Influence of supplementation with branched-chain amino acids in combination with resistance exercise on p70S6 kinase phosphorylation in resting and exercising human skeletal muscle

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Abstract

Aim: Skeletal muscle growth is thought to be regulated by the mammalian target of rapamycin (mTOR) pathway, which can be activated by resistance exercise and branched-chain amino acids (BCAA). The major aim of the present study was to distinguish between the influence of resistance exercise and BCAA on key enzymes considered to be involved in the regulation of protein synthesis, including p70S6 kinase (p70S6k).

Methods: Nine healthy subjects (four men and five women) performed unilateral resistance exercise on two occasions separated by 1 month. Subjects were randomly supplied either a mixture of BCAA or flavoured water. Muscle biopsies were taken from both resting and exercising muscle before, after and 1 h after exercise.

Results: Phosphorylation of Akt was unaltered by either resistance exercise and/or BCAA supplementation whereas mTOR phosphorylation was enhanced (P < 0.05) to a similar extent in both exercising and resting muscle following exercise in the absence (70–90%) and presence of BCAA supplementation (80–130%). Phosphorylation of p70S6k was unaffected by resistance exercise alone; however, BCAA intake increased (P < 0.05) this phosphorylation in both legs following exercise. In resting muscle, a 5- and 16-fold increase in p70S6k was observed immediately after and 1 h after exercise, respectively, as compared to 11- and 30-fold increases in the exercising muscle. Phosphorylation of eukaryotic elongation factor 2 was attenuated 1 h after exercise (P < 0.05) in both resting (10–40%) and exercising muscle (30–50%) under both conditions.

Conclusion: The present findings indicate that resistance exercise and BCAA exert both separate and combined effects on the p70S6k phosphorylation in an Akt-independent manner.

Keywords mTOR, resistance exercise, translation initiation.

Repeated performance of resistance exercise for longer periods of time causes hypertrophy of skeletal muscle, probably as a result of the cumulative effect of each individual exercise session on the turnover of muscle protein. Following an acute bout of resistance exercise, muscle protein synthesis can be elevated up to 48 h (Phillips et al. 1997). However, for a net increase to occur, muscle protein synthesis must surpass muscle protein breakdown (Biolo et al. 1995, Phillips et al. 1997) and for this to take place, additional nutritional...
stimulus is necessary (Rasmussen et al. 2000, Fujita et al. 2007). Thus, when essential amino acids are added to the equation, the hypertrophic response is enhanced resulting in a net increase in muscle protein (Biolo et al. 1997, Tipton et al. 1999).

The molecular mechanisms underlying these effects of exercise and nutrition on the turnover of muscle protein have only recently begun to unravel. Protein synthesis is thought to be regulated primarily at the level of translation involving changes in signal transduction. Several lines of evidence implicate the pathway involving the mammalian target of rapamycin (mTOR) as a major regulator of the synthesis of muscle protein (Bolster et al. 2004). The synergy observed between resistance exercise and amino acids in this context (Biolo et al. 1997, Tipton et al. 1999) suggests that these stimuli may act via a common pathway (Bolster et al. 2004). Indeed, investigations on both animals (Anthony et al. 1999, 2000a,b, Baar & Esser 1999, Bolster et al. 2003, Parkington et al. 2003) and humans (Liu et al. 2001, 2004, Cuthbertson et al. 2005, Dreyer et al. 2006, Mascher et al. 2008) have revealed that amino acids and resistance exercise are each capable of independently inducing signalling through the mTOR pathway. Of the essential amino acids, the branched-chain amino acids (BCAA) and leucine in particular, are most potent in this respect (Anthony et al. 2000b).

In our previous study, ingestion of BCAA in connection with a single session of resistance exercise was found to cause pronounced elevation of p70S6K kinase (p70S6K) and S6 phosphorylation, both downstream of mTOR, 1 and 2 h but not immediately after termination of the exercise (Karlsson et al. 2004). These results indicate that both resistance exercise and BCAA, in combination, are required for stimulation of p70S6K. However, this stimulatory effect could have been exerted by the BCAA alone, as resistance exercise itself did not activate this pathway.

Therefore, the major aim of the experiments described here was to distinguish between the effects of resistance exercise and BCAA on p70S6K phosphorylation. In addition, a more intense work-load was examined as some evidence suggests that heavier resistance exercise can activate the mTOR pathway in the absence of nutritional factors (Dreyer et al. 2006). For these purposes, the phosphorylation of proteins in the mTOR pathway together with effectors of this pathway, including phosphoinositide-dependent protein kinase (PDK1) and the mTOR-inhibitor AMP-activated protein kinase (AMPK) was determined in both exercising and resting muscle tissue from subjects who performed unilateral resistance exercise with and without BCAA supplementation. Our hypotheses were that BCAA would elicit a stronger signalling response in the exercising than in the resting leg and that an enhanced workload would result in phosphorylation of key components of the mTOR pathway even without ingestion of BCAA.

Materials and methods

Subjects

Nine healthy subjects, four men and five women, participated in the study. They were informed both orally and in writing of the purpose of this study and of all potential associated risks prior to giving their oral consent. None of the subjects performed resistance exercise on a regular basis, although they were recreationally active, participating in activities such as running, cycling and acrobatics once or twice a week. For the men and women, the mean (±SE) age was 27 (±1) and 24 (±2) years; height 180 (±4) and 162 (±2) cm; weight 73 (±7) and 51 (±2) kg; and maximal oxygen uptake 3.18 (±0.23) and 2.16 (±0.04) L min⁻¹, respectively. This study was approved by the Ethics Committee of the Karolinska Institutet and performed in accordance with the principles outlined in the Declaration of Helsinki.

Pre-study

Prior to initiation of the actual experiments, the subjects participated in three preparatory tests on a leg press machine (243 Leg press 45°; Gymleco, Stockholm, Sweden) after warming up on a cycle ergometer for 10 min. The first test was designed to determine each subject’s one repetition maximum (1 RM) performed with one leg. The exercising leg was randomly assigned for each subject. The 1 RM was assessed by gradually increasing the load until the subject was unable to perform no more than one single repetition (90–180° knee angle).

The purpose of the second and third tests was to familiarize the subjects with the intensity and frequency of repetitions involved in the actual experimental protocol. These tests began with a warm-up set of 10 repetitions with no load. Thereafter, two more warm-up sets of five repetitions at approx. 25% and 50% of 1 RM were carried out. Following these warm-ups, each subject performed the actual protocol of resistance exercise (see below). These two tests were conducted approx. 1 week apart and the actual experiment itself approx. 10 days after the last pre-test.

Maximal oxygen uptake was determined on a mechanically braked cycle ergometer (Monark 839E, Vansbro, Sweden). The work rate was gradually increased until exhaustion as described by Åstrand & Rodahl (1986). Oxygen uptake was measured continuously utilizing an
Experimental protocol

The subjects were instructed to refrain from any type of intense physical activity the 2 days prior to the experiment, as well as to follow a standardized diet during these same 2 days. This diet contained 17 energy (E) % protein, 26 E % fat and 57 E% carbohydrate and approx. 2100 kcal for the women and 2700 kcal for the men, based on reported activity levels.

On the day of the experiment, the subjects came to the laboratory at 07:30 hours following an overnight fast from 21:00 hours the evening before. Upon arrival, the subjects were asked to lie down and a catheter was inserted into the antecubital vein for repeated blood sampling. They rested for 30 min prior to the collection of a resting blood sample and after subsequent administration of local anaesthesia, resting biopsies were collected from the vastus lateralis muscle of both legs using a Weil-Blakesley conchotome (AB Wisex, Mölndal, Sweden), as described previously by Henriksson (1979).

Prior to the resistance exercise, subjects warmed up for 10 min on a cycle ergometer (Monark Ergomedic 839E) at a level of 100 W for the men and 60 W for the women. Following this initial warm-up, they were seated in the leg press machine and performed three warm-up sets of five repetitions at 0, 25 and 50% of 1 RM using one leg. Thereafter, the subjects performed four sets of 10 repetitions at 80% of 1 RM, followed by four sets of 15 repetitions at 65% of 1 RM, with 5 min of rest following each set. The entire session of resistance exercise lasted approx. 40 min.

Blood samples were collected in heparinized tubes at rest prior to warm-up, immediately before resistance exercise, after the fifth set (following approx. 25 min of exercise) and immediately after termination of exercise and following 15 and 45 min of recovery. Both drinks were lemon flavoured, contained salts and artificial sweetener and were indistinguishable in taste. The subjects were provided with a total of 85 mg BCAA kg⁻¹ body weight in 900 mL of flavoured water which was the same amount given per unit of time as in our previous study (Karlsson et al. 2004). In this randomized, double-blind, cross-over investigation, the two experiments were separated by a period of 4 weeks. A schematic overview of the experimental protocol is presented in Figure 1.

Tissue processing

Muscle biopsy specimens were freeze-dried, freed of blood and connective tissue, and then homogenized in ice-cold buffer (80 μL mg⁻¹ dry weight) containing 2 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl₂, 50 mM β-glycerophosphate, 1% TritonX-100, 1 mM Na₃VO₄, 2 mM dithiothreitol, 20 μg mL⁻¹ leupeptin, 50 μg mL⁻¹ aprotinin,
1% phosphatase inhibitor cocktail (Sigma P-2850; St Louis, MO, USA), 40 μg mL⁻¹ PMSF. Homogenates were then centrifuged at 10 000 g for 10 min at 4 °C in order to remove cellular debris and the resulting supernatant was stored at −80 °C.

Protein concentrations were determined in aliquots of supernatant diluted 1 : 10 in distilled water using a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL, USA). Samples were diluted in Laemmli sample buffer (Bio-Rad Laboratories, Richmond, CA, USA) and homogenizing buffer to obtain a final protein concentration of 1.5 μg μL⁻¹. Following dilution, the samples were heated at 95 °C for 5 min to denature proteins present in the supernatant. Samples were then kept at −20 °C until further analysis.

**Immunoblot analysis**

Samples containing 30 μg total protein were separated by SDS-PAGE on Criterion cell gradient gels (Bio-Rad Laboratories) consisting of 7.5% acrylamide for separation of mTOR Ser2448, p70S6k Thr389, rpS6 Ser235/236, eukaryotic elongation factor 2 (eEF2) Thr56, AMPK Thr172, PDK1 Ser241 or 4–20% in the case of Akt. Subsequently, the membranes were incubated overnight with specific antibodies (Cell Signaling Technology, Danvers, MA, USA) and horseradish peroxidase (diluted 1 : 10 000; Cell Signaling Technology). The membranes were then washed serially (twice for 1 min followed by three times for 15 min) with TBS-T containing 2.5% non-fat dry milk or with TBS-T alone (for Akt Ser473), followed by four additional washes with TBS for 5 min each. Finally, membranes with the antibodies bound to the phosphorylated proteins were visualized by chemiluminescent detection on a Molecular Imager ChemiDoc™ XRS system and the bands were analysed using Quantity One® version 4.6.3 software (Bio-Rad Laboratories).

**Plasma analysis**

The blood samples were drawn from the venous catheter into heparinized tubes and then centrifuged (9000 g for 2 min) and the plasma stored at −80 °C. For amino acid measurements, plasma samples were deproteinized by precipitation with 5% trichloroacetic acid (1 : 5), centrifuged at 9000 g for 2 min and the supernatant stored at −80 °C. The concentration of amino acids in the supernatant was measured by reversed-phase high-performance liquid chromatography according to Pfeifer et al. (1983), with orthophthalaldehyde as the derivatizing agent. Plasma insulin concentrations were measured using a radioimmunoassay kit in accordance with the manufacturer’s protocol (Millipore, Billerica, MA, USA). Glucose and lactate concentrations were analysed as described by Bergmeyer (1974).

**Statistical analysis**

Parametric statistical procedures were employed to calculate the means and standard errors of the mean (SE). Unless indicated, the values presented in the text are means ± SE. A two-way repeated measures ANOVA was used to compare changes in plasma concentration of BCAA, insulin, glucose and lactate in the two conditions. A three-way repeated measures ANOVA was used to evaluate changes over time, differences between supplements and the two legs. The factors were Supplement (placebo and BCAA), Time (before, after and after 1 h) and Leg (rest and exercise). In case of a significant interaction, simple main effects tests were performed, i.e. effects of one factor holding the level/levels of the other factor/factors fixed. If no significant interactions with factor Time were present and the sphericity assumption was met, a Fisher’s LSD *post hoc* test was performed to determine the effects from baseline, provided that the sphericity assumption was met.
assumptions were met; otherwise, planned comparisons were performed. For some positively skewed distributed variables, log-transformation was performed before the formal analyses. \( P < 0.05 \) was considered statistically significant.

**Results**

**Task performance**

All of the subjects completed the first part of the exercise protocol (four sets of 10 repetitions at 80% of 1 RM) on both occasions. In the second part of the exercise protocol (four sets of 15 repetitions at 65% of 1 RM), one subject performed 57 and another subject performed 50 repetitions on both occasions.

**Protein signalling**

Phosphorylation of Akt, an immediate upstream effector of mTOR, remained unaltered in both legs during both trials (Fig. 2a,b). In contrast, after exercise and following 1 h of recovery, phosphorylation of mTOR at Ser\(^{2448}\) was enhanced 50–90% in both legs in the placebo condition and 65–160% in both legs in the BCAA condition; a main effect of time \( (P < 0.01) \) but no interaction between time and supplement was obtained in the ANOVA (Fig. 2c,d). There was, however, a trend for main effect of supplement \( (P = 0.073) \) in the ANOVA.

Phosphorylation of p70S6k at residue Thr\(^{389}\) remained unaltered in both legs during the entire placebo trial, but with BCAA supplementation, this phosphorylation was elevated 5 to 11-fold in both legs after exercise and further elevated (16 to 30-fold) after 1 h of recovery. The three-way ANOVA showed main effects of time \( (P < 0.01) \), supplement \( (P < 0.05) \) and leg \( (P < 0.01) \), and an interaction between time and supplement \( (P < 0.05) \). Planned comparisons also showed a trend \( (P = 0.13) \) for an interaction between supplement and leg (Fig. 3a,b). Exercise led to an 8 to 45-fold increase in phosphorylation of S6 that persisted for 1 h of recovery in the exercising leg and a 3 to 10-fold increase in the resting leg under both conditions, although this increase was more pronounced in the exercising leg. The three-way ANOVA showed main effects of time \( (P < 0.01) \), supplement \( (P < 0.05) \) and leg \( (P < 0.01) \), a significant interaction between time and leg \( (P < 0.01) \), and an interaction between time and supplement \( (P < 0.05) \).

![Figure 2](image-url) Phosphorylation of Akt at Ser\(^{473}\) (a, b) and mTOR at Ser\(^{2448}\) (c, d) in resting and exercising muscle during placebo and branched-chain amino acid (BCAA) trials. Representative immunoblots from one subject are shown above each graph. Bands have been rearranged to fit the illustrated bars. Values in graphs are arbitrary units (means \( \pm \) SE for nine subjects). *\( P < 0.05 \) vs. before exercise.
indication \( (P = 0.16) \) for interaction between time and supplement (Fig. 3c,d). Phosphorylation of p90RSK at Thr573 increased following exercise in both conditions; main effect of time \( (P < 0.05) \) was obtained in the ANOVA (Fig. 3e,f).

Phosphorylation of AMPK (Fig. 4a,b), and PDK1, another upstream activator of the mTOR pathway (Fig. 4c,d), was unaltered in either leg by exercise with or without BCAA. Phosphorylation of the eukaryotic elongation factor 2 (eEF2) at Thr56 was unchanged immediately after exercise in both legs under both conditions, but was attenuated 15–50% in both the resting and exercising legs 1 h after exercise under both conditions (Fig. 4e,f). ANOVA revealed a main effect of time \( (P < 0.05) \) but no interaction between time and supplement was obtained.

**Plasma data**

Plasma concentrations of the BCAA increased rapidly following supplementation and were higher at all time points compared to before exercise, as well as in comparison with the placebo condition (Fig. 5a). In the latter case, these concentrations exhibited a small but statistically significant reduction during the recovery period (Fig. 5a, Table 1).

Under both conditions, plasma concentrations of lactate were elevated rapidly during exercise, peaking at
a level of approx. 7.5 mmol L\(^{-1}\) immediately after exercise and returning to near-basal levels after 1 h of recovery (Fig. 5b).

Plasma insulin concentrations increased over time in both conditions; however, there was no difference between the two (Fig. 5c). Plasma glucose concentrations remained unchanged at approx. 5.5–6.0 mmol L\(^{-1}\) during the entire experimental period under both conditions (data not shown).

No differences were seen between male and female subjects for any of the variables measured, except for the concentration of plasma lactate, which was higher during and after exercise in the male subjects. The average concentration was 4.5 and 6.9 mmol L\(^{-1}\) during exercise and 5.8 and 8.7 mmol L\(^{-1}\) after exercise for the females and males respectively.

**Discussion**

The primary and novel finding of the present investigation is that ingestion of BCAA enhances phosphorylation of p70\(^{S6k}\) in both resting and exercising skeletal muscle, an effect which was not observed without BCAA supplementation. The phosphorylation level of

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**Figure 4** Phosphorylation of AMPK at Thr\(^{172}\) (a, b), PDK1 at Ser\(^{241}\) (c, d) and eEF2 at Thr\(^{56}\) (e, f) in resting and exercising muscle during placebo and branched-chain amino acid (BCAA) trials. Representative immunoblots from one subject are shown above each graph. Bands have been rearranged to fit the illustrated bars. Values in graphs are arbitrary units (means ± SE for nine subjects). *P < 0.05 vs. before exercise.
p70S6k increased to a greater extent in all subjects in the exercising muscle as compared to the resting muscle, although the statistical analysis did not reveal a significant interaction between supplement and leg (P = 0.13). In a previous study, we observed that a combination of resistance exercise and BCAA was necessary for the stimulation of the mTOR pathway (Karlsson et al. 2004). However, our earlier study design did not allow us to eliminate the possibility that this stimulatory effect was exerted by BCAA alone. Here, we demonstrate for the first time that ingestion of BCAA and resistance exercise synergistically enhances p70S6k phosphorylation (Fig. 3a,b). Furthermore, in the present study, BCAA intake led to an elevation immediately after exercise, which was not seen in the previous study (Karlsson et al. 2004). This suggests that despite a rapid increase in plasma levels of BCAA, a certain time is required before any measurable effect on p70S6k can be detected. The longer exercise duration (40 min vs. 20 min in the previous study) could in fact have made the difference. This is supported by the finding that when amino acid availability is increased by intravenous infusion to human subjects, the rate of muscle protein synthesis increased 30–60 min after the start of the infusion, while no change in synthesis was detected during the first 30 min of infusion (Bohé et al. 2001).

The phosphorylation pattern of ribosomal protein S6 on Ser235/236 observed here closely resembled that of p70S6k at Thr389 in the BCAA trial, with enhancement immediately after resistance exercise and a further elevation after 1 h of recovery. At this same time point, S6 phosphorylation was also increased in the placebo trial, which is in contrast with our previous findings that resistance exercise alone had no influence on S6 phosphorylation (Karlsson et al. 2004). This discrepancy may be due to the greater amount of work performed in the present study. To the workload employed previously, we added four sets of 15 repetitions at 65% of 1 RM. However, despite this additional workload, we observed no change in p70S6k phosphorylation at Thr389 at this same time point. This finding was unexpected as p70S6k has long been considered to be the major upstream effector of ribosomal protein S6 (Meyuhas 2008) and, consequently, phosphorylation of S6 has been thought to be a reliable indicator of p70S6k activity. However, it has recently been confirmed that p90 ribosomal S6 kinase (p90RSK) phosphorylates specific residues on S6 in a mTOR-independent manner (Roux et al. 2007). Furthermore, the extracellular signal-regulated kinase (ERK1/2) pathway, including p90RSK, is activated in young men immediately after resistance exercise (Williamson et al. 2003). Thus, the increase in S6 phosphorylation observed here after exercise in the placebo trial may therefore be a result of increased signalling through the ERK1/2 pathway. Furthermore, although p90RSK phosphorylation increased over time in both conditions (Fig. 3e,f), we could not detect any
Table 1 Plasma concentrations of individual branched-chain amino acids (BCAA) at rest, before, during and after exercise

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Condition</th>
<th>Before exercise</th>
<th>Exercise</th>
<th>Recovery, min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before</td>
<td>During</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Placebo</td>
<td>64 ± 6</td>
<td>61 ± 5</td>
<td>62 ± 7</td>
</tr>
<tr>
<td></td>
<td>BCAA</td>
<td>54 ± 5</td>
<td>86 ± 11*</td>
<td>127 ± 7*</td>
</tr>
<tr>
<td>Leucine</td>
<td>Placebo</td>
<td>120 ± 11</td>
<td>114 ± 9</td>
<td>113 ± 12</td>
</tr>
<tr>
<td></td>
<td>BCAA</td>
<td>109 ± 10</td>
<td>163 ± 19*</td>
<td>232 ± 15*</td>
</tr>
<tr>
<td>Valine</td>
<td>Placebo</td>
<td>228 ± 15</td>
<td>219 ± 18</td>
<td>218 ± 17</td>
</tr>
<tr>
<td></td>
<td>BCAA</td>
<td>206 ± 19</td>
<td>257 ± 22*</td>
<td>331 ± 20*</td>
</tr>
</tbody>
</table>

Values are expressed in μmol L⁻¹ (means ± SE for nine subjects).

*P < 0.05 vs. before exercise. In the BCAA condition, plasma concentrations of individual BCAAs were different at all time points, except before exercise, when compared with the placebo condition.

differences between resting and working muscle which is in contrast to S6. Therefore, the relationship between the MAPK pathway and S6 phosphorylation in human skeletal muscle remains to be clarified.

In contrast to the situation with p70S6k and ribosomal protein S6, phosphorylation of mTOR at Ser²⁴⁴⁸ was enhanced to a similar extent in both resting and working muscle in the BCAA trial and this elevation persisted for at least 1 h after termination of the exercise. Furthermore, a similar pattern of phosphorylation was observed in the placebo trial. This apparent disconnect between the phosphorylation of mTOR and p70S6k is unexpected as it is in disagreement with the general view that mTOR activates p70S6k via a linear signalling sequence (Glass 2003). The differential signalling pattern between mTOR and p70S6k suggests that p70S6k is activated via a pathway other than the one involving mTOR, provided that Ser²⁴⁴⁸ phosphorylation represents an activation of mTOR. However, this may not be the case as it has been shown that point mutation of the Ser²⁴⁴⁸ residue on mTOR has no effect on p70S6k activity (Sekulic et al. 2000). The role of mTOR phosphorylation at Ser²⁴⁴⁸ is unclear. Phosphorylation of this site is blocked by rapamycin (Kimball et al. 1999, Anthony et al. 2000b, Drummond et al. 2009), suggesting that it can either be autophosphorylated or can be phosphorylated by p70S6k (Holz & Blenis 2005). In practice, this site is likely targeted by a number of different kinases. Considering the similarity of the recognition sequence of p70S6k and p90RSK and the similar pattern of mTOR at Ser²⁴⁴⁸ and p90RSK phosphorylation reported here, it is possible that in response to BCAA this site is phosphorylated by p90RSK. However, a clear interpretation of mTOR phosphorylation at Ser²⁴⁴⁸ is difficult at the present time.

PDK1, an upstream activator of Akt (Alessi et al. 1997), has been suggested to be a potential activator of p70S6k (Alessi et al. 1998, Pullen et al. 1998). However, PDK1 remained unchanged at all time points and under all conditions, a finding in agreement with the conclusion that PDK1 is constitutively active (Mora et al. 2004) and is therefore unlikely to play a specific role in the BCAA-stimulated increase in p70S6k phosphorylation.

Phosphorylation of Akt, a direct upstream activator of mTOR (Navé et al. 1999), was unaltered in both resting and exercising muscle immediately following resistance exercise and remained unchanged after 1 h of recovery, both with and without BCAA supplementation. These findings are in agreement with some (Coffey et al. 2006, Deshmukh et al. 2006, Eliasson et al. 2006) but no all (Creer et al. 2005, Blomstrand et al. 2006, Dreyer et al. 2006, Deldicque et al. 2008, Terzis et al. 2008) earlier studies of contractile activity on Akt phosphorylation. These apparent discrepancies may reflect differences in study design, e.g. in the volume, intensity and/or duration of the exercise or in the time points at which the biopsies were taken. In our previous investigation, Akt phosphorylation was actually found to be attenuated immediately after resistance exercise (Blomstrand et al. 2006), which has also been observed by others (Deldicque et al. 2008, Terzis et al. 2008). The results lend further support to the proposal that, under certain conditions, activation of the mTOR pathway in human skeletal muscle is in response to resistance exercise and amino acids is mediated in an Akt-independent manner.

It has been proposed that signalling through the mTOR pathway is inhibited during conditions of energetic stress, which is thought to involve activation of AMPK, which is referred to as a cellular energy sensor on the basis of its ability to recognize diminishing levels of cellular ATP (Winder & Hardie 1999). In rodents, pharmacological activation of AMPK reduces signalling through the mTOR pathway in both resting (Bolster et al. 2002) and exercising muscle (Thomson et al. 2008). In human subjects, resistance exercise was followed by enhanced AMPK activity, however, with...
concomitant elevations of mTOR phosphorylation (Dreyer et al. 2006, Wilkinson et al. 2008). Furthermore, in both studies, resistance exercise stimulated protein synthesis at the same time as AMPK activity was elevated. Under all conditions in the present investigation, AMPK phosphorylation was unaltered (Fig. 4e, f) while the extent of mTOR phosphorylation increased over time (Fig. 2c, d). The amount of work performed here was similar to that in the study by Dreyer et al. (2006); however, the resting period between the sets was longer in the present study (5 min vs. 3 min in the study by Dreyer et al. 2006), which may have been sufficient to restore the muscle energy state and thus eliminating the effect on AMPK. Therefore, the differences in study design offer a potential explanation for the divergent results of the two studies.

Whereas the signalling proteins discussed above affect primarily initiation of translation, eEF2 regulates the elongation phase of protein synthesis. Unlike the situation for most signalling proteins, phosphorylation of eEF2 inhibits its activity (Ryazanov & Davydova 1989, Redpath et al. 1993), thus preventing it from binding to the ribosome (Carlberg et al. 1990). Here, eEF2 phosphorylation was reduced to a similar extent in both resting and exercising muscle after 1 h of recovery under both conditions compared to before exercise. This attenuation of eEF2 phosphorylation may be related to the simultaneous elevation in p70S6k phosphorylation at Thr^{389} in the BCAA condition, as activated p70S6k promotes phosphorylation of eEF2 kinase (Wang et al. 2001), rendering this enzyme inactive and thereby relieving its inhibitory effect on eEF2. However, a reduction in eEF2 phosphorylation also occurred in the placebo trial despite the absence of an increase in p70S6k phosphorylation, which may suggest an alternative activation pathway of eEF2 in the absence of nutritional supply. In a recent study, Rose et al. (2009) showed that elevated Ca^{2+} levels in rat skeletal muscle increased eEF2 phosphorylation during contraction through a Ca^{2+}-calmodulin-dependent activation of eEF2 kinase. However, a decrease in eEF2 phosphorylation due to reduced Ca^{2+} signalling seems unlikely as the reduction occurred 1 h after exercise, a time point at which intracellular Ca^{2+} levels should be low. The similarities in mTOR and eEF2 phosphorylation in resting and exercising muscle under both conditions suggest that phosphorylation of these enzymes may be promoted by a systemic rather than a contraction-induced stimulus. A systemic effect on p38 MAPK phosphorylation has previously been reported during one-leg cycle ergometer exercise (Widegren et al. 2001), supporting the view that systemic factors as well as changes within the muscle are involved in stimulating signalling pathways in muscle. Insulin may play a role as a systemic factor as the hormonal levels increased over time during both conditions. However, although the rise in circulating insulin was statistically significant, the actual increase was small and within the range of fasting levels. Furthermore, phosphorylation of Akt, an immediate target of insulin signalling, was unaltered during both trials. Therefore, it is unlikely that the small rise in insulin can account for the similarities in mTOR and eEF2 signalling in resting and working muscle.

The findings documented here indicate that ingestion of BCAAs activates p70S6k in both resting and exercising human skeletal muscle. One obvious limitation associated with our experiments is the absence of direct assessment of protein synthesis. At the same time, an expanding body of evidence links the activation of p70S6k to a simultaneous increase in the synthesis of muscle protein. For example, Dreyer et al. (2008) reported that resistance exercise alone or in combination with supplementation with essential amino acids and carbohydrates elevated protein synthesis in muscle and enhanced signalling via mTOR and p70S6k. In addition, Cuthbertson et al. (2005) demonstrated that in both young and old subjects, supplementation with essential amino acids under resting conditions stimulated both muscle protein synthesis and signalling through both mTOR and p70S6k. Furthermore, in a recent study by Kumar et al. (2009), the level of p70S6k phosphorylation was found to be a good predictor of increased rates of muscle protein synthesis in young men following a session of acute resistance exercise. Altogether, this supports the view that enhanced levels of p70S6k phosphorylation reflect an increase in protein synthesis during supplementation with BCAA; however, this needs to be confirmed in future studies.

In conclusion, in connection with unilateral resistance exercise, the combined stimuli of BCAA ingestion and resistance exercise increases p70S6k phosphorylation to a greater extent than does the sum of the two when exerted separately, indicating the existence of a synergistic effect of these two stimuli on p70S6k. In contrast, phosphorylation of mTOR is enhanced to a similar extent in both resting and exercising muscle and is not significantly influenced by BCAA ingestion. In addition, the increase in mTOR and p70S6k phosphorylation appears to be independent of Akt, as no effect on this enzyme was noted. Phosphorylation of mTOR, p90RSK and eEF2 was altered to a similar extent in both resting and exercising muscle, suggesting a systemic rather than a contraction-induced regulation of these enzymes.

Conflict of interest

The authors have no conflicts of interest.

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