Integrin-Mediated Collagen Gel Contraction by Cardiac Fibroblasts

Effects of Angiotensin II

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Abstract Angiotensin II (Ang II), a vasoactive octapeptide, has been implicated in cardiac growth and the development of hypertrophy and fibrosis secondary in hypertensive disease. These consequences of Ang II imply an effect on the function and morphology of cardiac interstitial cells (fibroblasts). The present investigation was designed to (1) determine whether neonatal heart fibroblasts (NHFs) possess functional Ang II receptors on their plasma membrane and (2) examine the effects of Ang II on NHFs in vitro using three- and two-dimensional (3D and 2D, respectively) cultures. Several analytic techniques were used to test the specific questions of the present study. Since cardiac fibroblast phenotype can be influenced by culture conditions, both 2D and 3D cultures were used in the present investigations. Reverse-transcriptase polymerase chain reaction and radioligand binding analysis were used to test for the presence of Ang II receptors on NHFs. Both revealed that NHFs in 2D culture possess Ang II receptor mRNA and Ang II receptors. When isolated NHFs were cultured in 3D collagen gels and treated with Ang II, gel contraction was stimulated by NHFs. This effect was attenuated by the specific Ang II receptor antagonist [Sar¹,Ala⁸]Ang II. Ang II-stimulated gel contraction was completely inhibited by extracellular matrix receptor (β₁-integrin) antibodies (P<.05), supporting previous studies indicating that collagen gel contraction is mediated via the integrins. Immunofluorescent staining was used to test the localization of cell-surface integrins. A more intense staining pattern for β₁-integrin in Ang II–treated versus control cells was observed. Immunoprecipitation of β₁-integrin revealed more β₁-integrin protein on the surface of Ang II–treated cells (P<.05). These results suggest that Ang II mediates collagen gel contraction via Ang II receptors and β₁-integrin, indicating a functional relation between Ang II and β₁-integrin on cardiac fibroblasts. We conclude that Ang II influences the ability of cardiac fibroblasts to contract 3D collagen gels and the regulation of membrane-bound integrins in both 2D and 3D cultures of NHFs. (Circ Res. 1994;74:291-298.)

Key Words • angiotensin II • fibroblasts • integrins • collagen gels • heart • AT₁ receptor

Recent evidence has suggested a role for angiotensin II (Ang II), a vasoactive octapeptide, as a growth-regulating factor important both in normal heart development and in hypertrophy from systemic hypertension.¹ Pressure-overload cardiac hypertrophy and neonatal development are characterized by increases in extracellular matrix (ECM) accumulation of collagen and associated glycoproteins as well as by increases in myocyte size and ventricular wall thickness.² The association of Ang II with the accumulation of interstitial matrix components (ie, collagen and elastin) in hypertrophy that occurs during development or disease has recently been given considerable attention. For example, reducing endogenous Ang II with angiotensin-converting enzyme inhibitors attenuates the normal accumulation of both collagen and elastin in the hearts of developing young rats,³ whereas increased Ang II levels stimulate collagen synthesis in cultured vascular smooth muscle cells.⁴ In vivo experiments cannot distinguish the variety of serum factors, such as hormones and growth factors, that may be partially responsible for the Ang II–associated effects on cardiac hypertrophy during development and disease. In addition, the most probable site of Ang II action must involve myocardial fibroblasts, since they are responsible for synthesizing the majority of ECM proteins; however, evidence for the presence of Ang II receptors on cardiac fibroblasts has only been recently demonstrated.²,³,⁵ Indeed, the direct effects of Ang II on cardiac fibroblasts warrant further research.

Integrins have been recognized as a widely expressed family of cell surface adhesion receptors that are noncovalently linked αβ heterodimers.⁶ Multiple integrins are expressed on the surface of the cellular components of the heart and play an important role as signaling receptors in a variety of cell types.⁶ A particularly important feature of integrins is that they undergo activation. For example, in adhesion processes, integrins provide the strong adhesion but only after activation by other stimuli, which can include soluble mediators such as hormones and cytokines.⁶ Activation of adhesion or signaling by integrins result in a variety of influences on the cell that play roles in development and cellular differentiation,⁷ platelet and lymphocyte function, fibroblast cell adhesion, T-cell activation and proliferation, and ECM regulation.⁶,⁷ Integrins are important regulators in early cardiac development, providing positional information for migrating and differentiating cells.⁸ They are also important regulators of the intimate actions between myocytes, ECM proteins, and cytoskel-
etal components in the adult heart. In an in vitro assay using three-dimensional (3D) collagen gels to assess cell function, \( \beta \)-integrin mediates gel contraction by neonatal heart fibroblasts (NHF5) by various growth factors.

Cell phenotype and function can be influenced by culture conditions. Cardiac fibroblasts in situ reside within a collagenous network and are elongate and bipolar in shape. Plated in two-dimensional (2D) culture, these cells will change from elongate to a flat shape with stellate projections and actively synthesize collagen. Fibroblasts plated in 3D collagen gels maintain their elongation and have less projections and lower rates of collagen synthesis (versus cells plated in 2D culture), which are characteristics similar to in vivo conditions. Collagen gel contraction assays are often used as an in vitro model for wound contraction and connective tissue morphogenesis, and the contraction process has been characterized in great detail. The interaction of fibroblasts with a 3D collagen lattice affects cellular activities such as protein synthesis and proliferation and thus provides a unique model for fibroblast responses in vitro. NHFs in attached collagen gels interact with the collagen via specific integrin receptors, and this interaction results in gel contraction. Cardiac fibroblasts in vivo interact with myocytes, and together, they regulate mechanical properties of the heart. Similarly, 3D collagen gels in vitro reestablish the interaction of fibroblasts with their collagen matrix. Because culture conditions influence cell phenotype and function, the present study examines NHFs in both 2D and 3D cultures.

In the present investigation, a variety of in vitro models were used to (1) determine the presence of functional Ang II receptors on NHFs and (2) determine the specific effects of Ang II on cardiac fibroblast morphology and function. A variety of techniques were used to examine the effects of Ang II on isolated NHFs in vitro in regard to modulation of collagen gel contraction and integrin regulation. The data from each of the techniques used support the presence of Ang II receptors on NHFs as well as the regulation of integrins by Ang II.

Materials and Methods

Animals

All animals were housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care with food and water ad libitum. The present investigation was conducted in conformity with the “Guiding Principles for Research Involving Animals and Human Beings,” as approved by the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health, Bethesda, Md). All experimental protocols involving the animals were approved by the University Institutional Animal Review Board.

Antibodies

A rabbit polyclonal antibody against rat \( \beta \)-integrin was used for immunofluorescent localization and immunoprecipitation of \( \beta \)-integrins and \( \beta \)-integrin-inhibited gel contraction experiments. The purification and characterization of this antibody have been previously described.

Cell Isolation

Neonatal Sprague-Dawley rats (3 to 5 days of age) were decapitated, and the hearts placed in ice-cold phosphate-buffered saline (PBS). NHFs were isolated by established procedures. Briefly, minced heart tissue was subjected to collagenase digestion, and the dissociated cells were plated in 150-cm\(^2\) culture flasks with Dulbecco’s modified essential media (DMEM) supplemented with 10% neonatal bovine serum, 5% fetal bovine serum, 0.5 U/mL penicillin G, 0.5 \( \mu \)g/mL streptomycin, and 2 \( \mu \)g/mL amphotericin B (10/5-DMEM). After \( \approx \) 1 to 2 hours, the medium was aspirated, and the attached cells were washed with Moscona’s saline. Forty milliliters of 10/5-DMEM was added, and cells were grown at 37\(^\circ\)C in 5% CO\(_2)/95%\) air until confluent. Cells from subsequent passages 3 to 10 were used for all experiments.

Ang II Receptors

Saturation binding analysis was used to test the presence of Ang II receptors on NHFs. Reverse-transcriptase (RT) polymerase chain reaction (PCR) was used to test whether NHFs possessed mRNA for Ang II receptors.

The presence of Ang II receptors on NHFs was determined using a modification of the method of Aceto and Baker. Briefly, NHFs were plated in 35-mm six-well culture plates at a density of 200,000 per well in 10/5-DMEM. After 24 hours, the attached cells were washed with Moscona’s saline and fed with 5 mL of serum-free DMEM. Cells were grown in serum-free DMEM for 4 to 5 days before conducting receptor-binding experiments. Cells were washed three times with 1 mL of an incubation buffer (50 mmol/L Tris, 120 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L CaCl\(_2\), 1 mmol/L MgCl\(_2\), 2 mg/mL dextrose, 10 \( \mu \)g/mL bacitracin, and 0.25% bovine serum albumin [BSA] at pH 7.5 and 25\(^\circ\)C). A saturation radioligand-binding assay was conducted as follows. One milliliter of incubation buffer was then added either alone or in combination with varying concentrations of \(^{125}\)I-Ang II. Nonselective binding was determined in the presence of 10 nmol/L unlabeled Ang II. The cells were incubated for 60 minutes at room temperature, after which the incubation mixture was removed by aspiration. The cells were then washed three times with incubation buffer, after which 200 \( \mu \)L of lysis solution (0.25% NaOH and 0.5% sodium dodecyl sulfate [SDS]) was added to each well. After a 10-minute incubation at room temperature, wells were washed with an additional 500 \( \mu \)L of the NaOH/SDS solution, and the solutions were transferred into vials for gamma counting. Identical wells for each condition were used for determination of protein content. Total protein was determined by the Pierce assay (Pierce Chemical Co, Rockford, Ill). Affinity (\( K_d \)) and receptor number (\( B_{max} \)) were determined using saturation analysis (0 to 8 nmol \(^{125}\)I-Ang II; specific activity, 2175 Ci/mmol) and calculated according to the method of Lundeen and Gordon. The data were then graphed as a Scatchard plot to demonstrate linearity of transformed data. Ang II receptor-binding experiments were repeated at least three times, and results are expressed as moles per milligram protein.

RT-PCR methodology determined whether NHFs were expressing mRNA for Ang II (type 1) receptors, because this receptor mediates all of the hemodynamic manifestations of Ang II. Total RNA was isolated from confluent NHFs in 2D culture using the one-step guanidinium thiocyanate method. RT-PCR was carried out with the GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, Conn) using 2 \( \mu \)g of total RNA per reaction. Briefly, Moloney murine leukemia virus RT and random primers were used for cDNA synthesis from the RNA. The cDNA was amplified with AmpliTag DNA polymerase and primers for the rat aortic vascular smooth muscle Ang II (type 1) receptor. Specifically, cDNA (rat) sequences nt 296 to 318 (ACCTATGTAAGATCGCTTCGGC) and nt 854 to 875 (TAGGCTATGCAGATGGTGATGG) were used. Primer Detective software (Clontech, La Jolla, Calif) was then used to select the Ang II primers for this experiment. These primers were synthesized by the Oligonucleotide Synthesis Facility at the University of South Carolina (Institute for Biological and Research Technology, Columbia, SC). Primers for \( \beta \)-actin

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Ang II receptors were attempted in 3D collagen gels. The collagen gels retained a large amount of radioactivity so that concentration differences could not be distinguished, and attempts to extract RNA from fibroblasts in collagen gels has not been successful to date in this laboratory.

Collagen Gel Contraction

The second set of experiments used collagen gel contraction to determine whether Ang II had an effect on NIH interaction with surrounding ECM components. Collagen gel contraction was used to test the direct effects of Ang II on NIHs in a 3D in vivo-like medium. Confluent NIHs in 15-cm2 polystyrene culture flasks were trypsinized (0.25% trypsin in 0.1% EDTA) and washed three times in serum-free DMEM. A total of 4 x 10⁶ cells per ml were trypsinized with 10 μg/mL Ang II, 10 μg/mL [Sar¹,Ala⁶]Ang II (an Ang II antagonist), both Ang II and [Sar¹,Ala⁶]Ang II, 10 μg/mL anti-β1-integrin IgG, both Ang II and β1-integrin IgG, or sterile water (control) for 30 minutes at 37°C. Cells were then plated in 3D collagen gels (neutral collagen contains one part 10 X minimum essential medium, one part 0.2 mol/L HEPES, and eight parts Vitrogen 100–purified collagen [Celtix Laboratories, Palo Alto, Calif]) in 24-well culture plates with equal concentrations of hormone, antibody, and/or water. After a 1-hour incubation at 37°C to polymerize the gel, 1 mL of serum-free DMEM and appropriate concentrations of hormone or antibody were added to the wells, and cells were incubated at 37°C for up to 96 hours. Images were captured at 24, 48, 72, and 96 hours, and gel areas were calculated from the diameter as measured via an Image-1/AT software program (Analytical Imaging Concepts, Irvine, Calif). Each of six experimental conditions (ie, Ang II, [Sar¹,Ala⁶]Ang II, Ang II and [Sar¹,Ala⁶]Ang II, anti-β1-integrin IgG, anti-β1-integrin IgG, or sterile water) were run in duplicate at least three times (n=6 per condition).

Immunofluorescence

The distribution of β1-integrin on NIHs was examined by confocal microscopy after indirect immunofluorescence staining of β1-integrin. Immunofluorescence was used to locate the presence of β1-integrins on the surface of NIHs, both in 2D and 3D cell culture. The collagen minigels were prepared identically to the 1-mL gels (described previously), except 50,000 cells per milliliter were treated with or without hormone, and total gel volume was 25 μL. At 24 and 96 hours after the addition of 10 μg/mL Ang II, NIHs cultured on laminin-coated coverslips in 15-mL culture plates or in collagen minigels were washed three times with PBS and fixed with 2% paraformaldehyde for 10 or 90 minutes, respectively, followed by three 5- or 90-minute washes in PBS. Fixed cells or gels were stored in PBS with 0.2% azide at 4°C in the dark until analysis.

Immunofluorescence procedures involved washing cells in 1% glycine in PBS for 15 or 90 minutes and rinsing three times for 5 or 90 minutes in PBS before the addition of the primary antibody (50 μg/mL β1-integrin IgG). Cells were incubated in primary antibody for 30 or 90 minutes at 37°C, followed by three 5- or 90-minute rinses in PBS with 0.5% BSA. Cells were then incubated in the presence of the fluorescein isothiocyanate–tagged secondary antibody (50 μg/mL) for an additional 30 or 90 minutes at 37°C and washed three times for 5 or 90 minutes with PBS/0.5% BSA. The coverslips were mounted on glass slides with PBS/10% glycerol (1:3). The minigels were set in well-labeled slides with 0.1% 1,4-diazabicyclo[2.2.2]octane in PBS/glycerol (1:1). Images of stained cells or gels were captured by laser-scanning confocal microscopy (MRC600 laser scanning confocal imaging system, Biorad, Cambridge, Mass), with the manual gain set identically for each image under all experimental conditions. Immunofluorescence experiments were repeated at least three times.

Iodination of Cell Surface and Immunoprecipitation

The upregulation of integrin by Ang II (as suggested by the immunofluorescence experiments) was examined by immunolabeling cultured cells for β1-integrin. Immunoprecipitation was used to compare relative amounts of surface-labeled β1-integrin protein in cells treated with Ang II versus control cells. For immunoprecipitation experiments, 100-mm culture dishes were plated with 2 x 10⁶ cells in 10/5-DMEM and allowed to adapt to culture conditions for 48 hours until confluent. After 48 hours, cells were washed once with Moscona’s saline and twice with serum-free DMEM and treated with Ang II (10 μg/mL) in 15 mL of serum-free DMEM. Media and hormone were replaced every 24 hours. After 24 and 96 hours, the confluent NIHs were surface-labeled with [125I]Labeled cells were extracted for 30 minutes with a solubilization buffer (1% Triton X-100, 1 mM/L MgCl₂, 1 mM/L CaCl₂, and 10 mM/L Tris, pH 8.0) and a protease inhibitor cocktail (1 mM/L phenylmethylsulfonyl fluoride, 1% aprotonin, 1 μg/mL pepstatin A, 2 μg/mL leupeptin, and 1 μg/mL benzamidine). The samples were centrifuged at 15,000g for 30 minutes, and the supernatant was used for immunoprecipitation. Supernatants were counted for incorporation of [125I] into cells before immunoprecipitation. Preimmune IgGs (100 μg/mL) and protease inhibitor cocktail were added to the supernatants and incubated for 4 hours in an end-over-end fashion, followed by the addition of 100 μL of protein A-Sepharose 4B (50% slurry in PBS) and protease inhibitor cocktail and incubation for 1 hour. This mixture was then centrifuged at 10,000g for 10 minutes, and the supernatant was incubated with anti-β1-integrin IgG (100 μg/mL) for 8 hours in the presence of protease inhibitor cocktail. Incubation was carried out an additional hour in the protein A-Sepharose slurry. The protein A-Sepharose with bound proteins was washed as follows: five times with buffer 1 (1% Triton X-100, 0.5 mol/L NaCl, 10 mM/L Tris, pH 7.4, and 1 mM/L MnCl₂), three times with buffer 2 (1% Triton X-100, 0.5% deoxycholate, 0.1 mol/L NaCl, 10 mM/L Tris, pH 7.6, and 1 mM/L MnCl₂), and two times with PBS. Samples were solubilized by boiling in SDS loading buffer and subjected to nonreducing SDS–polyacrylamide gel electrophoresis and autoradiography. Bands for β1-integrin were determined by comparison with size standards and quantitated by soft laser densitometry. The blots were exposed for 2, 4, 6, and 8 hours to ensure that scanning was performed within the linear range of densitometry. Relative absorbance was divided by total incorporated counts for each respective sample in an effort to standardize relative quantitation. Immunoprecipitation experiments were performed at least three times.

Statistics

Data from saturation experiments were subjected to Lundon and Scatchard analysis for determination of receptor Kᵣ and Bᵣ, where A * X (condition × time) ANOVA was used to test differences in area between Ang II–treated and control cells over 24, 48, 72, and 96 hours in the collagen gel contraction experiments. Immunoprecipitation studies were analyzed by a two-way (condition) ANOVA using the densitometry units (peak area per total counts incorporated) for each integrin band from the autoradiographs. When a main effect was significant (P<.05), Student-Newman-Keuls post hoc analysis
Fig 1. Left, Scatchard plot of a typical saturation binding experiment for angiotensin II (Ang II) receptors on neonatal heart fibroblasts (NHFs) is shown. In this experiment, NHFs were exposed to increasing amounts of $^{125}$I-Ang II for 60 minutes, after which cells were washed (to stop the binding reaction), scraped, and counted on a gamma counter. Linear regression analysis revealed an $r$ value of .90. Saturation occurred at $7 \text{nmol/L}$ (inset). Right, Reverse-transcriptase polymerase chain reaction gel shows amplification of Ang II receptor mRNA in NHFs. The number 558 represents the size of the band (in base pairs [bp]) for Ang II receptor mRNA, with 540 bp for $\beta$-actin mRNA. All samples were run with RNA isolated from NHFs in two-dimensional culture. The lanes are as follows: lane 1, NHF RNA with primers for Ang II receptor; lane 2, NHF RNA with primers for Ang II receptor with non-specific primer; lane 3, NHF RNA with primers for $\beta$-actin; and lane 4, NHF RNA with primers for $\beta$-actin without reverse-transcriptase enzyme. Both samples were run with reverse-transcriptase enzyme to control for contaminating DNA (Ang II receptor, lane 2; $\beta$-actin, lane 4). Sizes were calculated from HindIII-digested dsDNA run in an additional lane of the gel.

was used to probe individual differences. Where appropriate, results are presented as mean±SEM.

**Results**

**Ang II Receptor Binding**

Receptor binding studies demonstrate the presence of Ang II receptors on NHFs by analysis of saturation experiments (Fig 1, left). Binding demonstrated saturation at $7 \text{nmol/L}$ (see Fig 1, left, inset). The $K_d$ for NHFs was $1 \text{nmol/L}$, and the $B_{max}$ was $24.4 \text{fmol/mg}$ protein as determined by the Lunden method. Competition binding experiments showed appropriate displacement of $^{125}$I-labeled Ang II by unlabeled hormone (data not shown). RT-PCR analysis showed positive expression for Ang II (type 1) receptor RNA (Fig 1, right). The Ang II (type 1) receptor band was of a size similar to that shown previously. Together, these data indicate the presence of Ang II receptors on isolated heart fibroblasts.

**Collagen Gel Contraction**

Collagen gel contraction experiments showed a significant main effect for area (enhanced contraction evidenced by decreased gel area) of the 3D collagen gels with the Ang II treatment versus control gels (Fig 2, left). Figures express change in gel area as percentage of control gel area. Student-Newman-Keuls post hoc analysis revealed that significant differences in gel contraction occurred at the 24-, 48-, 72-, and 96-hour time points (Fig 2, right; $P<.05$). When collagen gels were incubated with [Sar$^1$,Ala$^3$]Ang II (an Ang II receptor antagonist) or both Ang II and Ang II receptor antagonist, gel contraction was attenuated at all time points examined (Fig 3, $P<.05$). When collagen gels were incubated with $\beta_1$-integrin antibody or both Ang II and 10 $\mu$g/mL $\beta_1$-integrin antibody, gel contraction was completely abolished, even after 96 hours (Fig 4, $P<.05$).

**Immunofluorescence**

Confocal microscopic localization of $\beta_1$-integrin shows more intense staining in the 2D Ang II–treated cells versus control cells (Fig 5). The more concentrated staining appears to be distributed in the bottom focal plane between the cell and the culture dish, with intense staining under the nucleus. The figures represent substrate-level cell-surface sections, where the gain was set for control conditions and held constant for all experimental images. Fig 6 shows more intense staining for $\beta_1$-integrin of NHFs plated in 3D collagen minigels. In Fig 6, the cells in the 3D gels have organized into a ring within the gel. This organization has been previously described. The fluorescence shows the ringlike organization of the cells within the gel in addition to the localization of the $\beta_1$-integrins. The micrographs represent a low magnification image of a portion of the cell ring. In both 2D and 3D culture conditions, Ang II–treated cells demonstrated a more intense staining pattern for $\beta_1$-integrin.

**Immunoprecipitation**

Results from immunoprecipitation experiments show a relative increase in $\beta_1$-integrin on the surface of Ang II–treated cells versus control cells (Fig 7) by gel electrophoresis. The arrows on Fig 7 mark the $\beta_1$-integrin bands. Identical incorporated counts of $^{125}$I were used for immunoprecipitation ($1.4\times10^5$ counts per condition). Although not quantitative, the immunoprecipitation procedure is used for relative comparisons in the present study. The bands directly above the $\beta_1$-integrin bands have migrated to molecular weights similar to the $\alpha_1$ or $\alpha_2$-integrin chains; however, monospecific antibody immunoprecipitation to those $\alpha$ chains was not performed. Analysis by laser densitometry revealed a relative 56% increase in the cell surface accumulation of $\beta_1$-integrin in Ang II–treated versus control cell extracts ($P<.05$).
Discussion

The ECM in the heart is important for contraction of the extracellular collagen network by tension.\(^5\)\(^6\)\(^7\) Directly involved in the regulation of ECM components and contraction are the cardiac fibroblasts, which are at least in part responsible for the normal ECM adaptation of the myocardium, which may in turn set the stage for pathological hypertrophy and ventricular dysfunction in hypertension and hypertrophy.\(^2\)\(^8\) Although Ang II has been implicated in regulating cardiac ECM morphology and function, the present study provides evidence for direct localization and effects of Ang II on cardiac fibroblasts, which are responsible for the synthesis of many ECM components in the myocardium.\(^21\)\(^22\)

The present study used both 2D and 3D culture systems to examine the effects of Ang II on neonatal heart fibroblasts to control for the phenotypic changes often seen when isolating cells into culture. The results
of most experimental procedures in the present study exhibited similar responses to Ang II in both 2D and 3D culture conditions. Specifically, both culture conditions resulted in a more dense staining pattern in NHFs for β₁-integrin, and immunoprecipitation for β₁-integrin in 2D culture revealed a greater number of integrins on the cell surface. These results may suggest an important role for Ang II in the remodeling of the ECM during pathological hypertrophy, where elevated levels of Ang II are present.

The present study demonstrates the presence of Ang II receptors on neonatal rat heart fibroblasts by saturation-binding analysis. Binding of Ang II to cardiac fibroblasts could be displaced by competitive agonists. RT-PCR also indicates that these cells are expressing mRNA for the Ang II (type 1) receptor. Until very recently, support for the presence of Ang II receptors on cardiac fibroblasts has been only indirect. Ang II receptors in cultured fibroblasts prepared from neonatal skin 1 day after birth were reduced by 80% compared with a 19-day fetus. Unfortunately, Millan et al. did not examine the presence of Ang II receptors on the fetal or neonatal heart cells. Other investigations have implied the presence of Ang II receptors on cardiac fibroblasts as evidenced by increased collagen accumulation, the presence of Ang II hormone in cultured rat cardiac fibroblasts, and fibroblast proliferation. Only very recently have binding studies on cardiac fibroblasts demonstrated the presence of Ang II receptors. As evidenced by the different receptor densities in the present study and that in the study of Schorb et al., culture conditions play an important role in the expression of AT₁ receptors on cardiac fibroblasts. The present study demonstrated enhanced collagen gel contraction by cardiac fibroblasts treated with Ang II, which was attenuated in the presence of Ang II receptor antagonist. This result indicates that Ang II stimulation of gel contraction is Ang II receptor-mediated. The
function of cardiac fibroblasts in the 3D culture system (ie, the ability of the fibroblasts to contract the collagen gels) can be altered by inhibition of Ang II receptors, implying that Ang II is important in mediating this aspect of cardiac fibroblast function. Previous investigations have shown that Ang II mediates cardiac fibroblast metabolism. For example, angiotensin-converting enzyme inhibitors will suppress normal accumulation of ECM proteins in the heart, whereas treatment with Ang II will promote collagen synthesis in vascular smooth muscle cells and murine mesangial cells. Enhanced remodeling of the ECM by Ang II treatment of neonatal heart fibroblasts in the collagen gel system implies that Ang II may in part be responsible for the dramatic reorganization of interstitial collagen observed in hypertrophy from growth and hypertension. For example, an increased Ang II concentration seen by the cardiac fibroblast in some forms of renovascular hypertension might stimulate collagen synthesis because of the enhanced metabolic action of this cell observed during normal cardiac growth and in hypertrophy resulting from pressure overload (hypertension). Although the present study did not examine specific mechanisms of action, it does imply that Ang II may impose an increased resistance to the contracting myocytes in the heart by causing fibroblasts to constrict the ECM around the contracting myocytes. Support for this speculation is that altered ECM tension, metabolism, and synthesis have been seen in disease states with elevated Ang II levels. For example, collagen accumulation in hypertensive disease has been held responsible for abnormal myocardial stiffness and impaired pumping capacity of the heart. In addition, administration of the angiotensin-converting enzyme inhibitor enalapril to rats after abdominal aortic constriction prevented the left ventricular hypertrophy usually seen with this model. Enalapril treatment of young growing rats suppressed accumulation of elastin and collagen in large and small arteries as well as total collagen in both left and right ventricles. Ang II has also been implicated in normal cardiac development. For example, Beinlich et al showed decreased left ventricular weight, RNA content, total RNA, and RNA/DNA ratio in left ventricle of newborn pig hearts treated with an Ang II antagonist.

Since fibroblasts produce ECM proteins (ie, collagen) that bind to their membrane-bound integrin receptor, the present study tested whether Ang II effects might be associated with the integrins. By culturing cardiac fibroblasts in substrata (such as hydrated collagen gels), these cells would bind with the substrate via the integrin and then contract by pulling on the collagen, releasing water and thus shrinking the gel. Such an event occurs in wound healing and tumor metastasis, as evidenced by the 3D collagen gel system. The observed increase in β1-integrin under Ang II treatment may explain the behavior of the fibroblasts in the collagen gel cultures. Specifically, increased β1-integrin in the Ang II–treated collagen gels would provide more adhesion sites for the cells, thus enhancing gel contraction. These results support a relation in the mechanism(s) of action of both Ang II and β1-integrin in influencing cardiac fibroblast function in the 3D culture system. Specifically, Ang II stimulation of collagen gel contraction may be due to the upregulation of β1-integrins by this vasoactive peptide.

The precise mechanism(s) by which Ang II regulates ECM morphology and function is yet to be determined. The present study demonstrated increased collagen gel contraction and β1-integrin localization and content in Ang II–treated cells. It is possible to speculate that Ang II, acting through its receptor in the plasma membrane, may induce these changes in integrins via a similar mechanism as that proposed by Katz. Specifically, binding of Ang II to its receptor increases phospholipase C activity through a mechanism that probably involves a G protein. Phospholipase C then hydrolyzes phosphatidylinositol 4,5-bisphosphate and forms diacylglycerol and inositol 1,4,5-trisphosphate (InsP3). InsP3 increases cytosolic calcium, which may explain the ability of Ang II to promote contraction in smooth and cardiac muscle. Protein kinase C (activated by diacylglycerol) and calcium appear to induce the c-fos proto-oncogene within the nucleus, and c-fos is believed to accelerate transcription and thus increase protein synthesis, possibly accounting for the growth-promoting effects of Ang II.

Integrins are intimately associated with the cytoskeleton and transmit extracellular signals across the sarcolemma to the internal milieu of the cell. This is especially true for mechanical signals. Ang II may provide a chemical signal to the cytoplasm, similar to platelet-derived growth factor (PDGF) and other growth factors, with the subsequent surfaceing of cytosolic β1-integrins and/or enhanced gene expression of β1-integrin. The mechanism proposed by Katz is supported by concomitant increases in integrin expression, protein synthesis, and cardiac hypertrophy in vivo. Previous studies have demonstrated a role for the α-integrin chains in recognition sites for ECM substrates, such as collagen. In addition, it is thought that the α-chains dictate integrin substrate specificity. Regulation of the α-chains by Ang II may provide more evidence of the relation and mechanisms of action between Ang II–mediated and integrin-mediated effects in cardiac fibroblasts. Characterization of the α-integrin chains in fibroblasts in response to Ang II treatment is currently in progress. Other factors have been shown to influence collagen gel contraction by cardiac fibroblasts.
PDGF stimulates collagen gel contraction via the integrins, whereas transforming growth factor-β exhibits no such effect (authors' unpublished data). Studies examining the effects of Ang II and other growth factors may serve to elucidate the precise mechanism(s) by which Ang II and cell surface integrins are related to the control of cardiac fibroblast function.

The present study documents the expression and presence of Ang II receptors on the surface of cardiac fibroblasts by pharmacologic, biochemical, and molecular techniques. There appears to be an interaction between Ang II receptors and integrins, as revealed by the correlation of the data from immunofluorescent staining, immunolabeling, and collagen contraction assays. Specifically, the neonatal rat heart fibroblast up-regulates its expression of surface β1-integrins by Ang II treatment. These results suggest a role for Ang II in mediating heart fibroblast function and may provide insight into the precise role of Ang II on heart fibroblasts in both health (physical training) and disease (hypertension).

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