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Cessation of cyclic stretch induces atrophy of C2C12 myotubes

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ABSTRACT

Cyclic stretch of differentiated myotubes mimics the loading pattern of mature skeletal muscle. We tested a cell culture model of disuse atrophy by the cessation of repetitive bouts of cyclic stretch in differentiated C2C12 myotubes. Myotubes were subjected to cyclic strain (12%, 0.7 Hz, 1 h/d) on collagen-I-coated Bioflex plates using a computer-controlled vacuum stretch apparatus (Flexcell Int.) for 2 (2dSTR) or 5 (5dSTR) consecutive days. Control cultures were maintained in the Bioflex plates without cyclic stretch for 2 d or 5 d. Additionally, some cultures were stretched for 2 d followed by cessation of stretch for 3 d (2dSTR3dCES). Cyclic stretching (5dSTR) increased myotube diameter and overall myotube area by ~2-fold ($P < 0.05$) compared to non-stretched controls, while cessation of stretch (2dSTR3dCES) resulted in ~80% smaller myotubes than 5dSTR cells, and 40–50% smaller than non-stretched controls ($P < 0.05$). Further, the calpain-dependent cleavage products of αII-spectrin (150 kDa) and talin increased (3.5-fold and 2.2-fold, respectively; $P < 0.05$) in 2dSTR3dCES myotubes, compared to non-stretched controls. The 1 h cyclic stretching protocol acutely increased the phosphorylation of Akt (+4.5-fold; $P < 0.05$) and its downstream targets, FOXO3a (+4.2-fold; $P < 0.05$) and GSK-3β (+1.8-fold; $P < 0.05$), which returned to baseline by 48 h after cessation of stretch. Additionally, nitric oxide production increased during stretch and co-treatment with the NOS inhibitor, L-NAME, inhibited the effects of stretch and cessation of stretch. We conclude that cessation of cyclic stretching causes myotube atrophy by activating calpains and decreasing activation of Akt. Stretch-induced myotube growth, as well as activation of atrophy signaling with cessation of stretch, are dependent on NOS activity.

1. Introduction

Skeletal muscle cells are extremely sensitive to mechanical forces allowing mature muscle fibers to hypertrophy or atrophy with increasing or decreasing mechanical loading, respectively. While generally adaptive, this property can produce dramatic muscle wasting and weakness during periods of physical inactivity, such as bed rest, limb immobilization, or space flight, thereby hindering functional recovery upon muscle reloading. A cell culture model of unloading-induced muscle cell atrophy would allow rapid analysis of the signaling mechanisms involved in the response to removal of mechanical tension without potential confounding effects of changes in neural recruitment, blood flow, or endocrine factors that occur with whole-body inactivity.

Reduced mechanical strain on the muscle membrane may impair signaling from force transducers in the extracellular matrix to the cytoskeleton. Disruption of the mechanotransduction proteins connecting the extracellular matrix to the cytoskeleton has been implicated in the initiation of proteolysis during muscle unloading [1,2], which occurs due to both a decrease in muscle protein synthesis and an increase in the rate of proteolysis [3]. Skeletal muscle proteolysis is initiated by calpain and caspase proteases triggered by accumulation of intracellular calcium [4], leading to upregulation of the E3 ubiquitin ligases, MAFbx and MuRF1, and the 26S proteasome to degrade myofibrillar proteins [5].

The dystrophin–glycoprotein complex (DGC) provides a transmembrane association between the extracellular matrix and various intracellular proteins and protein complexes [6]. Disruption of the DGC, such as occurs in Duchenne's muscular dystrophy or the mdx mouse, results in repetitive skeletal muscle injury/regeneration and muscle atrophy. Proteins involved in transmembrane signaling function as mediators of survival signaling and protein synthesis, often working through PI3K and one of its downstream effectors, a serine/threonine kinase known as Akt [7]. Akt activation has a protective effect, blocking induction of apoptosis in differentiated muscle cells [8]. Events that may mediate cell survival downstream of Akt include inhibitory phosphorylation of GSK-3β and transcription factors in the Forkhead family (FOXO) [9]. Additionally, through its action on the mammalian target of rapamycin (mTOR), FOXO, and other intermediates, Akt maintains protein synthesis and inhibits the proteolytic pathways [10].

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Evidence suggests that the DGC works as a regulator of muscle atrophy and serves as a scaffold for anti-atrophic signal transduction [1]. Therefore, routine deformation of the sarcolemma by stretch and/or contraction would maintain signaling through the DGC and preserve muscle mass. Neuronal NOS (nNOS) is a peripheral member of the DGC and may play a role in transducing mechanical signals. Sarcolemmal-associated nNOS is reported to be a necessary signaling molecule for satellite cell activation, glucose uptake, muscle contraction, and vasodilation [11–14]. Further, NOS activity appears to be necessary for muscle hypertrophy [15]. Neuronal NOS expression and activity has been shown to increase with mechanical activity [16], training [17], and electrical stimulation [18], and to decrease with unloading [16]. More recently, Wozniak and cohorts demonstrated that a 10% mechanical stretch applied to primary cultured myotubes transiently increased fluorescence of the NO probe, DAF-2DA; whereas myotubes from mdx mice, which contain little nNOS at the sarcolemma, exhibited much less NO release after stretch [19]. These results suggest that mechanical loading, such as stretch, is a positive regulator of NOS expression and activity in myotubes and fully differentiated muscle.

In this report, we examine an *in vitro* model of disuse atrophy, where cells are cyclically stretched for 1 h/d for 2 or 5 days or stretched for 2 days and then remain inactive for three subsequent days, so we can evaluate the effect of stretch and the effect of cessation of stretch independent of external factors. The necessity of NO for myotube adaptations during and after stretch was assessed using a non-specific NOS inhibitor. We examined several key players (e.g., Akt, FOXO, calpain, caspase) implicated in many *in vivo* studies to determine if our *in vitro* atrophy model produced similar responses. We hypothesized that stretch-induced NO production contributes to myotube hypertrophy through Akt signaling, and cessation of stretch will cause atrophy by upregulating proteolysis. Our results demonstrate that cessation of repeated bouts of cyclic stretching induces atrophy and activation of calpain-dependent proteolysis in cultured myotubes. Further, we have previously shown that NO is involved in Akt regulation in response to calcium ionophore [20]. This study extends our observations to show that functional stretch-induced Akt activation is also NO-dependent.

2. Materials and methods

2.1. Myogenic culture

We used an immortal cell line derived from mouse skeletal muscle, called C2C12 (ATCC, Manassas, VA). These cells were originally obtained by Yaffe and Saxel through selective serial passage of myoblasts cultured from the thigh muscle of C3H mice 70 h after a crush injury [21]. These cells are capable of differentiation and are a widely used model to study differentiated skeletal muscle cells. Myoblasts were cultured on 100 mm dishes in Dulbecoo's Modified Eagle's Medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 0.1% funzigone at 37 °C in the presence of 5% CO2 until 50–60% confluence was reached as visualized by light microscopy. The cultures were then trypsinized and replated at equal density to 6-well Flexcell plates for mechanical stimulation as described below. Once the Flexcell plate cultures reached 60–80% confluency, myotube differentiation was initiated by switching to DMEM supplemented with 2% horse serum, 1% penicillin/streptomycin, and 0.1% fungizone.

2.2. Mechanical stimulation using cyclic strain

Myoblasts were plated on type I collagen-coated flexible-bottom plates (Bioflex plates, Flexcell International, McKeesport, PA) and incubated at 37 °C in an incubator maintained at 5% CO_2 until 90% myotube population was visualized. Immediately prior to the initiation of stretch, the medium was aspirated and changed to differentiation medium supplemented with or without 5 mM L-NAME. The cells were subjected to cyclic strain at 0.7 Hz for 1 h using a computer-controlled vacuum stretch apparatus (FX-4000T Tension Plus System, FlexCell International) with a vacuum pressure that is sufficient to generate to predetermined percentage of mechanical strain. Replicate control samples and cells undergoing cessation of stretch were maintained under static conditions with no applied cyclic strain.

The Flexcell system used for our stretch apparatus employed equibiaxial deformations to the membranes on the bottom of each culture plate. Equibiaxial stretch is the preferred model of *in vitro* deformation because a myotube adhered in any direction is subject the same shape change. Although the Flexcell Bioflex software uses percentile stretch to calibrate the vacuum necessary to deform the elastic membrane, each cell is not exposed to the same percent of stretch. The percent stretch would depend on the orientation and length of the myotube. Larger myotubes would be stretched a smaller percent than those of a lesser size. Specifically, the 12% stretch corresponds to a 4.2 mm stretch equal in all directions. While the orientation of the myotube on the membrane would still affect the direction of deformation, the myotubes had a tendency to align in the same plane with maturation and exposure to stretch as observed previously [22]. We have previously reported that cyclic stretching or L-NAME treatment of C2C12 cells does not reduce cell viability as measured by trypan blue exclusion [23]. Further, in the present study, counting of DAPI-stained nuclei indicate no differences in the total number of nuclei between groups (data not shown).

2.3. Imaging and immunohistochemistry

Twenty-four hours after the last bout of stretch, myotubes were fixed in 2% paraformaldehyde for 10 min and washed twice with PBS. All cultures were viewed and digital images captured using a Zeiss inverted microscope (Axiovert 200). Three digital images per culture were captured. The images were analyzed for myotube length, diameter and area using ImageJ imaging software (NIH).

After fixing in paraformaldehyde, myotubes were washed twice with PBS, and membranes were permeabilized for 15 min with 0.2% Triton-X. Myotubes were blocked in 5% goat serum for 30 min, followed by incubation in primary antibody diluted in 0.5% BSA for dystrophin (Lab Vision Corporation), MHC type IIa (1:50; N2.261 Developmental Studies Hybridoma Bank), nNOS (1:133; BD Transduction Labs), eNOS (1:133; BD Transduction Labs), or iNOS (1:133; BD Transduction Labs) for 1 h. Myotubes were then incubated in secondary antibody diluted in 5% Pierce Super Blocker (Pierce Biotechnology) for Rhodamine (1:40, Invitrogen) or Alexa 488 IgG (1:133, Invitrogen) for 1 h and stained with DAPI-containing mounting media (Vector Laboratories). All cultures were viewed on a Zeiss microscope with rhodamine, FITC, and DAPI filters.

2.4. Western blot analysis

Cells were harvested in ice-cold non-denaturing lysis (NDL) buffer containing 30 mM Tris–HCl (pH 7.5), 0.7% Triton-X, 150 mM NaCl, 3.5 mM EDTA, 10 mg/ml NaN_3, 1 μM Na_3VO_4, 0.05% vol/vol protease inhibitors and 0.5% vol/vol phosphatase inhibitors (Sigma, St. Louis). Lysates were then centrifuged at 4 °C for 10 min at 1000*g*. Protein concentrations were measured using the D_C Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). Aliquots of whole cell lysate were run on SDS–PAGE gels and proteins were transferred to nitrocellulose membranes and blocked with Odyssey

blocking buffer for 1 h. The membranes were incubated at 4 °C overnight in primary antibody diluted with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE), TBS, and 0.01% Tween-20 for phospho-GSK-3β (Santa Cruz; Santa Cruz, CA), total GSK (Santa Cruz; Santa Cruz, CA), phospho-AKT (Santa Cruz; Santa Cruz, CA), total AKT (Santa Cruz; Santa Cruz, CA), phospho-FOXO3a (Ser253; Cell Signaling Technology #9466) and β-actin (Abcam; Cambridge, MA). Membranes were washed with TBS-T three times and incubated for 35 min in secondary antibody, Odyssey blocking buffer, and TBS-T. The secondary antibodies were IR Dye conjugated secondaries obtained from LICOR detectable at wavelengths of 680 or 800 nm. Membranes were washed three times with TBS-T and once with TBS before being scanned and detected using the Odyssey infrared imaging system (LI-COR). Boxes were manually placed around each band of interest, which returned near-infrared fluorescent values of raw intensity with intra-lane background subtracted using Odyssey 3.0 analytical software (LiCor, Lincoln, NE).

2.5. Nitric oxide production

Intracellular NO was monitored with DAF-FM diacetate (Invitrogen, Carlsbad,CA), a pH-insensitive fluorescent dye that emits increased fluorescence after reaction with an active intermediate of NO formed during the spontaneous oxidation of NO to NO_2. Myotubes were incubated at 37 °C for 30 min in phenol red-free, serum-free DMEM containing 10 μM of DAF-FM diacetate. After loading was completed, cells were rinsed three times with phenol red-free, serum-free DMEM and subjected to 1 h of mechanical strain. Cells were harvested in deionized water, centrifuged at 12,000*g* and 100 μl aliquots of lysate were quantified on a microplate fluorometer (Molecular Devices) using excitation and emission wavelengths of 488 and 520 nm, respectively.

2.6. Statistical analysis

Group sample size was determined with power analysis of our preliminary data. Comparisons between groups were made by a 3-way full factorial ANOVA, and when appropriate, Tukey's HSD test was performed post hoc. Intracellular NO assay was compared by linear regression. Significance was established at $P < 0.05$.

3. Results and discussion

3.1. Myotube characteristics

After 6 d of differentiation, immunohistochemistry revealed that C2C12 myotubes express type IIa myosin heavy chain (Suppl. Fig. 1). This is in agreement with other studies in C2C12s demonstrating that myotubes begin expressing all isoforms of MHC after 3 d of differentiation (40). Dystrophin staining is also evident throughout the C2C12 myotubes (Suppl. Fig. 1). Dystrophin and the glycoprotein complex with which it is associated play a fundamental role in mechanotransduction during stretch and provide structural integrity for the cell. Thus, the presence of dystropin in normally differentiated C2C12 myotubes was critical for testing our hypotheses of myotube adaptation to cyclic strain and inactivity. The presence and localization of the nitric oxide synthases in C2C12s are shown by immunostaining for nNOS, iNOS, and eNOS after 6 d of differentiation (Suppl. Fig. 1). Both nNOS and iNOS appear to be localized in clusters near the cell membrane whereas eNOS is expressed more diffusely throughout the cytosplasm.

3.2. Effect of cyclic stretch on nitric oxide production

Because cyclic stretch in culture and mechanical strain *in vivo* have been shown to increase nNOS expression and NO production [16,24], we sought to determine if different magnitudes of cyclic stretch would increase NO production in C2C12 myotubes. To test this hypothesis, myotubes were loaded with DAF-FM diacetate for 30 min and subjected to 1 h of mechanical strain at various magnitudes. The results show that there is a stepwise increase of NO production with increasing magnitudes of stretch from 6 to 18% (Fig. 1). Only 18% stretch was significantly higher than no-stretch controls (+1.3-fold, $P < 0.05$). However, it has been suggested that 18% stretch may induce inflammatory pathways and iNOS expression in culture [25,26], so we chose to use 12% stretch for the remainder of the experiments.

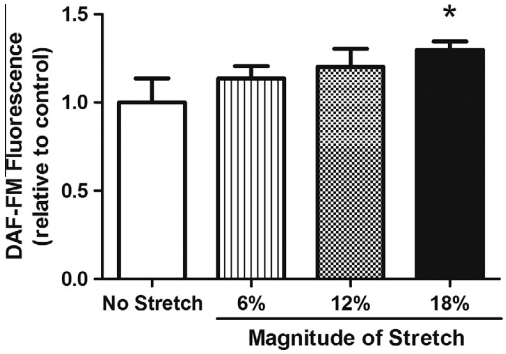

Fig. 1. Average 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate fluorescence in C2C12 myotubes after 1 h of cyclic stretch at various magnitudes. Values represent the mean ± SEM ($n = 3$). *Significantly different from no-stretch control ($P < 0.05$).

3.3. Effect of cyclic stretch and cessation of stretch on myotube size

Two days of 12% cyclic stretch for 1 h/d did not alter average length, diameter or area of C2C12 myotubes (Suppl. Fig. 2 and Fig. 2); however, cells undergoing stretch for 5 days experienced a significant increase in myotube diameter and area (>2-fold,

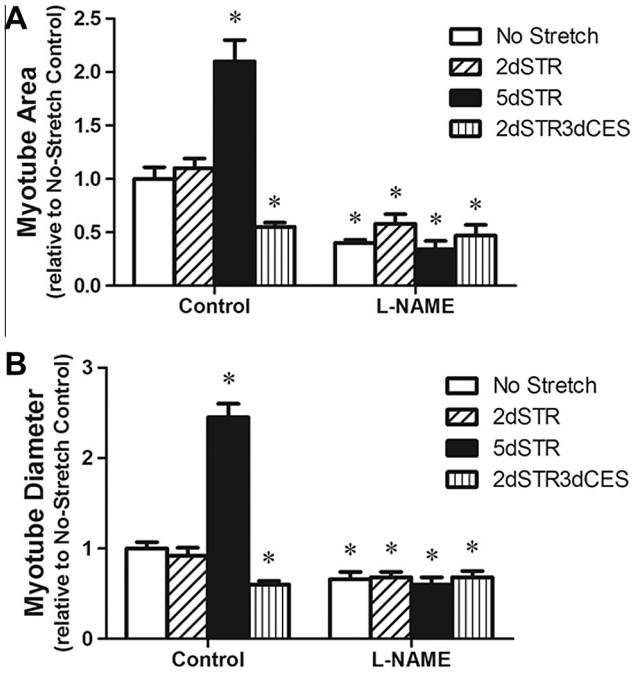

Fig. 2. Image analysis of C2C12 myotubes: (A) average myotube area, (B) average myotube diameter. Values represent the mean ± SEM ($n = 35$). *Significantly different from no-stretch control ($P < 0.05$).

$P < 0.05$). Importantly, cessation of stretch for 3 days caused a significant reduction in myotube diameter and area in comparison to all no-stretch control and stretched groups, representing skeletal muscle myotube atrophy. Treatment with the NOS inhibitor L-NAME led to diameter and area measurements similar to those of atrophied myotubes, which were significantly lower than non-treated cells across all groups. The L-NAME results suggest that NOS activity is essential for myotube growth independent of stretch.

3.4. Effect of stretch cessation on protein degradation

Muscle atrophy is predominantly due to increased proteolysis [3]. Because image analysis of C2C12 myotube morphology (i.e. myotube area and diameter) showed evidence of myotube atrophy and activation of calpain proteases is believed to initiate muscle catabolism [4], we sought to determine if calpain activity was elevated in myotubes during cessation of stretch. Two cytosketetal muscle proteins important for sarcomeric structural integrity and susceptible to cleavage by calpains, αII-spectrin and talin, were measured. The 150 kDa cleavage product of αII-spectrin and the 190 kDa cleavage product of talin were significantly elevated following 3 days of mechanical inactivity *in vitro* (2dSTR3dCES, Fig. 3). L-NAME prevented the increase in αII-spectrin and talin cleavage products in the 2dSTR3dCES group implicating excess NO production in the activation of proteolysis during inactivity, which has been previously suggested by Suzuki et al. [1]. Conversely, L-NAME treatment increased talin cleavage in 2-d cultures, which agrees with another report showing that NO can prevent calpain-mediated talin proteolyisis in C2C12 cells [27]. Nitric oxide has been shown to inhibit calpain activity via S-nitrosylaton of an active site cysteine, and the NO donor sodium nitroprusside was sufficient to prevent vinculin and talin degradation after calcium ionophore-treatment in C2C12 myoblasts [27]. Thus, endogenous nitric oxide production may be protective against calpain-induced proteolysis. Additionally, since nitric oxide production is enhanced by cyclic stretch (Fig. 1), this may partly explain why cytoskeletal proteolysis by calpain was reduced by mechanical activity in our model. This unusual effect of L-NAME (i.e. preventing cleavage of αII-spectrin and talin during cessation of stretch, while increasing cleavage of talin in control and 2 d stretch cultures) suggests a dual role of nitric oxide, inhibiting calpain proteolysis in basal or active conditions, and activating calpain proteolysis during inactivity.

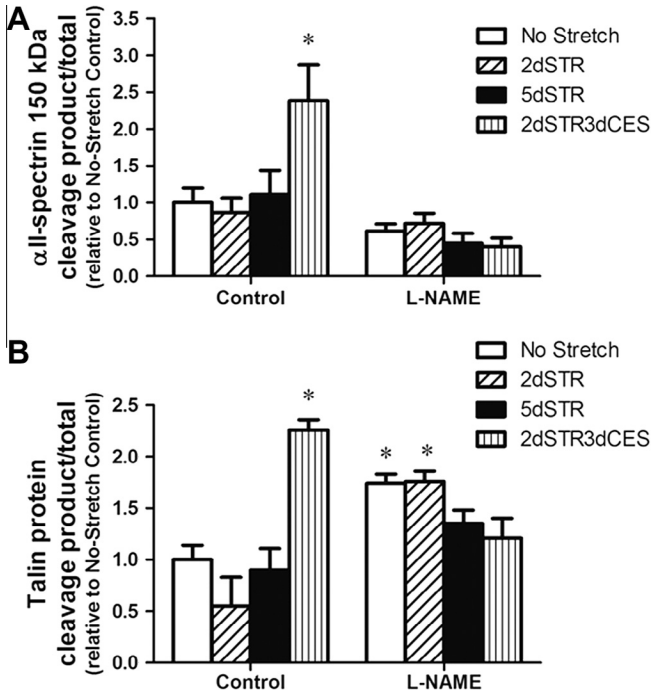

Fig. 3. Cytoskeletal protein degradation products in of C2C12 myotubes using the atrophy protocol. Graphs represent ratio of cleaved to intact protein: (A) calpain-specific 150 kDa αII-spectrin cleavage product, (B) calpain-specific 190-kDa talin cleavage product. Values represent the mean ± SEM ($n = 5$). *Significantly different from no-stretch control ($P < 0.05$).

Although our data provide evidence for an increase in proteolysis with cessation of stretch, there was no change in protein content in myotubes after withdrawal from activity (Suppl. Fig. 3) and no change in protein concentrations with stretch. On the other hand, treatment of myotubes with L-NAME significantly reduced protein content in the 2dSTR3dCES + LN group. This result is surprising considering that, collectively, our data indicate that 2 days of stretch followed by 3 days of inactivity is sufficient to induce cytoskeletal protein degradation and visual atrophy of C2C12 myotubes.

3.5. Effect of cessation of stretch on Akt signaling

The Akt pathway is known to regulate muscle protein synthesis and is a master controller of skeletal muscle size by also playing a role in skeletal muscle atrophy [9,28], and NO has also been shown to be involved in Akt activation in a PI3-kinase dependent manner [20]. To determine if Akt signaling is altered with changes in stretch activity *in vitro*, phosphorylated-to-total Akt and its downstream targets, FOXO3a and GSK-3β, were measured for 48 h following 2 d of repeated bouts of stretch.

Akt activation was increased ~5-fold following the 2 consecutive days of cyclic stretch (Fig. 4A, stretched). This elevation was transient as it returned to baseline after 48 h of cessation of stretch. Notably, NOS inhibition significantly blunted activation of Akt at all time points, indicating that phosphorylation of Akt in C2C12 myotubes is NOS-dependent. The results for phosphorylated-FOXO3a (p-FOXO3a) closely resemble the pattern for p-Akt (Fig. 4B), which was expected because FOXO3a is phosphorylated and inactivated by Akt. Immediately after the stretch bout on day two, p-FOXO3a was significantly elevated and tapered as time elapsed. Nitric oxide synthase inhibition completely abrogated the increase in FOXO3a phosphorylation, again demonstrating the importance of NO in this signaling pathway. GSK-3β is activated by its dephosphorylation and translocation to the nucleus where it is known to trigger numerous transcription factors. Many of its targets are thought to promote transcription of atrophy-related genes [29]; thus, we sought to determine if GSK-3β activation was altered in our stretch model. Two days of 12% cyclic stretch significantly induced inhibitory GSK-3β phosphorylation, while the ratio of phosphorylated-to-total GSK-3β tended to decrease over time with cessation of stretch (Fig. 4C). Interestingly, L-NAME administration to the media prevented stretch-induced phosphorylation of GSK-3β until 48 h post-stretch. Altogether, the anabolic effects of the Akt pathway are upregulated after 2 d of moderate magnitude cyclic stretch, and the upregulation of Akt with stretch is dependent on NOS activity, as L-NAME blocked both phosphorylation of Akt and its substrate GSK-3β. In the absence of muscle activity for 48 h, Akt is inactivated, leading to dephosphorylation of FOXO transcription factors. As a result, dephosphorylated FOXOs enter into the nucleus and activate atrophy-inducing genes [9]. During cessation of cyclic stretch, we observed a decline in p-Akt with a concomitant increase in dephosphorylated FOXO3a, which suggests that reduced activity *in vitro* turns off signaling for muscle hypertrophy, also required for maintenance of muscle mass, and turns on signaling for protein degradation and muscle atrophy

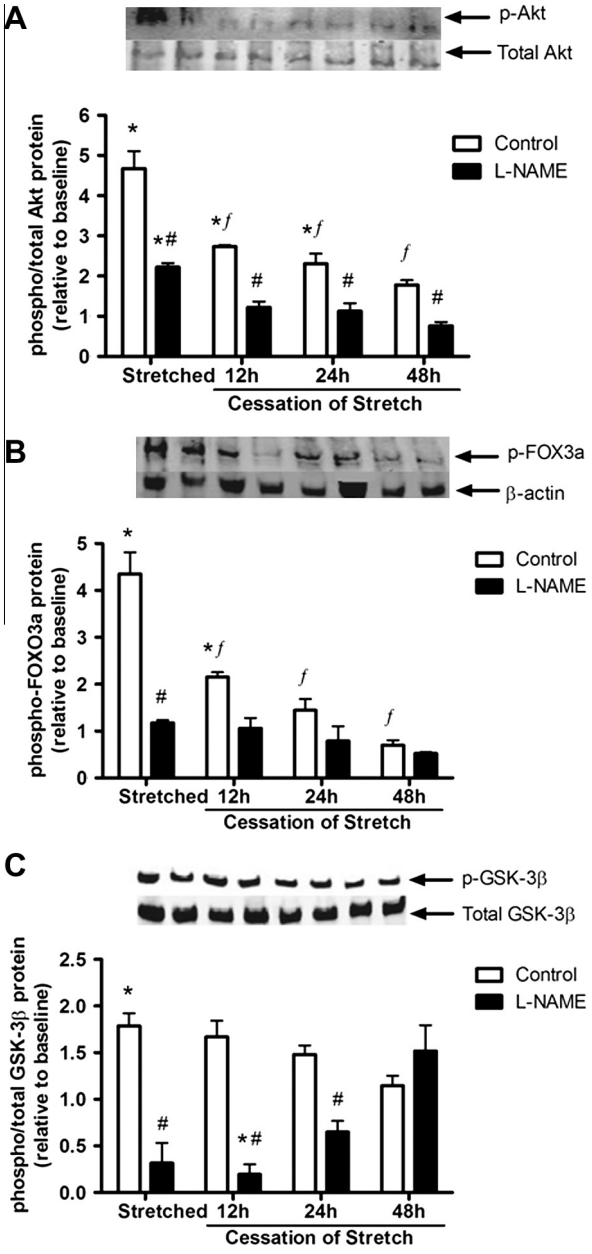

Fig. 4. Protein expression of Akt, FOXO3a, and GSK-3β in C2C12 myotubes 12, 24, and 48 h after 2 d of 12% stretch: (A) ratio of phosphorylated-to-total Akt, (B) phosphorylated-FOXO3a, (C) phosphorylated-to-total GSK-3β. Values represent the mean ± SEM ($n = 3$). *Significantly different from baseline, #Significantly different from control, ^{f}Significantly different from stretched control ($P < 0.05$).

[30]. Treatment with the NOS-inhibitor, L-NAME, accelerated the Akt/FOXO3a response; again, suggesting a role for NO in regulation of muscle size.

In conclusion, this study describes an intrinsic model of *in vitro* skeletal muscle myotube atrophy by withdrawal from daily bouts of cyclic stretch. First, we show that cyclic mechanical stretch mimics muscle loading in cultured C2C12 myotubes, and increases myotube size through NOS-dependent induction of Akt signaling. Second, cessation of cyclic stretch causes protein degradation, a reduction in myotube size, and downregulation of Akt signaling. Our results demonstrate an *in vitro* model of atrophy independent of external factors and provide evidence to better understand the signaling pathways involved during changes in skeletal muscle loading patterns.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.048.

References

[1] N. Suzuki, N. Motohashi, A. Uezumi, S. Fukada, T. Yoshimura, Y. Itoyama, M. Aoki, Y. Miyagoe-Suzuki, S. Takeda, NO production results in suspension-induced muscle atrophy through dislocation of neuronal NOS, J. Clin. Invest. 117 (2007) 2468–2476.
[2] T.A. Rando, The dystrophin–glycoprotein complex, cellular signaling, and the regulation of cell survival in the muscular dystrophies, Muscle Nerve 24 (2001) 1575–1594.
[3] D.B. Thomason, R.B. Biggs, F.W. Booth, Protein metabolism and beta-myosin heavy-chain mRNA in unweighted soleus muscle, Am. J. Physiol. 257 (1989) R300–R305.
[4] S.K. Powers, A.N. Kavazis, J.M. McClung, Oxidative stress and disuse muscle atrophy, J. Appl. Physiol. 102 (2007) 2389–2397.
[5] S.C. Bodine, E. Latres, S. Baumhueter, V.K. Lai, L. Nunez, B.A. Clarke, W.T. Poueymirou, F.J. Panaro, E. Na, K. Dharmarajan, Z.Q. Pan, D.M. Valenzuela, T.M. DeChiara, T.N. Stitt, G.D. Yancopoulos, D.J. Glass, Identification of ubiquitin ligases required for skeletal muscle atrophy, Science 294 (2001) 1704–1708.
[6] S. Frisch, E. Ruoslahti, Integrins and anoikis, Curr. Opin. Cell Biol. 9 (1997) 701–706.
[7] Y. Sonoda, S. Watanabe, Y. Matsumoto, E. Aizu-Yokota, T. Kasahara, Fak is the upstream signal protein of the phosphatidylinositol 3-kinase-Akt survival pathway in hydrogen peroxide-induced apoptosis of a human glioblastoma cell line, J. Biol. Chem. 274 (1999) 10566–10570.
[8] K. Langenbach, T. Rando, Inhibition of dystroglycan binding to laminin disrupts the PI3K/AKT pathway and survival signaling in muscle cells, Muscle Nerve 26 (2002) 644–653.
[9] M. Sandri, C. Sandri, A. Gilbert, C. Skurk, E. Calabria, A. Picard, K. Walsh, S. Schiaffino, S.H. Lecker, A.L. Goldberg, Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy, Cell 117 (2004) 399–412.
[10] T.N. Stitt, D. Drujan, B.A. Clarke, F. Panaro, Y. Timofeyva, W.O. Kline, M. Gonzalez, G.D. Yancopoulos, D.J. Glass, The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors, Mol. Cell 14 (2004) 395–403.
[11] J.E. Anderson, A role for nitric oxide in muscle repair: nitric oxide-mediated activation of muscle satellite cells, Mol. Biol. Cell 11 (2000) 1859–1874.
[12] G.J. Etgen Jr., D.A. Fryburg, E.M. Gibbs, Nitric oxide stimulates skeletal muscle glucose transport through a calcium/contraction- and phosphatidylinositol-3-kinase-independent pathway, Diabetes 46 (1997) 1915–1919.
[13] L. Kobzik, M.B. Reid, D.S. Bredt, J.S. Stamler, Nitric oxide in skeletal muscle, Nature 372 (1994) 546–548.
[14] R.W. Grange, E. Isotani, K.S. Lau, K.E. Kamm, P.L. Huang, J.T. Stull, Nitric oxide contributes to vascular smooth muscle relaxation in contracting fast-twitch muscles, Physiol. Genomics 5 (2001) 35–44.
[15] L.W. Smith, J.D. Smith, D.S. Criswell, Involvement of nitric oxide synthase in skeletal muscle adaptation to chronic overload, J. Appl. Physiol. 92 (2002) 2005–2011.
[16] J.G. Tidball, E. Lavergne, K.S. Lau, M.J. Spencer, J.T. Stull, M. Wehling, Mechanical loading regulates NOS expression and activity in developing and adult skeletal muscle, Am. J. Physiol. 275 (1998) C260–C266.
[17] T.W. Balon, J.L. Nadler, Evidence that nitric oxide increases glucose transport in skeletal muscle, J. Appl. Physiol. 82 (1997) 359–363.
[18] P.J. Reiser, W.O. Kline, P.L. Vaghy, Induction of neuronal type nitric oxide synthase in skeletal muscle by chronic electrical stimulation in vivo, J. Appl. Physiol. 82 (1997) 1250–1255.
[19] A.C. Wozniak, J.E. Anderson, The dynamics of the nitric oxide release-transient from stretched muscle cells, Int. J. Biochem. Cell Biol. 41 (2009) 625–631.
[20] J.A. Drenning, V.A. Lira, Q.A. Soltow, C.N. Canon, L.M. Valera, D.L. Brown, D.S. Criswell, Endothelial nitric oxide synthase is involved in calcium-induced Akt signaling in mouse skeletal muscle, Nitric Oxide 21 (2009) 192–200.
[21] D. Yaffe, O. Saxel, A myogenic cell line with altered serum requirements for differentiation, Differentiation 7 (1977) 159–166.
[22] A.M. Collinsworth, C.E. Torgan, S.N. Nagda, R.J. Rajalingam, W.E. Kraus, G.A. Truskey, Orientation and length of mammalian skeletal myocytes in response to a unidirectional stretch, Cell Tissue Res. 302 (2000) 243–251.
[23] Q.A. Soltow, V.A. Lira, J.L. Betters, J.H. Long, J.E. Sellman, E.H. Zeanah, D.S. Criswell, Nitric oxide regulattes strtetch-induced proliferation in C2C12 myoblasts, J Muscle Res Cell Motil. 31 (2010) 215–225.
[24] J.S. Zhang, W.E. Kraus, G.A. Truskey, Stretch-induced nitric oxide modulates mechanical properties of skeletal muscle cells, Am. J. Physiol. Cell Physiol. 287 (2004) C292–299.
[25] S. Agarwal, J. Deschner, P. Long, A. Verma, C. Hofman, C.H. Evans, N. Piesco, Role of NF-kappaB transcription factors in antiinflammatory and proinflammatory actions of mechanical signals, Arthritis Rheum. 50 (2004) 3541–3548.
[26] S. Agarwal, P. Long, A. Seyedain, N. Piesco, A. Shree, R. Gassner, A central role for the nuclear factor-kappaB pathway in anti-inflammatory and proinflammatory actions of mechanical strain, FASEB J. 17 (2003) 899–901.

[27] T.J. Koh, J.G. Tidball, Nitric oxide inhibits calpain-mediated proteolysis of talin in skeletal muscle cells, Am. J. Physiol. Cell Physiol. 279 (2000) C806–C812.

[28] B. Leger, R. Cartoni, M. Praz, S. Lamon, O. Deriaz, A. Crettenand, C. Gobelet, P. Rohmer, M. Konzelmann, F. Luthi, A.P. Russell, Akt signalling through GSK-3beta, mTOR and Foxo1 is involved in human skeletal muscle hypertrophy and atrophy, J.Physiol. 576 (2006) 923–933.

[29] L. Zhao, J.Y. Lee, D.H. Hwang, The phosphatidylinositol 3-kinase/Akt pathway negatively regulates Nod2-mediated NF-kappaB pathway, Biochem. Pharmacol. 75 (2008) 1515–1525.

[30] J. Zhao, J.J. Brault, A. Schild, P. Cao, M. Sandri, S. Schiaffino, S.H. Lecker, A.L. Goldberg, FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells, Cell Metab. 6 (2007) 472–483.