Alterations in the expression of mRNAs and proteins that code for species relevant to eIF2B activity after an acute bout of resistance exercise


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Kubica, Neil, Scott R. Kimball, Leonard S. Jefferson, and Peter A. Farrell. Alterations in the expression of mRNAs and proteins that code for species relevant to eIF2B activity after an acute bout of resistance exercise. J Appl Physiol 96: 679–687, 2004. The focus of the study described herein was to examine the relative expression levels of mRNAs and proteins relevant to the regulation of translational initiation, and hence protein synthesis, in the time course after an acute bout of resistance exercise in male Sprague-Dawley rats. Significant increases in the relative abundance of the mRNAs coding for the epsilon (33%) and gamma (26%) subunits of eukaryotic initiation factor 2B were observed 48 h after the exercise bout. Furthermore, the mRNA coding for the delta subunit of eIF2B was also significantly increased, both 24 h (46%) and 48 h (44%) postexercise. There was a relative decrease in three eIF2Bε kinase mRNAs, namely sequences coding for glycogen synthase kinase 3β (49%), casein kinase I (48%), and casein kinase II (42%) 48 h into the recovery period. Additionally, there was a significant decrease in expression of the mRNAs coding for eIF2ε (28% 24 h postexercise) and one of its regulatory kinases, double-stranded RNA-activated protein kinase (33% 48 h postexercise). Finally, an increase in eIF2B total protein (124%) was observed within 3 h postexercise. These results suggest that there may be rapid translational regulation of mRNAs coding for species relevant to translational initiation after an acute bout of resistance exercise. Furthermore, transcription of these mRNAs is altered further into the recovery period, and this might play a role in protein synthetic capacity on subsequent bouts of resistance exercise. eukaryotic initiation factor 2ε; eukaryotic initiation factor 2β; glycogen synthase kinase 3β; casein kinase I; casein kinase II; double-stranded RNA-activated protein kinase
to exercise is entirely absent. To gain a more complete understanding of protein synthesis in response to exercise, a more dynamic view of eIF2B regulation is needed, including experiments designed to address transcriptional regulation of the initiation factor itself and its proposed upstream signaling molecules.

We suggest that, although enhanced translational efficiency appears to be the initial response to acute resistance exercise, alterations in transcription of genes that code for translationally relevant proteins will be an essential component of the adaptive process associated with exercise training. Booth and Kirby (5) suggest that mRNA species relevant to training-related adaptations will accumulate during exercise training. However, it has also been shown that alterations in mRNA levels for these training-related species are often altered after a single bout of acute exercise (7). The present study addresses this issue by examining alterations in mRNA expression of the aforementioned eIF subunits, and their upstream kinases, during the 48 h after an acute bout of resistance exercise. Second, we hypothesized that total protein levels of eIF2B and eIF2 would be altered in unison with directional changes in their respective relative mRNA abundance.

MATERIALS AND METHODS

Animal care. All experimental procedures were approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine. Male Sprague-Dawley rats (~300–350 g) were housed in temperature- and humidity-controlled holding facilities on a 12:12-h light-dark cycle. Rats were fed a standard rodent diet (Harlan-Teklad Rodent Chow, Madison, WI), and both food and water were provided ad libitum.

Acute resistance exercise protocol. Details of the exercise protocol have been described previously (13). Briefly, male Sprague-Dawley rats were operantly conditioned to stand on their hindlimbs and touch an illuminated bar located high on the wall of a Plexiglas cage. This movement was reinforced with the use of a mild foot shock (~2 mA, 60 Hz) over the course of four familiarization sessions. Once this learning process was completed, weighted Velcro vests were strapped over the scapulae and the animals were required to touch the overhead bar 50 times during a given exercise session. The “acute” resistance exercise protocol consisted of four separate sessions with 1 day of rest between sessions. The rats performed 50 repetitions in each session with 0.2 (day 1), 0.4 (days 2 and 3), and 0.6 (day 4) g weighted vest/g body wt, respectively.

It is important to note that even the 60% of body weight intensity level is insufficient to cause muscle hypertrophy with repeated exercise bouts. In fact, a progressive overload paradigm culminating in several weeks of resistance exercise with a load in excess of 1 g/kg body wt is necessary to induce muscle hypertrophy (unpublished results). However, the 60% of body weight exercise session is considered to be representative of resistance exercise because such a load has been shown in numerous published studies to cause an increase in muscle protein synthesis in untrained rats. Conversely, the 20 and 40% of body weight sessions are included simply as a training tool to teach the rats to maintain their balance while performing the desired exercise motion. These exercise stimuli are insufficient to induce increases in muscle protein synthesis and thus are not considered part of a short-term training paradigm. Thus the penultimate exercise session is considered to be an example of acute resistance exercise.

Exercise sessions occurred under red light in the late afternoon. On all four training days, sedentary control animals were placed in the cage and given five mild shocks to stimulate the stress experienced by the exercised animals. One of the shock control sessions took place 3 h before death. Although the shock received by the control group only correlates to the effect of the foot shock on the earliest time point examined, shock-matched controls were previously performed for animals killed 16 h after the final bout of resistance exercise and no effects were observed on any of the end points examined. Additionally, shock dose-response curves showed that there was no correlation between the number of shocks administered and postexercise rates of protein synthesis (unpublished data). Exercised animals were killed at 3, 24, or 48 h after the last acute resistance exercise session depending upon their inclusion in specific treatment groups. The animals were fasted for 5 h before death. At the time of death, all rats were anesthetized with isoflurane, and gastrocnemius tissue was excised and immediately snap frozen in liquid nitrogen.

Isolation of total RNA. Gastrocnemius tissue was frozen between aluminum blocks cooled to the temperature of liquid nitrogen, pulverized with a mortar and pestle under liquid nitrogen, and stored at −80°C until processing. Total RNA was isolated by using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol specific for muscle, heart, and skin tissue. To reduce contamination by genomic DNA, samples were treated on the spin columns with RNase-free DNaseI (Qiagen). Samples were analyzed for RNA abundance and quality via spectrophotometry on a Beckman Coulter DU 640 (Fullerton, CA). Additional quality control analyses were conducted by use of an Agilent Technologies 2100 bioanalyzer lab chip system (Palo Alto, CA) in the Genomics Core Facility of The Pennsylvania State University College of Medicine. All samples were adjusted to a concentration of 1 μg RNA/μl.

Real-time PCR for eIF2Be, eIF2α, and eIF2β. The real-time PCR reactions for eIF2Be, eIF2α, and eIF2β were conducted at the Nucleic Acid Core Facility at The Pennsylvania State University (University Park, PA). Real-time PCR was conducted by using the Perkin-Elmer/ Applied Biosystems Division 7700 sequence detector (Foster City, CA). The methodology used at Penn State’s Nucleic Acid Facility has been previously reported (15). Briefly, total RNA samples were reverse transcribed with the use of the following reaction mix: 0.5 μl RNase inhibitor (40 U/μl), 2.0 μl 10X TaqMan universal master mix buffer, 3.6 μl MgCl₂ (25 mM), 1.0 μl specific reverse primer (gene of interest or GAPDH), 1.0 μl random hexamers (50 μl), 1.0 μl of each nucleotide triphosphate (adenine, thymidine, cytosine, and guanine) (10 mM), and 5.0 μl deionized, diethyl pyrocarbonate-treated H₂O. This mixture was used in the no-template control wells after addition of murine leukemia virus reverse transcriptase (0.44 μl; 50 μU/μl). Total RNA samples (40 ng/μl) were heated at 65°C for 5 min, centrifuged, and placed on ice. A sample of the reaction mix was then added to the microamplification tube and run in a thermocycler using the following conditions: 1 h at 42°C, 5 min at 72°C, and 2 min at 95°C.

The cDNA product of this RT reaction (20 ng/μl) was then used in the real-time PCR reaction. PCR reactions were optimized for Mg²⁺ concentration and primer concentration for each specific primer/probe set. The reaction mix for the PCR reaction included the following: 5.0 μl 10× TaqMan universal master mix buffer, MgCl₂ (25 mM), gene-specific upper primer (10 μM), gene-specific lower primer (10 μM), gene-specific probe, 1.0 μl of each nucleotide triphosphate (10 mM), 0.25 μl Taq Gold (5 U/μl), 8.0 μl cDNA, and deionized H₂O. Specific quantities of some reaction components were omitted because of variation based on optimization of the reactions for individual primer/probe sets.

The primer/probe sets used for each of the respective eukaryotic initiation factor genes were as follows: eIF2Be upper primer, 5'-TTCGCCATCTCCAAAGGCC-3'; lower primer, 5'-TCGATCCAGCGGCATCTG-3'; probe, 5'-CCTGGGTTTGGCCAATCTC-3'; eIF2α upper primer, 5'-GGGGCTTGCGGTCTTTCATC-3'; lower primer, 5'-TGGAGGCCCTGTAATCTTCT-3'; probe, 5'-TGGTCTCCTGATTGCGACCTGTTA-3'; eIF2β upper primer, 5'-AAGTGGCCCTCAGGCTGCTA-3'; lower primer, 5'-ATGGTGAGTTACCAATAGTTAG-3'; probe, 5'-AGGCAACGGCAGA-

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CAGCTCCGT-3'. All probes were purchased from Biosearch Technologies and labeled at the 5' end with the 6-carboxyfluorescein aminohexyl amidite and at the 3' end with Black Hole Quencher (Biosearch Technologies, Novato, CA). The thermocycler conditions for the PCR reactions were 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 s at 95°C followed by 1 min at 60°C.

After the PCR run, Sequence Detector version 1.7 software (Applied Biosystems) was used to analyze the fluorescence data. A suitable threshold was set above the noise, and the cycle number at which the fluorescence exceeded the threshold was determined for each sample. Data for eukaryotic initiation factor mRNA expression were normalized to GAPDH expression, which was determined using a primer/probe kit from Applied Biosystems.

**Real-time PCR for eIF2Bα, eIF2Bβ, and the initiation factor kinases.** First-strand cDNA synthesis was conducted with the use of 1 μg of total RNA from each sample. Total RNA was reverse transcribed by using the Invitrogen SuperScript One-Step RT-PCR kit with platinum Taq polymerase and oligo(dT) primers (Invitrogen, Carlsbad, CA). The RT-PCR was conducted for 1 h at 42°C. Real-time PCR was conducted with the Qagen QuantiTech SYBR green PCR kit. Again, expression of the genes of interest were normalized to GAPDH expression. Because of the possibility of different amplification efficiencies between the reference gene and the gene of interest, the cycle number was set above the noise, and the cycle number at which the fluorescence exceeded the threshold was determined for each sample. Data for eukaryotic initiation factor mRNA expression were normalized to GAPDH expression, which was determined using a primer/probe kit from Applied Biosystems.

**RESULTS**

*EIF2B mRNA levels before and after resistance exercise.*** There was no significant difference in relative mRNA expression levels of GAPDH in the time course examined, thus validating the use of this housekeeping gene to normalize expression of other mRNA species in the present study (Fig. 1). There was a significant increase in the relative abundance of eIF2Bα, -γ, and -δ mRNAs in the gastrocnemius muscle after resistance exercise. The relative level of eIF2B mRNA in the 48-h postexercise group was significantly greater than that in the sedentary group (33% increase; P < 0.01; Fig. 2A). Similar to transcripts coding for the e-subunit, relative mRNA levels of eIF2Bβ were also significantly elevated compared with the control only 48 h into the recovery period (26% increase; P < 0.05; Fig. 2B). Relative expression of eIF2Bβ mRNA was also increased in the recovery period after exercise. This increase became statistically significant 24 h after the final exercise bout (46% increase; P < 0.01; Fig. 2C) and remained elevated 48 h into the recovery period (44% increase; P < 0.01; Fig. 2C).

**Relative mRNA expression of eIF2B2 mRNA levels.** The mRNAs encoding each of the protein kinases examined in this study that have eIF2B as a common substrate decreased significantly over the time course studied. The relative level of...
GSK-3β mRNA was significantly decreased 48 h after the exercise bout compared with the sedentary group (49% decrease; \( P < 0.01 \); Fig. 3A). CKIIβ mRNA, which codes for the regulatory subunit of this kinase, was also decreased 48 h postexercise vs. the sedentary control group (42% decrease; \( P < 0.01 \); Fig. 3C).
Relative mRNA expression of eIF2α/H9251 and eIF2α/H9252.

In contrast to eIF2B subunit mRNAs, there was a statistically significant decrease in the relative eIF2α/H9251 mRNA transcript levels over the time course examined. This decrease was transient and statistically significant only 24 h after the completion of the exercise bout (28% decrease; P < 0.05; Fig. 4A). No statistically significant alteration in eIF2α/H9252 mRNA was observed over the time course examined (Fig. 4B).

Relative mRNA expression of eIF2α and eIF2β kinases.

The relative mRNA quantity for the eIF2α kinase PKR was significantly decreased during the time course after the exercise bout (33% decrease 48 h postexercise vs. control; P < 0.01; Fig. 5A). The relative mRNA quantities for the eIF2α kinase HCR (Fig. 5B) were unchanged throughout the time course examined. Likewise, the mRNA levels of eIF2β kinase PKA (Fig. 5C) were not significantly altered by resistance exercise during the time course examined.

Relative protein expression of eIF2B/e and eIF2α total proteins.

On the basis of Western blot analysis of the e-subunit, there was a significant increase in the total amount of eIF2B protein present in the gastrocnemius muscle after an acute bout of resistance exercise. This increase peaked at 3 h postexercise (2.2-fold vs. control, P < 0.01) and remained significantly elevated at both 24 and 48 h postexercise (Fig. 6A). It is important to note that this increase in total eIF2B protein occurred before any alteration in expression of mRNAs coding for eIF2B subunits. In contrast to these findings, there was no significant change in total eIF2 protein in the time course examined after acute resistance exercise as determined by Western blot analysis of the α-subunit (Fig. 6B).
of control and shown as means ± SEM. Sedentary animals (n = 4) are compared with animals killed 3 h (n = 4), 24 h (n = 4), or 48 h (n = 4) after acute resistance exercise. A: total eIF2B protein. B: eIF2α protein. Values are expressed as a fraction of control and shown as means ± SEM. Sedentary animals (n = 4) are compared with animals killed 3 h (n = 4), 24 h (n = 4), or 48 h (n = 4) after acute resistance exercise. *Values for the 3 h (P < 0.01), 24 h (P < 0.05), and 48 h (P < 0.05) postexercise groups are significantly greater than those for the sedentary control group.

**DISCUSSION**

The most immediate effect observed in the present study was a significant increase in the quantity of eIF2B protein 3 h after resistance exercise (2.2-fold). This increase remained statistically significant at both 24 and 48 h postexercise. We focused on the ε-subunit because this subunit catalyzes the guanine nucleotide exchange reaction on eIF2; however, previous work suggests that all subunits of eIF2B holoprotein are present in a stoichiometric complex (25, 35). Therefore, we speculate that these results are indicative of an increase in the total protein expression of the total eIF2B complex. This approach has been used previously to quantify total eIF2B protein (25).

The increase in protein appeared before an increase in the relative quantity of the mRNA species coding for subunits of eIF2B. This result suggests that eIF2B is translationally regulated after an acute bout of resistance exercise and that, temporally, transcriptional regulation of eIF2B is a secondary response in the postexercise period. It is important to note that we cannot eliminate decreased degradation of eIF2B protein as an alternative explanation for the present results; however, because of the long half-life of this protein under basal conditions, this explanation is considered less likely.

Unlike the alterations in eIF2B, eIF2 protein did not change significantly in the postexercise period examined. Therefore, translational regulation of eukaryotic initiation factors appears to be specific to particular proteins after resistance exercise. Although the mechanism of such discrimination is not known, this hypothesis is teleologically attractive because eIF2B is present in limiting quantities relative to the number of ribosomes present in some cell types (18, 40), whereas eIF2 is believed to be more abundant. Oldfield et al. (35) found that eIF2 was 5.6 times more abundant than eIF2B in rabbit reticulocytes and >10 times more abundant in the heart. Furthermore, computer predictions of secondary structure in the rat eIF2B 5′-untranslated region (free energy = −30.79 kcal/mol, RNA structure 3.71, unpublished results) (32) suggest the possibility of translational regulation of this mRNA sequence. Thus, because eIF2B activity may be the most important determinant of increased translational initiation after an acute bout of resistance exercise, an increase in available eIF2B in the gastrocnemius could lead to an increased capacity of skeletal muscle to elevate postexercise global protein synthesis. Interestingly, the initial increase in eIF2B total protein occurred before the time of the increase in protein synthesis previously reported (17). Thus the increase in eIF2B could play a role in acute increases in protein synthesis after load-bearing activity; however, the increase in eIF2B protein alone is insufficient to explain such a response.

Whereas changes in mRNA translation appear to be the dominant process for modulating eIF2B abundance immediately after exercise, transcriptional processes could potentially be important later in the postexercise period. There was a significant increase in mRNA coding for the ε-, γ-, and δ-subunits of eIF2B in the postexercise time course examined. The five subunits of eIF2B are categorized into two families on the basis of amino acid sequence homology. The α-, β-, and δ-subunits comprise one family, whereas the ε- and γ-subunits make up the other family. Therefore, mRNA species encoding proteins belonging to both families were increased by the resistance exercise protocol.

The changes observed in the mRNAs encoding eIF2B ε kinases were also in line with the initial hypotheses. GSK-3β mRNA levels were significantly reduced in the 48-h postexercise group compared with the sedentary group and the 3 h postexercise group. Although Dholakia and Wahba (9) found increases in eIF2B activity with phosphorylation of the ε-subunit by GSK-3, the majority of published studies have found the exact opposite to be true. Price and Proud (39) and Webb and Proud (49) proposed that the activity of eIF2B is increased by the dephosphorylation of eIF2Be, therefore suggesting that phosphorylation of eIF2Be at the GSK-3 site would actually inhibit exchange activity. There is some evidence to suggest that this hypothesis may be correct in the case of exercise. First, Markuns et al. (31) showed that GSK-3ε and GSK-3β activity were both reduced after treadmill running, an exercise protocol that has been shown to induce skeletal muscle hypertrophy (23). Furthermore, a recently published study by Vyas et al. (48) showed that GSK-3β activation negatively regulated skeletal muscle hypertrophy in myotubes. The present results predict that the quantity of GSK-3 protein in the cell may be limited via downregulation of mRNA transcription, thereby
creating a decreased potential for suppression of eIF2 activity. Because GSK-3 has other actions (8, 14) in skeletal muscle, it is also possible that the kinase contributes to alterations in eIF2 activity through less direct mechanisms.

The argument presented above can be extended to the interpretation of the decrease in mRNA levels of CKI and CKII observed in this study. Both CKIγ and CKIβ mRNA were significantly reduced 48 h postexercise compared with the control sedentary animals. The casein kinases phosphorylate sites on eIF2β that are different from one another and different from the GSK-3 site. CKI is believed to phosphorylate Ser464, whereas CKII phosphorylates sites in the extreme COOH terminus (Ser712, 713). This pattern of phosphorylation creates the possibility of differential effects of these phosphorylation events on eIF2 activity. There is no consensus on the effects of casein kinase phosphorylation of the eIF2β subunit on eIF2 activity. According to Singh et al. (44), phosphorylation by CKI and CKII activates eIF2B; however, other groups have shown no change in eIF2 activity associated with these phosphorylation events. As with the GSK-3 data, the results concerning the mRNA levels of the casein kinases suggest that the protein abundance of these kinases may be reduced with resistance exercise training. Because both CKI and CKII are kinases with a wide range of cellular functions, including Wnt signaling, circadian rhythm, nuclear import, and cell survival (10, 30, 33, 38, 47), it is certainly possible that these alternate functions might also play a role in skeletal muscle adaptations to resistance exercise.

Surprisingly, mRNA coding for eIF2α was significantly decreased 24 h after our resistance exercise protocol relative to control mRNA expression levels. This result is contrary to the hypothesis that transcriptional alterations after resistance exercise support an increase in translation capacity. Although phosphorylation of the α-subunit is known to decrease eIF2 activity, an increase in the eIF2 protein (potentially regulated via increased transcription of mRNAs coding for subunits of the protein) would be expected under conditions of increased capacity of the translation apparatus. It is possible that the transient decrease in eIF2 mRNA is not additive on multiple bouts of resistance exercise and therefore does not have any effect on the available pool of eIF2 protein. Second, it is possible that eIF2 is not limiting relative to the number of ribosomes present in the gastrocnemius and/or to the amount of eIF2 present during the time course studied. Finally, this result might represent an experimental artifact because the mRNA coding for eIF2β and the amount of eIF2α protein did not change in this study.

The alteration in expression of mRNA coding for the eIF2α kinase PKR was consistent with the initial hypothesis. Because eIF2α phosphorylation results in negative regulation of eIF2 activity, decreases in transcription of PKR could blunt signaling through this pathway, thus preserving increases in eIF2 activity after resistance exercise. These findings are the first implication that eIF2α phosphorylation may play a role in regulating eIF2 activity after exercise. Because PKR is activated in response to proinflammatory signals, the effects of proinflammatory cytokines on translational initiation in acute vs. chronic resistance exercise could be a potentially interesting area of future investigation. PKR has also been shown to be a positive regulator of muscle differentiation in vitro (28, 41). However, extensive satellite cell invasion is unlikely to occur in the acute resistance exercise model employed in the present study.

The results of the present study do not preclude the possibility that these mRNA transcripts are regulated in an alternative manner as well. It is possible that existing mRNA transcripts are preferentially shifted into polysomes after an acute bout of resistance exercise. Resistance exercise (17), electrical stimulation (3), and stretch (4) have been shown to increase the activity of p70S6K, which is known to upregulate the translation of mRNA sequences that contain a 5′-oligopyrimidine tract (5′-TOP) (20, 21, 29, 46). Such sequences are present in mRNAs encoding other members of the translational apparatus, including elongation factors and ribosomal proteins, but 5′-oligopyrimidine tract sequences have not currently been identified in eukaryotic initiation factors.

The arguments presented above with regard to the functional consequences of the observed alterations in mRNA expression are all contingent on transcriptional contributions to total protein expression. It was not particularly surprising that the increases or decreases in gene expression were seemingly minor (<2-fold). Exercise is a unique physiological perturbation in that to obtain a “training” effect many bouts must be performed with an appropriate amount of recovery in between each bout. Therefore, our results provide direction for future inquiries. In particular, it will be interesting to determine whether the completion of several acute sessions of resistance exercise results in an additive increase or decrease in the mRNA species examined. Dissecting the relative contribution of translational regulation vs. transcriptional regulation of these species in acute vs. chronic exercise paradigms will be essential to understanding regulation of eIF2B protein capacity and eIF2B upstream signaling capacity. Because the acute protein synthetic response is attenuated by 8 wk of resistance exercise training (11), it will be interesting to explore the temporal nature of the alterations in the context of short- vs. long-term exercise training protocols. Overall the results demonstrate the need for a more clear understanding of the effect of eIF2B abundance (and subsequent posttranslational control) on eIF2B activity and the effect of prolonged training on gene expression of translationally relevant mRNA species.

In summary, there was a rapid increase in the amount of total eIF2B protein 3 h after an acute bout of resistance exercise. Furthermore, a modest but significant increase in mRNA levels of species coding for the e-, γ-, and δ-subunits of the eIF2B protein was observed after an acute bout of resistance exercise, but after the postexercise time when an increase in eIF2B protein was observed. To our knowledge, this is the first evidence that gene expression of mRNAs coding for proteins involved in translation initiation is altered by exercise. These results suggest a rapid increase in eIF2B mRNA translation followed by a slower elevation in eIF2B gene transcription. Furthermore, mRNAs coding for the eIF2β kinase GSK3, CKI, and CKII were all decreased over this time course. These results implicate both translational and transcriptional regulation of eIF2B subunit mRNAs and its regulatory kinases as a potentially important mechanism of protein synthesis regulation after resistance exercise. Transcriptional regulation may be especially important late in the recovery period, or perhaps on the completion of subsequent bouts of exercise (i.e., exercise training). Further studies will be required to assess the functional significance of these findings in rat skeletal muscle.
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