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Cyclic stretch induces the release of growth promoting factors from cultured neonatal cardiomyocytes and cardiac fibroblasts

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Abstract

Growth factors and hormones may play an autocrine/paracrine role in mechanical stress-induced cardiac hypertrophy. Using an *in vitro* model of mechanical stress, i.e. stretch of cardiomyocytes and cardiac fibroblasts, we tested the involvement of growth factors and hormones in this process.

We found that conditioned medium (CM) derived from 4 h cyclicly (1 Hz) stretched cardiomyocytes increased the rate of protein synthesis in static cardiomyocytes by 8 ± 3%. Moreover, CM derived from 2 h stretched fibroblasts increased the rate of protein synthesis in static fibroblasts as well as in static cardiomyocytes by 8 ± 2 and 6 ± 2%, respectively. Analysis of CM using size-exclusion HPLC showed that cardiomyocytes and fibroblasts released at least three factors with MW ≤ 10 kD, their quantities being time-dependently increased by stretch. Subsequent analyses using immunoassays revealed that cardiomyocytes released atrial natriuretic peptide (ANP) and transforming growth factor-beta1 (TGFβ_1) being increased by 45 ± 17 and 21 ± 4% upon 4 h of stretch, respectively. Fibroblasts released TGFβ_1 and very low quantity of endothelin-1 (ET-1). The release of TGFβ_1 was significantly increased by 18 ± 4% after 24 h of stretch in fibroblasts. Both cell types released no detectable amount of angiotensin II (Ang II).

In conclusion, upon cyclic stretch cardiomyocytes and fibroblasts secrete growth factors and hormones which induce growth responses in cardiomyocytes and fibroblasts in an autocrine/paracrine way. TGFβ secreted by cardiomyocytes and fibroblasts, and ANP secreted by cardiomyocytes are likely candidates. We found no evidence for the involvement of Ang II and ET-1 in autocrine/paracrine mechanisms between cardiac cell types. (Mol Cell Biochem **208**: 89–98, 2000)

Key words: stretch, autocrine/paracrine mechanisms, transforming growth factor-β, endothelin-1, atrial natriuretic peptide, myocardial cells

Introduction

Mechanical stress is a key factor in the development of hemodynamic overload-induced cardiac hypertrophy [1, 2]. Via integrins and/or the cytoskeleton, the mechanical signal may trigger signal transduction pathways resulting in gene reprogramming and acceleration of protein synthesis [3–6]. The mechanical signal may also trigger the myocardium to produce and release factors that initiate myocardial growth [7].

In literature, it is reported that endothelin-1 (ET-1), angiotensin II (Ang II) and transforming growth factor-beta1 (TGFβ_1) play a role in the mechanism of cardiac hypertrophy during hemodynamic overload *in vivo* [8–13]. In culture, cardiomyocytes and fibroblasts are capable to release various growth factors, hormones and/or cytokines, such as Ang II,

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ET-1, and TGFβ_1 [11, 14–19]. These factors can induce a hypertrophic response in cardiomyocytes, thereby supporting the hypothesis that upon mechanical stress growth-promoting factors are released which induce hypertrophy.

A suitable *in vitro* model of mechanical stress-induced hypertrophy is available which allows neonatal rat cardiomyocytes to be subjected to passive stretch [20]. Using such a model, we and others have shown that upon passive stretch cardiomyocytes secrete factors which induce hypertrophy in an autocrine and/or paracrine way [21–25]. The involvement of fibroblasts in this process has been hypothesized.

In the present study, we investigated whether autocrine and/or paracrine mechanisms are involved in mechanical stress-induced hypertrophy. First, we tested whether conditioned medium (CM) derived from stretched cardiomyocytes and fibroblasts was capable of inducing growth in static cardiomyocytes and fibroblasts. Then, we tried to identify the factors that were released upon stretch. We found that upon stretch of cardiomyocytes and fibroblasts transferable factors were released which induced a growth response in an autocrine and paracrine way.

Materials and methods

Materials

DMEM, Ham's F10, heat-inactivated fetal bovine serum, heat-inactivated horse serum, and trypsine/EDTA solution were purchased from Gibco (Life Technologies BV, Breda, The Netherlands). Bovine serum albumin, that was essentially fatty acid free, was supplied by Sigma (Veenendaal, The Netherlands). The leucine-free DMEM was from ICN Biomedicals BV (Zoetermeer, The Netherlands). Leucine-free Ham's F10 was manufactured on request by Gibco. Collagenase was supplied by Worthington Biochemical Corporation (Aristoforma Interchema, Amsterdam, The Netherlands). Natrium-penicillin was from Yamanouchi (Leiderdorp, The Netherlands) and streptomycin was from Radiumfarma-Fisiopharma (Naples, Italy). The flexible-bottomed 6-well culture plates coated with collagen I were purchased from Flexcell Int. Corp. (Dunn Laboratory, Asbach, Germany). The primaria-coated 6 cm ∅ culture dishes and the conventional 12-well plates were from Falcon (Micronics BV, Lelystad, The Netherlands). Tritiated leucine (L-[4,5-^{3}H]-leucine) with a specific activity of 120–190 Ci/mmol was purchased from Amersham-Pharmacia Biotech Benelux (Den Bosch, The Netherlands). Coomassie Plus Protein Assay was supplied by PIERCE (Brunschwig Chemie, Amsterdam, The Netherlands). Ang II, ET-1, and the EIAH kits for detection of ANP and ET-1 were purchased from Peninsula Laboratories Europe (St. Helens, Merseyside, UK). The E_{max}™ Immunoassay system for determination of TGFβ_1 was from Promega (Leiden, The Netherlands).

Cell culture

Cultures of cardiomyocytes and fibroblasts were prepared as described below. Hearts were dissected from 16 neonatal (1–2 day-old) rat heart ventricles, minced and washed in solution A (0.02% phenol red, 137 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.6 mM D-glucose and 20 mM HEPES, pH 7.3) at room temperature. Then, the heart tissue was dissociated using a digestion solution containing 450 U collagenase and 14 U DNase per ml solution A, in an erlenmeyer containing glass beads, and put in a shaking water bath (at 37°C). Pooled cell suspensions from two dissociations were cooled on ice, centrifuged at 1000 rpm for 15 min, resuspended in Ham's F10 medium supplemented with 10% foetal bovine serum (FBS) and 10% horse serum (HS), and plated onto 12 primaria-coated 6 cm ∅ culture dishes (Falcon). The cells were plated for 45 min to allow preferential attachment of fibroblasts to the bottom of the culture dishes. Non-adherent cells (representing the cardiomyocytes) were collected and adherent cells (mainly fibroblasts) were supplied with Dulbecco's modified Eagle medium (DMEM) containing 10% FBS and antibiotics (100 U/ml penicillin and 0.1 g/L streptomycin). Fibroblasts were grown to confluence and subsequently passaged and plated onto 10 collagen-I coated 6-well stretch plates (Flexcell Int. Corp.) that have flexible bottoms, or onto 3 conventional 12-well plates (Falcon). The collected cardiomyocytes were directly plated at a density of 1×10^5 cells/cm^2 onto 8 collagen-I coated 6-well stretch plates, or 3 conventional 12-well plates, and cultured in Ham's F10 and DMEM (1:1) supplemented with 8% HS and antibiotics. Cardiomyocytes and fibroblasts were incubated at 37°C at 5% CO_2 in a humidified incubator. The culture medium of both cell types was refreshed every 2–3 days.

Studies were conducted on fibroblasts (passage 1) that were grown to subconfluence in serum-containing media for 5 days. Then they were growth-arrested in serum-free medium being DMEM supplemented with 25 mM HEPES and antibiotics for 24 h before the experiment. After 3 days of culturing cardiomyocytes were put on serum-free medium being DMEM/Ham's F10 (1:1, v/v) supplemented with 12.5 mM HEPES, 2 g/L BSA, and antibiotics for 24 h.

Preparation of stretch-conditioned medium

Cells were grown on collagen I-coated stretch plates, consisting of 6 wells of 5 cm^2 each with 1 ml of culture medium.

Twenty-four h before start of the experiments the cells were put on serum-free medium. In addition, when the conditioned medium was used for determination of protein synthesis rate, the medium was serum-free and leucine-free, being Ham's F10 without leucine for cardiomyocytes and DMEM without leucine for fibroblasts. Then, they were submitted to stretch in a stretch device (Flexercell Strain® Unit FX2000, Flexcell Int. Corp.) that applies stretch to the cells by pulling the flexible bottoms using vacuum [26]. Cyclic stretch was applied with 1 Hz (per cycle 0.5 sec elongation and 0.5 sec relaxation) and a peak elongation of 20%. After the cells had been stretched, the stretch-conditioned medium was collected and transferred to static cells (rate of protein synthesis) or analysed for the presence of growth-promoting factors (determination of Ang II, ET-1, ANP and $TGF\beta_1$).

Stretch-conditioned medium (CM) is defined as cell culture medium collected from cardiomyocytes (CM-Mc) and fibroblasts (CM-Fb) cyclicly stretched for 2, 4, 6, 18, and 24 h and abbreviated as CM2h, CM4h, CM6h, CM18h, and CM24h, respectively. Conditioned medium derived from static cells (CMCo) that were grown on identical culture plates and maintained under identical circumstances served as control medium.

Rate of protein synthesis

For these experiments the cells were seeded onto the conventional 12-well plates and put on serum-free medium 24 h before start of the experiments. Cardiomyocytes were incubated for 6 h with CM derived from 0 (control), 4, and 6 h stretched cardiomyocytes and with CM derived from 0 (control), 2, and 6 h stretched fibroblasts. Fibroblasts were incubated for 6 h with CM derived from 0 (control), 2, and 6 h stretched fibroblasts. To measure the rate of protein synthesis, ^{3}H-leucine (1 μCi/well) was added for the final 2 h during which it incorporates into newly synthesized proteins. Subsequently, the cells were treated with 10% trichloracetic acid to precipitate cellular protein, that was later dissolved in 0.2 M NaOH. The quantity of incorporated ^{3}H-leucine was determined by counting an aliquot of sample in a β-scintillation counter (LKB Wallac, Turku, Finland). An aliquot was used to determine total cellular protein using Coomassie plus protein assay. The incorporated ^{3}H-leucine was corrected for cell density by dividing the radioactivity in counts per min by the total amount of cellular protein. Finally, the rate of protein synthesis in cells incubated with stretch-conditioned medium was expressed as percentage of control, i.e. as percentage of that in cells incubated with CM derived from static cells (control medium).

Size-exclusion High Pressure Liquid Chromatography (HPLC)

The stretch-conditioned media (20 μl) were loaded on a TSK-Gel G2000SW column (30 cm × 7.5 mm, TosoHaas, Montgomeryville, PA, USA) preceded by a BIOSEP SEC guard column (50 × 7.5 mm, Phenomenex, Torrance, CA, USA). This set-up of columns elutes high molecular weight proteins first, followed by lower molecular weight proteins. Fractions were eluted with 0.1 mM NaH_2PO_4, pH 6.8 at a speed of 1 ml/min. The use of the NaH_2PO_4-solution prevented the baseline from rising during the run, at the expense of detection of ANP. Peaks were detected using the adsorption at 280 nm (A_{280nm}) with control medium, derived from static cultures, as reference.

The peak areas in chromatograms from control media were subtracted from those with comparable retention times of stretch-conditioned media. After subtraction, residuals were expressed as percentage of peak area in control medium.

Measurements of ANP, ET-1, $TGF\beta_1$, and Ang II

Presence of immunoreactive atrial natriuretic peptide (ANP) and endothelin-1 (ET-1) in stretch-conditioned media was measured in triplicate using competitive enzyme immunoassay (EIAH) kits from Peninsula. The quantity of ANP and ET-1 in the stretch-conditioned media was corrected for the quantity of total cellular protein. The anti-ANP antibody showed no cross-reactivity with either ET-1 or brain natriuretic peptide (BNP). The anti-ET-1 antibody showed very low cross-reactivity with endothelin-3 (0.005%) and no cross-reactivity with Ang II, ANP and BNP. The range of detection is 0.04–25 ng/ml for the standard curve of ANP and ET-1.

The levels of transforming growth factor-beta$_1$ ($TGF\beta_1$) in the stretch-conditioned media were determined in triplicate using an E_{max}™ immunoassay system from Promega. $TGF\beta_1$, *in vivo*, is processed from a latent form to a bioactive form of the protein. Only the bioactive from of $TGF\beta_1$ is immunoreactive and detectable by the anti-$TGF\beta_1$ antibody used in the immunoassay. $TGF\beta_1$ is produced by most cultured cells in a latent form [27]. Therefore, we treated the samples at pH 2.6 at room temperature for 20 min and then neutralized them. The anti-$TGF\beta_1$ antibody showed very low cross-reactivity with other TGFβ isoforms: 11.5% cross-reactivity with $TGF\beta_{1.2}$ (a heterodimer containing a $TGF\beta_1$ and a $TGF\beta_2$ chain), 1.6% with $TGF\beta_2$, and 0.7% with $TGF\beta_3$. The range of detection is 15.6–1000 pg/ml for the standard curve of $TGF\beta_1$.

Statistics

Statistical analysis was performed by using the Student's *t*-test and by analysis of variance wherever appropriate. Data were expressed as mean ± S.E.M.

Results

Rate of protein synthesis

To test whether factors with growth-promoting capacities are released upon stretch, cardiomyocytes and fibroblasts were submitted to cyclic stretch for 0 (control), 2, 4, and 6 h. Then, the stretch-conditioned medium was collected and transferred to static cells. These cells were incubated for 6 h with the stretch-conditioned medium. In the last 2 h of incubation, the rate of proteins synthesis was determined by adding ^{3}H-leucine.

The conditioned medium of 4 and 6 h stretched cardiomyocytes increased the rate of protein synthesis slightly but significantly with 8 ± 3 and 4 ± 2% ($p \leq 0.05$), respectively, in static cardiomyocytes (Fig. 1a). The conditioned medium of 2 and 6 h stretched fibroblasts increased the rate of protein synthesis in static fibroblasts with 8 ± 2% ($p \leq 0.01$) (Fig. 1b) as well as in static cardiomyocytes with 6 ± 2% ($p \leq 0.05$) and 4 ± 4% (not significant) (Fig. 1a). Thus, the stretch-conditioned media of cardiomyocytes as well as fibroblasts contained growth-promoting factors.

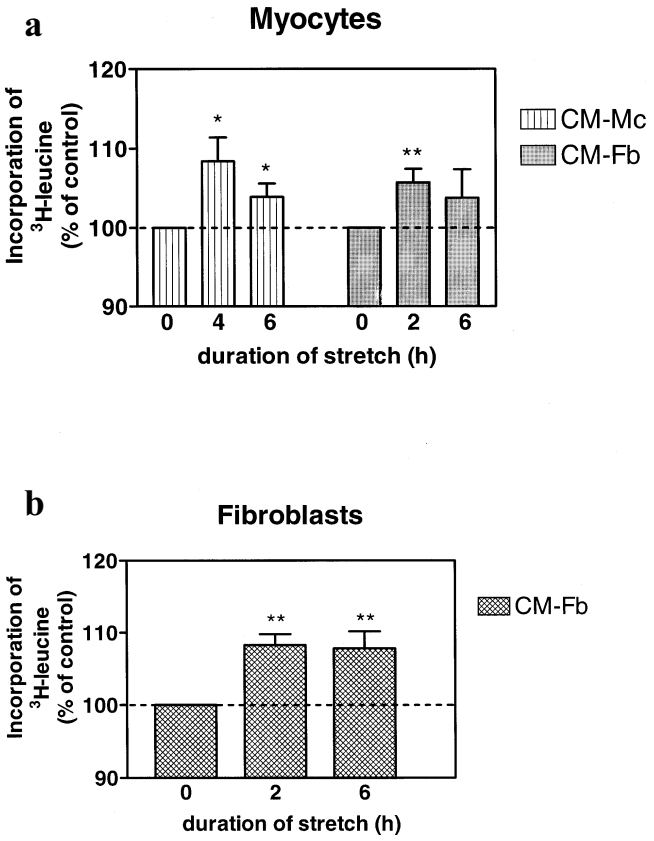

Fig. 1. Rate of protein synthesis in cardiomyocytes and fibroblasts incubated for 6 h with stretch-conditioned medium (CM). (a) shows the rate of protein synthesis in cardiomyocytes incubated with CM derived from 0 (control), 4 and 6 h stretched cardiomyocytes and with CM derived from 0, 2, and 6 h stretched fibroblasts; (b) shows the rate of protein synthesis in fibroblasts incubated with CM derived from 0, 2 and 6 h stretched fibroblasts. Rate of protein synthesis was assessed by incorporation of ^{3}H-leucine, followed by scintillation counting. Data were normalised to their controls (cells incubated with CM derived from static cells) within each experiment. The data are represented as means ± S.E.M. from 8 experiments. * $p \leq 0.05$; ** $p \leq 0.01$ vs. incubation with control CM.

HPLC analysis

The factors present in the stretch-conditioned media were separated according to their molecular size using size-exclusion chromatography. Of each sample the peak areas of the separated factors on the chromatogram were calculated. Using a set of molecular weight markers the apparent molecular weights of the identified peaks were calculated.

In stretch-conditioned medium of cardiomyocytes six peaks were found with a molecular weight of less than 10 kD (Fig. 2a). Of these peaks, only the areas of three peaks increased significantly compared to control medium, having a retention time of 11.1–11.4, 13.5–13.6, and 15.6–15.7 min (about 8–9, 3, and 1 kD, respectively) (Fig. 3). The peak with a retention time of 11.1–11.4 min was increased with 8 ± 2 and 17 ± 6% in CM4h and CM6h, respectively. The peak of 13.5–13.6 min was increased with 17 ± 6%, and the peak of 15.6–15.7 min with 12 ± 4% in CM4h. The peak with a retention time of 12.4–12.6 min (about 5 kD) increased with the duration of stretch, but not significantly. The peak with a retention time of 11.1–11.4 min very likely consists of two peaks. However, since only a few chromatograms allowed us to discriminate between these two peaks, we decided to consider these peaks as one peak.

In stretch-conditioned medium of fibroblasts, six peaks were found with a molecular weight of less than 8 kD (Fig. 2b). The peak with a retention time of 11.5–11.9 min consists very likely of three peaks, which could rarely be identified separately. The peak areas of three peaks showed significant increases with duration of stretch, i.e. the peaks with a retention time of 11.5–11.9, 12.5–12.6, and 15.5–15.7 min (about 6–7, 5, and 1 kD, respectively) (Fig. 4). The peak with a retention time of 11.5–11.9 min was increased with 9 ± 3 and 13 ± 3%, the peak of 12.5–12.6 with 12 ± 3 and 19 ± 6%, and the peak of 15.5–15.7 min with 7 ± 2 and 11 ± 3% in CM2h and CM6h, respectively.

When we spiked the samples with rat angiotensin II, a peak with a retention time of about 12.9 min was detected. There

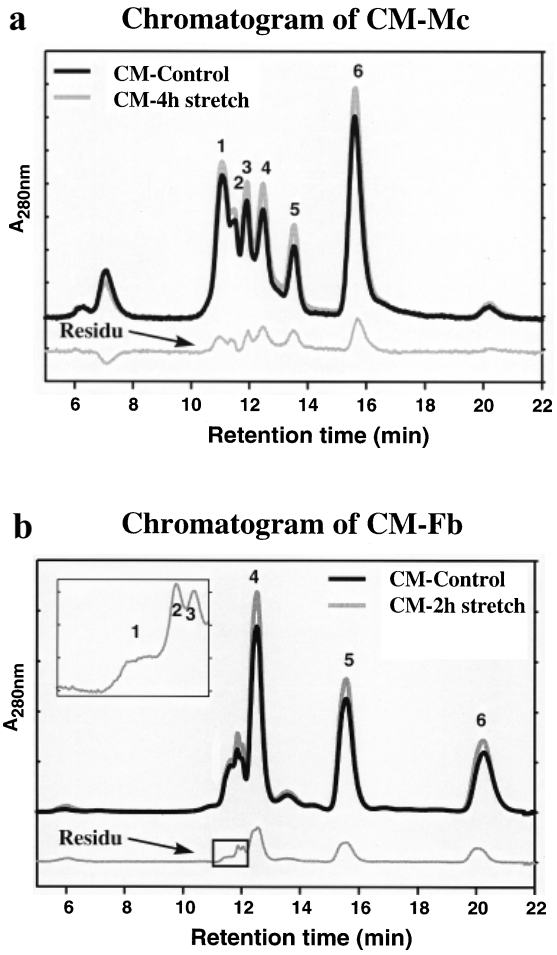

Fig. 2. HPLC chromatogram of conditioned medium. The CM samples were fractionated using size-exclusion HPLC and peaks were detected by the absorption at 280 nm. For details of the conditions used see the Materials and methods section. The black line represents the chromatogram of control medium and the grey line represents the chromatogram of stretch-conditioned medium. The lower grey line represents the residue after subtraction of the control medium from the stretch-conditioned medium. (a) shows a typical HPLC chromatogram of CM derived from cardiomyocytes. Peaks 1 and 2 were rarely identified separately and have a retention time of 11.1–11.4 min, peak 3 has a retention time of 11.9–12.1 min, peak 4, 12.4–12.6 min, peak 5, 13.5–13.6 min and peak 6, 15.6–15.7 min; (b) shows a typical HPLC chromatogram of CM derived from fibroblasts. Peaks 1–3 could not be identified separately having a retention time of 11.5–11.9 min, peak 4 has a retention time of 12.5–12.6 min, peak 5, 13.4–13.6 min, and peak 6, 15.5–15.7 min.

was no such peak present in the conditioned media, suggesting that stretched cardiomyocytes and fibroblasts do not secrete angiotensin II. Furthermore, we spiked with rat endothelin-1 which gave a peak with a retention time of about 12.6 min. In the CM of stretched fibroblasts and cardiomyocytes a peak with comparable retention time was found, although in the CM of stretched fibroblasts only the area of this peak increased significantly compared to control.

Detection of immunoreactive ANP

Since fibroblasts are unable to synthesize and secrete atrial natriuretic peptide (ANP), we measured the presence of immunoreactive (ir)ANP in the stretch-conditioned medium of cardiomyocytes only. Upon stretch, the cardiomyocytes secreted irANP (Fig. 5). The amount of irANP, however, was only significantly different from the control after 4 and 24 h of stretch (by 45 ± 17 and 60 ± 18%, respectively). If all values obtained at stretch times of 4, 6, 18, and 24 h were pooled, mean irANP was increased by 56% over control ($p < 0.001$).

Determination of ET-1

ET-1 was not detectable in the conditioned medium of stretched cardiomyocytes, despite the use of a highly sensitive assay with a detection limit of 0.04 ng per ml of conditioned medium, i.e. ET-1 levels were below 0.04 ng per about 0.5×10^6 cells. In the conditioned medium of stretched fibroblasts, ET-1 levels were just detectable, although the values were below the range of the standard curve, i.e. about 0.02 and 0.03 ng/ml after 2 and 24 h of stretch, respectively.

Detection of TGFβ_1

Cultured cells are probably not capable to transform the latent form of TGFβ_1 into the active form themselves [27]. Since the immunoassay system recognizes only bioactive TGFβ_1, the samples were treated with pH 2.6 for 20 min at room temperature, followed by neutralization.

In conditioned medium of stretched cardiomyocytes we found higher levels of TGFβ_1 as compared with control (Fig. 6a). After 2 and 4 h of stretch the quantities of TGFβ_1 in the CM of cardiomyocytes were increased by 21 ± 2 and 21 ± 4% over control, respectively ($p < 0.01$). In conditioned medium of stretched fibroblasts we found no induction of TGFβ_1 in the first 6 h of stretch (Fig. 6b). After 24 h of stretch, however, the quantities of TGFβ_1 increased by 18 ± 4% over control ($p = 0.008$).

Discussion

In the present study, we tested whether autocrine and/or paracrine mechanisms are involved in the development of mechanical stress-induced hypertrophy of cardiomyocytes. To this purpose, we cyclicly stretched cardiomyocytes and fibroblasts derived from neonatal rat ventricles and tested the growth-promoting capacities of the stretch-conditioned medium (CM), followed by analysis of CM for the presence of growth factors and/or hormones.

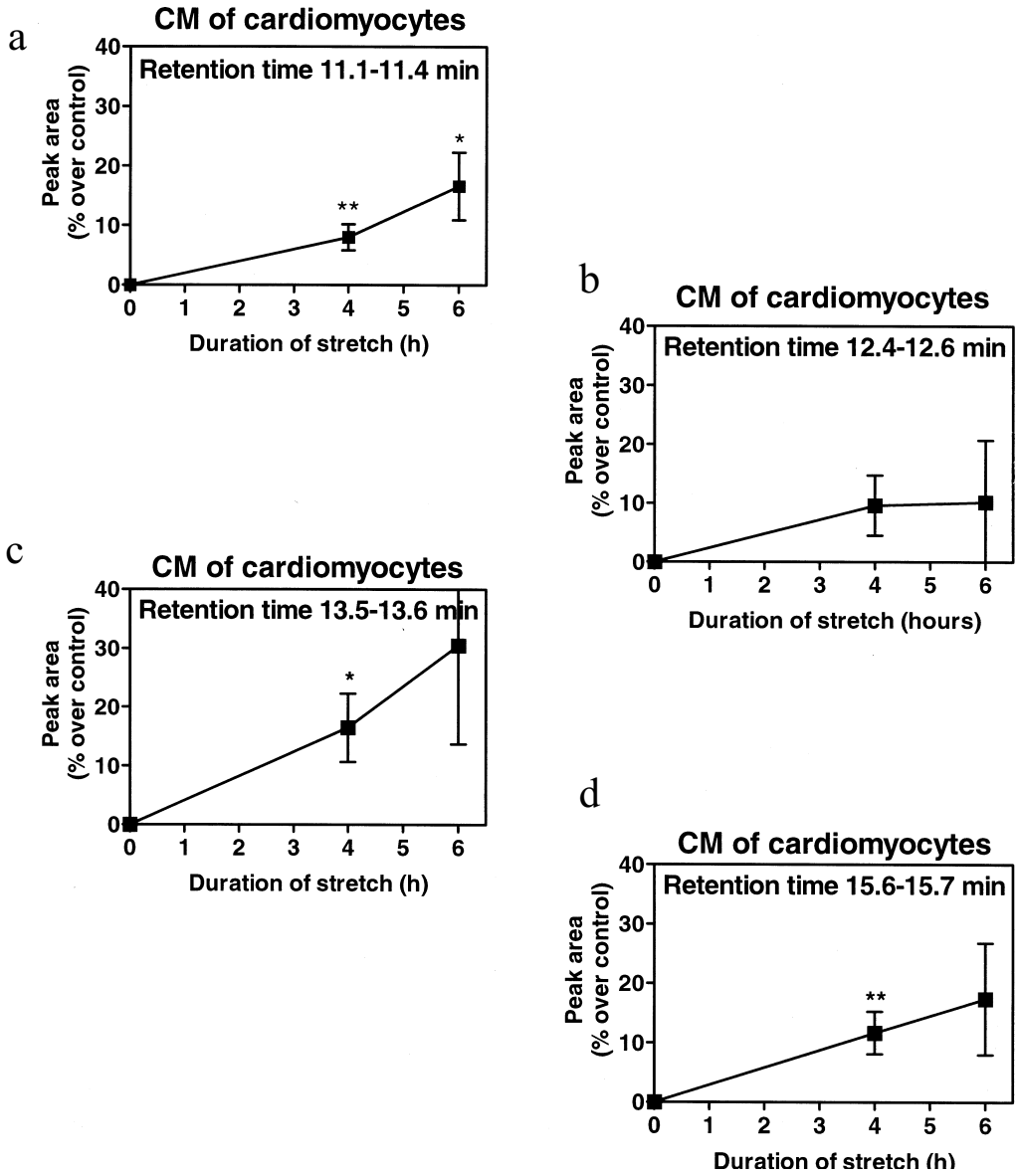

Fig. 3. Peak areas of chromatograms from conditioned medium (CM) derived from 0 (control), 4, and 6 h stretched cardiomyocytes. Shown are (a) the peak area with a retention time of about 11.1–11.4 min (about 8–9 kD); (b) the peak area with a retention time of about 12.4–12.6 min (about 5 kD); (c) the peak area with a retention time of about 13.5–13.6 min (about 3 kD); and (d) the peak area with a retention time of about 15.6–15.7 min (about 1 kD). The CM were analysed using size-exclusion HPLC followed by detection of the adsorption at 280 nm. Peak areas in chromatograms from control media were subtracted from those of stretch-conditioned media. After subtraction, residual peak areas were expressed as percentage of peak area in control medium. The data are represented as means ± S.E.M. from 6 experiments. *$p \leq 0.05$; **$p \leq 0.01$ vs. control CM.

In cardiomyocytes and fibroblasts incubated for 6 h with CM of cardiomyocytes and fibroblasts, respectively, the rate of protein synthesis increased. Furthermore, in cardiomyocytes incubated for 6 h with CM of fibroblasts the rate of protein synthesis increased also. These findings strongly suggest that upon stretch of cardiomyocytes these cells secrete factors that have autocrine effects on cardiomyocytes. Moreover, it appears that fibroblasts secrete factors that have autocrine and paracrine effects on fibroblasts and cardiomyocytes, respectively.

If CM of cardiomyocytes was analysed by size-exclusion HPLC, three peaks with a MW ≤ 10 kD had significantly increased after 4 h of stretch, and one of these peaks remained increased significantly after 6 h of stretch. In the CM of fibroblasts, three peaks with a MW ≤ 8 kD were detected that increased time-dependently during stretch. These findings suggest the existence of at least three growth factors in the CM of stretched cardiomyocytes as well as in the CM of stretched fibroblasts.

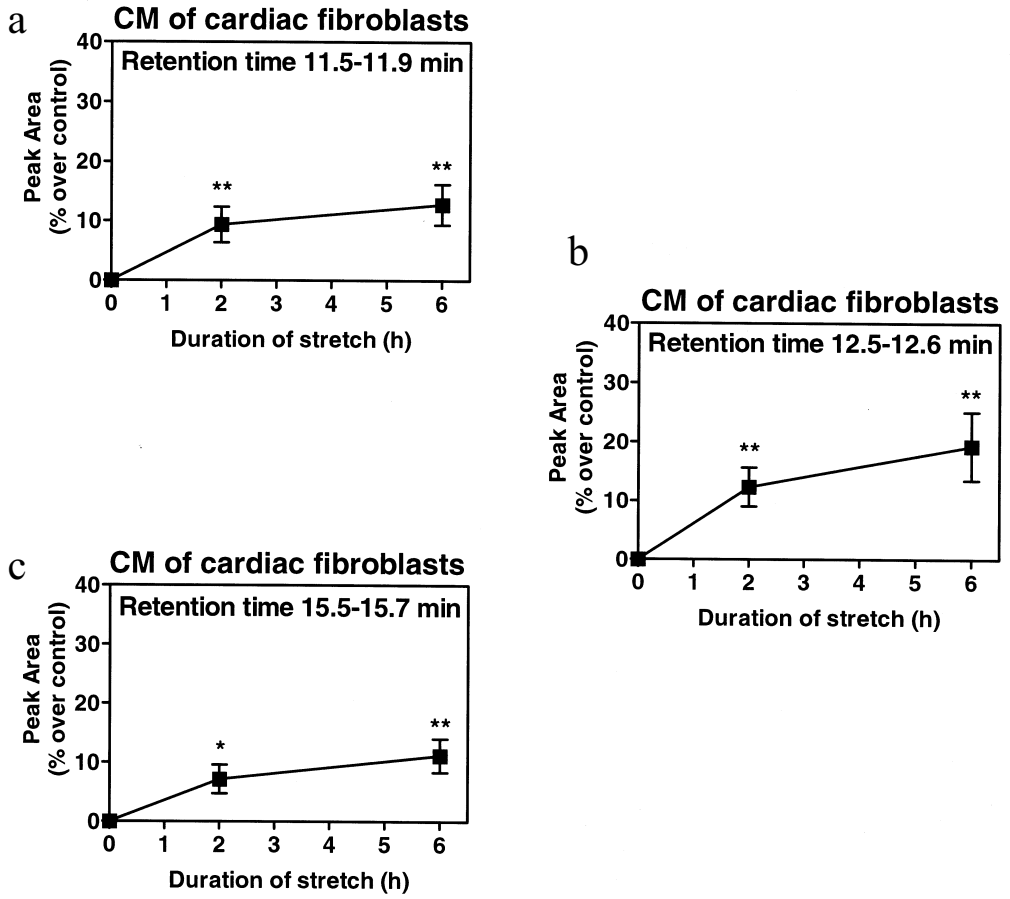

Fig. 4. Peak areas of chromatograms from conditioned medium (CM) derived from 0 (control), 2, and 6 h stretched fibroblasts. Shown are (a) the peak area with a retention time of about 11.5–11.9 min (about 6–7 kD); (b) the peak area with a retention time of about 12.5–12.6 min (about 5 kD); and (c) the peak area with a retention time of about 15.5–15.7 min (about 1 kD). The CM were analysed using size-exclusion HPLC followed by detection of the adsorption at 280 nm. Peak areas in chromatograms from control media were subtracted from those of stretch-conditioned media. After subtraction, residual peak areas were expressed as percentage of peak area in control medium. The data are represented as means ± S.E.M. from 6 experiments. *$p \leq 0.05$; **$p \leq 0.01$ vs. control CM.

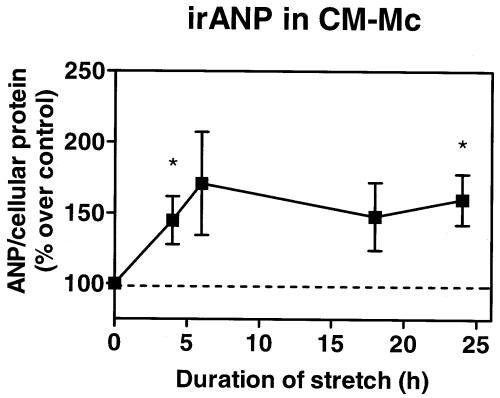

Fig. 5. Quantity of immunoreactive (ir)ANP in conditioned medium derived from stretched cardiomyocytes. Release of irANP from 0 (control), 4, 6, 18, and 24 h stretched cardiomyocytes. The quantity of irANP in the CM was determined using an immunoassay. The data are expressed as percentage of the quantity of irANP in control CM. Data represent means ± S.E.M. from 6 independent experiments. *$p \leq 0.05$; **$p \leq 0.01$ vs. control CM.

Subsequently, we tested the presence of angiotensin II (Ang II), endothelin-1 (ET-1), atrial natriuretic peptide (ANP), and transforming growth factor beta-1 ($TGF\beta_1$) in CM of cardiomyocytes and fibroblasts. In chromatograms of CM of cardiomyocytes and fibroblasts, we found no peak that may represent Ang II, and one peak that may represent ET-1. Using an immunoassay, we found no detectable ET-1 in the CM of cardiomyocytes. In the CM of fibroblasts ET-1 was just detectable after 2 and 24 h of stretch, although such low levels are considered irrelevant. $TGF\beta_1$ was found in CM of both cardiomyocytes and fibroblasts. In CM of cardiomyocytes the release of TGFβ1 increased rapidly within the first 4 h of stretch, whereas in CM of fibroblasts $TGF\beta_1$ levels increased later (significantly increased after 24 h of stretch). The quantity of ANP in CM of cardiomyocytes was increased upon stretch for at least 24 h.

Our results suggest that $TGF\beta_1$ may be involved in mechanical stress-induced hypertrophy in an autocrine and/

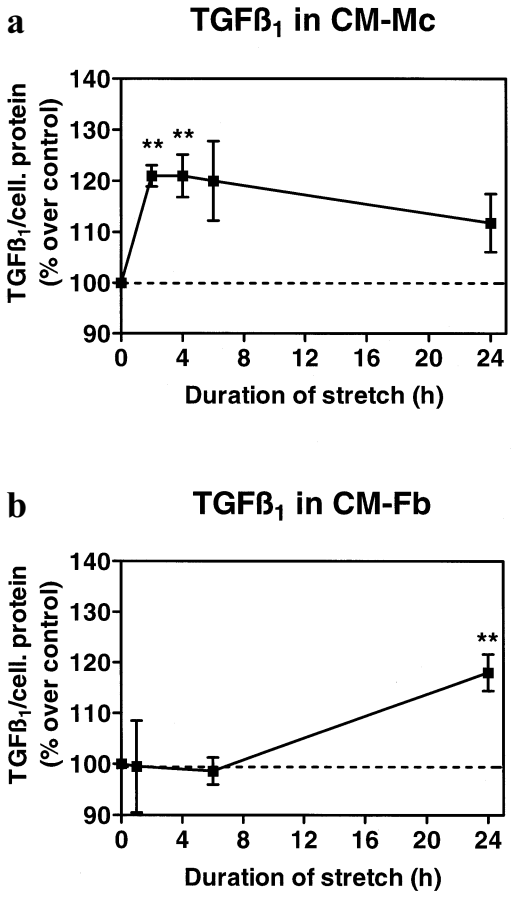

Fig. 6. Quantity of TGFβ$_1$ in conditioned medium from stretched cardiomyocytes and fibroblasts. (a) shows the quantity of TGFβ$_1$ in CM derived from 0 (control), 2, 4, 6, and 24 h stretched cardiomyocytes; (b) shows the quantity of TGFβ$_1$ in CM of 0, 1, 2, 6, and 24 h stretched fibroblasts. Samples of the CM were treated at pH 2.6 for 20 min at room temperature to transform the TGFβ$_1$ from a latent into an active form that can be recognized by the antibody used in the immunoassay. The data are expressed as percentage of the quantity of TGFβ$_1$ in control CM. Data represent means ± S.E.M. from 4 independent experiments. **p ≤ 0.01 vs. control CM.

or paracrine way. This result is in agreement with several reports. Li *et al*. induced myocardial stretch in an isolated perfused Langendorff preparation by inflation of an intraventricular balloon, and observed increased concentrations of TGFβ$_1$ in the perfusate [28]. In addition, Takahashi *et al*. showed that aortic constriction in rats caused ventricular hypertrophy associated with increases in TGFβ$_1$ mRNA in the myocyte fraction, but not in the non-myocyte fraction [11]. Calderone and colleagues reported that in rats with hypertrophy due to pressure and volume overload, the mRNA levels of TGFβ$_1$ are increased in the cardiomyocyte fraction, but remained unchanged in the non-myocyte fraction [29]. Furthermore, Seko *et al*. reported that stretched cardiomyocytes released TGFβ that induced the expression and release of vascular endothelial growth factor (VEGF) [30]. Thus, stretched cardiomyocytes secrete TGFβ which may induce hypertrophy in an autocrine way.

We found no detectable (or very low levels) of ET-1 and Ang II in the CM of stretched cardiomyocytes and fibroblasts. This finding is in contradiction with several previous reports [19, 24, 25]. A possible explanation is a difference in serum source or duration of serum incubation as shown by van Kesteren *et al*. [31]. These authors reported that in response to stretch cardiomyocytes and fibroblasts produce and release Ang II only in the presence of serum, since these cells use the (pro)renin provided by the serum for production of Ang II. On the other hand, our findings corroborate the work of several groups. Corda *et al*. showed that pericardial fluid (PF, an ultrafiltrate of plasma) has trophic effects on adult cardiomyocytes and was positively correlated with left ventricular mass of overloaded hearts from which the PF was obtained [32]. This LV mass-dependent trophic effect was independent of Ang II, ANP, and ET-1. Furthermore, Harada and colleagues reported that Ang II is not essential for the development of cardiac hypertrophy [33, 34]. These authors showed that in Ang II type 1_A receptor knock-out mice, pressure overload produced by aortic constriction still induced cardiac hypertrophy. In addition, Kent and McDermott showed that stretch-induced acceleration of protein synthesis in cardiomyocytes was unaffected by an antagonist peptide to Ang II [35]. Stretch-induced activation of mitogen-activated protein kinases (MAPKs), a signaling pathway involved in stretch-induced hypertrophy, was not prevented by the combined blockade of the ET_A receptor and the Ang II type 1_A receptor [24]. Taken together, these results indicate that there are other mechanisms, independent of Ang II and ET-1, by which stretch can induce hypertrophy.

The fact that we failed to identify a growth-promoting factor in the CM of fibroblasts that is released stretch-dependently, indicates that other factors than Ang II, ET-1 and TGFβ$_1$ are involved in the autocrine/paracrine mechanisms induced by stretched fibroblasts. Our HPLC results suggested that these factors have a molecular weight of less than 10 kD. There is a factor called insulin-like growth factor-I (IGF-I), a polypeptide isolated from serum, that has a molecular weight of 7.6 kD [36]. IGF-I mediates the anabolic and cardiovascular actions of growth hormone *in vivo* [37]. IGF-I was shown to be involved in volume- and pressure-overload induced cardiac hypertrophy [13]. Upon these hemodynamic overloads, mRNA levels for IGF-I were elevated and mainly expressed in cardiomyocytes [13]. In addition, transgenic mice overexpressing IGF-1 showed increased heart weight and increased number of cardiomyocytes [38]. Furthermore, in cultured cardiomyocytes, IGF-I induced hypertrophy via the MAPK pathway [39, 40]. Thus, IGF-1 may possibly represent one of the growth-promoting factors of 8–9 kD that we detected in CM of stretched cardiomyocytes and fibroblasts.

In conclusion, stretch of cardiomyocytes and fibroblasts induces the release of growth-promoting factors that act in an autocrine/paracrine way. One of the factors that stretched cardiomyocytes and fibroblasts release is TGFβ_1. We detected no significant quantities of Ang II and ET-1 released upon stretch. HPLC analyses detected three growth factors of a MW ≤ 10 kD that showed increased levels with increased duration of stretch in the CM of cardiomyocytes and another three in the CM of fibroblasts. Taken together, these results suggest that yet unidentified factors are involved in the autocrine/paracrine mechanism of stretch-induced hypertrophy. Discovery of the identity of these factors warrants further investigation.

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