Optimizing Mechanical Stretching Protocols for Hypertrophic and Anti-apoptotic Responses in H9c2 Cardiomyocytes

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Abstract

Background: Cardiomyocytes are sensitive to mechanical loading, possessing the ability to respond to mechanical stimuli by reprogramming their gene expression. In this study, signaling as well as expression responses of myogenic, anabolic, inflammatory, atrophy and pro-apoptotic genes to different mechanical stretching protocols were examined in differentiated cardiomyocytes.

Methods: H9C2 cardiomyoblasts were cultured on elastic membranes up to their 5th day of differentiation (myotubes) and then subjected to three different stretching protocols by altering their strain, frequency and duration characteristics, using an *in vitro* cell tension system. cells were harvested and lysed 24 hours after the completion of each stretching protocol and Real Time-PCR was used to monitor changes in mRNA expression of myogenic regulatory factors (MyoD, Myogenin, MRF4), the IGF-1 isoforms (IGF-1Ea, IGF-1Eb), as well as atrophy (Atrogin-1), pro-apoptotic (FoxO, Fuca), and inflammatory (IL-6) factors in response to the different mechanical loading conditions. The activation of Akt and Erk 1/2 signaling proteins following the various stretching protocols was also evaluated by Western blot analysis.

Results: We documented that the low strain (2.7% elongation), low frequency (0.25 Hz) and intermediate duration (12 hrs) stretching protocol was overall the most effective in inducing beneficial responses in differentiated cardiomyoblasts as it increased the expression of IGF-1 isoforms and phosphorylation of Akt and Erk1/2 ($p<0.05$), while it provoked the downregulation of all the other factors examined ($p<0.05$-0.001).

Conclusion: These findings demonstrated that a low strain, low frequency of intermediate duration stretching protocol is the most effective in inducing a hypertrophic and anti-apoptotic response in H9C2 cardiomyotubes, *in vitro.*

Background

Cardiomyocytes are mechanosensitive cells having the ability to transduce, mechanical stimuli to intracellular chemical and electrical signals [1–3]. Mechanotransduction and its downstream responses mediate effective adaptation mechanisms of cardiomyocytes to mechanical loading [4, 5]. However, under abnormal loading conditions this process can become maladaptive, leading to altered physiological function and the development of pathological cardiac hypertrophy and heart failure [6, 7].

Mechanosensitive cellular and extracellular structures have been shown to mediate the transduction of mechanical signals into the cell nucleus [8]. These mechanotransduction elements comprise cell-cell adhesions, intracellular receptors, and extracellular matrix sensing receptors, including integrins, cytoskeletal filaments and ion channels [8–12]. Through these mechanosensors mechanical loading can activate a complex network of downstream signal transduction pathways that induce protein synthesis and increase the autocrine production and release of growth factors by the mechanosensitive cells [1, 4, 6, 8]. Moreover, essential cellular processes, such as proliferation, differentiation survival and apoptosis, are also modulated by mechanical stimuli [13–17]. Indeed, mechanical signals that regulate

cardiomyocyte growth, in both physiological and pathological conditions [8, 10] can also affect their differentiation [13, 14], which is driven by multiple signal transduction pathways that coordinate the balance between protein synthesis and protein degradation, or muscle growth and atrophy [18].

Specifically, myogenic differentiation is regulated by four transcription factors, the myogenic regulatory factors (MRFs) MyoD, Myf5, Myogenin and MRF4 [19, 20]; these transcriptional activators share the ability to convert various differentiated cell types to myogenic [21], while recent studies have documented that they play a similar role in cardiomyocyte myogenic differentiation [22, 23]. In addition, insulin-like growth factor-1 (IGF-1) signaling has been implicated in skeletal and cardiac cell growth through the activation of extracellular signal-regulated kinases (Erk) 1/2 [24] and in the loading-induced adaptive cardiac hypertrophy through the activation of Akt (protein kinase B) [11, 25, 26]. Moreover, potentially differential actions of IGF-1 isoforms in cardiac myoblasts growth as well as in the myocardial repair/remodeling process have been proposed [27–29].

However, depending on its specific characteristics, mechanical loading can have detrimental effects on cardiomyocytes by activating apoptotic pathways; mechanical stretching can activate important pro-apoptotic factors, such as FoxO and subsequently its targets genes Fuca, muscle-specific ubiquitin ligase Atrogin-1 (MaFbx), and interleukin (IL)-6 [30, 31], which have been negatively implicated in cell growth and survival [18, 32], while FoxO also inhibits Akt and Erk1/2 activation, inducing cardiac muscle wasting and promoting heart failure [15–17].

Thus, it remains a challenge to better understand the contribution of the particular inputs of such factors on cardiac cell adaptation to mechanical loading, depending on the specific loading characteristics. Given the complexity of the *in vivo* models of cardiac adaptations [33] in response to mechanical loading, *ex vivo and in vitro* models of muscle mechanical loading [34] applied on myocardial cells are crucial for understanding the cellular and molecular mechanisms that mediate loading-induced adaptations.

The aim of the present study was to investigate and compare the effects of various *in vitro* mechanical stretching protocols on signaling and gene expression responses of differentiated H9C2 cardiomyoblasts, associated with anabolic/atrophy, pro-apoptotic and inflammation-related factors that are involved in their myogenic lineage. We set the hypothesis that the signaling and expression responses elicited would differ depending on the loading characteristics of the protocols used, thus indicating loading-specific, detrimental or beneficial effects on cardiomyotubes *in vitro*.

Materials And Methods

H9C2 Cell Culture

The H9C2 cell line of embryonic rat heart-derived ventricular cells was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as previously described [25]. Briefly, cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS),

1% penicillin/streptomycin at 37^{o}C in a humidified atmosphere of 5% CO_2 in air, while medium was changed every other day. The H9C2 cardiomyoblasts were seeded onto 6-well flexible-bottomed culture plates coated with Collagen I (Flex I Culture Plates Collagen I; Flexcell International, Hillborough, NC, USA) and maintained in growth media until 70–80% confluent, then switched to differentiation media (2% horse serum, 1% of penicillin/streptomycin in DMEM). Cardiomyoblasts were allowed to differentiate into multinucleated myotubes for a 5-day period during which media was changed every other day before stretching, as described below.

Cardiomyocyte mechanical loading

Differentiated H9C2 (myotubes) were stretched using the Flexcell FX-4000 strain unit (Flexcell International) that produces isotropic two-dimensional (biaxial) strain of cells cultured on the flexible surface (silicone membrane) of the culture plates, again at 37 °C in a humidified atmosphere of 5% CO_2. Briefly, cardiomyotubes were subjected to three different stretching protocols: a) 12.7% elongation (strain), at a frequency of 0.5 Hz for 15 min (high strain/short duration protocol); b) 2.7% elongation, 0.25 Hz for 12 h (low strain/intermediate duration protocol), or c) 2.7% elongation, 0.25 Hz for 24 h (low strain/long duration protocol). It is worth mentioning that in order to use experimental approaches the more physiological possible, in each protocol the pattern (waveform) of the tension applied on the cardiomyocytes in each stretching cycle was mimicking the pressure fluctuations of a heart beat *in vivo*.

Cell lysis and RNA extraction

Cell extracts were obtained by cell lysis using NucleoZOL (Mecherey-Nagel, German) 12 hrs after the completion of the stretching protocol, while control (non-stretched) myotubes were also harvested 12 hrs after the end of each stretching protocol used for the stretched cardiomyotubes. Total RNA was isolated from the lysates according to the manufacturer's recommendations. The extracted RNA was dissolved in RNAases free water (Invitrogen) and the concentration and purity were determined spectrophotometrically (ThermoNanodrop 2000) by absorption at 260 and 280 nm. Integrity of total RNA was confirmed by visual inspection of the electrophoretic pattern of 18S and 28S ribosomal RNA in ethidium bromide-stained 1% agarose gels under ultraviolet (UV) light. The total RNA samples were stored at − 80 °C until further analyses for the determination of the mRNA levels of the genes of interest by reverse transcription and semi-quantitative real-time PCR procedures.

Reverse Transcription and Real-time PCR

Total RNA from each sample was used for the production of single-stranded cDNA by reverse transcription using reverse transcriptase ProtoScript II (NEB) and the resultant cDNAs were utilized in real-time PCR. More specifically, for the reverse transcription 1 μg of total RNA from each sample was mixed with random primers mix (300 ng/reaction), oligod$(T)_{23}$VN (300 ng/reaction) and nuclease-free water in a total volume of 8 μl, heated at 65^{o}C for 5 min and then placed on ice. Next, the samples were mixed with 10 μl ProtoScript II Reaction Mix and 2 μl Protoscript II Enzyme mix and incubated consecutively at 25^{o}C for 5 min and at 45^{o}C for 1 hour according to manufacturer's recommendations. At the final step of the

reverse transcription, the samples were heated at 80^{o}C for 5 min, to inactivate the reaction, and stored at -20^{o}C.

Real-time PCR analyses were performed using the Bio-Rad 96-well iCycler thermal cycler (Bio-Rad iQ5 Real-Time PCR Detection System, Hercules, CA, USA) and Bio-Rad reagents (iQ™ SYBR Green Supermix). The primer set sequences used for the specific detection of IGF-1 isoforms (IGF-1Ea, IGF-1Eb), myogenic regulatory factors (MyoD, Myogenin, MRF4), pro-apoptotic (Foxo, FUCA), atrophy (Atrogin-1) and inflammation-related (IL-6) factors are shown in Table 1. To prevent detection of genomic DNA, the primer sets were designed to lie within different exons while, particularly, each set of primers for the detection of the IGF-1 isoforms was specific to detect only one IGF-1 transcript variant. Each PCR reaction contained 50 ng of cDNA, 12.5 μl SYBR green master mix, 0.4 μM of each primer, and nuclease free water to a total volume of 20 μl. The real-time PCR parameters were the following: initial denaturation at 95 °C for 5 min followed by 40 cycles of 30 sec at 95 °C, 30 seconds at 62 °C for annealing, and 30 seconds at 72 °C for extension. Transcript levels of the genes of interest were assessed by automatically calculating the threshold cycle (Ct) as the number of cycles at which the measured fluorescence exceeds the threshold for detection. To normalize the amount of total RNA present in each PCR reaction and the mRNA expression (relative quantification-dCt) of the genes of interest, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene (internal standard). Each sample was analyzed in duplicate, and the resulting data were averaged. A melting curve (Tm) was also generated by the Bio-Rad iQ5 Real-Time PCR Detection System software after the final cycle for each experimental sample, by continuous monitoring the Bio-Rad SYBR fluorescence throughout the temperature ramp from 70 °C to 95 °C. The specificity of the primers for the corresponding transcript was also confirmed by the melting curve analysis of samples, where there was only one melting curve for each sample, while electrophoretic analysis of the real-time PCR products further verified the specificity of the transcript of each gene of interest. Control for specificity included cDNA-free and template-free reactions.

Table 1
The sequence of the specific sets of primers used for RT-PCR analyses.

Target Gene	5'-3' (forward) Primer Sequence	3'-5' (reverse) Primer Sequence
GAPDH	CAA CTC CCT CAA GAT TGT CAG CAA	GGC ATG GAC TGT GGT CAT GA
MYOD	TGC TCC TTT GAG ACA GCA GA	AGT AGG GAA GTG TGC GTG CT
MYOGENIN	AGG AGA GAA AGA TGG AGT CCA GAG	TAA CAA AAG AAG TCA CCC CAA GAG
MRF4	AGG GCT CTC CTT TGT ATC CAG	TGG AAG AAA GGC GCT GAA GA
IGF-1Ea	GTG GAC GCT CTT CAG TTC GT	GCT TCC TTT TCT TGT GTG TCG ATA G
IGF-1Eb	GTC CCC AGC ACA CAT CGC G	TCT TTT GTG CAA AAT AAG GCG TA
FUCA	TTT GGT CGG TGA GTT GGG AG	CCA TTC CAA GAG CGA GTG GT
FOXO	AGT GGA TGG TGA AGA GCG TG	GAA GGG ACA GAT TGT GGC GA
IL-6	CCT TCC TAC CCC AAT TTC CAA T	AAC GCA CTA GGT TTG CCG AGT A
ATROGIN-1	AAC AAG GAG GTA TAC AGT AAG G	AAT TGT TCA TGA AGT TCT TTT G

Protein extraction and Immunoblotting analysis

Total proteins were extracted from H9C2 myotubes as previously described [27]. Briefly, cardiomyotubes were washed with ice-cold PBS before lysing in 150 µL of RIPA buffer (Cell signaling) supplemented with protease and phosphatase inhibitors cocktails (Cell signaling). Lysates were incubated on ice under shaking for 20 minutes in order to ensure complete lysis of the cells, centrifuged at 15,000 rpm for 20 min at 4 °C and the supernatants retained. Protein content was determined using a BCA protein assay kit (Thermo Scientific). Samples were stored in aliquots at − 80˚C until Western blot analysis as previously described [27, 35]. Briefly, equal amounts of protein extracts (30 µg) from H9C2 myotubes were mixed with a loading buffer (Invitrogen), denatured at 95 °C for 5 minutes, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [10% (w/v) separating gel and 4% (w/v) stacking gel] and vertically electrophoresed at 100 V for 3 hours. They were then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) at 100 V for 3 hours at 4 °C. Membranes were incubated with a blocking solution containing 5% bovine serum albumin (BSA) in Tris phosphate-buffered saline (TBS; 10 mM Tris, pH 7.6; 100 mM NaCl) plus Tween (0.1% v/v Tween 20) (TBS-T) at room temperature for 1 h. After three washes with TBS-T for 10-min each, blots were incubated with the following primary antibodies overnight at 4 °C under gentle shaking for the immunodetection of Phospho-Akt and Phospho-Erk 1/2 proteins: rabbit monoclonal anti- Phospho-Akt (1:2000 dilution with 5% BSA in TBS-T) (#4060; Cell Signalling) anti-Phospho-p44/42 MAPK (Erk1/2) (1:2000 dilution with 5% BSA in TBS-T) (#4370; Cell Signalling), respectively. After overnight incubation and three washes with TBS-T, membranes were incubated with a horseradish peroxidase-conjugated secondary anti-rabbit IgG (goat anti-rabbit, 1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-mouse IgG (goat anti-mouse, 1:2000 dilution; Santa Cruz Biotechnology) in TBS-T containing 2.5% BSA, for 1 h at room temperature. The expected bands

were visualized by exposure of the membranes to x-ray film after incubation with an enhanced chemiluminescent substrate for 3 min (ECL Supersignal west picoThermo scientific). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:2,000 dilution; Santa Cruz Biotechnology) was used as an internal standard to correct for potential variation in the protein loading and to normalize the protein measurements on the same immunoblot. Band intensity was then semi-quantified using the Image J software.

Statistical analysis

One-way analysis of variance (ANOVA) with Dunn's Multiple Comparison post-hoc test was used for statistical analyses of gene expression and Student's t test for signaling data analyses, utilizing GraphPad Prism 5. All experiments were performed in triplicate and data are presented as mean ± standard error of the mean (SE). The level of statistical significance was set at $p < 0.05$.

Results

Myogenic Regulatory Factors

In order to investigate the potential effects of mechanical loading on the myogenic lineage of differentiated cardiomyoblasts, we examined the expression levels of both early (MyoD) and late (Myogenin, MRF4) differentiation factors in cardiomyocytes. We found that only the low frequency (0.25 Hz), low elongation (2.7%) of longer durations stretching protocols induced significant changes in the expression levels of those MRFs compared either to control (no stretch) or the higher elongation/frequency and short duration condition (Fig. 1A-C). Moreover, the 24 hrs stretching resulted in a significant upregulation of MyoD and Myogenin compared to the intermediate duration (12 hrs) stretching protocol (Fig. 1A, B). Interestingly, the 12 hrs protocol induced a significant decrease in the expression of MRF4 compared to both the control and the higher elongation/frequency and short duration condition (Fig. 1C).

IGF-1 isoforms

IGF-1 is a key factor in the regulation of cardiomyocytes development and growth; thus, we examined the effects of different stretching protocols on the particular expression of IGF-1 isoforms in differentiated cardiomyocytes. Interestingly, a similar profile was revealed for both IGF-1 isoforms regarding their responses to the different mechanical loading protocols. We found that the low frequency (0.25 Hz), low elongation (2.7%) of longer durations stretching protocols induced significant increases in the expression levels of both isoforms compared to the higher elongation/frequency and short duration condition (Fig. 2A, B). However, only the 12 hrs loading protocol induced a significant increase in the expression of IGF-1Eb isoform compared to control (Fig. 1B).

Pro-apoptotic factors

In parallel with the effects of mechanical loading on the anabolic/survival factor IGF-1, we also examined the effects of different stretching protocols on the expression of apoptosis-related factors in cardiomyotubes. It was found that all three mechanical loading protocols resulted in a similar, significant downregulation of FoxO compared to the control condition (Fig. 3A). Moreover, the intermediate duration (12 hrs) protocol induced a significant decrease in the expression levels of Fuca compared to the long duration condition (Fig. 3B).

Muscle atrophy and inflammation-related factors

We further examined the loading-induced regulation of Atrogin-1 and IL-6 in cardiomyotubes. Similarly to the responses of the pro-apoptotic factor Fuca to the various mechanical loading protocols, the 12 hrs stretching of cardiomyoblasts resulted in a significant downregulation of the cardiac and skeletal muscle atrophy factor Atrogin-1 and the inflammation factor IL-6 compared to the 24 hrs duration stretching as well as to control condition (Fig. 4A, B). Particularly for IL-6, the long duration (24 hrs) and low elongation (2.7%)/frequency (0.25 Hz) protocol induced significantly higher responses compared to the short duration (15 min) and higher elongation (12.7%)/frequency (0.5 Hz) stretching protocol (Fig. 4A).

Activation of the signaling proteins Akt and Erk 1/2

Along with the various gene expression responses to mechanical stretching of cardiomyotubes, we also investigated the effects of the different loading protocols on the phosphorylation of important intracellular signaling mediators. Interestingly, only the intermediate duration (12 hrs) and low elongation (2.7%)/frequency (0.25 Hz) stretching protocol induced the activation of both signaling proteins, Akt and Erk1/2, while a tendency towards the downregulation of phosphorylation of these proteins was observed after either the short duration (15 min) and higher elongation (12.7%)/frequency (0.5 Hz), or the long duration (24 hrs) and low elongation (2.7%)/frequency (0.25 Hz) stretching protocol (Fig. 5A, B).

Discussion

This study examined and compared the effects of 3 different *in vitro* cell stretching protocols on gene expression and signalling responses associated with the myogenic lineage of differentiated H9C2 cardiomyoblasts, in order to reveal potential loading-specific, detrimental or beneficial effects on cardiac myotubes, depending on the loading characteristics of the different protocols. The expression of myogenic, anabolic, atrophy, pro-apoptotic and inflammatory factors, as well as the activation of major intracellular signaling cascades were measured 12 hours after the completion of each stretching protocol, to check durable, persistently triggered rather than short signaling and transcriptional responses. Our main findings revealed that a low strain (2.7% elongation), low frequency (0.25 Hz) of an intermediate duration (12 hrs) mechanical stretching protocol was overall the most effective in inducing a hypertrophic response in cardiac myotubes, by increasing the expression of the anabolic factor IGF-1 and the phosphorylation of Akt and Erk 1/2 signaling proteins, while downregulating atrophy, pro-apoptotic and inflammation-related factors. Furthermore, the present study revealed that the late myogenic factor MRF4

exhibited differential responses to mechanical loading compared to the other two MRFs examined, MyoD and Myogenin.

The ability of cardiomyocytes to sense external mechanical stimuli (mechanosensing) and convert them into electrochemical and biochemical signals is critical for the maintenance of their homeostasis as well as for cardiac muscle tissue adaptation to mechanical loading [1, 2, 4, 5]. In this context, *in vitro* models of cell stretching are virtually the main if not the only experimental approach to meticulously study the intracellular molecular events in cardiomyocytes as a result of mechanical stimuli [36, 37].

Mechanical loading of skeletal and cardiac muscle cells both *in vivo* and *in vitro* can lead to the upregulation of many growth factors, including IGF-1, and the activation of signaling pathways associated with protein synthesis and cell growth, eventually leading to muscle hypertrophy [10, 24–26, 38, 39]. Indeed, IGF-1 upregulation and signaling have been implicated in the mechanical loading-induced adaptive cardiac hypertrophy [10, 25, 26], while potentially differential actions of IGF-1 isoforms in myocardial repair/remodeling process have been proposed [27–29, 40]. To the authors' best knowledge, this is the first study investigating the distinct expression profiles of IGF-1 isoforms following mechanical loading of cardiac myotubes, *in vitro.* Interestingly, our findings showed that both isoforms were upregulated by low strain/frequency and long durations stretching protocols, with the more pronounced responses being exhibited after the intermediate duration (12 hrs) protocol. Inversely, the high strain/frequency of short duration stretching of cardiomyotubes resulted in a tendency of decreased IGF-1 isoforms expression (Fig. 2). These findings suggest that both IGF-1 isoforms need prolonged, low strain/frequency loading to be activated in differentiated cardiac cells, *in vitro*.

Furthermore, two primary mechanosensitive intracellular pathways have been associated with the IGF-1 actions in skeletal and cardiac muscle physiology [26, 34]; the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, the activation of which is involved in cellular processes such as protein synthesis, hypertrophy and protection from apoptosis, and the Ras/Raf/MEK/Erk 1/2 signaling pathway, which has been shown to increase muscle cell proliferation. The outcomes of these two pathways are based on complex interactions that require comprehensive identification, since in some cell types, PI3K and Erks appear to act in concert, e.g., both PI3K and Erks are possibly required for the myogenic differentiation of myoblasts [26, 41]. In particular, the PI3K/Akt has been implicated in myocardial cells survival and their protection against reperfusion-induced injury [42], while both the IGF-1 receptor (IGF-1R)/PI3K/Akt [43] and the Ras/Raf/Erk 1/2 signaling pathways have been shown to be essential for myocardial hypertrophy [24].

Our findings showed a loading-specific activation of these two pathways in cardiomyotubes *in vitro*; specifically and similarly to the uperegulation of IGF-1, only the intermediate duration (12 hrs) low strain/frequency protocol induced the activation of both signaling proteins, Akt and Erk1/2 (Fig. 5). These findings suggest that the loading-induced activation of these signaling mediators appears not to be mutually exclusive and may be depended on the loading characteristics of mechanical stretching and possibly on the stretching-induced IGF-1 upregulation. Further studies are needed to determine whether these pathways are activated through the same or different mechanosensors of cardiac muscle cells.

Overall, we found that the low strain/frequency of intermediate duration stretching protocol was the more effective in inducing an anabolic/anti-apoptotic response in the differentiated cardiomyocytes.

In parallel with highlighting the anti-apoptotic/anabolic profile of the cardiac myotubes in response to different loading conditions, this study also examined the expression responses of pro-apoptotic and muscle atrophy genes to the various mechanical stimuli. While many studies have suggested potentially beneficial effects of mechanical stretching on cardiomyocytes structure and function [1, 44–46], nevertheless, excessive mechanical stimuli have been reported to induce cardiac cell apoptosis and maladaptive hypertrophy, which promote upregulation of atrophy and inflammation factors [7, 36, 47].

Various pro-apoptotic factors may potentially be involved in the myogenic program of myoblasts; FoxO is a fate decider within the myogenic lineage as opposed to an inducer of the myogenic differentiation [48], while Fuca inhibits cell growth and induces cell death [32]. Moreover, muscle-specific atrophy genes, such as Atrogin-1, are considered to play an important role in driving an atrophic phenotype through the ubiquitin-proteasome pathway [49], although the defined mechanisms of their action remain to be fully elucidated.

Interestingly and in contrast with the anabolic signaling and IGF-1 responses, our study showed that the stretching protocol characterized by low strain/frequency for an intermediate duration resulted in decreased expression of the atrophy (Atrogin-1), pro-apoptotic (FoxO, Fuca) and inflammation-related (IL-6) genes examined. Moreover, it is worth mentioning that increasing the duration of the low strain/frequency stretching led to significant increase in the expression of Fuca, Atrogin-1 and IL-6 compared with the intermediate duration protocol (Figs. 3 and 4).

These findings suggest a multiple beneficial effect of the low strain/frequency of intermediate duration mechanical stretching, which simultaneously upregulates anabolic/survival program and downregulates muscle atrophy and pro-apoptotic factors in advanced differentiation cardiomyocytes [17]. Moreover, our findings indicate that there might be a threshold (or range) of duration of low strain/frequency mechanical loading for the induction of beneficial or detrimental effects on cardiomyotubes [45, 47], (Fig. 2–5).

The differentiation of myoblasts into myotubes has become a model for understanding the molecular mechanisms that regulate the antagonistic phenomena of cell proliferation and differentiation. Myogenic differentiation of myoblasts is regulated by MRFs [50] and it has been established that MyoD is already present in the proliferating myoblasts and is involved in the myogenic determination, while Myogenin and MRF4 are expressed in a subsequent phase and are involved in the terminal differentiation of myoblasts into non-proliferating myotubes. Nevertheless, MyoD can further trigger muscle differentiation by activating the expression of myogenin and other muscle-specific genes that are key factors of the myogenic lineage progression [22, 23, 51–53]. Moreover, studies have revealed that mechanical stimuli affect the expression of these myogenic determination factors [54, 55]. Nevertheless, the specific responses of MRFs to mechanical loading in cardiomyocytes remain largely unknown.

In our study, MRFs exhibited differential responses to the various stretching protocols applied on the differentiated cardiomyotubes. Specifically, the 12-hr, low strain/frequency mechanical loading resulted in significant decrease in the expression of the late differentiation factor MRF4, while the same low strain/frequency protocol applied for a longer duration (24 hrs) led to the upregulation of MyoD and Myogenin. Interestingly, the MRFs responses to low strain/frequency loading in differentiated cardiomyocytes and, thus, the regulation of their myogenic lineage appears also to be time-dependent (Fig. 1A-C).

The effects of the higher strain/frequency for a short duration (15 min) loading protocol on signaling and gene expression responses of cardiomyoblasts found, overall, to be mild and limited, resulting only in the downregulation of the pro-apoptotic factor FoxO. Nevertheless, this appeared to be a common effect of all the stretching protocols used in the present study, regardless of their specific loading characteristics (Fig. 3A).

Conclusions

Cardiomyocytes, as skeletal muscle cells, are mechanosensitive and respond to mechanical signals in order to maintain their homeostasis and adapt to external loading. The development of *in vitro* models of cell mechanical loading can greatly contribute to shed more light on the cellular and molecular responses of cardiac muscle cells to loading. The present study demonstrated that varying the characteristics of mechanical loading (i.e., strain, frequency, duration) applied on advanced differentiation cardiomyocytes *in vitro* resulted in different effects on their myogenic lineage, indicating specific features of loading for regulating the anabolic/survival program in these cells. These findings may be a valuable resource for developing more focused *in vitro* experimental designs to characterize the particular inputs of specific factors and mechanotransduction pathways on cardiac cell adaptation to mechanical loading.

Declarations

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' contributions

EZ designed and performed the experiments, analyzed the data and wrote the manuscript; AP and MK conceived or designed the experiments, analyzed data and wrote the manuscript; AM performed the experiments and analyzed the data; AC analyzed data and reviewed the manuscript. All Authors have read and approved the final manuscript.

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Figures

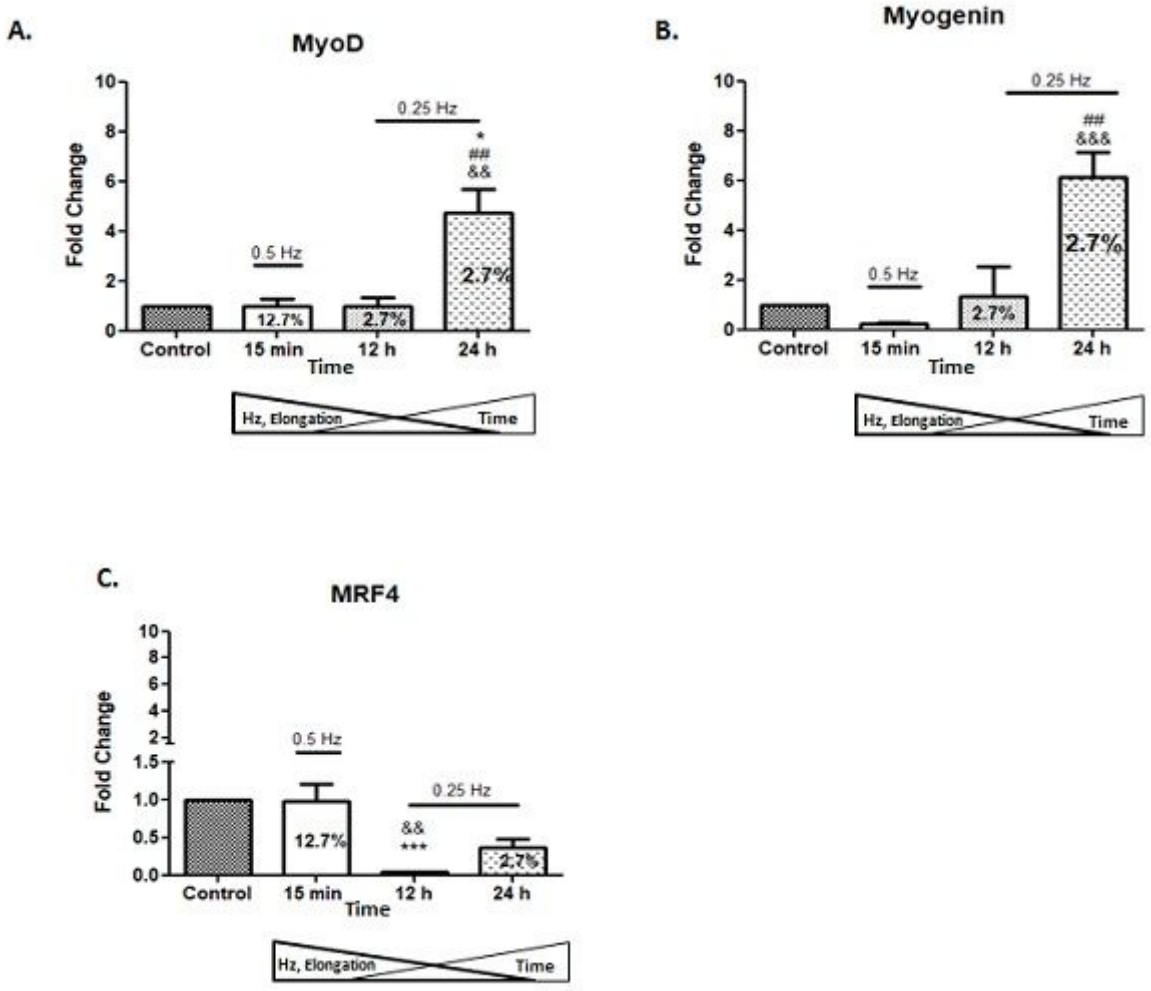

Figure 1

Effects of cyclic mechanical stretch on the expression of Myogenic Regulatory Factors (MRFs). Quantitative analysis of (A) MyoD, (B) Myogenin and (C) MRF4 mRNA expression in cardiomyotubes subjected to mechanical stretch compared to control (non-stretched myotubes); The mRNA values of MRFs in stretched myotubes have been normalized to the corresponding GAPDH mRNA and are expressed as fold changes compared to control. Percentages on the columns represent the degree of elongation of the cell culture surface. Note that in the selected stretching protocols as the elongation and frequency (Hz) decrease the duration (Time) increases. Significantly different compared to control, *: p <0.05; **: p <0.01; ***: p <0.001; Significantly different compared to 12.7%/0.5Hz/ 15min, &&: p <0.01;

&&&: p <0.001; Significantly different compared to 12hrs duration, ##: p <0.01; Mean+SE of 3 independent experiments performed in triplicate.

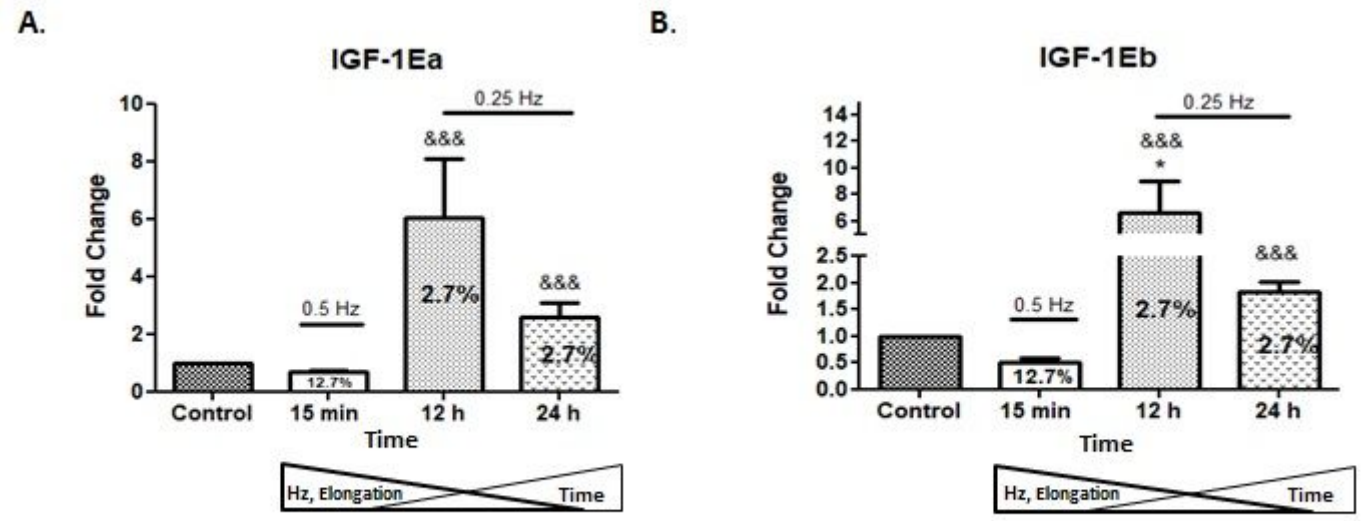

Figure 2

Effects of cyclic mechanical stretch on the expression of IGF-1 isoforms. Quantitative analysis of(A) IGF-1Ea and (B) IGF-1Eb mRNA levels in cardiomyocytes subjected to mechanical stretch compared to control (non-stretched myotubes); The mRNA values of IGF-1 isoforms in stretched cardiomyocytes have been normalized to the corresponding GAPDH measurement and are expressed as fold changes compared to control. Percentages on the columns represent the degree of elongation of the cell culture surface. Note that in the selected stretching protocols as the elongation and frequency (Hz) decrease the duration (Time) increases. Significantly different compared to control, *: p <0.05; Significantly different compared to 12.7% elongation/ 0.5Hz/ 15min, &&&: p<0.001; Mean+SE of 3 independent experiments performed in triplicate.

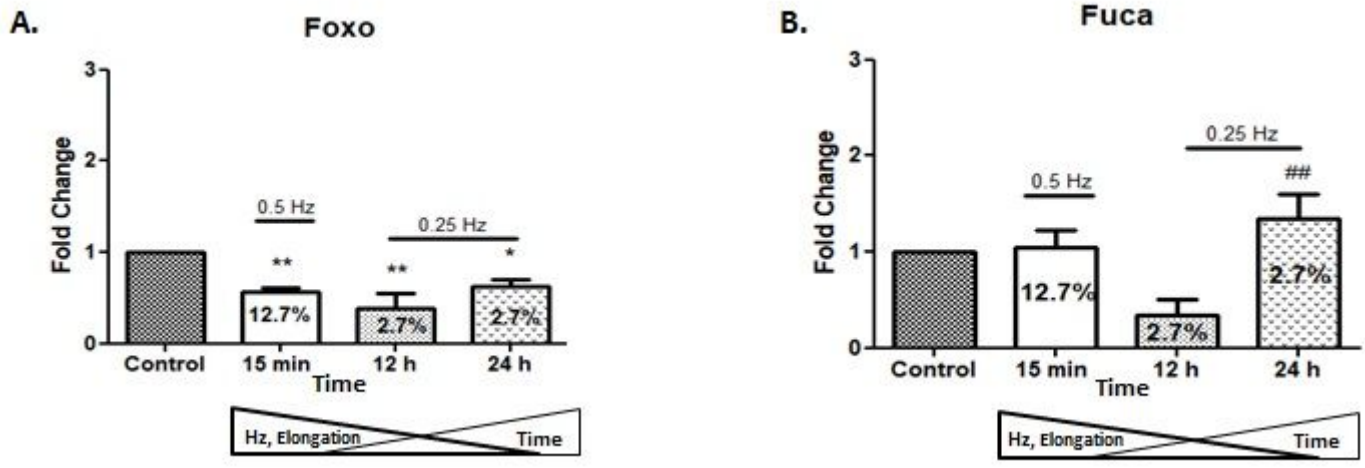

Figure 3

Effects of cyclic mechanical stretch on the expression of pro-apoptotic factors. Quantitative analysis of (A) FoxO and (B) Fuca mRNA expression in cardiomyotubes subjected to mechanical stretch compared to control (non-stretched myotubes); The mRNA values of apoptotic factors in stretched myotubes have been normalized to the corresponding GAPDH mRNA and are expressed as fold changes compared to control. Percentages on the columns represent the degree of elongation of the cell culture surface. Note that in the selected stretching protocols as the elongation and frequency (Hz) decrease the duration (Time) increases. Significantly different compared to control,*: p <0.05; **: p <0.01; Significantly different compared to 12hrs duration, ##: p <0.01; Mean+SE of 3 independent experiments performed in triplicate.

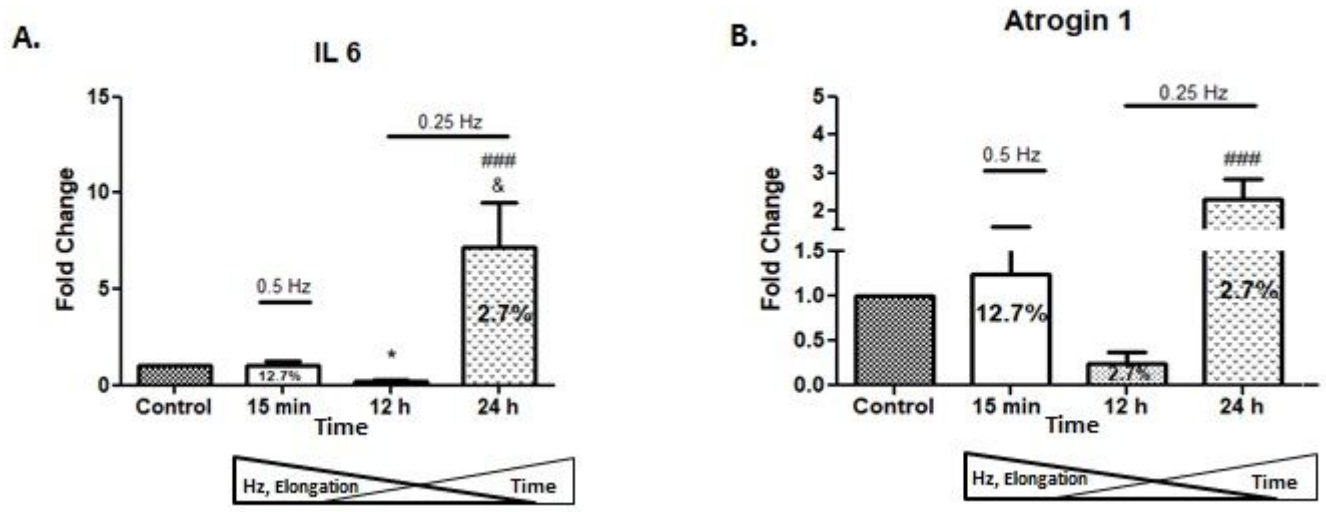

Figure 4

Effects of cyclic mechanical stretch on the expression of IL-6 and Atrogin-1. Quantitative analysis of (A) IL-6 and (B) Atrogin-1 mRNA expression levels in cardiomyocytes subjected to mechanical stretch compared to control (non-stretched myotubes); The mRNA values of IL-6 and Atrogin-1 in stretched cardiomyocytes have been normalized to the corresponding GAPDH mRNA and are expressed as fold changes compared to control. Percentages on the columns represent the degree of elongation of the cell culture surface. Note that in the selected stretching protocols as the elongation and frequency (Hz) decrease the duration (Time) increases. Significantly different compared to control, *: p <0.05; Significantly different compared to 12.7%/ 0.5Hz/ 15min, &: p <0.05; Significantly different compared to 12hrs duration, ###:p <0.001; Mean+SE of 3 independent experiments performed in triplicate.

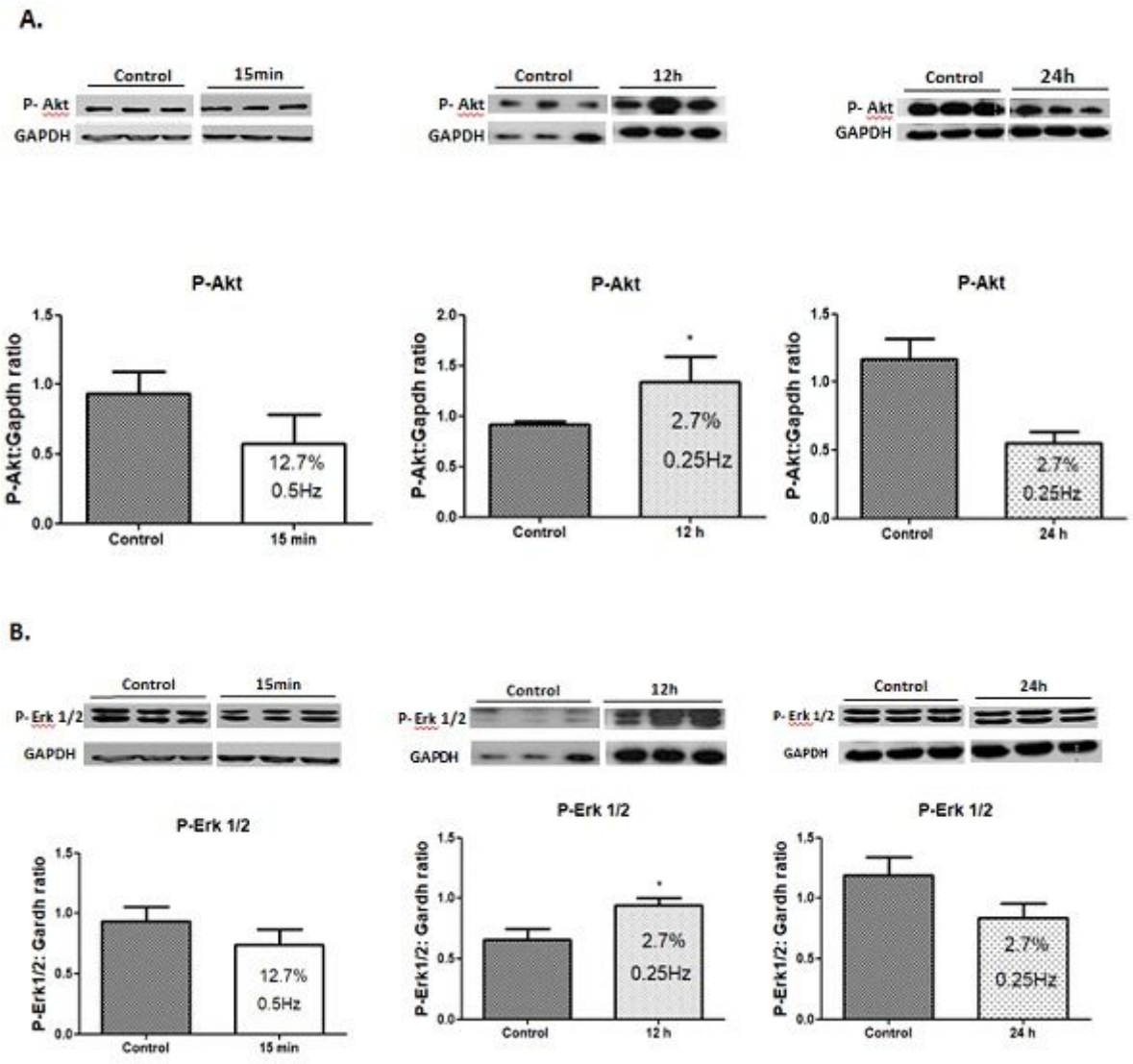

Figure 5

Effects of cyclic mechanical stretch on the activation of the signaling proteins Akt and Erk 1/2. Representative Western blots and Immunoblotting quantification of phosphorylated (A) Akt and (B) Erk 1/2 in cardiomyoblasts subjected to different mechanical stretching protocols compared to control (non-stretched myotubes) the values of the phosphorylated proteins were normalized to each corresponding GAPDH on the same immunoblot. Significantly different compared to control, *:p <0.05; Mean+SE of 3 independent experiments performed in triplicate.