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# Eukaryotic initiation factors and protein synthesis after resistance exercise in rats

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**Farrell, Peter A., Jazmir M. Hernandez, Mark J. Fedele, Thomas C. Vary, Scot R. Kimball, and Leonard S. Jefferson.** Eukaryotic initiation factors and protein synthesis after resistance exercise in rats. *J. Appl. Physiol.* 88: 1036–1042, 2000.—Translational control of protein synthesis depends on numerous eukaryotic initiation factors (eIFs) and we have previously shown (*Am. J. Physiol. Endocrinol. Metab.* 276: E721–E727, 1999) that increases in one factor, eIF2B, are associated with increases in rates of protein synthesis after resistance exercise in rats. In the present study we investigated whether the eIF4E family of initiation factors is also involved with an anabolic response to exercise. Male Sprague-Dawley rats either remained sedentary ( $n = 6$ ) or performed acute resistance exercise ( $n = 6$ ), and rates of protein synthesis were assessed in vivo 16 h after the last session of resistance exercise. eIF4E complexed to eIF4G (eIF4E·eIF4G), eIF4E binding protein 1 (4E-BP1) complexed to eIF4E, and phosphorylation state of eIF4E and 4E-BP1 ( $\gamma$ -form) were assessed in gastrocnemius. Rates of protein synthesis were higher in exercised rats compared with sedentary rats [ $205 \pm 8$  (SE) vs.  $164 \pm 5.5$  nmol phenylalanine incorporated·g muscle<sup>-1</sup>·h<sup>-1</sup>, respectively;  $P < 0.05$ ]. Arterial plasma insulin concentrations were not different between the two groups. A trend ( $P = 0.09$ ) for an increase in eIF4E·eIF4G with exercise was noted; however, no statistically significant differences were observed in any of the components of the eIF4E family in response to resistance exercise. These new data, along with our previous report on eIF2B, suggest that the regulation of peptide chain initiation after exercise is more dependent on eIF2B than on the eIF4E system.

translation; contractions; insulin

EARLY STEPS IN THE TRANSLATION of mRNA are regulated by cytosolic proteins referred to as eukaryotic initiation factors (eIFs). These factors facilitate the formation of a translationally competent polyribosome through a series of reactions collectively called peptide chain initiation. Peptide chain elongation and termination are also important to the overall control of protein synthesis; however, under several conditions, including diabetes

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(29) and exercise (1, 14), the control of peptide chain initiation can be rate limiting for overall protein synthesis. Control of mRNA translation is not only complex from a biochemical standpoint but this process is also differentially regulated depending on the physiological status of the organism. For example, a reduction in rates of skeletal muscle protein synthesis due to severe diabetes (a state of muscle catabolism) is associated with reductions in the activity of specific eIFs as well as changes in the state of phosphorylation of several eIFs or their binding proteins (26, 29, 34, 38). The activity of eIF2B decreases (22), the association of eIF4E with eIF4G (eIF4E·eIF4G) decreases (26), and the association of eIF4E with binding protein 1 (4E-BP1) increases as protein synthesis declines. Exogenous treatment of a stress such as diabetes or sepsis or endogenous adaptations by the animal (18, 34, 41) result in elevations in rates of protein synthesis, and eIFs return to normal. Each of these changes in initiation factors is compatible with regulation of protein synthesis.

A role for eIFs in regulating translation initiation when rates of protein synthesis are elevated above basal values is less clear. The exception to this is the pathophysiology of tumor formation (36) in which rates of protein synthesis are elevated and the eIF4E system seems to have a dominant role. The physiological perturbation of acute resistance exercise elevates rates of protein synthesis, and such elevations are associated with enhanced activity of eIF2B (9), but no studies have examined the effect of resistance exercise on the eIF4E system. The present study determined whether changes in specific eIFs were associated with elevations in protein synthesis above basal levels.

## METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University. Male Sprague-Dawley rats were housed in temperature- and humidity-controlled holding facilities with lights on at 0700 and off at 1900. Rats were fed ad libitum a standard rodent diet (PMI Feeds 5001) that contained an estimated 24% protein, 12% fat, 50% carbohydrate, 7% ash, 6% fiber, and vitamins. Twelve rats [randomly assigned to exercised ( $n = 6$ ) or sedentary ( $n = 6$ ) groups] were studied 16 h after the last bout of acute resistance exercise (described in *Resistance exercise*). Rats were fasted for 5 h before tissue procurement.

### Procedures

**Resistance exercise.** Rats performed resistance exercise as previously described (12). Briefly, rats were operantly conditioned to touch an illuminated bar low on a Plexiglas exercise cage and then were taught to stand and touch an illuminated bar that was located high on the opposite wall of the cage. Electrical foot shock (<2 mA, 60 Hz) was used to reinforce these movements. Once the learning process was completed (3–4 sessions), weighted vests were strapped over the scapulae and the rats were required to touch the high bar 50 times during one acute exercise session. We defined “acute” resistance exercise as four separate sessions with 1 day of rest between sessions. Rats performed 50 repetitions each day with 0.2 (on *day 1*), 0.4 (on *days 2 and 3*) and 0.6 (on *day 4*) g weighted vest/g body wt. Previous work had shown that rats that were naive to the lifting procedure would not lift the 0.6 g/g body wt on the first day weights were applied to the vest. This protocol can be considered as acute because it does not result in changes in body or muscle weight (12). We are fully aware that some metabolic adaptations occur after only a few bouts of exercise. Exercise sessions occurred in the dark (red light) at fixed times during the day. Sedentary rats were placed in the lifting cages at least three times during the week of acute exercise and were given five electric shocks to simulate some of the stress experienced by the exercised group. One of these shock control sessions occurred 16 h before tissue procurement.

**Rates of protein synthesis.** Sixteen hours after the last bout of exercise, rats were anesthetized with methoxyflurane and placed on a heating pad, and the left carotid artery and right jugular vein were catheterized. Rates of protein synthesis were estimated by using a flooding dose (16) of L-[2,3,4,5,6-<sup>3</sup>H]phenylalanine (1 mCi/rat; Amersham Life Science, Arlington Heights, IL) in unlabeled phenylalanine (150 mM; 1 ml/100 g body wt, total volume) that was injected into the venous catheter over a 15-s period. The flooding dose method has been validated for *in vivo* use by Davis et al. (4) because the tissue, blood, and phenylalanyl-tRNA specific radioactivity is equilibrated in young pigs 30 min after infusion. Similar validation has been provided during *in situ* hindlimb perfusions in rats (3). Arterial blood (1 ml) was obtained at 6 and 10 min after infusion of the flooding dose (labeled and unlabeled phenylalanine infused simultaneously), and immediately after the last blood sample the left gastrocnemius muscle was excised, dropped into liquid nitrogen, and stored at –70°C until processing for phenylalanine incorporation into protein. Gastrocnemius from the other leg was immediately trimmed of fat and connective tissue, weighed, and homogenized in 7 vol of an ice-cold buffer containing (in mM) 20 HEPES (pH 7.4), 100 KCl, 0.2 EDTA, 2 EGTA, 1 dithiothreitol, 50 NaF, 50 β-glycerophosphate, 0.1 phenylmethylsulfonyl fluoride, 1 benzamide, and 0.5 sodium vanadate. The homogenate was centrifuged and the supernatant stored at –70°C until analyzed for initiation factors as described below.

**ASSAYS.** Frozen muscles were stored at –70°C until phenylalanine incorporation into trichloroacetic acid-precipitable protein was analyzed as radioactivity measured by liquid scintillation counting with appropriate use of quench curves. Plasma phenylalanine was measured by using dabsylation of the amino acid and measurement on a HPLC (5). Radioactivity in the phenylalanine peak was measured by liquid scintillation counting with appropriate correction for quench. Protein determinations were made by using the biuret method. Rates of muscle protein synthesis were calculated by using the method of Garlick et al. (16).

The association of eIF4E with 4E-BP1 or eIF4G was quantitated exactly as described previously (24, 25). Briefly, eIF4E and the eIF4E·4E-BP1 and eIF4G·eIF4E complexes were immunoprecipitated from aliquots of muscle homogenate by using an anti-eIF4E monoclonal antibody. The eIF4E antibody was raised against recombinant human eIF4E as described previously (26). The efficiency of eIF4E immunoprecipitation was >88% under all conditions. The antibody-antigen complex was collected by incubation for 1 h with goat anti-mouse Biomag IgG beads (PerSeptive Diagnostics). Before use, the beads were washed in 1% nonfat dry milk in *buffer C* (20 mM Tris·HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% β-mercaptoethanol, and 0.5% Triton X-100). The beads were captured by using a magnetic stand and were washed twice with *buffer C* and once with *buffer D* (50 mM Tris·HCl, pH 7.4, 500 mM NaCl, 5 mM EDTA, 0.04% β-mercaptoethanol, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS). Protein bound to the beads was eluted by resuspending the beads in SDS-sample buffer and boiling the sample for 5 min. The beads were collected by centrifugation, and the supernatants were subjected to electrophoresis either on a 7.5% polyacrylamide gel for quantitation of eIF4G or on a 15% polyacrylamide gel for quantitation of 4E-BP1 and eIF4E. Proteins were then electrophoretically transferred to a polyvinylidene difluoride membrane as described previously (27). The membranes were incubated with a mouse anti-human eIF4E antibody, a rabbit anti-rat 4E-BP1 antibody, or a rabbit anti-human eIF4G antibody for 1 h at room temperature. The antibodies against 4E-BP1 and eIF4G were raised against the recombinant rat and human proteins, respectively, by the method described previously (28). The blots then were developed by using an enhanced chemiluminescence Western blotting kit as described in PROTEIN IMMUNOBLOT ANALYSIS.

**EXAMINATION OF 4E-BP1 PHOSPHORYLATION.** Aliquots of cell homogenates were immunoprecipitated by using a monoclonal anti-4E-BP1 antibody by using the method described in ASSAYS for immunoprecipitation of eIF4E. The 4E-BP1 antibody was raised against the rat recombinant protein by the method described previously (27). The immunoprecipitates were solubilized with SDS-sample buffer and then subjected to protein immunoblot analysis by using a rabbit anti-rat 4E-BP1 antibody. Subunits of phosphorylated 4E-BP1 were visualized, and the amount of the γ-subunit that was phosphorylated was quantified as described in PROTEIN IMMUNOBLOT ANALYSIS.

**PROTEIN IMMUNOBLOT ANALYSIS.** Blots were developed by using an Amersham enhanced chemiluminescence Western blotting kit as described previously (27). Films were scanned by using a Microtek ScanMaker IV scanner equipped with a transparent media adapter connected to a Macintosh PowerMac 9600 computer. Images were obtained by using the ScanWizard Plugin (Microtek) for Adobe Photoshop and quantitated by using National Institutes of Health Image software.

Plasma glucose was measured in duplicate by using a Beckman model 2 autoanalyzer, which measures products of the glucose oxidase reaction. Plasma insulin was measured in duplicate by using a double-antibody radioimmunoassay (33) as modified in our laboratory (6) that could detect 10 pmol of insulin/tube and has an interassay coefficient of variation of 3%.

### Statistical Analysis

Statistical differences between sedentary and exercised groups were analyzed by using *t*-test for unpaired comparisons. Mean values were considered significantly different at  $P < 0.05$ .

Table 1. *Physical and physiological characteristics of the rats*

	Weight, g	Hct, %	Hb, g/dl	Insulin, pM	Glucose, mg/dl
Sedentary	360 ± 12	43.2 ± 0.6	13.9 ± 0.3	531 ± 91	216 ± 26
Exercised	358 ± 18	42.2 ± 0.5	14.0 ± .24	576 ± 77	204 ± 37

Values are means ± SD for 6 rats in each group. Hct, hematocrit.

## RESULTS

Physical and physiological characteristics of rats are provided in Table 1. There were no significant differences between groups for any variable.

Figure 1 provides data for rates of protein synthesis. Rates were higher in the exercised group, and the percent increase above values observed in sedentary animals (25%) was similar to increases that we have previously reported using this model (7–9). Figure 2 shows that there were no significant differences in the amount of eIF4E bound to 4E-BP1 between the two groups. The amount of eIF4E bound to eIF4G (Fig. 3), the degree of 4E-BP1 phosphorylation (Fig. 4), and eIF4E phosphorylation (Fig. 5) also were not different between exercised and sedentary groups. Although there was a trend ( $P = 0.09$ ) toward a greater amount of the eIF4E in the eIF4E·eIF4G complex in muscle after exercise, the difference was not statistically significant.

## DISCUSSION

The present data provide new insights into the regulation of protein synthesis after resistance exercise. We originally hypothesized that changes in the eIF4E family of initiation factors would be consistent with elevations in rates of protein synthesis. These predictions were based on studies in which rates of protein synthesis were accelerated from depressed values rather than being elevated above normal levels. For example, rates of protein synthesis in several

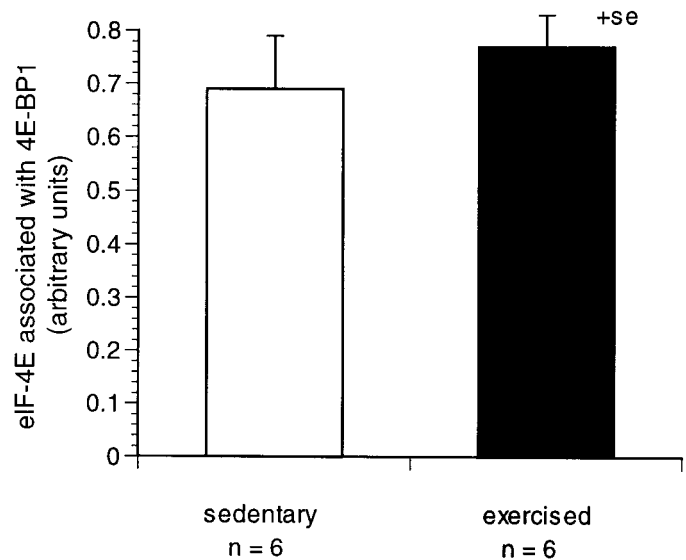


Fig. 2. Amount of eukaryotic initiation factor 4E (eIF4E) associated with eIF4E binding protein 1 (4E-BP1) in mixed gastrocnemius. Rats were studied 16 h after last bout of acute exercise. Values are means + SE; *n*, no. of rats.

different skeletal muscles are “elevated” from depressed to normal basal values when severely diabetic rats are treated with insulin (11, 29, 35, 40).

The availability of eIF4E is partially dependent (37) on whether this factor is complexed to its binding protein (4E-BP1). When 4E-BP1 is phosphorylated, it dissociates from eIF4E, and such dissociation is observed when rates of protein synthesis are restored from reduced to normal values (25, 39). Thus we expected less eIF4E to be associated with 4E-BP1 when rates of protein synthesis were elevated by exercise. Data presented in Fig. 2 suggest that the amount of eIF4E associated with 4E-BP1 is not altered by prior exercise. In a similar manner, Kimball et al. (25) re-

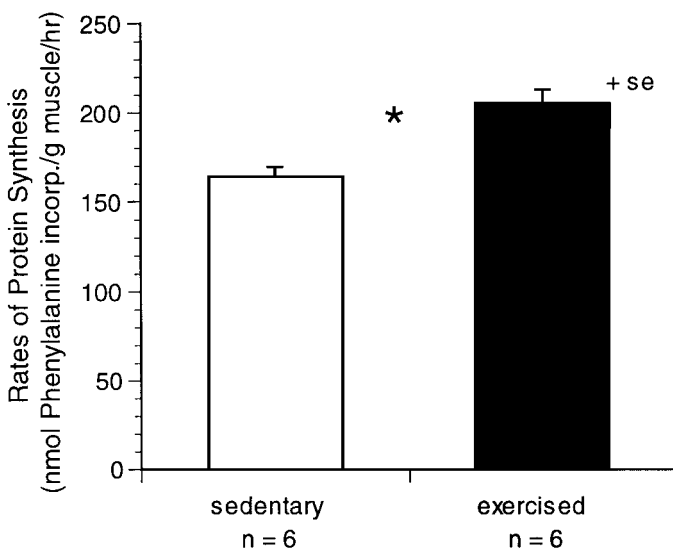


Fig. 1. Rates of protein synthesis in gastrocnemius for exercised and sedentary rats. Rats were studied 16 h after the last bout of acute exercise. Values are means + SE; *n*, no. of rats. incorp, Incorporated. \*Groups are significantly different at  $P < 0.05$ .

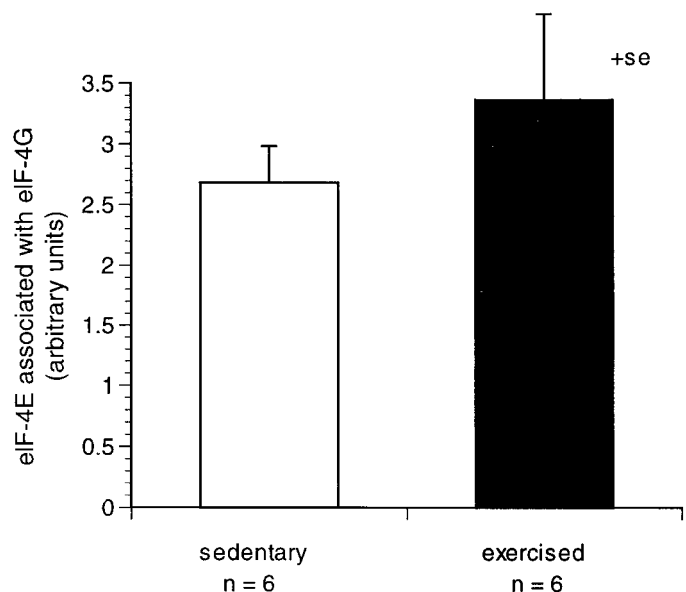


Fig. 3. Amount of eIF4E associated with eIF4G in mixed gastrocnemius. Rats were studied 16 h after the last bout of acute exercise. Values are means + SE; *n*, no. of rats.

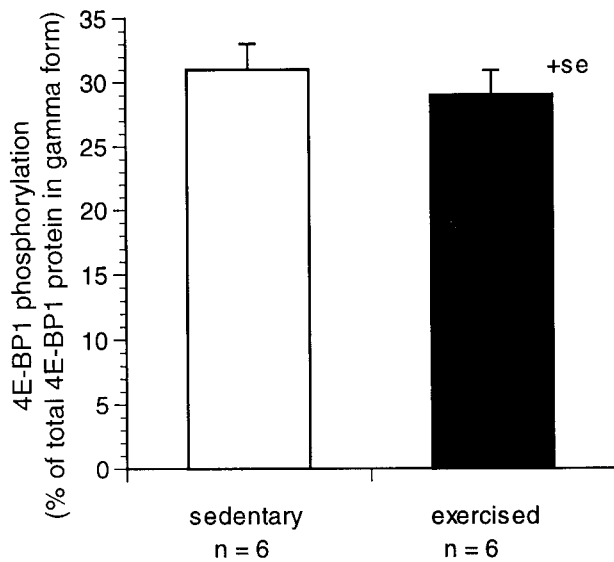


Fig. 4. Relative amount of 4E-BP1 phosphorylated in the  $\gamma$ -form in mixed gastrocnemius. Rats were studied 16 h after last bout of acute exercise. Values are means + SE; *n*, no. of rats.

ported reductions in the phosphorylation of 4E-BP1 when rates of protein synthesis are depressed by diabetes and increases in the phosphorylation of 4E-BP1 during recovery from diabetes (treatment with insulin). In contrast, data presented in Fig. 4 show that when rates of protein synthesis were elevated above basal levels there was no difference between groups in the phosphorylation of 4E-BP1.

After exercise eIF4E·eIF4G was higher in exercised animals, but this difference was not statistically significant. In situ hindlimb perfusions of gastrocnemius (26) with and without insulin show that perfusion with a medium lacking insulin reduces protein synthesis and the amount of the eIF4E·eIF4G complex but the addition of insulin to the medium restores both protein synthesis and eIF4E·eIF4G to basal values. Such

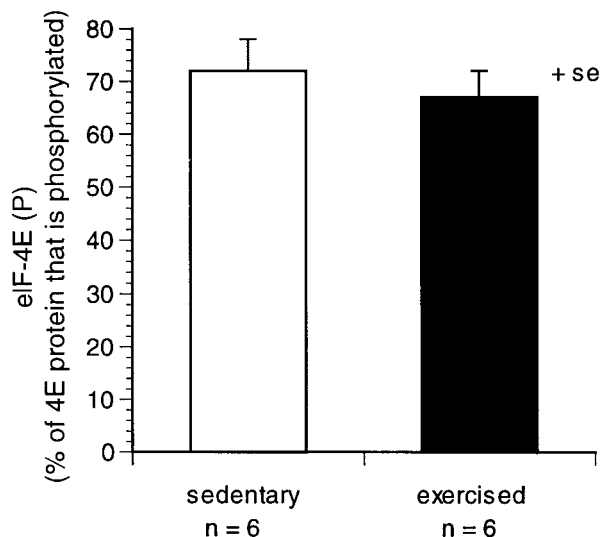


Fig. 5. Relative amount of eIF4E in the phosphorylated form [eIF-4E(P)] in mixed gastrocnemius. Rats were studied 16 h after the last bout of acute exercise. Values are means + SE; *n*, no. of rats.

relationships have also been found by using isolated L6 myocytes (23) when the presence or absence of leucine was used to increase or decrease protein synthesis, respectively. Thus we expected to find increases in eIF4E·eIF4G when rates of protein synthesis were elevated by exercise. Data in Fig. 3 show that there was no such change in this complex.

Increases in eIF4E phosphorylation have been reported to be associated with increases in protein synthesis (21); however, other studies suggest that such phosphorylation is not consistently linked to changes in protein synthesis (17, 24, 25). The state of phosphorylation of eIF4E did not change after exercise (Fig. 5). This finding in skeletal muscle is in contrast to cardiac muscle, in which changes in the phosphorylation state of eIF4E due to mechanical overload of the heart were linked (42) to increased rates of protein synthesis in cardiocytes.

Only one prior study using exercise as a model evaluated the eIF4E system; however, it is difficult to compare our data with those of that study because Gautsch et al. (17) used endurance exercise to alter rates of protein synthesis, and they studied muscle 1 h after exercise when rates of protein synthesis were reduced as a result of exercise. At that time, the amount of eIF4E associated with 4E-BP1 in the gastrocnemius-plantaris muscle complex was elevated. Once rates of protein synthesis returned to control values (as a result of feeding a nutrient-complete meal after exercise), there was no difference in eIF4E associated with 4E-BP1 between sedentary and treadmill exercised rats. It is important to note that rates of protein synthesis in the study of Gautsch et al. were never elevated above basal levels as a result of exercise, perhaps because the observations were limited to 1 h postexercise. If the amount of the eIF4E associated with 4E-BP1 were the dominant controller of peptide chain initiation, we would have expected a decrease in that complex in exercised rats, but this was not the case. Thus the amount of eIF4E associated with 4E-BP1 is a regulatory factor during conditions when synthesis is normalized to control levels but not necessarily when synthesis is elevated by a physiological stimulus to levels above those observed in the basal state.

It is possible that changes in eIFs occur at times before those assessed in the present paper. Although we cannot totally discount this possibility, several reasons make it unlikely. Increases in rates of protein synthesis after resistance exercise are delayed (2) and may be model dependent or dependent on the duration of the exercise performed. Using the same exercise model as in the present study, Hernandez et al. (19) have reported that rates of protein synthesis do not increase for at least 6 h after exercise. Finally, simultaneous changes in eIFs and rates of protein synthesis are probably quite rapid. As an example, elevations in eIF4E binding to 4E-BP1 due to diabetes are completely reversed 2 h after treatment with insulin *in vivo* (25). Thus it seems unlikely that changes in the eIF4E system of initiation factors occurred early after exer-

cise. We limit this interpretation to the specific stress of resistance exercise, the time of assessment after exercise, and the feeding conditions used in this study.

Mechanisms explaining the regulation of protein synthesis during recovery after exercise remain unclear. The results of the present study indicate that increases in eIF4E activity are most likely not involved in the stimulation of protein synthesis in skeletal muscle after resistance exercise. We have reported a likely candidate for regulating elevations in protein synthesis in our exercise model when rats were studied 16 h after exercise; i.e., the activity of eIF-2B (9) is consistently higher in exercised rats. In contrast to the eIF4E system, which regulates interactions between mRNA and ribosomal subunits, eIF2B regulates the exchange of GTP for GDP which is complexed to eIF2 (34). Such an exchange is necessary for repeated formation and cycling of the ternary complex at the start of peptide chain initiation. eIF2B may be especially important for regulating global rates of protein synthesis (23).

Some mRNAs are more difficult to translate compared with others (32, 34). Terms such as global vs. specific protein synthesis or quantitative vs. qualitative protein synthesis are used, and discussions of the purported factors that coalesce to make an mRNA easy or difficult to translate have been provided (31, 34). As applied in the present study, the flooding dose technique measures global rates of protein synthesis (15), i.e., synthesis of most or all proteins in the muscle. Recent *in vitro* data using L6 myocytes (23) show that the association of eIF4E with eIF4G may not be a primary regulator of rates of global protein synthesis. In that report, decreases in rates of protein synthesis due to leucine deprivation were associated with reductions in eIF4E associated with eIF4G. However, histidine deprivation also reduced protein synthesis, but the amount of eIF4E associated with eIF4G was not altered. That same report suggests that another initiation factor, eIF2B, may have greater importance in regulating global protein synthesis than does eIF4E availability. Because acute resistance exercise increases the activity of eIF2B (9), our *in vivo* data strengthen previous *in vitro* work in proposing that eIF2B may have a greater role than does the eIF4E system in regulating global rates of protein synthesis.

It is possible that the involvement of eIF4E system is not elevated until high strains are placed on muscle fibers whereas the eIF2B system is activated by lower strains. A previous report, however, showed that when nondiabetic rats lifted 100% of the body weight, less of an elevation in rates of protein synthesis occurred compared with lifting 60% of the body weight (8). Additionally, moderately diabetic rats could not increase rates of protein synthesis after severe intensity (100% body weight) exercise. Finally, previous work by Wong and Booth (43) demonstrated that both high- and low-intensity resistance exercise elevated rates of protein synthesis in rat gastrocnemius muscle of nondiabetic rats, suggesting that regulators of protein synthesis may not depend on the intensity of contrac-

tions. No studies have investigated the effect of intensity of exercise on regulators of peptide chain initiation, and such studies may provide insights into characteristics of the mechanical environment that stimulate these eIFs.

It is unlikely that changes in circulating insulin were responsible for stimulation of protein synthesis after exercise. Arterial plasma insulin concentrations were not different between exercised and sedentary groups. This was an expected finding because the rats were fed identically before exercise and this type of exercise does not alter circulating insulin concentrations (9), whereas it does increase insulin secretion from isolated islets of Langerhans (12). These data agree with those of our previous work showing that elevations in protein synthesis after similar exercise are independent of insulin concentrations as long as a low but critical amount of insulin is available *in vivo* (10). Such a concept is compatible with many studies (11, 22) demonstrating that severe diabetes or the absence of insulin in an *in situ* hindlimb perfusion model (20) markedly reduces basal rates of protein synthesis.

A complete understanding of intracellular cell signaling pathways that activate eIFs is not available, and the studies that address this issue predominantly use *in vitro* cell culture models (30). Related to resistance exercise, however, Baar and Esser (1) recently demonstrated that *in situ* stimulation of hindlimb muscles of rats results in elevations in the activity of the 70-kDa ribosomal S6 protein kinase (p70<sup>S6k</sup>) in plantaris, tibialis anterior, and extensor digitorum longus muscles. Tibialis anterior and extensor digitorum longus muscles are subjected to forced-lengthening contractions in the model of resistance exercise used in the referenced study. Such elevations in p70<sup>S6k</sup> activity were sustained after exercise and correlated to elevations in muscle mass that occur after resistance exercise training (1). In our model, gastrocnemius muscle contractions occur in the rat's movement going from quadrupedal to bipedal and back to quadrupedal, and we have recorded significant electromyographic activity (data not shown) during both movements. Thus forced lengthening contractions occur in gastrocnemius in our model. We have recently shown (19) that p70<sup>S6k</sup> activity is elevated 12–24 h after exercise at the same time rates of protein synthesis are also elevated. Our data strengthen the suggestion by Baar and Esser (1) that p70<sup>S6k</sup> is important for anabolic responses to resistance exercise. Additionally, when L6 myocytes are deprived of leucine and then this amino acid is resupplemented, protein synthesis, eIF2B activity, eIF4E availability, and p70<sup>S6k</sup> phosphorylation all increase. In contrast, when histidine is resupplemented in histidine-deprived L6 myocytes, the eIF4E system is not involved in the elevations (from reduced levels) in rates of protein synthesis (24). We recognize that amino acid deprivation and muscle contractions are not similar stresses, yet both studies show that it is not obligatory for the eIF2B and eIF4E systems to be elevated simultaneously.

In summary, many eIFs are known to regulate rates of protein synthesis when the animal (or cell line) is

recovering from a stress that results in reduced protein synthesis. In the present study, the eIF4E system did not change in a manner that was consistent with elevations in rates of protein synthesis caused by exercise. Because previous work has shown that elevations in eIF2B are associated with increased rates of protein synthesis after the physiological stress of exercise, we suggest that this factor may be more important to the anabolic response to resistance exercise than the eIF4E system.

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