Isometric resistance exercise fails to counteract skeletal muscle atrophy processes during the initial stages of unloading

F. Haddad, G. R. Adams, P. W. Bodell, and K. M. Baldwin

Department of Physiology and Biophysics, University of California, Irvine, Irvine, California

Submitted 20 September 2005; accepted in final form 13 October 2005

Haddad, F., G. R. Adams, P. W. Bodell, and K. M. Baldwin. Isometric resistance exercise fails to counteract skeletal muscle atrophy processes during the initial stages of unloading. *J Appl Physiol* 100: 433–441, 2006. First published October 20, 2005; doi:10.1152/japplphysiol.01203.2005.—This study tested the hypothesis that an isometric resistance training paradigm targeting the medial gastrocnemius of adult rodents is effective in preventing muscle atrophy during the early stages of hindlimb unloading by maintaining normal activation of the insulin receptor substrate-1 (IRS-1)/phosphoinositide-3 kinase (PI3K)/Akt signaling pathway. This pathway has been shown to simultaneously create an anabolic response while inhibiting processes upregulating catabolic processes involving expression of key enzymes in the ubiquitination of proteins for degradation. The findings show that during the 5 days of unloading *1*) absolute medial gastrocnemius muscle weight reduction occurred by ~20%, but muscle weight corrected to body weight was not different from normal weight-bearing controls ($P < 0.05$); *2*) normalized myofibril fraction concentration and content were decreased; and *3*) a robust isometric training program, known to induce a hypertrophy response, failed to maintain the myofibril protein content. This response occurred despite fully blunting the increases in the mRNA for of atrogin-1, MURF-1, and myostatin, e.g., sensitive gene markers of an activated catabolic state. Analyses of the IRS-1/PI3K/Akt markers indicated that abundance of IRS-1 and phosphorylation state of Akt and p70S6 kinase were decreased relative to normal control rats, and the resistance training failed to maintain these signaling markers at normal regulatory level. Our findings suggest that to fully prevent muscle atrophy responses affecting the myofibril system during unloading, the volume of mechanical stress must be augmented sufficiently to maintain optimal activity of the IRS-1/PI3K/Akt pathway to provide an effective anabolic stimulus on the muscle.

hindlimb suspension; protein degradation; anabolic stimuli; insulin-like growth factor-1; insulin receptor substrate-1

RESISTANCE EXERCISE HAS BEEN shown to be an important, effective modality to induce muscle hypertrophy in both human and animal models involving a variety of contraction paradigms (1, 12, 14, 19, 37). In rodents our laboratory has demonstrated approximately equivalent gains in muscle mass and protein accumulation after relatively short duration training programs (e.g., within 2–3 wk) (1). These adaptations occur essentially independently of the contraction mode used to induce the mechanical stimulus (i.e., isometric, shortening, or lengthening actions during muscle loading). Thus various types of high-force-output stimuli applied intermittently are effective in providing an anabolic stimulus to the target muscle group.

In contrast to hypertrophic adaptations, when lower limb muscle groups are subjected to unloading conditions, as during hindlimb suspension involving rodents, the target muscle groups rapidly atrophy with significant changes seen in as little as 4–8 days (18, 38, 39). Given the above contrasting responses that can be induced in skeletal muscle, we tested the primary hypothesis that resistance-training (RT) paradigms, similar to those used to induce hypertrophy (1), can serve as an effective countermeasure to retard the atrophy process when the exercise protocol is initiated during the onset of inducing the atrophy stimulus. Furthermore, we postulated that the exercise paradigm exerts its primary physiological effect by blunting expression of specific gene markers (e.g., atrogin-1, MURF-1, myostatin) that define a catabolic state (5, 16, 33) while upregulating a key signaling pathway (IRS-1/PI3K/Akt) known to be essential for inducing anabolic processes favoring a positive protein balance in skeletal muscle (11, 29).

Contrary to these hypotheses, the findings reported herein suggest that whereas the training paradigm used in the present study was indeed effective in blunting key markers of catabolism, it was ineffective in maintaining net myofibril protein balance in the target muscle and also failed to activate the Akt/p70S6k pathway to a level comparable to that seen in normal weight-bearing muscles.

METHODS

Experimental design. This study was conducted in conformity with the American Physiological Society's *Guiding Principles in the Care and Use of Animals*, and the protocol was approved by the University of California, Irvine IACUC. Young adult female Sprague-Dawley rats weighing 258 ± 5 g were randomly assigned to two experimental groups (*n* = 7 each), designated as normal control (NC) and hindlimb suspended (HS). Rats were grouped housed in standard vivarium cages on a 12:12-h light-dark cycle and were allowed access to food and water ad libitum. The experiment lasted a total of 6 days with both HS and the first day of training being performed on designated *day 0*. The animals then were suspended and trained for 2 more days followed by a day of rest from exercise, and then they were trained for 2 additional days while the suspension continued before being killed on *day 6* (i.e., 24 h after the last training session). This time frame was selected because pilot studies establish that during the first week of suspension, the mixed-fiber-type medial gastrocnemius (MG) muscle undergoes its steepest atrophy response followed by a more gradual decline in muscle weight. Thus the objective was to ascertain whether the intermittent training stimulus we employed was sufficient to blunt the atrophy response during its most intensive stage.

Hindlimb suspension. Animals designated to be HS were treated with a noninvasive tail casting technique as described previously (38). This technique utilized a swivel harness system incorporated into the casting materials, which was attached to a hook on the top of the cage. The hook was adjusted to allow only the front legs of the animal to

Address for reprint requests and other correspondence: F. Haddad, Dept. of Physiology and Biophysics, Univ. of California, Irvine, Irvine, CA 92697 (e-mail: fhaddad@uci.edu).

reach the floor. Suspended animals were free to move about the cage using their front legs for obtaining food and water.

Muscle activation and RT exercise. For each training bout the rats were anesthetized with ketamine-xylazine-acepromazine (30:4:1 mg/kg). Stimulation electrodes consisting of Teflon-coated stainless steel wire were introduced into the subcutaneous region adjacent to the popliteal fossa via 22-gauge hypodermic needles. The needles were then withdrawn leaving the wire in place. Prior to electrode insertion a small section of Teflon coating was removed to expose the wire. Wire placement was lateral and medial of the location of the sciatic nerve allowing for field stimulation of the nerve. The stimulation wires were then attached to the output poles of a Grass stimulus isolation unit interfaced with a Grass S8 stimulator. This allowed for the delivery of current to the sciatic nerve resulting in muscle contraction. The rats were then positioned in a specially built training platform described previously (6). The right leg was positioned in a footplate attached to the shaft of a Cambridge model H ergometer. The voltage and stimulation frequency (54 ± 0.9 Hz) were adjusted to produce maximal isometric tension. Previous studies indicated that this approach resulted reproducible torque production within and between rats over multiple training sessions (1). During each training session NC rats were anesthetized similarly to the HS groups except that they were not mounted on the training platform.

Each training bout consisted of a series of four sets of contractions with 5 min of recovery between sets. Each set consisted of a series of 10 maximal isometric contractions lasting 2 s each with 20 s of rest in between contractions. Thus each training session lasted for 27 min, during which the muscle was activated for a cumulative time of 80 s. This protocol resulted in less than 25% fatigue on the basis of force output comparison between the first set and the last set. After each training session the electrodes were withdrawn. The training protocols were controlled by computer via a digital-to-analog board (DDA-06, Keithley Instruments) used to control footplate excursion and to trigger the stimulus. A separate analog-to-digital board (DAS-16) was used to acquire force measurements (100-Hz acquisition). Data acquisition, control of stimulus triggering, and footplate excursion were programmed by use of LabTech Notebook (Laboratory Technologies). Data analysis was conducted with AcqKnowledge software (Biopac Systems). Force output was monitored in real time on the computer screen during each contraction. Contractions were performed in the isometric mode with positioning of the right foot on the footplate with an angle of ~44° relative to the tibia. During muscle stimulation there was no change in the footplate angle. Rats were trained ~4 h after the beginning of their standard light cycle.

Tissue collection. Twenty-four hours after the last training bout the rats were killed via an injection of Pentosol euthanasia solution (Med-Pharmex) at a dose of 0.4 ml/kg ip (~160 mg/kg pentobarbital). At the cessation of heart beat, a skin incision is made and the MG muscles of both legs were dissected free of connective tissue, weighed, snap-frozen between blocks of dry ice, and stored at −80°C for later analysis.

Biochemical and molecular analyses. A preweighed portion of each mixed MG muscle sample was homogenized in 20 volumes of a homogenization buffer, which contained 250 mM sucrose, 100 mM KCl, 5 mM EDTA, and 10 mM Tris·HCl, pH 7.0. Myofibrillar proteins were quantitatively extracted from a known volume of the total homogenate by a modification of the original procedure described by Solaro et al. (34) and were suspended into a known volume of 100 mM KCl, 10 mM Tris, and 1 mM EDTA, pH 7.4. Protein concentration in the homogenate and myofibril suspension was determined by use of the Biorad Protein assay with gamma globulin as a standard. Muscle protein and myofibril content were calculated on the basis of the homogenized muscle piece weight and total muscle weight. Muscle total DNA concentration calculation was based on total DNA concentration in the total homogenate and was determined using a fluorometric assay using the DNA-specific fluorescent Hoechst 33258 dye (25).

Total RNA isolation. Total RNA was extracted from preweighed frozen muscle samples of mixed-fiber-type MG (comprising the belly of the MG) using the TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the company's protocol. This procedure is based on the method described by Chomczynski (7). Extracted RNA was precipitated from the aqueous phase with isopropanol, and, after being washed with ethanol, the extract was dried and suspended in a known volume of nuclease-free water. The RNA concentration was determined by optical density at 260 nm (using an OD260 unit equivalent to 40 μg/ml). The muscle total RNA concentration was calculated on the basis of total RNA yield and the weight of the analyzed sample. The RNA samples were stored frozen at −80°C and were used subsequently in relative RT-PCR procedures.

RT. One microgram of total RNA was reverse transcribed for each muscle sample by use of 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 20-μl total reaction volume at 45°C for 50 min, according to the provided protocol. At the end of the RT reaction, the tubes were heated at 90°C for 5 min to stop the reaction and then they were stored at −80°C until used in the 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-I (IGF-I), IGF-I binding protein 4 (IGF1BP4), myostatin, atrogin 1, and MURF-1. The sequence for the primers used for the specific target mRNAs is shown in Table 1. These primers were purchased from Operon Biotechnologies (Huntsville, AL). In each PCR, 18S ribosomal RNA was coamplified with the target cDNA (mRNA) to serve as an internal standard and to allow correction for any differences in starting amounts of total RNA.

For the 18S amplification we used the Alternate 18S Internal Standards (Ambion), which yields a 324-bp product. The 18S primers were mixed with competimers at an optimized ratio that could range from 1:4 to 1:10, depending on the abundance of the target mRNA. Inclusion of 18S competimers was necessary to bring down the 18S signal, which allows its linear amplification to be in the same range as the coamplified target mRNA (Ambion, Relative RT-PCR kit protocol).

Table 1. *Primer sequences for RT-PCR mRNA analyses*

Target mRNA	PCR Primer sequence 5′→3′	PCR Product Size, bp	GenBank Accession No.
IGF-I	Fwd: GCATTGTGGATGAGTGTTGC Rev: GGCTCCTCCTACATTCTGTA	202	M15480
IGF-BP4	Fwd: CCTGGGCTTGGGGATGC Rev: AGGGGTTGAAGCTGTTGTTGG	212	AY686592
Myostatin	Fwd: CAAACAGCCTGAATCCAACTTAG Rev: CCGTGAGGGGGTAGCGACAG	215	NM_019151
Atrogin-1	Fwd: CAGAACAGCAAAACCAAAACTC Rev: GCGATGCCACTCAGGGATGT	218	NM_133521
MURF-1	Fwd: TACCGAGAGCAGTTGGAAAAGT Rev: CTCAAGGCCTCTGCTATGTTT	215	AY059627

For each target mRNA, the RT and PCR were carried out under identical conditions by using the same reagent premix for all the samples to be compared in the study. To validate the consistency of the analysis procedures, at least one representative sample from each group was included in each RT-PCR run.

One microliter of each RT reaction (0- to 10-fold dilution depending on target mRNA abundance) was used for the PCR amplification. PCRs were carried out in the presence of 2 mM $MgCl_2$ using standard PCR buffer (Bioline), 0.2 mM dNTP, 1 μM specific primer set, 0.5 μM 18S primer/competimer mix, and 0.75 unit of Biolase DNA polymerase (Bioline, Genesee, San Diego, CA) in 25-μl total volume. Amplifications were carried out in a Stratagene Robocycler with an initial denaturing step of 3 min at 96°C, followed by 25 cycles of 1 min at 96°C, 1 min at 55°C (55–60°C depending on primers), 1 min at 72°C, and a final step of 3 min at 72°C. PCR products were separated on a 2.5% agarose gel by electrophoresis and stained with ethidium bromide. The ultraviolet light-induced fluorescence of stained DNA bands was captured by a digital camera, and the band intensities were quantified by densitometry with ImageQuant software (GE Healthcare) on digitized images and were reported as arbitrary scan units. In this approach, each specific mRNA signal is normalized to its corresponding 18S. For each primer set, PCR conditions (cDNA dilutions, 18S competimer/primer mix, $MgCl_2$ concentration, 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.

Western blot analyses. Immunoblotting was used for the analyses of expression and/or phosphorylation states of specific protein involved in muscle intracellular signaling. These include analyses of insulin receptor substrate-1 (IRS-1), the p70-S6 kinase (p70S6K) and its phosphorylation at Thr 389, Akt/protein kinase B and its phosphorylation at Thr 308. These protein- and phospho-protein-specific antibodies were purchased from Cell Signaling Technology (Beverly, MA). Phosphorylation at the specified sites has been shown to strongly correlate with the kinase biological activity in vivo (Cell Signaling). Muscle samples were extracted by homogenization in seven volumes of ice-cold buffer A (50 mM Tris·HCl pH 7.8, 2 mM potassium phosphate, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 1 mM DTT, 3 mM benzamidine, 1 mM sodium orthovanadate, 10 μM leupeptin, 5 μg/ml aprotinin, 200 μg/ml soybean trypsin inhibitor, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride) by use of a motor-driven glass pestle. The homogenate was immediately centrifuged at 12,000 g for 30 min at 4°C. The supernatant was immediately saved in aliquots at −80°C for subsequent use in immunoblotting. The supernatant protein concentration was determined using the Bio-Rad protein assay with BSA as the standard. Approximately 50 μg of supernatant proteins were subjected to SDS-PAGE (12.5% T), according to standard protocol (26), and then electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P) using 10% methanol, 1 mM orthovanadate, 25 mM Tris, 193 mM glycine, pH 8.3. 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/Image Quant). For each specific antibody, all the samples were run under identical (previously optimized) conditions, including the transfer on the membrane, the reaction with the first and secondary antibodies, washing conditions, enhanced chemiluminescence detection, and film exposure. To ensure the consistency of this analysis, at least one representative sample from each group was included in each gel run and Western analysis. In addition, a positive control, provided by the antibody supplier, was run on each gel to allow for normalization. For each set of Western blotting and detection conditions, the detected signal was directly proportional to the amount of protein loaded on the gel over a range 20–150 μg (data not shown).

Data Presentation and Statistical Analysis

Because the hindlimb-suspension model is associated with significant body mass loss, muscle mass, muscle protein content, and myofibril content are normalized to body mass to assess whether the changes observed in the whole muscle are reflective of whole body mass change or whether they are specific to the muscle. All values are reported as means and SE. Treatment effects were determined by one-way ANOVA with post hoc testing (Newman-Keuls multiple comparison tests) using the Prism software package (Graphpad). Additionally, one-tailed paired t-tests were performed in comparing trained vs. nontrained legs in the suspension group; these paired t-test comparisons were done to detect the effectiveness of RT in altering the parameters being studied in the hindlimb suspension independently of the weight-bearing NC group. For all statistical tests the 0.05 level of confidence was accepted for statistical significance.

RESULTS

Body and Muscle Weight and Protein Analyses

The 5-day suspension program induced a significant 9% loss in body weight (Fig. 1A). This is a consistent finding with this
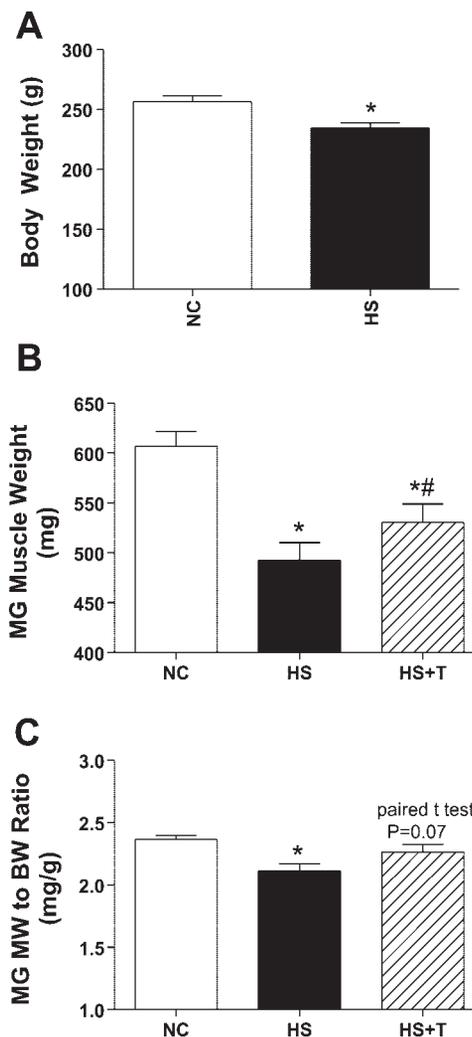

Fig. 1. Body weight (BW; *A*) and absolute (*B*) and relative (*C*) medial gastrocnemius (MG) muscle weights (MW) in normal control (NC) hindlimb suspension (HS) and hindlimb suspension plus trained (HS + T) muscle groups. Values are means ± SE (*n* = 7 animals/group). *Different from NC group $P < 0.05$ by unpaired t-test in *A* and 1-way ANOVA and post hoc Newman-Keuls t-tests in *B* and *C*. #Significant difference between HS and HS + T as analyzed by a paired t-test.

model during the initial stages of suspension; therefore examining the normalized muscle weight to body weight becomes important to assess muscle atrophy. The absolute and normalized MG muscle weights were significantly reduced by 20 and 11% via the suspension stimulus relative to the NC group, respectively (Fig. 1). Although the RT program had a positive effect on maintaining relative muscle weight at a higher level compared with the HS group (8%; $P = 0.07$), this response could have been due, in part, to edema, because total protein concentration was slightly lower (7%, $P < 0.05$) in the HS + T compared with the HS group (Fig. 2A). Thus the RT paradigm fell short in maintaining true muscle weight at the NC level (Fig. 1). Also, there were small decreases in the myofibril protein concentration in the HS and HS + T muscle groups (5 and 10%, respectively, $P > 0.05$); and this response became further exaggerated by expressing the myofibril data in terms of myofibril content (concentration × normalized muscle weight) in which the myofibril pool was diminished by 16 and 14%, respectively, in both the HS and HS + T muscle groups

Fig. 3. MG muscle myofibrils. Myofibril concentration (A) and content (B) in NC, HS, and HS + T muscle groups. Also C shows the myofibril content as normalized to body weight. Values are means ± SE (n = 7 animals/group). *Significantly different from NC (ANOVA, Newman-Keuls; $P < 0.05$).

(Fig. 3). This response clearly shows the negative impact that HS had on the muscle system by demonstrating that the myofibril pool was indeed a primary target of the atrophy response. Also, similar to the muscle weight response, the RT program failed to prevent the reductions in content of the myofibril protein pool.

Total RNA and DNA Concentration

Hindlimb suspension induced a significant decrease in the total RNA concentration of the MG (24% HS vs. NC, $P < 0.05$) (Fig. 4). Because the majority of the RNA is thought to be ribosomal and most of the muscle RNA originates in the myofibers, as they represent the primary cell type in muscle tissue, this response is indicative that the machinery for protein translational events in the myofibers is compromised. Interestingly, this deficit was prevented by the RT program, although this response appeared to be ineffective in establishing normal protein balance in the muscle as demonstrated above. No changes in DNA concentration were noted across the three groups.
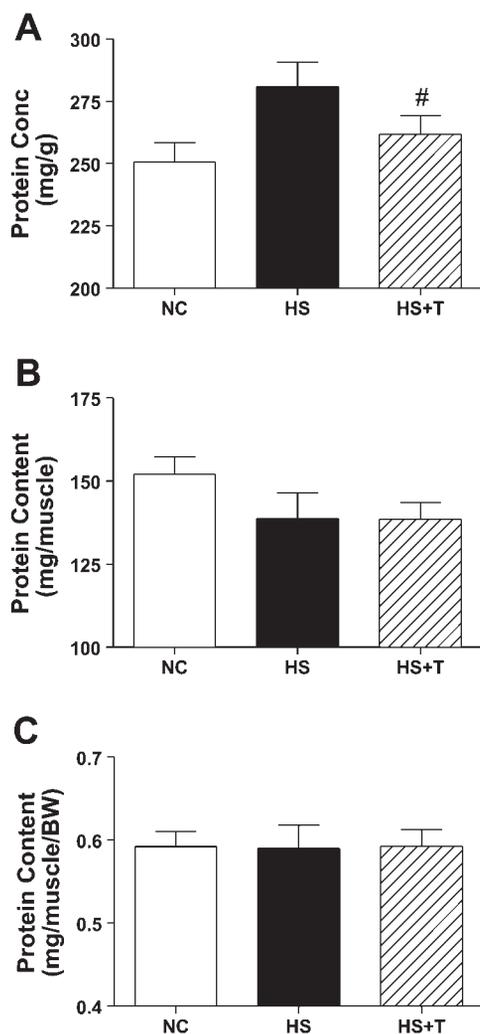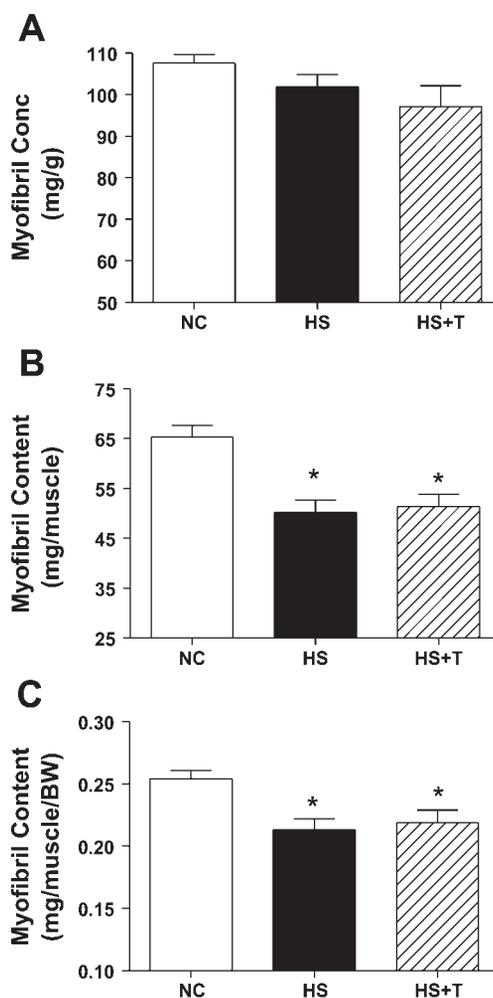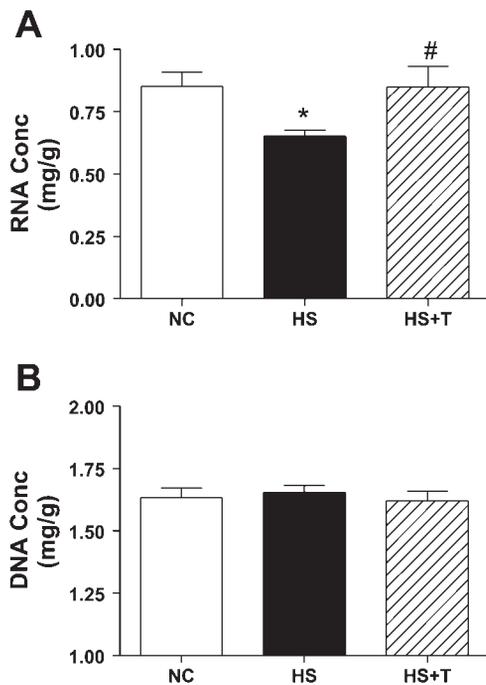
Fig. 2. MG muscle protein. A: total protein concentration (Conc, mg/g). B: total protein content (mg per whole MG muscle). C: total protein content normalized to BW in NC, HS, and HS + T muscle groups. Values are means ± SE (n = 7 animals/group). #Significant difference between HS and HS + T group(s) paired t-test.

Fig. 4. RNA (*A*) and DNA (*B*) concentration in NC, HS, and HS + T muscle groups. Values are means ± SE (*n* = 7 animals/group). *Different from the NC group (ANOVA, Newman-Keuls; *P* < 0.05). #Significant difference between HS and HS + T group by paired *t*-test comparison.

translation and elongation processes. Moreover, components of this pathway have been shown to be downregulated in the model of hindlimb suspension, further indicating that this pathway is an essential player in regulating both muscle mass and protein balance. In support of these findings we show that both IRS-1 abundance (Fig. 6) and phosphorylation state of Akt and of p70S6 kinase (Fig. 7) were significantly reduced in the HS MG compared with NC animals. Interestingly, we also show that the RT paradigm was ineffective in maintaining the
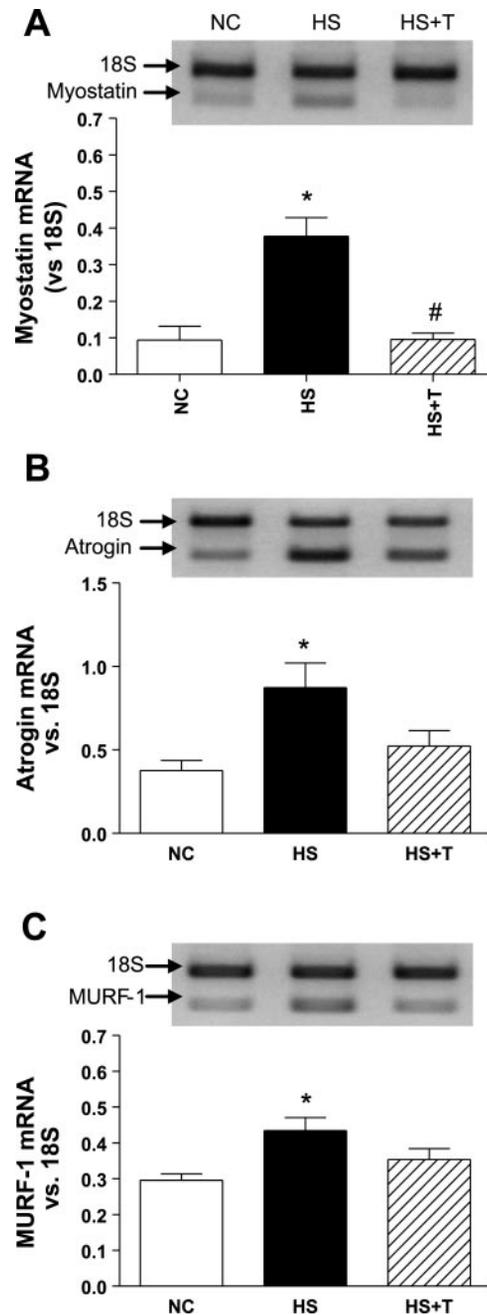
Catabolic Markers of Protein Balance

In recent years evidence has accumulated that two newly discovered ubiquitin ligases, muscle ring finger 1 (MURF-1) and muscle atrophy F-box (Mafbx), also known as atrogin 1, are probable causative factors contributing to the majority of muscle wasting models that have been studied to date, including hindlimb suspension (9, 15, 16, 31). Furthermore, myostatin, a transcription factor that serves as a skeletal muscle antigrowth factor, is also upregulated during conditions of muscle wasting [see review by Jackman and Kandarian (20)]. Thus we examined mRNA expression of these factors as markers for a catabolic state of the muscle. As presented in Fig. 5, there was significant upregulation of these three factors in the HS muscles relative to NC conditions, which is in line with an increased catabolic state in the unloaded HS MG muscle. Interestingly, the RT paradigm was effective in blunting this collective response. For example, the myostatin mRNA in HS MG was 400% that of the NC, and training brought it down to normal control level (Fig. 5*A*). Atrogin and MURF-1 mRNA levels were 230 and 140% that of NC levels (Fig. 5, *B* and *C*), respectively (*P* > 0.05, one-way ANOVA). The RT brought these signals down to become not significantly different from control (Fig. 5), thus reverting the muscle to a more normal metabolic state in this regard.

Anabolic Markers of Protein Balance

In 2001, the seminal work of Bodine et al. (4) demonstrated the importance of the IGF-I/PI3K/Akt pathway and its downstream effectors (e.g., p70S6k, 4EBP-1) as an essential pathway for inducing hypertrophy (a state of positive protein balance) by modulating several factors essential to protein

Fig. 5. MG muscle myostatin (*A*), atrogin (*B*), and MURF-1 (*C*) mRNA in NC, HS, and HS + T muscle groups. Also shown are representative ethidium bromide-stained gels with PCR products corresponding to 18S rRNA and target mRNA. Values are means ± SE (*n* = 7 animals/group). *Different from the NC group as analyzed by ANOVA, Newman-Keuls, *P* < 0.05. #Significant difference between HS and HS + T group as analyzed by paired *t*-test.
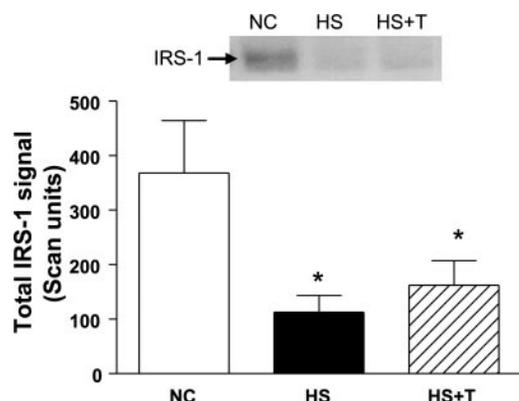
Fig. 6. MG muscle total insulin receptor substrate-1 (IRS-1) substrate abundance in NC, HS, and HS + T muscle groups. Also shown are representative IRS-1 immunoblots from 1 animal/group. Values are means $\pm$ SE ($n = 7$ animals/group). *Different from the NC group (ANOVA, Newman-Keuls; $P < 0.05$).

activation of the markers in this pathway at a level mimicking that seen in NC muscle (Figs. 6 and 7). These changes occurred despite evidence that the resistance-exercise stimulus had a positive impact on stimulating increased expression of muscle derived IGF-I and especially IGF-I binding protein 4 mRNA (Fig. 8), which have been shown to be upregulated in models of muscle hypertrophy (3). Apparently, the elevation of these factors was inconsequential given the negative impact on IRS-1, which is pivotal in the chain of signaling impacting protein translational events.

DISCUSSION

The findings reported herein are interesting for the fact that the imposed exercise countermeasure failed to prevent muscle atrophy, despite blunting of catabolic indexes associated with unloading. Thus, although blunting the degradation markers, RT was not sufficient to blunt the atrophy response because the anabolic pathway was defective, more likely owing to IRS-1 deficit in the unloaded muscle. This finding is intriguing and shows that protein deficit countermeasure effectiveness not only is the result of blunting the degradation response but also requires the interactive involvement of the anabolic response.

Recent studies involving both in vitro and in vivo experiments targeting manipulation of the IRS-1/PI3K/Akt pathway suggest that this particular pathway is pivotal for regulating protein balance, i.e., protein synthesis and degradation processes in muscle cells, in an antithetical fashion (9, 27, 29). Activation of this pathway by treatment with either IGF-I or mechanical stimulation (an event also causing increased gene expression of IGF-I) results in an anabolic response affecting increased phosphorylation of proteins such as p70S6K and eukaryotic initiation factor 4 binding protein (4EBP-1), which play key roles increasing protein translational efficiency. In addition, via Akt, activity of this pathway inhibits the activation and translocation of FOXO1, a member of the forkhead family of transcription factors, that are necessary for the expression of essential components of the ubiquitin-proteasome system, e.g., atrogin-1 and MURF-1 in muscle cells (31, 36). Conversely, it has been shown that conditions of muscle wasting, as examined in cell systems, as well as in animal
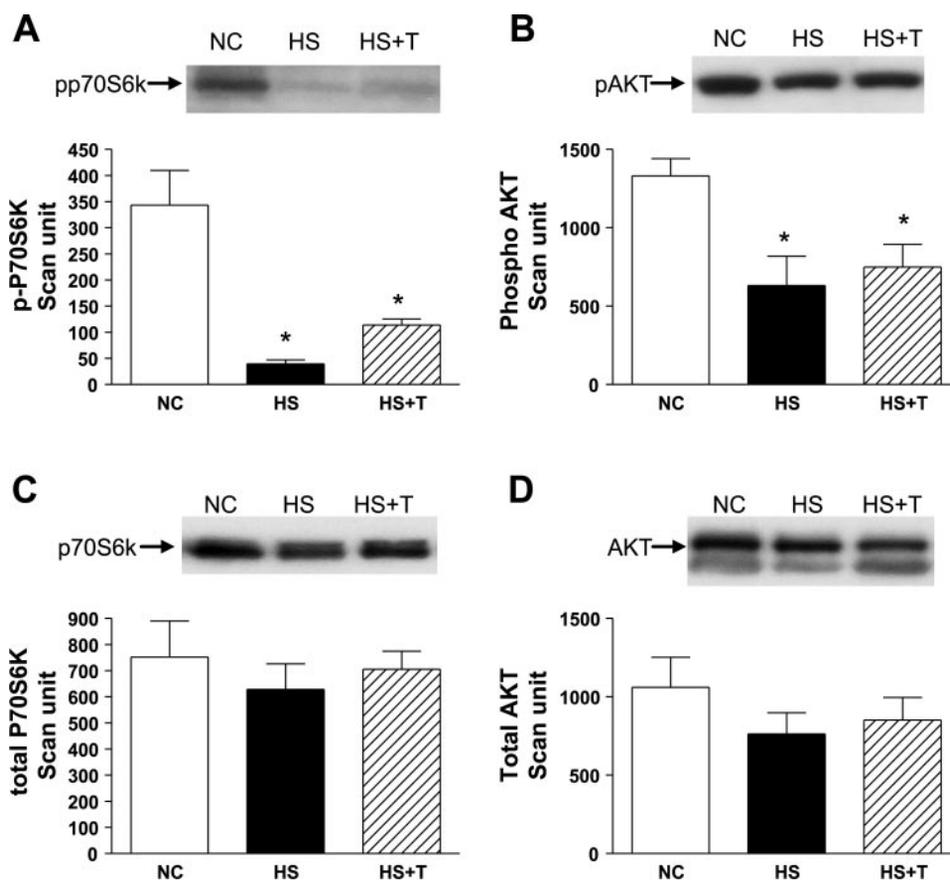

Fig. 7. Phosphorylation and expression levels of p70S6 kinase (p70S6K) and AKT in NC, HS, and HS+T muscle groups. A: Phospho-p70S6 kinase (pp70S6K); B: total p70S6K; C: phospho-AKT; D: total AKT. Also shown are representative immunoblots for corresponding antibodies in the 3 groups. Values are means $\pm$ SE ($n = 7$ animals/group). *Different from the NC group (ANOVA, Newman-Keuls; $P < 0.05$).
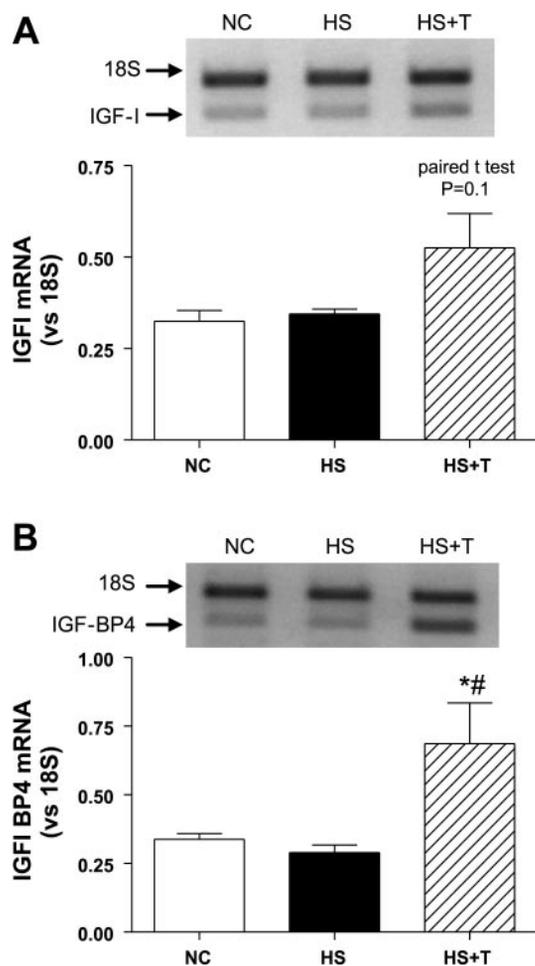
Fig. 8. MG IGF-I (*A*) and IGF-I binding protein-4 (IGF1 BP4; *B*) mRNA in NC, HS, and HS + T muscle groups. Also shown are representative ethidium bromide-stained gels with PCR products corresponding to 18S rRNA and target mRNA. Values are means ± SE (*n* = 7 animals/group). *Different from the NC group (Anova, Neuman-Keuls, *P* < 0.05). #Significant difference between HS and HS+T as analyzed by paired *t*-test.

response is complemented by the observations that atrogin-1 and MURF-1 expression is upregulated at the mRNA level. In additional experiments we have noted that such changes can occur in as little as 2 days after the onset of suspension (unpublished observations). Thus the suspension model induces a reduction in protein accumulation capacity (also taking into consideration the reduction in total RNA concentration), which is coupled with the activation of key elements in the protein degradation system linked to the ubiquitin-proteasome axis.

One of the more dramatic effects of the limb unweighting protocol involved a significant decrease in the amount of IRS-1 protein present in the unloaded muscle (Fig. 6). IRS-1 participates in a pivotal step in the transmission and amplification of signaling via the insulin and IGF-I receptors. Upon ligation, receptor initiated tyrosine phosphorylation of IRS-1 promotes signaling via the phosphoinositide-3 kinase (PI3K) pathway leading to the activation of downstream effectors such as Akt, mammalian target of rapamycin, and S6K1 (17).

Although it is not clear at what level the amount of IRS-1 would become limiting for signal transduction, the results of the present study provide evidence that the decrease in this protein may have contributed significantly to the inability of the unloaded muscles to maintain sufficient anabolic drive. This result appears to have been manifested in the decline in Akt and S6K1 phosphorylation seen in the unweighted muscles and in the inability of the RT countermeasure to significantly ameliorate this effect.

The literature clearly supports a critical role for the involvement of IRS-1 activity as a regulator of signaling flux through the PI3K pathway. A wide variety of signaling pathways and mediators converge on IRS-1 acting in a regulatory mode (10, 13, 17, 24). One interpretation of the present results is that IRS-1 inhibition and degradation may be a very early response to a substantial decrease in loading. In this scenario, the initial loss of IRS-1 activity would result in an alteration in the baseline capacity to activate the PI3K pathway that appears to be difficult to ameliorate with just 80 s of contractile activity per day, which was the case in the present study. This response would hold true even when this high force loading is implemented early on during the unloading exposure. If this interpretation has validity, this would suggest that one or more of the mediators that regulate IRS-1 may be more sensitive to the cumulative amount of loading imposed on muscle rather than to some function of the peak load seen by the muscles.

We and others have previously reported that the isometric mode training is very effective in producing muscle hypertrophy (e.g., Refs. 1, 14, 21, 22, 40). However, it is possible that unloading induces changes in skeletal muscle that are uniquely refractory to the anabolic stimulus provided by isometric exercise. In this case the failure of the RT protocol in the present paper to prevent muscle atrophy would be a function of the contraction mode. However, there is evidence that isometric contractions in humans and rodents can ameliorate some of the muscle atrophy induced by unloading indicating that there is probably not a unique insensitivity to this contraction mode (e.g., Refs. 2, 8, 23, 30).

The present results indicate that isometric RT did positively impact a number of catabolic and anabolic responses over the short duration of this study. This suggests that the continued application of this training protocol would eventually amelio-

models such as starvation, insulin deficiency, and glucocorticoid treatment not only inhibit the IRS-1/PI3K/Akt pathway in skeletal muscle but they also increase the activation of FOXO-1 and thus increase proteolysis as evidenced by the activation of atrogin-1 and MURF-1 [see papers reviewed in Du and Mitch (9)]. Of further relevance is the finding that caspase-3, which is thought to regulate the breakdown of actin-myosin complexes in the myofibril system is negatively modulated by activation of the IRS-1/PI3K/Akt pathway and vice versa (35). Collectively, these findings point to a pivotal level of regulation involving this key signaling pathway under various conditions likely to induce muscle wasting vs. that of muscle hypertrophy.

The findings reported herein are thus consistent with the dogma concerning the role the IRS-1/PI3K/Akt under conditions of muscle unloading as initially reported by Bodine et al. (5). Here we provide strong evidence that several key components of this pathway (IRS-1 abundance, Akt and p70S6K phosphorylation levels) were significantly reduced during a critical stage in which the muscle rapidly goes into protein deficit, especially involving the myofibril fraction (Fig. 3). This

rate the loss in contractile protein. Further studies, imposing RT during extended unloading, will be necessary to determine whether the observed anabolic inputs will completely reverse this initial loss in muscle mass or whether some portion of the deficit will remain as a new steady state is established.

Regardless of the mechanism of decreased muscle anabolic drive, the results of this study indicate that there appears to be a thresholdlike event that occurs early on in the response to decreased loading that leads to a change in some baseline parameters, such as IRS-1 protein. This event thus causes the muscle to be refractory to the stimulus of RT. This response is in contrast to normally loaded muscle, which has a higher baseline of signaling activity. Thus, under the latter condition muscle hypertrophy would be the logical end result to appropriate signaling stimuli. One challenge is then to elucidate the mechanisms that respond so powerfully to unloading and devise countermeasures that prevent the crossing of this threshold, thereby protecting existing muscle mass rather than attempting to rebuild what has been lost.

Practical Considerations

From a practical perspective muscle atrophy is an important problem with negative impact concerning human performance during long-term space travel, chronic inactivity such as bed rest, and as a result of the aging process. It is apparent that physical exercise with high-resistance loading can be a logical countermeasure to such challenging deficits in muscle structure and function. Studies with humans point to the fact that exercise modalities can be an important strategy to reduce atrophy in different muscle groups. To that issue it is becoming apparent that in training various muscle groups in humans, the calf region appears to be more challenging than for example training the quadriceps muscle group in terms of inducing hypertrophy as well as preventing atrophy (28, 32). It is uncertain whether this factor was a confounding issue in the case of this study concerning identification of an optimal training paradigm for the MG. Therefore, questions remain as to what are the appropriate prescriptions (contraction modes, number of contractions per set and per training session, and the time intervals between sessions) that are necessary to significantly blunt the marked atrophy that occurs under conditions of chronic muscle unloading. The results of this study clearly suggest that *1*) in the case of the HS model of rodent muscle atrophy one must use physiological approaches of training rat skeletal muscle that are in line with the modalities and approaches used for humans, and *2*) it is apparent that more accumulative stress on muscle be applied to oppose the apparent deficits in the IRS-1/PI3K/Akt pathway that lead to the net catabolic state as seen herein for the MG muscle. Regarding the rat training model, this challenge does not require a greater activation state of the muscle because our technical design involved fully activating and recruiting all of the muscle fibers while using an isometric contraction mode that optimizes the full "turning on" of the active state of the muscle for generating optimal force output via cross-bridge interaction. Rather, it implies that either more contractions per set, more sets per session, and/or more sessions per day must be invoked to maintain a mechanical stimulus that activates the signaling pathways that produce an anabolic stimulus along with a simultaneous stimulus that prevents the upregulation of pro-

cesses that blunt augmented proteolytic processes. Our findings suggest that although our paradigm was effective in causing the latter, it clearly fell short in effectively impacting the former.

REFERENCES

1. **Adams GR, Cheng DC, Haddad F, and Baldwin KM.** Skeletal muscle hypertrophy in response to isometric, lengthening, and shortening training bouts of equivalent duration. *J Appl Physiol* 96: 1613–1618, 2004.
2. **Akima H, Kubo K, Kanehisa H, Suzuki Y, Gunji A, and Fukunaga T.** Leg-press resistance training during 20 days of 6 degrees head-down-tilt bed rest prevents muscle deconditioning. *Eur J Appl Physiol* 82: 30–38, 2000.
3. **Awede B, Thissen J, Gailly P, and Lebacq J.** Regulation of IGF-1, IGFBP-4 and IGFBP-5 gene expression by loading in mouse skeletal muscle. *FEBS Lett* 461: 263–267, 1999.
4. **Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, and Glass DJ.** Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* 294: 1704–1708, 2001.
5. **Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, and Yancopoulos GD.** Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol* 3: 1014–1019, 2001.
6. **Caiozzo VJ, Ma E, McCue SA, Smith E, Herrick RE, and Baldwin KM.** A new animal model for modulating myosin isoform expression by altered mechanical activity. *J Appl Physiol* 73: 1432–1440, 1992.
7. **Chomczynski P.** A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* 15: 532–534, 536–537, 1993.
8. **Diffee GM, Caiozzo VJ, McCue SA, Herrick RE, and Baldwin KM.** Activity-induced regulation of myosin isoform distribution: comparison of two contractile activity programs. *J Appl Physiol* 74: 2509–2516, 1993.
9. **Du J and Mitch WE.** Identification of pathways controlling muscle protein metabolism in uremia and other catabolic conditions. *Curr Opin Nephrol Hypertens* 14: 378–382, 2005.
10. **Evans JL, Maddux BA, and Goldfine ID.** The molecular basis for oxidative stress-induced insulin resistances. *Antioxid Redox Signal* 7: 1040–1052, 2005.
11. **Franch HA and Price SR.** Molecular signaling pathways regulating muscle proteolysis during atrophy. *Curr Opin Clin Nutr Metab Care* 8: 271–275, 2005.
12. **Fry AC.** The role of resistance exercise intensity on muscle fibre adaptations. *Sports Med* 34: 663–679, 2004.
13. **Gao Z, Zuberi A, Quon MJ, Dong Z, and Ye J.** Aspirin inhibits serine phosphorylation of insulin receptor substrate 1 in tumor necrosis factor-treated cells through targeting multiple serine kinases. *J Biol Chem* 278: 24944–24950, 2003.
14. **Garfinkel S and Cafarelli E.** Relative changes in maximal force, EMG, and muscle cross-sectional area after isometric training. *Med Sci Sports Exerc* 24: 1220–1227, 1992.
15. **Glass DJ.** Skeletal muscle hypertrophy and atrophy signaling pathways. *Int J Biochem Cell Biol* 37: 1974–1984, 2005.
16. **Gomes MD, Lecker SH, Jagoe RT, Navon A, and Goldberg AL.** Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proc Natl Acad Sci USA* 98: 14440–14445, 2001.
17. **Gual P, Le Marchand-Brustel Y, and Tanti JF.** Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. *Biochimie* 87: 99–109, 2005.
18. **Haddad F, Qin AX, Zeng M, McCue SA, and Baldwin KM.** Interaction of hyperthyroidism and hindlimb suspension on skeletal myosin heavy chain expression. *J Appl Physiol* 85: 2227–2236, 1998.

19. **Hather BM, Tesch PA, Buchanan P, and Dudley GA.** Influence of eccentric actions on skeletal muscle adaptations to resistance training. *Acta Physiol Scand* 143: 177–185, 1991.

20. **Jackman RW and Kandarian SC.** The molecular basis of skeletal muscle atrophy. *Am J Physiol Cell Physiol* 287: C834–C843, 2004.

21. **Jones DA and Rutherford OM.** Human muscle strength training: the effects of three different regimens and the nature of the resultant changes. *J Physiol* 391: 1–11, 1987.

22. **Kanehisa H, Nagareda H, Kawakami Y, Akima H, Masani K, Kouzaki M, and Fukunaga T.** Effects of equivolume isometric training programs comprising medium or high resistance on muscle size and strength. *Eur J Appl Physiol* 87: 112–119, 2002.

23. **Kawakami Y, Akima H, Kubo K, Muraoka Y, Hasegawa H, Kouzaki M, Imai M, Suzuki Y, Gunji A, Kanehisa H, and Fukunaga T.** Changes in muscle size, architecture, and neural activation after 20 days of bed rest with and without resistance exercise. *Eur J Appl Physiol* 84: 7–12, 2001.

24. **Kim HJ, Higashimori T, Park SY, Choi H, Dong J, Kim YJ, Noh HL, Cho YR, Cline G, Kim YB, and Kim JK.** Differential effects of interleukin-6 and -10 on skeletal muscle and liver insulin action in vivo. *Diabetes* 53: 1060–1067, 2004.

25. **Labarca C and Paigen K.** A simple, rapid, and sensitive DNA assay procedure. *Anal Biochem* 102: 344–352, 1980.

26. **Laemmli UK.** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685, 1970.

27. **Lai KM, Gonzalez M, Poueymirou WT, Kline WO, Na E, Zlotchenko E, Stitt TN, Economides AN, Yancopoulos GD, and Glass DJ.** Conditional activation of akt in adult skeletal muscle induces rapid hypertrophy. *Mol Cell Biol* 24: 9295–9304, 2004.

28. **Rittweger J, Frost HM, Schiessl H, Ohshima H, Alkner B, Tesch P, and Felsenberg D.** Muscle atrophy and bone loss after 90 days' bed rest and the effects of flywheel resistive exercise and pamidronate: results from the LTBR study. *Bone* 36: 1019–1029, 2005.

29. **Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD, and Glass DJ.** Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat Cell Biol* 3: 1009–1013, 2001.

30. **Rozier CK, Elder JD, and Brown M.** Prevention of atrophy by isometric exercise of a casted leg. *J Sports Med Phys Fitness* 19: 191–194, 1979.

31. **Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH, and Goldberg AL.** Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell* 117: 399–412, 2004.

32. **Shackelford LC, LeBlanc AD, Driscoll TB, Evans HJ, Rianon NJ, Smith SM, Spector E, Feeback DL, and Lai D.** Resistance exercise as a countermeasure to disuse-induced bone loss. *J Appl Physiol* 97: 119–129, 2004.

33. **Sharma M, Langley B, Bass J, and Kambadur R.** Myostatin in muscle growth and repair. *Exerc Sport Sci Rev* 29: 155–158, 2001.

34. **Solaro RJ, Pang DC, and Briggs FN.** The purification of cardiac myofibrils with Triton X-100. *Biochim Biophys Acta* 245: 259–262, 1971.

35. **Song YH, Li Y, Du J, Mitch WE, Rosenthal N, and Delafontaine P.** Muscle-specific expression of IGF-I blocks angiotensin II-induced skeletal muscle wasting. *J Clin Invest* 115: 451–458, 2005.

36. **Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, Gonzalez M, Yancopoulos GD, and Glass DJ.** The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol Cell* 14: 395–403, 2004.

37. **Tesch PA, Ekberg A, Lindquist DM, and Trieschmann JT.** Muscle hypertrophy following 5-week resistance training using a non-gravity-dependent exercise system. *Acta Physiol Scand* 180: 89–98, 2004.

38. **Thomason DB, Herrick RE, Surdyka D, and Baldwin KM.** Time course of soleus muscle myosin expression during hindlimb suspension and recovery. *J Appl Physiol* 63: 130–137, 1987.

39. **Vermaelen M, Marini JF, Chopard A, Benyamin Y, Mercier J, and Astier C.** Ubiquitin targeting of rat muscle proteins during short periods of unloading. *Acta Physiol Scand* 185: 33–40, 2005.

40. **Welsh L and Rutherford OM.** Effects of isometric strength training on quadriceps muscle properties in over 55 year olds. *Eur J Appl Physiol* 72: 219–223, 1996.