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ANGIOTENSINOGEN GENE EXPRESSION IS INDUCED BY CYCLICAL MECHANICAL STRETCH IN CULTURED RAT CARDIOMYOCYTES

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The effect of cyclical mechanical stretch on angiotensinogen gene expression was examined using a neonatal rat cardiocyte culture system. Cultured cardiocytes grown on a flexible membrane base were stretched by vacuum to 20% of maximum elongation, at 60 cycles/min. The angiotensinogen gene was activated 2 to 5 fold after stretch for 3 to 24 hr, as shown by quantitative reverse transcription polymerase chain reaction. The 5'-flanking region of the angiotensinogen promoter was activated after stretch for 24 hr. This gene expression could be completely suppressed by losartan, a specific antagonist of angiotensin II receptor. These results indicate that (1) cyclical mechanical stretching of cardiocytes is a good model for the study of cardiac hypertrophy-related gene expression; (2) cyclical stretch up-regulates expression of the angiotensinogen gene and (3) the increase in promoter activity may contribute to the induction of angiotensinogen mRNA by cyclical stretch.

The renin-angiotensin system (RAS) plays an important role in the control of cardiovascular and renal homeostasis (1,2). Previously, this system was considered to be an endocrine system, but recently, a local tissue RAS in the heart has been

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Abbreviations: bp, basepair(s); CAT, chloramphenicol acetyltransferase; RT-P C R, reverse-transcription polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase.

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found (3,4). Since angiotensinogen is the only precursor of the angiotensin peptide, definite evidence of the RAS requires demonstration of local synthesis of angiotensinogen. In pressure-overload cardiac hypertrophy in rats, angiotensinogen mRNA is upregulated, suggesting that a cardiac RAS may be activated (5). Mechanical stretch causes hypertrophy of cardiac myocytes and induction of immediate early genes (6,7). This mechanical load directly regulates gene transcription in cardiocytes without the participation of humoral factors. Static mechanical stretch also causes release of angiotensin II from cultured cardiocytes and up-regulates angiotensinogen mRNA (8). However, the effect of mechanical stretch on the regulation of angiotensinogen gene itself is unkown. The rat angiotensinogen gene has been isolated and sequenced (9,10). Its 5'-flanking region which contains regulatory sequences is responsive to a variety of hormones (11). How mechanical stretch affects regulation of the angiotensinogen gene at the 5'-flanking region in the cardiac myocytes is still not clear.

Cells in the cardiovascular system are continually subjected to mechanical forces due to changes in pressure and volume. The cyclical strain model system subjects cultured cells to repetitive stretch-relaxation at rates comparable to dynamic stretch overload in vivo. This model has been applied widely to study the molecular mechanisms of gene expression and signal transduction in endothelial cells and smooth muscle cells (12- 14). The effect on cardiocytes of cyclical stretch, which represents the rhythmic deformation of myocardium associated with the oscillation of systole and diastole, has not been well characterized.

In the present study, we show that cyclical mechanical stretch upregulates the level of angiotensinogen mRNA and activates transcription of the angiotensinogen gene, as assessed by the chloramphenicol acetyltransferase (CAT) reportor gene.

Materials and Methods

Cardiocyte culture: Neonatal cardiac myocytes were obtained from Wistar rats aged 2-3 days by trypsinization. Cardiocyte isolation method has been described previously (15). Cardiocytes in the medium were plated into 6-well culture plates (Flex 1, Flexcell Co., McKeesport, PA) at a cell density of 1.6×10^6 cells/well. After 2 days in culture, the medium was replaced by serum-free medium.

In vitro cyclical mechanical stretch: The cyclical strain unit (Flexcell I FX-2000, Flexcell International Co.) consists of a vacuum unit linked to a valve controlled by a computer program. Neonatal cardiocytes, cultured on plates with a flexible membrane base (Flex I) coated with type 1 rat collagen were subjected to cyclical strain. A vacuum of approximately 13 kPa was repetitively applied at a frequency of 60 cycles/min (1 Hz, 0.5 sec on-time) to the flexible membrane via the baseplate.

Application of the vacuum results in maximal elongation of 20% to cells at the periphery of the well, with strain declining towards the center (15). The cardiocytes were placed in a humidified incubator with 5% CO_2 at 37°C. Cell viability after application of strain was constantly monitored by trypan blue staining.

Reverse transcription polymerase chain reaction (RT-PCR) assay: Total RNA from cardiocytes was isolated by a single-step procedure (16). Two µg RNA was reverse transcribed and amplified according to the manufacturer's instructions (Promega). The reactions were stopped by incubation at 95°C for 10 min. The PCR amplifications were carried out in 100 µl reaction buffer with 5mM $MgCl_2$, 1mM dNTP, specific primers of angiotensinogen (40 pmoles) and of GAPDH (15 pmoles), 2.5 unit Taq DNA polymerase and 10 ul cDNA. Primer oligonucleotides were prepared by an ABI synthesizer (model 380B). The sense primer, 5'-TTGTTGAGAGCTTGGGTCCCTTCA-3', and antisense primer, 5'-CAGACACTGAGGTGCTGTTGTCCA-3', were used to generate a 263 base pair PCR product, spanning bases 699 to 962 of rat angiotensinogen cDNA (17) . The sense primer, 5'-TCCATGACAACTTTGGCATCGTGG-3', and antisense primer, 5'-GTTGCTGTTGAAGTCACAGGAGAC-3', were used to generate a 366 base pair PCR product, spanning bases 556 to 922 of rat GAPDH cDNA (18). The ratio of angiotensinogen and GAPDH primers (8:3) was used to obtain the optimal uniform coamplification (Figure 1). Amplification was performed with a DNA thermal cycler (Perkin- Elmer Cetus, Norwalk, Conn., USA). A cycle profile consisted of 30 sec at

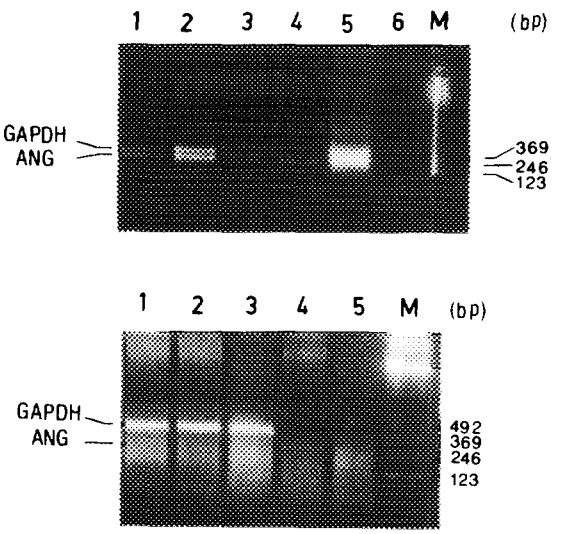


Figure 1. Simultaneous amplification of angiotensinogen and GAPDH cDNAs. 2 µg of reverse-transcribed RNA was amplified for 35 cycles, using 40 pmoles of angiotensinogen and 15 pmoles of GAPDH (upper panel, lanes 1 and 2), 40 pmoles of angiotensinogen and 10 pmoles of GAPDH primers (upper panel, lanes 3 and 4), and 40 pmoles of angiotensinogen and GAPDH primers (lower panel, lanes 1, 2 and 3). 2 µg rat liver RNA was reverse-transcribed and amplified for 35 cycles as the positive control in lane 5 of upper panel. cDNA was amplified using 40 pmoles of angiotensinogen primers, without GAPDH primers, in lane 4 of lower panel. Lane 6 in the upper and lane 5 in the lower panels were negative control. No RNAs were added in the lane 6 of upper and lane 5 of lower panels. Lane M contains 123 bp markers. The PCR products were separated on 1.2% agarose gels which were then stained with ethidium bromide and photographed under UV light.

95°C for denaturation, 30 sec at 58°C for annealing, and 30 sec at 72°C for extension. This procedure was carried out for 35 cycles followed by a final extension for 10 min at 72°C. The absence of contaminants was regularly checked by RT-PCR assays of control samples that contained no reverse transcriptase, or no RNA or DNA.

cDNA probe: A 738-bp product of rat angiotensinogen cDNA was obtained by RT-PCR using synthesized oligonucleotide primers derived from angiotensinogen cDNA, 5'-CCTTGGATCGTTGGATCC-3' (411-418) and 5'-CTGCAGGTTGTAGGATCC-3'(1126-1143). The sequence of PCR product was analyzed by the dideoxy chain termination method and was subcloned into pGEM vector. The sequencing results revealed that the PCR product was identical to part of the rat angiotensinogen cDNA cloned by Ohkubo *et al* (17). The cDNA inserts were excised with Bam HI, then purified by separation in a low-melting point agarose gel. cDNA for rat GAPDH was a 1.25 kb Pst I fragment subcloned into pIBI 30 vector.

Analysis of RT-PCR products: Thirty µl of RT-PCR products was separated on 1.2% agarose gels ,transferred to nylon membranes by vacuum (VacuGene XL, Pharmacia LKB Biotechnology, Uppsala, Sweden), and immobilized by UV irradiation. The membranes were prehybridized at 42°C in buffer containing 50% formamide, 5x SSC, 100 µg/ml salmon sperm DNA, 5x Denhardt's solution , and 0.5% SDS for at least 4 hours. Hybridization was carried out in the same buffer containing [^{32}P] labeled 0.7-kb angiotensinogen cDNA probe, or 1.2-kb GAPDH cDNA probe. After overnight incubation at 42°C, the membrane was washed in 5x SSC-1%SDS at room temperature for 15 min, 1x SSC-1%SDS at room temperature for 30 min, and 0.1x SSC-1%SDS at room temperature for 30 min, and then analyzed by autoradiography followed by scanning densitometry. The hybridization signals of angiotensinogen were normalized to those of GAPDH.

Construction of angiotensinogen promoter chimeric plasmid and chloramphenicol acetyltransferase (CAT) assay: The 5'-flanking region of the angiotensinogen gene was generated by PCR, using rat chromosomal DNA as template. The sense primer, 5'- TATCACGGATCCACCCGTCTCATT-3', and antisense primer, 5'-CTTTAGCTCGAGCGCTGTCAAGC-3', were used to generate a 712 bp product, spanning bases -688 to +24 of the rat angiotensinogen gene. The PCR product was verified by DNA sequencing and subsequently ligated to the chloramphenicol acetyltransferase (pBLCAT$_3$) reporter gene pre-digested with Bam H1 and Xho I. Transfection was performed by the calcium phosphate precipitation method as described previously (19). Briefly, 2µg of angiotensinogen CAT reporter gene construct and 0.75 µg of plasmid pSV-ß-galactosidase expression were cotransfected per well in serum-free medium. Forty-eight hours after transfection, the cells were stretched for 24 hours. Cardiocyte extracts were prepared and the CAT activity was assayed as described previously (19). The CAT activity was calculated after normalization to protein content and transfection efficiency. Control and stretching experiments on all constructs were carried out simultaneously in the same batch of cultured cardiocytes.

Results

The levels of angiotensinogen mRNA in cultured cardiocytes were determined by Northern blot analysis. However, no signals were detected even when 50 µg of each total RNA sample was loaded (data not shown). We therefore used quantitative

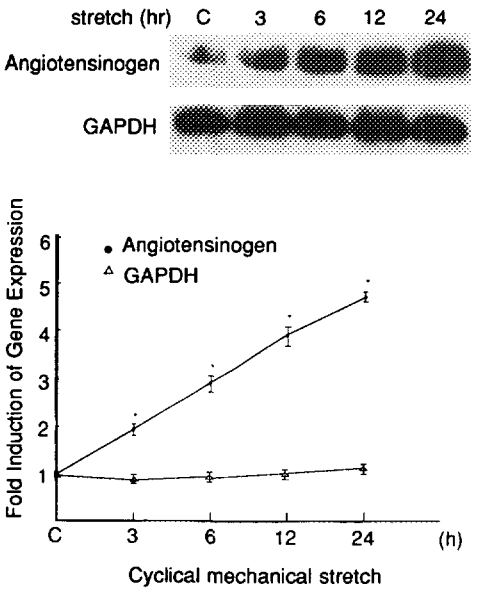
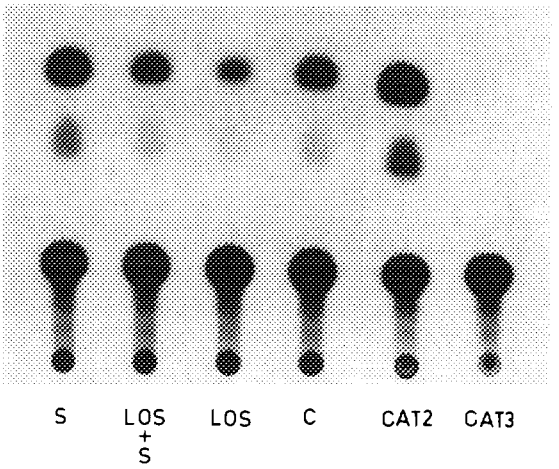


Figure 2. Cyclical mechanical stretch induces angiotensinogen gene expression. The upper panel is a representative Southern blot showing induction of angiotensinogen and GAPDH by cyclical stretch. 20% cyclical stretch was applied to the cardiac myocytes for the time indicated above. The lower panel shows cyclical stretch induces angiotensinogen gene expression in a time-dependent manner. Results were expressed as relative levels of angiotensinogen or GAPDH gene in comparison to non-stretched cells. Data were expressed as mean ± S.E.M. from 4-5 separate experiments. * <0.01 as compared with non-stretched cardiac myocytes.

RT-PCR method to study the effect of cyclical stretch on the low level of angiotensinogen mRNA expression. Using this method, total RNA was reverse transcribed and amplified. An increase in the level of the angiotensinogen gene was observed as early as 3 hr after stretch, reaching 4.8 fold over controls in 24 hours. As shown in figure 2, the expression of the angiotensinogen gene was stimulated by cyclical stretch in a time-dependent manner, with angiotensin transcript levels in stretched cells increasing significantly between 3 to 24 hours of stretch.

The effect of cyclical stretch on expression of the 5'-flanking region of the angiotensinogen gene was determined by transfection experiments using the angiotensinogen promoter-CAT chimeric construct. The PCR generated promoter region of the rat angiotensinogen gene including the 688 bp 5'-flanking region, the start site of transcription and the 24 bp first exon was ligated to the CAT reporter gene. This chimeric construct was cotransfected with psv-ß-galactosidase into cultured cardiocytes, and the cell extract was subsequently used to determine CAT activity. As shown in Figure 3 (upper panel) induction of angiotensinogen promoter



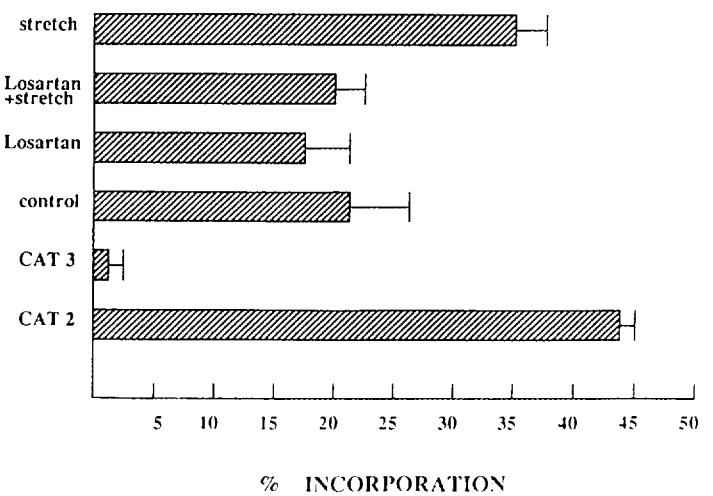


Figure 3. Effect of cyclical mechanical stretch on angiotensinogen promoter activity. The cultured cardiac myocytes were transfected with CAT fusion genes of angiotensinogen promoter by Ca^{2+} precipitation for 48 hours. Cultured cardiocytes were subjected to cyclical stretch for 24 hours in the presence or absence of losartan (100 nM), applied 30 minutes before stretch. Results derived from the auotradiograms of 4 separate experiments were analyzed by scanning densitometer and expressed as mean $\pm$ S.E.M. ANOVA with Scheffe's comparison shows significant difference between stretched and control (non- stretched), stretched and losartan only (nonstretched), stretched and losartan plus stretched ($p<0.05$). The top panel is a typical autoradiogram of CAT assay of angiotensinogen promoters. CAT2 and CAT3 are shown as positive and negative control.

activity from 21% in nonstretched cells to 36% was observed after 24 hrs of cyclical stretch. However, pretreatment of cells with losartan (100 nM) under similar stretch completely suppressed CAT activity. This observation was reproducible and found to be statistically significant ($P < 0.05$) (Figure 3 - lower panel).

Discussion

Myocardial hypertrophy is an important problem in clinical cardiology. Mechanical stress is a major cause for cardiac hypertrophy. A major component of myocardial hypertrophy is hypertrophy of cardiocytes, a heterogenous growth process that varies both quantitatively and qualitatively (20). The local RAS is functionally involved in cardiac hypertrophy (21). Thus, it is important to define the level of gene regulation of RAS in cardiac hypertrophy. While cultured cardiocytes have been used to study the morphological changes following cyclical stretch (22,23), it is not known whether this cyclical mechanical stretch model system is suitable for the study of molecular mechanisms of gene expression in cultured myocytes. In this study, we used a cultured cardiocyte system to elucidate the mechanism of regulation of the angiotensinogen gene, one of the components in the RAS, by applying cyclical mechanical stretch which mimics hemodynamic overload in vivo. We have also demonstrated that the cyclical mechanical stretch system is a good model for the study of cardiac hypertrophy-related gene expression.

Although angiotensinogen mRNA has been detected in the neonatal rat heart by PCR and in situ hybridization (24), it is difficult to detect this mRNA in the rat ventricle using Northern blot analysis and S1 nuclease protection assay (25) due to its low abundance. Quantitative RT-PCR is a powerful approach which allows the investigator to measure a wide range of target mRNA using a small amount of material. The levels of housekeeping genes, GAPDH mRNA are not affected by stretch (8), and represent a reliable internal control for studies of gene expression.

Most previous studies concerning angiotensinogen gene expression in cardiocytes were done only at the protein and mRNA level (8). Passive stretch of cardiocytes cultured on silicone membrane was previously reported to induce the release of angiotensin II, as well as enhance expression of angiotensinogen mRNA (8). In this study, cyclical stretch induced the expression of angiotensinogen mRNA. This finding suggests that there is a local positive feedback mechanism in the stretch-induced cardiac hypertrophy. We have also demonstrated that the -658 bp 5'-flanking sequence of the rat angiotensinogen gene contains the necessary elements to drive its expression in response to cyclical stretch. However, how this stretch element in the 5' promoter regions of the angiotensinogen gene interacts with trans-acting factors in order to enhance transcription of the angiotensinogen gene remains to be elucidated. This stretch-induced angiotensinogen promoter activity was suppressed by the AT1 receptor-selective antagonist losartan which is known to block the actions

of angiotensin II. This finding suggests that cyclical stretch is acting through the AT1 receptor and that angiotensin II may be the initial mediator of positive-feedback regulation of cardiac hypertrophic response by inducing the expression of the angiotensinogen gene.

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