Gene expression during inactivity-induced muscle atrophy: effects of brief bouts of a forceful contraction countermeasure

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Kim SJ, Roy RR, Kim JA, Zhong H, Haddad F, Baldwin KM, Edgerton VR. Gene expression during inactivity-induced muscle atrophy; effects of brief bouts of a forceful contraction countermeasure. J Appl Physiol 105: 1246–1254, 2008. First published July 24, 2008; doi:10.1152/japplphysiol.90668.2008.—Anabolic and catabolic markers of muscle protein metabolism were examined in inactivity-induced atrophying muscles with and without daily short-duration, high-resistance isometric contractions. Inactivity was achieved via spinal cord isolation (SI), which results in near inactivity of the hindlimb musculature without compromising the motoneuron-muscle connectivity. Adult rats were assigned to a control (Con) or SI group in which one limb was stimulated (SI-Stim, 5 consecutive days of brief bouts of high-load isometric contractions) while the other served as a SI control (SI). Both the medial gastrocnemius (MG) and soleus weights (relative to body weight) were ~71% of Con in the SI, but maintained at Con in the SI-Stim group. Activity of the IGF-1/phosphatidylinositol 3-kinase (PI3K)/Akt pathway of protein synthesis was similar among all groups in the MG. Expression of atrogin-1 and muscle RING finger-1 (MuRF-1), markers of protein degradation, were higher in the MG and soleus of the SI than Con and maintained at Con in the SI-Stim. Compared with Con, the anti-growth factor myostatin was unaffected in the MG and soleus in the SI but was lower in the MG of the SI-Stim. These results demonstrate that upregulation of specific protein catabolic pathways plays a critical role in SI-induced atrophy, while this response was blunted by 4 min of daily high-resistance electromechanical stimulation and was able to preserve most of the muscle mass. Although the protein anabolic pathway (IGF-1/PI3K/Akt) appears to play a minor role in regulating mass in the SI model, increased translational capacity may have contributed to mass preservation in response to isometric contractions.

SKELETAL MUSCLE MASS is highly adaptive to changes in the level of neuromuscular activity and mechanical loading. For example, periodic resistance exercise training can promote muscle hypertrophy (8), whereas periods of reduced activity and/or loading result in muscle atrophy (1, 2, 14, 34). Furthermore, resistance exercise has been shown to be an effective strategy to counteract muscle atrophy in models of reduced neuromuscular activity (11, 14, 20, 32). These adaptations in muscle mass are achieved via a modulation of the molecular signaling cascades that influence both protein catabolic and anabolic pathways.

We hypothesize that forceful contractions play an important role in maintaining protein balance in skeletal muscle. Activation of the IGF-1/phosphatidylinositol 3-kinase (PI3K)/Akt pathway has been identified as a key signaling cascade in the hypertrophy response to exercise by upregulating the protein translational capacity of the muscle (7, 30). The importance of this signaling pathway in the regulation of muscle mass is further demonstrated by its downregulation under muscle atrophying conditions, e.g., hindlimb unloading (HU) (14, 18). Skeletal muscle atrophy in response to HU and denervation is also associated with an upregulation in the expression of catabolic markers, specifically atrogin-1, muscle RING finger-1 (MuRF-1), and myostatin (6, 14, 34). Atrogin-1 and MuRF-1 are two key E3 ligases involved in the ubiquitin proteasome pathway required for identifying and attaching ubiquitin to proteins to be degraded (6). In contrast, UBE3C, another E3 ligase abundantly expressed in skeletal muscle, is not altered by HU or denervation-induced muscle wasting (6) and may not be a general marker of atrophy. Furthermore, denervation-induced muscle atrophy is partially blunted in mice deficient in atrogin-1 and MuRF-1 gene expression (6). Together, these data suggest that both anabolic and catabolic pathways have a profound impact on the net protein status of a muscle and that both mechanisms are important in the regulation of muscle mass.

The purpose of the present study was to identify potential candidate molecular markers that may be involved in the atrophic processes induced by an almost total absence of electromechanical events and the subsequent response of these same markers when reintroducing very brief electromechanical activity that is sufficient to blunt atrophy (20). Atrophy of the medial gastrocnemius (MG) and soleus was induced using the spinal cord isolation model (SI). SI results in a near-zero baseline of electromechanical activity in the hindlimb musculature by surgically preventing hindlimb motoneurons from receiving electrical input while maintaining the integrity of the motoneuron muscle connection (33). Electromechanical stimulation was used to administer a high-load isometric contraction paradigm with a total activity duration of 4 min/day to prevent atrophy. The IGF-1/PI3K/Akt pathway and its downstream effectors [p70S6k and eukaryotic initiation factor 4E binding protein (4EIBP)] were examined as anabolic markers of protein regulation. The ubiquitin ligases, atrogin-1 and MuRF-1, and the antigrowth transcription factor myostatin were examined as catabolic markers of protein regulation as these are key markers upregulated under atrophic conditions (6, 14, 34).
Based on previous electromechanical stimulation studies (14, 20, 32), we hypothesized that a high-force isometric stimulation training countermeasure would be effective in blunting SI-induced atrophy. Furthermore, we hypothesized that SI-induced atrophy would be characterized by a marked downregulation in the activity of anabolic markers and an upregulation in the expression of catabolic markers of protein regulation, thus resulting in a net catabolic state. We also hypothesized that very brief periods of electromechanical activations would be sufficient to reverse these adaptations and maintain the expression of protein anabolic and catabolic markers near control levels, thus maintaining a more normal homeostatic balance in protein turnover and accounting for the amelioration in muscle atrophy. The results demonstrate that in contrast to other atrophy models, e.g., HU and denervation, the IGF-1/PI3K/Akt pathway appears to play a less obvious role in SI-induced atrophy. The adaptations in the expression of catabolic markers following atrophy and stimulation training were consistent with our hypotheses.

MATERIALS AND METHODS

Experimental groups. Adult female Sprague-Dawley rats (235 ± 1 g body wt, Charles River, Wilmington, MA) were assigned randomly to a normal control (Con, n = 6) or SI (n = 6) group. SI rats underwent spinal cord isolation surgery and were implanted unilaterally with a wireless microstimulator (Alfred Mann Foundation, Santa Clarita, CA). The implanted limb of each rat in the SI group received electromechanical stimulation training (SI-Stim group) beginning the day after surgery, while the contralateral hindlimb served as an internal control (SI group). A previous study demonstrated that the implant had no adverse effects on the hindlimb muscles (20).

Surgical procedures and animal care. The SI surgery procedure was a modification (13, 20) of the original protocols of Tower (38). Briefly, the spinal cord was transected completely at a midthoracic and a high-sacral spinal cord level, and a subdural bilateral dorsal rhizotomy was performed between the two transection sites. During the same surgery, SI rats were implanted unilaterally along the sciatic nerve with a BION microstimulator as described previously (20). The proper placement of the BION was verified for each rat in a terminal experiment. All surgical procedures were performed under aseptic conditions.

Postsurgical care and maintenance procedures for spinal cord-injured animals have been detailed previously (20). The animals had access to food and water ad libitum throughout the study. Throughout the study there was no response to reflex testing or toe pinching, and the hindlimbs of the SI rats remained completely flaccid. All procedures were approved by the UCLA Chancellor’s Animal Research Committee and followed the American Physiological Society Animal Care Guidelines.

Electromechanical stimulation. The BION microstimulator (Alfred Mann Foundation) is a wireless implantable (2-mm diameter × 16-mm length) device designed for functional electrical stimulation (24). It receives power and stimulation commands (pulse width, pulse amplitude, frequency, stimulation duration, and cycle time) via a 2-MHz magnetic field generated by an external radiofrequency coil.

During electromechanical stimulation training of the SI rats, the limb implanted with a BION microstimulator (SI-Stim) was secured in a removable cast (ankle at ~90° and knee at ~120°) to optimize isometric contractions of the muscles functioning at the ankle and/or knee. A force transducer embedded in the plantar surface of the cast enabled quantification of the torque at the ankle produced by each stimulation and was used to monitor tension production to ensure consistent contractions throughout each stimulation bout. During each bout of activity, pulses were delivered once every 30 s for 5 min, followed by 5 min of rest (during which the limb was removed from the cast). Three consecutive bouts of activity were administered twice per day, separated by a 9-h interval. Each stimulation was delivered at a frequency of 100 Hz and a duration of 4 s. The stimulation pulse width was maintained at 50 μs, and the threshold pulse amplitude (μA) was determined daily by doubling the minimum pulse amplitude necessary to produce a maximum twitch response. This resulted in a total activity duration of 4 min/day. Daily stimulation training began the day after surgery and continued for 5 consecutive days.

Tissue harvesting. Approximately 24 h after the last stimulation treatment, the MG and soleus muscles were removed bilaterally, trimmed of fat and connective tissue, and weighed (wet weight). All muscles were quick-frozen between blocks of dry ice and stored at −80°C until processed.

Total protein and DNA isolation. As previously described by Haddad et al. (15), a portion from the midbelly of the MG (mixed region) was cut, weighed, and homogenized in 20 μl of ice-cold homogenization buffer (250 mM sucrose, 130 mM KCl, 5 mM EDTA, and 10 mM Tris–HCl, pH 6.8). A known volume of the total homogenate was used for myofibrillar protein extraction using a modification of the original protocols of Solaro et al. (37). The protein concentration of the total homogenate and myofibrillar suspension were determined by the Bio-Rad protein assay using gamma globulin as a standard and used to calculate the muscle total protein and myofibrillar protein concentrations. DNA concentration was measured using a volume of the total homogenate and a fluorometric assay (21) and then used to calculate the total DNA concentration. Total protein, myofibrillar, and DNA content were determined based on their concentration values multiplied by the MG whole muscle wet weight. Due to the small size of the soleus muscle, it was only processed for total RNA extraction and specific mRNA analysis via RT-PCR (see below).

Total RNA isolation. Total RNA was extracted from a preweighed portion of the midbelly of the frozen MG and soleus as previously described by Haddad et al. (15) using the TRI reagent (Molecular Research Center, Cincinnati, OH) according to the company’s protocol. Extracted RNA was precipitated from the aqueous phase with isopropanol, washed with ethanol, dried, and then suspended in a known volume of nuclease-free water. The RNA concentration was determined by optical density at 260 nm. The muscle total RNA concentration was calculated based on total RNA yield and the weight of the muscle portion analyzed. The extracted RNA samples were stored at −80°C until used for RT reactions.

RT. For each MG and soleus muscle sample, 1 μg of total RNA was reverse transcribed using the SuperScript II RT from Invitrogen (Carlsbad, CA) and a mix of oligo(dT) (100 ng/reaction) and random primers (200 ng/reaction) in a total reaction volume of 20 μl at 44°C for 50 min, according to the manufacturer’s protocol. At the end of the RT reaction, the tubes were heated for 5 min at 90°C to stop the reaction and stored at −80°C until used for PCR reactions for specific mRNA analyses.

PCR. A relative RT-PCR method, using 18S as an internal standard (Ambion, Austin, TX), was applied to study the expression of specific mRNAs for insulin-like growth factor-1 (IGF-1), IGF binding proteins (BP-4 and BP-5), mechanogrowth factor (MGF), atrogin-1, MuRF-1, and myostatin. The sequences for the various primers used for the specific target mRNAs have been described previously (14, 16) and were purchased from Operon Biotechnologies (Huntsville, AL). In each PCR reaction, the 18S ribosomal RNA was coamplified with the target cDNA (mRNA) as an internal standard.

The PCR procedures and quantification methods were conducted as described previously with all samples run in duplicates (1, 14). Briefly, for each target mRNA, the reactions were carried out under identical conditions by using the same reagent premix for all samples. One to two microliters of each RT reaction (0 to 15-fold dilution depending on target mRNA abundance) was used for the amplifica-
tion. PCRs were carried out in the presence of MgCl2, dNTP, the specific primer set, the 18S primer/competimer mix, and Biolase DNA polymerase (Bioline, Boston, MA) in 25-μl total volume. Amplifications were carried out in a Stratagene Robocycler. For each primer, the PCR conditions (premix concentrations and annealing temperature) were optimized so that both the target mRNA and 18S product yields were in the linear range of the semilog plot when the yield is expressed as a function of the number of cycles. PCR products were separated on a 2.5% agarose gel by electrophoresis and stained with ethidium bromide. Band intensities were quantified by densitometry with ImageQuant software and reported as arbitrary units (AU).

The 18S rRNA PCR signal was found to be similar across all experimental groups and therefore can be used as an internal reference. It was noted that although the total RNA concentration for some groups was decreased (soleus in the SI group) or increased (MG in the SI-Stim group), the corresponding signal for 18S rRNA per unit of total RNA remained the same, indicating that the rRNA expression ratio remained the same extent as the total RNA in these muscles.

Western blot analyses. Immunoblotting was used to analyze the expression of specific proteins [insulin receptor substrate-1 (IRS-1) (no. 2382) and its phosphorylation at Ser 307 (no. 2381), Akt/protein kinase B (no. 9272) and its phosphorylation at Thr 308 (no. 4056), Akt/protein kinase B (no. 9234), and 4EBP (sc-6034)] involved in muscle intracellular signaling. p-Thr 308 Akt (4) and p-Thr 389 p70S6K (31) have been implicated with increased biological activity, whereas p-Ser 307 IRS-1 (3) has been implicated with inhibition of insulin signaling. These antibodies were purchased from Cell Signaling Technology (Beverly, MA), with the exception of 4EBP which was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylation at the specified sites has been shown to strongly correlate with the biological activity in vivo. Muscle samples from the MG were extracted by homogenization using a motor-driven glass pestle as previously described (14). The enhanced chemiluminescence method was used for signal detection (Amersham, Piscataway, NJ) after incubations with the primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Signal intensity was determined by laser scanning densitometry (Molecular Dynamics/ImageQuant). For each specific antibody, all the samples were run under identical (previously optimized) conditions. The specificity of each antibody was judged based on comparison with the positive control to match the molecular weight of the target protein, as well as on the absence of the band in the negative control immunoblot, which consisted of incubation with preimmune serum instead of the specific antibody. Due to the number of samples analyzed, the samples were run on more than one gel for immunoblotting. Each gel contained representatives from each group with all samples run in duplicates, a positive control provided by the antibody supplier, and some repeated samples on each gel. The signal intensity from the latter was used for comparative purpose to allow normalization among the gels.

Statistical analyses. All data are presented as means ± SE. Group differences were determined by one-way ANOVA followed by the Newman-Keuls post hoc test using the Prism software package (GraphPad). The level of significance was set at P ≤ 0.05.

RESULTS

Body weights. Initial mean body weights were similar between the Con (235 ± 1 g) and SI (236 ± 1 g) groups. At the end of the 7-day study, the mean body weight of the Con group was unchanged (246 ± 3 g), whereas that of the SI group was decreased significantly (208 ± 4 g). Thus the rats in the SI group were significantly smaller than the Con group at the end of the study.

Muscle weights. The MG (Fig. 1A) and soleus (Fig. 1C) absolute weights were 41 and 39% smaller in the SI than Con group, respectively. Stimulation blunted atrophy by 57 and 50% in the MG and soleus muscles, respectively, and the absolute weights were significantly higher in the SI-Stim than SI group. The absolute weights of the SI-Stim group, however, remained smaller than Con. When the difference in body weight between groups was considered, the relative MG (Fig. 1B) and soleus (Fig. 1D) weights were 30 and 28% smaller in the SI than Con group, respectively, and were maintained at Con levels in the SI-Stim group. The MG and soleus relative weights were 39 and 32% larger in the SI-Stim than SI group, respectively.

Protein analyses of the MG. Both total protein (Fig. 2A) and myofilament protein (Fig. 2C) concentrations in the MG were similar among the Con, SI, and SI-Stim groups. When expressed per muscle weight, the total protein (Fig. 2B) and myofilament protein (Fig. 2D) contents were 44 and 50% lower in the SI than Con group, respectively. Stimulation had a positive effect on these measurements. In the SI-Stim group, total protein content was lower than Con, but 45% higher than in the Con group (Fig. 2B). The myofilament protein content in the SI-Stim group was higher than in the SI group and similar to that of the Con group (Fig. 2D).

RNA and DNA analyses. The total RNA concentration of the MG was unaffected in the SI group (Fig. 3A), whereas RNA content per muscle was 34% lower than in the Con group (Fig. 3B).
In contrast to the MG, both the total RNA concentration (Fig. 3C) and content (Fig. 3D) in the soleus were lower than Con, i.e., by 23 and 53%, respectively. Daily stimulation training had different effects on the MG and soleus muscles. In the MG of the SI-Stim group, the RNA concentration (Fig. 3A) was higher than in the other two groups, and the protein content (Fig. 3B) was at Con levels and higher than in the SI group. These values were less affected by stimulation in the soleus, i.e., RNA concentration (Fig. 3C) was similar in the SI-Stim and SI groups, whereas RNA content (Fig. 3D) was higher in the SI-Stim than SI group, but lower than in the Con group. DNA concentration in the MG (Fig. 3E) was 34% higher and DNA content (Fig. 3F) 21% lower in the SI compared with Con group. Both of these values remained at Con levels in the SI-Stim group.

Catabolic markers of protein balance. The mRNA levels of key catabolic markers of atrophy, atrogin-1 (Fig. 4A) and MuRF-1 (Fig. 4B), were four- to fivefold higher in both the MG and soleus of the SI than Con group. Both of these values were similar to Con in the SI-Stim group, with the exception of atrogin-1 in the soleus, which was lower than in the SI group but higher than in the Con group. In both muscles, the myostatin levels were similar in the SI and Con groups, whereas these levels were lower in the MG of the SI-Stim than in the other two groups (Fig. 4C).

Anabolic markers of protein balance. The IGF-1/P13K/Akt pathway and its downstream effectors (p70S6k and 4EBP) have been shown to be important players in the regulation of muscle mass via their ability to induce a positive protein balance. In the present study, despite the pronounced atrophy observed in the muscles of the SI group, the mRNA expression levels of IGF-1 (Fig. 5A) and its binding proteins BP-4 (Fig. 5C) and BP-5 (Fig. 5D), and its splice variant MGF (Fig. 5B) were similar to Con in the MG of both the SI and SI-Stim groups. In contrast, the expression levels in the soleus of each of these mRNAs were higher in the SI than Con group. In addition, the soleus IGF-1 (Fig. 5A) and MGF (Fig. 5B) mRNA levels in the SI-Stim group were higher than in the Con group, while BP-4 and BP-5 were similar to Con.

In the MG muscle, the phosphorylated levels of IRS-1 and Akt were similar across Con, SI, and SI-Stim groups (Figs. 6A and 7A). However, total expression of these proteins changed with atrophy. Total IRS-1 levels were 36% lower in the SI than Con group, and the expression levels were not different from Con and higher than in the SI group in the SI-Stim group (Fig. 6B). Total Akt levels were 56% and 34% higher in the SI and SI-Stim than Con group, with these levels being lower in the SI-Stim than SI group (Fig. 7B). The IRS-1 phosphorylated state (as determined by the ratio of phosphorylated to total levels) was ~3-fold higher in the SI than in the Con and SI-Stim groups (Fig. 6C). The phosphorylated state of Akt was lower in both the SI and SI-Stim groups compared with Con, and higher in the SI-Stim compared with SI group (Fig. 7C). The levels of phosphorylated p70S6k (Fig. 8A), the total p70S6k (Fig. 8B), the phosphorylated state of p70S6k (Fig. 8C), and total 4EBP (Fig. 8D) were similar among the three groups. The phosphorylated state of IRS-1 (as determined by the ratio of phosphorylated to total levels) was 3-fold higher in the SI than in the Con and SI-Stim groups (Fig. 6C). The phosphorylated state of Akt was lower in both the SI and SI-Stim groups compared with Con, and higher in the SI-Stim compared with SI group (Fig. 7C). The levels of phosphorylated p70S6k (Fig. 8A), the total p70S6k (Fig. 8B), the phosphorylated state of p70S6k (Fig. 8C), and total 4EBP (Fig. 8D) were similar among the three groups. The phosphorylated state of IRS-1 (as determined by the ratio of phosphorylated to total levels) was 3-fold higher in the SI than in the Con and SI-Stim groups (Fig. 6C). The phosphorylated state of Akt was lower in both the SI and SI-Stim groups compared with Con, and higher in the SI-Stim compared with SI group (Fig. 7C). The levels of phosphorylated p70S6k (Fig. 8A), the total p70S6k (Fig. 8B), the phosphorylated state of p70S6k (Fig. 8C), and total 4EBP (Fig. 8D) were similar among the three groups.

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groups, except for a lower level (14%) of total p70S6k in the SI-Stim than SI group (Fig. 8B).

DISCUSSION

The primary aim of the present study was to identify protein regulatory markers involved in skeletal muscle mass adaptations in response to a chronic absence of neuromuscular activity induced by SI and in the atrophy blunting effect of short bursts of activation. Brief bouts (a total of 4 min/day) of high-load, isometric contractions significantly ameliorated inactivity-induced atrophy of the MG and soleus hindlimb muscles. The increase in protein catabolic markers (i.e., atrogin-1 and MuRF-1) is consistent with their role in SI-induced atrophy (Fig. 9). This is further supported by the normalization of these transcripts in both the MG and soleus in response to very brief periods of electromechanical stimulation. Surprisingly, SI-induced atrophy of the MG was not associated with a downregulation of the protein anabolic IGF-1/PI3K/Akt pathway. Furthermore, the positive impact of stimulation on MG mass occurred independent of an upregulation of this known positive protein balance cascade (7, 30). However, the compromised cellular properties of the MG (decreased total protein, myofibril protein, RNA, and DNA content) associated with SI were blunted by the electromechanical stimulation paradigm. In addition, since ~85% of the RNA pool is ribosomal (rRNA) (5), the protein translational processes that are compromised during SI-induced atrophy were likewise positively impacted with the brief bouts of high-load contractions. This suggests that since the activity level of the IGF-1/PI3K/

Fig. 4. Expression levels of atrogin-1 (A), muscle RING finger-1 (MuRF-1; B), and myostatin (C) mRNAs in the MG and soleus muscles from Con, SI, and SI-Stim groups. Values are means ± SE. *Significant difference from Con group (P ≤ 0.05). †Significant difference from SI group (P ≤ 0.05).

Fig. 5. Expression levels of IGF-1 (A), mechanogrowth factor (MGF; B), and IGF binding proteins BP-4 (C) and BP-5 (D) mRNAs in the MG and soleus muscles from Con, SI, and SI-Stim groups. Values are means ± SE. *Significant difference from Con group (P ≤ 0.05). †Significant difference from SI group (P ≤ 0.05).
Akt pathway was not different from control in the SI-Stim group, it may have improved translational capacity. Together, these results highlight a significant role for both catabolic and anabolic processes in muscle mass regulation.

Regulation of myofibrillar protein. The negative protein balance of SI muscles is reflected in the reduced myofibrillar content, which primarily consists of myosin heavy chain (MHC) and actin proteins. This myofibril loss has been shown in the soleus to result from reduced levels of the transcriptional (pre-mRNA) and pretranslational (mRNA) capacity of the most abundant myofibrillar proteins, i.e., type I MHC and actin, at 7 days post-SI (16). Similar reductions have been observed at the same time point in all MHC and actin pre-mRNA and mRNA levels in the plantaris, a muscle which expresses both slow and fast adult MHC isoforms (Baldwin and Haddad, unpublished observations). These findings suggest that there is less substrate available for translation, and combined with the increased expression of atrogenes, leads to the net loss in protein content per fiber in muscles of SI rats. Also contributing to the negative protein balance is the decrease in total RNA content in both the MG and soleus muscles post-SI, reducing the machinery available to translate the already low levels of myofibrillar substrate. Resistance exercise is effective in partially maintaining the myofibrillar pool per muscle by blunting the increase in catabolic markers and increasing the MHC and actin transcriptional capacity to maintain sufficient substrate for translation (Baldwin and Haddad, unpublished observations). Furthermore, the RNA pools are maintained at a more optimal level, increasing translational capacity and creating a more positive protein balance as reflected in muscle mass and myofibril content.

Protein degradation markers. Atrogin-1 and MuRF-1 are ubiquitin ligases that have been identified as important players in protein degradation processes and have a critical role in the regulation of skeletal muscle mass. SI-induced atrophy upregulated atrogin-1 and MuRF-1 in the fast MG and slow soleus extensor muscles. The increased expression of these molecules...
contributing to muscle wasting is consistent with several other atrophy models, e.g., immobilization (6), HU (6, 14), and denervation (6, 34), and has been shown previously with SI (16, 34). The significance of atrogin-1 and MuRF-1 in regulating muscle mass is further demonstrated in that their maximal levels of expression correlate to the period during which the muscles underwent the most rapid loss of mass, i.e., the first 7–10 days (34).

Overexpression of atrogin-1 has been shown to be sufficient to induce muscle atrophy, whereas the genetic knockout of atrogin-1 and MuRF-1 partially attenuates denervation-induced muscle wasting (6). In the present study, daily administration of the electromechanical stimulation paradigm, which blunted muscle mass loss, maintained the expression of atrogin-1 and MuRF-1 at control levels in both the MG and soleus muscles. Thus the capability of the applied stimulation countermeasure to prevent the increased expression of atrogenes contributes to the positive effect on mass. Similar results were observed in muscles of HU rats that received a resistance exercise countermeasure (14). The regulation in expression of these atrogenes in response to exercise also has been shown in human skeletal muscles (25). These data demonstrate that the expression levels of these ubiquitin ligases correlate with changes in muscle mass, i.e., atrophy increased the expression of atrogin-1 and MuRF-1, whereas ameliorating the atrophy via electromechanical stimulation blunted this increase in atrogen gene expression.

A previous study has suggested fiber type specificity to MuRF-1 activity. Specifically, MuRF-1 has been implicated as a critical ligase for degrading the slower type I and IIA MHC isoforms, those found in the predominantly slow soleus muscle (12). The response of these markers to SI and stimulation, however, were similar in a predominantly slow and a predominantly fast extensor muscle in the present study. The myofibrillar atrophy observed in the present study in both the soleus and the MG muscle, which expresses a higher percentage of the faster IIx and IIB MHCs, suggests that the MuRF-1 system targets all adult MHC isoforms.

Myostatin is a transcription factor that serves as an anti-growth signal and is upregulated in atrophying muscles (14). When the activity of myostatin is compromised, either by gene disruption or pharmacological inhibition in mice, the skeletal muscles become significantly larger, resulting from fiber hypertrophy and/or hyperplasia (28, 39). Myostatin expression appears to have fiber type specificity, with higher expression levels in predominantly slow than fast muscles (35). Myostatin expression in the MG and soleus muscles was unaffected by SI, which is in stark contrast to the increase observed in the MG muscle after 5 days of HU (14). Muscle-specific differences in myostatin were observed in response to resistance exercise training in the SI model. High-load isometric contractions reduced myostatin expression below Con and SI levels in the fast MG whereas no change was observed in the soleus muscle. At this short 7-day period, differences in myostatin expression do not appear to have a major role in the muscle sparing response as the mass of both the soleus and MG was spared to a similar extent.

Protein synthesis markers. Several studies have identified the IGF-1/PI3K/Akt cascade and its downstream effectors (p70S6K and 4EBP) as positive modulators of protein synthesis and muscle mass. For example, activity of this pathway is

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**Fig. 8.** Phosphorylation of p70S6K (A), total p70S6K (B), the ratio of phosphorylated to total p70S6K (C), and total 4EBP (D) in the MG muscle from Con, SI, and SI-Stim groups as determined by Western blot analysis. Values are means ± SE. *Significant difference from Con group (P ≤ 0.05). †Significant difference from SI group (P ≤ 0.05).

**Fig. 9.** Schematic representation of the role of the IGF-1/phosphatidylinositol 3-kinase (PI3K)/Akt pathway and atrogin-1 and MuRF-1 in SI-induced atrophy and in the amelioration of atrophy by high-load isometric contractions. SI had no effect on p level or p state of mTOR signaling (p70S6K and 4EBP). The p state of Akt, however, was decreased, which may be suggestive of inactivation of its signaling pathway. Akt inactivation leads to activation of Foxo action on gene transcription of catabolic markers, i.e., atrogin-1 and MuRF-1 (dashed line). SI was associated with a decreased level of total IRS-1, and increased phosphorylation (p) state for IRS-1 at Ser307, leading to a generalized resistance to IGF-1 signals. SI had no effect on p level or p state of mTOR signaling (p70S6K and 4EBP). The p state of Akt, however, was decreased, which may be suggestive of inactivation of its signaling pathway. Akt inactivation leads to activation of Foxo action on gene transcription of catabolic markers, i.e., atrogin-1 and MuRF-1 (dashed line). SI was associated with increased mRNA levels of atrogin-1 and MuRF-1, possibly via activation of the Foxo system. Electromechanical stimulation (Stim action) appears to be effective in opposing all SI induced alterations. Boldface arrows, activation; boldface blunt bars, inhibition.
upregulated during muscle hypertrophy induced by isometric resistance exercise, acute bouts of contractile activity, and compensatory hypertrophy, and is downregulated during HU and denervation-induced atrophy (7, 14, 18, 30). Furthermore, inhibition of this pathway by rapamycin, a selective blocker of the downstream kinase mammalian target of rapamycin (mTOR) (29), partially blocks the hypertrophy response of regenerating muscles in response to innervation and chronic electrical stimulation, while activation of this pathway induces hypertrophy. Thus activation of the IGF-1/Pi3K/Akt cascade is not limited to inducing hypertrophy but also is sufficient to ameliorate atrophy (29).

HU, denervation, and SI all induce muscle atrophy and involve the upregulation in the expression of protein catabolic markers. However, there are clear differences in the role of the IGF-1/Pi3K/Akt cascade among these atrophy models and across muscle types during the short-term period of atrophy. For instance, while activity of this cascade is downregulated during an early period (from 5 to 14 days) of HU in the fast MG (7, 14) and of denervation in the slow soleus muscle (18), 7 days of denervation increased the percent phosphorylation of p70S6K in the fast extensor digitorum longus muscle (18). In the present study, the overall activity levels of the downstream markers p70S6K and 4EBP in the MG were similar in the Con and SI groups (Figs. 8 and 9). SI-induced atrophy did increase the upstream p-Ser 307 IRS-1/total IRS-1 ratio. Phosphorylation at Ser 307 has been shown to inhibit insulin signal transduction (3), and consistent with this role, the phosphorylated state of the downstream marker Akt was lower than Con in the SI group. Stimulation maintained the p-Ser 307 IRS-1/total IRS-1 ratio at Con levels. These changes in the phosphorylated state of IRS-1 at Ser 307 did not translate to the downstream effectors p70S6K and 4EBP. The regulation of IRS-1 activity, however, still may be playing an important role by affecting other signaling cascades activated by IRS-1, i.e., Ras-Raf (9, 17). Although the IGF-1/Pi3K/Akt cascade does not appear to be upregulated in the MG of SI-Stim rats, the elevated levels of RNA concentration and content suggest an increase in the translational capacity of the muscle. Thus the normal activity level of the anabolic markers, particularly that of Ser 307 IRS-1, likely contributed to the amelioration of atrophy in the stimulated muscle, and combined with the downregulation of the atrogenes, provides a better net protein balance in the SI-Stim vs. SI group.

In addition to its influence on protein synthesis, the IGF-1/Pi3K/Akt cascade also exerts a regulatory role on protein degradation, and as such, plays a pivotal role in protein metabolism. Specifically, phosphorylated Akt can inactivate a subfamily of forkhead transcription factors (Foxo). Downregulation of the IGF-1/Pi3K/Akt cascade and decreased levels of active Akt leads to activation of Foxo, which translocates into the nucleus and can increase transcription of atrogin-1 and MuRF-1 (Fig. 9) (26, 36). The phosphorylated state of Ser 307 on IRS-1 was elevated in the SI rats, which would inhibit the downregulatory effect of IRS-1 on the Foxo system (3). Thus the increase in the Ser 307 p-IRS-1/total IRS-1 combined with the decrease in the p-Akt/total Akt level may account for the decrease in atrogin-1 and MuRF-1 expression during atrophy. Accordingly, the maintenance of the Ser 307 p-IRS-1/total IRS-1 level at Con values and the higher level of p-Akt/total Akt in the SI-Stim than Con group is consistent with the decrease in atrogin-1 and MuRF-1 observed in the fast MG muscle.

The protein anabolic and catabolic pathways examined in skeletal muscle in the present study also play an important role in cardiac muscle size. Activation of the Pi3K pathway affects cardiac contractility (19) and induces cardiac hypertrophy (10, 27). In mice cardiac muscle, the expression of activated Akt is sufficient to induce hypertrophy (27), suggesting that Akt is a crucial regulator of certain forms of cardiac myopathy. The atrogin-1 ubiquitin ligase also plays a critical role in cardiac muscle (40). It has been shown to inhibit cardiac hypertrophy in response to pathological stimuli (23). Similar to its function in skeletal muscle, the regulation of the Pi3K and atrogenic systems may be an important mechanism through which cardiac muscle size can be regulated. Expression of IGF-1 mRNA was upregulated in the soleus muscle in the SI group. Because of the small size of the soleus, however, the levels of the downstream effectors were not determined in the present study. A previous report showing a similar increase in the level of IGF-1 mRNA in the rat soleus muscle after 8 days of SI demonstrated that it was not sufficient to increase the activity of downstream markers p70S6K and 4EBP (16). Although differences in the expression of upstream markers, i.e., IGF-1, exist between the soleus and MG muscles, the levels of the downstream targets of the IGF-1/Pi3K/Akt anabolic cascade appear to be unaffected in both fast and slow muscles of SI rats. Therefore, it appears that a downregulation of p70S6K and 4EBP is not necessary for SI-induced muscle loss during the early rapid phase of atrophy.

Perspectives

The present study demonstrates a high level of responsiveness of the protein metabolic processes to changes in electromechanical activity. The elevated expression of the measured protein catabolic markers during inactivity-induced atrophy is blunted with a reintroduction of only a small fraction of the activity duration lost with SI. These changes in protein catabolic marker expression appear to play a key role in the regulation of muscle mass as selected proteins of the anabolic cascade (IGF-1/Pi3K/Akt) were similar to control in the atrophying and stimulated muscle. The response of the anabolic markers to SI-induced atrophy contrasts with other atrophy models in which these cascade proteins are reduced when the atrophy is induced by unloaded muscle contractions and the absence of a motoneuron-muscle connection, i.e., denervation. The inability to increase activity of this protein anabolic pathway above control levels may have contributed to the partial atrophy still observed in SI stimulated muscles. A countermeasure that activates this anabolic pathway beyond control levels may contribute to a complete maintenance of muscle mass. Although forceful muscle contractions applied at the onset of an atrophy perturbation represent a relatively small fraction of the activity and loading duration found in normal controls, they can have a great impact in ameliorating atrophic adaptations at the whole muscle and cellular level.

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