J Appl Physiol 93: 394–403, 2002.
First published February 8, 2002; 10.1152/japplphysiol.01153.2001.

highlighted topics

Exercise Effects on Muscle Insulin Signaling and Action Selected Contribution: Acute cellular and molecular responses to resistance exercise

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Received 21 November 2001; accepted in final form 5 February 2002

Haddad, Fadia, and Gregory R. Adams. Selected Contribution: Acute cellular and molecular responses to resistance exercise. *J Appl Physiol* 93: 394–403, 2002. First published February 8, 2002; 10.1152/japplphysiol.01153.2001.—Training protocols apply sequential bouts of resistance exercise (RE) to induce the cellular and molecular responses necessary to produce compensatory hypertrophy. This study was designed to *1*) define the time course of selected cellular and molecular responses to a single bout of RE and *2*) examine the effects of interbout rest intervals on the summation of these responses. Rat muscles were exposed to RE via stimulation of the sciatic nerve in vivo. Stimulated and control muscles were obtained at various time points post-RE and analyzed via Western blot and RT-PCR. A single bout of RE increased intracellular signaling (i.e., phosphorylations) and expression of mRNAs for insulin-like growth factor-I system components and myogenic markers (e.g., cyclin D1, myogenin). A rest interval of 48 h between RE bouts resulted in much greater summation of myogenic responses than 24- or 8-h rest intervals. This experimental approach should be useful for studying the regulatory mechanisms that control the hypertrophy response. These methods could also be used to compare and contrast different exercise parameters (e.g., concentric vs. eccentric, etc.).

rodent; translation initiation; myogenic; insulin-like growth factor-I; mechano-growth factor; cyclin D1

MAMMALIAN SKELETAL MUSCLE is a highly plastic tissue that readily adapts to changes in loading state. Increasing the load imposed on skeletal muscles elicits adaptations that result in increased muscle size and changes in contractile characteristics. In mammals, the majority of these adaptations appear to take the form of increases in myofiber size (e.g., Ref. 17) and alterations in the mix of contractile and energy metabolism proteins present in these cells (e.g., isoform shifts, etc.; Ref. 9). A prime example of these processes is seen in the muscle hypertrophy and functional gains that result from efficacious resistance training programs in humans. Clearly, alterations in the types and amounts of cellular proteins present in myofibers must involve a number of intra- and possibly intercellular regulatory mechanisms to ensure that the process is coordinated. Accordingly, the processes associated with compensatory muscle hypertrophy would be expected to include regulation of cellular mechanisms such as mRNA transcription, accumulation, and translation. In fact, in vitro and in vivo studies have identified a number of regulatory and/or signaling pathways that have the potential to impact these fundamental cellular and molecular processes in skeletal muscles (10, 14, 20, 28, 34, 37, 38, 42, 51).

To date, the majority of the cellular level research aimed at elucidating these adaptive processes in vivo have been confined to evaluation of the "end state," i.e., examining the extent of changes at a time when the adaptation is well underway. Such end-state measurements might include the analysis of contractile protein isoform changes as well as changes in the amounts of various proteins present and/or some measurement of the size of the myofibers. However, it would be difficult to deduce the timing and identity of the regulatory events that orchestrated the observed adaptations from these end-point measures. In addition, this lack of knowledge of the mechanisms that are internal to the adaptation process would prevent a clear understanding of how the individual loading events interacted to produce the adaptation.

Further insights into the mechanisms of skeletal muscle adaptation will require the collection and analysis of data with much higher temporal resolution than has generally been applied in the past. Ideally, it would be possible to investigate the cellular and molecular responses of skeletal muscle cells after discrete loading events. With the identification of cellular and molecular responses to acute loading events, it should then be

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possible to fully understand the regulatory components that control and coordinate adaptation.

On the basis of both in vitro and in vivo studies, it has become clear that two primary processes are involved with the compensatory hypertrophy response in mammalian skeletal muscles. The first and most obvious would be anabolic processes necessary for the accretion of protein to support myofiber enlargement. The second process involves the proliferation of satellite cells, which appears to be necessary to provide additional myonuclei to the enlarging myofibers (5, 45, 46, 48–50, 54). In each case, the cellular and molecular mechanisms underlying these processes have been extensively characterized in a number of cell types, particularly in cell culture settings. In particular, the steps associated with the initiation of cellular proliferation are generally well known (e.g., can be found in cell biology textbooks).

The principles of resistance training implicitly assume that multiple bouts of exercise, imposed within an appropriate time frame, must stimulate and reinforce cellular and molecular processes that lead to a compensatory hypertrophy response. In the present study, we hypothesized that relatively well-characterized events, for example the increased expression of cell cycle regulatory components, that we have previously determined to be activated during the development of muscle hypertrophy would be useful in detecting the initiation of a response to increased loading in rat skeletal muscles (1, 2). We postulated that the levels of key cellular and molecular markers would increase and then decline over time in response to a single bout of resistance exercise. Furthermore, we hypothesized that the imposition of a second bout of exercise would result in the summation of these responses with regard to magnitude and/or duration. The in vivo validation of loading-sensitive cellular and molecular responses will help to elucidate the processes that have been assumed to take place as a result of resistance training programs but until now have not been systematically investigated. This understanding will in turn allow for the further elucidation of the regulatory mechanisms that control and coordinate the suite of adaptations that comprise the compensatory hypertrophy response and, at least in a laboratory setting, provide a mechanism by which different training protocols and modalities (e.g., static vs. dynamic, concentric vs. eccentric, etc.) could be objectively evaluated. To our knowledge, this is the first investigation to systematically evaluate a spectrum of cellular and molecular markers that are relevant to the development of compensatory hypertrophy with regard to the response time course and the potential summation of responses after the imposition of one or two bouts of resistance exercise.

METHODS

Animal Care and Experimental Design

Ninety female Sprague-Dawley rats were purchased from Taconic Farms (Germantown, NY). The body weights of these animals were 226 ± 5 g. All animals were housed in standard vivarium cages and allowed food and water ad libitium. All treatment protocols were approved by the institutional animal research committee.

Unilateral muscle exercise protocol. Rats were anesthetized with ketamine and acepromazine (40 and 2 mg/kg, respectively), and a small incision was made along the posterior aspect of the right lower limb to allow the placement of fine wire platinum electrodes adjacent to the sciatic nerve. The rats were then positioned in a specially built training platform described previously (11). The right leg was positioned in a foot plate attached to an isometric force transducer interfaced with a computer. The electrodes were connected to a Grass S-48 stimulator via a stimulus isolation unit. The voltage (6–9 V), stimulation frequency (35–40 Hz), and foot position (e.g., ankle ~85–90°) were adjusted to produce maximal isometric tension. For exercise bouts, the stimulation parameters were 3 maximal isometric contractions per minute, 4-s duty cycle and 16 s of rest, for 30 min. The timing of the contractions was controlled by the interfaced computer. Force output was monitored on the computer screen during each contraction. After the exercise bout, the electrodes were withdrawn and the incision was closed with a single wound clip.

An isometric training protocol was chosen to minimize the potential for muscle injury. Isometric mode resistance exercise is known to result in muscle hypertrophy in humans (e.g., Ref. 29). We have previously used similar isometric training protocols to produce muscle hypertrophy or prevent muscle atrophy in rats (18, 19, 25).

Single exercise bout. Six groups of rats (n = 6 per group) completed the 30-min exercise protocol described above. One group was then killed at each of 0, 2, 6, 12, 24, and 40 h postexercise. Tissue was collected as described under *Tissue Collection*.

Two exercise bouts. Rats assigned to this experiment completed two consecutive bouts of resistance exercise. These rats were randomly assigned to one of three groups (n = 18 per group), which experienced either 8, 24, or 48 h of rest between the two exercise bouts. From each of these three groups, six rats were killed at 16, 24, and 40 h after the second bout of resistance exercise. Tissue was collected as described below. The rest periods were purposely chosen to span a range that, on the basis of the literature, would have been predicted to be too short through optimum (8 h to ~2 days) (22).

Tissue Collection

At the time points indicated above, the rats were killed via an injection of Euthi-6. The medial gastrocnemius muscles of both the exercised and contralateral legs were dissected free of connective tissue, weighed, and snap-frozen between blocks of dry ice.

Biochemical and Molecular Analyses

Tissue samples were analyzed for total protein content as described previously (1).

Total RNA isolation. Total RNA was extracted from preweighed frozen muscle samples by using the TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the company's protocol, which is based on the method described by Chomczynski and Sacchi (12). Extracted RNA was precipitated from the aqueous phase with isopropanol and, after being washed with ethanol, 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 extracted muscle piece. The RNA

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

Target mRNA	PCR Primer Sequence 5′→3′	Product Size (bp)	GenBank Accession No.
IGF-I (all)	5′ sense: GCATTGTGGATGAGTGTTGC	202 all	X06043
	3′ antisense: GGCTCCTCCTACATTCTGTA	254 MGF	
MGF	5′ sense: GCATTGTGGATGAGTGTTGC	163	X06108
Load-sensitive IGF-I	3′ antisense: CTTTTCTTGTGTGTCGATAGG		
IGFR1	5′ sense: CGGCTTCTCTGCAGTAAACACA	245	L29232
	3′ antisense: ACTGGGAAGCGGAGAAAAGAGA		
IGFBP5	5′ sense: CACGCCTTCGACAGCAGTAAC	214	NM_012817
	3′ antisense: GTCGGGAATGGGGAGTGTCT		
IGFBP4	CCTGGGCTTGGGGATGC	212	X76066
	AGGGGTTGAAGCTGTTGTTGG		
Myogenin	5′ sense: ACTACCCACCGTCCATTCAC	233	M24393
	3′ antisense: TCGGGGCACTCACTGTCTCT		
MyoD	5′ sense: CTACAGCGGCGACTCAGACG	563	M84176
	3′ antisense: TTGGGGCCGGATGTAGGA		
Cyclin D	5′ sense: AAGTGCGTGCAGAGGGAGAT	267	D14014
	3′ antisense: GGGGCGGATAGAGTTGTCAG		
p21	5′ sense: CCCGTGGACAGTGAGCAGTT	233	U24174
	3′ antisense: AGCAGGGCCGAGGAGGTA		

IGF, insulin-like growth factor; MGF, mechano-growth factor; IGFR1, IGF-I receptor; IGFBP5 and IGFBP4, IGF binding proteins 5 and 4, respectively.

samples were stored frozen at −80°C to be used subsequently in determining the specific mRNA expression via relative RT-PCR procedures.

Reverse transcription. One microgram of total RNA was reverse transcribed for each muscle sample by using the SuperScript II RT from GIBCO BRL 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 were stored at −80°C until used in the PCR reactions for specific mRNA analyses.

Polymerase chain reaction. A relative RT-PCR method using 18S as internal standard (Ambion, Austin, TX) was applied to study the expression of specific mRNAs of interest including insulin-like growth factor (IGF)-I, IGF-I receptor (IGFR1), IGF binding proteins (IGFBP4 and IGFBP5), myogenin, cyclin D1, and p21. The sequences for the various primers used for the specific target mRNAs are shown in Table 1. These primers were purchased from GIBCO Life Technologies. Primers were designed by using the Primer Select computer program (DNASTar), except for the IGF-I (all) primer set, which was previously used by Reiss et al. (43). It is important to note that using the primer set for IGF-I (all) gives two bands, one 202 and one 254 bp. The 254 bp corresponds to mechano-growth factor (MGF), the variant isoform splice, which contains an additional 52 bp corresponding to an alternate splice of exon 5 of the IGF-I gene (44, 59). For the MGF-specific primer set, the 5′ primer is common for both IGF (all) and MGF, and it corresponds to a sequence from exon 3. The 3′ antisense PCR primer for MGF is complementary to a sequence from the exon 5 splice contained only in MGF. Under the PCR conditions used to detect IGF-I, MGF cannot be detected under low-expression conditions, i.e., the normal control samples give only one IGF-I band: 202 bp. Larger amounts of template cDNA were used for the MGF PCR detection/quantification. For IGF-I RT-PCR, 1 μl of a 1:10 dilution of cDNA (RT reaction) was used, whereas for MGF detection, 1 μl of undiluted cDNA (RT reaction) was used in a 25-μl PCR reaction.

Note that, for IGFBP4, the primer sequence is based on the mouse X76066 sequence. These mouse primers were selected on the basis of regions that are highly similar to the human IGFBP4 cDNA, and they proved to be effective with rat mRNA. All the primers listed in Table 1 were tested for their compatibility with the alternate 18S primers.

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 differences in starting amounts of total RNA.

For the 18S amplification, we used the alternate 18S internal standard (Ambion), which yields a 324-bp product.

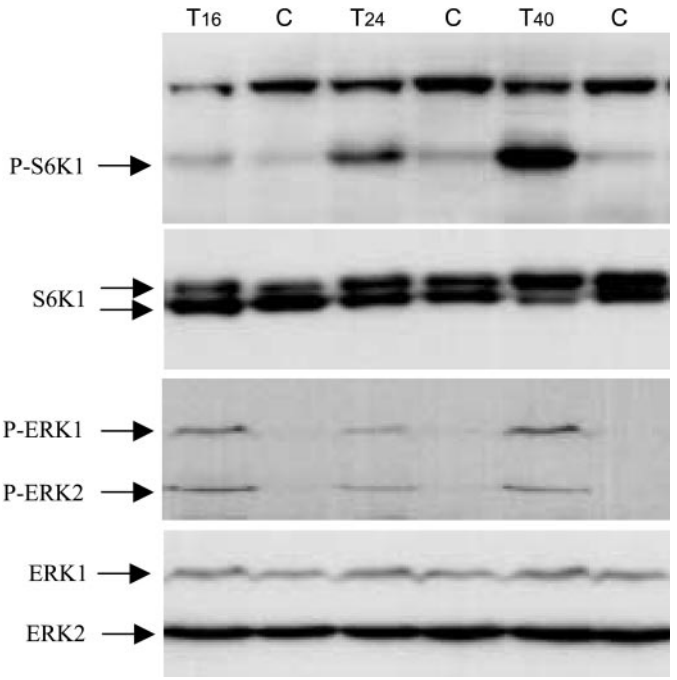

Fig. 1. Increased phosphorylation of p70-S6 kinase (S6K1) and extracellular response kinase (ERK) 2 after resistance exercise. Representative Western blots probed with antibodies to total (S6K1, ERK1, and ERK2) or phosphospecific (P-S6K1 and P-ERK) protein in the nonstimulated contralateral muscles (C) and at 16 (T16), 24 (T24), and 40 h (T40) after 2 bouts of exercise with a 48-h rest interval are shown. Total and phosphoprotein blots are from the same membrane, which was washed and reprobed.

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 the same range as the coamplified target mRNA (Ambion, relative RT-PCR kit protocol).

For each specific target mRNA, the RT-PCR reactions 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 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 $MgCl_2$ by using standard PCR buffer (GIBCO), 0.2 mM dNTP, 1 μM specific primer set, 0.5 μM 18S primer/competimer mix, and 0.75 unit *Taq* DNA polymerase (GIBCO) in 25 μl of 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 2–2.5% agarose gel by electrophoresis and stained with ethidium bromide, and signal quantification was conducted by laser scanning densitometry, as reported previously (58). 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 set to optimal conditions, so that both the target mRNA and 18S product yields are in the linear range of the semilog plot when the yield is expressed as a function of the number of cycles.

Phosphorylation State of Intracellular Signaling Proteins

The phosphorylation states of the p70-S6 kinase (S6K1) and extracellular response kinase 1 and 2 (ERK1 and ERK2) were examined by immunoblotting using phosphospecific antibodies (Cell Signaling Technology, Beverly, MA). The antibodies used detected changes in phosphorylation of residue Thr^{389} of S6K1 and Thr^{183} and Tyr^{185} of rat ERK2. In each case, phosphorylation at these sites is critical for increased activity in vivo (40, 57). 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 dithiothreitol, 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 HCl] 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 by using the Bio-Rad protein assay with BSA as standard. Approximately 50 μg of supernatant proteins were subjected to SDS-PAGE [12.5% T, according to standard protocol (32)] and then electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P) with the use of 10% methanol, 1 mM orthovanadate, 25 mM Tris, and 193 mM glycine, pH 8.3. Phospho-ERK1/2 and phospho-S6K1 were detected by using phosphorylation state-specific antibodies (9204 and 9101, Cell Signaling Technology) and the enhanced chemiluminescence method of detection (Amersham). Signal intensity was determined by laser scanning densitometry (Molecular Dynamics/ImageQuant). For each specific antibody, all the samples were run under identical (previously optimized) conditions, including the transfer on the membrane, the reaction with the first and secondary antibodies, washing conditions, enhanced chemiluminescence detection, and the 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 blot analysis was performed. In addition, a positive control, provided by the antibody manufacturer, 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 of 20–150 μg (data not shown).

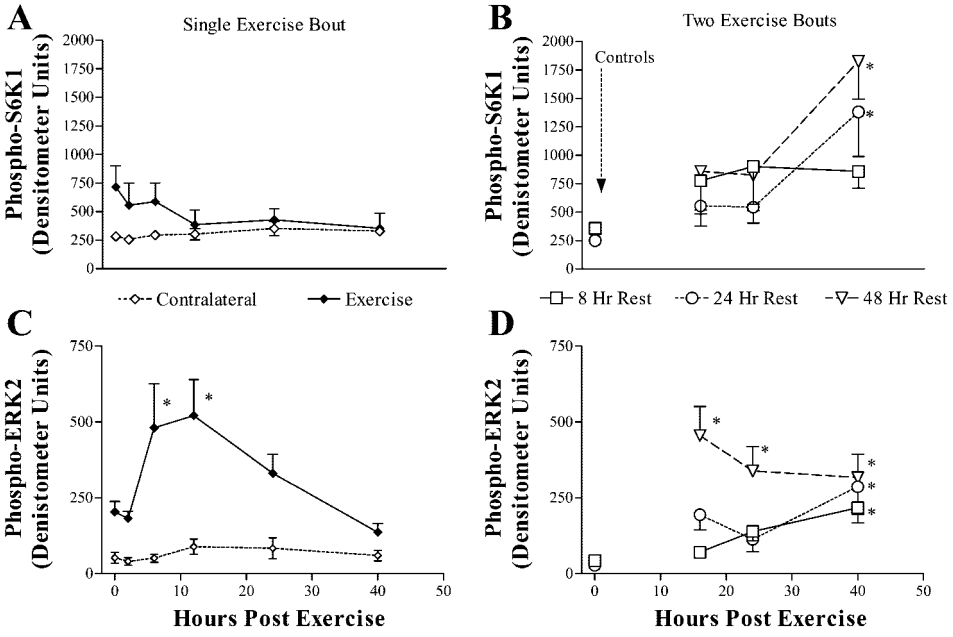

Fig. 2. Changes in the phosphorylation of S6K1 and ERK2 in the medial gastrocnemius (MG) muscle at different time points after resistance exercise. There appeared to be a trend toward increased S6K1 phosphorylation after a single bout of exercise (*A*). S6K1 phosphorylation was significantly increased at 40 h after 2 bouts of exercise spaced either 24 or 48 h apart (*B*). ERK2 phosphorylation was increased at several time points after either a single (*C*) or 2 bouts of resistance exercise (*D*). *Statistically significant difference ($P < 0.05$) vs. the contralateral muscle at the indicated time point. Contralateral values did not differ between groups (see *A* and *C*); therefore, a single value representing the mean ± SE for all contralateral muscles has been used as the 0 time point to reduce the complexity of *plots B* and *D*. However, statistical analysis was conducted by using the individual contralateral time points.

Statistical Analysis

All values are reported as means ± SE. For each time point, treatment effects (exercise vs. contralateral) were determined by ANOVA with post hoc testing (Student-Newman-Keuls) by using the Prism software package (Graphpad). For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

AUC. It is possible that visual examination of multiple data plots might not allow for a clear evaluation of the results obtained. Accordingly, area under the curve (AUC) measurements have been provided for the mRNA data in an attempt to provide a semiquantitative estimate of the differences and similarities between the responses to the different treatments. In each case, the baseline was set at the mean of the contralateral values (mean from all data collection time points). In the two-bout experiments, data were not collected at time points before 16 h postexercise to reduce the number of animals used. As a result, the AUC values cannot be rigorously tested because the span from the "0" time point to 16 h does not reflect actual data. AUC is generally used as a cumulative measure of the effects of some agent. With the assumption that some of the measured mRNAs were transcribed, this estimate incorporates the temporal aspects of the summation of responses and may provide insights into the overall effect of the exercise protocols employed.

RESULTS

Muscle Mass, Protein, and RNA Content

In general, electrically stimulated contractile activity had no overt effects on skeletal muscles. For example, the muscle weight and total protein content of the stimulated muscles were unchanged relative to that of the contralateral muscles. However, when two exercise bouts were imposed, there appeared to be a trend toward an increase in total RNA (~10%) in the stimulated muscles at 24 and 40 h postexercise (data not shown).

Effects of a Resistance-Type Exercise on Posttraining Intracellular Signaling

Representative Western blots are shown in Fig. 1. Phosphorylation of S6K1 is associated with an increase in translation and is known to occur with increased muscle loading and/or subsequent to IGF-I receptor ligation (31). Compared with the contralateral muscle, there appeared to be a trend, e.g., increased 2.5-fold immediately postexercise ($P < 0.07$) for the phosphorylation of S6K1 to be increased at early time points after a single bout of exercise (Fig. 2*A*). A second bout of exercise induced a significant increase in S6K1 phosphorylation in stimulated muscles of the 24- and 48-h rest interval groups that were collected 40 h after the exercise (Fig. 2*B*). Exercise had no effect on the total amount of S6K1 protein detected by Western blot (Fig. 1).

Increased phosphorylation of the ERKs is known to occur after the ligation of a number of growth factor receptors including IGFR1 (41). The ERKs are activated by phosphorylation, and they can in turn phosphorylate and activate a number of transcription factors (30, 61). The phosphorylation, of ERK2 was transiently increased after a single bout of resistance-type exercise, returning to baseline by 40 h postexercise (Fig. 2*C*). A second bout of exercise appeared to prolong the increase in ERK2 phosphorylation (Fig. 2*D*). In particular, the 48-h rest period resulted in a marked prolongation of this ERK2 phosphorylation response (Fig. 2*D*).

Effects of a Resistance-Type Exercise on IGF-I-Related mRNA Expression Posttraining

IGF-I mRNA and peptide have previously been shown to increase during compensatory skeletal muscle hypertrophy in rats (e.g., Refs. 1, 2, 6). More recently, MGF has been identified as an IGF-I isoform that is sensitive to mechanical loading (24, 36). Increases in IGF-I and MGF expression most likely indicate the activation of a local (i.e., autocrine/paracrine) growth factor signaling process associated with hypertrophy-like responses. Representative RT-PCR results for MGF and IGF-I are presented in Fig. 3. The expression or accumulation of the mRNAs for IGF-I and MGF was significantly increased (range of 1.5- to 2.5-fold) after a single bout of exercise (Fig. 4, *A* and *C*). The two-exercise-bout protocol also increased MGF and IGF-I expression (Fig. 4, *B* and *D*). The magnitudes of these changes were similar to those seen with a single bout of exercise for the 8-h rest groups. However, the response of these mRNAs in the 24- and most notably the 48-h rest group was substantially greater than the increases seen with the other training paradigms. For example, an estimation of the MGF AUC for a single bout of exercise is 2.2 AUC units vs. 4.7 AUC units for the 48-h group.

IGFBP4 and IGFBP5 are known to be produced by skeletal muscle cells (35) and would be expected to modulate the effects of IGF-I via regulation of the free IGF-I concentration in muscle and possibly via competition with the IGFR1 for IGF-I (8). The expression or accumulation of IGFBP4 mRNA was increased significantly and similarly after either one or two bouts of resistance exercise (Fig. 5 and Fig. 6, *A* and *B*). There were no significant changes in the levels of mRNA for IGFBP5 (data not shown). The level of IGFR1 mRNA was also increased significantly and similarly after all resistance exercise protocols (Fig. 6, *C* and *D*).

Effects of a Resistance-Type Exercise on Myogenic Marker mRNA Expression Posttraining

The cell cycle is regulated in part by the cyclin-dependent kinases (cdk), which are in turn regulated by the presence or absence of various cyclins as well as inhibitory proteins such as p21 (51). Increased cyclin D1 expression suggests that some cell population(s) within the muscle is (are) becoming mitotically active. The mRNA for cyclin D1 has previously been shown to be increased in overloaded skeletal muscles (2). Representative RT-PCR results for cyclin D1 and myogenin are presented in Fig. 7. There was an apparent trend toward an increase in cyclin D1 mRNA after a single bout of resistance exercise (Fig. 8*A*). Two bouts of exercise resulted in significant increases in cyclin D1 mRNA, with longer rest periods apparently enhancing this response (Fig. 8*B*).

To date, the preponderance of published results suggest that, in innervated skeletal muscle, myogenin expression appears to be associated with the differen-

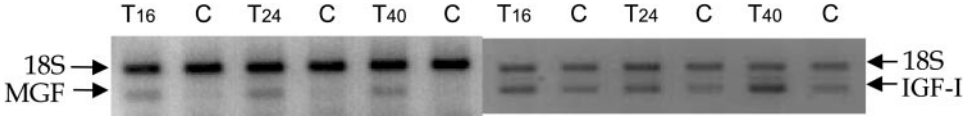

Fig. 3. Increased mechano-growth factor (MGF) and insulin-like growth factor (IGF)-I mRNA after resistance exercise. Representative PCR gel scans demonstrate the observed changes in MGF and IGF-I mRNA in the nonstimulated contralateral muscles (C) and in muscles at 16 (T16), 24 (T24), and 40 h (T40) after 2 bouts of exercise with a 48-h rest interval.

tiation of satellite cells or myogenic precursor cells (2, 21, 27, 33, 47, 51, 62). Myogenin mRNA increased in all muscles after resistance exercise (Fig. 8, *C* and *D*). The absolute magnitude of this increase was similar after both the one- and two-bout protocols. However, two bouts of exercise and in particular those involving 24 and 48 h of rest would appear to have resulted in this parameter being elevated for a greater proportion of the 40-h period over which data were collected.

The cdk inhibitor p21 inhibits progression through the cell cycle and initiates the process of differentiation. We previously reported that cdk inhibitor p21 mRNA increased in parallel with myogenin mRNA during compensatory hypertrophy (2). In the present study, the increase in the mRNA for cdk inhibitor p21 mirrored that seen for myogenin and, as reported previously, was highly correlated with the observed changes in myogenin ($r = 0.86$, $P < 0.0001$; data not shown).

DISCUSSION

In humans, the adaptation of skeletal muscle to increased loading may occur in occupational settings, usually as an unintended consequence, or can be deliberately elicited via resistance exercise training. In the latter case, a program that imposes acute bouts of loading is generally instituted. Such programs include manipulation of the frequency, intensity, and duration of the individual training bouts. In general, the characteristics of these three parameters are determined on the basis of observations from previously efficacious training programs. As a result, a number of differing permutations of frequency, intensity, and duration may be applied, some of which may prove effective in simulating desirable adaptations. However, regardless of the protocol employed, the effectiveness of a training program will not be evident until an appreciable period of time has elapsed, allowing for the completion of a relatively large number of training sessions. This reliance on end-point measures, i.e., overt measures of hypertrophy, precludes understanding of how each of the three parameters and more specifically the individual exercise bouts interacted to stimulate the cellular and molecular mechanisms that mediate the adaptive responses.

In the present study, we started with the simple hypothesis that a preselected set of markers of cellular and molecular processes would respond measurably to acute bouts of resistance exercise. Building on the observations from the first set of experiments (single exercise bout), we postulated that changes in these markers would be useful in discriminating between different training parameters on the basis of the summation of the observed responses. The primary training variable manipulated in the second set of studies was the frequency of exercise training, i.e., the rest period between training bouts. We purposely chose rest periods that we predicted (on the basis of the literature) would span a range that would probably be too short (8 h) through optimum (~2 days). The cellular

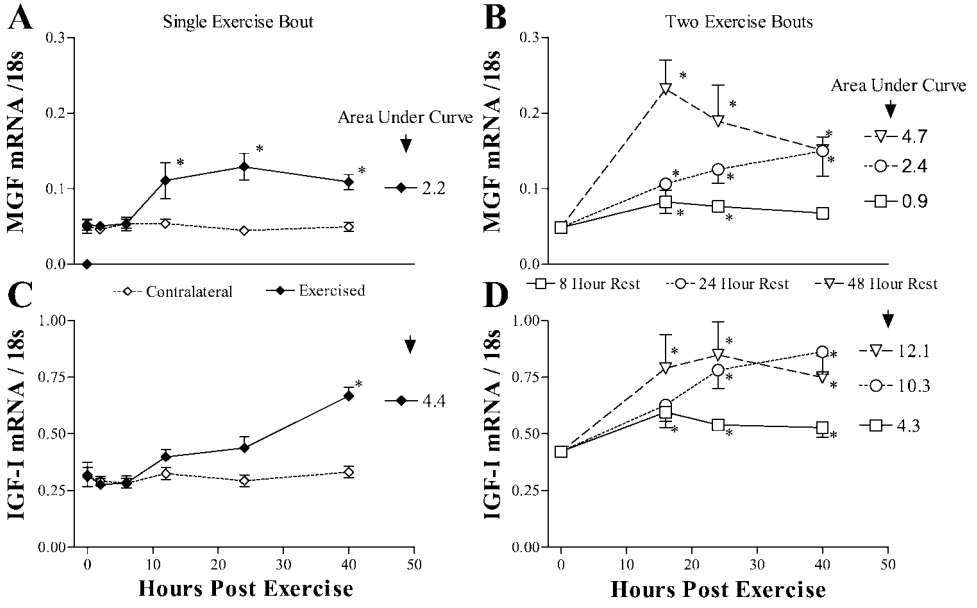

Fig. 4. MGF and IGF-I mRNA expression in MG muscle at different time points after resistance exercise. mRNA for MGF and IGF-I increase at several time points after a single bout of exercise (*A* and *C*) and 2 successive bouts with either 8, 24, or 48 h of rest between bouts (*B* and *D*). The area under the curve was calculated by using the mean of the control (*time 0*) time point as the baseline. $*P < 0.05$ vs. the contralateral muscle.

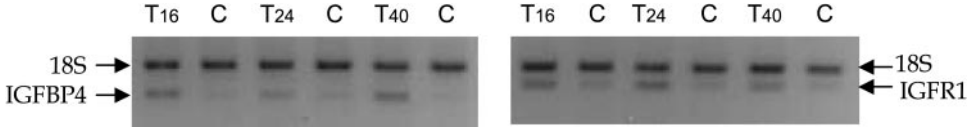

Fig. 5. Increased IGF binding protein 4 (IGFBP4) and IGF-I receptor (IGFR1) mRNA after resistance exercise. Representative PCR gel scans demonstrate the observed changes in IGFBP4 and IGFR1 mRNA in the nonstimulated contralateral muscles (C) and in muscles at 16 (T16), 24 (T24), and 40 h (T40) after 2 bouts of exercise with a 48-h rest interval.

and molecular markers chosen had been previously identified as being responsive to increased loading in a variety of in vitro or in vivo models (e.g., Refs. 2, 15, 39).

Cellular Signaling Responses

The activation of S6K1 has a relatively modest, positive impact on translation in general, but more importantly it increases the translation of specific mRNAs that encode components of the translational apparatus itself (26, 55). The contribution of this regulatory step in supporting anabolic responses would therefore be of great importance (15). In the present study, two consecutive bouts of exercise spaced either 24 or 48 h apart resulted in a significant increase in S6K1 phosphorylation (5.5- to 6.3-fold increase, Fig. 2*B*). Nader and Esser (39) have recently reported that a single bout of resistance exercise resulted in significant increases in S6K1 phosphorylation at 3 and 6 h postexercise. In the present study, there was a trend toward a similar response.

The phosphorylation of the ERKs is thought to be a result of activation of the Ras-Raf pathway, subsequent to the ligation of various growth factor receptors (23). In muscle cell culture, the cellular signaling pathways that include ERK activation have been reported to be important in promoting cellular proliferation (14). A single bout of resistance exercise stimulated a significant increase in ERK2 phosphorylation at 6 (ninefold) and 12 (sixfold) h postexercise (Fig. 2*C*). Nader and Esser (39) reported increased ERK phosphorylation at earlier time points after resistance exercise. Two bouts of resistance exercise, particularly with a 48-h rest interval, appear to prolong the elevation of ERK2 phosphorylation (Fig. 2*D*).

Together, these results demonstrate that intracellular signaling pathways, which have been reported to be associated with hypertrophy, are responsive to acute bouts of increased loading. These responses appear to exhibit temporal and/or quantitative summation that is sensitive to the duration of the rest interval between the exercise bouts.

IGF-I System Responses

There is an extensive body of literature that indicates that IGF-I and the loading-sensitive IGF-I isoform, MGF, are important components of the system by which skeletal muscle responds to increased loading (e.g., Refs. 1, 3, 7, 13, 24, 56, 60). For example, we and others had previously demonstrated that IGF-I mRNA and peptide levels increase during compensatory hypertrophy (1, 2, 6, 16) and that increased muscle IGF-I levels can induce muscle hypertrophy in the absence of apparent changes in loading state (3, 13). In the present study, we found that a single bout of resistance exercise stimulated a relatively rapid increase in MGF mRNA and, to a lesser extent, that of IGF-I (Fig. 4, *A* and *C*). Two bouts of exercise resulted in sustained increases in MGF and IGF-I mRNAs that were more

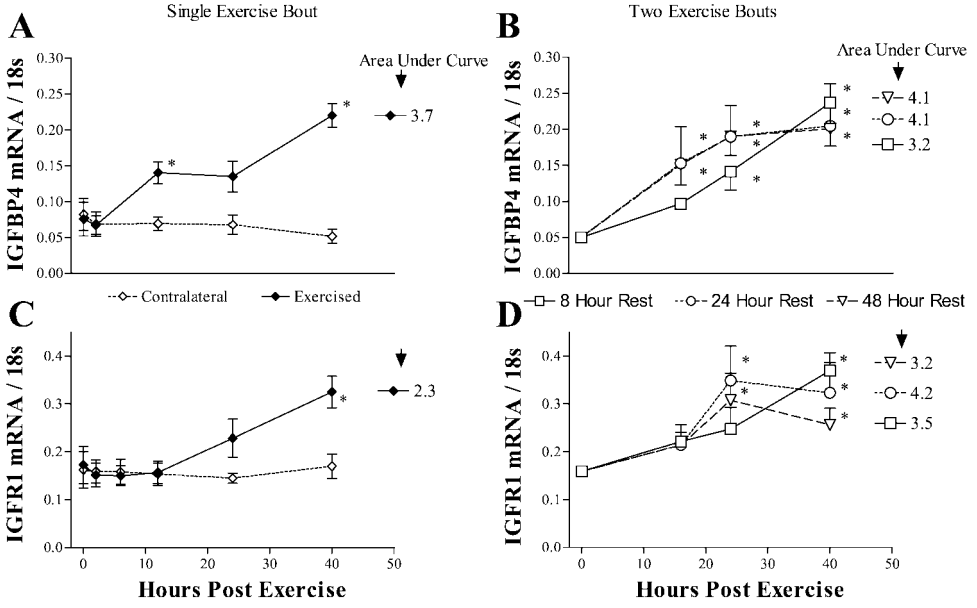

Fig. 6. IGFBP4 and IGFR1 mRNA expression in MG muscle at different time points after resistance exercise. mRNAs for IGFBP4 and the IGFR1 were significantly increased at a number of time points after both a single bout of exercise (*A* and *C*) and 2 successive bouts with either 8, 24, or 48 h of rest between bouts (*B* and *D*). $*P <$ 0.05 vs. the contralateral muscle.

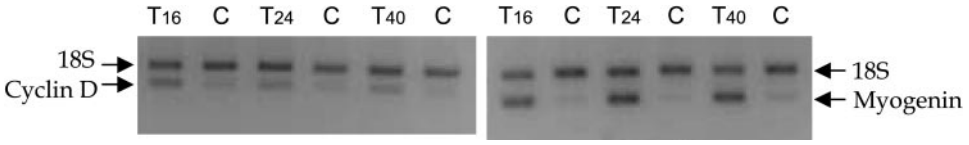

Fig. 7. Increased cyclin D1 and myogenin mRNA after resistance exercise. Representative PCR gel scans demonstrate the observed changes in cyclin D1 and myogenin mRNA in the nonstimulated contralateral muscles (C) and in muscles at 16 (T16), 24 (T24), and 40 h (T40) after 2 bouts of exercise with a 48-h rest interval.

pronounced when the rest intervals were longer (Fig. 4, *B* and *D*). Resistance exercise also induced significant increases in the mRNAs for components of the IGF-I system such as IGFBP4 and the primary IGF-I receptor (Fig. 6). IGF-I actions in skeletal muscles are reported to include stimulation of the proliferation and differentiation of satellite cells as well as insulin-like anabolic effects (41). In the present study, the IGF-I-related changes appear to be consistent with cellular responses that, if sustained, would be expected to contribute to the development of hypertrophic adaptations.

Markers of Cellular Proliferation and Differentiation

The expression or accumulation of the mRNA for cyclin D1 was increased, in some cases more than twofold, after two bouts of resistance-type exercise. This would suggest that cells within the muscle were preparing to enter the cell cycle. In light of the large amount of data, which indicates that satellite cells proliferate during skeletal muscle hypertrophy, this result may reflect the initial activation steps in these cells. In addition, a hypertrophy response would most likely entail increases in connective tissue and other structures necessary to support increased muscle size and force-generation capability. These responses might also be expected to include proliferation in nonmuscle cell types as well. Thus the indication of proliferative responses most likely represents the initiation of the coordinated suite of responses within the muscle necessary for the adaptation to increased loading.

As noted above, an increase in myogenin expression is generally thought to reflect the differentiation of satellite cells and/or myogenic precursor cells in intact mammalian skeletal muscle. In the present study, both the single- and dual-exercise-bout protocols resulted in significant increases in myogenin mRNA. As we had reported previously (4), the increases seen in myogenin were paralleled by, and highly correlated with, those in cdk inhibitor p21 in this study. This result would support the idea that myogenin expression was occurring in muscle lineage cells that were preparing to withdraw from the cell cycle and to begin differentiating.

Summation of Cellular and Molecular Responses

Although it has not generally been addressed in terms of cellular-level events, the need for summation of responses, most likely in both the muscular and neural systems, is implicitly acknowledged in the design of most resistance training programs, which generally include manipulations of exercise frequency as well as the intensity and duration of bouts. To the best of our knowledge, this is one of the first descriptions of cellular and molecular response summation induced by resistance exercise. In particular, the observation that the magnitude of cellular and molecular response is sensitive to a training parameter such as bout frequency appears to be novel if not unexpected. In the present study, a single bout of resistance-type exercise generally resulted in increased mRNA levels, which

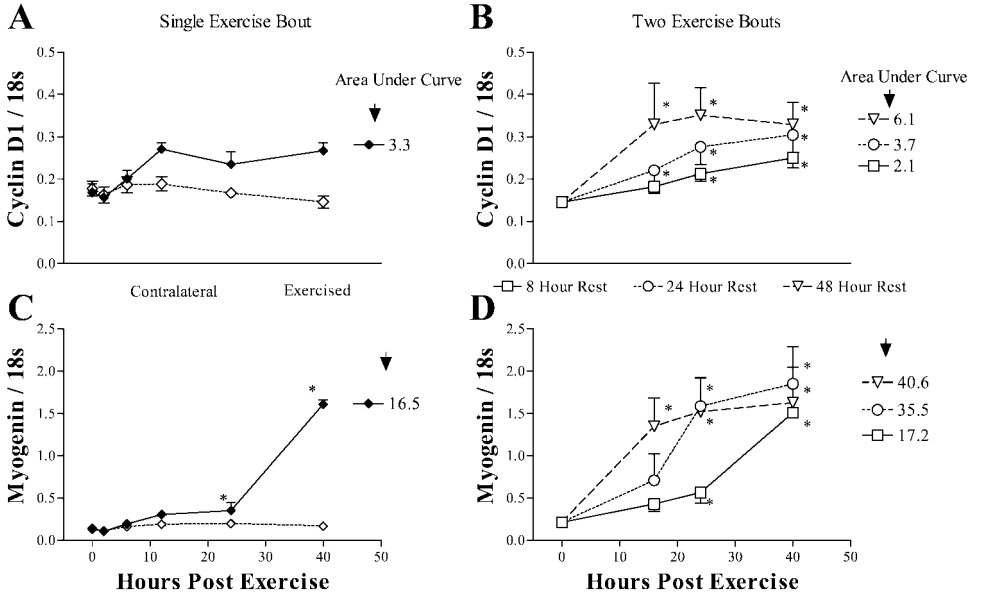

Fig. 8. Cyclin D1 and myogenin mRNA expression in MG muscle after resistance exercise. There appeared to be a trend toward an increase in the mRNA for cyclin D1 after a single bout of exercise (*A*). mRNA for cyclin D1 was significantly increased after 2 successive bouts of resistance exercise (*B*). Myogenin mRNA was increased by a similar magnitude by both a single bout of exercise (*A* and *C*) and 2 successive bouts with either 8, 24, or 48 h rest between bouts (*D*). $*P < 0.05$ vs. the contralateral muscle.

were not completely resolved at 40 h postexercise. However, in the cases of the phosphorylation of S6K1 and the increase in cyclin D1 expression, the single-exercise-bout responses did not reach statistical significance. With the possible exception of IGFBP4 and the IGFR1, the imposition of a second bout of exercise, at time points when the response to the initial bout was ongoing, appears to have substantially increased the magnitude and/or duration of the increase in mRNAs. In most cases, the 48-h rest interval resulted in a substantially greater increase and/or prolongation of the observed response. Twenty-four hours of rest were also fairly effective, whereas the 8-h interval was, in most cases, clearly inferior to the longer rest times in terms of stimulating increases in signaling or mRNA. It is not immediately clear why a second bout of exercise imposed 8 h after the first would be so markedly inferior in producing an enhanced response compared with 24 or 48 h of rest (i.e., MGF, IGF-I, myogenin, cyclin D1 mRNA). One explanation might be that some signaling mechanisms enter a refractory period after an exercise bout, thereby blunting the response to a second exercise stimulus.

Summation of cellular and molecular responses to exercise most likely represents a key process for the modulation of adaptation to changes in loading state. For example, a requirement for the summation of responses would protect the cells from entering a costly process of adaptation after a novel stimulus that is not repeated.

Application of Findings

The results of this study suggest that cellular and molecular events that underlie the response to individual exercise training bouts can be measured and their time course can be characterized. This capability will allow for the study of the regulation of the hypertrophy response with a very high level of temporal resolution. For example, assuming that the observed changes in mRNA reflect increased transcription, the elucidation of loading-sensitive transcription factors can be conducted after a single bout of exercise and can be, at least initially, focused on transcription factors that regulate the few genes identified herein.

In addition to the use of exercise bouts to characterize molecular and cellular mechanisms, the ability to characterize the time course of presumably hypertrophic responses after one or a few exercise bouts will also allow for the objective evaluation of training protocol parameters. In the present study, only the simplest of parameters were manipulated. However, this investigative approach would be equally useful for the evaluation of other parameters such as repetition number or timing, maximal vs. submaximal contractions, dynamic vs. isometric contractions, and so on.

In summary, programmed resistance training employs sequential bouts of exercise to stimulate adaptation. The results of the present study indicate that a number of cellular and molecular processes respond to exercise bouts and are sensitive to the manipulation of training parameters such as between-bout rest intervals. The methods used in this study should be useful in the study of regulatory mechanisms as well as the evaluation of exercise training parameters.

The authors thank Angi Qin, Ming Zeng, and Sam McCue for technical assistance.

This work was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-45594.

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