Angiotensin II Induces Fibronectin Expression Associated With Cardiac Fibrosis in the Rat

Dennis C. Crawford, A.V. Chobanian, Peter Brecher

Abstract Fibronectin expression was studied in the heart of rats given a continuous infusion of angiotensin II (Ang II). Northern blot analysis showed that left ventricular fibronectin steady-state mRNA increased fivefold to eightfold in response to pressor doses of Ang II after 24 hours. Accumulation of immunodetectable fibronectin in the ventricles occurred after the mRNA levels increased. The changes in fibronectin expression were reversible when Ang II treatment was withdrawn. The Ang II-induced increase in fibronectin mRNA accompanied similar increases for collagen type I, collagen type IV, and atrial natriuretic factor steady-state mRNA. Interstitial and perivascular fibrosis was identified in both ventricles of angiotensin-treated rats within 3 days. In situ hybridization identified cells associated with areas of fibrosis as the principal site of fibronectin mRNA accumulation in treated animals. By comparison, normal hearts showed fibronectin expression primarily within ventricular vascular tissue and the atrial endocardium. A dose-dependent reduction of fibronectin expression followed treatment with losartan, indicating an Ang II type 1 receptor-mediated effect. Normalization of blood pressure during Ang II infusion by either hydralazine or prazosin had different effects on the level of fibronectin steady-state mRNA, indicating that blood pressure elevation was not the principal factor responsible for fibronectin induction. Concurrent administration of angiotensin-converting enzyme inhibitors with Ang II attenuated the increased fibronectin expression. Our data indicate that Ang II induces an acute fibrotic response within the heart and suggests that Ang II stimulates fibronectin expression within nonmyocytes cardiac cells by a direct action. (Circ Res. 1994;74:727-739.)

Key Words • hypertension • cardiac hypertrophy • extracellular matrix • wound healing

The effects of angiotensin II on the heart are diverse, and the underlying mechanisms that function in vivo have been difficult to study because of the need to dissociate indirect effects, such as blood pressure, from direct effects of the polypeptide hormone on the different cardiac cell types. Several in vivo studies have suggested that angiotensin II causes a trophic response, both on blood vessels and the heart, that is independent of hemodynamic influence. Cardiac hypertrophy, whether induced by angiotensin II or by other established methods, is often accompanied by varying degrees of fibrosis, defined by a proliferation of interstitial fibroblasts and biosynthesis of extracellular matrix components. The mechanisms thought to induce the fibrosis have been the subject of recent reviews. Evidence linking cardiac fibrosis with angiotensin II has emerged from studies in rats showing that chronic angiotensin II infusion at subpressor doses induced fibrosis, even after only 2 days of treatment. In that study, it was suggested that the angiotensin-induced fibrosis was causally related to myocyte necrosis. The possibility that angiotensin II acts directly on cardiac myocytes was suggested on the basis of in vitro studies using neonatal myocytes, but there is little evidence showing a trophic influence of angiotensin II on adult rat myocytes, although such effects on vascular cells including smooth muscle and fibroblast are well documented and recently reviewed.

Fibronectin is a dimeric glycoprotein located in the extracellular matrix of most tissues that, through an interaction with cell surface receptors, has been shown to influence diverse cellular properties including adhesion, growth, and wound repair. Fibronectin has been identified immunohistochemically in the interstitium of cardiac tissue and appears in the normal adult heart predominantly within small groups of myocardial cells and in connective tissue surrounding the microvasculature. A role for fibronectin in connecting cardiocytes to the collagen fibers and a functional influence on myocardial compliance have been suggested on the basis of these structural relations. Although a functional role for fibronectin in either the normal or hypertrophic heart has not been definitively established, its importance in experimental models of wound healing has been described previously. The remodeling that occurs during the development of cardiac hypertrophy with fibrosis is considerably analogous to wound healing processes, implicating a role for fibronectin. In several previous studies, we have shown that increased fibronectin expression is associated with both vascular and cardiac hypertrophy. Within the heart, we have shown increased mRNA and protein levels for fibronectin after ischemia, triiodothyronine treatment, and mineralocorticoid-induced hypertension. These levels are also increased in spontaneously hypertensive rat hearts. Samuel et al used in situ hybridization to show that fibronectin mRNA is associated with vascular smooth muscle cells in small coronary arteries and that, after aortic coarctation, fibronectin production is localized to focal areas of fibrosis acutely induced in this pressure-overload model. In a separate study, also using aortic coarctation, cardiac fibronectin mRNA increased

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within 12 hours after surgery, as measured by Northern blot analysis.  

The present study was performed to determine the interrelation between the renin-angiotensin system, blood pressure, and fibronectin expression during cardiac fibrosis. An experimental model was used in which angiotensin II was chronically infused into rats at a pressor dose by use of osmotic minipumps. Northern and Western blot procedures were used to measure steady-state mRNA and protein content of cardiac fibronectin during the induction of a mild fibrosis. In addition, in situ hybridization was used to localize the spatial distribution of fibronectin mRNA. We found that fibronectin expression occurred most prominently in the coronary vasculature and within the atrial endocardium. Angiotensin-induced increases in fibronectin expression were associated with areas of fibrosis and were not solely dependent on blood pressure elevation.

**Materials and Methods**

**Drugs, Hormones, and Reagents**

Human (Val)\(^{\text{a}}\)angiotensin II acetate salt and hydralazine HCl were purchased from Sigma Chemical Co, St Louis, Mo; prazosin HCl, from Research Neurochemicals, Natick, Mass; and Nembutal, from Abbott Laboratories, Chicago, Ill. Trandolapril and losartan were generously provided by Knoll Pharmaceutical, West Point, PA, and DuPont/Merck, Wilmington, Del, respectively. [\(\alpha^{32}\Pd]dCTP, [\(\alpha^{35}\S]UTP, and [\text{methyl-}
\text{\textsuperscript{3}H}]thyidine (1 mCi/mL) were obtained from DuPont/NEN Corp, Boston, Mass.

**Experimental Animal Models**

Male Wistar rats 8 to 10 weeks of age from Charles River Breeding Laboratories, Wilmington, Mass, were allowed 1 week to adjust to facilities before all experimental protocols. Angiotensin II was dissolved in 0.15 mol/L NaCl containing 0.01N acetic acid and infused subcutaneously via Alzet osmotic minipumps (models 1000D or 2001, Alza Corp, Palo Alto, Calif). Control rats received vehicle alone. When \text{[H]}thyidine was given, a total of 250 \muCi per animal was injected intraperitoneally at 8-hour intervals, beginning 24 hours before killing.

Antihypertensive drugs were given ad libitum via the drinking water, with treatment routinely initiated 1 day before pump implant. The dosages used were as follows: hydralazine, 2 mg/kg per day; prazosin, 0.5 mg/kg per day; losartan, 1 or 10 mg/kg per day; and trandolapril, 0.5 mg/kg per day. Systolic blood pressures were obtained by tail-cuff plethysmography. Blood pressure levels were determined before drug treatment, 1 day after implant, and 4 to 6 hours before killing. In all experiments, the reported value at each time point is an average of multiple (four to six) recordings. Nembutal was used as surgical anesthesia (50 mg/kg) and for overdosing (0.5 g/kg) the rats. Cardectomy was followed by retrograde aortic perfusion with phosphate-buffered saline (PBS) containing 4% (wt/vol) paraformaldehyde before histochemical analysis. For biochemical analysis, hearts were dissected by removal of the pericardium. Ventricle free walls, septum, and atria were separated from fibrous septum and then washed in cold PBS. Samples were flash-frozen in liquid N\(_2\) for subsequent extraction of RNA or protein.

**RNA Isolation and Northern Blot Hybridization**

Total RNA from cardiac tissue was extracted by the method of Chomczynski and Sacchi\(^{22}\) with a 20-fold volume of guanidinium thiocyanate buffer for the initial homogenization. Northern blot analysis was performed as described by Mamuya and Brecher.\(^{17}\) cDNA probes were generated using a random-prime nucleotide synthesis kit (Amersham International, UK), and hybridization was performed at 55°C for atrial natriuretic factor (ANF) and at 62°C for all other cDNA probes.

**Tissue Preparation and In Situ Hybridization**

Fixation of tissue for in situ hybridization, autoradiography, or staining followed procedures described by Wilkinson et al.\(^{25}\) Briefly, perfused hearts were cut transversely at three parallel locations, vacuum-fixed for 16 to 24 hours in PBS containing 4% paraformaldehyde at 4°C, and then washed, dehydrated, and embedded in Paraplast (Oxford Laboratories, St Louis, Mo). Serial sections (4 to 6 \mu)m were transferred to gelatin-coated slides for in situ hybridization. Staining with hematoxylin and eosin or Masson's trichrome followed standardized protocols. In situ hybridization was performed essentially as described by Sassoon et al.\(^{24}\) Briefly, slides were hydrated, pretreated with paraformaldehyde, and incubated at 4°C with 20 \mug/\muL proteinase K. Sections, fixed again with PBS/4% paraformaldehyde, were treated with acetic anhydride before

### Table 1. Effect of Angiotensin II Treatment on Wistar Rats

<table>
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<tr>
<th>Treatment</th>
<th>Time, d</th>
<th>n</th>
<th>Blood Pressure, mm Hg</th>
<th>Heart Weight, g</th>
<th>Heart Weight/Body Weight, ×1000</th>
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<td>3</td>
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<tr>
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<tr>
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</tr>
<tr>
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<tr>
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<td>15</td>
<td>131±1</td>
<td>1.03±0.01</td>
<td>3.32±0.08</td>
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</tbody>
</table>

\(n\) indicates the number of rats in each group; \(d\), experimental duration in days; and PI, postinfusion period. Values are mean±SEM.

\(\dagger\)Ang II was administered at a dosage of 0.75 mg/kg per day, unless otherwise indicated.

\(\text{vs age-matched control rats.}\)

\(\text{vs 3-day sham-operated rats.}\)
rapid dehydration and drying. Hybridization with the 35S-labeled riboprobe at a concentration of 35000 cpmp/µL was performed at 52°C for 16 hours in a buffer containing 50% formamide, 0.3 mol/L NaCl, 20 mmol/L Tris, 5 mmol/L EDTA, 10% dextran sulfate, 1x Denhardt’s solution, 0.25 mg/mL yeast RNA, and 10 mmol/L dithiothreitol. After hybridization, slides were sequentially washed in 5x standard saline citrate (SSC) and 10 mmol/L dithiothreitol at 50°C and then in 50% formamide, 2x SSC, and 0.1 mol/L dithiothreitol at 65°C. Digestion of unbound probe with 20 µg/mL RNase A/T1 (Sigma) at 37°C for 1 hour was followed by washing in 2x and 0.1x SSC. Dehydrated slides were diped in Kodak NTB-2 emulsion and exposed for 3 to 10 days. A 2-week exposure was performed for autoradiography to detect tritium after an identical protocol. Sections were developed with Kodak D19. Slides were counterstained with toluidine blue, and analysis and photography were performed by use of a Nikon Optiphot 2 system.

Protein Fractionation and Western Blot Analysis

Tissue samples were homogenized in 1:20 (wt/vol) PBS containing (mmol/L) phenylmethylsulfonyl fluoride 2, EDTA 2, N-ethylmaleimide 2, and sodium iodoacetate 2 at 4°C. An aliquot of homogenate was centrifuged for 15 minutes at 16,000g and 4°C, and the supernatant was removed and saved as PBS fraction 1. The pellet was resuspended in 4% sodium dodecyl sulfate (SDS) and 2 mol/L urea with 2 mmol/L protease inhibitors (as above), incubated at 100°C for 5 minutes, and then centrifuged (16,000g) at ambient temperature. This supernatant SDS fraction 2 was stored at −70°C. Fractional protein concentrations were measured using a BCA protein assay reagent kit (Pierce Chemical Co, Rockford, III). Protein samples were separated by SDS–polyacrylamide gel electrophoresis, as described by Laemmli,25 with a Mini-Protein II dual slab cell apparatus (Bio-Rad Laboratories, Richmond, Calif). Protein was immobilized by transfer to a nitrocellulose membrane (Transblot, Bio-Rad) at 4°C for 14 to 18 hours at 150 to 200 mA in 20 mmol/L Tris and 200 mmol/L glycine, pH 8.3. Immunobinding with 35 µg/mL goat anti-rabbit polyclonal antibody against rat plasma fibronectin (The Binding Site Limited, Birmingham, UK) was used to selectively identify purified fibronectin (Sigma) and tissue-extractable fibronectin after blocking with 10% skim milk for 1 hour at 37°C. Anti-goat IgG (144 ng/mL) conjugated to horseradish peroxidase (Sigma) permitted detection of each sample with chemiluminescence emitted from oxidized luminal as described for the enhanced chemiluminescence detection kit (Amersham). Signals were recorded by exposing prefocused hyperfilm (Amersham) to membranes for 2 to 5 minutes at 23°C. Laser densitometry was performed on each image using a 300-A computing densitometer (Molecular Dynamics, Sunnyvale, Calif) to quantify the relative signal intensity of the bands obtained.

Statistical Analysis

All values are expressed as mean±SEM. Values for body and heart weights, blood pressures, densitometric quantita-
tions, and subsequent calculations based on these measurements were subject to a global test for statistical significance. Calculations using ANOVA by either one-way or two-way methods were applied to each data set on the basis of the number of independent variables. Multiple-comparison procedures were accomplished by using Scheffé’s F-test method of ANOVA.26

**cDNA**

Rat fibronectin probes for Northern blot analysis were made from the plasmid 1-rlf-1, provided by R.O. Hynes (Schwartzbauer et al.27). cDNA for rat collagen I α-1 (pal-R1) was obtained from D. Rowe (Genovese et al.28), and collagen IV α-2 cDNA was provided by M. Kurkinen (Kurkinen et al.29). Glyceraldehyde-3-phosphate dehydrogenase cDNA was purchased from American Type Culture Collection, Rockville, Md. Murine prepro-ANF cDNA was a gift of C.E. Seidman (Seidman et al.30). For in situ hybridization, fibronectin antisense riboprobe was synthesized from a cDNA representing all isoforms of fibronectin (p-SR270), previously described by F. French-Constant and Hynes.31 The plasmid was linearized with HincII and incubated with 35S-labeled UTP in the presence of SP6 polymerase. Sense probes were made by linearization with EcoRI in the presence of T7 polymerase.

**Results**

Continuous subcutaneous infusion of 125 to 150 ng/min (0.75 mg/kg per day) angiotensin II produced hypertension within 24 hours, and this was maintained throughout the treatment period (Table 1). Systolic blood pressure for these animals was increased by day 1 to 170±4 mm Hg, remained near this level during the infusion period, and reached 181±3, 172±7, and 195±5 mm Hg at days 3, 5, and 7, respectively. The dose of 50 ng/min (0.30 mg/kg per day) angiotensin II failed to produce hypertension. Despite this difference, a significant growth of the heart, as indexed by both heart weight and the heart weight–to–body weight ratio, was seen after 3 days of treatment with either dosage. If treatment was continued with a pressor dose of angiotensin II, the difference in the weight of treated hearts compared with age-matched control hearts increased. When animals were treated with angiotensin II for 3 days and then withdrawn from treatment for a 1-week postinfusion period, blood pressure became hypertensive and subsequently returned to normotensive levels. Coincident with this was a regression of hypertrophic indexes to values no different from control for both heart weight and heart weight–to–body weight ratios.

Northern blot analysis was performed on left ventricular RNA taken from animals treated with pressor (0.75 mg/kg per day) and subpressor (0.30 mg/kg per day) doses of angiotensin II for 3 days (Fig 1A). Steady-state mRNA levels for fibronectin were increased between 10- and 20-fold after treatment with the higher dose. No increase in fibronectin expression was found in the left ventricle from animals treated with the subpressor level of angiotensin II. In contrast to the left ventricle, steady-state mRNA levels for fibronectin did not change in left atrial tissue taken from animals treated with a pressor dose of angiotensin II for 3 days (Fig 1B). The changes occurring in the left ventricle in response to angiotensin II infusion for 3 days at a pressor dose also were found in the right ventricle and were quantitatively comparable (data not shown). Further analysis was performed on left ventricular RNA taken from animals treated with angiotensin II (125 ng/min) for 1 day or 3 days or for 3 days followed by a 7-day recovery period without treatment (Fig 1C). Steady-state mRNA levels for fibronectin were increased more than fivefold after
levels between 175 and 205 mm Hg during the 7-day period. The increased fibronectin mRNA that was observed at day 3 was consistently reduced by day 5 and day 7, indicating that this modification of fibronectin mRNA was transient when the infusion period was prolonged. Although reduced after day 3, the fibronectin mRNA levels remained significantly above the control levels. Similar changes in mRNA for collagen type I and type IV followed this temporal pattern. In contrast, ANF transcription levels were increased markedly by the third day and remained so during the treatment period. Increased ANF expression is considered a marker for ventricular myocyte changes during hypertrophy; thus, the changes in ANF mRNA suggest that myocyte hypertrophy occurs during the angiotensin II treatment period.

Data obtained from multiple Northern hybridization analysis, involving 4 to 10 individual samples for each time point, are summarized in Fig 2, center and right panels. ANF mRNA, which was almost undetectable in RNA samples from control ventricles, was increased more than 20-fold after 3 days of angiotensin II treatment and, like fibronectin, did not reach a maximum expression level by the first day. After the recovery period, ANF mRNA returned almost to control levels, similar to fibronectin and both collagen type I and type IV. Unlike the pattern of ANF induction, neither fibronectin, collagen type I, nor collagen type IV maintained a maximum level of steady-state mRNA during continuous infusion.

A Western blot measuring immunodetectable fibronectin in ventricular extracts is shown in Fig 3A. A major band was identified corresponding to ≈220 kD, which consistently migrated faster than plasma fibronectin (not shown) and indicated extractable cellular fibronectin. Increased protein was obvious after 3 days of treatment, the average increase being 1.67±0.1-fold for six separate animals (Fig 3B). This increase in immunodetectable fibronectin during the 7-day period indicated a dissociation between fibronectin mRNA and protein between days 3 and 7. Of particular interest was the ventricular fibronectin content after the recovery period, during which the levels were significantly below the values for animals treated for 3 days. Thus, angiotensin II treatment induced reversible changes in fibronectin expression at the level of both mRNA and protein.

In situ hybridization was used to study the localization of fibronectin mRNA within the rat heart. Histological analysis of control and angiotensin II–treated ventricular tissue (Fig 4A and 4C) indicated that, after 3 days of infusion with angiotensin II, there was a mild to moderate fibrosis. The regions of fibrosis were often interstitial, but perivascular fibrosis also was obvious. The fibrotic regions were focal and were distributed both in left and right ventricular tissue with no obvious selective distribution either in endocardial or myocardial regions. In the control ventricle, in situ hybridization indicated fibronectin mRNA almost exclusively associated with small arteries, with the vascular smooth muscle cells as the major source (Fig 4B). After 3 days of angiotensin II infusion, in situ analysis showed a strong signal for fibronectin mRNA associated with interstitial fibroblasts primarily in the regions of fibrosis (Fig 4D), and no significant signal appeared within
myocytes relative to background. The signals in Fig 4 represent antisense riboprobe recognizing all isoforms of fibronectin mRNA. When a sense riboprobe was used, no distinct pattern over background was seen either in control or treated tissue.

In a separate series of experiments (data not shown) in which \[^{3}H\]thymidine was injected into rats treated with angiotensin II for up to 3 days, the thymidine label was found almost exclusively in nuclei of interstitial and perivascular fibroblasts within fibrotic regions. Occasional label was associated with interstitial fibroblasts outside these areas of fibrosis and within mesothelial cells. Of note was the virtual absence of label within other cell types of the heart, including myocytes and vascular smooth muscle cells. Thus, the interstitial fibroblasts appeared to be the principal cell type induced to proliferate or enter S phase after hormone administration. With in situ hybridization, we found no detectable fibronectin mRNA in cell types other than vascular smooth muscle cells and proliferating interstitial fibroblasts in either control or angiotensin II–treated animals. Although we cannot rule out the possibility that myocytes do express low levels of fibronectin, it would appear that the increased steady-state fibronectin mRNA levels detected by Northern blot analysis correspond in large part to the interstitial fibroblasts associated with fibrosis, consistent with the recent findings of Samuel et al.,\(^{20}\) who observed fibronectin that was associated with fibrotic regions induced by acute pressure overload after aortic coarctation.

In situ hybridization of atrial tissue from untreated or treated rats showed fibronectin mRNA associated with the connective tissue component of the endocardium. A trichrome stain of atria (Fig 5A) showed the extracellular matrix associated with both endocardium and epicardium. A comparison of serial sections stained either with hematoxylin and eosin (Fig 5B) or analyzed by in situ hybridization for fibronectin (Fig 5C) showed dense accumulation of silver grains primarily in the endocardial regions. The intensity of the signal within the endocardial region was considerably greater than that associated with the vascular smooth muscle cells within the atria, suggesting that the relatively high amount of steady-state fibronectin mRNA within the atria was due primarily to connective tissue cells within the endocardium. Angiotensin II infusion produced no obvious qualitative or quantitative change in atrial expression of fibronectin by in situ hybridization or histological evidence of fibrosis (data not shown), consistent with Northern blot analysis shown in Fig 1B.

To distinguish the role of angiotensin II in fibronectin regulation from the effect of blood pressure elevation, antihypertensive drugs were given during angiotensin II infusion. The effects of antihypertensive drugs on Wistar rats treated with pressor doses of angiotensin II are summarized in Table 2. Coadministration of either 5 mg/kg per day of the vasodilator hydralazine, 0.2 mg/kg per day of the \(\alpha_1\)-adrenergic receptor antagonist prazosin, or 10 mg/kg per day of angiotensin II type 1 (AT1) receptor antagonist losartan was effective in normalizing blood pressure. However, only the high-dose losartan was shown to completely block the increase in heart weight after 3 days of angiotensin administration. A lower dose of losartan, 1 mg/kg per day, did not return systolic pressure to normal, although this dose of losartan did significantly reduce heart weight gain.

The effect of losartan on fibronectin mRNA expression during angiotensin infusion is summarized in Fig 6. A dose-dependent attenuation of fibronectin expression was found 3 days after coadministration of losartan with angiotensin II. At 10 mg/kg per day, losartan blocked the increase in systolic blood pressure (132±4 mm Hg) expected after angiotensin II infusion and completely eliminated the increase in fibronectin expression. By use of 1 mg/kg per day of losartan, an intermediate level of blood pressure control was achieved (158±5 mm Hg) in animals given angiotensin II. In the left ventricle from these animals, increases in fibronectin expression were at a level between control and angiotensin II–treated animals. Changes comparable to those shown for fibronectin mRNA were found when mRNA for collagen types I and IV was analyzed in the ventricles of losartan-treated rats (data not shown).

Both hydralazine and prazosin administered during angiotensin II treatment lowered blood pressure significantly below hypertensive levels. Fig 7 shows a representative Northern blot of left ventricles from rats treated with angiotensin II for 3 days along with either hydralazine or prazosin. The steady-state level of fibronectin mRNA in ventricles from animals receiving hydralazine during angiotensin II treatment was no different from control animals after the third day of infusion. In contrast, animals whose blood pressures were normalized by prazosin during angiotensin II treatment had left ventricular fibronectin levels typical of rats made hypertensive after 3 days of angiotensin II. Changes in collagen type I mRNA reflected the changes seen with fibronectin. Taken together, these data indicate that elevated blood pressure is not the principal regulator of fibronectin induction in the left ventricle. In addition, it suggests that pharmacologic intervention may influence the mechanism responsible for regulating fibronectin expression.

To examine the potential influence of converting enzyme inhibitors on angiotensin II–mediated fibronectin induction, trandolapril was administered during angiotensin II treatment. Fig 8 shows a reduced steady-state mRNA level of fibronectin in ventricular tissue from rats treated with angiotensin II plus trandolapril when compared with tissue from rats treated with angiotensin II alone. This effect appeared to be specific for fibronectin among the genes examined under these conditions. Unlike fibronectin, neither collagen type I, collagen type IV, nor ANF expression was changed by trandolapril. Trandolapril treatment did not lower blood pressure in angiotensin II–treated rats (204±10 mm Hg); thus, the suppressive effect on fibronectin mRNA was independent of any change in blood pressure.

Fig 4. In situ hybridization showing the distribution of fibronectin mRNA in the ventricle of normal and 3-day angiotensin II–treated rats. Serial 4- to 6-\(\mu\)m sections of left ventricle from control rats (A and B) and rats treated with angiotensin II for 3 days (C and D) are shown (original magnification \(\times 26\)). Photomicrographs are of representative sections stained with hematoxylin and eosin (A), with Masson’s trichrome (C), or under dark-field illumination after in situ hybridization for fibronectin (B and D).
FIG 5. In situ hybridization showing that atrial endocardium is the principal site of fibronectin mRNA. A, Masson's trichrome stain of left atria from a normal 275-g Wistar rat (original magnification ×50). B and C, Representative images of serial 4- to 6-μm sections of left atria either stained with hematoxylin and eosin (B) or under dark-field illumination after in situ hybridization for fibronectin (C) (original magnification ×50). No difference in atrial fibronectin distribution was seen when angiotensin was infused.
Table 2. Effect of Antihypertensive Drugs on Angiotensin II–Treated Wistar Rats

<table>
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<th>Treatment</th>
<th>Time, d</th>
<th>n</th>
<th>Blood Pressure, mm Hg</th>
<th>Heart Weight, g</th>
<th>Heart Weight/Body Weight, x1000</th>
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<tr>
<td>Control</td>
<td>3</td>
<td>5</td>
<td>122±1</td>
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<td>Ang II</td>
<td>3</td>
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<td>184±4*</td>
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</table>

n indicates the number of rats in each group; Ang II, angiotensin II. Values are mean±SEM. Ang II was administered at a dosage of 0.75 mg/kg per day. Antihypertensive drugs were given in the drinking water at the doses shown.

*P<.05 vs sham-operated and control rats.
†P<.05 vs 3-day Ang II–treated rats.

Discussion

A role for the renin-angiotensin system in the development of hypertension-induced cardiac hypertrophy has long been suspected, yet it has been difficult to establish whether a causative role for angiotensin II in cardiac hypertrophy is due to direct or indirect effects of the hormone. In the present study, we subcutaneously administered angiotensin II to adult rats to study the interrelations between this hormone, cardiac fibronectin expression, and the cellular changes associated with hypertrophy. Our results support the interpretation that fibronectin expression in nonmyocytic cells of the ventricular myocardium undergoes an increase in response to pressors levels of angiotensin II. This increase apparently leads to an accumulation of fibronectin associated with elevated collagen expression, fibrosis, and myocyte hypertrophy. In addition, we suggest that angiotensin II may be acting directly on cardiac fibroblasts and that pharmacologic intervention may modify this process via a mechanism distinct from blood pressure reduction.

The transient increase in fibronectin steady-state mRNA in ventricular tissue during a 7-day angiotensin II administration period contrasted with the progressive accumulation of immunoreactive fibronectin. This dissociation between cardiac fibronectin mRNA and protein was also documented in the rat after induction of cardiac hypertrophy by triiodothyronine administration.17 In that study, mRNA levels rose and fell over a 10-day treatment period, with maximum induction between 3 and 6 days, whereas immunoreactive protein was severalfold more abundant after 10 days. In a recent study by Villarreal and Dillmann,21 cardiac hypertrophy

![Fig 6. Losartan attenuates angiotensin II (All)-mediated increase in ventricular fibronectin (FN) expression. Northern blot analysis using total RNA (10 μg per lane) from pooled pairs of left ventricles compares control rats and rats treated with All alone for 3 days (3d All) or in combination with one of two dosage levels of losartan. Steady-state mRNA levels of FN and glyceraldehyde-3-phosphate dehydrogenase (GAPD) are shown. Mean systolic blood pressure (SBP) on day 3, for the animals represented, is indicated below each group.](http://circres.ahajournals.org/)

![Fig 7. Northern blot analysis of total RNA (10 μg per lane) from left ventricles of individual rats. Expression of fibronectin (FN), collagen type I α-1 (CI), and glyceraldehyde-3-phosphate dehydrogenase (GAPD) in age-matched control rats is compared with expression in rats treated with angiotensin II (All) for 3 days (3d All) or subjected to both 3 days of All treatment plus antihypertensive drugs. Groups treated with All and either 2 mg/kg per day of hydralazine (3d All+hydralazine) or 0.5 mg/kg per day of prazosin (3d All+prazosin) are compared. Mean systolic blood pressure (SBP) on day 3, for the animals represented, is indicated below each group.](http://circres.ahajournals.org/)
was induced by aortic coarctation, and a similar transient increase was found in fibronectin mRNA 1 to 3 days after surgery, followed by a progressive reduction to control levels during the subsequent 10 days. Although fibronectin itself was not determined in those studies, Northern blot analysis showed that induction of fibronectin mRNA preceded that for fibrillar collagen types, consistent with the findings in the present study. In a study by Chapman et al., a pressure-overload model involving constriction both of aorta and right renal artery was used to study collagen gene expression in the rat heart. It was found that a transient increase in collagen types I and III occurred 3 days after surgery and returned to control levels after 7 days. These changes were identified as being produced in cardiac fibroblasts, and distinctions between regulatory events occurring in myocytes and fibroblasts were made during the hypertrophic response in this model.

We found elevated ventricular levels of both fibronectin mRNA and protein by the third day of angiotensin II infusion. Increase in collagen type I also occurred but was not induced as rapidly. These increases were consistently found within cardiac tissue undergoing hypertrophy and a mild interstitial fibrosis. Increased fibronectin expression followed by collagen deposition and fiber formation is a major feature of wound healing in diverse tissue types. It is thought that fibronectin, through interactions with specific binding domains, may influence the organization of collagen bundles as healing progresses. In normal wound healing, during the formation of evolving granulation tissue, collagen deposits remain to form fibrils, whereas fibronectin slowly resolves from the wound area. In contrast, keloid lesions, which are characterized by abnormal collagen metabolism and scar formation, contain fibroblasts that maintain a fourfold increase in fibronectin biosynthesis corresponding to an increased level of fibronectin mRNA. In the present study, we report a potentially reversible accumulation of fibronectin within the ventricle undergoing fibrosis, which appears to depend on the presence of angiotensin II. The loss of immunoreactive fibronectin after cessation of treatment with angiotensin II could reflect increased degradation of the protein, perhaps by the action of specific proteases induced by growth factors or by other posttranscriptional mechanisms.

In two previous studies of the rat heart after acute cardiac injury due to either ischemia or acute pressure overload, increases in fibrillar collagen types I and III were preceded by fibronectin accumulation, a temporal relation we found in response to angiotensin II. In the failing human heart, fibronectin was colocalized with collagen types I, III, and IV, and a positive correlation was established between increased fibronectin and other extracellular matrix proteins by electron microscopy and immunofluorescence. Moreover, in the spontaneously hypertensive rat, which exhibits cardiac hypertrophy with associated fibrosis, the normal decrease in cardiac fibronectin seen with aging does not occur, implying increased fibronectin production. Specifically how fibronectin accumulation influences collagen deposition and extracellular matrix structure during the abnormal fibrous tissue response found in the heart of hypertensive animals has not been clearly established.

Fibrosis occurred rapidly after angiotensin II administration and appeared to be responsible for the increased expression of fibronectin mRNA measured by Northern blot analysis. In situ hybridization showed that the major difference between treated and control animals was the fibronectin mRNA associated with proliferating interstitial fibroblasts, particularly in perivascular regions. These findings are consistent with those recently reported by Samuel et al., showing fibronectin expression associated with the fibrosis induced by aortic coarctation. In those studies, the fibrosis also occurred rapidly and was attributed to acute pressure overload because of the predominant response in the left ventricle.

Tan et al. used a subpressor angiotensin infusion model to produce cardiac fibrosis. In those studies, evidence for fibrosis was found after 1 day of treatment, and most of the cellular proliferation occurred within a 2-day period, subsequently declining to control levels. These findings were correlated with myocyte loss and were induced in a normotensive animal model. Our experimental model differed from that of Tan et al. mainly in the dose and route of administration of the angiotensin II. Subcutaneous (0.75 mg/kg per day) rather than intraperitoneal (0.96 to 1.44 mg/kg per day) minipump implantation resulted in a more effective pressor response despite the lower dose. Histological analysis in the present study showed the fibrosis to be obvious even after only 1 day of treatment with this lower dose, yet the fibrotic regions remained sparsely dispersed throughout the ventricles and did not appear to progress or worsen after the third day. Autoradiographic analysis of thymidine incorporation also was consistent with a rapid response that did not progressively increase during the initial 3 days of treatment. Despite the
differences in protocols, the similar findings with respect to fibrosis in animals given both pressor or subpressor doses does suggest a direct effect of angiotensin II on cardiac cells.

The presence of angiotensin receptors throughout the rat heart has been described, suggesting the possibility that this peptide can act directly on cells of this tissue. Using losartan, a receptor subtype-1 antagonist, in combination with angiotensin II, we described a dose-dependent reduction in fibronectin expression. Although correlated with a dose-dependent reduction in blood pressure, animals treated with low-dose losartan remained hypertensive yet had less fibronectin expression than hypertensive animals without losartan treatment. Thus, competitive inhibition of the AT1 receptor may attenuate angiotensin II–mediated increases in fibronectin expression even in the presence of elevated systolic blood pressure.

The relative importance of hemodynamic factors versus circulating hormones in promoting myocyte hypertrophy and ventricle fibrosis is of considerable interest. To distinguish between these possible influences on fibronectin expression, we administered a number of antihypertensive drugs to rats during concurrent angiotensin II infusion. When prazosin was given, it lowered blood pressure by equal or greater amounts than losartan treatment yet had no effect on the increase in fibronectin. In contrast, hydralazine lowered pressures to normotensive levels and prevented fibronectin mRNA accumulation. These data show that induction of fibronectin is not necessarily dependent on elevated blood pressure and that therapeutic interventions can modify fibronectin expression during the growth of the heart. The specific mechanisms involved in the different responses to prazosin and hydralazine with respect to fibronectin expression cannot be explained at present. However, a possible regulatory role for nitric oxide could be proposed on the basis of the possibility that hydralazine would influence nitric oxide and cGMP levels in the cardiac microvasculature. Alternatively, direct effects of these drugs on cardiac fibroblasts could also occur.

Administration of the angiotensin-converting enzyme inhibitor trandolapril in combination with angiotensin II yielded a consistent reduction of ventricular fibronectin, despite the presence of hypertension. Angiotensin-converting enzyme inhibitors are known to influence cell growth and differentiation. and numerous studies indicate a powerful effect by these drugs on prevention and regression of hypertrophy. Reversal of cardiac fibrosis, and survival after experimental and clinical myocardial infarction. In addition, the presence and function of a tissue renin-angiotensin system within the heart supports the hypothesis that local angiotensin II generation acts during cardiac growth and that converting enzyme inhibitors may interfere with this process. Alternatively, by inhibiting the degradation of bradykinin, this class of drugs may modify the cardiac response to angiotensin II via a mechanism separate from the renin-angiotensin cascade. Whichever was the mechanism responsible for altering fibronectin expression, it had limited influence on the acute induction of either collagen or ANF mRNA. This distinction may relate to in vitro studies using cardiac cell explants, which suggest that both collagen type III and ANF mRNA can be upregulated by mechanical stimulation. In addition, the present study shows that when blood pressure was reduced during angiotensin II infusion with hydralazine or losartan, both collagen type I and type IV mRNA levels were reduced (data not shown).

We selected the ANF gene as an indicator of a cell-type-specific phenotypic shift, since ANF gene induction in the adult rat ventricle occurs in cardiac myocytes during hypertrophy. In a genetic rat model of biventricular hypertrophy, Lee et al described an equivalent level of ANF induction in both left and right ventricles. Other investigators using models of altered cardiovascular hemodynamics have demonstrated a marked induction of left ventricular ANF with expression localized to myocytes within the myocardium. In the present study, ANF expression was elevated >20-fold by the third day of angiotensin II treatment, indicating a phenotypic shift characteristic of myocytes in the hypertrophic state. ANF expression may represent a direct myocyte response to angiotensin II, an adaptive component of myocyte growth, or a general gene-reprogramming coincident with hypertrophic growth. The temporal pattern that we observed of ANF induction during cardiac hypertrophy and the subsequent reduction in fibronectin and collagen expression may reflect a paracrine influence by the hypertrophic myocytes on interstitial cells. In support of this hypothesis, a number of studies have shown that ANF can reduce hypertrophy and block cell proliferation induced by angiotensin II. Similarly, in vitro application of ANF to cultured vascular endothelial cells reduced expression, translation, and secretion of endothelin 1, a growth factor shown to stimulate cardiac myocyte hypertrophy.

Several in vitro studies also suggest that angiotensin II mediates both stimulation of 3T3 cell division and increases cardiac fibroblast expression of fibronectin and collagen through the AT1 receptor. In addition, studies by Baker and Aceto have shown direct effects of angiotensin II on cardiac myocytes and have suggested that local production of transforming growth factor-β (TGF-β) may precede and perhaps cause the increase in fibronectin expression. TGF-β has been shown to be an important regulator of extracellular matrix proteins, including fibronectin, in diverse cell types and recent speculation concerning the potential role of TGF-β in self-limiting fibrotic processes suggests a role for this growth factor in cardiac fibrosis. Villarreal and Dillmann recently showed that steady-state mRNA levels for TGF-β, but not TGF-β, in the heart increased very rapidly (within 12 hours) after thoracic banding and remained elevated for 2 weeks. This rapid increase preceded increases in both fibronectin and collagen mRNA, suggesting a functional role for this growth factor in the development of hypertrophy. Which growth factors specifically modulate the progression and regression of the hypertrophic response remains an important question in the understanding of cardiac biology. Our data suggest that angiotensin II has a role in promoting the expression of fibronectin during the remodeling of the ventricle. How these alterations in ventricular fibronectin functionally affect ventricular performance is unknown at present. However, if indeed pharmacologic intervention modifies the response of...
nonmyocytic cells to angiotensin II, this in vivo model can provide an approach in the study of intercellular signaling mechanisms that regulate ventricular remodeling in situ.

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