Stress and Signaling Responses of Rat Skeletal Muscle to Brief Endurance Exercise During Hindlimb Unloading: a Catch-Up Process for Atrophied Muscle

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Key Words

Akt1 • FoxO • p70/S6K • Proteolysis • Soleus muscle
• Treadmill running • Ubiquitin E3 ligase

Abstract

At times, exercise accompanied by its anabolic effects is not a tractable countermeasure to muscle atrophy. Instead, training is often attempted after the affected muscle has atrophied greatly as a result of unloading. This study was designed to elucidate stress and signaling mechanisms underlying a process of muscle catch-up growth as a result of transitory exercise during unloading. Rats were exercised daily with a routine of 20- or 40-minute treadmill running (at 60% of maximum oxygen uptake) during the second week of a two-week hindlimb suspension. We examined the expression and activation of heat shock proteins and anabolic and proteolytic markers in the rat soleus muscle. Muscle mass relative to body mass decreased 2.4-fold in the unloaded group (HU) with respect to controls but decreased only 1.7-fold in the 40-min trained group (HT40) ($P < 0.05$) - equivalent to a 1.4-fold increase in the relative muscle mass over HU. Immunoblotting analyses on whole-tissue lysates demonstrated the following: (1) HSP72 and

α B-crystallin were upregulated 7- and 2.5-fold, respectively, in HT40 versus HU; (2) phosphorylation of Akt1 and p70/S6K decreased only slightly in HU; (3) when compared to HU, HT40 phosphorylation of Akt1, S6K, and FoxO1 increased 1.4- to 3.0-fold while phosphorylation of FoxO3 was unchanged; and (4) activities of the ubiquitin E3 ligases, calpain 1 and caspase-3 increased 2- to 4-fold in the unloaded groups regardless of exercise duration. These results suggest that the significant upregulation of chaperones and anabolic markers (e.g., HSP72, p-Akt1, p-S6K) in HT40, along with the lack of the training effect on proteolytic activity, is likely crucial for muscle mass catch-up in the unloaded muscle.

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Introduction

Muscle wasting is an inevitable, non-pathological phenomenon resulting from disuse, e.g., bed rest and spaceflight. Such conditions cause a severe loss in muscle tone and work capacity [1]. Endurance exercise has been considered an effective countermeasure against muscle loss. However, a precise exercise regimen that leads to

muscle mass regrowth has yet to be thoroughly investigated [2, 3]. Recent reports have revealed that muscle mass is finely controlled by several counteracting cellular mechanisms that balance protein synthesis and degradation [4]. In terms of protein synthesis, heat shock proteins (HSPs) play a crucial role in cytoprotection as well as anabolic regulation by actively assisting in protein folding, repair, and regeneration [5, 6]. Moreover, the stress-responsive signaling pathway involving class I phosphoinositide-3 kinase (PI3K) and Akt/protein kinase B (PKB) plays a major role in the stimulation of protein synthesis. This pathway stimulates activation of downstream proteins including mammalian target of rapamycin (mTOR) and p70/S6K [4, 7, 8]. Phosphorylation of mTOR and S6K has been implicated in elevated muscle protein synthesis by increasing mRNA translation through the phosphorylation of ribosomal protein components (e.g., S6) [7, 8].

During muscle disuse, these cellular responses are reversed, i.e., HSPs and anabolic signaling proteins are generally downregulated [4, 9]. In addition, several proteolytic pathways have been reported to be activated and induce muscle atrophy in the unloaded muscle. The ubiquitin-proteasomal pathway, which includes the muscle-specific E3 ligases, atrogin-1/muscle atrophy F-box (MAFbx) and muscle RING Finger 1 (MuRF1), is known to be a powerful contributor to muscle proteolysis [10, 11]. In this pathway, the transcription factor forkhead box O (FoxO) is a critical regulator of proteolysis, as decreased activation of Akt leads to FoxO-stimulated expression of the ubiquitin E3 ligases [4, 11]. These ligases then shuttle damaged or unnecessary proteins to the proteasome for degradation [12, 13]. Furthermore, the level of FoxO is likely to be regulated by the F-box protein S-phase kinase-associated protein 2 (Skp2), a component of the Skp/Cullin/F-box (SCF) ubiquitin ligase complex [14]. Skp2 was found to ubiquitinate and degrade phosphorylated FoxO in the proteasomal system and thus might indirectly contribute to myofibrillar degradation upon unloading [15, 16]. Parallel to the FoxO-ubiquitin ligase pathway, both calpain and caspases are also important regulators of muscle wasting in unloaded muscle [17, 18]. The activity of calpain depends on the release of intracellular calcium ions from the sarcoplasmic reticulum (SR) upon damage by oxidants during unloading or overloading [18, 19]. The activated calpain or other factors, such as cytochrome C released from damaged mitochondria, trigger the caspase cascade. Once active, the caspase cascade mediates DNA fragmentation and apoptosis in the unloaded tissue [18].

At times, exercise, accompanied by its anabolic effects, is not a tractable countermeasure. Instead, training is often attempted after the affected muscle has atrophied greatly as a result of unloading, as in hospitalization or spaceflight. In these instances, timely exercise can be delayed due to initial immobilization or engaged missions. Once exercise is feasible, it is usually scheduled on a daily basis; the transitory nature may be problematic since the unloaded muscle returns to the detrained condition after a brief exercise overload. For such transitory exercise to be effective, protein synthesis should be sufficiently high to overcome proteolysis. Furthermore, subsequent exercise must be conducted before the anabolic effect of a previous routine diminishes in order to allow muscle mass regrowth [17]. To date, the effectiveness of brief exercise for such a catch-up process during unloading has been poorly evaluated.

The current study was designed to elucidate stress and signaling mechanisms underlying muscle catch-up growth as a result of transitory exercise during unloading. We examined the expression and activation of relevant proteins in the soleus muscle of rats exposed to daily treadmill training at a moderate intensity during the second week of a two-week hindlimb suspension. The overall goal of this study was to define activity patterns of key stress and signaling markers that mediate the catch-up process in order to find a proper exercise protocol to counteract muscle atrophy.

Materials and Methods

Animals

This study was approved by the Yonsei University Animal Care and Use Committee. Sprague-Dawley rats were purchased from a local supplier at five weeks of age (~100 g) and reared at 22 ± 1 °C in a 12:12 h light-dark cycle. Standard Purina rat chow and water were provided ad libitum. At the end of five weeks, the rats were randomly divided into five groups and subjected to one of the following conditions for two weeks (Fig. 1): (1) controlled (no exercise, no hindlimb unloading) for the full two weeks (Con), (2) forced treadmill training for 20 min per day for the second week (CT20), (3) hindlimb unloading (HU) for the full two weeks, (4) hindlimb unloading with treadmill training for 20 min per day for the second week (HT20), and (5) hindlimb unloading with treadmill training for 40 min per day for the second week (HT40).

Hindlimb suspension

The general procedure for hindlimb suspension (HS) was described previously [20]. In the current study, we modified the model by using a wire hook to conveniently move the subject between the suspension and the treadmill exercise. The

Fig. 1. (A) Summary of the experimental design of the five groups of Sprague-Dawley rats over the two-week study period. (B) A hypothesized outcome depicting the catch-up response of the skeletal muscle by the 20- or 40-min transitory overloading during unloading.

wire hook was made of 1-mm thick Teflon-coated stainless steel and was attached to the tail at a spot one third from its base. On the first day of week six, all the rats including Con and CT20 were weighed and caged separately. The tail and the hook on each animal were covered with a sterilized cotton pad and wrapped firmly with stretch tape. For the animals in the three HS groups (HU, HT20, and HT40), a flexible Tygon tubing (5.6-mm OD) was placed along the tail. One end of the tubing was connected to the wire hook and the other end was connected to a tether set at the top of the cage. This resulted in the subject being suspended at an inclination of approximately 35° in a head-down orientation. The animal was free to move around the cage on its front feet. The small-to-moderate animal mass allowed the hooking model to be supportive enough to keep the subject suspended for the two weeks. At the time of treadmill training, the animal was freed from the suspension mode by disconnecting the wire hook from the Tygon tubing and placed on the treadmill pad for exercise.

Treadmill exercise

Overall features of the treadmill and oxygen consumption measurements were previously described in great detail [21–23]. The treadmill ($l \times w \times h = 430 \text{ mm} \times 95 \text{ mm} \times 165 \text{ mm}$) was caged with 10-mm thick plexiglass plates in an air-tight seal for oxygen consumption measurements and was set at 0° incline during the exercise trials. The oxygen consumption rate was

measured with an Oxymax System (Columbus Instruments, Columbus, OH, USA) that included an automated, open-circuit equal flow system set at 1500 mL min^{-1} [22]. Incurrent air flow was controlled by an air supply regulator (overall capacity of $0\text{--}2450 \text{ mL min}^{-1}$). Excurrent subsamples of air (500 mL min^{-1}) were diverted through desiccant columns and, finally, to the O_2 sensor. The gas analyzer signals were transmitted every two minutes through the Oxymax A/D converter to an IBM-compatible PC.

The subjects assigned to exercise (CT20, HT20, and HT40) were habituated to treadmill training at a speed of 15 m min^{-1} for 20 min over the first five days during week five. The temperature in the treadmill was maintained at $20\text{--}22^\circ\text{C}$ during the experiment. The maximum oxygen consumption rate ($\text{VO}_{2\text{max}}$) was determined using an extra set of vivarium rats that were age-matched at the first day of week seven. These animals also had a wire hook attached to their tail. The treadmill speed was increased by approximately 2 m min^{-1} every two minutes until the rats refused to run further. The $\text{VO}_{2\text{max}}$ was defined as the point where oxygen uptake leveled off, even if the rats were able to run at higher speeds. Once the $\text{VO}_{2\text{max}}$ ($4.95 \pm 0.06 \text{ L kg}^{-1} \text{ h}^{-1}$, $n = 6$) was obtained, individuals in the three exercise groups were trained at a treadmill speed of 12 m min^{-1} for the designated duration (20 or 40 minutes) during the active period every day during week seven. At this treadmill speed, oxygen consumption rate was $60 \pm 3\%$ of the $\text{VO}_{2\text{max}}$. The treadmill speed was a jogging level for these animals and has been used often for studies on animal models of acute ischemic stroke or cardiovascular aging [23, 24].

Muscle samplings

To test whether the effect of daily training would accumulate or diminish, we collected muscle tissues 24 h after the last treadmill trial. The animals were weighed and anesthetized with pentobarbital sodium (injected intraperitoneally at 50 mg kg^{-1}). Since the soleus muscle has been popularly examined as an anti-gravity muscle [1], the tissues of both hindlimbs were quickly removed, weighed to the nearest 0.1 mg, and frozen in liquid nitrogen for immunoblotting analysis.

Immunoblotting Analysis

The muscle preparation for immunoblotting was described previously [20]. The frozen soleus muscle ($1 \text{ mL}/0.1 \text{ g}$) was homogenized in tissue lysis buffer [20 mM HEPES (pH 7.4), 75 mM NaCl, 2.5 mM MgCl_2 , 0.1 mM EDTA, 0.05% (v/v) Triton X-100, 20 mM β -glycerophosphate, 1 mM Na_3VO_4 , 10 mM NaF, 0.5 mM DTT, and the protease inhibitor cocktail Complete Mini (Roche, Penzberg, Germany)]. The homogenized tissue was incubated for 15 min on ice and centrifuged for 30 min at $12,000 \text{ g}$ at 4°C . The supernatant whole-tissue soluble lysate was collected and its protein concentration was determined using a Bradford assay kit (Bio-Rad, Hercules, CA).

Whole-tissue lysate ($50 \mu\text{g}$) was subject to SDS-PAGE on 8–12% gels to detect HSP72, HSP60, HSP27, α B-crystallin, Akt1, phospho-Akt1, S6K, phospho-S6K, FoxO1, phospho-FoxO1, FoxO3, phospho-FoxO3, Skp2, atrogin-1, MuRF1, calpain-1, and caspase-3. The proteins were transferred from the gel to a

Table 1. Body and muscle masses of five groups of Sprague-Dawley rats. Data: mean $\pm$ SEM.

Variables	Groups				
	Con	CT20	HU	HT20	HT40
Body mass (g)	248 $\pm$ 3.3	233 $\pm$ 3.4	195 $\pm$ 6.4	182 $\pm$ 4.5	191 $\pm$ 6
Muscle mass (mg)	95.9 $\pm$ 2.3	93.5 $\pm$ 2.4	30 $\pm$ 2.0	35 $\pm$ 3.9	41.3 $\pm$ 2.66
n	6	6	6	5	6

Fig. 2. Intergroup comparison of the soleus muscle mass relative to body mass. Data are presented as mean $\pm$ SEM ($n = 5$ to 6). Actual values for the muscle and body masses are summarized in Table 1. Significant differences at $P < 0.05$ (one-way ANOVA and Scheffe's *post hoc* multiple comparison tests) of *a* versus *b* and *c* versus *d*.

nitrocellulose membrane. The membrane was incubated in blocking buffer (1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk) for 1 h at room temperature and was washed with 15 mL TBS/T for three 5-min intervals. The membrane was subsequently incubated overnight at 4°C with primary antibodies of appropriate dilution against the aforementioned proteins in 10 mL primary antibody dilution buffer (1:500 to 1:10,000). The membrane was then incubated with an HRP-conjugated secondary antibody (1:1,000 to 1:20,000) in 10 mL of blocking buffer with gentle agitation for 1 h at room temperature. The membrane was washed 3×5 min with 15 mL TBS/T between incubations. Immune complexes were detected with the ECL system (GE Healthcare, Fairfield, CT), and the bands were quantified using a densitometer (Bio-Rad). Proteins' densities were normalized to GAPDH. For detection of additional proteins, the membranes were stripped with 15 mL of Restore™ Western Blot Stripping Buffer (Thermo scientific, IL). The membranes were incubated for 15 min at room temperature and were washed 3×5 min with 15 mL TBS/T. Primary antibodies used for the assays: mouse anti-HSP72 and HSP60 monoclonal antibodies from Stressgen (Victoria, BC, Canada), mouse anti-HSP27 monoclonal antibody and rabbit anti- α B-crystallin, atrogin-1, MuRF1, Skp2 polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), mouse anti-Akt1 monoclonal antibody, rabbit anti-phospho-Akt1 (at Ser⁴⁷³), S6K, phospho-S6K (at Thr³⁸⁹), FoxO1, phospho-FoxO1 (at Ser²⁵⁶), FoxO3, phospho-FoxO3 (at Ser²⁵³), calpain 1, caspase-3 polyclonal antibodies (Cell Signaling Technology, Beverly, CA) and mouse monoclonal antibody to GAPDH (Abcam, Cambridge, UK). The secondary antibodies were HRP-conjugated anti-mouse IgG, anti-rabbit IgG (Cell signaling), and anti-rabbit IgG, anti-goat IgG (Santa Cruz).

Data analysis

All data are presented as mean $\pm$ SEM, unless otherwise noted. Statistical differences in the muscle properties among the five groups were tested by one-way analysis of variance (ANOVA) and Scheffe's *post hoc* multiple comparison tests. In the Results, the CT20 average was only compared to that of Con to maintain clarity even though outcomes of the statistical tests were shown in graphs. Statistical analyses were performed with SPSS/PC+ at the significance level $P = 0.05$.

Results

Muscle mass

The ratio of the soleus muscle mass to total body mass was 3.81 ± 0.08 ($\times 10^{-4}$) for the Con group (Fig. 2). The average relative mass for animals in the HU group decreased 2.4-fold compared to Con while only a 1.7-fold decrease was measured for HT40 ($P < 0.05$); in other words, hindlimb unloaded animals that underwent a 40-min exercise routine exhibited 1.4-fold, or 40%, additional muscle mass to total body mass over unexercised hindlimb unloaded subjects. The relative mass did not statistically differ between Con and CT20 or between HU and HT20.

Chaperone proteins

Four HSPs, including HSP72, HSP60, HSP27, and α B-crystallin, were investigated in this study (Fig. 3). The expression level of HSP72 did not differ between Con and CT20 ($P > 0.05$), but decreased 4.4-fold in HU animals. On the other hand, HSP72 expression was rescued in the HT20 group and, interestingly, upregulated in HT40 animals ($P < 0.05$). When compared to the HU group, HT20 rescue and HT40 upregulation correspond to 5.5- and 6.8-fold increases, respectively ($P < 0.05$; Fig. 3B). For HSP60 and HSP27, there were no intergroup differences in the cellular levels (Fig. 3C and 3D). For α B-crystallin, the trend in expression was similar to that for HSP72 except that HT40 was not statistically upregulated (Fig. 3E).

Fig. 3. Differential expression of heat shock proteins (HSPs) in the soleus muscle in response to unloading or transitory overloading during unloading. Whole-tissue soluble lysates were used to determine the levels of HSPs by immunoblot analysis with respective antibodies. (A) Representative gel images of the four HSPs. GAPDH was used for normalization of protein densities. (B through E) Intergroup comparisons of the levels of HSP72, HSP60, HSP27, and α B-crystallin, respectively. Data are presented as mean $\pm$ SEM (n = 5 to 6). Statistical analyses were performed using one-way ANOVA and Scheffe's multiple comparison tests: *a* versus *b*, *a* versus *c*, and *b* versus *d*, $P < 0.05$.

Fig. 4. Effect of unloading or overloading on activation of Akt1 and p70/S6K in the soleus muscle. Whole-tissue soluble lysates were used to determine unphosphorylated and phosphorylated status of these proteins by immunoblot analysis with respective antibodies. Intergroup comparisons of Akt1, *p*-Akt1, and the ratio of *p*-Akt1/Akt1 (A-B), and S6K, *p*-S6K, and *p*-S6K/S6K (C-D). Mean $\pm$ SEM (n = 5 to 6). *a* versus *b*, $P < 0.05$ (one-way ANOVA and Scheffe's multiple comparison tests).

Signaling responses

Akt1 and p70/S6K (Fig. 4) - The level of Akt1 was significantly lower in HT40 compared to Con while the level of phosphorylated Akt1 (*p*-Akt1) was rescued to

Con levels from the 2.0-fold decrease observed for HU ($P < 0.05$). In fact, the *p*-Akt1/Akt1 ratio increased 3.0-fold between HU and HT40 ($P < 0.05$). There were no statistical differences in the levels of Akt1, *p*-Akt1, and

Fig. 5. Activities of the transcription factors FoxO1 and FoxO3 in the soleus muscle in response to unloading or the transitory overloading during unloading. Intergroup comparisons of FoxO1, *p*-FoxO1, and *p*-FoxO1/FoxO1 (A and B) and FoxO3, *p*-FoxO3, and *p*-FoxO3/FoxO3 (C and D). Data are presented as mean ± SEM (n = 5 to 6). *a* versus *b*, *P* < 0.05.

Fig. 6. Upregulation of atrogen-1 (A and B), MuRF1 (A and C), and Skp2 (A and D) in the soleus muscle in response to unloading or the transitory overloading during unloading. Data are presented as mean ± SEM (n = 5 to 6). *a* versus *b* and *c* versus *d*, *P* < 0.05.

p-Akt1/Akt1 between Con and CT20 or between HU and HT20. There were no intergroup differences in the levels of S6K, *p*-S6K, and *p*-S6K/S6K, except that the level of *p*-S6K decreased 1.5-fold from Con to HU and that the *p*-S6K/S6K ratio increased

1.4-fold from HU to HT40 (*P* < 0.05).

FoxO1 and FoxO3 (Fig. 5) - The level of FoxO1 was found to be 1.9-fold greater in HT40 than Con, and the *p*-FoxO1 level in HT40 was 2.3- and 4.0-fold greater than Con and HU, respectively (*P* < 0.05). The *p*-FoxO1/

Fig. 7. Upregulation of calpain 1 (A and B) and caspase-3 (A and C) in response to unloading or the transitory overloading during unloading. Data are presented as mean $\pm$ SEM ($n = 5$ to 6). *a* versus *b*, *a* versus *c*, and *d* versus *e*, $P < 0.05$.

FoxO1 ratio decreased 2.9-fold from Con to HU whereas the ratio increased 2.5-fold from HU to HT40 ($P < 0.05$).

The FoxO3 level increased 2.4-fold from HU to HT40 whereas the level of *p*-FoxO3 decreased 4.0-fold from Con to HU ($P < 0.05$). The *p*-FoxO3/FoxO3 ratio decreased 2.7- to 3.1-fold from Con to all three HS groups ($P < 0.05$).

For both FoxO1 and FoxO3, the 20-minute training did not result in any statistical difference in the levels of unphosphorylated and phosphorylated forms and the ratios between Con and CT20 or between HU and HT20 ($P > 0.05$).

Ubiquitin E3 ligases (Fig. 6) - The levels of the three ligases (atrogin-1, MuRF1, Skp2) did not differ significantly between Con and CT20. There was

significant upregulation in these ligases between Con and HU (1.9-fold for atrogin-1; 3.7-fold for MuRF1; 3.0-fold for Skp2) ($P < 0.05$). The levels of the three proteins remained consistent within the three HS groups ($P > 0.05$).

Calpain 1 and caspase-3 (Fig. 7) - The level of calpain 1 did not change between Con and CT20, but increased 3.2- to 3.8-fold between Con and the three HS groups ($P < 0.05$). Caspase-3 was upregulated 1.5-fold between Con and CT20 and 2.8- to 3.4-fold between Con and the three HS groups ($P < 0.05$). For neither proteases was there any statistical difference in the levels among the three HS groups ($P > 0.05$).

Discussion

Endurance training has been the most feasible solution to overcome unloading-induced muscle atrophy for bed rest patients or spaceship crews who remain immobilized or weightless for a period of time [25, 26]. Because both physical and spatial limitations are inherent in these cases, moderate-intensity exercise would be a practical countermeasure. To date, however, the effectiveness of transitory, moderate exercise during unloading on promoting muscle mass catch-up growth has been overlooked.

Our data show that with no exposure to exercise the soleus muscle lost 2.4-fold of its relative mass during the two-week unloading. The tissue restored its mass 1.4-fold as a result of daily 40-min training intervals during the second week of the two-week unloading period (Fig. 2). Daily exercise for 20 minutes failed to result in muscle growth during the same period, although the overload had a positive effect on muscle retention. The 20-min trials for the controls also failed to invoke hypertrophy as seen in the comparison between Con and CT20. After observing the potential of daily 40-min exercise for muscle catch-up, we attempted to identify major molecular mechanisms that set the HT40 group apart from HU and HT20.

Our principal findings can be summarized by four major observations: (1) HSP72 and α B-crystallin were remarkably upregulated (up to 7-fold) by transitory exercise conducted in the middle of unloading (Fig. 3), (2) phosphorylation of Akt1 and S6K decreased only slightly from Con to HU (Fig. 4), (3) phosphorylation of FoxO1 and FoxO3 was differentially regulated in response to the transitory overloading (Fig. 5), and (4) expression of proteolytic markers (namely atrogin-1, MuRF1, Skp2, calpain 1, and caspase-3) all increased significantly in

the three HS groups compared to Con, irrespective of the duration of the transitory exercise (Figs. 6 and 7).

HSPs are known to play crucial roles in cytoprotection against environmental stresses like heat or hypoxia. As molecular chaperones, HSPs are important for myofibrillar organization and contractile properties [5, 6, 27]. Our results in the current study illustrate that the transitory overload stress during unloading enabled the muscle to upregulate expression of HSP72 and α B-crystallin (but not HSP60 and HSP27), which appeared to correlate with muscle mass growth (Figs. 2 and 3). Interestingly, the unloaded muscle was much more responsive than the control tissue to the overload stress. This is demonstrated by the remarkable increase in levels of HSP72 and α B-crystallin between HU and HT20 (as well as HT40) whereas there was little change in the levels of CT20 with respect to Con (Fig. 3B and 3E). Further works (e.g., HSP knockdown or HSP inhibitor treatment) will affirm the roles of these chaperones in myofibrillar anabolism during the overloading trials.

The Akt-mTOR-S6K axis has been well established as the main pathway associated with muscle protein synthesis [7, 28]. In the present study, phosphorylation of Akt1 and S6K was only slightly affected during unloading. This suggests that the dramatic wasting of the muscle in the HU group is not satisfactorily explained by the lack of anabolic activity, but rather is associated with the remarkable elevation of proteolytic markers (e.g., E3 ligases, calpain, caspase-3) during unloading. Because the levels of proteolytic proteins remained unaltered in the three HS groups, the upregulation of *p*-Akt1 and *p*-S6K in HT40 is likely crucial for muscle mass catch-up in this group. Importantly, such elevated anabolism versus balanced proteolysis in HT40 seems to be ascribed to differential regulation of FoxO proteins.

FoxO1 and FoxO3 have been shown to regulate Akt-dependent protein degradation [11, 29]. The FoxO proteins are normally phosphorylated and remain in the sarcoplasm under physical stress or Akt1 activation [4, 30]. During unloading, however, FoxO proteins translocate to the nucleus and increase transcriptional activities of their target genes through a direct interaction with forkhead binding elements. This interaction is known to stimulate expression of ubiquitin E3 ligases [11]. Our current study demonstrates that the unphosphorylated state of both FoxO proteins remained unchanged in hindlimb-unloaded rats (Fig. 5). Interestingly, while unphosphorylated FoxO1 was present in similar amounts in HU and HT40, phosphorylated FoxO1 was significantly greater in HT40, resulting in a significant elevation of *p*-FoxO1/FoxO1

from HU to HT40. This is contrasted by the lack of FoxO3 phosphorylation, which results in the considerable decrease in *p*-FoxO3/FoxO3 in HT40 compared to Con (Fig. 5). Thus, the overall pattern of intergroup variation in *p*-FoxO1/FoxO1 paralleled that of upstream *p*-Akt1/Akt1, whereas the variation pattern of *p*-FoxO3/FoxO3 inversely correlated with that of downstream E3 ligases (atrogin-1, MuRF1, and Skp2). Differential regulation of the FoxO1 and FoxO3 was also observed in skeletal muscular responses to various metabolic and loading conditions [4, 31]. Our data thus suggest that the 40-min exercise exerted dual effects on the FoxO transcription factors: upregulation of phosphorylated FoxO1 in HT40, which mimicked Akt1 activation, appears to be reflected in anabolic muscle growth for this group. In contrast, upregulation of unphosphorylated FoxO3 (and hence diminishing *p*-FoxO3/FoxO3) that paralleled the upregulated atrogens seems to contribute to muscle wasting. The question of how the same transitory exercise created differences in the phosphorylation states of the two FoxO proteins is beyond the scope of this study. There might be complicated interactions during transition of the loading conditions between the FoxOs and the F-box ligases, such as atrogins and Skp2, which are known to regulate FoxO phosphorylation [14, 32]. This issue will be investigated further in order to better understand the mechanism of muscle catch-up recovery.

Lastly, the powerful proteolytic regulators (ubiquitin E3 ligases, calpain 1, and caspase-3) showed similar remarkable upregulation in the three HS groups (Figs. 6 and 7). The comparable levels of these proteases irrespective of exercise duration might, in part, be limited by the corresponding downregulated *p*-FoxO3/FoxO3 in the HS groups. The similar pattern in the expression levels suggests a global cross-talk among these and other relevant signaling molecules [17, 33]. Calpain and caspases are known to be proteasome-dependent for proteolytic activity, with concurrent reduction in Akt and mTOR activation and HSP90 expression [32, 34, 35]. Calpain proteolysis was also shown to be prevented by mild oxidative stress that induced activation of p38 mitogen-activated protein kinase (MAPK) and HSP25/27 in ischemia-reperfused hearts of rats [34]. Another study reported that activation of caspases and subsequent apoptosis was promoted by increased activity of atrogins through elevation of c-Jun and c-Jun N-terminal kinase (JNK) 1/2 [32]. Together, these studies suggest that muscle proteolytic activity should be comprehended on the basis of integrative global linkages of anabolic and catabolic molecules.

In conclusion, our study shows that a jogging level of exercise longer than 20 minutes is sufficient to reduce the soleus muscle wasting during unloading. Significant upregulation of chaperones and anabolic markers (e.g., HSP72, *p*-Akt1, and *p*-S6K) between HU and HT40, along with the lack of the training effect on proteolytic activities, are likely to contribute to muscle mass recovery during unloading. Differential regulation of FoxO proteins and potential cooperative interactions among HSPs, FoxOs, and ubiquitin E3 ligases are worth further investigation to better understand their roles in muscle protein regulation during endurance exercise.

Acknowledgements

We would like to thank two anonymous reviewers for their special concern and comments to improve the manuscript, and Mr. Min Jae Park for assistance of laboratory procedures including animal management. This research was supported by the National Space Lab (NSL) program through the Korea Science and Engineering Foundation funded by the Ministry of Education, Science and Technology (grant No. 2008-03190) awarded to I. Choi.

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