Stretch-induced hypertrophy of isolated adult rabbit cardiomyocytes

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Stretch-induced hypertrophy of isolated adult rabbit cardiomyocytes. Am J Physiol Heart Circ Physiol 299: H780–H787, 2010. First published July 16, 2010; doi:10.1152/ajpheart.00822.2009.—Both mechanical and humoral triggers have been put forward to explain the hypertrophic response of the challenged cardiomyocyte. The aim of the present study was to investigate whether cyclic equibiaxial stretch is a direct stimulus for isolated adult rabbit cardiomyocytes to develop hypertrophy and to explore the potential involvement of the autocrine/paracrine factors ANG II, transforming growth factor (TGF)-β1, and IGF-I in this process. Isolated cardiomyocytes were exposed to 10% cyclic equibiaxial stretch (1 Hz) for up to 48 h or treated with ANG II (100 nM), TGF-β1 (5 ng/ml), IGF-I (100 ng/ml) ANG II type 1 (AT1) receptor blockers, or conditioned medium of stretched fibroblasts. Cyclic stretch significantly increased cell surface area (+3.1%), protein synthesis (+21%), and brain natriuretic peptide (BNP) mRNA expression (6-fold) in cardiomyocytes. TGF-β1 expression increased (+42%) transiently at 4 h, whereas cardiomyocyte IGF-I expression was not detectable under all experimental conditions. The AT1 receptor blockers candesartan and irbesartan (100 nM) did not prevent the stretch-induced hypertrophic response. Direct exposure to ANG II, TGF-β1, or IGF-I did not enhance cardiomyocyte BNP expression. In cardiac fibroblasts, stretch elicited a significant approximately twofold increase in TGF-β1 and IGF-I expression. Conditioned medium of stretched fibroblasts increased BNP expression in cardiomyocytes (~2-fold, P = 0.07). This study clearly indicates that cyclic stretch is a strong, direct trigger to induce hypertrophy in fully differentiated rabbit cardiomyocytes. The present findings do not support the notion that stretch-mediated hypertrophy of adult rabbit cardiomyocytes involves autocrine/paracrine actions of ANG II, TGF-β1, or IGF-I.

Chronic stretch induces cardiomyocyte hypertrophy, concomitant with the secretion of ANG II (33, 43). Exposure of quiescent cardiomyocytes to ANG II also induces hypertrophy (31), although the results are not unequivocal (16, 42). Furthermore, exposure of cardiomyocytes to transforming growth factor (TGF)-β1 (29, 46) or IGF-I (17) elicits a hypertrophic response.

Extrapolation of these findings to the intact adult heart in situ is not straightforward. First, previous experiments have been commonly performed on cardiomyocytes isolated from neonatal rat hearts. Neonatal cardiomyocytes are not fully differentiated (41) and still have a relatively high expression of fetal genes, such as β-myosin heavy chain (33). Moreover, hearts from rodents differ substantially from the human heart with respect to action potential duration, excitation-contraction coupling, and Ca2+ handling (23, 44) as well as the force-frequency relationship (15). Finally, static stretch, although generally applied in in vitro experiments, does not mimic the rhythmic changes in workload during systole and diastole of the heart in situ.

The aims of the present study were to investigate whether cyclic equibiaxial stretch in vitro is a direct stimulus for adult rabbit cardiomyocytes to develop hypertrophy and to determine to what extent the autocrine/paracrine actions of ANG II, TGF-β1, or IGF-I are involved in the hypertrophic response. Therefore, fully differentiated adult rabbit cardiomyocytes were either subjected to cyclic equibiaxial stretch or treated with ANG II, TGF-β1, IGF-I, and/or specific ANG II type 1 (AT1) receptor blockers. Changes in cell morphology, protein synthesis, and mRNA expression of the hypertrophic marker gene brain natriuretic protein (BNP) were assessed. Cardiomyocyte mRNA expression of atrial natriuretic factor (ANF), TGF-β1, and IGF-I was also determined. In addition, isolated adult rabbit heart fibroblasts were exposed to cyclic equibiaxial stretch, and TGF-β1 and IGF-I mRNA expression were measured. Finally, the effect of conditioned medium of stretched and nonstretched fibroblasts on cardiomyocyte BNP and ANF mRNA expression was assessed.

Materials and Methods

Animals. Cardiomyocytes and fibroblasts were isolated from adult male white New Zealand rabbits (~3 kg). Experiments were performed with approval of the Animal Ethical Committee of Maastricht University and conformed with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996).

Isolation of adult rabbit cardiomyocytes. Isolation of cardiomyocytes was performed according to modified Mitcheson procedures (27). Briefly, animals were anesthetized with ketamine (35 mg/kg, Eurovet Animal Health, Bladel, The Netherlands) and xylazine (5 mg/kg, Produlab Pharma, Raamsdonksveer, The Netherlands) via an intramuscular injection followed by the intravenous administration of 2,500 units of heparin (Leo Pharma, Breda, The Netherlands). Rabbits were sacrificed by cervical dislocation and the hearts were removed and placed in cold Tyrode solution containing 0.07 units/ml of heparin (Leo Pharma, Breda, The Netherlands).
were killed by cervical dislocation, and hearts were excised and rinsed in ice-cold modified Krebs-Henseleit buffer A (pH 7.4), which contained (in mM) 133 NaCl, 5 KCl, 2 MgCl₂, 1.2 Na₂HPO₄, 10 glucose, 10 HEPES, 6 taurine, and a low Ca²⁺ concentration (10 μM CaCl₂). After the removal of noncardiac tissue, the aorta was cannulated, and the heart was retrogradely perfused with oxygenated Krebs-Henseleit buffer A (25 mL/min) at 37°C for 10 min. Thereafter, the perfusion buffer was switched to a collagenase-containing digestion buffer consisting of 150 mL fresh Krebs-Henseleit buffer A supplemented with BSA [1% (wt/vol), fraction V, Roche Diagnostics, Mannheim, Germany] and 290 U/mL collagenase type II (Worthington, Lakewood, NJ). The digestion buffer (pH 7.4, 37°C) was oxygenated and recirculated for 8–10 min. Atrial and right ventricular tissue were then removed, and the left ventricle (LV) was incubated in Krebs-Henseleit buffer B (pH 7.4), which contained (in mM) 120 NaCl, 5 KCl, 2 MgCl₂, 1.2 Na₂HPO₄, 10 glucose, 10 HEPES, and 6 taurine supplemented with 10 μM CaCl₂ and 1% (wt/vol) BSA (fraction V) at room temperature for 10 min. Butanedione monoxime (20 mM) was added to prevent cardiomyocyte contraction. Thereafter, the LV was transferred to fresh Krebs-Henseleit buffer B at room temperature and cut in small pieces. Gentle shaking resulted in the complete dissociation of ventricular tissue. Dissociated cells were filtered through nylon gauze and allowed to recover at room temperature for 20 min. Thereafter, Ca²⁺ was gradually increased from 10 to 50, 75, 100, 150, 200, 400, 800, and 1,200 μM, with each step separated by 3 min. Cardiomyocytes were subsequently collected by a mild centrifugation step at 50 g at room temperature for 3 min. The supernatant was discarded, and cardiomyocytes were resuspended in 25 mL of “recovery medium,” which consisted of medium 199 (no. 31153, Gibco, Invitrogen, Breda, The Netherlands) supplemented with FBS [5% (vol/vol), Gibco], L-carnitine (final concentration: 2 mM), taurine (5 mM), penicillin (100 U/mL), streptomycin (100 mg/mL), and gentamicin (0.05 mg/mL). To prevent noncardiomyocyte proliferation, culture medium contained cytosine-β-d-arabinofuranoside ( AraC; 10 μM). Cells were counted using a Burker Turk chamber, and cell viability was assessed by trypan blue exclusion.

Isolation of adult rabbit heart fibroblasts. After collagenase perfusion, right ventricular tissue was dissected and cut in small pieces in DMEM (no. 22320, Gibco) supplemented with FBS [10% (vol/vol)] and gentamicin (0.05 mg/mL) (DMEM*). The suspension was shaken for 3 min to remove cardiomyocytes. Fibroblasts were collected from the supernatant by a subsequent centrifugation at 300 g at room temperature for 5 min. Pelleted fibroblasts were resuspended in DMEM* and cultured in 75-cm² plastic cell culture flasks in a humidified 95% air-5% CO₂ incubator at 37°C. After the removal of noncardiac tissue, the aorta was cannulated, and hearts were excised and rinsed in ice-cold modified Krebs-Henseleit buffer A (pH 7.4), which contained (in mM) 133 NaCl, 5 KCl, 2 MgCl₂, 1.2 Na₂HPO₄, 10 glucose, 10 HEPES, and 6 taurine supplemented with 10 μM CaCl₂ and 1% (wt/vol) BSA (fraction V) at room temperature for 10 min. Butanedione monoxime (20 mM) was added to prevent cardiomyocyte contraction. Thereafter, the LV was transferred to fresh Krebs-Henseleit buffer B at room temperature and cut in small pieces. Gentle shaking resulted in the complete dissociation of ventricular tissue. Dissociated cells were filtered through nylon gauze and allowed to recover at room temperature for 20 min. Thereafter, Ca²⁺ was gradually increased from 10 to 50, 75, 100, 150, 200, 400, 800, and 1,200 μM, with each step separated by 3 min. Cardiomyocytes were subsequently collected by a mild centrifugation step at 50 g at room temperature for 3 min. The supernatant was discarded, and cardiomyocytes were resuspended in 25 mL of “recovery medium,” which consisted of medium 199 (no. 31153, Gibco, Invitrogen, Breda, The Netherlands) supplemented with FBS [5% (vol/vol), Gibco], L-carnitine (final concentration: 2 mM), taurine (5 mM), penicillin (100 U/mL), streptomycin (100 mg/mL), and gentamicin (0.05 mg/mL). To prevent noncardiomyocyte proliferation, culture medium contained cytosine-β-d-arabinofuranoside (AraC; 10 μM). Cells were counted using a Burker Turk chamber, and cell viability was assessed by trypan blue exclusion.

Experimental protocols. Silicone Flexcell membranes (6-well Bioflex plates, precoted with collagen-I, Flexcell Dunn Labortechnik, Asbach, Germany) were coated with 15 μg/mL laminin (Invitrogen). Cardiomyocytes were plated at a density of 7.5 × 10⁴ cells/well on the coated Bioflex plates. Cells were cultured in a humidified 95% air-5% CO₂ incubator at 37°C. After 2–3 h, the medium was aspirated and refreshed to remove nonattached, nonviable cells. The next day, the recovery medium was refreshed once more. The day thereafter, 1 h before the experiment, the recovery medium was replaced with a comparable medium containing 0.5% FBS instead of 5% FBS ("experimental medium").

When fibroblast cultures were confluent (>90%, passage 0), cells were detached using 0.25% trypsin-EDTA (no. 25200, Gibco) and collected in 100 mL of "recovery medium" without AraC. Thereafter, 2 mL of the cell suspension were added per well of the collagen (type I)- and laminin-coated 6-well Bioflex plates. The next day, the recovery medium was aspirated and refreshed. The following day, fibroblasts were 50–70% confluent. One hour before the experiment, the recovery medium was replaced with 3 mL of experimental medium without AraC.

Cardiomyocytes and fibroblasts were subjected to 10% cyclic equibiaxial stretch, which was produced by a Flexcell FX-4000 strain unit (Dunn Labortechnik) with the computer-controlled application of sinusoidal negative pressure (1 Hz) for up to 48 h. Control, nonstretched cells were subjected to identical conditions; stretch was prevented using FlexStops (Dunn Labortechnik). In a subset of experiments, cardiomyocytes were cultured on collagen (type I)- and laminin-coated Silicone Flexcell membranes (6-well plates) and exposed to TGF-β₁ (5 ng/mL, R&D Systems, Minneapolis, MN), IGF-I (100 ng/mL, R&D Systems), ANG II (100 nM, Sigma, Zwijndrecht, The Netherlands) or preincubated with the AT₁ receptor blockers candesartan [100 nM, CV 11974 (the active form of candesartan), AstraZeneca, Zoetermeer, The Netherlands] or irbesartan (100 nM, Haorui Pharma-Chem, Edison, NJ) 30 min before the start of the stretch experiments. TGF-β₁, IGF-I, ANG II, and candesartan were dissolved in PBS, and irbesartan was dissolved in 1% ethanol (final ethanol concentration: 1%). Test experiments revealed that 1% ethanol did not affect the experimental outcome. With respect to ANG II, positive control tests were performed to explore the responsiveness of isolated rabbit cardiac fibroblasts and cardiomyocytes to ANG II doses at the time intervals applied in the present study. Exposure of fibroblasts and cardiomyocytes to 100 nM ANG II for 48 h resulted in a 35 ± 5% increase and a 51 ± 8% decline in collagen (type I) expression, respectively, clearly indicating that the cells were able to respond to a single dose of exogenous ANG II. Moreover, the mRNA expression of IGF-I was approximately twofold increased in isolated rabbit cardiac fibroblasts exposed to ANG II. Irbesartan (100 nM) completely blocked the ANG II-induced upregulation of IGF-I expression.

Fibroblast-conditioned medium. Stretched fibroblast-conditioned medium was collected after 48 h of cyclic stretch. Control fibroblast-conditioned medium was prepared from nonstretched fibroblasts and from stretched plates without cells ("control medium") after 48 h of incubation. Collected, pooled medium was stored at −20°C before being used. Cardiomyocytes were exposed to conditioned media for up to 48 h. Before application, the conditioned and control media were 1:1 diluted with experimental medium to a total volume of 2 mL.

RNA isolation and real-time quantitative PCR. After the experiment, cardiomyocytes and fibroblasts were washed twice with PBS, and total RNA was extracted with TRI Reagent (Sigma). RNA concentration and purity were determined using Nanodrop ND-1000 (Witec, Luzern, Switzerland). Total RNA (100 ng) was used for DNAse I treatment (Sigma) to remove contaminating genomic DNA and, subsequently, for cDNA synthesis (Iscript cDNA synthesis kit, Bio-Rad, Hercules, CA). Gene expression analysis was performed by real-time quantitative PCR on an iCycler Real-Time PCR detection system (Bio-Rad) using SYBR green supermix (Bio-Rad). Primer sets are shown in Table 1. Gene expression differences were normalized to the reference gene cyclophilin-A.

[^H]leucine incorporation. Cardiomyocytes were exposed to [¹⁴C]leucine (1 μCi/mL, GE Healthcare, Amersham, Little Chalfont, UK) for 48 h. Thereafter, cardiomyocytes were washed twice with ice-cold PBS, and proteins were precipitated with 4% trichloroacetic acid at 4°C for 60 min. Proteins were subsequently scraped from the plate and concentrated by centrifugation (10,000 g, 4°C, 15 min). Pellets were dissolved in 0.5 M NaOH for liquid scintillation counting.

Cardiomyocyte dimensions. Phase-contrast micrographs were taken using a digital Nikon-Coolpix-990 camera. Cell width and length were measured using ImageJ software (~150 randomly chosen cells/well, NIH, Bethesda, MD). Cell surface area (in μm²) was calculated by multiplying cell length and cell width.

Statistical analysis. Measurements were performed on cardiomyocytes and fibroblasts from three independent isolations, respectively, with two or three wells per isolation. For each set of control wells per isolation, the mean was set to 1.0; subsequently, the individual values

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obtained in control and time-matched treated wells, respectively, were normalized to the mean of the control values. Thereafter, the normalized values obtained in the six or nine individual wells were expressed as means ± SE. Finally, data were analyzed by a two-tailed Student t-test to compare differences evoked by stretch or treatment with growth factors or blockers. P values of <0.05 were considered statistically significant.

RESULTS

Yield of cardiomyocyte isolation and cell viability. The present procedures, modified from previously published protocol of Mitcheson and colleagues (27), resulted in a relatively high yield of adult rabbit cardiomyocytes, i.e., 20–25 million cells per LV per isolation. Commonly, 70–80% of the freshly isolated cardiomyocytes were viable, Ca²⁺ tolerant, and rod shaped. After being seeded on the silicone Flexcell membranes, 96 ± 3% of the cardiomyocytes remained attached during 48 h of preincubation. The percentages of cells that remained attached to the silicone membrane subjected to nonstretch or cyclic stretch (10% cyclic equibiaxial stretch at 1 Hz) for another 48 h averaged 90% and 77%, respectively. Representative photographs of cardiomyocytes attached to Flexcell membranes after 48 h of rest or cyclic stretch are shown in Fig. 1, A–D.

Stretch-induced cardiomyocyte hypertrophy and protein synthesis. The width, length, and surface area of nonstretched control cardiomyocytes amounted to 17.7 ± 0.3 μm, 118.0 ± 1.2 μm, and 2.09 ± 0.05 μm² × 10³, respectively. Cyclic stretch (10%) for 48 h resulted in a significant increase (3.1%) in cardiomyocyte surface area (Fig. 2A). The increase in surface area was predominantly caused by the increased cell width (data not shown). Cyclic stretch also significantly increased (+21%) protein synthesis (Fig. 2B). These findings indicate that cyclic stretch is a potent and direct trigger to elicit a hypertrophic response in isolated adult rabbit cardiomyocytes.

Stretch-induced alterations in cardiomyocyte gene expression. Expression of the hypertrophy marker BNP was significantly increased (6-fold) after 48 h of stretch (Fig. 2C). BNP expression showed a moderate (+55%) increase at 24 h of cyclic stretch (data not shown). Cyclic stretch resulted in a transient increase (+47% at 4 h) in ANF mRNA expression. No changes in ANF mRNA expression were observed after 48 h of cyclic stretch (data not shown). Stretch only transiently increased TGF-β₁.
expression (42%) at 4 h (Fig. 3). IGF-I mRNA expression in cardiomyocytes remained undetectable (data not shown).

**Effect of ANG II and AT1 receptor blockers on cardiomyocyte dimensions and gene expression.** In contrast to cyclic stretch, treatment of quiescent rabbit cardiomyocytes with ANG II for 48 h did not significantly increase cardiomyocyte surface area (Fig. 4A, ANG II and stretch results). Stretch of cardiomyocytes in the presence of the bona fide AT1 receptor blockers candesartan and irbesartan resulted in almost identical percent increases in cell surface area as stretch in the absence of AT1 receptor blockers, strongly suggesting that AT1 receptor blockade fails to prevent the stretch-induced hypertrophic response (Fig. 4A). Treatment of cardiomyocytes with ANG II did not alter BNP mRNA expression. As previously shown, cyclic stretch substantially increases the mRNA expression of the hypertrophy marker BNP (Fig. 4B, ANG II and stretch results). Treatment of cardiomyocytes with the AT1 receptor blockers candesartan and irbesartan failed to mitigate the expression of BNP during stretch conditions (Fig. 4B). ANF mRNA expression was not altered in ANG II-treated adult rabbit cardiomyocytes, nor did the two AT1 receptor blockers affect ANF mRNA expression (data not shown).

**Stretch-induced alterations in gene expression in isolated fibroblasts.** To explore a potential paracrine role of fibroblast-derived growth factors in cardiomyocyte hypertrophy, cyclic stretch was also applied on adult rabbit cardiac fibroblasts. Exposure of fibroblasts to cyclic stretch for 48 h significantly increased TGF-β1 and IGF-I expression, by 60% and 100%, respectively (Fig. 5, A and B).
Effect of TGF-β1, IGF-I, and fibroblast-conditioned medium on cardiomyocyte gene expression. Neither 4 nor 48 h of TGF-β1 or IGF-I treatment evoked cardiomyocyte BNP mRNA expression (Fig. 6). Exposure of adult rabbit cardiomyocytes to TGF-β1 or IGF-I resulted in a small and transient increase in ANF mRNA expression of cardiomyocytes, by ~30% at 4 h (P < 0.05; data not shown). Treatment of cardiomyocytes with the conditioned medium of stretched fibroblasts resulted in an approximately twofold (P = 0.07) increase in cardiomyocyte BNP mRNA expression at 48 h. Control medium and conditioned medium of nonstretched fibroblasts did not alter cardiomyocyte BNP mRNA expression (Fig. 7). Similar treatments had no effect on cardiomyocyte ANF mRNA expression (data not shown).

Fig. 5. Effect of cyclic stretch for 4 and 48 h on the mRNA expression of TGF-β1 (A) and IGF-I (B) in adult rabbit cardiac fibroblasts, respectively. mRNA levels of TGF-β1 or IGF-I in stretched fibroblasts (n = 6) are expressed relative to those of nonstretched, time-matched control cardiac fibroblasts (n = 6). *P < 0.05 vs. the corresponding time-matched controls.

Fig. 6. Effect of 4 and 48 h of exposure to TGF-β1 (n = 9) or IGF-I (n = 9) on cardiomyocyte BNP mRNA expression. mRNA levels of treated cardiomyocytes are expressed relative to those of time-matched control cardiomyocytes (n = 9). Treatment with growth factors failed to induce a significant effect.

Fig. 7. Effect of 4 or 48 h of exposure of adult rabbit cardiomyocytes to conditioned medium from nonstretched (CM-NS) or stretched (CM-S) adult rabbit fibroblasts on BNP mRNA expression. mRNA levels of cardiomyocytes treated with conditioned medium from either 48-h nonstretched fibroblasts (n = 6) or stretched fibroblasts (n = 6) are expressed relative to corresponding time-matched control cardiomyocytes, which were treated with control medium (M; n = 6). $P = 0.07$ compared with the corresponding time-matched control.

DISCUSSION

Stretch-induced cardiomyocyte hypertrophy. The present study shows that cyclic stretch is a potent, direct trigger to induce hypertrophy in fully differentiated cardiomyocytes derived from the adult rabbit heart. This conclusion is based on the observations that cellular surface area is increased, protein synthesis enhanced, and BNP mRNA expression upregulated when cardiomyocytes are exposed to 48 h of cyclic stretch in vitro. Indications are lacking that the hypertrophic response is caused by direct autocrine and/or paracrine actions of ANG II, as treatment of cardiomyocytes with specific AT1 receptor blockers did not abrogate the stretch-induced hypertrophic response and exposure of adult rabbit cardiomyocytes to ANG II did not evoke enhanced expression of the molecular hypertrophy marker BNP. Moreover, a possible autocrine/paracrine action of TGF-β1 and IGF-I in stretch-induced hypertrophy of rabbit cardiomyocytes is also less likely since exposure of cardiomyocytes to these two growth factors did not result in increased BNP mRNA expression. Furthermore, cyclic stretch did not elicit enhanced cardiomyocyte expression of IGF-I, and there was only a small, transient induction of TGF-β1 mRNA expression during 4 h of stretch. We cannot exclude that in addition to a direct effect of stretch on cardiomyocyte hypertrophy, an as-yet-unidentified growth factor released from fibroblasts is able also to exert a hypertrophic effect on the adult cardiomyocyte, because the conditioned medium from cardiac fibroblasts exposed to 48 h of cyclic stretch evoked an approximately twofold increase in cardiomyocyte BNP expression.

Throughout the investigation, mRNA expression of BNP, rather than ANF, was considered a bona fide and sensitive molecular marker for cardiomyocyte hypertrophy. This notion corroborates previous studies (3, 25) in rabbit hearts showing the superiority of BNP in diagnosing overload-induced LV dysfunction. Comparable conclusions were drawn in studies
Ang II is involved in the transition from cardiac hypertrophy (30), which was supported by experimental observations (42), and the responsiveness to ANG II during the process of maturation has been extensively investigated by Liu and coworkers (24). They have shown that cell width and cell thickness increase to the same extent, implying that the enlarged cell surface area indeed reflects an increase in cell volume.

**Role of ANG II and AT1 receptors in stretch-induced cardiomyocyte hypertrophy.** Although previous studies (29, 34) have indicated that ANG II could act as an autocrine or paracrine factor in stretch-induced hypertrophy of cardiomyocytes, the present study failed to reveal these actions of ANG II. Exposure of quiescent cardiomyocytes to ANG II did not increase cellular dimensions or BNP expression (Figs. 4, A and B, respectively). Theoretically, the lack of effect could be due to the rapid degradation of ANG II supplemented to the incubation medium. However, Van Kesteren and colleagues (42) measured the half-life time of ANG II after its addition to the incubation medium of isolated cardiomyocytes. The half-life time amounted to 7 h. The corollary of their finding is that, in our study, during the first 24 h, the concentration of exogenous ANG II dropped to 10 nmol/l, which was still sufficiently high to evoke a response, if any. Moreover, it can be deduced from the fact that ANG II at the applied dose and time intervals affected collagen (type I) mRNA levels in both cardiac fibroblasts and cardiomyocytes (see MATERIALS AND METHODS) that adult rabbit cardiomyocytes are indeed intrinsically able to respond to this growth factor but that ANG II did not elicit a hypertrophic growth response. The main difference between the present experimental setup and previous ones is that in the past, experiments were mainly performed on immortalized H9C2 cell lines (38) or on cultured neonatal cardiomyocytes, predominantly from the rat origin. The contrasting properties of neonatal and adult cardiomyocytes are substantiated by experiments showing that cardiomyocytes reduce their responsiveness to ANG II during the process of maturation (31). A critical autocrine role of ANG II in the cardiomyocyte hypertrophic response has also been disputed in a recent review (30), which was supported by experimental observations (42, 48). Moreover, Wenzel and colleagues (47) suggested that ANG II is involved in the transition from cardiac hypertrophy to failure rather than in the primary hypertrophic process.

The observation that the AT1 receptor blockers candesartan and irbesartan failed to inhibit stretch-induced cardiomyocyte hypertrophy also rules out the possibility that cardiac hypertrophy is caused by the direct stretch activation of AT1 receptors. This conclusion contrasts with previous findings of Zou and colleagues (52), who showed that stretch imposed on wild-type rat neonatal cardiomyocytes and on adult mouse cardiomyocytes lacking the angiotensinogen gene induced cardiomyocyte hypertrophy via the activation of AT1 receptors in the absence of ANG II and that candesartan effectively blocked the hypertrophic response. A later study performed by the same investigators indicated that stretch-induced conformational changes of the receptor elicited this hypertrophic response. Candesartan maintained the receptor in its inactive conformation (50). The apparent discrepancy between these findings (50, 52) and our results could be explained by species differences. Our findings do not, however, exclude an intermediary role of ANG II in stretch-induced cardiac hypertrophy. For instance, stretch of cardiomyocytes in situ may increase their endogenous production and release of ANG II, which, in turn, may prompt the release of other growth factors from noncardiomyocytes in the intact heart (14, 35). Theoretically, these growth factors may modulate the stretch-induced hypertrophic response of the challenged cardiomyocytes.

**Potential autocrine or paracrine actions of TGF-β1 and IGF-I in rabbit cardiomyocyte hypertrophy.** With respect to the potential role of other growth factors, stretch induced a small but significant increase in TGF-β1 expression in the adult rabbit cardiomyocyte (Fig. 3), whereas stretch of cardiac fibroblasts resulted in a substantially enhanced expression of this growth factor (Fig. 5A). However, since direct exposure of the cardiomyocytes to exogenous TGF-β1 for 4 and 48 h did not result in enhanced BNP mRNA expression (Fig. 6) and evoked only a minor and transient increase in ANF expression, it is unlikely that TGF-β1 plays an autocrine or paracrine role in the hypertrophic response of stretched adult rabbit cardiomyocytes.

The autocrine involvement of IGF-I in the hypertrophic process is even less likely, because stretch of cardiomyocytes did not result in a measurable increase in IGF-I mRNA expression. Although stretched fibroblasts increased significantly their expression of IGF-1, no convincing proof was obtained for a paracrine role as direct exposure of adult rabbit cardiomyocytes to IGF-I for 4 and 48 h did not elicit a hypertrophic response in terms of enhanced BNP expression (Fig. 6).

In contrast to our findings, previous studies (5, 11) have indicated that TGF-β1 and IGF-I may act as potent trophic factors on cardiomyocytes. The discrepancy between previous observations and ours is most likely due to inherent differences between neonatal and adult cardiomyocytes in addition to species differences, i.e., the rat versus the rabbit. Terminally differentiated cardiomyocytes are known to respond differently to IGF-I than neonatal cardiomyocytes (9). Furthermore, the IGF-I mediated hypertrophic response is associated with activation of the phosphatidylinositol 3-kinase/Akt/PI3K signaling pathway, which is linked to cardiomyocyte survival, regeneration, and physiological hypertrophy rather than to maladaptive, pathological hypertrophy (8, 36). The latter might explain the absence of substantially enhanced BNP mRNA expression in adult rabbit cardiomyocytes exposed to IGF-I, because increased expression of this marker gene is associated with maladaptive hypertrophy (49).

**Additional role for fibroblasts in stretch-induced cardiomyocyte hypertrophy.** Despite the lack of convincing evidence for a paracrine action of TGF-β1, IGF-I, and ANG II, it should be emphasized that exposure of cardiomyocytes for 48 h to the conditioned medium of stretched cardiac rabbit fibroblasts induced a borderline significant approximately twofold increase in BNP mRNA expression (Fig. 7). This finding might infer the presence of as-yet-identified factors released by stretched fibroblasts, adding to the direct hypertrophic effect of stretch on cardiomyocytes. It cannot be excluded that the hypertrophic effect of conditioned media of stretched cardiac...
fibroblasts, as observed in the present experimental setup, is underestimated as a consequence of dilution in the incubation medium in contrast to the in situ situation in the cardiac interstitial compartment. The present study also does not exclude that other cell types, such as endothelial cells and smooth muscle cells, could produce paracrine factors in the mechanically challenged heart in situ (7, 37).

**Potential mechanisms of stretch-induced cardiomyocyte hypertrophy.** It should be emphasized that the present findings strongly suggest that cyclic stretch exerts a direct effect on the adult cardiomyocyte resulting in a hypertrophic response. As the underlying mechanisms were not studied, one may speculate about the identity of the stretch sensors and nature of the signaling pathways involved. Regarding the stretch sensors, several models have been put forward to explain the sensing of changes in stretch of cardiac muscle cells (4, 21). Stretch-activated ion channels in the sarcolemma are potential candidates for mechanosensing by modulating the intracellular Ca^{2+} concentration. In addition, integrins and associated proteins, such as melusin and integrin-linked kinase, have been postulated to transmit the mechanical signal to the cytoskeleton. Moreover, sarcolemmal non-receptor-type tyrosine kinases have been linked to stretch sensing. Furthermore, Z-disc proteins, such as titin and related compounds, have been shown to act as molecular structures sensing cardiomyocyte deformation (18, 21, 22). A plethora of intracellular signaling pathways has been proposed to transmit the mechanical signal, sensed by the stretch sensor, to the nucleus of the challenged cardiomyocyte, eventually resulting in enhanced protein synthesis, increased cell volume, and the expression of specific molecular markers of cardiomyocyte hypertrophy (1, 16, 18, 22, 34).

**Summary.** The collective findings indicate that sustained cyclic stretch is a potent, direct trigger to evoke a hypertrophic response in isolated adult rabbit cardiomyocytes. In the present experimental setup, no strong indications were found for autocrine and/or paracrine effects of ANG II, TGF-β1, and IGF-I on cardiomyocyte hypertrophy. The moderate, borderline significant hypertrophic effect of the conditioned medium of stretched fibroblasts suggests that certain biochemical factors secreted by fibroblasts might play an (at most) additional role in the induction of cardiomyocyte hypertrophy. It is of note that our findings were obtained in experiments performed on isolated adult rabbit cardiomyocytes instead of commonly used immortalized cardiac muscle cell lines or cultures of neonatal cardiomyocytes (12, 32, 34, 43). This crucial difference in experimental design, in addition to cyclic rather than static stretch as a mechanical trigger, might explain the deviant findings emerging from the present study with respect to the role of growth factors such as ANG II, TGF-β1, and IGF-I in the onset of cardiac hypertrophy. Adult rabbit cardiomyocytes are considered to be a more appropriate cardiomyocyte preparation when attempting to extrapolate the experimental findings to the human heart. This notion is based on the fact that adult rabbit cardiomyocytes are fully differentiated and differ from cardiomyocytes obtained from small rodents with respect to action potential duration, excitation-contraction coupling, Ca^{2+} handling, and the force-frequency relationship (15, 23, 44).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**REFERENCES**


