Effects of 3 days unloading on molecular regulators of muscle size in humans


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Gustafsson T, Osterlund T, Flanagan JN, von Waldén F, Trappe TA, Linnehan RM, Tesch PA. Effects of 3 days unloading on molecular regulators of muscle size in humans. J Appl Physiol 109: 721–727, 2010. Changes in skeletal muscle mass are controlled by mechanisms that dictate protein synthesis or degradation. The current human study explored whether changes in activation of the phosphoinositide 3-kinase (PI3K)-Akt1, p38, myostatin, and mRNA expression of markers of protein degradation and synthesis occur soon after withdrawal of weight bearing. Biopsies of the vastus lateralis muscle (VL) and soleus muscle (Sol) were obtained from eight healthy men before and following 3 days of unilateral lower limb suspension (ULLS). Akt1, Forkhead box class O (FOXO)-1A, FOXO-3A, p38, and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) phosphorylation and protein levels and myostatin protein level were analyzed by Western blot. Levels of mRNA of IGF1, FOXO-1A, FOXO-3A, atrogin-1, MuRF-1, caspase-3, calpain-2, calpain-3, 4E-BP1, and myostatin were measured using real-time PCR. The amounts of phosphorylated Akt1, FOXO-1A, FOXO-3A, and p38 were unaltered (P > 0.05) after ULLS. Similarly, mRNA levels of IGF1, FOXO-1A, FOXO-3A, atrogin-1, MuRF-1, caspase-3, calpain-2, calpain-3, 4E-BP1, and myostatin were measured using real-time PCR. The amounts of phosphorylated Akt1, FOXO-1A, FOXO-3A, and p38 were unaltered (P > 0.05). The mRNA levels of atrogin-1 and MuRF-1, as well as the mRNA and protein phosphorylation of 4E-BP1, increased (P < 0.05) in VL but not in Sol. Both muscles showed increased (P < 0.05) myostatin mRNA and protein following ULLS. These results suggest that pathways other than PI3K-Akt1 stimulate atrogin-1 and MuRF-1 expression within 3 days of ULLS. Alternatively, transient changes in these pathways occurred in the early phase of ULLS. The increased myostatin mRNA and protein expression also indicate that multiple processes are involved in the early phase of muscle wasting. Further, the reported difference in gene expression pattern across muscles suggests that mechanisms regulating protein content in human skeletal muscle are influenced by phenotype and/or function.

atrophy; proteasome degradation; simulated spaceflight; ubiquitin

The phosphoinositide 3-kinase (PI3K)-Akt1 pathway has been put forth as the principal signaling protein cascade regulating muscle protein content (7, 8, 15, 28, 38). This pathway is initiated by the activation of lipid kinase PI3K, which phosphorylates serine/threonine kinase Akt1 (protein kinase B), leading to the regulation of glycogen synthase kinase 3β (GSK3β) and mammalian target of rapamycin (mTOR) kinases. Many of the effects mediated by IGF-1 on muscle growth are activated through this pathway (15, 38, 43). The signaling endpoint involves phosphorylation of several key regulatory proteins such as eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) resulting in enhanced protein translation and processing (7, 28). Other targets of PI3K-Akt1 phosphorylation are the family members of Forkhead box class O (FOXO) transcription factors. Phosphorylation of FOXO-1A and -3A results in cytoplasmic retention and inhibition of its nuclear translocation activating transcription factors involved in the ubiquitin-proteosome (Ub-P) protein degradation pathway (8, 28, 40, 43). Thus the activity level of the PI3K-Akt1 pathway appears to dictate both skeletal muscle protein synthesis and degradation. In such a scenario, attenuated activity in the PI3K-Akt1 pathway facilitates muscle atrophy through both reduced protein synthesis and augmented protein degradation.

The Ub-P pathway is a concerted action of three ubiquitin ligase enzymes, i.e., E1–E3, which function by attaching ubiquitin cofactors to proteins, thus rendering them targets for the proteasome degradation pathway (4). It has been shown that atrogin-1 and MuRF-1, two E3 ubiquitin ligase subtypes, play central roles in the muscle atrophy process (6, 18, 26, 39, 42). Both are regulated by members of the FOXO family and upregulated in conditions resulting in muscle loss (43). Given that Ub-P only degrades monomeric actin and myosin, other proteases, e.g., caspase-3, calpain-2, and calpain-3, are required to assist in Ub-P protein degradation (4, 14, 20, 25).

The picture is, however, more complex, and other pathways are known to influence muscle mass as well. For example, members of mitogen-activated protein kinase (MAPK) pathways, e.g., p38, are activated and initiate protein degradation processes in response to muscle unloading (29). Myostatin, a member of the transforming growth factor (TGF)-β superfamily, has also emerged as an important regulator of muscle size (21, 27). Loss of myostatin function promotes marked hyper trophy in both human and animal skeletal muscle (24, 41), and systemic administration of myostatin induces severe muscle wasting (34, 49). Furthermore, overexpressing myostatin results in cachexia in mice accompanied by increased atrogin-1 and MuRF-1 expression in mice (34). Recent studies in rodent

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The dynamic balance between anabolic and catabolic events ultimately determines human skeletal muscle protein content. Muscle hypertrophy occurs when the rate of protein synthesis exceeds degradation. Atrophy, resulting in net muscle loss, is prompted by protein degradation that is greater than the concurrent synthesis. Although the cause of atrophy is multifactorial, the prevailing theory proposes a common mechanism where the main stimulus provokes a negative protein balance (15).
skeletal muscle have also linked myostatin activity to the PI3K-Akt1 pathway (3, 10).

Our research has shown that unilateral lower limb suspension (ULLS) triggers proteolysis after 3 days (45), and robust whole muscle atrophy is evident within just a few weeks of ULLS (5, 19, 44). The present investigation explored the effects of 3 days of ULLS on potential candidate signaling pathways in weight-bearing muscles of healthy adult men. We hypothesized that 3 days of ULLS induces muscle wasting through reduced PI3K-Akt1 activation and increased myostatin expression. Because the triceps surae show greater atrophy than the quadriceps muscle in response to long-term bed rest (1, 46), we also studied signaling pathways and gene expression in the two weight-bearing vastus lateralis (VL) and soleus (Sol) muscles before and after 3 days of ULLS. We hypothesized that signaling pathways that initiate processes leading to muscle atrophy would be more pronounced in Sol.

METHODS

Subjects. Eight healthy adult men (mean ± SD; 25 ± 5 yr, 183 ± 3 cm, 76 ± 8 kg) were recruited from the Stockholm metropolitan area. The subject sample ranged from physically active to sedentary individuals. Before the experiments, subjects were screened for any history of lower limb pathology, neuromuscular disorder, or cardiovascular disease. Written consent was obtained from each subject after being informed of the procedures, risks, and potential benefits associated with the experiments. The study protocol was approved by the Ethics Committee at the Karolinska Institute.

Unloading. ULLS was accomplished as previously described (44, 45). In brief, upright or ambulatory activities were aided by short-length crutches with handgrip and forearm support distal to the elbow (Swereco Rehab AB, Sollentuna, Sweden). The right foot was equipped with a shoe outfitted with a 10-cm-thick sole in order to remove weight-bearing load from the left unloaded limb. There were no straps attached to the shoe restraining ankle or knee joint movement. The subjects lived at home and maintained their normal occupational tasks throughout the experimental period. Compliance was encouraged through daily interaction with one of the investigators. Two weeks before the ULLS intervention, all subjects underwent four sessions to practice walking on crutches and daily tasks associated with the ULLS intervention. The subjects refrained from any strenuous physical activity 3 days before the onset of ULLS, yet maintained their normal dietary habits. However, 24 h before any measurements, diet was provided as standardized meals consisting of pasta and vegetables.

Collection of muscle samples. Muscle biopsies were obtained from the right leg before ULLS and from the left leg after completing 3 days (72 h) of ULLS before resuming any weight-bearing activity. Following an overnight fast and after injection of local anaesthetic (Carbocain) and skin incision, muscle biopsies were obtained from the VL and Sol using a 5-mm Bergström needle (Bergström 1962). Muscle samples were cleansed of excess blood, connective tissue, and fat and then frozen in liquid nitrogen and stored at −80°C until further analysis.

Protein extraction and Western blot. Tissue samples were homogenized with a glass homogenizer in 2 mM HEPES (pH 7.4) buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10 mM MgCl₂, 25 mM β-glycerol phosphate, 8 mM DTT, 1 mM PMSF, 1 mM Na₃VO₄, leupeptin (20 μg/ml), aprotonin (20 μg/ml), and 1× complete protease inhibitor cocktail (Roche Diagnostics). The protein homogenate was gently rotated at 4°C for 60 min followed by centrifugation at 4°C for 10 min (14,000 g). The supernatant was collected and protein concentrations determined by Bradford protein assay. Homogenates were further diluted in the homogenization buffer (see above) to a final concentration of 2 μg/μl. The samples, a protein ladder, and a positive control were run on SDS gels. Following electrophoresis the proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratory, Hercules, CA) and reversibly stained with Ponceau (Sigma, St. Louis, MO) to verify equal loading and transfer. Incubation with primary antibodies was performed overnight at 4°C. The primary antibodies used (diluted 1:1,000) were phospho-Akt1 (Ser473), total Akt1, phospho-FOXO-1A (Ser256), total FOXO-1A, phospho-FOXO-3A (Ser253), total FOXO-3A, phospho-4E-BP1 (Thr37/Thr46), total 4E-BP1, phospho-p38 MAP kinase (Thr180/Tyr182), and total p38 MAP kinase. All antibodies were commercially available from Cell Signaling Technology (Beverly, MA). Myostatin antibody (sc-6885-R, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to detect full-length myostatin monomer at ~50 kDa. GAPDH (sc-47724, Santa Cruz Biotechnology, Santa Cruz, CA) antibody was used for normalization. Secondary anti-rabbit IgG horseradish peroxidase-linked antibody was used (diluted to 1:5,000) (Cell Signaling Technology), and proteins were visualized using enhanced chemiluminescence (ECL) reagents (Amersharm, Buckinghamshire, UK) according to the manufacturer’s protocol. The quantification was made using the Quantity One software (Bio-Rad Laboratories).

RNA isolation, reverse transcription, and real-time (RT)-quantitive PCR. Total RNA was extracted from muscle biopsies using TRIzol (Invitrogen Life Technologies Carlsbad, CA) according to manufactu-her’s protocol. Two micrograms of the RNA was synthesized into cDNA using reverse transcriptase (Superscript II RNase H, Invitrogen Life Technologies) and random primers (Roche Diagnostics) in a total volume of 20 μl. Real-time PCR was performed on ABI-PRISM 7700 Sequence Detector System (Perkin-Elmer Applied Biosystems, Foster City, CA). For each reaction, 5 μl of the diluted single-stranded cDNA was mixed with 12.5 μl of the 2× TaqMan PCR Mastermix, 1.25 μl gene-specific primers/probe set, and 6.25 μl sterile dH₂O. The thermal cycling protocol was as follows: 2 min at 50°C and 10 min at 90°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The predescribed sequence-specific primers/probes sets (Taqman Gene Expression Assays from Applied Biosystems) were atrogin-1, Hs00369714_m1; calpain-2, Hs00156251_m1; calpain-3, Hs 00181057_m1; caspase-3, Hs00234387_m1; FOXO-1A, Hs00231161_m1; FOXO-3A, Hs00818121_ m1; IGF1, Hs00153126_m1; MuRF-1, Hs00822397_m1; myostatin, Hs00193363_m1; and 4E-BP1, Hs0067050_m1. There were no systematic changes in the selected housekeeping genes, and thus almost identical results were obtained with 18S or GAPDH as the housekeeping gene. The GAPDH/18S ratio did not change with ULLS in either muscle, and the GAPDH/18S ratio was similar in VL and Sol (data not shown). Control experiments revealed very similar efficiency over different starting template concentrations for target genes and endogenous control. For any individual, all samples were analyzed simultaneously in a single assay run. Measurements of the relative distribution of target genes were performed for each individual; a ΔΔCT value was obtained by subtracting the 18S or GAPDH Ct value from the corresponding target Ct value. The expression of each target was then determinate by 2−ΔΔCT, which provides the number of target gene copies per each copy of the housekeeping gene.

Statistics. Following a test for normality, a nonparametric Wilcoxon paired-samples test was employed to assess the effects of time (pre; post) and muscle (VL; Sol) on protein phosphorylation and mRNA expression (Statistica, StatSoft, Tulsa, OK). The p-pre differences in VL were tested against the pre-post differences in Sol to identify interaction between the two muscles. Significance was accepted at P < 0.05. Figures show data expressed as ratio of post to pre values (fold change) and presented as median and range, unless otherwise stated.
RESULTS

To control for antibody functionality, dilution curves were performed for all of the used antibodies. There were no differences in protein or phosphorylation levels of Akt1, FOXO-1A, FOXO-3A, and p38 proteins, or between VL and Sol before and after ULLS \((P > 0.05; \text{Fig. 1})\). Before ULLS, IGF1, FOXO-1A, FOXO-3A, caspase-3, and calpain-2 and -3 mRNA expressions were greater for Sol than VL \((P < 0.05; \text{data not shown})\). Neither muscle showed changed expression of these factors in response to ULLS \((P > 0.05; \text{Fig. 2})\). There were no differences \((P > 0.05)\) in baseline mRNA expression of atrogin-1, MuRF-1, 4E-BP1, or myostatin between muscles. Atrogin-1 tended to be higher in Sol compared with VL \((P = 0.12; \text{data not shown})\). Atrogin-1 and MuRF-1 increased in VL, but not Sol, after ULLS \((\text{interaction time and muscle } P < 0.05; \text{Fig. 3})\). 4E-BP1 mRNA and protein phosphorylation increased in VL \((P < 0.05)\) but not in Sol \((\text{Fig. 4})\). Myostatin mRNA and protein levels increased in both muscles following ULLS \((P < 0.05; \text{Fig. 5})\).

DISCUSSION

Skeletal muscle breakdown is evident within days (45) and robust whole muscle atrophy occurs within weeks in healthy individuals subjected to ULLS (5, 19, 44). Given these observations it was hypothesized that gene expression and signaling pathways controlling the atrophic processes in muscle cell culture and animal models would be altered in human skeletal muscle after only a few days of non-weight bearing. Indeed, we showed increased mRNA levels of atrogin-1 and MuRF-1, key
factors in the Ub-P pathway activation, after 3 days ULLS. Albeit parallel changes in Akt1 phosphorylation did not occur, there were concomitant increases in myostatin mRNA and protein levels.

The two muscle-specific E3 ubiquitin ligase proteins, atrogin-1 and MuRF-1, are activated in experimental models that induce muscle wasting. Hence, these factors have been designated markers of skeletal muscle degradation (4, 6, 18, 26, 39, 42). In a recent study we reported enhanced actin and myosin proteolysis (45), and the present investigation showed increased atrogin-1 and MuRF-1 mRNA levels after 3 days ULLS. Collectively, these findings suggest skeletal muscle proteolysis occurs early in the atrophy process via activation of the Ub-P pathway. Yet, at the mRNA level, “upstream” factors in the protein degradation process, i.e., caspase-3, calpain-2, and calpain-3, were unchanged following ULLS. This observation accords with the general idea that these enzymes become proteolytically activated through cleavage of preformed proteins (4, 17, 25). Thus future ULLS studies exploring changes in skeletal muscle enzyme activity or content are warranted.

Muscle atrophy may also be facilitated by compromised protein synthesis (6, 15, 16, 42). While regulation of protein synthesis is mediated through a number of translational factors (32, 37), it appears dephosphorylation of 4E-BP1 controls protein synthesis rate by inhibiting protein translation through enhanced binding to eIF-4E. In the present study 4E-BP1 mRNA and the phosphorylation to protein level ratio increased in parallel. The significance of a change in a single factor should, however, not be overstated but does not support reduced protein synthesis in the early phase of ULLS. This contrasts the well-documented finding of atten-

Fig. 2. Fold changes in mRNA expression of IGF1, FOXO-1A, and FOXO-3A (A) and of caspase-3, calpain-2, and calpain-3 (B) in VL and Sol. mRNA expressions are normalized to the GAPDH housekeeping gene. Box plots show 25–75 percentiles and median values (n = 8).

Fig. 3. Fold changes mRNA expression of atrogin-1 and MuRF-1 in VL and Sol. Atrogin-1 is normalized to both 18S and GAPDH to demonstrate that changes in target gene/housekeeping gene ratios were independent of changes in a specific housekeeping gene. #Difference (P < 0.05) pre-ULLS vs. post-ULLS between VL and Sol; *Difference within VL. Box plots show 25–75 percentiles and median values (n = 8).
uated protein synthesis following a more extended time of muscle disuse (35).

Given that increased skeletal muscle contractile activity augments Akt1 signaling (12), we hypothesized that 3 days ULLS would blunt Akt1 activity. However, its phosphorylation state was unchanged in response to ULLS. The mRNA expression of the upstream Akt1 activator IGF1 (15, 38, 43), as well as downstream targets, i.e., the FOXO family member (8, 40, 43) mRNA, protein, and phosphorylation state were unaltered after ULLS. Growing evidence suggest that changes in PI3K-Akt1 pathway activity are not necessarily correlated with altered expression of the E3 ubiquitin ligase factors atrogin-1 and MuRF-1 (11, 23, 28, 30, 33). p38 activation is an alternative pathway promoting increased MuRF-1 transcription (15, 22, 29, 31). However, similar to Akt1, phosphorylation was unaltered following 3 days ULLS. Thus these findings offer no evidence to support changes in atrogin-1 and MuRF-1 are due to attenuated PI3K-Akt1 pathway or augmented activity of p38. The present data should be viewed with caution, and potential transient changes and different time courses for protein phosphorylation and gene expression should be acknowledged. In fact, and consistent with our observation, recent reports show early downregulation (<48 h) with unloading in PI3K-Akt1 pathway activity (47) that is normalized within ~72 h (13). This appears

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**Fig. 4.** A: fold changes of eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) mRNA levels in VL and Sol. mRNA are normalized to GADPH as housekeeping gene. B: fold changes in phosphorylated 4E-BP1 are normalized total protein levels in VL and Sol. *Difference (P < 0.05) pre-ULLS vs. post-ULLS between VL and Sol. #Difference within VL. Box plots show 25–75 percentiles and median values (mRNA, n = 8; protein, n = 6).

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**Fig. 5.** A: fold changes in myostatin mRNA levels in VL and Sol. mRNA is normalized to GADPH as housekeeping gene. B: fold changes in myostatin protein levels in VL and Sol. *Significant difference (P < 0.05) pre-ULLS vs. post-ULLS without any differences between VL and Sol. Box plots show 25–75 percentiles and median values as indicated by the schematic box plot (n = 8).
to contrast a more sustained upregulation of atrogin-1 and MuRF-1 past 10 days (13, 23).

Myostatin was analyzed in an effort to further explore alternative candidate pathways controlling human skeletal muscle wasting processes. The physiological regulatory significance of the myostatin gene is supported by data obtained at the mRNA level (2, 9, 36, 49). We showed sustained and concomitant increases in mRNA and myostatin protein expression following 3 days ULLS, which accords with results shown in the rat following 10 days of hindlimb suspension (48). Muscle atrophy appears to be more prominent in Sol than VL in response to long-term bed rest (1, 46). Given this, we hypothesized that factors facilitating the atrophy process would be more robust in the Sol muscle following short-term ULLS. With exception of myostatin, only VL showed changes in mRNA for factors involved in Ub-P protein degradation and/or protein synthesis. Hence, the present study could not identify any mechanism(s) explaining the more rapid and substantial atrophy seen in Sol with muscle disuse. Yet, the findings of increased myostatin noted in both muscles, accompanied by overall higher basal mRNA levels in the Sol compared with VL, would suggest that protein turnover and pretranslational responses to short-term unloading are different across the two muscles.

In conclusion, the present data indicate that pathways other than PI3K-Akt stimulate atrogin-1 and MuRF-1 expression within 3 days of ULLS. Alternatively, transient changes occur in these pathways in the early phase of ULLS. Moreover, the increases in myostatin mRNA and protein expression suggest that processes initiating global muscle loss involve multiple pathways. Gene expression patterns across the human Sol and VL muscles imply that protein turnover mechanisms may vary depending on muscle function and/or phenotype.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**REFERENCES**

SHORT-TERM UNLOADING ALTERS GENE EXPRESSION


