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Combined isometric, concentric, and eccentric resistance exercise prevents unloading-induced muscle atrophy in rats

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Adams GR, Haddad F, Bodell PW, Tran PD, Baldwin KM. Combined isometric, concentric, and eccentric resistance exercise prevents unloading-induced muscle atrophy in rats. *J Appl Physiol* 103: 1644–1654, 2007. First published September 13, 2007; doi:10.1152/japplphysiol.00669.2007.—Previously, we reported that an isometric resistance training program that was effective in stimulating muscle hypertrophy in ambulatory rats could not completely prevent muscle atrophy during unloading (Haddad F, Adams GR, Bodell PW, Baldwin KM. *J Appl Physiol* 100: 433–441, 2006). These results indicated that preventing muscle atrophy does not appear to be simply a function of providing an anabolic stimulus. The present study was undertaken to determine if resistance training, with increased volume (3-s contractions) and incorporating both static and dynamic components, would be effective in preventing unloading-induced muscle atrophy. Rats were exposed to 5 days of muscle unloading via tail suspension. During that time one leg received electrically stimulated resistance exercise (RE) that included an isometric, concentric, and eccentric phase. The results of this study indicate that this combined-mode RE provided an anabolic stimulus sufficient to maintain the mass and myofibril content of the trained but not the contralateral medial gastrocnemius (MG) muscle. Relative to the contralateral MG, the RE stimulus increased the amount of total RNA (indicative of translational capacity) as well as the mRNA for several anabolic/myogenic markers such as insulin-like growth factor-I, myogenin, myoferlin, and procollagen III-α-1 and decreased that of myostatin, a negative regulator of muscle size. The combined-mode RE protocol also increased the activity of anabolic signaling intermediates such as p70S6 kinase. These results indicate that a combination of static- and dynamic-mode RE of sufficient volume provides an effective stimulus to stimulate anabolic/myogenic mechanisms to counter the initial stages of unloading-induced muscle atrophy.

anabolic; catabolic; IGF-I; myogenin; eukaryotic initiation factor 4E binding protein; p70 S6 kinase; myoferlin; procollagen

RESISTANCE EXERCISE has been shown to be an important, effective modality to induce muscle hypertrophy in both human and animal training paradigms (17, 45). Our recent findings on both acute and prolonged rodent resistance exercise paradigms (4, 32), as well as those involving humans, as comprehensively reviewed by Wernbom et al. (64), suggest that an enhancement of an anabolic state resulting in muscle hypertrophy appears to be equivalent among different contraction modes (e.g., isometric, concentric, and eccentric). These findings suggest that it is likely the combination of factors such as *1*) volume (number of repetitions), *2*) intensity (relative force generated during contraction), and *3*) frequency (number of training sessions spanning the program) within a given resistance exercise protocol that collectively interact to enhance muscle mass and net protein accumulation (4, 32, 37, 64).

On the other hand, when specific muscle groups are subjected to unloading conditions, as occurs during hindlimb suspension involving rodents (33, 37) or flight crews during spaceflight (2), the target muscle groups rapidly atrophy with significant changes observed in as little as 5–7 days (33, 37). In an attempt to counteract this response, we recently ascertained the effectiveness of an isometric resistance exercise paradigm that had been shown previously to be effective in inducing muscle hypertrophy (37). The chief results of that study demonstrated that although the training program used was indeed effective in blunting key markers of catabolism, it was ineffective in maintaining net myofibril protein balance in the target medial gastrocnemius muscle. Moreover, the paradigm also failed to sufficiently maintain the Akt/p70S6k signaling pathway to a level comparable to that seen in the normal weight-bearing muscle (37). This particular pathway has been implicated in the induction of hypertrophic responses in both cardiac and skeletal muscle (13, 16, 35). These findings suggested that to fully prevent the muscle atrophy response the total volume of mechanical stress, as integrated over the training period, needed to be augmented to optimize anabolic signaling processes and ameliorate the rapid atrophy response that occurs in the hindlimb suspension model (33, 37, 58).

Subsequent to the completion of our previous study (37) noted above, additional observations have occurred impacting the thrust of the present study. First, we have demonstrated that different contraction modes (i.e., isometric, concentric, eccentric) of equivalent force output produce similar cell signaling outcomes (32), as well as degrees of hypertrophy (4), suggesting that the variable of force rather than contractile mode is likely a key factor in creating anabolic events in the loading of skeletal muscle (32). However, in interactions with the National Aeronautics and Space Administration (NASA) Human Countermeasure Research Program at Johnson Space Center by K. M. Baldwin, Muscle Team Leader of the National Space Biomedical Research Institute (a subsidiary of NASA), it has become evident that the agency favors an integrated contraction regimen involving a combination of dynamic action modes as a central feature of the conditioning program as prescribed in the "American College of Sports Medicine Position Stand on Resistance Training for Healthy Adults" (45). Given these events, the following training study was undertaken with the objective of *1*) increasing the duration of each contraction cycle from 2 to 3 s, thereby approximately doubling the force output of the muscle during each contraction to increase the

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volume of force activity accumulated during each training session; and *2*) imposing a systematic combination of muscle actions on the muscle utilizing an isometric, concentric, and eccentric sequence of actions (see Fig. 1). Since this particular contraction paradigm, to our knowledge, has not been studied in terms of its impact on a variety of cell signaling processes, an additional objective was to ascertain the effect of this training paradigm, in the context of an unloading stimulus, on a variety of markers indicating that an anabolic stimulus on the target skeletal was sufficient to blunt the atrophy response. The central findings of this study demonstrate that, in contrast to previous findings using a lower volume of isometric-only contraction, an integrated combination of muscle actions of sufficient volume was effective in blunting the atrophy response.

METHODS

Experimental design and treatment protocol. This study was conducted in conformity with the *Guiding Principles in the Care and Use of Animals* of the American Physiological Society, and the protocol was approved by the University of California, Irvine, Animal Use Committee. This study used young adult female Sprague-Dawley rats weighing 266 ± 7 g ($n = 20$). Rats were on a 12:12-h light-dark cycle and were allowed access to food and water ad libitum. Rats were hindlimb suspended (tail cast method, see below) for a total of ~ 5 days ($n = 12$). Immediately before tail casting, the rats experienced a bout of electrically stimulated unilateral leg resistance training (see below). Resistance training was repeated on each of the subsequent days of hindlimb suspension. The experiment was terminated, and the animals were euthanized for tissue collection 24 h after the last resistance exercise session. The contralateral limbs served as an internal control, and data values are therefore designated as "Contra" (suspended contralateral) or "Train" (suspended trained).

To establish a normal loading baseline, a separate group of rats that were obtained in the same batch of animals was handled in parallel with the treatment rats (e.g., single housed, anesthetized, etc.) but did not experience hindlimb suspension or resistance training [sham ground control (GC) group, $n = 8$].

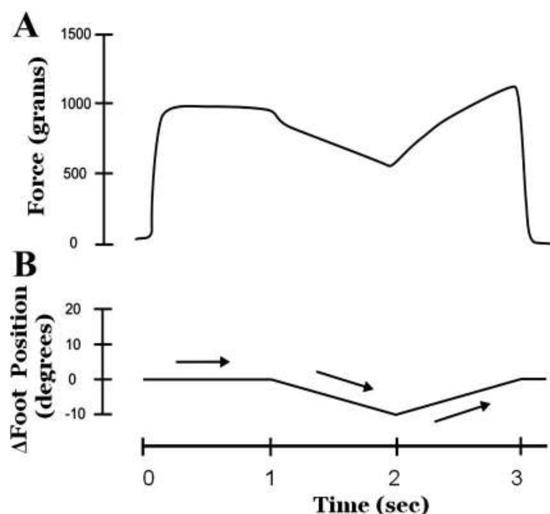

Fig. 1. Combined isometric, concentric, and eccentric muscle action. Rat leg muscles were electrically stimulated via the sciatic nerve to induce maximal activation. Activation was for 3 s. *A*: representative force output captured by computer during 1 activation. *B*: schematic representation of muscle length changes (i.e., foot movement relative to the tibia) during activation.

Hindlimb suspension. Animals were treated with a noninvasive tail-casting technique as described previously (63). 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 reach the floor. Suspended animals were free to move about the cage using their front legs for locomotion and obtaining food and water.

Muscle activation and resistance exercise. For each training bout, the rats were lightly anesthetized with ketamine-xylazine-acepromazine (30/4/1 mg/kg). Stimulation electrodes consisting of 40-gauge insulated nickel chromium wire (Stablohm 800B, CA Fine Wire) were introduced into the subcutaneous region adjacent to the popliteal fossa via 27-gauge hypodermic needles. The needles were then withdrawn leaving the wire in place. Before electrode insertion a small section of insulation 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 (without any direct contact of the nerve) to induce muscle contraction. The rats were then positioned in a specially built training platform described previously (18). The left leg was positioned in a foot plate attached to the shaft of a Cambridge model H ergometer. The voltage and stimulation frequency (60 ± 2 Hz) were adjusted to produce maximal isometric tension. Previous studies indicated that this approach resulted in reproducible torque production within and between rats over multiple training sessions (4). During each training session, GC rats were anesthetized similarly to the hindlimb-suspended trained groups except that the GC were not mounted on the training platform.

The stimulation technique used in this study should activate both the anterior and posterior muscles of the leg. This being the case, the antagonistic actions of the anterior compartment might be expected to counter some of the force produced by the posterior muscle groups. In pilot studies we determined that, for isometric, eccentric (lengthening), and concentric (shortening) muscle actions, tenotomy of the primary antagonistic muscle, the tibialis anterior (TA), resulted in <10% difference in force output measured pre- and posttenotomy (unpublished observations). This result is similar to that originally reported by Wong and Booth (65).

Training protocol. In keeping with the programmatic goals of the NASA Human Countermeasure Research Program, one of the key aims of this study was to ascertain if a combination of actions routinely used in muscle training with free weights and other training devices is effective in creating an anabolic state sufficient to offset the atrophy response that is typical of the hindlimb suspension model. To accomplish this aim the foot was initially positioned at an angle of ~44° relative to the tibia, and no change in the foot-plate angle was allowed at the onset of the induced contraction (this consisted of an isometric contraction, and it lasted for 1 s). The isometric phase was immediately followed by the concentric phase, which also lasted for 1 s. Because of the inherent drop in force output as the muscle shortens (see Fig. 1), the concentric-mode contraction was limited to 10° relative to the starting position (e.g., 44° to 54°). This phase of the contraction was immediately followed by an eccentric action, which returned the muscle back to its initial position. To complete all phases of contraction the parameters required 3 s of stimulation. A period of 27 s of rest was allowed between each contraction. Rats performed 10 contractions per set. A pilot study demonstrated that sets of five contractions, which resulted in a duty cycle similar to that of the previous isometric study (37), were not sufficient to counter the muscle atrophy. Five minutes of recovery was allowed between sets. On *day 1*, e.g., the 1st day of training, the session was limited to three sets, which were immediately followed by the initiation of the suspension protocol as described above. Following the initial training session, the animals received four, four, five, and five sets of contractions on the subsequent days after initiation of suspension, with the

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animals being killed 24 h after the last training session. The above training protocol resulted in <30% fatigue (maximum force generated during the last set vs. first set of exercise) during each training session. Since this reduction in force over the training session was similar to our previous study using a 2-s isometric contraction mode (37), we calculated that there was a ~47% increase in the relative force that was accumulated in the present study relative to our previous isometric straining study by merely increasing the duration of each individual contraction from 2 to 3 s. Following 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 foot plate 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 foot plate excursion were programmed using LabTech Notebook (Laboratory Technologies). Data analysis was conducted using AcqKnowledge software (Biopac Systems). Force output was monitored in real time on the computer screen during each contraction. Rats were trained ~5 hours after the beginning of their standard light cycle, during each training session.

Tissue collection. Twenty-four hours after the last exercise bout (5th session), the rats were killed via an injection of Pentosol euthanasia solution (Med-Pharmex) at a dose of 0.4 ml/kg (~160 mg/kg pentobarbital) intraperitoneally. At the cessation of heart beat, a skin incision was made, and the medial gastrocnemius muscles (MG) 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.

In this and previous studies, we have chosen to focus our analyses on the medial gastrocnemius muscle (MG). This is a weight-bearing/locomoter muscle in both humans and rodents, which demonstrates a mixed fiber type phenotype. In humans, the gastrocnemius muscle appears to be particularly sensitive to decreased loading (2). We and others have observed that rat MG muscles experience a significant degree of unloading-induced atrophy in response to unloading (23, 37, 58; and Adams, unpublished observation).

Tissue processing. A preweighed portion of each mixed MG muscle sample was homogenized in 20 vol of a homogenization buffer, which contained 250 mM sucrose, 100 mM KCl, 5 mM EDTA, and 10 mM Tris·HCl, pH 7.0.

Protein concentration in the homogenate was determined using the Bio-Rad Protein assay with gamma globulin as a standard. Muscle total protein content was calculated on the basis of the homogenized muscle piece weight and total muscle weight.

Muscle total DNA concentration was calculated on the basis of total DNA concentration in the total homogenate and was determined using a fluorometric assay using the DNA-specific fluorescent Hoechst 33258 dye (47).

Total RNA isolation. Total RNA was extracted from an additional preweighed frozen muscle sample of mixed MG fiber-type (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 and Sacchi (20). Extracted RNA was precipitated from the aqueous phase with isopropanol, and after washing with ethanol, the extract was dried and suspended in a known volume of nuclease-free water. The RNA concentration was determined by optical density (OD) at 260 nm (using an OD 260 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.

Reverse transcription. One microgram of total RNA was reverse transcribed for each muscle sample using the SuperScript II RT from Invitrogen (Carlsbad, CA) and a mix of oligo(dT) (100 ng/reaction) and random primers (200 ng/reaction) in a 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 (see below).

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 IGF-I, mechanogrowth factor (MGF), IGF-I binding protein-4 (IGF-BP4), atrogin 1, procollagen III-α-1 (Col-3), cyclin D1, MyoD, myogenin, p21, myoferlin, and myostatin. With the exception of myoferlin, the sequences for the primers used for the specific target mRNAs have been published previously (32). Myoferlin PCR primers were designed based on GenBank accession no. XM_220031, forward 5′-TTGAAAAGAAACCACCAGAAAAGA-AGC-3′ and reverse 5′-TGTATTGAGTTGTTGATGCCAGAGGTT-3′, which amplify a 225-bp PCR product. All PCR primers were designed from regions spanning exon-intron boundaries. This way genomic DNA either will not amplify with the cDNA or it will result in a much larger PCR product such that it is easy to identify on the gel. Furthermore, PCR primers were designed from regions of least similarity to other mRNA of the same gene family, and their specificity was checked using nucleotide blast analyses.

All primers were purchased from Operon Biotechnologies, (Huntsville, AL). In each PCR reaction, 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, Austin, TX), 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).

For each target mRNA, the RT and PCR reactions were carried out under identical conditions 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. The PCR reactions 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 U 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 semi-log plot when the yield is expressed as a function of the number of cycles (15).

Western blot analyses. Immunoblotting was used for the analyses of total expression and to determine the phosphorylation states of specific proteins involved in muscle intracellular anabolic signaling. These include analyses of p70-S6 kinase (S6K1) and its phosphorylation at Thr 389, and the hyperphosphorylation of eukaryotic initiation factor 4 binding protein (4E-BP). These protein- and phosphoprotein-specific antibodies were purchased from Cell Signaling Tech-

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nology (Beverly, MA). Thr 389 phosphorylation of S6K1 has been shown to strongly correlate with the kinase biological activity in vivo (Cell Signaling), while hyperphosphorylation of 4E-BP removes it inhibiting effects on translation initiation (29).

Muscle samples were extracted by homogenization in 7 vol 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 AEBSF) using 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 was subjected to SDS-PAGE (12.5% T), according to standard protocol (48), then electrophoretically transferred to a PVDF membrane (Immobilon-P) using 10% methanol, 1 mM orthovanadate, 25 mM Tris, 193 mM glycine, pH 8.3. The enhanced chemiluminescence (ECL) 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, ECL 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).

Hyperphosphorylation of the 4E-BP1 protein was determined on the basis of the gel migration pattern (38). The reported data represent the proportion of the higher molecular weight band (gamma) relative to total.

Data presentation and statistical analysis. The experimental design of this study involved comparisons between the untrained (Contra) and trained (Train) muscles of the same rats following 5 days of continuous hindlimb suspension and intermittent resistance training, as well as comparisons to the muscles from nonsuspended ground control animals (GC group). Statistical analyses consisted of a one-way ANOVA with Newman-Keuls post hoc testing using the Prism software package (Graphpad). Pearson correlation analysis was used to assess the relationships in pairs of variables, e.g., myoferlin and myogenin correlation (Prism, Graphpad). For all statistical tests, the 0.05 level of confidence was accepted for statistical significance. All values are reported as means and SE.

RESULTS

Body weight, muscle mass, and protein content. As is common for this treatment, hindlimb suspension resulted in a ~7% decrease in body weight relative to that of nonsuspended (GC) rats (257 ± 14 vs. 238 ± 18 g).

The resistance exercise paradigm used in the present study resulted in significantly greater MG muscle mass than that of the nontrained muscle and was similar to the mean value seen in nonsuspended animals (Fig. 2*A*). The absolute mass of the untrained was lower compared with that of nonsuspended animals, reflecting the overall decline in body mass (Fig. 2*B*). The content of myofibrillar proteins of the resistance-trained MG muscles was significantly greater than that of the untrained

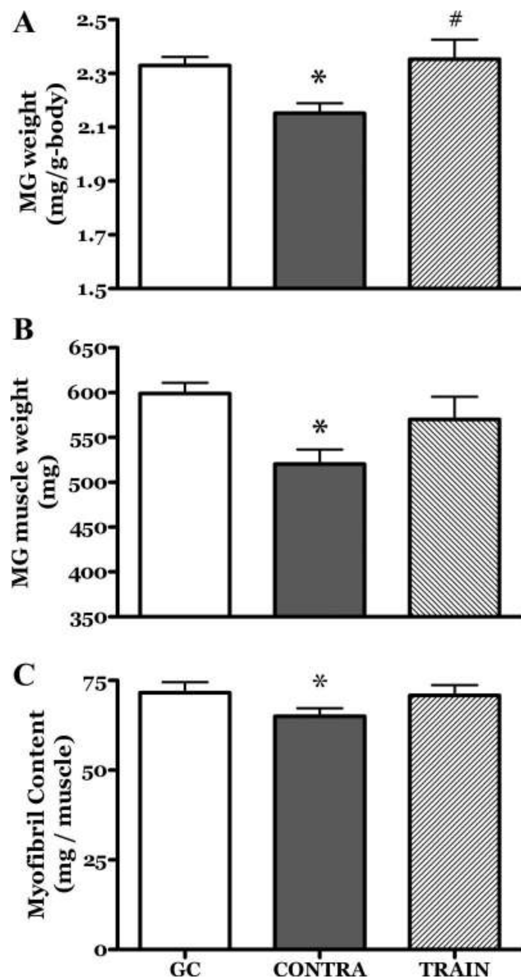

Fig. 2. Effects of combined-mode resistance training on muscle weight and myofibrillar protein content during 5 days of hindlimb suspension-induced muscle unloading. Open bars, mean for the right medial gastrocnemius (MG) muscle from the ground control (GC) rats (n = 8); filled bars, contralateral (Contra) nontrained MG muscles of the hindlimb-suspended rats; hatched bars, data from the resistance-trained MG muscles (Train) of the same rats (n = 12). *A*: muscle mass normalized to body weight was significantly greater in the trained muscles of hindlimb-suspended rats and not differ from that of the GC rats. *B*: absolute muscle mass of the Contra MG muscles was significantly lower that of the of the GC rats. *C*: myofibrillar protein content of the Contra muscles was significantly lower than that of the GC rats. *$P < 0.05$ vs. GC; #$P < 0.05$ vs. Contra.

contralateral muscles and was not different from that of the GC animals (Fig. 2*C*).

RNA and DNA content. Both the RNA concentration (GC 0.77 ± 0.02, Contra 0.76 ± 0.02, Train 0.85 ± 0.03 μg/mg) and content (Fig. 3*A*) were significantly increased in the trained MG muscle relative to the contralateral untrained muscle and that of the GC group.

The DNA content of the trained muscles was significantly higher than that of the untrained contralateral muscle and was not different from the muscles of the GC group (Fig. 3*B*). The DNA concentration was not different between the contralateral and trained muscles and was also similar to that seen in weight-bearing rats (Contra 0.78 ± 0.02, Train 0.81 ± 0.03, GC 0.79 ± 0.02 μg/mg).

IGF-1, MGF, and IGF-BP4. In the present study, the combined-mode resistance exercise protocol imposed during mus-

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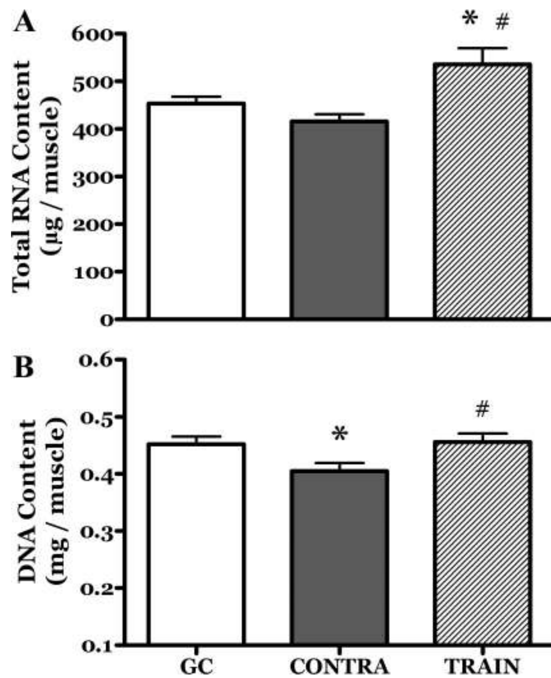

Fig. 3. Effects of combined-mode resistance training on muscle total extracted RNA and DNA content during 5 days of hindlimb suspension-induced muscle unloading. *A*: muscle total RNA was significantly greater in the trained muscles of hindlimb-suspended rats. *B*: DNA content of the Contra muscles was lower than that of the MG muscles from the GC rats and of the trained MG muscles. *$P < 0.05$ vs. GC; #$P < 0.05$ vs. Contra.

cle unloading significantly increased the production and/or accumulation of the mRNA for IGF-1, MGF, and IGF-BP4 (Fig. 4).

Collagen 3 α-1 and cyclin D1. Col-3 mRNA was increased approximately fivefold in the trained vs. contralateral MG muscle of hindlimb-suspended rats (Fig. 5*A*). Col-3 mRNA levels in the contralateral MG muscle was not sensitive to unloading (Fig. 5*A*).

Similar to Col-3, resistance exercise significantly increased the expression and/or accumulation of the mRNA for cyclin D1 (Fig. 5*B*), while unloading did not appear to effect its level.

Markers of myogenesis. In MG muscles unloaded via hindlimb suspension, resistance exercise significantly increased both myogenin and MyoD mRNA levels (Fig. 6, *A* and *B*).

A similar pattern, i.e., increased levels with resistance exercise, was seen in the mRNA for both the cyclin-dependent kinase inhibitor p21 and for myoferlin, which was also increased as a result of resistance exercise (Fig. 6, *C* and *D*).

Myostatin and atrogin mRNA. Myostatin is a negative modulator of muscle mass/growth (34). In the present study, myostatin mRNA levels were increased with unloading, and this increase was prevented by the combined resistance training protocol (Fig. 7*A*).

Atrogin is a muscle-specific ubiquitin ligase that is known to play a significant role in most muscle-wasting disorders (26, 31). In the present study, unloading resulted in a increase in atrogin mRNA. Interestingly, the combined-mode resistance exercise protocol actually resulted in an further increase in atrogin mRNA relative to the unloaded contralateral muscles (Fig. 7*B*).

Anabolic signaling. Both S6K1 and 4E-BP are downstream effectors of mammalian target of rapamycin (mTOR) signaling (27, 42, 55, 56). In the present study, the hyperphosphorylation of 4E-BP and the activating phosphorylation of S6K1 (Thr 389) were significantly increased relative to the nontrained, unloaded contralateral muscles (Fig. 8). There were no significant changes in the total amounts of either 4E-BP (GC 697±113, Contra 801 ± 79, Train 695 ± 52; Denistometer units) or S6K1 (GC 662 ± 111, Contra 779 ± 111, Train 742 ± 95) in any of the muscles.

DISCUSSION

Unloading of skeletal muscle acts as a powerful stimulus for adaptations leading to a decrease in muscle size and strength (2). We have previously observed that a high-force resistance training program was not able to counter the loading-induced muscle atrophy in hindlimb-suspended rats (37). This result was somewhat surprising in that isometric exercise had previously proven to be effective in inducing muscle hypertrophy in ambulatory rats (4). In that study, the isometric resistance exercise appeared to be equally effective in promoting hyper-

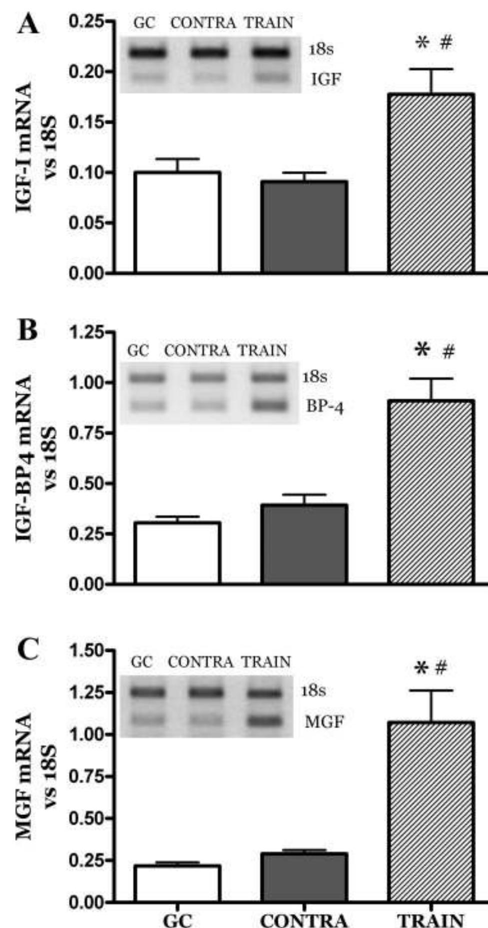

Fig. 4. Effects of combined-mode resistance training on the IGF-I system mRNA levels during 5 days of hindlimb suspension-induced muscle unloading. The mRNA levels for IGF-I (*A*), IGF-I binding protein-4 (BP-4) (*B*), and mechanogrowth factor (MGF) (*C*) were significantly greater in the trained muscles of hindlimb-suspended rats. *Insets*: representative ethidium bromide-stained gels with PCR products corresponding to 18S rRNA and target mRNA. *$P < 0.05$ vs. GC; #$P < 0.05$ vs. Contra.

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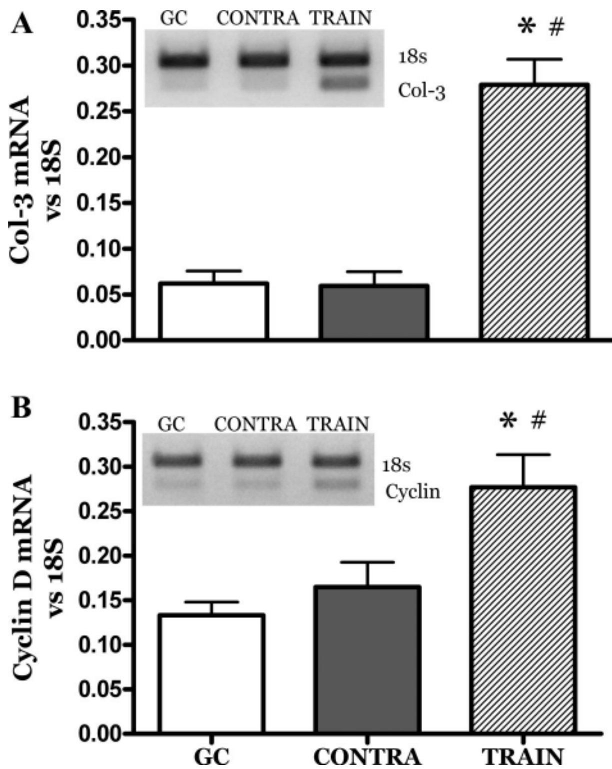

Fig. 5. Effects of combined-mode resistance training on the mRNA for procollagen-III-α1 (Col-3) and cyclin D1 levels during 5 days of hindlimb suspension-induced muscle unloading. The mRNA levels for Col-3 (*A*) and cyclin D1 (*B*) were significantly greater in the trained muscles of hindlimb-suspended rats. *Insets*: representative ethidium bromide-stained gels with PCR products corresponding to 18S rRNA and target mRNA. *$P < 0.05$ vs. GC; #$P < 0.05$ vs. Contra.

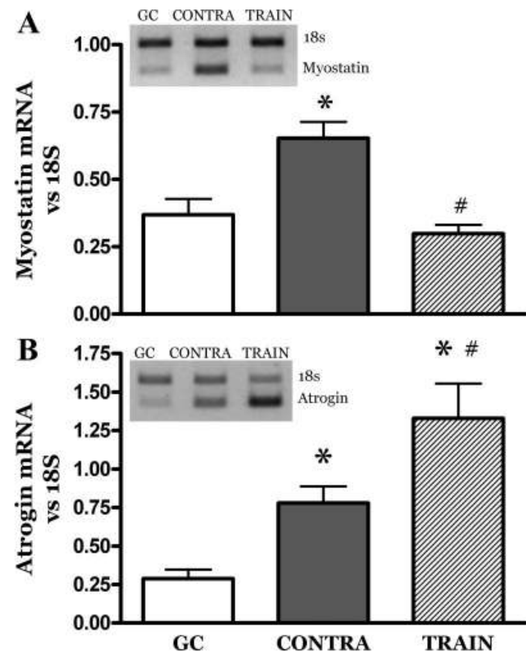

Fig. 7. Effects of combined-mode resistance training on the mRNA levels for myostatin and atrogin during 5 days of hindlimb suspension-induced muscle unloading. *A*: mRNA levels for myostatin were significantly depressed relative to the Contra muscles in response to resistance exercise. *B*: relative to the GC group, the mRNA for atrogin was significantly elevated in the Contra MG muscles. Resistance training resulted in a further, significant increase in atrogin mRNA (Train). *Insets*: representative ethidium bromide-stained gels with PCR products corresponding to 18S rRNA and target mRNA. *$P < 0.05$ vs. GC; #$P < 0.05$ vs. Contra.

trophy as pure concentric or eccentric training protocols (4). Taken together, the results of these previous studies suggest that the prevention of muscle atrophy using resistance exercise may have characteristics that differ significantly from the generation of a hypertrophy response. In the present study, we tested the hypothesis that combining all three resistance exercise modes, isometric, concentric, and eccentric (Fig. 1), would provide a sufficient anabolic stimulus to counteract the atrophy response induced by muscle unloading. In the context of using this paradigm, we purposely increased the per-set duty cycle (total time of activation) to 150 s. In a pilot study, we found that a combined-mode training program with the same duty

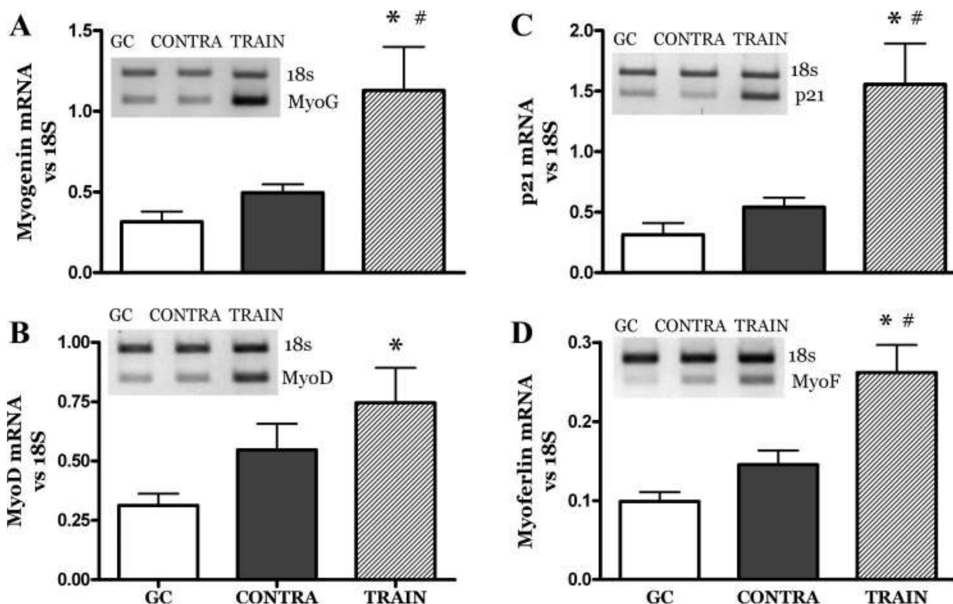

Fig. 6. Effects of combined-mode resistance training on the mRNA levels for myogenic regulatory factors (myogenin, MyoD), the cyclin-dependent kinase inhibitor p21, and myoferlin during 5 days of hindlimb suspension-induced muscle unloading. Amount of myogenin mRNA (*A*) in the trained MG muscle was significantly greater than that of the MG muscles of the GC rats. The mRNA levels for MyoD (*B*) as well as those for p21 (*C*) and myoferlin (*D*) were significantly greater in the trained muscles of hindlimb-suspended rats. *Insets*: representative ethidium bromide-stained gels with PCR products corresponding to 18S rRNA and target mRNA. *$P < 0.05$ vs. GC; #$P < 0.05$ vs. Contra.

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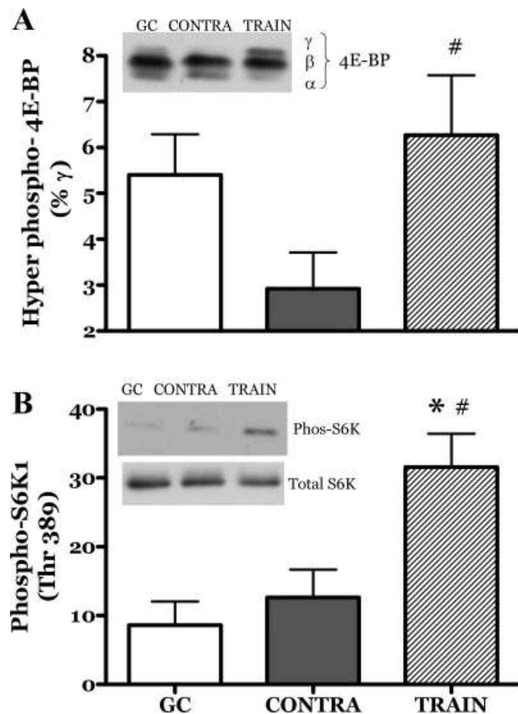

Fig. 8. Effects of combined-mode resistance training on components of the anabolic signaling pathway during 5 days of hindlimb suspension-induced muscle unloading. *A*: hyperphosphorylation of eukaryotic initiation factor 4E binding protein (4E-BP) was significantly increased in the trained MG muscles relative to the Contra muscles. The activating phosphorylation (Thr 389) of p70 S6 kinase (S6K1) was significantly increased by the resistance training protocol (*B*). *Insets*: representative immunoblots. $*P < 0.05$ vs. GC; $\#P < 0.05$ vs. Contra.

cycle (75 s) as our previous isometric-only study (37) was no more effective in stimulating the various anabolic markers or in preventing atrophy. We found that the 75-s duty cycle using a combined-mode contraction paradigm failed to elicit the robust changes in key anabolic markers seen in the present study. For example, IGF-I mRNA levels increased only 10% vs. the approximately twofold increase seen in the present study. This observation suggests that the critical difference between the previous isometric study and the present combined-mode study resides in the increase in training volume as opposed to some mode-specific effects.

The results of the present study clearly indicate that this high-volume, combined-mode protocol represents an effective countermeasure to unloading-induced muscle atrophy. Relative to body weight, the mass of the trained muscles was significantly greater than that of the contralateral, nontrained MG and was essentially the same as values seen for weight-bearing animals (Fig. 2*A*). Similarly, the total and myofibrillar protein content of the trained muscles appeared to be conserved by the resistance exercise intervention. These results contrast with our previous work in which resistance training using only isometric activation did not provide a stimulus sufficient to maintain muscle size (37). However, it is important to note that the absolute weight of the trained MG muscle was still somewhat lower than that found in matched ambulatory animals (Fig. 2*A*). This result suggests that the somatic regulatory mechanisms that are impacted by reduced activity, resulting in an overall reduction in body size, may be distinct from those that regulate muscle adaptation in the context of mechanical loading.

The majority of the total RNA pool present in skeletal muscle consists of ribosomal RNA (39). As a result, total RNA represents a rough approximation of the translational capacity of muscles. Our observations suggest that the RNA machinery is sensitive to unloading (Ref. 37; Fig. 3*A* of present study), whereas the combined resistance training protocol used in the present study resulted in significantly greater levels of RNA than were seen in the contralateral muscles and similar to that seen in the ambulatory rats (Fig. 3*A*). Since the large changes in total RNA most likely represent alterations in the amount of rRNA, this finding indicates that the combined resistance training protocol was sufficiently effective in maintaining the some of the synthetic machinery necessary to maintain muscle protein.

In the present study, the concentration of DNA present in MG muscles was not different among groups (not shown). Similarly, analysis of the DNA-to-protein ratios of the muscles from this study indicates that there were no differences in any group (data not shown). In light of the fact that the unloaded contralateral muscles weighed significantly less than the trained MG, this suggests that there was a net loss of DNA from these muscles. This result is reflected by the greater DNA content measured in the trained vs. contralateral MG muscles (Fig. 3*B*). It has previously been reported that unloaded skeletal muscle can experience a loss of myonuclei apparently via an apoptosis-like process, e.g., a loss of myonuclei but not cell death (9, 10, 11, 41). This would be consistent with unchanged DNA concentration and therefore decreased DNA content seen in the contralateral muscles in the present study (Fig. 3*B*). The results in the trained MG muscles indicates that the combined-mode resistance training protocol was effective in maintaining this parameter, either by preventing unloading-induced nuclear apoptosis or promoting cell proliferation within the muscles (Fig. 3).

There is a substantial body of literature indicating that various isoforms of IGF-I, as well as components of the IGF-I system (e.g., binding proteins), respond to increased loading of skeletal muscles (1). We have previously reported that IGF-I mRNA and peptide, as well as MGF mRNA, are increased significantly by increased loading (3, 4, 6, 35). Similarly, IGF-BP4 mRNA has been reported to be sensitive to loading in both rats and humans (3, 14, 15, 36, 54). The sensitivity of this system to loading is such that significant increases in the mRNA for IGF-I, MGF, and IGF-BP4 can be detected after a single training bout of resistance exercise (36). The unloading of skeletal muscle does not appear to significantly affect these components of the IGF-I system (Ref. 37; and Fig. 4 of present study). However, resistance training during muscle unloading resulted in significant increases in the mRNA for IGF-I, IGF-BP4, and MGF (Fig. 4). We and others have reported results indicating that increased provision of IGF-I within skeletal muscles can stimulate a hypertrophic response (8, 21, 51). The observation that resistance training during unloading increases these components of the highly anabolic IGF-I system suggests one potential mechanism by which resistance exercise might counter the atrophy associated with unloading. However, it should be noted that in our previous study in which isometric training failed to prevent muscle atrophy, there were apparent increases in IGF-I mRNA that approached significance (~84%

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increase). For comparison, the training-induced increase in IGF-I mRNA was ~97% in the present study. Taken together, these results would suggest that factors other than IGF-I changes induced by pure mechanical stimuli most likely contributed to the increased effectiveness of the combined-mode resistance exercise used in the present study.

It has been established that increased skeletal muscle loading gives rise to an enlargement of tendon tissue and to an increase in the collagen synthesis at both the protein and mRNA levels (12, 49). We previously reported that increased muscle loading results in a significant increase in muscle tendon mass (54). One hallmark of this adaptation appears to be a rapid, early increase in tendon procollagen III mRNA levels. In the present study we found that the combined resistance training protocol resulted in an approximately fivefold increase in procollagen III-1α (Col-3) mRNA levels (Fig. 5*A*). This result clearly indicates that this exercise protocol results in a very robust stimulus in the connective tissue components of the trained skeletal muscle cytoskeletal structures.

The cyclin-dependent kinases (cdk) function primarily to regulate and coordinate the passage of cells through the cell cycle (53, 62). Various cyclin proteins pair with cdks to regulate their activity. The cyclin D family are the first to be expressed when quiescent cells are stimulated, and the presence of at least one D cyclin is required for cells to complete the G1 phase (53). In light of this role, we have traditionally measured changes in cyclin D1 mRNA as a potential indicator of proliferative activity in skeletal muscle. However, recent work has suggested that, in addition to its role in regulating the proliferation processes, cyclin D1 may promote cell growth stimulation via pathways involving transcription of ribosomal RNA genes to promote increases in ribosomal RNA (52). In the present study, mRNA for cyclin D1 was significantly increased as a result of the resistance exercise protocol (Fig. 5*B*). This result could be interpreted as being consistent with the observed increase in total RNA content seen in the resistance-trained muscles (Fig. 3*A*).

MyoD and myogenin are members of the myogenic regulatory factor (MRF) family that regulates the expression of key muscle specific proteins during muscle development (61). As part of this process, the expression of MyoD appears to represent a commitment step, while myogenin is upregulated during myoblast differentiation. The role of MyoD and myogenin in adult skeletal muscle has yet to be fully elucidated. Based on developmental and cell culture results, it is clear that MyoD and myogenin participate in the activation and maturation of satellite cells. For example, Schultz et al. (59) demonstrated that, in growing muscle, MyoD was expressed in cells that were proliferating. However, there is evidence that MyoD and myogenin are expressed in mature myofibers, as well as satellite cells (43, 44). In general, increased expression of myogenin is often associated with committed stages of myoblast differentiation and fusion (30, 40, 57, 60, 66). We have previously reported that the mRNA for myogenin and MyoD increases in response to increased muscle loading in both mature rats and humans (6, 14, 28).

In MG muscles unloaded via hindlimb suspension, resistance exercise significantly increased both myogenin and MyoD mRNA levels (Fig. 6, *A* and *B*). While these changes demonstrate the efficacy of the resistance exercise protocol in creating an anabolic/myogenic state, the absence of specific information on the cell types expressing these changes within muscle makes it difficult to assess the specific role that their expression is playing. As such, our findings raise the necessity for future studies on this issue.

In an effort to further elucidate processes that might be associated with the observed changes in the levels of MRF mRNAs in the present study we measured changes in the levels of two mRNAs associated with events that would be expected to occur after satellite cell proliferation: *1*) changes in the levels of mRNA for the cyclin-dependent kinase inhibitor p21 and *2*) changes in myoferlin mRNA.

In the context of increased muscle loading, increased p21 expression is thought to indicate that cells are exiting from the cell cycle and differentiating (e.g., 44). In the present study, resistance exercise induced a significant increase in p21 mRNA (Fig. 6*C*). As we have observed in previous studies, in both rats and humans (e.g., 6, 14), loading-induced increases in the levels of p21 and myogenin mRNA (Fig. 6, *A* and *C*) were highly correlated ($r = 0.71$, $P < 0.0001$), suggesting that increased myogenin expression was associated with myogenic differentiation. Neither p21 nor myoferlin demonstrated correlations with MyoD levels (data not shown).

Under developmental conditions, myoferlin is thought to be important for the fusion of myoblasts to form myofibers (24, 25). In adult muscle, myoferlin appears to be upregulated to mediate the fusion of myoblasts with existing myofibers (24, 25). This is a logical process that would follow satellite cell differentiation. In the present study, myoferlin mRNA increased significantly in resistance-trained MG muscles compared with levels in the untrained contralateral muscle (Fig. 6*D*). Similar to the results with p21, we also observed that the levels of myoferlin mRNA were correlated with those of myogenin ($r = 0.57$, $P < 0.0031$).

Taken together, the results presented in Fig. 6 suggest that the predominating myogenic response of the otherwise unloaded muscles to resistance training was similar to that observed during a hypertrophy response in muscles from ambulatory animals or humans, i.e., increases in myogenic regulatory factors and in markers of differentiation (e.g., 6, 14, 15, 36). In the muscles of ambulatory animals, it would be reasonable to expect that resistance training-stimulated responses might include preparations for the inclusion of satellite cell-derived myoblasts (e.g., myogenin, p21, and myoferlin expression). The results of the present study suggest that this response is maintained in myofibers that were concurrently experiencing unloading in conjunction with brief bouts of resistance training.

To effectively ameliorate unloading-induced muscle atrophy, an intervention, in this case resistance exercise, would ideally both promote anabolic responses and suppress catabolic processes. In the present study we measured changes in myostatin mRNA, a known inhibitor of muscle growth, and the muscle-specific ubiquitin E3 ligase atrogin, known to participate in breaking down contractile proteins to assess the effectiveness of resistance exercise in blunting atrophy.

We have reported previously that myostatin expression is inhibited by resistance exercise (32, 35), whereas expression is increased during muscle unloading (37). In the present study, unloading resulted in a ~78% increase in myostatin mRNA levels (Fig. 7*A*). The combined resistance exercise program used in the present study reversed this effect by significantly

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depressing the levels of myostatin mRNA relative to the untrained muscle (Fig. 7*A*), essentially normalizing this response. However, it is not clear that loading-induced increases in myostatin are a primary driver of the atrophy process since we previously observed that isometric exercise, which did not prevent the majority of the atrophy, also restrained unloading-induced increases in myostatin mRNA (37).

Similar to myostatin, we previously reported that the mRNA for atrogin was increased during muscle unloading (37). However, in that previous study, using an isometric exercise countermeasure, atrogin mRNA was kept at levels observed in the muscles of ambulatory rats (37). In contrast, in the present study, the combined resistance exercise intervention resulted in a further increase in atrogin mRNA (Fig. 7*B*). While this may seem to be a contradictory finding, it is important to note that we have previously reported that resistance exercise in-and-of itself can result in an increase atrogin mRNA levels (35). In that study, two bouts of isometric exercise on separate days resulted in a significant increase in atrogin mRNA at 48 but not at 24 h postexercise (35). One potential interpretation of increased atrogin expression following resistance exercise may be that myofibrillar structures are being disassembled to facilitate phenotype shifts, for example, in preparation for the incorporation of different myosin heavy chain isoforms into the contractile structure, a process that is commonly associated with resistance training (e.g., 7). If this is the case, the difference in response seen in the present study using the combined resistance exercise protocol and the previous study using isometric exercise (37) may be functionally relevant to the differing outcomes with regard to the efficacy of muscle remodeling in the face of an atrophic stimulus.

The muscle signaling pathway that includes mTOR is known to have powerful anabolic effects in skeletal muscle (16, 22, 46, 52). Two of the primary downstream effectors of mTOR-mediated anabolic activity are S6K1 and 4E-BP. The hyperphosphorylation of 4E-BP removes the inhibition of the eukaryotic initiation factor 4E, thereby promoting translation initiation (55, 56). Similarly, mTOR stimulated phosphorylation of S6K1 results in the removal of inhibition of translation initiation (42), in addition to producing an increase in translational capacity by promoting ribosomal biogenesis (27, 55, 56). A number of studies have reported that the phosphorylation of these proteins is markedly increased in response to increased muscle loading (3, 13, 16, 35, 36, 46). In the present study, the hyperphosphorylation of 4E-BP was twofold higher that that of the contralateral untrained muscles and was essentially returned to normal levels (Fig. 8*A*). Similarly, the resistance training resulted in a significant increase in activating phosphorylation of S6K1 (2.5-fold) relative to the nontrained, unloaded contralateral muscles (Fig. 8).

These results suggest that the combined mode-resistance training countermeasure allowed the muscles to maintain translational activity. In particular, the elevated activation of S6K1 may have contributed to the increased RNA levels (Fig. 3*A*), indicating that the ribosomal pool and therefore translational capacity was protected during muscle unloading.

Study limitations. The analyses conducted for this study were limited to whole muscle measurements of mRNA or protein constituents. As a result, it was not possible to distinguish what cells within the muscles were contributing to the observed differences. As noted above, this was problematic when attempting to interpret certain results, especially those involving the MRF levels in the context of cell signaling as it is not known whether changes occur in myofibers or satellite cells. However, it is important to note that the overwhelming majority of the muscle consists of myofibers, and therefore primary measurements such as protein, mass, and RNA would be dominated by myocytes.

In humans unilateral resistance training is thought to result in some contralateral gains in strength (50). However, the current consensus appears to indicate that this effect is primarily mediated by central neural mechanisms (19). Since the present study used unilateral stimulation of the sciatic nerve in lightly anesthetized rats, we think that it is unlikely that a contralateral strength-training effect occurred. However, were this the case, the training-induced effects would have been even greater relative to the contralateral muscles as the anabolic markers demonstrate.

Summary. The results of the present study indicate that a resistance exercise protocol that combines isometric, concentric, and eccentric modes of contraction with an accumulated volume of training greater than that reported in our previous study results in a suppression of catabolic signaling (myostatin) and the maintenance of anabolic processes such that the muscle atrophy response to unloading can be effectively blunted.

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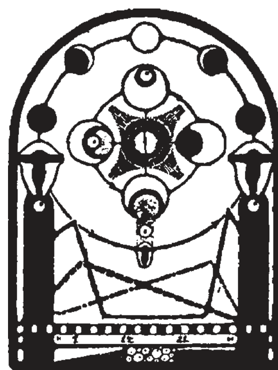

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