Regulation of Protein Metabolism in Exercise and Recovery

In conclusion, the elevation of p70S6k and the reduction of eEF2 although the level was markedly higher in type II fibers (P < 0.05). The molecular mechanisms by which resistance exercise enlarges muscle mass, particularly the mass of fast-twitch type II fibers, are likely to involve enhanced phosphorylation/activation of key enzymes regulating protein synthesis. The hypothesis is that resistance exercise influences the phosphorylation of such key signaling proteins to a greater extent in type II than in type I fibers. Six recreationally active male subjects performed four sets of six maximal lengthening contractions with one leg. Muscle biopsies were taken from the vastus lateralis before and immediately after exercise and following 1 and 2 h of recovery. Samples were freeze-dried, and individual muscle fibers were dissected out and identified as type I or type II after staining for myosin ATPase. Phosphorylation of p70S6k on Thr389 and S6 in type II fibers was increased three- to fourfold and six- to ninefold (P < 0.05), respectively, 1 and 2 h after exercise, whereas phosphorylation in type I fibers remained unchanged. Phosphorylation of Akt, mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) was unaltered in both fiber types, whereas that of eukaryotic elongation factor 2 (eEF2) was attenuated 20–45% (P < 0.05) immediately after exercise, and p38 MAPK phosphorylation was increased three- to fourfold (P < 0.05) for as long as 1 h after exercise in both types of fibers, although the level was markedly higher in type II fibers (P < 0.05). In conclusion, the elevation of p70S6k and the reduction of eEF2 phosphorylation in the type II fibers following resistance exercise suggest stimulation of protein synthesis, which may contribute to the more pronounced enlargement of these fibers. Our findings also suggest that p70S6k is activated, at least in part, via pathways not involving Akt-mTOR and MAPK.

Maximal lengthening contractions induce different signaling responses in the type I and type II fibers of human skeletal muscle

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HUMAN SKELETAL MUSCLE contains a mixture of fibers exhibiting different contractile and metabolic properties. In general, the slow-twitch type I fibers possess a greater capacity for oxidative energy production, whereas the fast-twitch type II fibers demonstrate a larger glycolytic capacity and, thereby, a greater ability to work under anaerobic conditions (12, 16). The increase in muscle size produced by regular performance of resistance exercise is due largely to an enhancement in the size of type II fibers (24). This is reflected in the observation that the rate of protein synthesis following a single bout of resistance exercise is elevated to a greater extent in human vastus lateralis muscle, the fibers of which are 50–60% of type II, than in the soleus muscle, which consists predominantly of type I fibers (39).

Although not yet fully understood, the molecular mechanisms underlying the anabolic effects of resistance exercise are likely to involve activation of signaling pathways that regulate protein translation (8), including activation of the enzyme p70S6 kinase (p70S6k), which should enhance translation of mRNAs encoding ribosomal proteins and elongation factors (22). There are some reports that resistance exercise activates p70S6k in human skeletal muscle via a protein kinase B (PKB/Akt)-independent pathway (11, 29, 37), whereas a few other studies have demonstrated increased phosphorylation of Akt following such exercise (6, 9). These divergent findings may be due not only to variations in the type and intensity of the exercise employed or the timing of muscle biopsy sampling but also to differences in the involvement of type I and type II fibers in force development. Investigations on experimental animals indicate that type I and type II fibers might respond differently to contractile activity, even though it was not always possible to distinguish fiber-specific effects from fiberspecific modes of contraction (2, 30, 31). Nonetheless, Parkington and coworkers (31) observed that both concentric and eccentric contractions resulted in extensive phosphorylation of mammalian target of rapamycin (mTOR) and p70S6k in the fast-twitch muscles of the rat, while no effect was detected on the slow-twitch soleus muscle after concentric contractions. Furthermore, in the fast-twitch tibialis anterior muscle, the enhancement in mTOR phosphorylation occurred only in a subgroup of type II fibers, the type IIA fibers.

To date, little information is available concerning the effect of resistance exercise on different types of fibers in human muscle. Employing immunohistochemical staining of cross sections of muscle fibers, Koopman and colleagues (26) observed more pronounced Ser424/Thr421 phosphorylation of p70S6k in type II than in type I fibers following resistance exercise. However, it should be emphasized that such an increase in phosphorylation at these sites does not necessarily lead to subsequent phosphorylation on the Thr389 residue of p70S6k, which is required for full activation of the enzyme (10, 33).

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Among the potential effectors of the Akt-mTOR signaling pathway, AMP-activated protein kinase (AMPK) is particularly responsive to the energy status of the cell. Activation of this enzyme in response to a reduction in the ATP/AMP ratio attenuates the activation of mTOR, as well as the rate of muscle protein synthesis, in experimental animals (5). In contrast, following resistance exercise in human subjects, mTOR was enhanced despite concomitant activation of AMPK (9). However, the specific effects of such exercise on AMPK in type I and type II fibers have not yet been examined.

An additional pathway that may participate in muscle response to hypertrophy is the signaling cascade involving mitogen-activated protein kinases (MAPKs). Rapid changes in phosphorylation of ERK1/2 and p38 MAPK in whole muscle of human subjects have been observed following resistance exercise, but, once again, the effect on different types of fibers remains unknown (7, 23, 42).

In our earlier study, maximal lengthening contractions were found to induce pronounced elevations in the phosphorylation of p70S6k and of the ribosomal protein S6 that persisted for 2 h of recovery, while maximal shortening contractions did not (11). Therefore, the goal of the present investigation was to test our hypothesis that maximal lengthening contractions result in more pronounced changes in the phosphorylation of enzymes involved in protein translation in type II than in type I fibers. To this end, phosphorylation of Akt, mTOR, p70S6k (both on residues Ser244/Thr241 and Thr389), S6, and the eukaryotic elongation factor 2 (eEF2) was examined in type I and type II muscle fibers taken from human subjects before and at repeated intervals following maximal lengthening contractions. Moreover, the potential effectors of the Akt-mTOR-p70S6k pathway, AMPK, and the MAP kinases ERK1/2 and p38, were also analyzed.

MATERIALS AND METHODS

Subjects. Six healthy male subjects who were recreationally active once or twice a week (i.e., jogging, jujutsu, soccer, or resistance exercise) participated in this study after being fully informed orally and in writing of its purpose and the possible risks involved. These individuals also took part in our previous study (11). Their mean ± SE age was 28 ± 1 yr, height 181 ± 3 cm, weight 75 ± 3 kg, body mass index 23 ± 0.7 kg/m², and maximal oxygen uptake 3.88 ± 0.26 l/min. The study protocol was approved by the Ethics Committee of the Karolinska Institutet.

Preparatory tests. Before performance of the actual experiment, the subjects carried out two preparatory tests on a modified leg press machine equipped with a force transducer (Nobel Elektronik KPG-4 T10; Vishay Nobel, Karlsga, Sweden). These preparatory tests were designed to determine the one-leg maximal force that could be exerted by each individual, as well as to familiarize the subjects with the exercise procedure. The appropriate settings on the machine were determined by each individual, as well as to familiarize the subjects with the exercise procedure. The appropriate settings on the machine were determined by each individual, as well as to familiarize the subjects with the exercise procedure. The appropriate settings on the machine were determined by each individual, as well as to familiarize the subjects with the exercise procedure. The appropriate settings on the machine were determined by each individual, as well as to familiarize the subjects with the exercise procedure. The appropriate settings on the machine were determined by each individual, as well as to familiarize the subjects with the exercise procedure. The appropriate settings on the machine were determined by each individual, as well as to familiarize the subjects with the exercise procedure.

Next the membranes were blocked in Tris-buffered Saline (TBS: 10 mM Tris, pH 7.6, in 100 mM NaCl) containing 5% nonfat dry milk (Akt, mTOR, p70S6k, phosphorylated at Ser244/Thr241, eEF2, p38, ERK 1/2, AMPK, and S6) or in StartingBlock blocking buffer (for p70S6k, phosphorylated at Thr389) (Pierce Biotechnology) for 1 h at room temperature and then incubated overnight at 4°C with commercially available phosopho-specific primary antibodies. The membranes were then washed and incubated with the appropriate secondary antibody for 1 h, and washed once again. Finally, the phosphorylated proteins were visualized by enhanced chemiluminescence quantified with densitometric scanning using a Gel Doc 2000 apparatus in combination with the Quantity One version 4.6.3 software (Bio-Rad Laboratories).

Total levels of each protein were determined by subsequently incubating the membranes in Restore Western blot stripping buffer.
Pierce Biotechnology) after which they were reprobed with appropriate polyclonal antibodies in the same manner as described above.

**Antibodies.** Primary polyclonal antibodies toward phospho-Akt (Ser473), phospho-p70S6k (Thr422/Ser424), phospho-mTOR (Ser2448), phospho-S6 (Ser235/236), phospho-p38 (Thr180/Tyr182), phospho-ERK1/2 (Thr202/Tyr204), phospho-eEF2 (Thr56), phospho-AMPK (Thr172), total S6, total p38, total ERK1/2, total AMPK, and total eEF2 were purchased from Cell Signaling Technology (Beverly, MA) and all diluted 1:1,000 before use. Polyclonal antibodies against phospho-p70S6k (Thr389; 1:2,000), total p70S6k (1:1,000) and total mTOR (1:1,000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). IgG horseradish peroxidase-conjugated secondary antibodies (diluted 1:10,000) were purchased from Cell Signaling Technology (anti-rabbit) and from Santa Cruz Biotechnology (anti-goat).

**Analysis of myosin heavy chains.** The pellets obtained by centrifugation of the homogenates of type I and type II muscle fibers were dissolved in 1 ml sample buffer (Laemmli, containing 5% β-mercaptoethanol; Bio-Rad Laboratories) by heating for 10 min at 60°C, and the protein concentrations of these solutions were determined using the RC DC Protein Assay (Bio-Rad Laboratories). Before electrophoresis, these samples were boiled for 5 min and allowed to cool, after which an aliquot containing 1.0 µg total protein was loaded onto each lane. Subsequent separation of the isoforms of myosin heavy chains (MHC) was achieved according to the procedure described by Talmadge and Roy (36), with minor modifications.

In brief, the gels were cast employing empty criterion cassettes 1 mm thick and containing 18 separate wells (Bio-Rad Laboratories). The separating and concentration gels contained 8% and 4% acrylamide, respectively, and β-mercaptoethanol (at a final concentration 0.16%) was added to the top running buffer, to improve the sharpness of the bands (4, 14, 24). Following electrophoresis for 20 h at a constant 130 V on ice in a cold room, the bands on the gels were visualized and quantified by silver staining (PlusOne Silver Staining Kit, Protein; Amersham Bioscience, GE Healthcare, Little Chalfont, UK) and scanning with a Gel Doc 2000 apparatus. Finally, the relative amounts of the different isozymes of MHC in relation to the total MHC content were determined utilizing the Quantity One version 4.6.3 software (Bio-Rad Laboratories).

**Statistical analyses.** The data are presented as means ± SE of the mean. To compare the levels of kinase phosphorylation at different time points in the two types of fibers, a two-way (fiber type, time) repeated-measures ANOVA was employed. When a significant overall effect was indicated, a Fisher least significant difference post hoc test was performed. In all cases P values of <0.05 were considered to be statistically significant.

**RESULTS**

The average force exerted by the subjects in the four sets of six maximal isokinetic eccentric contractions was similar, i.e., 2,540 ± 181, 2,460 ± 104, 2,490 ± 118, and 2,450 ± 116 N. The exercise was associated with a three- to sevenfold elevation in the level of p70S6k phosphorylation on Ser473/Thr421 in the type II fibers immediately after exercise, as well as after 1 and 2 h of recovery, whereas no change in this parameter was detected in type I fibers (Fig. 1A). In a similar manner,

![Phosphorylation of p70S6k at residues Ser473/Thr421 (A) and Thr389 (B) and of S6 (C) in type I and type II fibers of human skeletal muscle before and immediately after resistance exercise and following 1 h and 2 h of recovery. The representative immunoblots depicted above each graph show the type I fibers (before, after, 1 h, and 2 h after exercise) followed by the type II fibers (before, after, 1 h, and 2 h after exercise). Values in the graph are arbitrary density units (means ± SE) for 6 subjects. *P < 0.05 compared with before exercise and # P < 0.05 compared with type I fibers.](http://jap.physiology.org/)

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phosphorylation of p70S6k on Thr389 was increased three- to fourfold and that of S6 was elevated six- to ninefold in the type II fibers 1 and 2 h into recovery, although in this case, not immediately after exercise, and, again, with no change being detected in the type I fibers (Fig. 1, B and C). In addition, the ANOVA analysis revealed a significant interaction between time and fiber type with respect to the phosphorylation of p70S6k at both sites and of S6.

In contrast, exercise exerted no significant effect on the phosphorylation of Akt, mTOR, or AMPK in either type of fiber (Fig. 2, A–C), although there was a tendency ($P = 0.11$) for the level of Akt phosphorylation in the type II fibers to be reduced immediately after exercise, and moreover, the ANOVA analysis revealed a significant interaction between time and fiber type with respect to this parameter. Phosphorylation of eEF2 was attenuated 20% immediately after exercise and 30–45% following 1 and 2 h of recovery in type II, but not type I fibers (Fig. 2D), with significant interaction between time and fiber type being demonstrated by the ANOVA. Before exercise, eEF2 phosphorylation was an average of 58% higher in type II compared with type I fibers; however, the difference was not statistically significant. The level was enhanced in five of the six subjects.

In both type I and type II fibers, phosphorylation of ERK1/2 was enhanced six- to sevenfold immediately after exercise but had returned to the preexercise level within 1 h of recovery (Fig. 3A). p38 MAPK phosphorylation was elevated three- to fourfold in both types of fibers directly after exercise and following 1 h of recovery, and in addition, this phosphorylation was greater in type II than in type I fibers at these time points following exercise (Fig. 3B).

Table 1 shows the content of the different MHC isoforms as percentage of the total MHC content in pools of type I and type II fibers identified by myosin ATPase staining. In the type I fiber pools, MHC II isoforms constituted an average of 9.8% and in the type II fiber pools, the MHC I isoform made up an average of 10.3% of the total MHC content. No significant correlations were found between the percent content of MHC IIa or MHC IIX fibers and the increases in p70S6k, S6, p38, or ERK1/2 phosphorylation 1 and 2 h after exercise.

**DISCUSSION**

The major finding of the present investigation is that a single session of maximal lengthening contractions causes a pro-
nounced increase in phosphorylation of p70S6k and S6 in type II human muscle fibers, with no effect on type I fibers. Phosphorylation of p70S6k on Thr389 activates the enzyme, which probably explains the enhanced degree of phosphorylation of its substrate, the ribosomal protein S6. Activation of p70S6k is likely to contribute to the elevated rate of protein synthesis observed in human muscle after a single session of resistance exercise as well as to the enlargement of type II fibers that results from resistance training (3, 17, 27, 32, 38).

The absence of any such effect on type I fibers was unexpected, since several reports have shown that the area of type I fibers is also enhanced by eccentric resistance training, although to a lesser extent than that of the type II fibers (18, 21, 41). In contrast, when nutritional supplements are given to subjects in connection with resistance exercise, phosphorylation of the ribosomal protein S6 increases in both type I and type II fibers, judging from immunohistochemical staining of cross sections of muscle fibers (25). Thus muscle contractions may not activate the relevant signaling pathways in type I fibers unless an additional stimulus such as nutrition is present as well. However, the lack of change in p70S6k and S6 phosphorylation in type I fibers in the present study may be due to the exercise protocol employed here. Therefore one or more of the parameters (volume, mode, number of repetitions, intensity, etc.) may require adjustment for a hypertrophic signaling response to occur in type I fibers. It is also possible that the type I fibers display a delayed signaling response and due to the timing of biopsy sampling a potential increase in signaling went undetected. Another possibility is that other pathways than those involving p70S6k are activated in type I fibers, i.e., that different signaling pathways are primarily responsible for the exercise-induced hypertrophy of type I and type II fibers.

The maximal eccentric contractions employed here did not cause any significant increase in the degree of phosphorylation of Akt and mTOR in type I or type II fibers, and indeed, Akt phosphorylation in type II fibers was actually reduced immediately after exercise in five of the six subjects. This observation provides further support for the proposal that p70S6k is activated via an Akt-independent pathway, although some studies on whole muscle have reported an exercise-induced enhancement in Akt phosphorylation (6, 9). In these studies, where the phosphorylation of Akt was elevated after 10 min and 1 h of recovery, it is possible that both the intensity and duration of the resistance exercise performed as well as the time following the exercise at which the biopsies were taken had an influence on the results obtained. The unchanged levels of mTOR phosphorylation observed after exercise here is consistent with the absence of any change in AMPK phosphorylation in either fiber type.

The elongation phase of translation, another important step at which protein synthesis is controlled, is regulated by the enzyme eEF2, which is, in turn, regulated by the eukaryotic elongation factor 2 kinase (eEF2k). This kinase is deactivated by phosphorylation at several sites, one of which is a target for

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Table 1. Content of MHC isoforms in the myosin ATPase identified type I and type II fiber pools

<table>
<thead>
<tr>
<th>Subject</th>
<th>Type I Fibers</th>
<th></th>
<th>Type II Fibers</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MHC I, %</td>
<td>MHC Ia, %</td>
<td>MHC Ix, %</td>
<td>MHC I, %</td>
</tr>
<tr>
<td>1</td>
<td>87 (85–94)</td>
<td>8.5 (4.3–12)</td>
<td>4.2 (1.4–6.0)</td>
<td>15 (7.9–25)</td>
</tr>
<tr>
<td>2</td>
<td>90 (83–98)</td>
<td>8.8 (1.4–16)</td>
<td>0.9 (0.4–1.6)</td>
<td>18 (16–21)</td>
</tr>
<tr>
<td>3</td>
<td>92 (79–86)</td>
<td>15 (12–18)</td>
<td>2.2 (1.2–3.5)</td>
<td>1.2 (0.0–2.4)</td>
</tr>
<tr>
<td>4</td>
<td>95 (88–99)</td>
<td>4.4 (1.0–11)</td>
<td>1.0 (0.2–2.3)</td>
<td>1.5 (0.8–2.0)</td>
</tr>
<tr>
<td>5</td>
<td>93 (88–95)</td>
<td>6.2 (3.0–12)</td>
<td>1.1 (0.1–2.1)</td>
<td>13 (11–16)</td>
</tr>
<tr>
<td>6</td>
<td>97 (93–99)</td>
<td>2.9 (5.0–9.5)</td>
<td>0.4 (0.0–1.0)</td>
<td>2.5 (1.9–3.2)</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>90.8 ± 2.0</td>
<td>7.6 ± 1.7</td>
<td>1.6 ± 0.6</td>
<td>8.7 ± 3.2</td>
</tr>
</tbody>
</table>

Values for each subject are means (range) for the 3 biopsy samples taken after exercise (directly after, and 1 h and 2 h following exercise). MHC, myosin heavy chain.
p70^{S6k} (40). Such inactivating phosphorylation of eEF2k leads to a dephosphorylation (at Thr^{37}) and activation of eEF2 (34). Thus our present finding that the level of eEF2 phosphorylation is reduced in type II fibers following maximal eccentric contractions is consistent with an elevation in the rate of protein synthesis.

For the time being, we can only speculate concerning the causes of these more pronounced responses of type II fibers to resistance exercise. For example, the MAPK-signaling cascade involving ERK1/2 and p38 may influence protein signaling in this context. In myogenic C2C12 cells, inhibition of ERK1/2 or p38 MAPK, in particular p38, led to a pronounced reduction in the phosphorylation state of p70^{S6k} at Ser^{424}/Thr^{421} (7). The authors suggest that p38 MAPK can phosphorylate p70^{S6k} on this site, an event that may facilitate subsequent phosphorylations at residues Thr^{389} and Thr^{240} and, thereby, activation of the enzyme (7). In the present study, phosphorylation of both ERK1/2 and p38 MAPK was found to be enhanced in both types of fibers following exercise, although to a greater extent in type II fibers in the case of p38 MAPK. That this stronger latter effect may have contributed to the activation of p70^{S6k} in type II fibers seems unlikely, since no effect at all on p70^{S6k} was seen in type I fibers, despite their elevated level of p38 MAPK.

It has been proposed that the hypertrophy of muscle fibers following exercise is related to the degree of Z-band streaming following exercise (35), and indeed, following dynamic lengthening exercise, more such streaming has been observed in type II fibers (13). Although Z-band streaming has often been considered to be indicative of muscle damage (13), more recent studies suggest that this phenomenon may actually be part of the remodeling of the muscle fiber that occurs in response to exercise (43, 44). Phospholipase D (PLD) is localized at the Z-bands in the muscle fiber, which are the critical sites for mechanical force transduction (20), and this enzyme may thus represent a possible link between muscle structure and intracellular signaling. Stretching of the muscle enhances PLD activity and raises the intracellular level of phosphatidic acid. The latter compound has recently been proposed to be a mediator of stretch-induced signaling, by giving rise to activation of the mTOR-p70^{S6k} pathway in an Akt-independent manner (20). Furthermore, Lehman and coworkers (28) recently demonstrated that phosphatidic acid can phosphorylate and activate p70^{S6k} in an mTOR-independent manner.

In the present study, the fibers were identified as either type I or type II; no histochemical stainings for subgroups of type II fibers were performed. Instead, the proportions of MHC Iia and IIX in the type II fiber pools were determined using gel electrophoresis in an attempt to explore a possible correlation between kinase phosphorylation and the subgroups of type II fibers. However, no such correlations were discovered, but it cannot be excluded that this is due to the fact that the proportion of type IIA and IIB fibers is influenced by the amount of hybrid fibers expressing the MHC I/Iia or I/Iia/IIX isoforms, which varied in the different samples. The relatively small content of hybrid fibers in the type I and type II fiber pools is, however, not likely to have influenced the different response regarding kinase phosphorylation in the two fiber types (Figs. 1, 2D, and 3B).

In conclusion, when recreationally active young men perform maximal voluntary eccentric contractions, the levels of phosphorylation of p70^{S6k} and the ribosomal protein S6 in human type II muscle fibers are greatly increased, whereas phosphorylation of eEF2 is reduced. The absence of any similar effects in type I fibers indicates that the initiation and elongation processes involved in protein translation may only be activated in type II fibers. Phosphorylation of ERK1/2 is elevated to a similar extent in both types of fiber immediately following exercise, and, in addition, the level of p38 MAPK is enhanced for at least 1 h of recovery in both fiber types, although to a larger extent in the type II fibers. The difference in activation of p70^{S6k} may explain, at least in part, why type II fibers hypertrophy to a greater extent than do type I fibers following eccentric resistance exercise.

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