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Intake of Branched-Chain Amino Acids Influences the Levels of MAFbx mRNA and MuRF-1 Total Protein in Resting and Exercising Human Muscle

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Running head: BCAA intake reduces levels of MAFbx mRNA

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Abstract

Resistance exercise and amino acids are two major factors that influence muscle protein turnover. Here, we examined the effects of resistance exercise and branched-chain amino acids (BCAA), individually and in combination, on the expression of anabolic and catabolic genes in human skeletal muscle. Seven subjects performed two sessions of unilateral leg press exercise with randomized supplementation with BCAA or flavored water. Biopsies were collected from the vastus lateralis muscle of both the resting and exercising legs before and repeatedly after exercise to determine levels of mRNA, protein phosphorylation and amino acid concentrations. Intake of BCAA reduced ($P<0.05$) MAFbx mRNA by 30% and 50% in the resting and exercising legs, respectively. The level of MuRF-1 mRNA was elevated ($P<0.05$) in the exercising leg, 2- and 3-fold under the placebo and BCAA conditions, respectively, while MuRF-1 total protein increased by 20% ($P<0.05$) only in the placebo condition. Phosphorylation of $p70^{S6k}$ increased to larger extent (~2-fold; $P<0.05$) in the early recovery period with BCAA supplementation, whereas the expression of genes regulating mTOR activity was not influenced by BCAA. Muscle levels of phenylalanine and tyrosine were reduced (13-17%) throughout recovery ($P<0.05$) in the placebo condition, and to a greater extent (32-43%; $P<0.05$) following BCAA supplementation in both resting and exercising muscle. In conclusion, BCAA ingestion reduced MAFbx mRNA and prevented the exercise-induced increase in MuRF-1 total protein in both resting and exercising leg, whereas resistance exercise differently influenced MAFbx and MuRF-1 mRNA expression suggesting both common and divergent regulation of these two ubiquitin ligases.

Key words: phenylalanine, resistance exercise, Rheb, ubiquitin ligases

Introduction

Resistance exercise and amino acids are each capable of stimulating human muscle protein synthesis and when combined the effect is enlarged (6, 33, 46, 52). Because resistance exercise also increases the rate of muscle protein breakdown (52) ingestion of amino acids is essential in order to achieve a positive net muscle protein balance (33, 57). With respect to the amino acids, the branched-chain amino acids (BCAA) and, in particular leucine, appear to be the most potent based on data from experimental animals (31). Furthermore, both resistance exercise and amino acids are thought to mediate their effects on protein synthesis through activation of the pathway involving the mammalian target of rapamycin (mTOR) (17, 23).

Activation of mTOR by phosphorylation exerts well characterized effects on several downstream proteins, including activation of $p70^{S6}$ kinase ($p70^{S6k}$) and the eukaryotic elongation factor-2 (eEF2) as well as inactivation of 4E-binding protein-1 (4E-BP1), thereby enhancing translational initiation and elongation (58). In contrast, the upstream regulation of mTOR has been less extensively investigated, although this regulation associated with exercise and nutritional stimuli appears to differ. Muscle contraction (17), as well as insulin (32), are capable of activating protein kinase B/Akt and thereby leading to mTOR activation, either directly (49) or via stimulation of GTP binding to the small G-protein Rheb (28). Moreover, muscle contraction has been proposed to activate mTOR through the second messenger phosphatidic acid (27) and by down-regulating the expression of REDD 1 and 2, two negative regulators of mTOR (15). The corresponding activation by amino acids is suggested to involve not only Rheb but also stimulation of human vacuolar protein sorting-34 (hVps34), a class III phosphoinositide-3-kinase (4, 50). Even though hVps34 activity has been shown to be regulated by both Ca^{2+}/CaM (25) and hVps15 (61) *in vitro,* the mechanisms by which amino acids augment protein synthesis through mTOR signaling *in vivo* are unidentified.

The molecular mechanisms underlying protein breakdown involve tagging of proteins with ubiquitin molecules and subsequent degradation by the 26S proteasome (36). MuRF-1 (Muscle RING-finger 1) and MAFbx (Muscle atrophy F-box), two muscle-specific ubiquitin ligases, are up-regulated in connection with muscle atrophy (10, 29) by the FOXO family of transcription factors. These factors are, in turn, regulated upstream by Akt in such way that activation of Akt down-regulates the expression of MuRF-1 and MAFbx and thereby counteracts atrophy (53, 55).

Whereas extensive evidence for the stimulatory effect of amino acids, either alone or in combination with exercise, on protein synthesis has been reported (33), their effect on protein breakdown is elusive. Most previous investigations involving ingestion of essential amino acids (EAA) in connection with resistance exercise have revealed no attenuating effect on protein breakdown (33, 57). In contrast, when BCAA or leucine alone is administered, results indicate that protein degradation in subjects at rest (39, 48) or performing eccentric endurance exercise is reduced (41). Moreover, in rats, supplementation of BCAA/leucine appears to attenuate the expression of MAFbx and MuRF-1 mRNA (5), suppress protein degradation and attenuate muscle atrophy (47, 56).

In light of our limited knowledge of the upstream regulation of mTOR evoked by exercise and nutritional stimuli as well as emerging indications that BCAA help attenuate muscle proteolysis, the present study was designed to examine the independent and combined effects of resistance exercise and BCAA on the expression of mRNA encoding proteins involved in the upstream regulation of mTOR, as well as the proteolytic factors MuRF-1 and MAFbx. In addition, phosphorylation of proteins in the mTOR pathway and levels of amino acids in resting and exercising muscle were determined. Our hypothesis was that BCAA would alter the mRNA expression of proteins that regulate mTOR in such manner that its activity is elevated, while at the same time reduce expression of the ubiquitin ligases.

Materials and Methods

Participants

Seven healthy participants, five men and two women, were included in the study. They were recreationally active once or twice per week but did not perform resistance exercise on a regular basis. Their mean (± SE) age was 27 (± 2) years; height 175 (± 5) cm; weight 67 (± 7) kg; and maximal oxygen uptake 46 (± 1) $mL^{-1} \cdot min^{-1} \cdot kg^{-1}$. Each subject was fully informed about the purpose and procedures of the study and their right to withdraw at any point. This study was approved by the Regional Ethical Review Board in Stockholm and performed in accordance with the principles outlined in the Declaration of Helsinki. Five of these same subjects (three men and two women) also took part in one of our earlier investigations (2).

Pre-test

Prior to initiation of the actual experiments, each participant underwent three preparatory sessions on a leg press machine (243 Leg Press 45°; Gymleco, Stockholm, Sweden) following a 10-min warm-up on a cycle ergometer. The first test was designed to determine each participant's one repetition maximum (1 RM) performed with one leg that was chosen randomly. The 1 RM was assessed by gradually increasing the load until the participant was unable to perform no more than one single repetition (90-180° knee angle)**.** The purpose of the second and third sessions was to familiarize our subjects with the intensity and frequency of repetitions involved in the actual experimental protocol. These sessions began with a warm-up set of 10 repetitions with no load, followed by two sets consisting of five repetitions at 25% and 50% of 1 RM. Following these warm-ups, each participant performed the actual protocol of resistance exercise (see below). These two sessions of familiarization were conducted 1 week apart, approximately 10 days prior to the actual experiment.

Utilizing continuous monitoring with an on-line system (Amis 2001; Innovision A/S, Odense, Denmark), maximal oxygen uptake was determined while exercising on a mechanically braked cycle ergometer (Monark Ergomedic 839E, Vansbro, Sweden) with a gradually increasing work rate until volitional exhaustion, as described earlier by Åstrand and Rodahl (3).

Experimental protocol

The experimental set-up involved a randomized, double-blind, cross-over design. Participants had been instructed to refrain from any type of intense physical activity and to consume a standardized diet during the two days preceding the experiment. This diet consisted of 17 energy (E) % protein, 26 E% fat and 57 E% carbohydrate and contained approximately 2100 kcal for the women and 2700 kcal for the men (values based on the subjects' reported levels of activity).

On the day of the experiment, the men and women arrived at the laboratory at 7:30 AM after having fasted since 9:00 PM the evening before. Upon arrival, the participants were asked to lie down and a catheter was inserted into the antecubital vein for repeated sampling of blood, the first of which was drawn after 30 min of rest. Subsequently, local anaesthesia was administered and resting biopsies (Pre-Ex) taken from the vastus lateralis muscle of both legs using a Weil-Blakesley conchotome (Ab Wisex, Mölndal, Sweden).

Prior to performing the resistance exercise, each participant warmed up for 10 min on a cycle ergometer (Monark Ergomedic 839E) at a work load of 100 W for the men and 60 W for the women. Thereafter, they were seated in the leg press machine where they performed three warm-up sets of five repetitions at 0, 25 and 50% of 1 RM, followed by four sets of 10 repetitions at 80% of 1 RM, and finally, four sets of 15 repetitions at 65% of 1 RM. All of the resistance exercise was performed with one leg only, with 5 min of rest after each set. The entire session lasted approximately 40 min. This experimental protocol was carried out twice by each subject, with 4 weeks between the two occasions.

Blood samples were collected in heparinized tubes during the rest period prior to warm-up (see above), immediately before beginning the resistance exercise, after the fifth set (i.e. after approximately 25 min of exercise), directly after termination of the exercise and following 15, 30, 60, 120 and 180 min of recovery. These blood samples were centrifuged at 9,000 x g for 3 min and the plasma thus obtained stored at -80°C for future analyses.

Muscle biopsies from both legs (starting with the exercising leg) were taken using a Weil-Blakesly conchotome (AB Wisex, Mölndal, Sweden) immediately after termination of exercise (Post-Ex) and following one (1h) and three hours (3h) of recovery. However, in two of the participants, biopsies were only taken prior to the exercise and following three hours of recovery. The first biopsy was taken approximately 8-9 cm above the mid-patella and the following biopsies 3-4 cm proximal to the previous one. Biopsy samples were immediately frozen in liquid nitrogen and stored at -80°C for later analysis.

Each participant ingested 150 mL of either a mixture of the three BCAA (45% leucine, 30% valine and 25% isoleucine; Ajinomoto, Kanagawa, Japan) in flavored water or flavored water alone at rest prior to the warm-up, directly before beginning the resistance exercise, during and immediately after exercise, and following 15 and 45 min of recovery, resulting in a total intake of 85 mg BCAA kg^{-1} body weight in 900 mL of flavored water. Both drinks were lemon flavored, contained salts and artificial sweetener and were indistinguishable in taste. The amount and timing of BCAA ingestion were similar and the composition of the BCAA mixture was the same as in a previous study that led to marked increases in mTOR signaling (30).

Plasma analyses

For determination of amino acids, plasma samples were first deproteinized by precipitation with ice-cold 5% trichloroacetic acid (1:5), maintained on ice for 20 min, centrifuged at 10,000 x g for

3 min and the supernatant obtained stored at -80°C for later analysis. The concentrations of free amino acids in the supernatants were measured by reversed-phase high performance liquid chromatography (HPLC, Waters Corporation, Milford, MA) employing orthophtaldehyde (OPA) as the derivatizing agent as previously described (9). Plasma glucose and lactate were analyzed as described by Bergmeyer (7).

Muscle amino acid analyses

The muscle biopsies were lyophilized and blood and connective tissue subsequently removed by dissection under a light microscope (Carl Zeiss, Germany). Following transfer of 2-4 mg of muscle tissue to Eppendorf tubes, the amino acids were extracted in ice-cold 5% trichloroacetic acid (40 µL per mg), and the tubes then maintained on ice for 30 min, centrifuged at 10,000 x g for 3 min and the resulting supernatant removed and stored at -80°C. The concentrations of amino acids in the supernatants were later analyzed as described above.

Immunoblot analysis

Freeze-dried and cleaned muscle samples were homogenized as previously described (2). Homogenates were centrifuged and the resulting supernatant was stored at -80°C. Protein concentrations were determined after which 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. Following dilution, the samples were heated at 95°C for 5 min and then kept at -20°C until further analysis.

Details of the western blotting procedures have been described elsewhere (2). Briefly, and with minor modifications, samples were separated by SDS-PAGE on Criterion cell gradient gels (4-20% acrylamide; Bio-Rad Laboratories) following which the gels were equilibrated in transfer buffer (25 mM Tris base, 192 mM glycine, and 10% methanol) for 30

min. The proteins were then transferred to polyvinylidine fluoride membranes (Bio-Rad Laboratories) which in turn were stained with MemCode™ Reversible Protein Stain Kit (Pierce Biotechnology) to confirm successful transfer of proteins. All samples from each subject were run on the same gel.

Membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS; 20 mM Tris base, 137 mM NaCl, pH 7.6) containing 5% non-fat dry milk. Thereafter, the membranes were incubated overnight with commercially available primary phosphospecific antibodies diluted in TBS supplemented with 0.1% Tween-20 containing 2.5% non-fat dry milk (TBS-TM). After incubation with primary antibodies, membranes were washed with TBS-TM and then incubated for 1 h at room temperature with appropriate secondary antibodies. Membranes were then washed serially prior to visualization of the phosphorylated proteins by chemiluminescent detection on a Molecular Imager ChemiDoc™ XRS system. All bands were analysed using the contour tool in the Quantity One® version 4.6.3 software (Bio-Rad Laboratories) and phosphorylated proteins were expressed as arbitrary units relative to α-tubulin.

Antibodies

Primary antibodies raised against phospho-mTOR (Ser^{2448}; 1:500), phospho-$p70^{S6k}$ (Thr^{389}; 1:1000), phosphor-Akt (Ser^{473}; 1:1000) and phospho-4E-BP1 ($Thr^{36/37}$; 1:1000) were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibody against MAFbx (1:1000) was purchased from Abcam (Cambridge, United Kingdom), for MuRF-1(1:1000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and for α-tubulin (1:5000) from Sigma-Aldrich (St. Louis, MO, USA). Secondary rabbit and mouse antibodies (1:10,000) were purchased from Cell Signaling Technology and secondary goat antibodies (1;5,000) from Abcam.

RNA extraction and real-time PCR

Total RNA was extracted from approximately 3 mg muscle that had been freeze-dried and cleaned (see above) by homogenization with a Polytron (Kinematica AG, Lucerne, Switzerland) in PureZOL RNA isolation reagent (Bio-Rad Laboratories AB, Sundbyberg, Sweden) in accordance with the manufacturer's instruction. The final amount and purity of the RNA was determined by spectrophotometry.

Two μg of the isolated RNA was subjected to reverse transcription utilizing the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Sweden) to produce 20 μL cDNA, with thermal cycling being performed in a Bio-Rad iCycler (Bio-Rad Laboratories, Sweden).

The mRNA was quantified using real-time quantitative PCR (RT-qPCR), with the housekeeping GAPDH mRNA as an internal control. To determine a suitable cDNA concentration, annealing temperature and PCR cycling protocol for each primer the pooled cDNA obtained from all of the participants was diluted (1:5, 1:25, 1:125, 1:625 and 1:3125) and RT-qPCR performed on a Bio-Rad iCycler (Bio-Rad Laboratories, Sweden). The standard curves for all of the primers exhibited high efficiency and an R^2 value > 0.99. In addition, single melting peaks were observed during melt curve analysis, confirming the presence of only a single product. The dilutions found to be suitable were 1:30 in the case of REDD 1 and 2 and hVps34, 1:40 for Rheb, 1:200 for MuRF-1 and MAFbx, and 1:600 for GAPDH.

The reliability of GAPDH mRNA as an internal control was validated by the $2^{-\Delta C't}$ method, where $\Delta C'_T = C_{T\ time\ x} - C_{T\ time\ 0}$, as described by Livak and Schmittgen (37). The mean $2^{-\Delta C't}$ values for the exercising leg in the placebo and BCAA condition were 0.84 ± 0.16 and 1.09 ± 0.24, respectively. The corresponding values in the resting leg were 0.94 ± 0.19 and 1.05 ± 0.19. A two-way repeated measures ANOVA (leg x supplement) revealed no main effect or interaction

($P > 0.43$), i.e. the level of GAPDH mRNA was not influenced by either exercise or supplementation and was therefore suitable as an internal control.

All analyses were performed in triplicate and all samples from each subject were analyzed on a single 96-well plate for direct relative comparisons. The 25-µL mixtures for RT-qPCR amplification contained 2 x SYBR Green Supermix (Bio-Rad Laboratories, Sweden), 11.5 µL template cDNA diluted in RNase-free water and 0.5 µL of 10 µmol/L forward and reverse primers (Cybergene, Stockholm, Sweden). The details concerning these primers are documented in Table 1. The RT-qPCR reactions were carried out in a Bio-Rad iCycler (Bio-Rad Laboratories), initially at 95°C for 3 min, followed by thermal cycling, 40x at 95°C, 58°C and 72°C for 30 seconds each. All of the C_T values obtained ranged between 18 and 29, and the same fixed threshold was employed for all genes of interest. Relative changes in mRNA expression for all genes of interest were quantified by the $2^{-\Delta\Delta Ct}$ method, described by Schmittgen and Livak (54) and the results are expressed as fold-changes in comparison to baseline (3h vs. Pre-Ex) for all conditions, i.e. resting and exercising leg with placebo or BCAA supplementation.

Statistical analyses

All data are expressed as means ± SE and were checked for normal distribution before performing parametric statistical analyses. For variables exhibiting a positively skewed distribution, log-transformation was performed prior to analysis. A two-way repeated measures ANOVA (leg and supplement) was employed to compare fold-changes (from Pre-Ex) in the levels of mRNA expression in the resting and exercising legs and with BCAA and placebo supplementation. In the case of MAFbx mRNA, an outlier value higher than six standard deviations above the mean was excluded from the analysis.

To detect changes in plasma concentrations of glucose, lactate and amino acids, a two-way repeated measures ANOVA was employed, with time and type of supplementation as factors. A

three-way repeated measures ANOVA was used to evaluate changes in muscle amino acid concentrations, in phosphorylation of enzymes in the mTOR pathway and in protein content of MAFbx and MuRF-1 with time (Pre-Ex, Post-Ex, 1 and 3 h of recovery), supplementation and leg as factors. In this latter analysis, the Post-Ex and 1h values for two participants were missing; however, exclusion of these same participants from the analysis had no significant impact on the mean values or P-values obtained. When a significant main effect and/or interaction was observed, Fisher's LSD *post hoc* test was employed to pinpoint where the differences occurred. All statistical analyses were performed with the Statistica 8.0 software (StatSoft Inc., Tulsa, USA) and a P-value < 0.05 was considered to be statistically significant.

Results

Resistance exercise

With either placebo or BCAA supplementation all subjects were able to perform the first four sets of 10 repetitions at 80% 1 RM. During the final four sets of 15 repetitions at 65% 1 RM one subject could only perform 14 and 13 repetitions in the last two sets, respectively, in both conditions. The average workload in the first and final four sets was 130 ± 15 kg and 105 ± 12 kg, respectively.

Plasma concentrations

The plasma concentrations of glucose with BCAA or placebo supplementation did not differ, but was reduced under both conditions ($P<0.05$) following 30 min of recovery and was attenuated throughout the remaining period of recovery. The initial glucose concentrations were 5.4 ± 0.2 and 5.1 ± 0.2 mmol · L^{-1} under the placebo and BCAA conditions, respectively, and did not fall below 4.5 mmol · L^{-1} at any time point. Plasma levels of lactate were significantly elevated ($P <$ 0.05) during resistance exercise, reaching a maximum of 8 ± 1.5 mmol · L^{-1} and returning to

basal levels following 60 min of recovery, with no differences between placebo and BCAA supplementation.

As expected and as illustrated in Fig. 1A and Table 2, supplementation with BCAA resulted in a rapid elevation of the plasma levels of these amino acids, with a 113% increase following 30 min of recovery, and this value remained higher than the corresponding values at rest and with placebo supplementation throughout the rest of the trial ($P < 0.05$). Following supplementation, the changes in plasma for the individual BCAA differed. The plasma concentration of valine peaked following 30 min of recovery at a value 76% higher than at rest and remained significantly elevated during the entire recovery period. The corresponding values for leucine and isoleucine at the same time-point were 150% and 175%, respectively, and the isoleucine concentration returned to basal levels after 120 min of recovery, whereas, the concentration of leucine was still significantly elevated following 180 min of recovery. In the placebo condition, the plasma concentration of BCAA declined slightly 15 min after exercise and was attenuated during the remaining 180 min of recovery ($P < 0.05$).

With both kinds of supplementation the plasma levels of phenylalanine fell ($P<0.05$) below baseline values directly after resistance exercise (Fig. 1B). In the case of BCAA supplementation this level was further reduced following 60 min of recovery and was lower than the corresponding placebo value the remainder of the recovery period ($P < 0.05$). As also depicted in Fig. 1C, the plasma levels of essential amino acids other than BCAA (EAA-BCAA), were lower ($P < 0.05$) compared to the levels at rest following 15 min of recovery than at rest and remained attenuated thereafter, with no difference between the placebo and BCAA conditions.

Muscle amino acids

Ingestion of BCAA also resulted in a significant enhancement of these amino acids in the vastus lateralis muscle of both the resting and exercising legs (Table 3, 4 and Fig. 2A). Analysis by

three-way ANOVA revealed main effects of time ($P < 0.05$) and the type of supplementation ($P < 0.01$), as well as an interaction between these two factors ($P < 0.01$). In the exercising and resting legs, the highest level of BCAA were observed immediately after exercise and following 1 h of recovery, respectively. The magnitude of the changes in muscle concentrations of valine, leucine and isoleucine (Tables 3 and 4) paralleled the corresponding changes in the plasma.

The muscle level of phenylalanine was not altered immediately after exercise with either supplementation or in either leg. However, following 1 h of recovery, this level was reduced ($P < 0.05$) by 15 and 20% in the exercising and resting legs, respectively, in the placebo condition. This was also the case with BCAA supplementation, however, to a significantly greater extent; 37 % and 45 % ($P < 0.05$), in the resting and exercising leg, respectively (Fig. 2B). Thereafter, the phenylalanine concentrations remained reduced in both legs under both conditions during the entire recovery period. Muscle levels of tyrosine exhibited a similar pattern, but slightly delayed.

Muscle levels of EAA-BCAA were unaltered immediately after exercise under both conditions, but reduced in both legs ($P < 0.05$) following 1 and 3 h of recovery with BCAA supplementation. At these same time-points these latter concentrations were also lower ($P < 0.05$*)* than in the corresponding leg in the placebo condition. At all time-points in the placebo condition values were unaltered in comparison to Pre-Ex values (Fig. 2C).

Protein signaling

The phosphorylation status of Akt at Ser^{473} was unaltered with both supplements and in both legs following 1 and 3 h of recovery from resistance exercise (data not shown), whereas the phosphorylation of mTOR at Ser^{2448} was increased 1.5 to 3-fold ($P < 0.05$) in both legs 1 and 3 h following exercise in both the placebo and the BCAA conditions (Fig. 3A, B). Phosphorylation of $p70^{S6k}$ at Thr^{389} was also enhanced in both legs 1 and 3 h after exercise ($P < 0.05$) and in addition, the level was 2.1 and 2.4-fold higher in the resting and exercising leg, respectively, in

the BCAA condition at 1 h of recovery ($P < 0.05$) (Fig. 3C, D). The phosphorylation of 4E-BP1 at $Thr^{36/37}$, a downstream target of mTOR remained unaltered in both legs during both conditions (data not shown).

The total protein level of MAFbx did not change significantly over time in any leg or in any condition, although there was a tendency for interaction between supplement and time ($P = 0.14$) (Fig. 4A, B). The protein content of MuRF-1 was increased 20-40% 3 h following exercise in both legs in the placebo condition ($P < 0.05$), whereas the level remained unchanged with the BCAA supplement (Fig. 4C, D). In addition, there was a strong trend ($P = 0.052$) for a lower expression of MuRF-1 total protein in both legs 3 h after exercise during BCAA supplementation in comparison to placebo (Fig. 4C, D).

Levels of mRNA expression

All of these data are expressed as fold-changes following 3 hours of recovery in comparison to Pre-Ex levels.

Proteolytic factors. Supplementation with BCAA resulted in levels of MAFbx mRNA that were 50% and 70% of those observed at Pre-Ex in the exercising and resting legs, respectively, whereas with placebo the levels were 1.4-fold of initial values in both legs (Fig. 4E). The levels of MAFbx mRNA were significantly lower ($P < 0.05$) with BCAA supplementation compared to placebo. The expression of MuRF-1 mRNA was significantly higher in the exercising compared to the resting leg ($P < 0.05$), 3-fold in the case of the placebo and 2-fold with BCAA (Fig. 4F) with no significant difference between these supplements, mainly due to one subject who deviated from the others and showed a considerably larger increase in the BCAA trial.

Proposed negative regulators of mTOR. Neither exercise nor any form of supplementation had any significant impact on the expression of REDD1 mRNA (Fig. 5A). In contrast, the

expression of REDD2 mRNA in the exercising leg tended to be reduced ($P = 0.07$), 32 and 24 % under the placebo and BCAA conditions, respectively (Fig. 5B).

Proposed positive regulators of mTOR. The levels of Rheb mRNA tended to be lower in both legs after BCAA supplementation compared to placebo ($P = 0.05$) (Fig. 5C). The fold increases in the BCAA condition were 1.2- and 1.5-fold compared to 1.6- and 2.4-fold in the placebo condition in the resting and exercising legs, respectively. The content of hVps34 mRNA was not significantly different in any condition (Fig. 5D).

There were no obvious differences between male and female subjects regarding the response to BCAA supplementation with regard to the analyzed variables; however, the number of subjects was too small to allow any comparative analyses between men and women.

Discussion

The present investigation was designed to evaluate the influence of resistance exercise and BCAA, alone or in combination on selective markers for muscle protein synthesis and breakdown. The major novel findings are that supplementation with BCAA reduced the expression of MAFbx mRNA in both resting and exercising muscle and prevented the increase in total protein expression of the other ubiquitin ligase MuRF-1, which in contrast to MAFbx exhibited an increase in both mRNA and protein expression after resistance exercise. Furthermore, muscle levels of phenylalanine and tyrosine were reduced during recovery from resistance exercise, most potently after BCAA supplementation, which also attenuated muscle levels of EAA (BCAA excluded). Phosphorylation of $p70^{S6k}$ increased to larger extent in the early recovery period when BCAA were ingested and the level was still elevated in both legs three hours after exercise, although at this time the level was similarly elevated in the placebo condition.

This reduction in the level of MAFbx mRNA by BCAA has not been reported previously in human muscle. However, no significant effect on total protein content was observed, suggesting that the three hours time span was too short to detect changes in MAFbx protein. In contrast, the levels of both MuRF-1 mRNA and total protein were increased in the exercised leg (Fig. 4), which is in accordance with results from Glynn et al. (24). Furthermore, intake of BCAA prevented the increase in total protein and attenuated (insignificantly) the increase in mRNA level. This concordance between mRNA expression and total protein of MuRF-1 might reflect the relatively large changes in the mRNA expression of MuRF-1 reported here and by others (24, 40, 43). Administration of BCAA or leucine alone has earlier been reported to reduce the levels of both MAFbx and MuRF-1 mRNA in starved C2C12 myotubes (26) and in atrophied rat muscle (5). Of interest in this context are the observations by Herningtyas and colleagues (26) that although BCAA reduces the expression of both of these genes in myotubes the effect on MAFbx mRNA was dependent on activation of mTOR, whereas the effect on MuRF-1 was not, indicating differential regulation of these two ubiquitin ligases.

The divergent response to exercise with respect to the levels of MAFbx and MuRF-1 mRNA observed here is in agreement with most previous studies (12, 13, 19, 40, 43). This may reflect the different functions of these two proteins, MAFbx targets the regulatory protein transcription factor MyoD (34) and the eukaryotic initiation factor-3f (eIF-3f) (35) which is of importance in mTOR-$p70^{S6k}$ signaling, whereas MuRF-1 appears to interact with structural proteins like titin and myosin light chains 1 and 2 (14, 22). Our current observation that the level of MAFbx mRNA decreases following BCAA supplementation, suggests that the corresponding protein attenuates the breakdown of regulatory proteins involved in hypertrophic signaling mediated by mTOR. The acute increase in MuRF-1 expression following resistance exercise is likely to reflect enhanced degradation of contractile proteins. The similar response to BCAA but not to exercise suggests the presence of both common and divergent regulation of these ubiquitin

ligases; however, we were not able to detect involvement of Akt activation in either case. The absence of effect on Akt phosphorylation directly after resistance exercise (2) as well as 1 and 3 h later despite intake of BCAA, may be explained by the minor elevation in insulin (2) which probably was insufficient to activate Akt. Although phosphorylation of both mTOR and $p70^{S6k}$ was elevated after exercise, the latter to larger extent with BCAA supplementation (Fig. 3), phosphorylation of 4E-BP1 remained unchanged in both conditions. This finding is in agreement with some (17, 59) but not all (18, 45) previous studies. The divergent effects seen on $p70^{S6k}$ and 4E-BP1 here and the discrepancy in the literature suggest that these two downstream targets of mTOR may be differentially regulated under certain conditions (11).

Ingestion of BCAA led to a more pronounced reduction in the concentration of the aromatic amino acids, tyrosine and phenylalanine, in both plasma and muscle as well as muscle EAA (BCAA excluded) during the recovery period (Table 3, 4), an effect which is likely to be caused by leucine as demonstrated by Eriksson et al. (21). The results here are in agreement with previous studies showing a reduction in plasma and muscle levels of tyrosine and phenylalanine at rest (1) or after endurance exercise (9) following administration of leucine or BCAA. The novel finding in the present study is that similar reductions were seen in resting and exercising muscle. Since tyrosine and phenylalanine are neither synthesized nor degraded in skeletal muscle, reduction in the levels of these amino acid could be indicative of an improved net muscle protein balance, i.e. an enhanced rate of synthesis and/or decreased rate of breakdown. In the study by Alvestrand and colleagues (1), the exchange of amino acids from the muscle was calculated from measurements of blood flow and arterial-venous concentration differences, and since no change in the net release of tyrosine and phenylalanine was detected the authors concluded that part of the observed fall in intracellular amino acids could be explained by incorporation into protein. These observations, together with our findings that ingestion of BCAA led to a more pronounced increase in $p70^{S6k}$ phosphorylation, attenuated MAFbx

expression and prevented the exercise-induced increase in MuRF-1 protein provide additional support to the conclusion that administration of BCAA has anabolic effects on human skeletal muscle (9, 39, 48).

The similar reductions in the levels of phenylalanine and EAA-BCAA during the recovery phase in both resting and exercising leg following BCAA supplementation (Fig. 2) was unexpected. Supplementation with amino acids influences $p70^{S6k}$ phosphorylation and the rate of protein synthesis more potently when combined with resistance exercise (2, 8, 46). However, Moore and colleagues (46) have reported that the rates of protein synthesis in resting and exercising human muscle in response to amino acid ingestion are similar during the initial 1-3 h of recovery. Thus, changes in muscle levels of phenylalanine and EAA-BCAA might have occurred later than the 3 h period of recovery monitored here. Other possible explanations for the similar effect of BCAA on resting and exercising muscle are that resistance exercise also increases the rate of protein breakdown, thus partially counterbalancing the enhanced synthesis (52) or that because our subjects were fasting, the availability of the remaining EAA may have been inadequate to further increase the rate of synthesis in the exercised muscle (60).

In the present investigation we observed a tendency for BCAA supplementation to attenuate the elevation in the level of Rheb mRNA in both resting (1.7-fold under the placebo versus 1.2-fold in the BCAA condition) and exercising muscle (2.4-fold versus 1.5-fold). Although we did not asses statistical differences from baseline (Pre-Ex), the 2.4-fold elevation (individual values ranging from 1.6 – 5.0) with placebo supplementation clearly indicates an enhancement of this expression following exercise, which was not observed in the case of BCAA supplementation (Fig. 5C). Drummond and coworkers (20) demonstrated a 1.5-fold increase in the level of Rheb mRNA above baseline following combined resistance exercise and amino acid ingestion, but did not examine the effect of exercise alone. Rheb is a low-molecular weight GTPase located immediately up-stream of mTOR and involved in its activation (42). The

mechanisms through which various stimuli influence Rheb-mTOR signaling have not yet been fully investigated, but present evidence indicates that growth factors stimulate binding of GTP to Rheb, whereas amino acids may affect the formation of a Rheb-mTOR complex (38). It is not known whether the levels of Rheb mRNA influence the Rheb-mTOR interaction. This interaction is rapidly inhibited in cultured cells during withdrawal of leucine from the incubation media (38), however, future studies are needed to clarify whether a reduction in Rheb mRNA expression influences the binding of Rheb to mTOR.

HVps34 which seems to play a pivotal role in the control of macroautophagy (51) has also been suggested to play a role in the activation of mTOR by amino acids (25). However, in agreement with others (20) here the mRNA expression of this protein was unaltered by either resistance exercise or amino acid supplementation suggesting that amino acids do not promote hVps34 signaling by elevating the level of this protein.

The stress/hypoxia-induced proteins REDD 1 and 2 act as negative regulators of mTOR (15) by modulating the tuberous sclerosis tumor suppressor protein-2 (TSC2) (16, 44), located upstream of mTOR. In the present investigation the level of REDD2 mRNA in the exercising leg tended to be lower (P=0.07) with both types of supplementation, with no effect on REDD1. This dissimilar effect of resistance exercise on REDD 1 and 2 is in agreement with the findings of Drummond and colleagues (20). Moreover, our findings suggest that exercise rather than amino acids is responsible for the lower expression observed here.

In conclusion, BCAA supplementation reduced the expression of MAFbx mRNA and prevented the increase in MuRF-1 total protein in both resting and exercising muscle. Resistance exercise caused an elevation in the level of MuRF-1 mRNA and total protein, without significantly influencing MAFbx, suggesting both common and divergent regulation of these two ubiquitin ligases. Furthermore, the reduction in the level of phenylalanine and tyrosine, as well as the sum of EAA (BCAA excluded) in both resting and exercising muscle during the three-

hour recovery period was more pronounced with BCAA supplementation. These observations, together with the BCAA-induced enlargement in $p70^{S6k}$ phosphorylation and attenuation of MAFbx expression and MuRF-1 total protein provide additional support for the view that BCAA has an anabolic effect on human skeletal muscle, an effect which appears to be similar in resting and exercising human muscle.

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Disclosure

No conflict of interest, financial or otherwise, are declared by the authors.

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224 **Figure legends**

Figure 1

The plasma concentration of BCAA (A), phenylalanine (B) and EAA-BCAA (C) under the placebo session (triangles) and the BCAA session (boxes). The values presented are means ± SE (n=7). *Different from Rest, $P < 0.05$; #different from placebo, $P < 0.05$.

Figure 2

Levels of BCAA (A), phenylalanine (B) and EAA-BCAA (C) in the vastus lateralis muscle of the exercising leg (filled boxes or triangles) and the resting leg (white boxes or triangles) following supplementation with BCAA or placebo. The values are means ± SE (n=7 for Pre-Ex and 3h and 5 for Post-Ex and 1h). *Different from Pre-Ex, $P < 0.05$; #different from placebo, $P < 0.05$.

Figure 3

Phosphorylation of mTOR at Ser^{2448} (A, B) and $p70^{S6k}$ at Thr^{389} (C, D) in the resting and exercising leg with placebo and BCAA supplementation. Data are expressed relative to total protein content of α-tubulin. Representative immunoblots from one subject is shown above each graph, the bands have been separated to fit the illustrative bars. The values are arbitrary units and presented as means ± SE (n=7 for Pre-Ex and 3h and 5 for Post-Ex and 1h). *Different from Pre-Ex, $P < 0.05$; #different from placebo, $P < 0.05$.

Figure 4

Total protein content of MAFbx (A, B) and MuRF-1 (C, D) and levels of mRNA encoding MAFbx (E) and MuRF-1 (F) in the vastus lateralis muscle of the exercising and resting leg with placebo and BCAA supplementation after 3 h of recovery. The mRNA values are fold-changes

from baseline (Pre-Ex) and presented as means ± SE (n=7 or 6 for MAFbx, see the section of Statistical analyses). Protein data are expressed relative to total protein content of α-tubulin. Representative immunoblots from one subject is shown above graphs A-D. *Different from Pre-Ex, $P < 0.05$; $^{\#}$different from placebo, $P < 0.05$; $^{(\#)}$ different from placebo, $P = 0.05$; †different from resting leg, $P < 0.05$.

Figure 5

Levels of mRNA encoding REDD 1 (A), REDD 2 (B), Rheb (C) and hVps34 (D) in the vastus lateralis muscle of the exercising and resting leg with BCAA or placebo supplementation after 3h of recovery. These values are fold-changes from baseline (Pre-Ex) and presented as means ± SE (n=7).

Table 1. Details of the primers employed for RT-qPCR

Gene	Function of the product	Forward primer	Reverse primer	No. in Genebank
MuRF-1	Regulator of proteolysis	GCCACCTTCCTCTTGACTG	ATTCTTCCTCTTCATCTGTC	NM_032588
MAFbx	Regulator of proteolysis	GAGCGACCTCAGCAGTTAC	GGCAGTTGAGAAGTCCAGTC	NM_148177
Rheb	Positive regulator of mTOR	TTTTTGGAATCTTCTGCTAAAGAAA	AAGACTTGCCTTGTGAAGCTG	NM_005614
hVps34	Nutrient regulator of mTOR	GAAGCAGATGGATCAGAACC	CCAGCCAATCTACTTTCACC	NM_002647
REDD1	Negative regulator of mTOR	CTGGAGAGCTCGGACTGC	TCCAGGTAAGCCGTGTCTTC	NM_019058
REDD2	Negative regulator of mTOR	CCCAGAGAGCCTGCTGCTAAGTG	TTGCTTTGATTTGGACAGACA	NM_145244
GAPDH	Housekeeping (control)	AACCTGCCAAATATGATGAC	TCATACCAGGAAATGAGCTT	NM_002046

MuRF-1, muscle RING finger-1; MAFbx, muscle atrophy F-box; Rheb, ras-homologue enriched in brain; hVps34, human vacuolar protein sorting-34; REDD1 and 2, regulated in development and DNA damage response-1 and 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Plasma concentrations of amino acids following BCAA or placebo ingestion

Amino acid	Condition	Rest	Pre-Ex	Mid -Ex	Post-Ex	15 min	30 min	60 min	120 min	180 min
Histidine	Placebo	90 ± 2	88 ± 4	90 ± 4	88 ± 3	80 ± 4*	84 ± 2*	83 ± 1*	84 ± 2*	82 ± 2*
	BCAA	91 ± 4	92 ± 4	94 ± 4	90 ± 4	82 ± 4*	84 ± 4*	85 ± 3*	84 ± 4*	83 ± 3*
Isoleucine	Placebo	65 ± 6	60 ± 5	65 ± 8	51 ± 5	47 ± 4	47 ± 4	45 ± 3*	47 ± 3	51 ± 3
	BCAA	57 ± 4	87 ± 12*#	135 ± 7*#	121 ± 9*#	114 ± 15*#	158 ± 23*#	146 ± 15*#	90 ± 7*#	70 ± 5#
Leucine	Placebo	124 ± 10	117 ± 10	122 ± 14	99 ± 8	92 ± 8*	93 ± 8*	89 ± 7*	94 ± 7	102 ± 7
	BCAA	118 ± 8	168 ± 19*#	250 ± 13*#	227 ± 15*#	217 ± 26*#	292 ± 40*#	275 ± 25*#	185 ± 14*#	151 ± 11*#
Lysine	Placebo	187 ± 15	186 ± 14	176 ± 15	172 ± 12	157 ± 12*	165 ± 16*	161 ± 15*	164 ± 16*	160 ± 14*
	BCAA	182 ± 16	185 ± 14	183 ± 15	177 ± 18	151 ± 20*	167 ± 18*	158 ± 16*	149 ± 11*	140 ± 13*
Methionine	Placebo	27 ± 1	25 ± 2	25 ± 3	25 ± 2	23 ± 2*	23 ± 2*	22 ± 2*	22 ± 2*	22 ± 2*
	BCAA	27 ± 1	28 ± 1	28 ± 1	26 ± 2	23 ± 2*	25 ± 1*	22 ± 1*	20 ± 1*	18 ± 1*
Phenylalanine	Placebo	57 ± 3	55 ± 3	52 ± 5	51 ± 3*	49 ± 3*	49 ± 4*	47 ± 4*	48 ± 3*	49 ± 3*
	BCAA	52 ± 3	53 ± 3	51 ± 3	48 ± 3*	45 ± 3*	46 ± 2*	39 ± 3*#	36 ± 3*#	38 ± 3*#
Threonine	Placebo	147 ± 4	139 ± 5	139 ± 8	138 ± 5	128 ± 7*	137 ± 6*	129 ± 8*	122 ± 7*	116 ± 6*
	BCAA	143 ± 5	144 ± 5	143 ± 7	138 ± 9	129 ± 8*	136 ± 8*	127 ± 9*	119 ± 9*	111 ± 8*
Tryptophan	Placebo	58 ± 2	58 ± 3	54 ± 3	53 ± 2*	50 ± 3*	52 ± 1*	51 ± 2*	48 ± 2*	46 ± 2*
	BCAA	55 ± 3	55 ± 4	52 ± 3	49 ± 2*	46 ± 2*	48 ± 2*	45 ± 3*	39 ± 3*	40 ± 3*
Tyrosine	Placebo	63 ± 5	59 ± 5	56 ± 8	57 ± 5*	51 ± 5*	53 ± 5*	50 ± 4*	49 ± 4*	48 ± 4*
	BCAA	61 ± 7	61 ± 6	59 ± 7	55 ± 7*	49 ± 6*	51 ± 5*	43 ± 5*#	37 ± 6*#	33 ± 4*#
Valine	Placebo	224 ± 10	210 ± 10	222 ± 15	192 ± 8	178 ± 9*	185 ± 9*	176 ± 8*	175 ± 9*	175 ± 9*
	BCAA	212 ± 14	255 ± 21*#	343 ± 19*#	331 ± 26*#	311 ± 26*#	372 ± 41*#	373 ± 24*#	302 ± 24*#	259 ± 16*#
BCAA	Placebo	413 ± 24	387 ± 24	409 ± 37	342 ± 20	316 ± 21*	326 ± 21*	310 ± 18*	316 ± 18*	328 ± 18*
	BCAA	387 ± 25	510 ± 51*#	728 ± 36*#	679 ± 40*#	642 ± 65*#	822 ± 99*#	795 ± 62*#	578 ± 44*#	479 ± 32*#
EAA - BCAA	Placebo	567 ± 19	551 ± 24	536 ± 35	528 ± 19	486 ± 24*	510 ± 27*	494 ± 29*	487 ± 29*	476 ± 25*
	BCAA	550 ± 17	557 ± 11	550 ± 21	529 ± 27	475 ± 25*	506 ± 25*	476 ± 28*	447 ± 23*	430 ± 24*

The values presented are means ± SE (μmol ·L^{-1}) for 7 subjects. BCAA: branched-chain amino acids, EAA-BCAA: essential amino acids excluding BCAA. Blood samples were collected at rest prior to warm-up, immediately before the resistance exercise (Pre-Ex), after the fifth set (Mid-Ex), directly after termination of the exercise (Post-Ex) and following 15, 30, 60, 120 and 180 min of recovery. *Different from Rest, $P < 0.05$; $^{\#}$Different from placebo, $P < 0.05$

Table 3. Levels of amino acids in the vastus lateralis muscle of the resting leg following supplementation with either BCAA or placebo.

Amino acid	Condition	Pre-Ex	Post-Ex	1h	3h
Histidine	Placebo	1220 ± 70	1580 ± 110	1430 ± 120	1370 ± 120
	BCAA	1290 ± 130	1330 ± 240	1400 ± 180	1160 ± 130
Isoleucine	Placebo	260 ± 30	210 ± 30	190 ± 10	220 ± 20
	BCAA	230 ± 20	410 ± 40*#	450 ± 40*#	280 ± 40*#
Leucine	Placebo	500 ± 50	380 ± 50	350 ± 30	430 ± 40
	BCAA	470 ± 40	810 ± 90*#	910 ± 70*#	600 ± 90*#
Lysine	Placebo	1990 ± 250	2190 ± 370	1980 ± 410	1950 ± 240
	BCAA	1900 ± 220	1730 ± 280	1610 ± 210	1300 ± 200
Methionine	Placebo	110 ± 20	100 ± 10	90 ± 10	110 ± 10
	BCAA	100 ± 10	110 ± 10	70 ± 10*#	70 ± 10*#
Phenylalanine	Placebo	210 ± 20	190 ± 20	170 ± 20*	180 ± 10*
	BCAA	190 ± 10	180 ± 20	120 ± 20*#	130 ± 10*#
Threonine	Placebo	1990 ± 250	2190 ± 370	1980 ± 410	1950 ± 240
	BCAA	1900 ± 220	1730 ± 280	1610 ± 210	1300 ± 200
Tryptophan	Placebo	60 ± 10	60 ± 5	60 ± 10	55 ± 5*
	BCAA	60 ± 10	60 ± 10	50 ± 10	40 ± 5*
Tyrosine	Placebo	250 ± 30	240 ± 40	230 ± 20	210 ± 20*
	BCAA	260 ± 30	240 ± 40	190 ± 50*#	150 ± 20*#
Valine	Placebo	660 ± 50	560 ± 50	540 ± 40	560 ± 40
	BCAA	620 ± 30	830 ± 70*#	970 ± 100*#	720 ± 70*#
BCAA	Placebo	1430 ± 120	1140 ± 110	1080 ± 70	1210 ± 90
	BCAA	1330 ± 80	2060 ± 200*#	2330 ± 210*#	1600 ± 200*#
EAA - BCAA	Placebo	5670 ± 400	6190 ± 490	5910 ± 490	5690 ± 260
	BCAA	5650 ± 460	5610 ± 530	5320 ± 480*#	4580 ± 310*#

The values presented are means ± SE (μmol · kg^{-1} dry muscle tissue) for 7 subjects, except for Post-Ex and 1h where the number of subjects is five. BCAA: branched-chain amino acids, EAA-BCAA: essential amino acids, excluding BCAA. Muscle biopsies were collected at rest prior to warm-up (Pre-Ex), directly after termination of the exercise (Post-Ex) and following 1h and 3h of recovery.; *Different from Pre-Ex, $P < 0.05$; #Different from placebo, $P < 0.05$

Table 4. Levels of amino acids in the vastus lateralis muscle of the exercising leg following supplementation with either BCAA or placebo.

Amino acid	Condition	Pre-Ex	Post-Ex	1h	3h
Histidine	Placebo	1280 ± 100	1570 ± 70	1390 ± 50	1240 ± 170
	BCAA	1120 ± 120	1260 ± 100	1170 ± 110	1140 ± 140
Isoleucine	Placebo	250 ± 30	220 ± 20	190 ± 10	210 ± 20
	BCAA	260 ± 10	440 ± 70*#	400 ± 50*#	290 ± 40*#
Leucine	Placebo	470 ± 50	390 ± 40	350 ± 30	400 ± 60
	BCAA	530 ± 30	850 ± 140*#	790 ± 80*#	630 ± 80*#
Lysine	Placebo	1970 ± 250	2180 ± 360	2000 ± 230	1820 ± 300
	BCAA	1680 ± 160	1790 ± 150	1430 ± 120	1290 ± 200
Methionine	Placebo	100 ± 10	110 ± 10	100 ± 10	100 ± 20
	BCAA	110 ± 10	110 ± 10	70 ± 10*#	80 ± 10*#
Phenylalanine	Placebo	200 ± 20	200 ± 10	170 ± 10*	170 ± 20*
	BCAA	220 ± 10	200 ± 20	120 ± 20*#	130 ± 10*#
Threonine	Placebo	1920 ± 90	2190 ± 140	2320 ± 120	2190 ± 70
	BCAA	2150 ± 130	2400 ± 150	1900 ± 60	2180 ± 240
Tryptophan	Placebo	60 ± 5	60 ± 5	60 ± 5	50 ± 5*
	BCAA	60 ± 5	70 ± 10	50 ± 5	40 ± 5*
Tyrosine	Placebo	240 ± 20	240 ± 40	210 ± 30	200 ± 20*
	BCAA	270 ± 30	260 ± 50	180 ± 40*#	160 ± 20*#
Valine	Placebo	610 ± 50	590 ± 40	550 ± 30	540 ± 40
	BCAA	660 ± 40	870 ± 100*#	830 ± 80*#	760 ± 50*#
BCAA	Placebo	1330 ± 120	1200 ± 100	1080 ± 50	1140 ± 110
	BCAA	1460 ± 70	2150 ± 320*#	2010 ± 200*#	1680 ± 160*#
EAA - BCAA	Placebo	5510 ± 400	6300 ± 510	6000 ± 330	5550 ± 400
	BCAA	5330 ± 300	5810 ± 100	4730 ± 240*#	4830 ± 320*#

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The values presented are means ± SE (μmol · kg^{-1} dry muscle tissue) for 7 subjects, except for Post-Ex and 1h where the number of subjects is five. BCAA: branched-chain amino acids, EAA-BCAA: essential amino acids, excluding BCAA. Muscle biopsies were collected at rest prior to warm-up (Pre-Ex), directly after termination of the exercise (Post-Ex) and following 1h and 3h of recovery. *Different from Pre-Ex, $P < 0.05$; #Different from placebo, $P < 0.05$

Figure 1

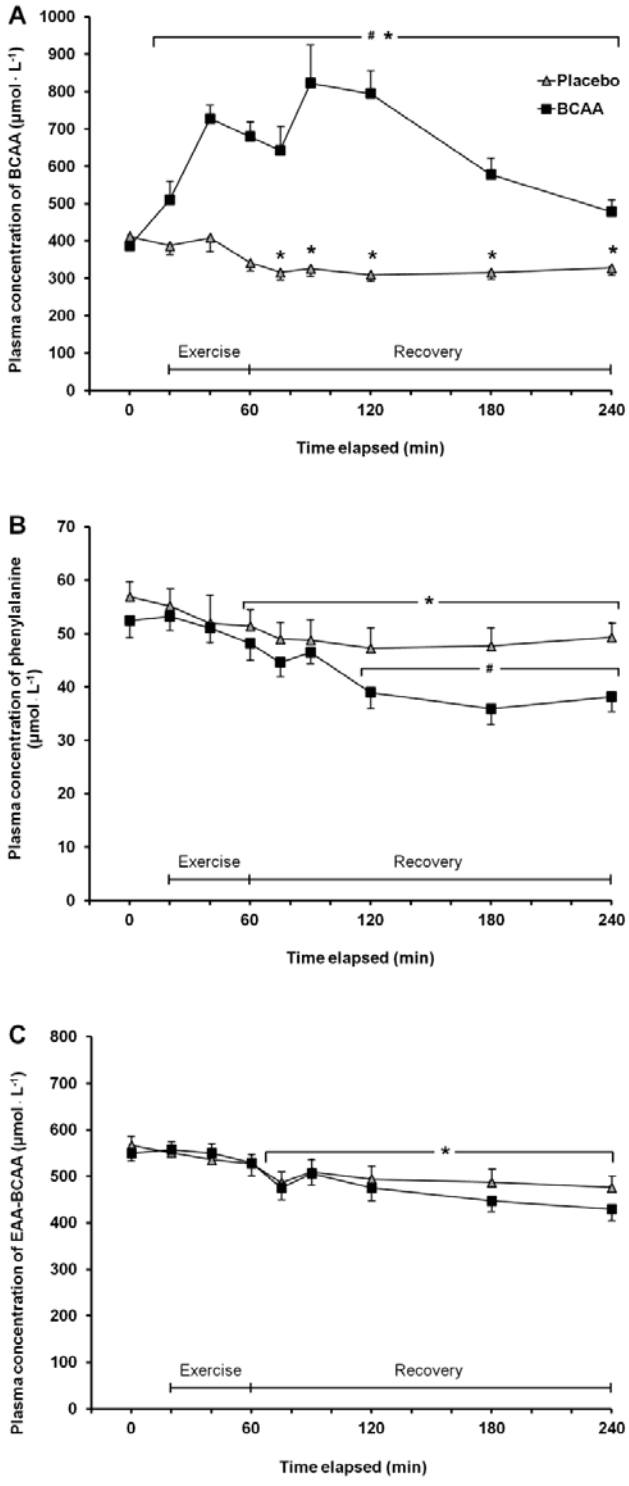

A
Plasma concentration of BCAA (μmol · L⁻¹)
Placebo
BCAA
Exercise
Recovery
Time elapsed (min)
B
Plasma concentration of phenylalanine (μmol · L⁻¹)
Exercise
Recovery
Time elapsed (min)
C
Plasma concentration of EAA-BCAA (μmol · L⁻¹)
Exercise
Recovery
Time elapsed (min)

Figure 2

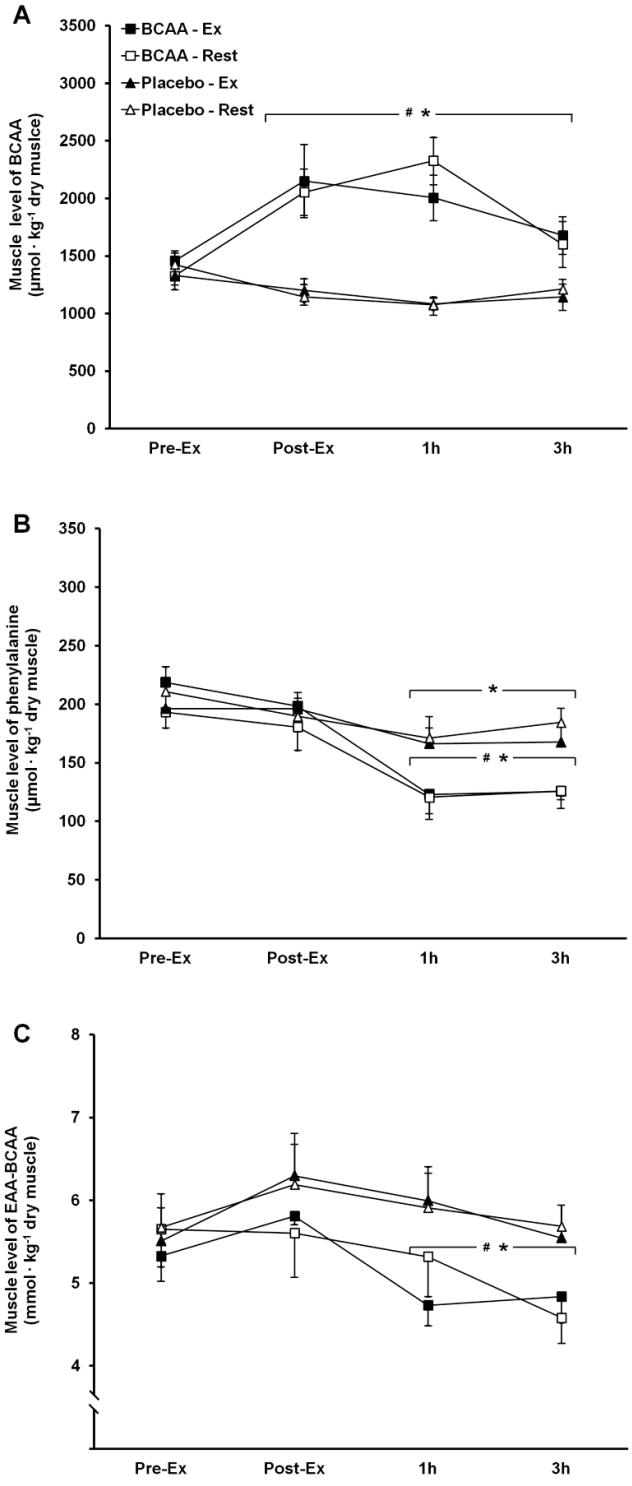

A
BCAA - Ex
BCAA - Rest
Placebo - Ex
Placebo - Rest
*
Muscle level of BCAA (μmol · kg-1 dry muslce)
3500
3000
2500
2000
1500
1000
500
0
Pre-Ex
Post-Ex
1h
3h
B
*
*
Muscle level of phenylalanine (μmol · kg-1 dry muscle)
350
300
250
200
150
100
50
0
Pre-Ex
Post-Ex
1h
3h
C
*
Muscle level of EAA-BCAA (mmol · kg-1 dry muscle)
8
7
6
5
4
Pre-Ex
Post-Ex
1h
3h

Figure 3

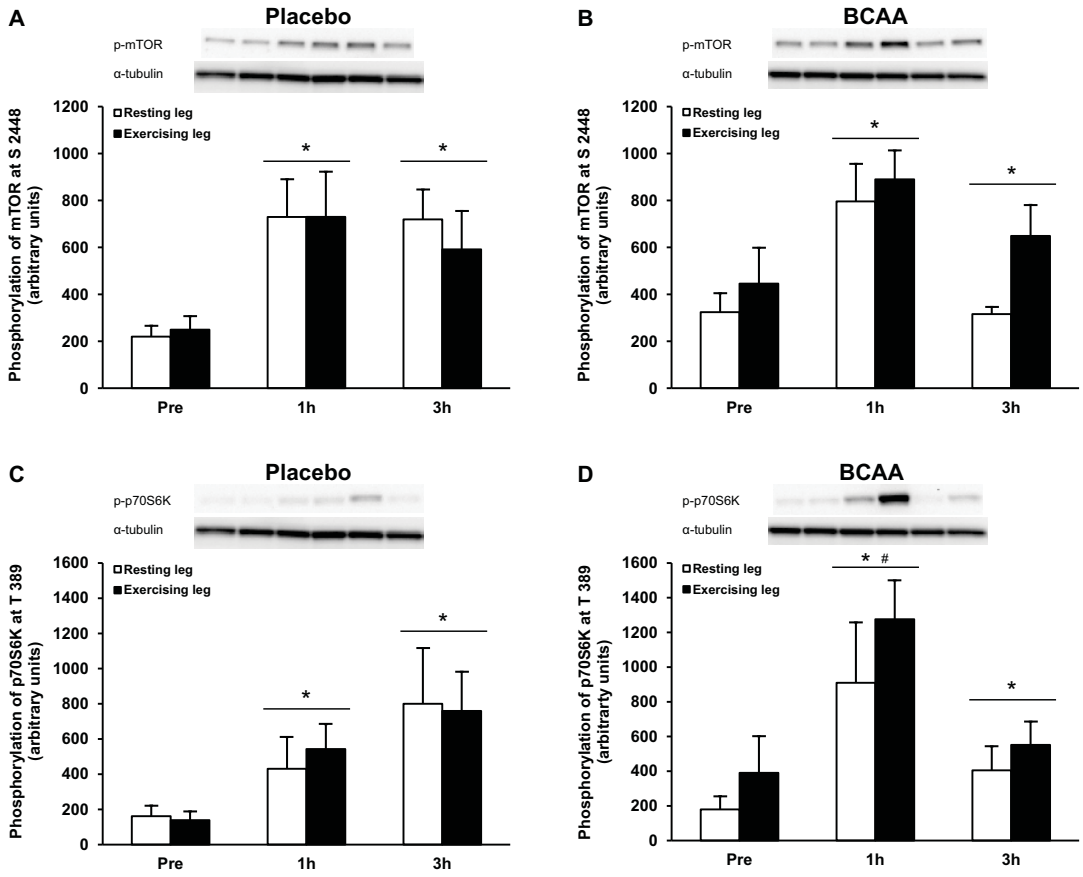

A
Placebo
p-mTOR
α-tubulin
Resting leg
Exercising leg
Phosphorylation of mTOR at S 2448 (arbitrary units)
Pre
1h
3h
B
BCAA
C
Placebo
p-p70S6K
Phosphorylation of p70S6K at T 389 (arbitrary units)
D
BCAA

Figure 4

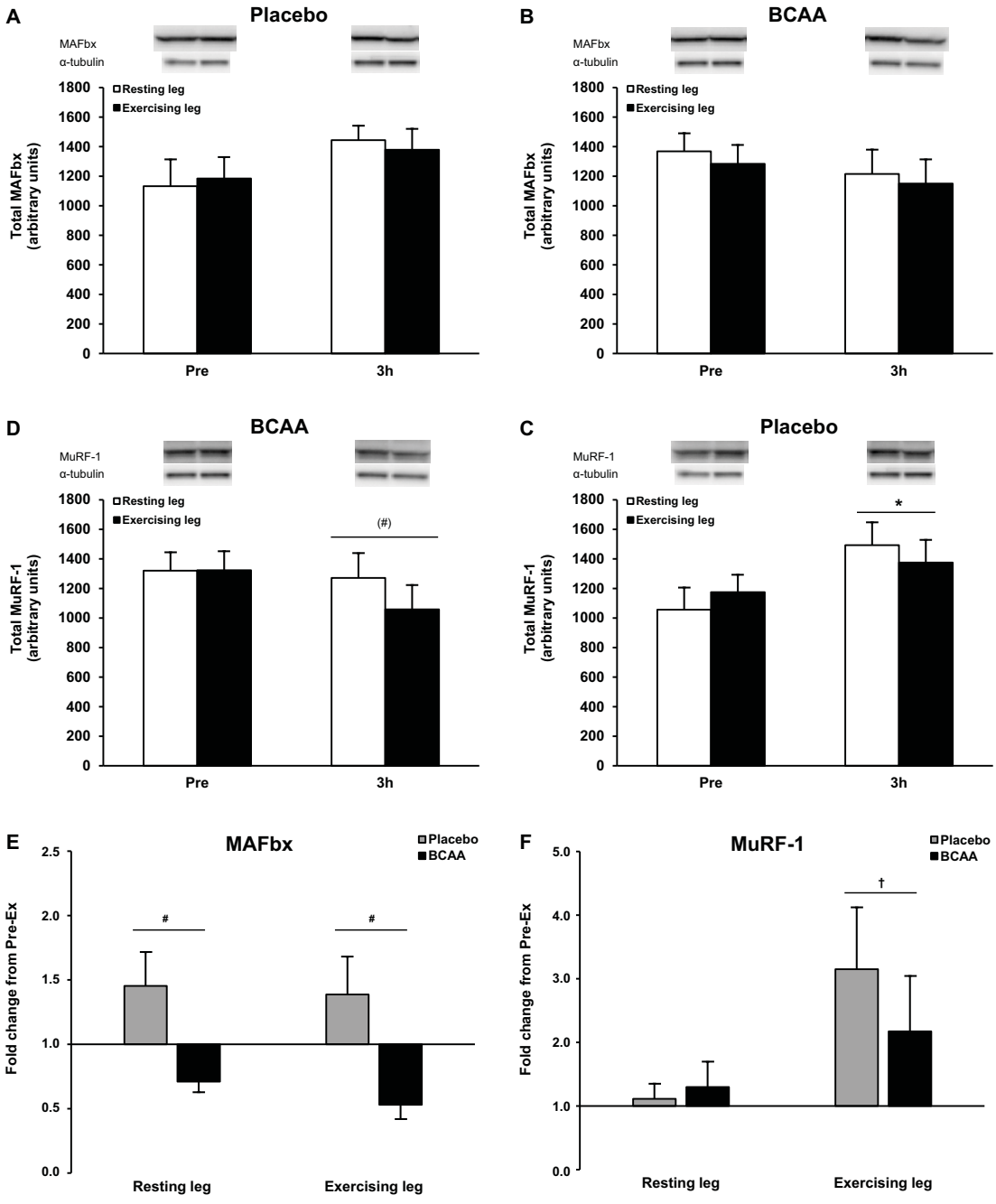
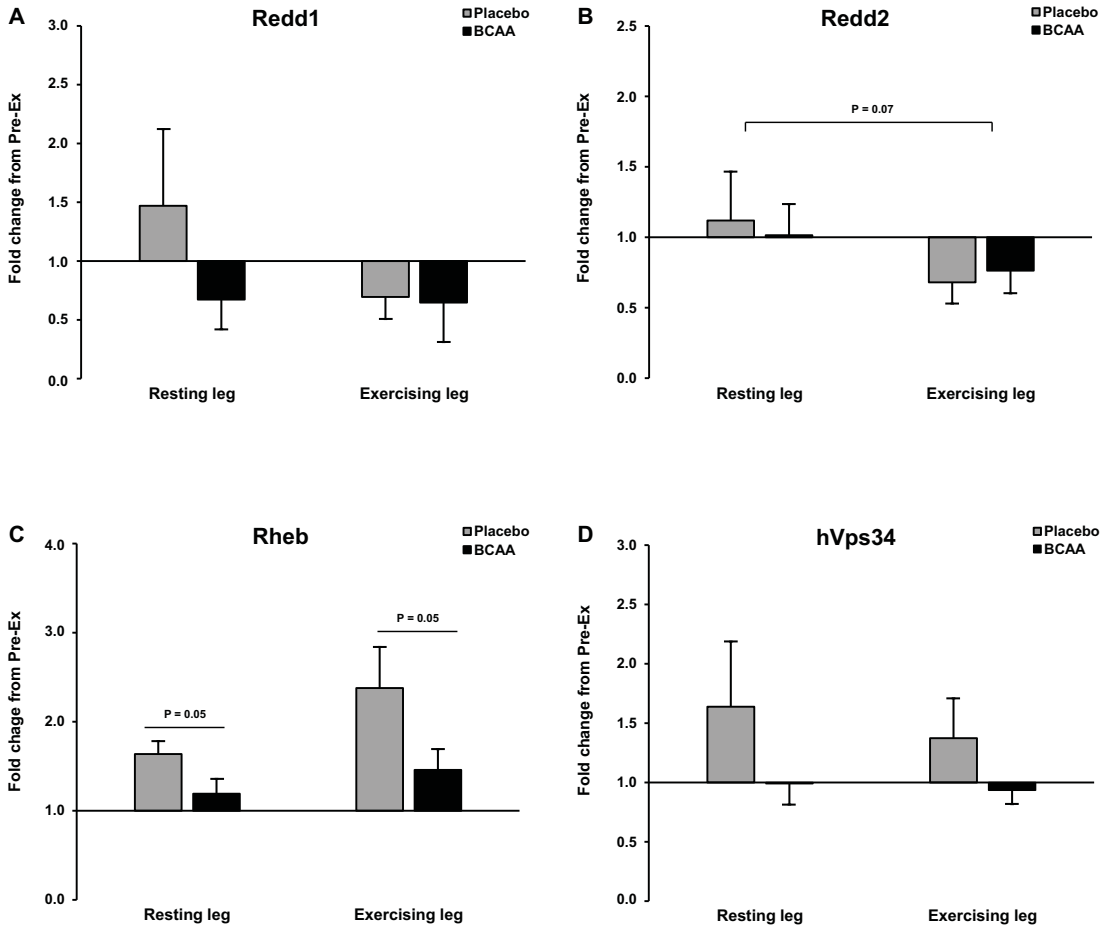

A
Placebo
MAFbx
α-tubulin
Resting leg
Exercising leg
Total MAFbx (arbitrary units)
Pre
3h
B
BCAA
D
BCAA
MuRF-1
(#)
Total MuRF-1 (arbitrary units)
C
Placebo
*
E
MAFbx
Placebo
BCAA
Fold change from Pre-Ex
#
Resting leg
Exercising leg
F
MuRF-1
†

Figure 5


A
Redd1
Placebo
BCAA
Fold change from Pre-Ex
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Resting leg
Exercising leg
B
Redd2
Placebo
BCAA
P = 0.07
Fold change from Pre-Ex
2.5
2.0
1.5
1.0
0.5
0.0
Resting leg
Exercising leg
C
Rheb
Placebo
BCAA
P = 0.05
P = 0.05
Fold chage from Pre-Ex
4.0
3.0
2.0
1.0
0.0
Resting leg
Exercising leg
D
hVps34
Placebo
BCAA
Fold change from Pre-Ex
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Resting leg
Exercising leg