losartan is a competitive receptor antagonist, which most likely explains the inability to completely block the 10 μM Ang II–stimulated increase in protein and DNA. The nonpeptide AT1 receptor antagonist EXP3174 provided effective noncompetitive blockade of the AT1 receptor.

Table 2. Effect of [Sar]Angiotensin II on Incorporation of Precursors Into DNA, Protein, and RNA

<table>
<thead>
<tr>
<th>Precursor</th>
<th>DNA Increase (%)</th>
<th>Protein Increase (%)</th>
<th>RNA Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Sar]Ang II</td>
<td>102.5±23.1*</td>
<td>57.8±8.9*</td>
<td>117.7±26.2*</td>
</tr>
<tr>
<td>[Sar]Ang II+EXP3174</td>
<td>17.3±3.7</td>
<td>−6.7±10.1</td>
<td>−2.3±19.4</td>
</tr>
</tbody>
</table>

Ang, angiotensin; EXP3174, noncompetitive AT1 receptor antagonist. Values are mean±SEM for data from three dispersions. Serum-deprived cultures of cardiac fibroblasts were treated for 24 hours with 1 μM [Sar]Ang II in the presence or absence of 10 μM EXP3174. Incorporation of precursors into DNA, protein, and RNA were measured over the final 3 hours by pulse labeling with [H]thymidine (2.5 μCi/ml), [3H]phenylalanine (0.25 μCi/ml), and [3H]uridine (0.2 μCi/ml), respectively.

*p<0.05 and *p<0.01 vs. control (nontreated) values by paired t test.
Growth-promoting effects of Ang II have been reported for other cell types, including cardiomyocytes, smooth muscle, a 3T3 fibroblast clone, and kidney proximal tubular cells. How the Ang II receptor is coupled with cellular growth has not been established, although Ang II has been shown to stimulate a number of intracellular signaling pathways that are also activated by other growth factors, such as phospholipase C and protein kinase C, elevation of cytosolic Ca\(^{2+}\), and the Na\(^{-}/\)H\(^{+}\) exchanger with subsequent intracellular alkalization. Alternatively, the growth-promoting effects of Ang II on cardiac fibroblasts could be mediated indirectly through the induction of a growth factor, such as PDGF, as recently shown for VSMCs.

Collagen accumulation in the adventitia of the intramyocardial coronary arteries and myocardial interstitium is a hallmark of the remodeling process seen in left ventricular hypertrophy. It has been proposed that this process may lead to diastolic and systolic dysfunction, ultimately leading to heart failure. Circumstantial evidence including the following has implicated Ang II in this process: 1) ACE inhibitors have been shown to improve left ventricular function of patients with severe congestive heart failure. 2) In spontaneously hypertensive rats, treatment with an ACE inhibitor caused a reversal of interstitial fibrosis and collagen accumulation. 3) Treatment with an ACE inhibitor prevented myocardial fibrosis in a rat model of renovascular hypertension. These studies have not established whether the beneficial effects of ACE inhibition are due to a reduction in preload and afterload or whether Ang II also exerts a direct effect on the cardiac remodeling process via stimulation of fibroblasts. Weber and coworkers have reported data suggesting that Ang II can stimulate in vivo fibrosis and collagen accumulation in rat hearts, independent of an increase in afterload. The results of our study support the hypothesis that Ang II directly stimulates fibroblasts to cause interstitial remodeling. Ang II has been implicated in the development of the neonatal pig heart. Treatment of piglets with an ACE inhibitor or losartan resulted in a decrease in the left ventricle/body weight ratio, total RNA content, and protein of the left ventricle when compared with vehicle-treated animals. In addition, treatment with an ACE inhibitor decreased ribosome formation and total protein synthesis. These data suggest that Ang II is an important factor in the growth of the neonatal heart.

Indirect fluorescent immunocytochemistry revealed some contamination of the cardiac fibroblast cultures with VSMCs. Therefore, one might argue that VSMCs contributed to the observed Ang II binding and the effects of Ang II on cellular growth. Several lines of evidence argue against this: 1) Total binding capacity of the fibroblast cultures for Ang II was twofold to fivefold greater than what was observed for cultured VSMCs. In contrast, rat VSMCs were reported not to respond to phorbol ester stimulation with enhanced DNA synthesis. It is also unlikely that contaminating cardiomyocytes contributed to the observed growth-promoting effects of Ang II, since enriched cultures of cardiomyocytes exhibit primarily a hypertrophic growth response to Ang II.

In summary, results of the present study demonstrate that cultured cardiac fibroblasts possess a single class of high-affinity G protein–linked AT\(_1\) receptors that are abundant and are coupled with stimulated increases in intracellular calcium and a proliferative response. Ang II enhanced incorporation of precursors into protein, RNA, and DNA and increased total protein, DNA, and cell number, whereas no effect was observed on cell area. These findings are consistent with the hypothesis that fibroblasts are a target for Ang II in the intact heart and may contribute to the Ang II–induced remodeling of the cardiac interstitium, which is associated with a variety of physiological and pathological conditions. Further studies are required to define the intracellular pathways involved in the Ang II–induced growth response and to assess whether Ang II regulates the genes involved in extracellular matrix production or remodeling.

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References


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