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Renin-angiotensin system in stretch-induced hypertrophy of cultured neonatal rat heart cells

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Abstract

Although it is well known that mechanical load to cardiac muscles causes cardiac hypertrophy, little is known about how mechanical load is transduced into the activation of intracellular signals which are linked to cell growth. We investigated whether the cardiac renin-angiotensin system was involved in stretch-induced hypertrophy of cultured neonatal rat heart myocytes. Myocytes were cultured with serum-free medium in a deformable silicon dish. Stretch of cardiac myocytes significantly increased the protein/DNA ratio at culture days 6 and 7, and the RNA/DNA ratio at culture days 4 and 5. Stretch significantly accelerated rates of protein synthesis by 15%. c-fos mRNA expression was significantly increased after stretch. The stimulatory effects of cell stretch on these parameters were significantly inhibited by the angiotensin converting enzyme inhibitor, captopril, or the type 1 angiotensin II receptor antagonist, losartan. The concentrations of angiotensin I and angiotensin II in culture media were significantly increased by stretch. Stretch did not change the angiotensin converting enzyme activity. These studies demonstrate that mechanical stretch activates the cardiac renin-angiotensin system in a autocrine and paracrine system which acts as an initial mediator of the stretch-induced hypertrophic growth.

Keywords: Cardiac hypertrophy; Myocyte; Stretch; Angiotensin

1. Introduction

External load plays an important role in determining muscle mass and its phenotype in vivo (Morgan and Baker, 1991; Vandenburgh, 1992). It has been demonstrated that cultured skeletal and cardiac muscles undergo hypertrophy in response to passive stretch of the substrate to which they are adhered (Komuro et al., 1990). Although it has become obvious that various hormones directly cause cardiac hypertrophy (Morgan and Baker, 1991), it is not known how cardiac cells sense the external load and convert this stimulus into intracellular signals that regulate cardiac hypertrophy. Hammond et al. (1982) studied tissue extracts from hypertrophying hearts and demonstrated that a tissue undergoing hypertrophy produces a soluble growth factor. The circulating renin-angiotensin system has been well characterized and a number of tissue-specific renin-angiotensin systems may be present in specific tissues (Campbell and Habener, 1986). Baker et al. (1990) and Schunkert et al. (1990) reported that angiotensin converting enzyme activity, angiotensin converting enzyme mRNA expression, angiotensinogen mRNA expression and rate of angiotensin II production in heart were increased by pressure overload. Recently, we reported that angiotensin II directly stimulated cellular hypertrophy through the type 1 angiotensin II receptor in cultured neonatal rat myocytes (Miyata et al., 1994). On the other hand, Ito et al. (1993) have reported that angiotensin II stimulated the release of endothelin-1 from cardiomyocytes and that angiotensin II-induced hypertrophic growth was blocked by an endothelin-1_A receptor antagonist. These authors suggested that endogenous endothelin-1 that was produced locally in cardiomyocytes played a potential role in angiotensin II-induced cardiac hypertrophy via an autocrine and paracrine system. Locally generated angiotensin II may be involved in paracrine or autocrine effects within the tissue (Sadoshima et al., 1993). However, the role of this system in the development of cardiac hypertrophy still remains to be elucidated. Sadoshima et al. (1993) have reported that mechanical stretch caused a direct release of angiotensin II from cardiac myocytes and that angiotensin II acted as an initial mediator of the stretch-induced hypertrophic response. However, it is not shown whether stretch actually increased RNA and protein contents in cardiomyocytes

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and whether stretch-induced hypertrophy was prevented by an angiotensin converting enzyme inhibitor and type 1 angiotensin II receptor antagonist. Therefore, we demonstrated in this study that stretch directly induced cardiac hypertrophy in cultured neonatal rat myocytes, and that this stretch-induced hypertrophy was prevented by an angiotensin converting enzyme inhibitor and type 1 angiotensin II receptor antagonist. Furthermore, we found that the factors secreted by stretch of cardiac myocytes were angiotensin I and angiotensin II and that this autocrine secretion of angiotensin I and angiotensin II played a critical role in the stretch-induced cellular hypertrophy.

2. Materials and methods

2.1. Myocyte culture

Monolayer cultures of 1–2-day-old neonatal Sprague-Dawley rat (Japan SLC) myocytes were prepared with modifications as described previously (Haneda and McDermott, 1991; Miyata et al., 1994). Briefly, minced ventricular myocardium was placed into Ca^{2+}- and Mg^{2+}-free Hanks' salt solution buffered with 30 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4. The cells were dissociated in a water jacketed Celstir apparatus (Wheaton Scientific) at 37°C with a mixture of partially purified trypsin (2.4 IU/ml, Worthington Biochemical), α-chymotrypsin (2.7 IU/ml) and elastase (0.94 IU/ml, Sigma Chemical). After each of five successive 20-min incubations, the dissociated cells were mixed with Eagle's minimal essential medium (MEM) (GIBCO) containing 10% newborn calf serum and were centrifuged and pooled. The dissociated cells were enriched for cardiomyocytes by the technique of differential adhesion for 90 min and plated onto laminin-coated (20 μg/ml) silicone dishes (2 × 4 × 1 cm) at a concentration of approximately 4×10^5 cells/dish (Komuro et al., 1990). We purchased elastic silicon dishes from Shin-Etsu Chemical Co., Japan. In the preliminary experiment, we studied the effect of laminin on protein/DNA, RNA/DNA and c-fos mRNA expression. There was no difference in these parameters between laminin coated-dish and laminin non-coated-dish. Cells were incubated in serum-free medium and 15% passive stretch was imposed on culture day 3. Cultures were incubated in a humidified 5% CO_2, 95% air atmosphere at 37°C. After an overnight incubation in MEM containing 10% newborn calf serum and 0.1 mM 5-bromo-2'-deoxyuridine (Sigma), the attached cells were rinsed and maintained in serum-free medium. Briefly, standard MEM was supplemented with MEM amino acids, vitamins, penicillin-streptomycin (GIBCO), and 2 mM glutamine. In addition, the medium contained 30 nM $NaSeO_4$, 2.5 μg/ml human insulin, 10 μg/ml human transferrin (Sigma), 0.25 mM ascorbic acid (Sigma) and 0.1 mM 5-bromo-2'-deoxyuridine to minimize the proliferation of non-myogenic cells. The medium was replaced every 2 days with fresh medium over the time course of the experiments. In the serum-free condition, cells spontaneously beat at the rate of about 10/min. The beating rate did not change after stretch. Stock solutions of an angiotensin converting enzyme inhibitor, captopril (a gift of Sankyou Pharma) or a type 1 angiotensin II receptor antagonist, losartan (a gift of Banyu Pharma) were prepared in MEM at a concentration of 10^{-2} M, respectively, stored at −20°C, and diluted in culture medium at the time of use.

2.2. Myocyte protein, DNA and RNA content

Each individual culture dish was rinsed three times with ice-cold phosphate-buffered saline (PBS). The cell layer was scraped from each dish with two 0.5 ml volumes of 1 × standard sodium citrate (SSC) containing 0.25% (wt/vol) sodium dodecyl sulfate (SDS) and frozen at −80°C. Before assay, the extracts were thawed and vortexed extensively. Protein content was assayed directly in aliquots of each extract by the method of Lowry using crystalline bovine serum albumin as a standard (Lowry et al., 1951). DNA content was measured fluorometrically in aliquots of each extract using calf thymus DNA as a standard (Cesarone et al., 1979). For RNA determination, a 750 μl aliquot from each extract was precipitated with an equal volume of ice-cold 1 N $HClO_4$. The material was centrifuged, and the pellet was washed three times with 0.5 N $HClO_4$. The pellet was hydrolyzed in 0.3 N NaOH for 24 h at 37°C, and the protein was reprecipitated by adding 4 N $HClO_4$ to a final concentration of 1 N. The supernatant was used to measure the absorbance at 260 and 232 nm following centrifugation. The RNA (μg/ml) concentration was calculated according to the following equation (Munro and Fleck, 1966)

$$\text{RNA} = (A_{260} \times 32.9) - (A_{232} \times 6.11)$$

where A_{260} and A_{232} are the absorbances at 260 and 232 nm, respectively.

2.3. Protein synthesis

The relative rate of protein synthesis was determined by assessing the incorporation of radioactivity into a 10% trichloroacetic acid insoluble fraction after incubation in medium that contained L-[^{14}C]phenylalanine ([^{14}C]phenylalanine; 1 μCi/ml, Amersham). In the determination of radioactivity incorporated into protein, cells were rapidly rinsed three times with ice-cold phosphate-buffered saline, and the protein was precipitated with 1 ml of 10% trichloroacetic acid for 1 h on ice and extracted overnight with 1 N NaOH (McKee et al., 1978; Morgan et al., 1971). This solution was used for determinations of radioactivity and protein. Radioactive samples were counted in a liquid scintillation counter (Beckman). Cell protein was determined by the Lowry method (Lowry et al., 1951).

2.4. RNA extraction and Northern blot analysis

Total RNA was extracted from myocytes stretched for 30 min using the acid guanidium-phenol-chloroform method (Chomczynski and Sacchi, 1987). Total RNA (15 μg) was separated electrophoretically in a 1.2% agarose gel, and the quantity of RNA in each track was verified by ethidium bromide staining before transfer. The RNAs were transferred to nylon membranes (Hybond-N, Amersham). A v-fos, 1.00 kb PstI/PvuII fragment containing FBJ murine osteosarcoma virus proviral DNA (Takara Co.) was used as the probe and was labeled by random priming with [α-^{32}P]dCTP. Hybridization to detect c-fos mRNA was carried out for 12–24 h at 42°C in 5 × Denhardt's, 0.5% SDS and 15 μg/ml of sonicated salmon sperm DNA. After washing, the membrane was subjected to autoradiography (X-Omat, Eastman Kodak, Rochester) at −80°C for 6 days.

2.5. Angiotensin converting enzyme-like activity

Angiotensin converting enzyme-like activity was measured by a modified radiometric assay system (Rosen et al., 1985; Ryan, 1988). Triplicate dishes of myocytes were rinsed twice with ice-cold buffer A (0.15 M NaCl, 0.05 M Hepes, 0.1% NaN_3, pH 8.0) and scraped into 3 ml of ice-cold buffer A. The cells were disrupted in a glass tissue homogenizer using a Teflon pestle and centrifuged twice at 10000 × g for 10 s on ice. The pellet was resuspended in 1.0 ml buffer A containing 0.6% Triton X-100 and frozen at −80°C until assayed. Hydolysis of a synthetic tripeptide [phenyl-4(n)-^{3}H]hippuryl-glycyl-glycine ([^{3}H]Hip-Gly-Gly) (Amersham) was used to measure angiotensin converting enzyme-like activity. Immediately prior to assay, 50 μl of the reaction mixture (16 mM [^{3}H]Hip-gly-gly, 0.1 M NaCl, 0.6 M Na_2SO_4, 0.05 M Hepes, pH 8.0) were added to a 50-μl aliquot from each extract. This solution was incubated in a water bath for 1 h at 37°C. When appropriate, the specificity of substrate hydrolysis by an angiotensin converting enzyme-like activity was verified by adding captopril to a final concentration of 1 μM. The reaction was stopped with 1.0 ml of 0.1 N HCl. The reaction product, [^{3}H]hippuric acid, was separated from the unreacted substrate by extraction with 1 ml of ethyl acetate following centrifugation at 1000 × g for 10 min. This solution was used for determinations of radioactivity and protein. Radioactive samples were counted in a liquid scintillation counter. Cell protein was measured by the Lowry method (Lowry et al., 1951). Angiotensin converting enzyme-like activity was expressed as nmol of hippuric acid formed/min/mg protein.

2.6. Angiotensin concentration

To measure the concentrations of angiotensin I and angiotensin II in the culture medium, 40 ml of conditioned media were collected in 50-ml siliconized polypropylene tubes after 1 h of treatment with a specific angiotensin II antagonist, saralasin (1 μM) (Dostal et al., 1992). Peptidase inhibitors (final concentrations of 10 μg/ml bacitracin, 100 μM phenylmethylsulfonyl fluoride, 3 mM ethylenediaminetetraacetic acid, 1 μM leupeptin, and 1 μM soybean trypsin inhibitor) were immediately added to the conditioned media. The conditioned media was freeze-dried and was resuspended in 2 ml of 1 mM HCl. The material was passed through C-18 Sep-Pac cartridge (Millipore) (Phillips and Stenstrom, 1985), and angiotensins were purified by high pressure liquid chromatography (CCP and 8010 model, TOSO, Japan) with a reverse phase column (TSKgel ODS-80T$_s$, 4.6 mm inner diameter × 250 mm length). The apparatus consisted of two pumps with a 200 μl sample loop. Solvent A was 0.1% trifluoroacetic acid and solvent B was 60% acetonitrile containing 0.05% trifluoroacetic acid. The column flow rate was adjusted to 1 ml/min, and fractions were collected every minute. The material was eluted with a gradient of 25–70% solvent B over 40 min. Angiotensin I and angiotensin II appeared in fractions that were collected at 20 min and 24 min, respectively. Quantities of angiotensin peptide in the fractions were determined by RIA using an assay buffer consisting of 19 mM NaH_2PO_4, 81 mM Na_2HPO_4, 50 mM NaCl, 1.0% bovine serum albumin (Sigma), 0.1% Triton X-100, and 0.1% NaN_3 (pH 7.4) as described prebiously (Sadoshima et al., 1993; Shimamoto et al., 1984). angiotensin I and angiotensin II standard (1–1000 pg) or HPLC fractions were mixed with 100 μl of angiotensin I or angiotensin II antiserum (final assay titers of 1 : 180000 and 1 : 90000, respectively) (Amersham), and the mixture was incubated overnight at 4°C. Radiolabeled angiotensin I or angiotensin II (1.0–1.5 pg of ^{125}I-angiotensin I or ^{125}I-angiotensin II, Amersham) were added to each reaction mixture, and the samples were incubated for an additional 24 h at 4°C. The primary antibodies were immunoprecipitated by adding 100 μl of goat anti-rabbit IgG serum (Amersham) and 100 μl of normal rabbit serum (Amersham) to the reaction mixture. After incubation for 2 h at 22°C, 0.5 ml of assay buffer was added to each assay tube, and the precipitates were sedimented by centrifugation at 1700 × g for 20 min at 4°C. The supernatants were aspirated, and the radioactivity associated with the protein pellets was counted in a gamma counter.

In this experimental system, angiotensin II concentration in the medium was at approximately 10^{-11} M level. We have previously reported that angiotensin II at concentrations of 10^{-7}–10^{-5} M level produces a growth response. Therefore, we think that not only stretch-induced autocrine release of angiotensin II but also the other mechanism might relate to the stretch-induced hypertrophic response. Captopril or losartan alone did not change the values of protein/DNA and RNA/DNA ratio under basal condition. This experimental system seems to be optimal for examining the stretch-induced hypertrophy.

2.7. Statistical analysis

All values are expressed as means ± S.E. Statistical significance was determined using unpaired Student's *t*-test or analysis of variance. The results were considered to be significant when $P < 0.05$.

3. Results

3.1. Effects of stretch, captopril and losartan on cellular hypertrophy

Subconfluent neonatal rat heart cells in culture were used to determine the effects of a 15% stretch on RNA content and myocyte growth. Myocytes were stretched on culture day 3, and protein/DNA and RNA/DNA ratios were measured at 24-h intervals over the next 4 days in culture. The protein/DNA ratio increased over the first 2 days after stretch (Fig. 1A). The protein/DNA ratios in stretched myocytes increased significantly by 18.6% and 20.5% compared with non-stretched myocytes at days 6 and 7, respectively. The RNA/DNA ratios increased significantly by 16.3% compared with non-stretched myocytes 1 day after stretch (Fig. 1B). However, a decline in the RNA/DNA ratios occurred after 2 days of stretch. These findings indicated that stretch rapidly increased the capacity for protein synthesis, as measured by RNA content and that greater capacity was followed by accelerated myocyte growth. Stretch did not change significantly the absolute values of DNA at culture days 4, 5, 6 and 7.

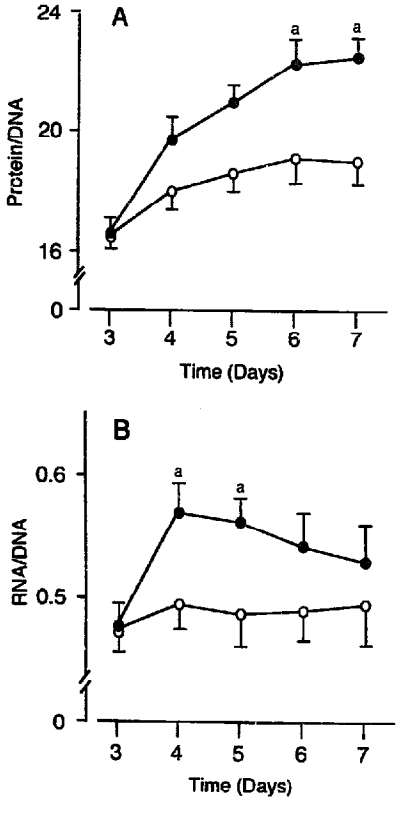

Fig. 1. Effects of stretch on cell growth and RNA content. Cardiomyocytes were incubated in serum-free medium and 15% stretch was imposed on day 3. Ratios of total protein/DNA (A) and RNA/DNA (B) were measured. Values at each time point are the means ± S.E. of 10 dishes in five different preparations; ○, control; ●, stretched myocytes. [a] $P < 0.05$, compared with control cells at the same point.

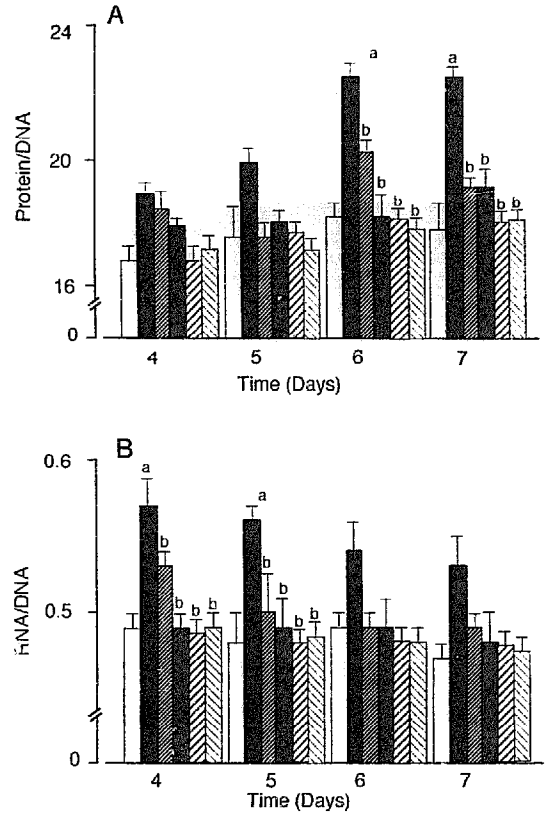

Fig. 2. Effects of captopril and losartan on stretch-induced increases in protein/DNA and RNA/DNA. Cardiomyocytes were incubated in serum-free medium. Captopril and losartan was added 30 min before stretch. Myocytes were stretched on culture day 3. Ratios of total protein/DNA (A) and RNA/DNA (B) were measured. Values are means ± S.E. of 10 dishes in five different preparations: columns: 1st, control; 2nd, stretched; 3rd, captopril + stretch; 4th, losartan + stretch; 5th, captopril; 6th, losartan myocytes. [a] $P < 0.05$, compared with control; and [b] $P < 0.05$, compared with stretched cells at the same point.

In the next experiments, to determine whether cardiac renin-angiotensin system was involved in stretch-induced hypertrophic growth, the effect of an angiotensin converting enzyme inhibitor, captopril, on stretch-stimulated increases in protein/DNA and RNA/DNA ratios was studied at 1, 2, 3 and 4 days after stretch, the times when significant increases in these ratios were observed (Fig. 1A,B). Captopril (10^{-6} M) was added 30 min before stretch. Captopril significantly inhibited the stretch-induced increases in protein/DNA ratios at culture days 6 and 7 and in RNA/DNA ratios at culture days 4 and 5 (Fig. 2). Stretch in the presence of captopril did not change values of DNA contents at culture days 4, 5, 6 and 7. Captopril alone did not change values of DNA contents or

protein/DNA ratios and RNA/DNA ratios (Fig. 2). Furthermore, the effect of a specific type 1 angiotensin II receptor antagonist, losartan, on stretch-stimulated increases in protein/DNA and RNA/DNA ratios was studied to determine which type of angiotensin II receptors was involved in stretch-induced hypertrophic growth. Losartan (10^{-6} M) was added 30 min before stretch. Losartan significantly inhibited the stretch-induced increases in protein/DNA ratios at culture days 6 and 7 and in RNA/DNA ratios at culture days 4 and 5 (Fig. 2). Stretch in the presence of losartan did not change values of DNA contents. Losartan alone did not change values of DNA contents or protein/DNA ratios and RNA/DNA ratios (Fig. 2).

We observed the morphologic changes of the cells by electronmicroscopy. The myofilaments of cardiocytes became better organized 24 h after stretch compared with non-stretched myocytes at the same time, and captopril and losartan did not change these myofilaments under basal conditions and prevented the change by stretching (data not shown).

3.2. Effects of stretch, captopril and losartan on protein synthesis

To determine whether the increase in cellular protein was the result of an increase in the rate of protein synthesis, rates of amino acid incorporation were determined by [^{14}C]phenylalanine incorporation into total protein (dpm/μg protein) (McKee et al., 1978; Munro and Fleck, 1966). Cultures were pulsed with [^{14}C]phenylalanine for 2 h to determine the rates. Rates of protein synthesis were measured at 2 days after stretch, when rapid increases in protein/DNA ratios were observed in this study (Fig. 1A). Stretch significantly increased rates of protein synthesis by 14.8 ~ 17.5% (Fig. 3). In the next experiments, the effects of captopril and losartan on preventing the acceleration of protein synthesis rates by stretch were studied. Captopril (10^{-9} ~ 10^{-5} M) or losartan (10^{-9} ~ 10^{-5} M) was added first, followed 30 min later by stretch. Stretch-induced increases in rates of protein synthesis were significantly inhibited by captopril (10^{-6} and 10^{-5} M) and losartan (10^{-7} ~ 10^{-5} M) (Fig. 3). As shown in Fig. 4, the inhibitory effect of losartan was larger than that of captopril at each concentration. Captopril and losartan did not change the rates of protein synthesis in the non-stretched myocytes.

3.3. Effects of stretch, angiotensin II and losartan on c-fos mRNA expression

In preliminary studies, maximal expression of c-fos mRNA was reached at 30 min after stretch. The effects of stretch, captopril and losartan on c-fos mRNA expression were evaluated at that time of exposure. As shown in Fig. 5, stretch markedly increased c-fos mRNA expression, and

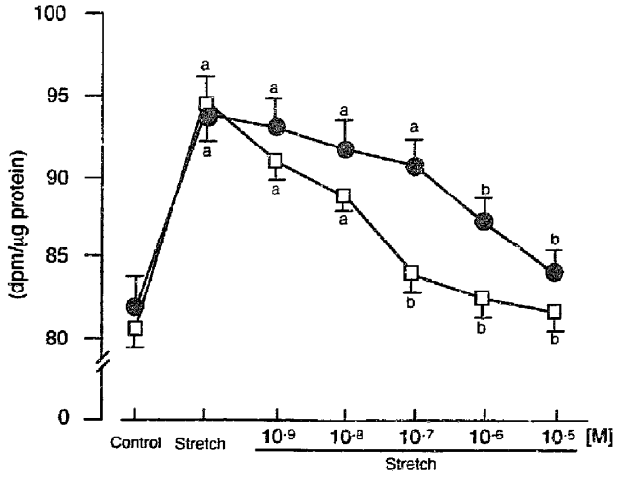

Fig. 3. Effects of captopril and losartan on stretch-induced increases in rates of protein synthesis. Cardiomyocytes were incubated in serum-free medium. Captopril or losartan was added on culture day 3 to the final concentrations indicated. The rates of protein synthesis were measured at day 5. Values are means ± S.E. of 16 dishes in four different preparations: ●, captopril-treated; □, losartan-treated myocytes. [a] $P < 0.05$, compared with control; and [b] $P < 0.05$, compared with stretched cells at the same point.

captopril (10^{-6} M) and losartan (10^{-6} M), when added to the medium 30 min before stretch, strongly inhibited the stretch-induced expression. Captopril and losartan did not affect the expression of c-fos mRNA in control cells.

3.4. Effect of stretch on secretion of angiotensin I and angiotensin II from cardiac myocytes

To determine whether stretch directly caused the secretion of angiotensin I and angiotensin II from myocytes into the culture medium, the conditioned media were collected from the same stretch dish before and after 10 min, 30

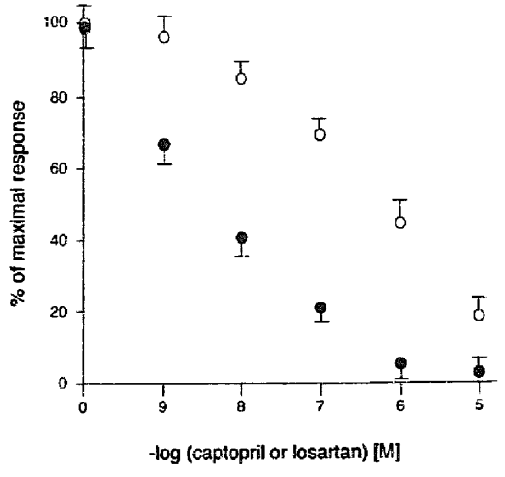

Fig. 4. Comparison between the inhibitory effects of captopril and losartan on stretch-induced accelaration of protein synthesis rates. Log [IC_{50}] of captopril-treated and losartan-treated myocytes are −6.16 and −8.32 ($P < 0.05$), respectively. Values at each time point are the means ± SE of 16 dishes in four different preparations: ○, captopril-treated myocytes; ●, losartan-treated myocytes.

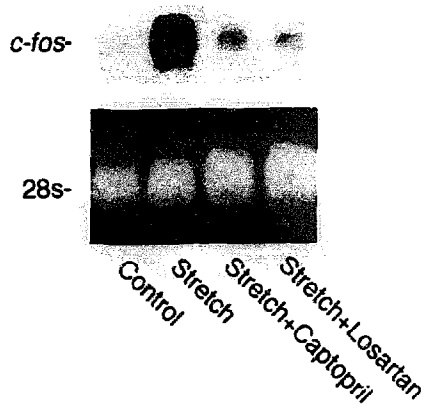

Fig. 5. Effects of stretch, captopril and losartan on c-fos mRNA expression in cardiomyocytes. Expression of c-fos mRNA was determined by Northern blot analysis. Total RNA (15 μg) was loaded in each lane. Cardiomyocytes were exposed to 15% stretch with or without captopril (10^{-6} M) or losartan (10^{-6} M) for 30 min. Ethidium bromide staining of 28s RNA showed equal amounts of RNA in each lane.

min, 1 h, 3 h and 24 h of stretch. As shown in Fig. 6, both angiotensin I and angiotensin II were not detected in the conditioned media before stretch and after 10 min of stretch in non-stretched and stretched myocytes. In the conditioned media of non-stretched myocytes, the levels of angiotensin I and angiotensin II immunoreactivity gradually increased during a 24 h period. Interestingly, stretch significantly increased the concentrations of angiotensin I in the conditioned media by 68% and 38% after 3 h and 24 h of stretch, respectively, compared with those in the media of non-stretched myocytes. Stretch significantly increased the concentrations of angiotensin II in the conditioned media by 77% after 24 h of stretch compared with the media of non-stretched myocytes.

3.5. Effects of stretch on angiotensin converting enzyme-like activity

In the next experiment, we measured the angiotensin converting enzyme activity in myocytes to examine whether stretch stimulated angiotensin converting enzyme activity in myocytes. The levels of angiotensin converting enzyme activity in stretched myocytes did not significantly change at 0, 5, 10, 15, 30, 60 min and 24 h (1.23 ± 0.07, 1.24 ± 0.09, 1.21 ± 0.07, 1.12 ± 0.10, 1.01 ± 0.06, 1.20 ± 0.10 and 1.21 ± 0.06 nmol/min/mg protein) after stretch compared with those before stretch.

4. Discussion

It has been reported that myocyte stretching directly elicites protein synthesis (Komuro et al., 1990). In the present study, we confirmed that stretch increases RNA content, protein synthesis and total protein in neonatal rat heart cell culture. However, it is not known which growth factor is the physiological mediator of stretch-induced cardiac hypertrophy, because the critical link between mechanical stress and the secretion of growth factors has not been defined.

We have reproted that angiotensin II directly produced a growth response via the type 1 angiotensin II receptor antagonist in neonatal rat heart cells (Miyata et al., 1994). Baker et al. (1990) and Schunkert et al. (1990) reported that cardiac renin-angiotensin system plays an important role in pressure overload-induced cardiac hypertrophy. Recently, Sadoshima et al. (1993) have reported that mechanical stretch causes hypertrophy of cardiac myocytes and that angiotensin II may act as an initial mediator of the stretch-induced hypertrophic response. These results and our own data strongly suggest that cardiac renin-angiotensin system plays an important role in stretch-induced hypertrophy. However, it is not known how cardiac cells sense the external overload and convert this change into intracellular signals that regulate cell growth and production of angiotensin II within the heart. The present experiments demonstrate that stretch-induced hypertrophic growth is inhibited by captopril and losartan and that the inhibitory effect of losartan on stretch-induced hypertrophic response is greater than that of captopril. These data indicate that stretch-induced hypertrophy is related to the activation of cardiac renin-angiotensin system and that inhibitory effects of captopril and losartan on stretch-in-

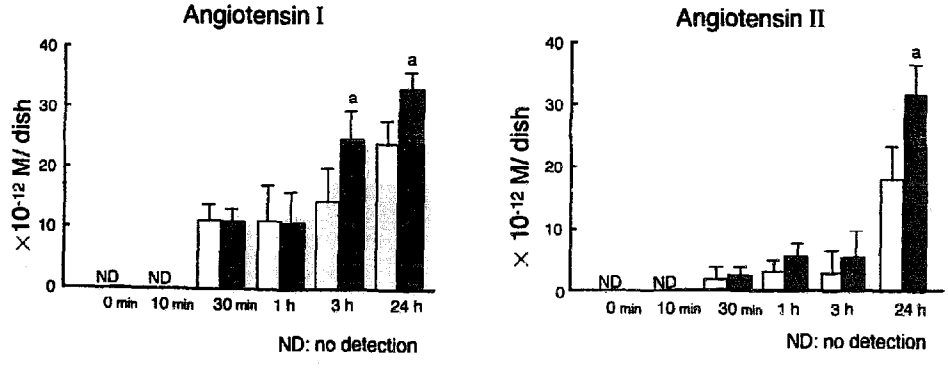

Fig. 6. Effect of stretch on angiotensin I and angiotensin II concentrations in the culture medium. Values at each time point are the means ± S.E. of 20 dishes in four different preparations; □, control cells; ■ stretched cells. [a] $P < 0.05$, compared with non-stretched cells at the same point.

duced hypertrophy may reflect a direct versus indirect effect on type 1 angiotensin II receptor occupacy.

The presence of autocrine and paracrine system has been proposed as one of the potential mechanisms that is involved in the pathogenesis of load-induced cardiac hypertrophy. Sadoshima et al. (1993) have reported that mechanical stretch causes a direct release of angiotensin II from cardiac myocytes and that the autocrine release of angiotensin II mediates the stretch-induced hypertropy. In the present study, we confirmed that the amounts of angiotensin I and angiotensin II are increased by stretch in cultured cardiomyocytes. Passive stretch increases the level of angiotensin I in the medium by 68% after 3 h and 38% after 24 h and the level of angiotensin II in the medium is increased by 80% over 24 h. On the other hand, there is the membrane anchored angiotensin converting enzyme (Hooper, 1991; Sibony et al., 1993) which may convert stretch-secreted angiotensin I to angiotensin II. Ruzicka and Leenen (1995) reported that angiotensin II can be generated from angiotensin I either by angiotensin converting enzyme or by an enzyme resistant to an angiotensin converting enzyme inhibitors and that the cardiac renin-angiotensin system acts a trophic factor. Schunkert et al. (1990) have reported that angiotensin converting enzyme activity was increased by pressure overload. We therefore examined whether stretch increases angiotensin converting enzyme activity. Stretch did not increase angiotensin converting enzyme-like activity in cultured myocytes. Captopril (10^{-6} and 10^{-5} M) or losartan(10^{-7}–10^{-5} M) significantly inhibited stretch-induced hypertrophic response to the same extent, but inhibitory effect of losartan was larger than that of captopril at each concentration. These data suggest that the increased angiotensin II in cultured medium was the result of two mechanisms, (1) angiotensin I secretion into medium and angiotensin I is converted to angiotensin II by angiotensin converting enzyme and (2) stretch directed secretion of angiotensin II into medium.

We have previously reported that angiotensin II at concentrations of 10^{-7} ~ 10^{-5} M increases intracellular free calcium concentration, protein kinase C activity and c-fos mRNA expression, and induces the cellular hypertrophy in neonatal rat heart cells (Miyata et al., 1994). In this experiment, angiotensin II concentration in the incubation medium was significantly increased by stretch but its concentration was at approximately 10^{-11} M level. Kojima et al. (1994) reported that some mechanisms other than a stimulation by endogenous angiotensin II may play a role in the signaling of cardiomyocyte hypertrophy provoked by stretch. Therefore, it is not safe to conclude that stretch-induced release of angiotensin II alone might directly cause hypertrophic growth. However, stretch actually induces hypertrophic growth, and both captopril and losartan inhibit this growth. It has been reported that the expression of the type 1A angiotensin II receptor mRNA and the number of angiotensin II receptors increase in the hypertrophied ventricle (Suzuki et al., 1993). Ito et al. (1993) have reported that angiotensin II stimulates the release of endothelin-1 from cardiomyocytes, suggesting that endogenous endothelin-1 that is produced locally in cardiomyocytes may play a key role in cardiac hypertrophy via an autocrine and paracrine system. Thus, the exact mechanisms of the stretch-induced hypertrophy need further studies to clarify whether the numbers of type 1 angiotensin II receptors are increased by stretch, whether stretch amplifies the intracellular signal transduction of angiotensin II, or whether angiotensin II is synergistic with stretch-induced secretion of growth factors such as endothelin-1.

In summary, we have demonstrated that (1) stretch causes accelerated protein synthesis and cell hypertrophy in neonatal rat myocytes; (2) stretch increases c-fos mRNA expression; (3) captopril and losartan inhibits these changes that are induced by stretch; (4) stretch increases angiotensin I and angiotensin II secretion into the conditioned media; and (5) stretch does not change angiotensin converting enzyme activity. Future experiments will be directed toward determining the autocrine and paracrine systems and the signal transduction systems that are responsible for the development of cardiac hypertrophy following a stretch.

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