Mechanical Stretch and Angiotensin II Differentially Upregulate the Renin-Angiotensin System in Cardiac Myocytes In Vitro

Ricky Malhotra, Junichi Sadoshima, Frank C. Brosius III, Seigo Izumo

Abstract—Pressure overload in vivo results in left ventricular hypertrophy and activation of the renin-angiotensin system in the heart. Mechanical stretch of neonatal rat cardiac myocytes in vitro causes secretion of angiotensin II (Ang II), which in turn plays a pivotal role in mechanical stretch–induced hypertrophy. Although in vivo data suggest that the stimulus of hemodynamic overload serves as an important modulator of cardiac renin-angiotensin system (RAS) activity, it is not clear whether observed upregulation of RAS genes is a direct effect of hemodynamic stress or is secondary to neurohumoral effects in response to hemodynamic overload. Moreover, it is unclear whether activation of the local RAS in response to hemodynamic overload predominantly occurs in cardiac myocytes or fibroblasts or both.

In the present study, we examined the effect of mechanical stretch on expression of angiotensinogen, renin, angiotensin-converting enzyme (ACE), and Ang II receptor (AT₁A, AT₁B, and AT₂) genes in neonatal rat cardiac myocytes and cardiac fibroblasts in vitro. The level of expression of angiotensinogen, renin, ACE, and AT₁A genes was low in unstretched cardiac myocytes, but stretch upregulated expression of these genes at 8 to 24 hours. Stimulation of cardiac myocytes with Ang II also upregulated expression of angiotensinogen, renin, and ACE genes, whereas it downregulated AT₁A and did not affect AT₁B gene expression. Although losartan, a specific AT₁ antagonist, completely inhibited Ang II–induced upregulation of angiotensinogen, renin, and ACE genes, as well as stretch-induced upregulation of AT₁A expression, it did not block upregulation of angiotensinogen, renin, and ACE genes by stretch. Western blot analyses showed increased expression of angiotensinogen and renin protein at 16 to 24 hours of stretch. The ACE-like activity was also significantly elevated at 24 hours after stretch. Radioligand binding assays revealed that stretch significantly upregulated the AT₁ density on cardiac myocytes. Interestingly, stretch of cardiac fibroblasts did not result in any discernible increases in the expression of RAS genes. Our results indicate that mechanical stretch in vitro upregulates both mRNA and protein expression of RAS components specifically in cardiac myocytes. Furthermore, components of the cardiac RAS are independently and differentially regulated by mechanical stretch and Ang II in neonatal rat cardiac myocytes. (Circ Res. 1999;85:137-146.)

Key Words: stretch ■ cardiac myocyte ■ renin-angiotensin system ■ angiotensin II ■ radioligand binding

The renin-angiotensin system has been described as an endocrine regulator of cardiovascular physiology. However, a number of recent biochemical and molecular biological studies point to the existence of a local renin-angiotensin system in the heart.1–3 The principal mediator of the renin-angiotensin system is the vasoactive peptide angiotensin II (Ang II). Ang II has been extensively implicated in the development of cardiac hypertrophy associated with hemodynamic overload, myocardial infarction, and hypertension.4–6 Previous studies have demonstrated that mechanical stretch of cultured rat cardiac myocytes causes a rapid secretion of Ang II7–10 and that this autocrine production of Ang II plays a critical role in many, if not all, stretch-induced hypertrophic responses.7,8 We, and others, have reported that Ang II causes hypertrophic responses and activates multiple protein kinases in neonatal rat cardiac myocytes.8,11,12

Several in vivo studies have demonstrated that mRNA expression of cardiac angiotensinogen,4,13 angiotensin-converting enzyme (ACE),14,15 and Ang II type I and type 2 receptors16–18 (AT₁ and AT₂, respectively) are upregulated in response to pressure overload or after myocardial infarction in various animal species. Similarly, cardiac renin activity...
and renin mRNA are increased in experimental animals having volume-overloaded cardiac hypertrophy.\textsuperscript{19,20} In a canine model of right ventricular hypertrophy and failure caused by tricuspid valve occlusion and progressive pulmonary artery constriction, upregulation of ACE and AT\textsubscript{2} mRNA was demonstrated recently.\textsuperscript{21}

Although these results indicate that mRNA expression of cardiac renin-angiotensin system genes is affected in response to hemodynamic overload in vivo, it is not known whether upregulation of renin-angiotensin system genes is due to the direct effect of hemodynamic stresses or is secondary to neurohumoral effects caused by hemodynamic overload. The main goal of this study was, therefore, to examine whether mechanical stretch (up to 24 hours) directly affects expression of angiotensinogen, renin, ACE, and Ang II receptor subtype (AT\textsubscript{1A}, AT\textsubscript{1B}, and AT\textsubscript{2}) genes in neonatal rat cardiac myocytes using the in vitro model of stretch-induced cardiac hypertrophy.\textsuperscript{22} In addition, we also addressed the following questions. First, given that mechanical stretch of neonatal rat cardiac myocytes causes a rapid secretion of Ang II, and Ang II mediates many, if not all, aspects of cardiac hypertrophic responses,\textsuperscript{7,22,23} did the Ang II secreted by stretch regulate subsequent expression of cardiac renin-angiotensin system genes? Second, given that a number of cell types are present in the heart, with cardiac myocytes and fibroblasts being the predominant cell types, did activation of the local renin-angiotensin system in response to hemodynamic overload in vivo occur primarily in cardiac myocytes or fibroblasts or both? Therefore, we separately made myocyte and fibroblast cultures on deformable silicone substrates and examined the effect of mechanical stretch on expression of renin-angiotensin system genes in the individual cell types.

We report that mechanical stretch is sufficient to upregulate expression of the local renin-angiotensin system genes, including angiotensinogen, renin, ACE, and AT\textsubscript{1A}, predominately in cardiac myocytes. In addition, we unexpectedly found that stretch-induced upregulation of angiotensinogen, renin, and ACE is mediated by an Ang II–independent mechanism.

Materials and Methods

Materials

All culture reagents were purchased from GIBCO-BRL. All radiochemicals were obtained from DuPont-New England Nuclear. 5-bromo-2′-deoxyuridine was generously gifts from DuPont Merck and Parke-Davis, respectively. Ang II was purchased from Peninsular Laboratories. All other chemicals were from Sigma.

Cell Culture

Primary cultures of neonatal rat cardiac myocytes were prepared as described.\textsuperscript{23} Cardiac myocytes were obtained from ventricular tissue of 1-day-old Wistar rats by 6 or 7 digestions, 15 minutes each, at 37°C in HEPES-buffered saline solution containing 0.1% collagenase IV, 0.1% trypsin, 15 μg/mL DNase I, and 1.0% chicken serum. The dissociated cells were collected by centrifugation and resuspended in DMEM/F-12 (GIBCO-BRL) (1:1, vol/vol) supplemented with 5% horse serum, 3 mmol/L pyruvic acid, 100 μmol/L ascorbic acid, 1 μg/mL transferrin, 10 ng/mL selenium, and 100 μg/mL ampicillin. To selectively enrich for cardiac myocytes, the differential attachment technique (preplating) for 1 hour was used. The resultant suspension of cardiac myocytes was plated onto the collagen-coated silicone sheet at a density of 1×10\textsuperscript{5} cells/cm\textsuperscript{2}.

Bromodeoxyuridine at a final concentration of 0.1 mmol/L was added during the first 36 hours to prevent nonmyocyte proliferation. After 36 hours, the culture medium was changed to serum-free DMEM/F-12. This procedure normally resulted in contractile myocardial cell cultures with \textasciitilde90% to 95% myocytes, as described previously.\textsuperscript{22} Highly enriched cultures of nonmyocytes (cardiac fibroblasts) were prepared by 2 passages of cells adhered to the culture dish during the preplating procedure.

Mechanical Stretch

The in vitro stretch device used in the present experiments has been previously described.\textsuperscript{22} A uniaxial strain was applied by stretching the silicone sheet in the Plexiglas support frame by 20%. All stretch experiments were performed 48 hours after changing the medium in cardiac myocyte and cardiac fibroblast cultures to serum-free medium.

Reverse Transcript–Polymerase Chain Reaction (RT-PCR)

Isolation and Analysis of RNA

Total cellular RNA was isolated from cardiac myocytes by harvesting the cells in 4 mol/L guanidinium thiocyanate, followed by ultracentrifugation through 5.7 mol/L cesium chloride solution.\textsuperscript{24} Genomic DNA was removed by incubation with RNase-free DNase (Promega) in 1× buffer (in mmol/L, Tris-HCl [pH 7.9] 40, NaCl 10, MgCl\textsubscript{2} 6, and CaCl\textsubscript{2} 10) at 37°C for 20 minutes. The mRNA was extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol. The integrity of the RNA was checked by electrophoresis through 1% formaldehyde-containing agarose gels.

Reverse Transcription

One microgram of RNA sample was reverse transcribed in 1× PCR buffer (50 mmol/L KCl and 10 mmol/L Tris-HCl, pH 8.3) in the presence of 5 mmol/L MgCl\textsubscript{2}, 1 mmol/L dNTPs, 1 U/μL RNase inhibitor, 2.5 μmol/L random hexanucleotide primers, and 2.5 μL Moloney murine leukemia virus reverse transcriptase (Perkin Elmer). The tubes were initially incubated at room temperature for 10 minutes, and then the reverse transcription reaction was carried out at 42°C for 60 minutes, followed by denaturation at 99°C for 5 minutes and cooling at 5°C for 5 minutes.

Design of Primers

The cDNA regions of rat angiotensinogen, renin, ACE, AT\textsubscript{1A}, AT\textsubscript{1B}, and AT\textsubscript{2} genes were amplified by the PCR of each gene. The PCR primers were designed in such a way that the sequences corresponding to the 2 primers were separated by introns. Thus, genomic DNA contamination, if present, in the PCR could be easily identified. The sequence of oligonucleotide primers used for PCR amplification of angiotensinogen, renin, ACE, AT\textsubscript{1A}, AT\textsubscript{1B}, and AT\textsubscript{2} genes and tubulin is shown in the Table. AT\textsubscript{1A} and AT\textsubscript{1B} were distinguished by using oligonucleotide primers specific for the respective 3′ noncoding region, as described previously.\textsuperscript{25}

Polymerase Chain Reaction

The 20-μL reverse transcription reactions were directly used for PCR amplifications of renin-angiotensin system and Ang II receptor genes. A typical PCR of 100 μL final volume was carried out in 1× PCR buffer (50 mmol/L KCl and 10 mmol/L Tris-HCl, pH 8.3) containing 2 mmol/L MgCl\textsubscript{2}, 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer), and 0.15 μmol/L of the respective primers. The PCR amplification profile for each of the genes examined involved an initial denaturation step at 94°C for 30 seconds to 1 minute. The primer-annealing step for the renin-angiotensin system and Ang II receptor genes was as follows: angiotensinogen, 63°C for 60 seconds; renin, 62°C for 30 seconds; ACE, 55°C for 30 seconds; and AT\textsubscript{1A}, AT\textsubscript{1B}, and AT\textsubscript{2}, 58°C for 45 seconds. The PCR products were extended for 30 to 90 seconds at 72°C, with a final elongation step at 72°C for 7 minutes. The numbers of PCR cycles for the 6 genes examined were as follows: angiotensinogen, 30; renin, 35; ACE, 25; and AT\textsubscript{1A}, AT\textsubscript{1B}, and AT\textsubscript{2}, 30. The size of the final PCR product is...
Design of Primers

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shown in the Table. The PCRs were carried out in the Perkin Elmer thermal cycler 480. The PCR products were extracted with phenol/ chloroform/isoamyl alcohol (25:24:1, vol/vol), and 10-μL products were analyzed in 1.5% to 2% agarose gels. To further ensure that RT-PCR products were not amplified from genomic DNA, all RNA samples were additionally amplified without reverse transcriptase. In all cases, PCR of RNA samples was negative in the absence of reverse transcriptase (data not shown).

**PCR–Southern Blot Analysis**

The specificity of PCR products was confirmed by Southern hybridization using a 5' end-labeled internal oligonucleotide probe. Briefly, the agarose was subjected to 20 to 30 minutes of successive treatment with denaturation (1.5 mol/L NaCl and 0.5 mol/L NaOH) and neutralizing (1.5 mol/L NaCl and 0.5 mol/L Tris-HCl, pH 7.0) solutions, followed by a distilled H2O rinse. The PCR products were transferred overnight to Hybond nylon N+ membranes (Amersham) in 10× SSC. The sequence of the oligonucleotide probes used in the present study is shown below:

**Angiotensinogen**: 5'-GGCAAGAGCTCGTGCGGCCCC-3'

**Renin**: 5'-TACCTGACAGCTACGACGCCAG-3'

**ACE**: 5'-ACCCAGAGCTCCAACTCCCTC-3'

**AT1A**: 5'-GACTAATGCTGCTGACAAA-3'

**AT1B**: 5'-AGAGAGCTCCTACTACAGGAAG-3'

**AT2**: 5'-ACCTGCGGCCCACCAACATGCTA-3'

The oligonucleotide probes (100 ng) were 5'-end labeled and purified on a nucleic acid chromatography system column (GIBCO-BRL), and they had a specific activity of ≥2×10^6 cpm/μL. The membranes were prehybridized (4 to 6 hours) and hybridized (10× Denhardt’s solution, 4× SSC, 10 mmol/L Tris-HCl [pH 7.5], 0.1% SDS, 1 mmol/L EDTA, and 100 μg/mL sheared and denatured salmon sperm DNA) for 10 to 12 hours at 20°C to 25°C below the estimated denaturing temperature of the respective oligonucleotide probes. The probe concentration used was 2 to 4×10^6 cpm/mL of hybridization buffer. After hybridization, the blots were washed 3 times at low stringency (2× SSC and 0.05% SDS at 22°C), followed by 2 washes at high stringency (4× SSC and 0.1% SDS at the respective hybridization temperature). Autoradiography was carried out at -70°C.

**Linearity of PCR Amplification**

To check whether our PCR conditions for the renin-angiotensin system genes were in the linear assay range, we performed experiments with varying amounts of total input RNA. As an internal control, we used primers for the ubiquitously expressed gene tubulin, which is coamplified along with the target RNA(s) of interest. Amplification of tubulin was monitored to determine sample-to-sample variations in RT and PCR conditions and the extent of degradation and recovery of RNA. Because of the relative abundance of tubulin expression in cells as compared with that of renin-angiotensin system genes, primers for tubulin were added to the PCR for only the last 22 cycles. A semiquantification of the renin-angiotensin system gene expression was achieved by the densitometric analysis of the signal strength obtained on the PCR-Southern autoradiograms. The densitometric score in the stretched state was expressed relative to a control or unstretched condition, which was arbitrarily fixed at 1. At fixed PCR cycle numbers, varying input RNA concentrations from 0.1 to 2.5 μg of all renin-angiotensin system genes resulted in a linear amplification range for each gene (Figure 1). These results indicate that our RT-PCRs were all performed in the linear range of the reaction.

![Figure 1. Linearity of PCR amplification. The linearity of RT-PCR to total input RNA (0.1 to 2.5 μg) for the renin-angiotensin system (angiotensinogen, renin, and ACE) and AT1A genes was examined. Each data point represents the mean of 2 independent observations. Straight lines represent best fit obtained by the linear regression analysis; r value indicates correlation coefficient.](http://circres.ahajournals.org/content/139/12/T185/F1.large.jpg)
Western Blot Analysis
Cardiac myocytes were grown on silicone membranes as described and stretched for 8 to 24 hours. The cells were rinsed with cold PBS (pH 7.4) and then scraped into hypotonic lysis buffer containing (in mmol/L) Tris (pH 7.4) 20, benzamidine 10, EDTA 10, β-mercaptoethanol 5, phenylmethylsulfonyl fluoride (PMSF) 1, and 1 mg/mL leupeptin and 0.1 mg/mL aprotinin for renin and radio-immunoprecipitation assay (RIPA) buffer (1 × PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors) for angiotensinsogen detection. The samples were further homogenized and sedimented at 100 000 × g for 40 minutes at 4°C. The supernatants were collected and stored at −70°C.

Proteins (50 μg) from control and stretched lysates were fractionated on 12.5% (for renin) and 10% (for angiotensinogen) SDS-PAGE as described.12 Blots were blocked by incubation for 1 hour at room temperature with Blotto (10 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, 0.5% nonfat dry milk, and 0.05% Tween 20). Mouse monoclonal antibody against rat angiotensinogen or renin (Swant, Swiss Antibodies) was diluted in Blotto (0.5 and 0.1 μg/mL, respectively) and incubated with the membranes overnight at 4°C. The blots were rinsed with TBS-T (10 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, and 0.05% Tween-20) and incubated for 1 hour in Blotto with a 1:10 000 dilution of horseradish peroxidase-conjugated anti-mouse secondary antibodies. Detection was performed using enhanced chemiluminescence (Amersham) followed by autoradiography.

Measurement of ACE-Like Activity
The ACE-like activity was measured by hydrolysis of synthetic tripeptide substrate N-(3-[2-furyl]acryloyl)phenylalanlynlglycylglycine followed by spectrophotometric determination of a decrease in absorbance at 340 nm as previously described.26 The ACE-like activity in the control and stretched cardiac myocytes samples was determined by comparing the sample reaction rate with that obtained with the ACE calibrator (Sigma).

Ang II Receptor Binding Assay
Radioligand binding assay for the Ang II receptor was performed as previously described.16 In addition, this assay was repeated on cardiac myocytes, which were highly enriched by centrifugation through Percoll gradient according to the method of Sheng et al.27 The cells were harvested in ice-cold buffer (0.25 mol/L sucrose and 25 mmol/L Tris, pH 7.5, containing 0.5 mmol/L EDTA, 0.5 mmol/L PMSF, 10 mg/L bacitracin, 4 μg/mL leupeptin, 4 μg/mL pepstatin, and 40 U/mL aprotinin [Trasylol]). The cells were subsequently disrupted with 2 freeze-thaw cycles and homogenized using a Polytron (twice for 30 to 60 seconds each). The homogenates were centrifuged twice at 10 000 × g for 10 minutes, and the supernatants were subjected to further centrifugation at 45 000 × g for 45 minutes. The pellet was resuspended in 25 mmol/L Tris, pH 7.5, containing 10 mmol/L MgCl2, 0.5 mmol/L PMSF, 4 μg/mL leupeptin, 4 μg/mL pepstatin, and 40 U/mL aprotinin. For binding experiments, 25 μg of membrane protein was incubated in 150 μL of assay buffer (25 mmol/L Tris, pH 7.5, containing 10 mmol/L MgCl2; 2 g/L BSA; 10 mg/L bacitracin; the peptidase inhibitors antipain, N-emamopranosylsuxoxyhydroxyphospharyl-Leu-Trp [Phosphoramidon], leupeptin, pepstatin, umbelvin [Bestatin], and amastatin, each at 1 μg/mL; and 0.5 mmol/L PMSF) at 22°C for 60 minutes with 0.05 to 10 nmol/L [125I]Tyr1-Ang II ([125I]Ang II) to determine saturation binding. The reaction was stopped by the addition of 1 mL of ice-cold 25 mmol/L Tris, pH 7.5, and the mixtures were immediately filtered through a Bio-Rad microfiltration apparatus by vacuum filtration after an initial rinse with 1 g/L BSA. The filters were washed twice with 5 mL of 25 mmol/L Tris, pH 7.5, and the trapped radioactivity was measured in a gamma counter. Nonspecific binding (in the presence of 1 μmol/L125 I-labeled Tyr4-Ang II) after preincubation with 10 μmol/L losartan for 30 minutes at 22°C, respectively. Ang II receptor densities were calculated from the Scatchard analysis of the saturation binding curves.

Statistics
Data are given as mean ± SEM. Statistical analysis was performed using ANOVA and an unpaired Student t test as appropriate. Significance was accepted at P < 0.05.

Results
Mechanical Stretch Upregulates Angiotensinogen, Renin, ACE, and AT1A mRNAs
We examined the effect of stretch on mRNA expression of angiotensinogen, renin, ACE, and AT1A, AT1B, and AT2 genes in cultured neonatal rat cardiac myocytes. Cardiac myocytes were cultured under serum-free conditions, and a 20% uniaxial stretch was applied for 8, 16, and 24 hours. Control myocytes were also plated on the stretch dish and harvested without stretch. Expression of each gene was determined by RT-PCR. We included primers for tubulin in the PCR as an internal control. The linearity of the PCR was confirmed for each gene (Figure 1) as described in Materials and Methods. The specificity of each PCR band was determined by the size as well as by Southern hybridization with an end-labeled internal oligonucleotide probe. Figures 2 and 3 show the results of RT-PCR analyses. The basal expression of angiotensinogen, renin, and ACE mRNA was very low in unstretched myocytes. Mechanical stretch of 16- to 24-hour duration resulted in a 3- to 5-fold upregulation in the expression of all 3 genes examined, as revealed by densitometric analyses of the autoradiograms (Figure 2).

Stretch also upregulated expression of the AT1A gene. Increased (8- to 10-fold) expression of the AT1A gene was observed at 8 hours after stretch was begun (Figure 3A). Interestingly, expression of the AT1A gene was not affected by stretch (Figure 3B). No RT-PCR signal for the AT2 transcript could be detected in cardiac myocytes or fibroblasts, although a distinct band was amplified from rat endothelial cell RNA (Figure 3C) used as a positive control for RT-PCR.28

Mechanical Stretch Also Upregulates Angiotensinogen and Renin Protein Expression and Increases ACE-Like Activities
To determine whether an increase in the mRNA expression of angiotensinogen, renin, and ACE genes by mechanical stretch is also translated into an increase in protein, we performed Western blot analyses using monoclonal antibodies specific for rat angiotensinogen and renin. We also measured the ACE-like activity in control and stretched conditions. Expression of both angiotensinogen and renin protein in control unstretched myocytes was undetectable. However, 16 to 24 hours of stretch significantly upregulated the protein expression of both angiotensinogen (Figure 4A) and renin (Figure 4B).

Furthermore, measurement of the ACE-like activity revealed that stretch of 24 hours significantly increased the ACE-like activity by almost 2-fold compared with that in control unstretched cardiac myocytes (unstretched control, 10.6 ± 2.8 mU/106 cells; after 24 hours of stretching, 23.7 ± 3.4 mU/106 cells [n = 3], P = 0.001).
Ang II Upregulates Angiotensinogen, Renin, and ACE but Downregulates AT1A Expression

We next examined whether Ang II, when exogenously applied, mimics the effect of stretch on expression of the renin-angiotensin system genes. Cells were grown on gelatin-coated dishes, serum starved, and stimulated with Ang II \((1 \times 10^{-7} \text{ mol/L})\) for various periods. Ang II caused a significant upregulation of angiotensinogen, renin, and ACE genes at 16 hours of stimulation. Ang II–induced upregulation of angiotensinogen, renin, and ACE genes was completely inhibited in the presence of the AT1 selective antagonist losartan \((1 \mu\text{mol/L})\) but not at all by the AT2 selective antagonist PD123319 (Figure 5), which suggests that Ang II–induced upregulation of angiotensinogen, renin, and ACE genes was mediated by AT1. In contrast, Ang II treatment significantly downregulated (by 70%) expression of AT1A, an effect that could be reversed by pretreatment of cells with losartan (Figure 5, right). Thus, both stretch and Ang II upregulate expression of angiotensinogen, renin, and ACE genes, whereas they differentially regulate mRNA expression of AT1A (upregulation by stretch and downregulation by Ang II).

Figure 2. Mechanical stretch of cardiac myocytes in vitro upregulates angiotensinogen, renin, and ACE. Total RNA \((1 \mu\text{g})\) was reverse transcribed, and the resultant cDNA was amplified with PCR using gene-specific primers. The specificity of PCR product was confirmed by Southern hybridization using end-labeled internal oligonucleotide probe as described in Materials and Methods. Primers for tubulin were used as an internal control. For renin gene expression, rat kidney RNA was used as a positive control. Southern hybridization of the PCR products for the renin-angiotensin system genes and ethidium bromide staining of tubulin PCR products are shown. The lower panels show the relative expression of the genes at different time intervals, obtained by densitometric analyses as described in Materials and Methods. The normalized value in the unstretched control was arbitrarily expressed as 1 unit. Data are mean±SEM of 4 separate experiments.

Figure 3. The effect of stretch on Ang II receptor expression in neonatal rat cardiac myocytes. Total RNA \((1 \mu\text{g})\) was reverse transcribed, and the resultant cDNA was amplified with PCR using gene-specific primers. A, Autoradiogram depicting mechanical stretch–mediated upregulation of AT1A gene expression at 8 to 24 hours. B, No change is observed in AT1B mRNA expression after myocyte stretching. C, Apparent absence of the AT2 transcript in control and stretched conditions. Rat endothelial cell total RNA was used as a positive control for AT2 expression. Ethidium bromide staining of tubulin signals used as an internal control is also shown. Bottom panels, relative expression of the genes at different time intervals, obtained by densitometric analyses. Level of expression before stretch (at time 0) was expressed as 1 arbitrary unit. Data are mean±SEM of 4 separate experiments.
of 20% was applied for 16 hours. Unexpectedly, stretch-induced upregulation of angiotensinogen, renin, and ACE was not suppressed by losartan (1 μmol/L) or PD123319 (1 μmol/L) (Figure 6). This suggests that stretch-induced upregulation of angiotensinogen, renin, and ACE genes in vitro is mediated by Ang II–independent mechanisms. In contrast, the stretch-mediated upregulation of AT1A mRNA was completely suppressed by losartan (Figure 6, right).

It has been reported that tyrosine kinases highly sensitive to genistein (20 μmol/L) are responsible for stretch-induced upregulation of AT1 in cardiac myocytes.29 Thus, we explored whether genistein-sensitive tyrosine kinases are responsible for stretch-induced upregulation of renin-angiotensin system genes. Cardiac myocytes were treated with genistein (100 μmol/L), and then a uniaxial stretch of 20% was applied for 16 hours. Stretch-induced increases in AT1A mRNA expression were inhibited by genistein, which was consistent with the previous observation.29 Interestingly, however, stretch-induced upregulation of angiotensinogen, renin, and ACE genes was not suppressed by genistein (Figure 7). This indicates that stretch-induced upregulation of angiotensinogen, renin, and ACE genes are mediated by a genistein-insensitive mechanism distinct from that for AT1A upregulation.

**Mechanical Stretch Induces an Increase in AT1 Protein Expression**

Our Ang II binding assay using [125I]Tyr4-Ang II demonstrated the presence of specific, saturable Ang II binding sites
on neonatal rat ventricular myocyte membranes (Figure 8A). Saturation binding studies of [125I]Tyr4-Ang II to the myocyte membranes were performed using 0.01 to 10 nmol/L [125I]Ang II. These data showed the binding constant (Kd) of Ang II for AT1 and AT2 on cardiac myocytes to be 0.7 nmol/L, which is in close agreement with the previously reported Kd of the Ang II receptors of neonatal rat cardiac myocytes.30,31 The total Ang II receptor density (Bmax) in the stretched cells increased nearly 3-fold as compared with those in the control unstretched cells (206±3 versus 72±3 fmol/mg protein). To characterize AT1 and AT2 subtypes, a competition binding was performed using their respective antagonists. The specific [125I]Ang II binding insensitive to losartan or PD123319 (10 μmol/L each) was estimated to be AT2 and AT1 binding, respectively. The proportion of receptor subtypes was 71±4% for AT1 and 29±3% for AT2 in the membranes prepared from control cells. In the membranes prepared from cells stretched for 16 hours, expression of AT1 was significantly increased by >3-fold as compared with those of controls, but that of AT2 was unchanged (Figure 8B).

Pretreatment of cells with losartan (1 μmol/L) for 30 minutes before stretch effectively reduced the increased density of the Ang II receptors to the values in the control cells (stretched myocytes, 69±7 fmol/mg protein; control myocytes, 72±3 fmol/mg protein). Reduced Ang II binding to stretched myocytes is not due to the remnant of losartan used for pretreatment, because we washed stretched myocytes extensively before we proceeded to the binding assay. These results indicate the effectiveness of AT1 antagonists in inhibiting mechanical stretch–induced increases in AT1 protein expression, which very closely correlates with the mRNA data on AT1A expression after mechanical stretch (Figure 8B).

Angiotensinogen, Renin, ACE, and AT1A Expression in Cardiac Fibroblasts Is Not Responsive to Stretching

We examined the possibility that a small (5% to 10%) contamination of fibroblasts in our myocyte-rich cultures could potentially contribute to the upregulation of renin-angiotensin system genes observed in stretched cardiac myocytes. To address this possibility, highly enriched cultures of cardiac nonmyocytes (mostly fibroblasts) were prepared by 2 passages of cells adhered to the culture dish during the preplating procedure.32 The cells were subsequently plated on the silicone substrate and then stretched for 0 to 24 hours. We
have previously shown that mechanical stretch causes an increase in cell number in this fibroblast culture, which indicates that cardiac fibroblasts do respond to mechanical stretch. As shown in Figure 9, however, the relative expression of angiotensinogen, renin, ACE, and AT1A mRNAs in these fibroblast-rich cultures was not affected by stretching up to 24 hours (Figure 9, middle). Interestingly, treatment of fibroblast-rich cultures with Ang II also did not result in any appreciable changes in expression of angiotensinogen, renin, and ACE genes, but it caused a significant downregulation of AT1A expression (Figure 9, right). Receptor binding studies indicate that densities of both AT1 and AT2 in cardiac fibroblasts did not significantly change after stretch (data not shown).

**Discussion**

The present study demonstrates that mechanical loading of cultured cardiac myocytes leads to upregulation of angiotensinogen, renin, ACE, and AT1A genes. Stretch-induced increases in mRNA expression result in increased protein expression of the renin-angiotensin system in cardiac myocytes. Because our results were obtained by stretching cultured cardiac myocytes in vitro, stretch, but not systemic endocrine or neuronal mechanism, is the primary stimulus that activates intracellular signaling mechanisms leading to upregulation of renin angiotensin system genes.

Recently, it was reported that stretch-induced upregulation of AT1 and AT2 mRNAs was further enhanced by pretreatment with the AT1 antagonist CV11974 (10 μmol/L), which suggests that (secreted) Ang II actually downregulates expression of AT1. On the other hand, in our study, losartan (1 μmol/L) completely blocked stretch-induced upregulation of AT1 mRNA. The reasons for the different results between our study and that by Kijima et al are not clear at the present time. It is possible that CV11974 and losartan differentially affect cell signals mediated by AT1A. Although our results suggest that stimulation of AT1 is required for stretch-induced upregulation of AT1 mRNA, Ang II is unlikely to be the sole mediator of this response, given that Ang II alone downregulates expression of AT1. This may be explained if both stretch and Ang II are required for upregulation of AT1. Coordinated activation of multiple sets of second messengers by stretch and Ang II may be necessary for upregulation of AT1. Alternatively, a factor, or factors, with strong stimulatory effects on AT1 expression may be upregulated only in the presence of both stretch and Ang II.

The direct intracellular signaling mechanisms that couple the stimulus of mechanical stress to enhanced gene expression, in the present context of the renin-angiotensin system genes, are not yet deciphered. However, among the possible factors that may mediate the effect of mechanical stress is Ang II. In fact, in the present observations, exogenously applied Ang II upregulated the expression of the angiotensinogen, renin, and ACE genes, but downregulated the expression of the AT1A gene. In another study, Ang II modestly upregulated (<2-fold) the AT1A promoter activity in a transient transfection assay using cultured neonatal rat cardiac myocytes. One possible explanation for this conflicting observation is that the AT1A promoter used in the study is short and does not contain all of the cis-acting DNA elements required for its complete activity in cultured cardiac myocytes. The fact that both mechanical stretch and Ang II upregulated mRNA expression of angiotensinogen, renin, and ACE genes initially led us to hypothesize a common mechanism, ie, an Ang II–dependent mechanism. Unexpectedly, however, losartan completely inhibited Ang II–induced, but not mechanical stretch–induced, upregulation of angiotensinogen, renin, and ACE genes. Therefore, in addition to the secreted Ang II, mechanical stretch seems either to directly activate unique intracellular signaling molecules or to cause release of additional growth factors, which lead to an activation of the renin-angiotensin system genes. Cyclic mechanical stretch of cultured rat cardiac myocytes also upregulates angiotensinogen gene expression, an effect completely suppressed by pretreatment with losartan, which suggests an Ang II–dependent effect. Thus, it seems the type (uniaxial or cyclic) and degree of mechanical stretch may also influence the expression of renin-angiotensin system genes and their susceptibility to inhibition by Ang II receptor antagonists.

Recently, it has been demonstrated that several growth factors besides Ang II are secreted from the heart in response to mechanical stress. For example, stretch of neonatal rat cardiac myocytes causes secretion of endothelin-1. Release of basic fibroblast growth factor has also been demonstrated from adult rat ventricular myocytes in vitro in response to increased mechanical activity. Thus, it is possible that stretch of cells causes secretion of a number of still-undefined growth factors. The role of these autocrine/paracrine factors in stretch-induced upregulation of the cardiac renin-angiotensin system remains to be determined.

The observation that mechanical stretch and Ang II independently and differentially regulate the expression of cardiac

![Figure 9. Effect of mechanical stretch and Ang II stimulation on renin-angiotensin system gene expression in cardiac fibroblasts in vitro.](image-url)
renin-angiotensin system genes suggests both AT_{1}-dependent and AT_{2}-independent effects. The results seem to be in close agreement with recent observations documenting both Ang II–dependent and Ang II–independent effects on some intracellular signaling molecules. For instance, activation of the JAK/STAT (just another kinase/signal transducer and activator of transcription) pathway by acute pressure overload\(^2\) and that of the stress-activated protein kinases by mechanical stretch\(^3\) have both Ang II–dependent and Ang II–independent components. Kijima et al.\(^2\) have reported that genistein-sensitive tyrosine kinases are involved in the Ang II–independent mechanisms, which mediate stretch-induced upregulation of AT_{1}. This pathway is apparently not essential for stretch-induced upregulation of angiotensinogen, renin, and ACE (see Figure 7).

In the present report, we could not detect any expression of AT_{2} mRNA by RT-PCR in cardiac myocytes and cardiac fibroblasts. Our PCR conditions seem to be satisfactory, as a clear band was observed in a positive control sample (rat endothelial cell RNA). At the protein level, the AT_{2} binding site could be detected, albeit at low levels, by radioligand binding assay, but it did not show any response to mechanical stretching. This result on AT_{2} mRNA and protein expression is different from that reported by Kijima et al.\(^2\) who reported upregulation of AT_{2} mRNA in cultured neonatal rat cardiac myocytes after mechanical stretch. A potential explanation for the apparent lack of AT_{2} mRNA expression in our study is the difference in culture conditions, which may directly or indirectly affect the rate of synthesis and stability of AT_{2} mRNA and protein. The apparent PD123319-sensitive component of the Ang II receptor in our preparation may be encoded by a DNA sequence distinct from that of the known AT_{2}. It is also pertinent to mention that Mukoyama et al.\(^4\) and Kambayashi et al.\(^4\) also could not detect any AT_{2} transcripts by Northern blot analyses using poly(A)\(^{+}\) RNA from rat hearts. AT_{2} appears to be abundantly expressed in fetal tissues and immature brain but present only at low levels in many adult tissues.\(^2\) Although Ang II has been shown to be sufficient to cause hypertrophic responses in neonatal rat cardiac myocytes in culture,\(^12,32\) some investigators have reported that Ang II has only weak hypertrophic effects in the same cell type.\(^4\) Because it has been reported that AT_{2} mediates antigrrowth effects on cardiac myocytes,\(^3\) vascular smooth muscle cells,\(^3\) and endothelial cells,\(^3\) it is possible that differences in densities of AT_{2} may explain discrepancies of the hypertrophic effects of Ang II in different cardiac myocyte preparations among investigators.

Another interesting observation made in this investigation was that stretch upregulates the renin-angiotensin system only in cardiac myocyte cultures but not in cardiac fibroblast cultures. We have previously shown that in vitro stretch causes acute secretion of Ang II from cardiac myocyte cultures but not from cardiac fibroblast cultures.\(^7\) Thus, it is likely that some stretch-sensitive signaling mechanisms are missing in fibroblasts. It should be noted that our results do not exclude the role of cardiac fibroblasts in stretch-induced cell responses, because our myocyte cultures are not pure myocyte cultures. It is possible that both basal and stretch-induced secretion of “paracrine” factors from the “contami-
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