Induction of hypertrophy in vitro by mechanical loading in adult rabbit myocardium

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Bupha-Intr T, Holmes JW, Janssen PM. Induction of hypertrophy in vitro by mechanical loading in adult rabbit myocardium. Am J Physiol Heart Circ Physiol 293:H3759–H3767, 2007. First published October 12, 2007; doi:10.1152/ajpheart.01267.2006.—To study myocardial hypertrophy under in vitro conditions, we developed an experimental system and protocol in which mechanical conditions of isolated multicellular myocardium can be controlled while function can be continuously assessed. This in vitro culture system now allows us to investigate how mechanical overload impacts on cardiac hypertrophy in the absence of systemic factors. In this system, small right ventricular rabbit trabeculae were subjected to different modes of mechanical load, while being electrically stimulated to contract at 1 Hz at 37°C. Muscles subjected to prolonged isometric contractions at high, but physiological, pre- and afterload showed a rapid induction of cardiac hypertrophy; overall muscle diameter increased by 4.3 ± 1.4 and 17.9 ± 4.0% after 24 and 48 h, respectively. This finding was confirmed at the cellular level; individual myocyte width significantly increased after 24 and 48 h. In muscles subjected to a low preload, or in the absence of afterload, this hypertrophic response was absent. Functionally, after 24 h of isometric contractions at high load, active developed tension had gradually increased to 168 ± 22% of starting values. Proteomic analysis of this cultured myocardium demonstrated reproducible changes in the protein expression pattern and included an upregulation of myofilament proteins, myosin light chain isoforms, α-b crystalline, and breast cancer 1 protein, and a downregulation of myoglobin. We conclude that multicellular myocardium can be stressed to undergo rapid hypertrophy in vitro, and changes in function and protein expression can be investigated during the transition from healthy myocardium to early hypertrophy.

trabeculae; remodeling; myocyte

CARDIAC HYPERTROPHY HAS BEEN shown to develop in a very short period of time under extreme conditions. In the Burmese python, cardiac mass can increase by >50% within 48 h after eating a large meal (3). Also, upon an in vitro stretch of isolated cardiomyocytes, new sarcomeres can be formed within hours of application of a high load (28). Still, it is unknown whether ultrarapid hypertrophy can occur in adult mammalian myocardium and whether this would reflect either a physiological or a pathophysiological remodeling event. Consequently, being able to assess molecular alterations that occur during the very early developmental stages of cardiac hypertrophy will provide us novel information to help better understand the pathological development of heart failure. Over the last decades, many studies have shown changes in molecular expression patterns after pressure overload-induced cardiac hypertrophy in animal models (8, 13, 22, 25, 42), as well as in patients suffering from cardiac hypertrophy and failure (24). Since the investigation of most models is done at a single time point during the disease process, obtained information combines both primary activation and compensatory processes, and the inability to distinguish between primary and compensatory effects has hampered the development of effective treatments. To understand the initial mechanisms responsible for cardiac hypertrophy, information from the earliest stages is, therefore, imperative in making discriminations between the primary and compensatory changes. However, an in vitro experimental model that would allow continuous, time-resolved investigation of healthy adult myocardium as it transitions into hypertrophic myocardium is currently lacking.

Load-induced cardiac hypertrophy involves multiple processes, including enhanced systemic and local neuroendocrine stimulation, as well as altered mechanical tension. Increases in the neuroendocrine stimulation by pressure overload have been established in vivo experiments (1, 33), and hypertrophic activity has been confirmed in cultured cardiac myocytes (38, 40, 44). The effect of increased mechanical load on the heart alone is, however, incompletely understood, as it cannot be easily uncoupled from the systemic neuroendocrine effects that are present in vivo. In the recent past, several in vitro attempts have been made to provide a system in which load-induced hypertrophy can be investigated. Cultured neonatal cardiac myocytes seeded on stretchable silicon backgrounds (35, 37, 40, 48) or engineering cardiac tissue (14) have been used to determine mechanical loading effects. Although these experiments have aided in the understanding of load-induced hypertrophy, neonatal cardiac myocytes have important differences in phenotype and growth response compared with adult cardiac myocytes (43). In addition to this limitation, proteomic investigations revealed that cells maintained in culture respond by alteration in their pattern of gene and protein expressions (29). Both lack of electrical stimulation as well as lack or incompleteness of cell-to-cell communication have been speculated to be at the basis of these changes (4, 47, 49). Electrical stimulation helps maintain the normal contractile function of culture-isolated cardiac myocytes (6) and maintains normal excitation-transcription coupling in those adult cardiac myocytes. Thus, to maintain the normal pattern of gene and protein expression in isolated cardiac myocytes, many investigations performed the culture of isolated cardiac myocytes under conditions of continuous electrical stimulation (12, 15). However, absence of normal communication to neighboring cells is another possible factor of different gene expression; HeLa cells

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transfected with the connexin 43 gene become more resistant to spontaneous, as well as chemically induced, genetic changes (49). Thus not only continuous electrical stimulation, but also cell-to-cell connections are deemed essential to preserve myocardial function in vitro.

We thus hypothesize that, if we keep isolated adult multicellular tissue in culture and electrically stimulate these multicellular preparations, we should be able to study load-induced hypertrophy in an in vitro setting, in the absence of systemic neuroendocrine intervention. This would allow for setting pre- and afterload conditions that are close to in vivo and possibly allow for determination of changes in morphology and protein expression. As a starting point of developmental studies, we used the multicellular myocardial culture system (20, 21) to characterize the effects of mechanical loading on physiological parameters and protein alteration. The results indicate that, using loaded intact contracting muscle preparations, we can indeed evoke rapid changes in the morphological level (increase in muscle mass), functional level (changes in contractile parameters), and changes in the protein expression pattern. The observed changes critically depend on the loading conditions and can be followed over time in adult myocardium.

MATERIALS AND METHODS

Multicellular myocardial culture. The cardiac trabecula culture system and protocol have been detailed previously (21). Briefly, New Zealand White rabbits (1.5–2.0 kg) were heparinized and anesthetized by infusion of pentobarbital sodium (50 mg/kg). The hearts were rapidly excised and retrogradely perfused in a modified Langendorff perfusion system with 2,3-butanedione monoxime (20 mM) containing low-calcium Krebs-Henseleit solution. Nonbranched trabeculae (average width 200 μm, average length 3 mm) from the free wall of the right ventricle were carefully dissected and mounted between the force transducer and a micromanipulator screw in the closed-circuit culture system (21). The solution was exchanged for Krebs-Henseleit buffer without 2,3-butanedione monoxime, and the muscles were electrically stimulated at 1 Hz. All of the animals were handled according to approved protocols of The Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

Experimental protocol. The solution was replaced for a modified cell culture medium (M199, Sigma) containing the following additions (in mM): 2.0 L-carnitine, 0.5 creatine, 5.0 taurine, 2.0 L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Extracellular Ca2+ concentration is equivalent to 1.75 mM. All muscles were initially mounted in the setup at a length without preload or afterload. After equilibration, the muscles were subjected to two loading conditions. In the first group, muscles were kept at extremely low preload (<1 mN/mm²) and experienced a moderate afterload during the isometric stimulation. The second group mimicked high preload by stretching to a passive tension of ~5–8 mN/mm², which was valued up to sarcomere lengths of ~2.2 μm, as previously described (26, 36). Contractile parameters, including relaxation kinetics, were continuously monitored for 48 h and stored digitally. A digital camera mounted onto a microscope (2-μm resolution) above the experimental chamber was used to capture the width of the muscles at various time points during the culture period. In high-load muscles, only trabeculae that showed an increase in developed force above the beginning value were selected. In addition, high-load muscles that developed force lower than 5 mN/mm² at 48 h were discarded.

Myocyte dimensions. After 24 or 48 h of culture, muscles were then fixed in 10% formalin for 48 h. For control, freshly isolated trabeculae were stretched to an identical preload level before fixation. To avoid assessment of potentially damaged ends, only the middle part of the fixed trabecula was rinsed in physiological salt buffer and incubated in 12.5 M KOH solution for 24 h, as previously described (45). Thereafter, myocytes were mechanically dissociated and imaged at ×300 magnification using light microscopy. Width and length of a large number of myocytes per studied muscle preparation were calculated using standard image analysis software. In addition to width and length, average sarcomere length of each myocyte was calculated using the repetitive striated sarcomere pattern.

Fig. 1. A: sample pictures of one single-rabbit cardiac trabeculae in the culture chamber before and after 48 h of high-load muscle culture. T₀, time 0. B: bar graph shows 24- and 48-h high load significantly increase in muscle diameter. Values are means ± SE of muscles from 48-h low-load (48-Low, n = 6), 24-h high-load (24-High, n = 8), 48-h high-load (48-High, n = 14), 48-h high-load without electrical stimuli (48-NoPace, n = 5), and 48-h nonfunction muscles (48-Death; n = 3). *P < 0.05, before vs. after treatment, by paired T-test.
group) were chosen for the protein identification. The sample spots were transferred to the MassPrep Station for automated in-gel protein digestion, following the protocol included with the Win PREP Multi-probe II software. Mass spectra of the digested peptide were assessed using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer in reflection mode. Resulting peptides were matched with their corresponding proteins with the ProFound database. Immunoblot analysis was performed using a standard protocol. Anti-sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) 2 antibody (1:2,000) and calsequestrin (1:5,000) were obtained from ABR, and anti-Na\(^{+}i\)/Ca\(^{2+}\) exchanger (NCX; 1:4,000) from Swant. Anti-cardiac troponin I (cTnI) (1:1,000) was purchased from Abnova. Amount of total protein loaded was 50 \(\mu\)g for SERCA, NCX, calsequestrin, GAPDH, and Egr-1, and 25 \(\mu\)g for TnT.

Statistical analysis. Data are presented as means \(\pm\) SE. Forces were normalized based on their dimension at initiation of culture and were not corrected during the experiment. Where applicable, a two-tailed paired \(t\)-test was used to determine the significance before and after treatment. One-way ANOVA was used for statistical analysis followed by Bonferroni’s test for multiple comparisons. \(P < 0.05\) was considered significant.

RESULTS

**High preload contractions induce hypertrophy in vitro.** To observe whether mechanical loading could induce a rapid hypertrophy and/or functional changes in mammalian myocardium, cardiac trabeculae were subjected to different pre- and afterload conditions at 1-Hz electrical stimulation (37°C, pH 7.4) for up to 48 h. Muscle diameters were compared before and after the experiments to assess overall cardiac hypertrophy (Fig. 1). In the high preload group, we observed a significant increase in muscle diameter by 4.3 \(\pm\) 1.4% \((n = 8, P < 0.05)\) and 17.9 \(\pm\) 4.0% \((n = 13, P < 0.01)\) of the initial diameter at 24 and 48 h, respectively. In sharp contrast, muscles in the low-load group did not change significantly in muscle diameter (101.6 \(\pm\) 2.3% of initial size, \(n = 6\)). To determine whether only mechanical pre-loading without muscle contraction can induce muscle hypertrophy, a group of high preloaded muscles was cultured without any electrical stimulation and thus experienced a high preload, but no afterload. After 48 h, survival of the muscles in this group was confirmed by the presence of normal levels of contractions in response to a brief electrical stimulation period right before the assessment of diameter. Interestingly, despite an identical level of preload as the high-load group, no significant change in the muscle dimension was found in this group (97.9 \(\pm\) 4.8%, \(n = 5\)). This result underlined that electrical-activated contractile motivation has profound effects on the morphological phenotype. In contrast to all of the successful experiments, we observed a decrease in muscle diameter (90.8 \(\pm\) 4.3%, \(n = 3\)) in nonfunctioning trabeculae (e.g., nonsurviving muscles) at 48 h.

![Fig. 2. Comparison of myocyte dimensions from fresh control trabeculae (Cont, \(n = 221\), 24-High \((n = 220)\), and 48-High \((n = 305)\). A: myocyte width; B: myocyte length; C: length-to-width ratio; D: surface area. Values are means \(\pm\) SE of myocytes from 5–6 muscles in each group. *\(P < 0.05\) vs. control by ANOVA.](http://ajpheart.physiology.org/)

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To confirm that high load induced hypertrophy at the cellular level, and as a second test to exclude effects of edema on muscle diameter assessment, width and length of individual isolated myocytes were measured (Fig. 2). After 24 h of high load, we observed significant increases in both cell width ($13.1 \pm 0.1 \mu m$) and length ($73.5 \pm 1.1 \mu m, n = 220$ myocytes from 5 muscles) compared with controls (width $12.2 \pm 0.1 \mu m$, length $69.9 \pm 1.1 \mu m, n = 221$ myocytes from 6 muscles). After a prolonged culture of 48 h where force development is declined vs. the 24-h values, a significant increase in cell width remained ($12.9 \pm 0.1 \mu m$), but not in cell length ($68.6 \pm 0.9 \mu m, n = 305$ myocytes from 5 muscles). These results underline that mechanical load can cause accelerated myocyte hypertrophy. Analysis of the individual myocyte images further revealed that the sarcomere length was identical in all groups ($1.6 \mu m$); thus a change in length directly indicates a change in the number of sarcomeres. Myocyte hypertrophy is consistent with a preliminary report by the group of Dr. Holmes (17), who reported rat myocytes isolated from stretched cultured rat papillary muscles were significantly larger than those in controls.

Mechanical loading alters active force development. Muscles with high pre- and afterload ("high-load") showed dramatic changes in developed force over time (Fig. 3A) compared with muscle with low preload (low-load). High-load conditions decreased contractile force immediately after initiation of culture. However, a couple of hours later (~5 h after initiation of culture), a slow, but continuous, and eventually substantial increase in developed force was observed. Developed force achieved a maximum at ~20–26 h after initiation of culture. Average developed force measured at 24 h was $168 \pm 22\%$ from starting values ($P < 0.01$). Thereafter, force started to progressively decline until the end of culture (48 h typically). Changes in time to peak force and time to 90% of relaxation were similar between muscles with a high preload and muscle with low load (Fig. 3, B and C). Time to peak force gradually increased after initiation, and, after 12 h, time to peak force kept constant until the end of the protocol in both groups.

Fig. 3. A: active developed force of cultured cardiac trabeculae, contracting once per second, continuously for 48 h at high pre- and afterload (○; high-load; $n = 12$) vs. no preload, but experienced afterload (●; low-load; $n = 5$). B: time to peak force. C: 90% of relaxation time (RT$_{90}$) changes slightly over time at the end. D: changes in diastolic force (●) overtime of high-load muscle. E: patterns of active developed force were compared between muscles cultured in media with penicillin/streptomycin (○, $n = 4$) and ampicillin (●, $n = 7$).
Passive force slightly decreased over time (Fig. 3D), but significantly increased after 40 h of culture.

In addition to increased sets of experiments, penicillin/streptomycin was replaced by 100 mg/l ampicillin to determine any effect of streptomycin, an aminoglycoside, on the stretch-activated ion channel (5). The patterns of force development between penicillin/streptomycin and ampicillin cultured medium were, however, not different, indicating that the observed effects were not specifically due to the presence of penicillin/streptomycin (Fig. 3E). Time at which peak force development was reached did not differ between the groups (30.6 ± 1.6 vs. 32.8 ± 1.7 h for penicillin/streptomycin vs. ampicillin response, \( P = 0.38 \)).

Proteomic analysis of high preload trabeculae. To determine the effect of mechanical load on molecular alterations after initiation of cardiac hypertrophy, protein expression was compared using two-dimensional electrophoresis (Fig. 4). A comprehensive study of the entire proteome was deemed well beyond the scope of this pioneering study at the present time. However, for proof of principle to test whether it is feasible to study the proteome in these small hypertrophied cultured muscles, we set out to detect reproducible changes in the protein expression pattern between high-preload muscles and controls. Results of matrix-assisted laser desorption/ionization time-of-flight analysis were matched with their corresponding proteins with the “ProFound” database, as shown in Table 1. Increases or posttranslational modification in ventricular myosin light chain isoforms, breast cancer 1 (BRCA1), and α-b crystallin were a few of the most obvious changes between the high and no-load groups using this analysis. Alterations in expression level of these proteins are in close accordance to those reported in various in vivo hypertrophy models (13, 22–24). Increases in immunoglobulin V lambda and arachidonate 12-lipoxygenase in high-loaded muscle were noticed of cardiac pathological hypertrophy (11, 32). There was also a decreased myoglobin expression in muscle with high preload compared with control muscle. This change in myoglobin likely indicated the alteration of cellular oxygen utility (30).

### Table 1. Forty-eight-hour-loaded changes of the cardiac trabeculae proteome

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<th>Protein Information and Sequence Analysis</th>
<th>Percent*</th>
<th>pI</th>
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<td>MYG_RABIT myoglobin</td>
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*Percent Coverage Profound = amount of protein sequence that was covered by the mass.

**Fig. 4.** Two-dimensional protein profiles from control (A) and high-load (B) cultured cardiac trabeculae. pI, Isoelectric point. Detailed information on the labeled protein spots is given in Table 1.

Protein identification is given after in-gel digest with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Expected molecular mass is shown in kilodaltons (kDa). Isoelectric point (pI) is according to the ProFound database. SSP, standard spot.
higher compared with calsequestrin (Fig. 5D). We detected a slight increase in Egr-1 in high-load muscle in 24 h, but barely detected one after 48 h (Fig. 6). Three out of four samples increased in Egr-1 expression by 11, 67, and 75% at 24 h, and one sample decreased by 25%. Although we observed substantial variability in our protein expression data, this could be due to the fact that the expression of many of these protein changes in a temporal fashion. In a related study, we observed that, for instance, connexin-43 was initially being upregulated and, thereafter, decays. Thus, when looking at an intermediate time point, in some muscles it may (still) be up compared with control, whereas in other it is (already) down. Future studies may address this issue by examining expression at multiple time points to investigate the temporal resolution of some of our observed changes.

DISCUSSION

Mechanical loading is a potent physical factor that can enhance or impair cardiac contraction and relaxation. We aimed to develop a system/protocol that allows the effects of mechanical loading to be studied in an in vitro, adult multicellular system. The data presented here show that multicellular adult myocardium can undergo rapid hypertrophy in vitro when stimulated to contract under high-loading conditions.

This experimental model allows for a continuous assessment of contractile force and kinetics, under near physiologically relevant conditions. In addition, the model also allows quantifying of morphology features and allows for quantification of protein expression patterns. Thus we here provide an in vitro experimental approach to investigate many aspects of the development of hypertrophy in adult myocardium under controlled, physiologically relevant in vitro conditions. Apart from the initial descriptive data that this developmental project rendered, our data strongly implicate the mechanistic finding that the sufficiency of mechanical loading, with a specific focus on afterload, caused the accelerated cardiac hypertrophy in the absence of a systemic impact.

The present study demonstrates that mammalian myocardium can hypertrophy in a very rapid timeframe. Despite that, in patients, in vivo hypertrophy develops over a much slower timeframe, many of the observed morphological, physiological, and proteomic features of our model are in concert with those observed in either patients in vivo, or in animal models of hypertrophy. The in vitro approach allows for the setting of loading conditions to such extremes (isometric contractions are basically of infinite afterload) (17), which cannot be achieved in vivo. In addition, the in vitro approach allows for a continuous assessment of contractile function, allowing the correla-

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**Fig. 5.** Immunoblot analysis demonstrates various protein expressions from fresh (Cont), 24-High, and 48-High muscles. Bar graph showed the relative arbitrary units of proteins normalized to cardiac troponin I (TnI) of the same gel. A: TnI is increased vs. calsequestrin (CQ). B: TnI is increased vs. GAPDH at 24 but not at 48 h. C: representative blot of protein expression of trabeculae at 3 different time points. D: *TnI, myosin heavy chain (MHC), troponin T (TnT), Na\(^+\)/Ca\(^{2+}\) exchanger (NCX), and TnT but not sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) were up-regulated after 24 h of high-load contractions compared with GAPDH and CQ. **TnI, MHC, TnT, NCX, and TnT but not SERCA were upregulated after 24 h of high-load contractions compared with CQ, but not compared with GAPDH.
ton of function with changes in morphology and/or gene product expression in the near future.

The observed increase in force during the first 24 h is due not only to an increase in muscle/myocyte mass, because the increases in force are much larger than the increase in contractile filament mass. Thus contractility increases as a result of the high-load condition. After ~30 h, we did observe a decline in force development, while the hypertrophy is increased, indicating a loss of contractility later in the protocol. Our laboratory has reported previously that a very high pre- and afterload clearly can induce a small amount of apoptosis after 24 h, and this becomes more prominent at 48 h (19). These findings are in line with our present result in which, after 24 h, both developed force and cell size increased. A decline was seen in developed force over time, accompanied by a decrease in cell size at 48 h. Although at this point we cannot rule out effects of possible inflammation, these results in many aspects appear to resemble the compensatory and decompensatory processes in patients with hypertrophy cardiomyopathy, but on a much faster temporal scale. Thus our present results provide a potential model to study or develop a therapeutic method targeted at the transition from the compensatory to decompensatory processes in hypertrophic cardiomyopathy.

The results show that, even from a small amount of tissue, typically 0.1 mg (ranging from 0.05 mg for short muscle to up to 0.5 mg for the longest ones), one can quantify protein expression after these muscles have been in culture for up to 48 h. Our laboratory has previously shown that, in this culture system, protein production persists throughout the culture period (21), and we here now show that this protein expression can be modulated by culturing under different loading conditions. The changes we observed in the expression of several proteins are consistent with our finding of myocyte hypertrophy. Some of the most profound changes seen when comparing muscles with high pre- and afterload with those without such a high contractile burden included a differential expression of myosin light chain isoforms and an increase expression of α-b crystalline. These may directly contribute to the alterations of the myofilamental and sarcomeric-structural properties, as it is well known that regulatory light-chain modulates cardiac contractility by influencing myofilament Ca2+ sensitivity and modifying cross-bridge cycling via phosphorylation (9), and that expression of different isoforms of myosin light chains may also influence contractility of cardiac tissue (16, 34). It has been shown that transgenic replacement of the atrial myosin light chain by the ventricular isoform improved the cardiac contractile property of atrial myocytes without any change in intracellular calcium (34), and replacement with the fast skeletal isoform impaired myocyte function (16). Therefore, changes in myosin light chain isoform expression cannot be ruled out to be involved in the initial cause-induced cardiac hypertrophy, rather than representing a compensatory response. Clearly, additional, more focused studies are warranted to investigate the significance of light-chain isoform expression during early hypertrophy. In addition, at this point, we cannot rule out that part of the protein changes observed are due to the transition from hypertrophy to failure, i.e., some proteolysis may already be detectable after 48 h. The observed increased expression of α-b crystalline indicates the change of subcellular structures. The α-b crystalline is a member of the family of small heat shock proteins (7) for which it is known that stress can induce expression via the MAPK kinase 6 pathway (18). In cardiac myocytes, α-b crystalline is associated with the myofilament structural protein titin at a discrete region of the I band. Bullard and coworkers (7) demonstrated that α-b crystalline helps stabilize titin from over-stretching, thereby preventing domain unfolding of titin in the I band. The increased expression of α-b crystalline in the present study may thus point to alterations of the elasticity of the heart muscle that occur early during cardiac remodeling. This possibility draws a parallel to a decrease in passive tension in the loaded muscles. However, since cardiac trabeculae are a complex structure containing extracellular matrix, vascular structure, and nonmuscle type cells, we could not, at this point, determine whether the small reduction of passive force in the preparations is (also) due to a change in expression or posttranslational modifications of other proteins such as titin. The α-b crystalline is also associated with the formation of sarcomere skeletal protein desmin (41, 46), and its mutations have been shown to be able to cause cardiomyopathy. These findings thus fit with our present observations and with a previous report (13) that cardiac myocytes respond to high mechanical loading by upregulation of α-b crystalline to stabilize the changes in myofibrillar and sarcomeric structure. It is not known, at present, how, or if, α-b crystalline expression is altered under neuroendocrine stimulation; α-b crystalline may be a specific indicator of mechanical over-load-induced early cardiac hypertrophy. Several other proteins showed changes in expression levels that are consistent with hypertrophy, including upregulation of arachidonate 12-lipoxygenase (32), downregulation of myoglobin (31), and the upregulation of BRCA1. Regarding the last observation, the significance of BRCA1 in cardiac muscle is presently unknown, and it is unresolved whether BRCA1 induces cell growth and/or has anti-apoptotic effects on the heart. However, specifically focused hypothesis-driven studies would be required to address this issue, and the protein expression changes we observed were performed for proof of principle to show that hypertrophic regulatory pathways are activated, resulting in a different protein expression pattern, and we can detect (some

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**A**

Egr-1

TnI

Cont. 24-High 48-High

**B**

Egr/ TnI

Cont. 24-High 48-High

Fig. 6. Immunoblot analysis demonstrated the expression of early growth response 1 (Egr-1) from Cont, 24-High, and 48-High muscles. A: immunoblots; B: bar graph. Values are means ± SE; n = 4/group. There was a slightly, but not significant, increase in Egr-1 expression at 24 h, but significant decrease after 48 h. *P < 0.05 vs. control by multiple comparison after ANOVA.
of) these changes when loading conditions change. The specific significance of the impact of these individual protein pattern changes was deemed well beyond the scope of the present work.

Both acute and slow responses of mechanical overload or stretch have been demonstrated to enhance myocardial contraction (2, 5, 27). Regarding the acute effect, it is well known that an increase in sarcomere length augments the possible number of myofilament cross-bridge interactions, thereby enhancing force development via the Frank-Starling mechanism. For the slow response, mechanical stretch can induce small changes in the intracellular Ca\(^{2+}\) transients (2, 27), as well as modulate myofilament Ca\(^{2+}\) sensitivity. The increased Ca\(^{2+}\) responsiveness of the myofilaments could be a result of the increased intracellular pH after stretch (2, 10). Cazorla and coworkers (9) reported that skinned cardiac myocytes showed an increased phosphorylation of myosin light chain 2B isofrom after a 1-min stretch was applied, but no changes in major isoform 2A or TnI phosphorylation were observed. All of these immediate factors induced by mechanical load could be important initiators playing in concert to induce cardiac hypertrophy when this acute effect is sustained for prolonged periods. However, in our present study, the increase in force contraction beginning 6 h after stretch points toward changes in protein synthesis and consequent adaptation of cellular morphology, rather than a stretch-induced immediate imbalance in ion homeostasis. In addition, our results show no effect of streptomycin, a well-known stretch-activated channel blocker, on load-induced hypertrophy, and this reduces the likelihood of a role of involvement of ion homeostasis via this mechanism.

Limitations of the technique. Noticeably, stretch does not affect only the cardiac myocyte, but also the other cardiac cells. Although clearly the bulk of the volume of the heart consists of myocytes, the other cells, including fibroblasts, endothelial cells, and vascular smooth muscle cells, may exert paracrine influences that can affect muscle hypertrophy (39). In addition, the cardiac myocyte itself could release some autocrine factors under stress conditions. Since trabeculae are multicellular preparations, these local physical and chemical factors cannot altogether be discarded. However, although this may pose a disadvantage of the model as opposed to a pure myocyte culture in some aspects, in other aspects the multicellular preparation culture may open the possibility for the future investigation of the cell-to-cell communication in vitro and more likely represents the in vivo situation where these various cell types coexist in the same ratio.

Another limitation is that, when the muscles in culture are too large, they may develop a hypoxic core. Thus only very thin muscles should ideally be used. These small preparations (0.1 mg typically) render only small amounts of tissue to be investigated. Due to size differences, each trabecula has a different protein content at the starting point, and at present no technique allows measurement of total protein content before and after stretch in the same preparation. Thus, as the myocytes hypertrophy, total protein content increases, and, due to the stoichiometric nature of the myofilament matrix, it is virtually impossible to quantify the increase in protein expression, and only changes as they relate to other proteins can be detected. Normalizing protein concentration to DNA content (based on the assumption that no cell numbers change) could be a possibility, but DNA extraction is also limited by size of the muscle. In the present study, we chose cTnI to normalize to other proteins. Therefore, if the muscle expressed the sarcomeric proteins in a strict stoichiometric ratio, normalizing by cTnI will show no change in proteins such as myosin heavy chain.

Our laboratory has previously shown that, under a low-loading condition, cardiac trabeculae can maintain their function for over 5 days in the culture setup (20). We limited the current experiments to 48 h, with a success rate of 70–80%. However, in the high-loading protocol, we have detected an increase in apoptotic signals over time (19), and, as the time axis of high-load experiments is extended, the success rate exponentially drops. Thus, although it is not impossible to prolong high-load experiments, given the extremely low success rate for 3- and 4-day experiments, collection of sufficient numbers for appropriate statistical analysis would not only be tedious, but may not remain unambiguous, as one may be studying the outliers rather than the mean response.

In conclusion, mechanical loading and likely specifically a reduction in myocyte shortening due to increased afterload in the absence of systemic factors are a potent activating factor of early-stage, overload-induced hypertrophy of adult myocardium. Our multicellular model demonstrates that mechanical overload activates many early molecular alterations in the cardiac contractile unit, subcellular structure, transcriptional factors, and oxygen metabolism, which alone or in synergy might subsequently induce cardiac hypertrophy. The rapid, highly reproducible in vitro induction of hypertrophy in cultured adult multicellular preparations represents a novel, in vitro model, and information so obtained provides us with a complementary tool to dissect and study time-resolved changes in morphology, function, and protein expression, thereby bridging the gap between information obtained from in vivo models and the basic experimental conditions of isolated cells in culture.

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