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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Borgenvik M, Apró W, Blomstrand E. Intake of branched-chain amino acids influences the levels of MAFbx mRNA and MuRF-1 total protein in resting and exercising human muscle. Am J Physiol Endocrinol Metab 302: E510–E521, 2012. First published November 29, 2011; doi:10.1152/ajpendo.00353.2011.—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 two- and threefold under the placebo and BCAA conditions, respectively, whereas MuRF-1 total protein increased by 20% (P < 0.05) only in the placebo condition. Phosphorylation of p70 S6 kinase (p70S6k) and the eukaryotic elongation factor-2 (eEF2) as well as inactivation of eIF4E-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, 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 downregulating the expression of REDD1 and -2, two negative regulators of mTOR (15). The corresponding activation by amino acids is suggested to involve not only Rheat 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 Ca2+/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 upregulated 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 downregulates the expression of MuRF-1 and MAFbx and thereby counteracts atrophy (53, 55).

Although extensive evidence for the stimulatory effect on protein synthesis of amino acids, either alone or in combination with exercise, 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 indepen-
dent 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 would be elevated, while at the same time reducing 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) yr; height 175 (±5) cm; weight 67 (±7) kg; and maximal oxygen uptake 46 (±1) ml·min⁻¹·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 Ethics Review Board in Stockholm and performed in accordance with the principles outlined in the Declaration of Helsinki. Five of these same subjects (3 men and 2 women) also took part in one of our earlier investigations (2).

Pretest. 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 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 wk apart, ~10 days prior to the actual experiment.

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

Experimental protocol. The experimental set-up involved a randomized, double-blinded, cross-over design. Participants had been instructed to refrain from any type of intense physical activity and to consume a standardized diet during the 2 days preceding the experiment. This diet consisted of 17 energy percent (E%) protein, 26 E% fat, and 57 E% carbohydrate and contained ~2,100 kcal for the women and 2,700 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 anesthesia 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 rate 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 ~40 min. This experimental protocol was carried out twice by each subject, with 4 wk 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 ~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 g for 3 min, and the plasma thus obtained was stored at −80°C for future analyses.

Muscle biopsies from both legs (starting with the exercising leg) were taken immediately after termination of exercise (Post-Ex) and following 1 and 3 h of recovery. However, in two of the participants, biopsies were taken only prior to the exercise and following 3 h of recovery. The first biopsy was taken ~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 body wt 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 BCAAs mixture was the same as in a previous study that had 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, and centrifuged at 10,000 g for 3 min, and the supernatant obtained was stored at −80°C for later analysis. The concentrations of free amino acids in

Table 1. Details of primers employed for RT-qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function of the Product</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>No. in GenBank</th>
</tr>
</thead>
<tbody>
<tr>
<td>MuRF-1</td>
<td>Regulator of proteolysis</td>
<td>GCACCTTTCTCTTGACTG</td>
<td>ATTCCTCCTCTTGATGTC</td>
<td>NM_032588</td>
</tr>
<tr>
<td>MAFbx</td>
<td>Regulator of proteolysis</td>
<td>GAGCAGCTGAGTCGCTAC</td>
<td>GGCACTGTTGAGATCCGC</td>
<td>NM_148377</td>
</tr>
<tr>
<td>Rheb</td>
<td>Positive regulator of mTOR</td>
<td>TTTTGTGAAATTTGCTCGAAAAGAA</td>
<td>AGAAGTCGCTGCTTAAAGCT</td>
<td>NM_005614</td>
</tr>
<tr>
<td>hVps34</td>
<td>Nutrient regulator of mTOR</td>
<td>GAAGCAGATGACCTGAACGA</td>
<td>CAACGAGAATCTACTTACCC</td>
<td>NM_002647</td>
</tr>
<tr>
<td>REDD1</td>
<td>Negative regulator of mTOR</td>
<td>CTGGAGAGCCCTGCGACTGC</td>
<td>TGGATAGGCGCTGATCT</td>
<td>NM_019058</td>
</tr>
<tr>
<td>REDD2</td>
<td>Negative regulator of mTOR</td>
<td>CGGCGGAGCCCGTGCTGAAAGTCG</td>
<td>TGGGCTTGTGATTTGAGACGAC</td>
<td>NM_145244</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Housekeeping (control)</td>
<td>AACAGTGCCGCTATGAGAC</td>
<td>TATGAGCTAGGATGGT</td>
<td>NM_002046</td>
</tr>
</tbody>
</table>

MuRF-1, muscle RING finger 1; MAFbx, muscle atrophy F-box; Rheb, ras-homolog 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.
the supernatants were measured by reversed-phase high-performance liquid chromatography (HPLC; Waters, Milford, MA) employing orthophthaldehyde (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/mg), and the tubes were then maintained on ice for 30 min and centrifuged at 10,000 g for 3 min, and the resulting supernatant was 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) 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 polyvinylidene difluoride 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% nonfat dry milk. Thereafter, the membranes were incubated overnight with commercially available primary phospho-specific antibodies diluted in TBS supplemented with 0.1% Tween-20 containing 2.5% nonfat 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 analyzed 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 β-tubulin.

**Antibodies.** Primary antibodies raised against phospho-mTOR (Ser2448, 1:500), phospho-p70 S6k (Thr389, 1:1,000), phospho-Akt (Ser473, 1:1,000) and phospho-4E-BP1 (Thr36/37, 1:1,000) were purchased from Cell Signaling Technology (Beverly, MA). Primary antibody against MAFbx (1:1,000) was purchased from Abcam (Cambridge, UK), for MuRF-1 (1:1,000) from Santa Cruz Biotechnology (Santa Cruz, CA), and for α-tubulin (1:5,000) from Sigma-Aldrich (St. Louis, MO). 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 ~3 mg of muscle that had been freeze-dried and cleaned (see above) by homogenization with a Polytron (Kinematica, Lucerne, Switzerland) in PureZOL RNA isolation reagent (Bio-Rad Laboratories, Sundbyberg, Sweden) in accordance with the manufacturer’s instruction. The final amount and purity of the RNA were determined by spectrophotometry.

Two micrograms of the isolated RNA was subjected to reverse transcription utilizing the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Sweden) to produce 20 μl of 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 1:3,125), and RT-qPCR was performed on a Bio-Rad iCycler. The standard curves for all of the primers exhibited high efficiency and an r² value of >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 REDD1 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−ΔΔCT method, where ΔΔCT = CT time − CT time 0, as described by Livak and Schmittgen (37). The mean 2−ΔΔCT values for the exercising leg in the placebo and BCAA condition were 0.84 ± 0.16 and 0.90 ± 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 × condition) 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 quantification by 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, initially at 95°C for 3 min, followed by thermal cycling, 40× at 95°C and 58°C and 72°C for 30 s each. All of the ΔCT 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−ΔΔCT method, as described by Schmittgen and Livak (54), and the results are expressed as fold changes compared with baseline (3 h 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 (time × condition) revealed no main effect or interaction (P > 0.35). 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× SYBR Green Supermix (Bio-Rad Laboratories, Sweden), 11.5 μl of 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, initially at 95°C for 3 min, followed by thermal cycling, 40× at 95°C and 58°C and 72°C for 30 s each. All of the ΔCT 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−ΔΔCT method, as described by Schmittgen and Livak (54), and the results are expressed as fold changes compared with baseline (3 h vs. Pre-Ex) for all conditions, i.e., resting and exercising leg with placebo or BCAA supplementation.

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 perform only 14 repetitions.
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 and 105 ± 12 kg, respectively.

Plasma concentrations. The plasma concentrations of glucose with BCAA or placebo supplementation did not differ, but were reduced under both conditions (P < 0.05) following 30 min of recovery and were attenuated throughout the remaining period of recovery. The initial glucose concentrations were 5.4 ± 0.2 and 5.1 ± 0.2 mmol/l under the placebo and BCAA conditions, respectively, and did not fall below 4.5 mmol/l at any time point. Plasma levels of lactate were significantly elevated (P < 0.05) during resistance exercise, reaching a

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Condition</th>
<th>Pre-Ex</th>
<th>Post-Ex</th>
<th>1 h</th>
<th>3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>Placebo</td>
<td>1,220 ± 70</td>
<td>1,580 ± 110</td>
<td>1,430 ± 120</td>
<td>1,370 ± 120</td>
</tr>
<tr>
<td></td>
<td>BCAA</td>
<td>1,290 ± 130</td>
<td>1,330 ± 240</td>
<td>1,400 ± 180</td>
<td>1,160 ± 130</td>
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<tr>
<td>Isoleucine</td>
<td>Placebo</td>
<td>260 ± 30</td>
<td>210 ± 30</td>
<td>190 ± 10</td>
<td>220 ± 30</td>
</tr>
<tr>
<td></td>
<td>BCAA</td>
<td>230 ± 20</td>
<td>410 ± 40*†</td>
<td>450 ± 40*†</td>
<td>280 ± 40*†</td>
</tr>
<tr>
<td>Leucine</td>
<td>Placebo</td>
<td>500 ± 50</td>
<td>380 ± 50</td>
<td>350 ± 30</td>
<td>430 ± 40</td>
</tr>
<tr>
<td></td>
<td>BCAA</td>
<td>470 ± 40</td>
<td>810 ± 90*†</td>
<td>910 ± 70*†</td>
<td>600 ± 90*†</td>
</tr>
<tr>
<td>Lysine</td>
<td>Placebo</td>
<td>1,990 ± 250</td>
<td>2,190 ± 370</td>
<td>1,980 ± 410</td>
<td>1,950 ± 240</td>
</tr>
<tr>
<td></td>
<td>BCAA</td>
<td>1,900 ± 220</td>
<td>1,730 ± 280</td>
<td>2,780 ± 120</td>
<td>3,300 ± 200</td>
</tr>
<tr>
<td>Methionine</td>
<td>Placebo</td>
<td>110 ± 20</td>
<td>100 ± 10</td>
<td>90 ± 10</td>
<td>110 ± 10</td>
</tr>
<tr>
<td></td>
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<td>110 ± 10</td>
<td>70 ± 10*†</td>
<td>70 ± 10*†</td>
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<tr>
<td>Phenylalanine</td>
<td>Placebo</td>
<td>210 ± 20</td>
<td>190 ± 20</td>
<td>170 ± 20*</td>
<td>180 ± 10*</td>
</tr>
<tr>
<td></td>
<td>BCAA</td>
<td>190 ± 10</td>
<td>180 ± 20</td>
<td>120 ± 20*†</td>
<td>130 ± 10*</td>
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<tr>
<td>Threonine</td>
<td>Placebo</td>
<td>1,990 ± 250</td>
<td>2,190 ± 370</td>
<td>1,980 ± 410</td>
<td>1,950 ± 240</td>
</tr>
<tr>
<td></td>
<td>BCAA</td>
<td>1,900 ± 220</td>
<td>1,730 ± 280</td>
<td>2,780 ± 120</td>
<td>3,300 ± 200</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Placebo</td>
<td>60 ± 10</td>
<td>60 ± 5</td>
<td>60 ± 10</td>
<td>55 ± 5*</td>
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<tr>
<td></td>
<td>BCAA</td>
<td>60 ± 10</td>
<td>60 ± 10</td>
<td>50 ± 10</td>
<td>40 ± 5*</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Placebo</td>
<td>250 ± 30</td>
<td>240 ± 40</td>
<td>230 ± 20</td>
<td>210 ± 20*</td>
</tr>
<tr>
<td></td>
<td>BCAA</td>
<td>260 ± 30</td>
<td>240 ± 40</td>
<td>190 ± 50*†</td>
<td>150 ± 20*†</td>
</tr>
<tr>
<td>Valine</td>
<td>Placebo</td>
<td>660 ± 50</td>
<td>560 ± 50</td>
<td>540 ± 40</td>
<td>560 ± 40</td>
</tr>
<tr>
<td></td>
<td>BCAA</td>
<td>620 ± 30</td>
<td>830 ± 70*†</td>
<td>970 ± 100*†</td>
<td>720 ± 70*†</td>
</tr>
<tr>
<td>BCAA</td>
<td>Placebo</td>
<td>1,430 ± 120</td>
<td>1,140 ± 110</td>
<td>1,080 ± 70</td>
<td>1,210 ± 90</td>
</tr>
<tr>
<td></td>
<td>BCAA</td>
<td>1,330 ± 80</td>
<td>2,060 ± 200*†</td>
<td>2,330 ± 210*†</td>
<td>1,600 ± 200*†</td>
</tr>
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<td>EAA-BCAA</td>
<td>Placebo</td>
<td>5,670 ± 400</td>
<td>6,190 ± 490</td>
<td>5,910 ± 490</td>
<td>5,000 ± 260</td>
</tr>
<tr>
<td></td>
<td>BCAA</td>
<td>5,650 ± 460</td>
<td>5,610 ± 530</td>
<td>5,320 ± 480*†</td>
<td>4,580 ± 310*†</td>
</tr>
</tbody>
</table>

Values presented are means ± SE (µmol/kg dry muscle tissue) for 7 subjects, except for Post-Ex and 1 h, where n = 5. Muscle biopsies were collected at rest prior to warm-up (Pre-Ex), directly after termination of exercise (Post-Ex), and following 1 and 3 h of recovery. *Different from Pre-Ex, P < 0.05; †different from placebo, P < 0.05.
maximum of $8 \pm 1.5$ mmol/l 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 for the remainder of the recovery period ($P < 0.05$). As also depicted in Fig. 1C, the plasma levels of EAA other than BCAA (EAA-BCAA), were lower ($P < 0.05$) than at rest compared with the levels at rest following 15 min of recovery 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 (Tables 3 and 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 levels 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 with both conditions but were 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 correspond-

Fig. 2. Levels of BCAA (A), phenylalanine (B), and EAA-BCAA (C) in vastus lateralis muscle of the exercising leg (filled boxes or triangles) and the resting leg (open boxes or triangles) following supplementation with BCAA or placebo. Values are means ± SE ($n = 7$ for Pre-Ex and $3 h$, $n = 5$ for Post-Ex and 1 h). *Different from Pre-Ex, $P < 0.05$; #different from placebo, $P < 0.05$. 

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ing leg in the placebo condition. At all time points in the placebo condition, values were unaltered compared with Pre-Ex values (Fig. 2C).

**Protein signaling.** The phosphorylation status of Akt at Ser473 was unaltered with both supplements and in both legs following 1 and 3 h of exercise from resistance exercise (data not shown), whereas the phosphorylation of mTOR at Ser2448 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. 3, A and B). Phosphorylation of p70^S6 kinase at Thr389 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 \((P < 0.05; \text{Fig. } 3, \text{C and D})\). The phosphorylation of 4E-BP1 at Thr36/37, 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 \((P = 0.14; \text{Fig. } 4, \text{A and B})\). 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. 4, C and 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 compared with placebo (Fig. 4, C and D).

**Levels of mRNA expression.** All of these data are expressed as fold changes following 3 h of recovery compared with 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 than with placebo. The expression of MuRF-1 mRNA was significantly higher in the exercising compared with the resting leg \((P < 0.05)\), threefold in the case of the placebo and twofold 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 with placebo \((P = 0.05; \text{Fig. } 5C)\). The fold increases in the BCAA condition were 1.2- and 1.5-fold compared with 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

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**Fig. 3.** Phosphorylation of mTOR at Ser2448 \((A \text{ and } B)\) and p70^S6 kinase at Thr389 \((C \text{ and } D)\) in 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 are shown above each graph. Values are arbitrary units and presented as means ± SE \((n = 7 \text{ for Pre-Ex and } 3 \text{ h, } n = 5 \text{ for Post-Ex and } 1 \text{ h})\). *Different from Pre-Ex, \(P < 0.05\); #different from placebo, \(P < 0.05\).
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 p70S6k increased to a larger extent in the early recovery period when BCAA were ingested, and the level was still elevated in both legs three h after exercise, although at that 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 3-h 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 accord 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 was earlier 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 et al. (26) that although BCAA reduces

![Fig. 4. Total protein content of muscle atrophy F-box (MAFbx; A and B) and muscle RING-finger 1 (MuRF-1; C and D) and levels of mRNA encoding MAFbx (E) and MuRF-1 (F) in vastus lateralis muscle of exercising and resting legs with placebo and BCAA supplementation after 3 h of recovery. mRNA values are fold changes from baseline (Pre-Ex) and presented as means ± SE (n = 7 or 6 for MAFbx; see Statistical analyses). Protein data are expressed relative to total protein content of α-tubulin. Representative immunoblots from one subject are 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.](http://ajpendo.physiology.org/)
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 eukaryotic initiation factor 3F (eIF-3F) (35), which is of importance in mTOR-p70S6k 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 p70S6k was elevated after exercise, the latter to a 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 p70S6k and 4E-BP1 herein 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 (Tables 3 and 4), an effect that 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 acids 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 et al. (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 p70S6k 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 legs following BCAA supplementation (Fig. 2) was unexpected. Supplementation with amino acids influences p70S6k phosphorylation and the rate of protein synthesis more potently when combined with resistance exercise (2, 8, 46). However, Moore et al. (46) reported that the rates of protein synthesis in resting and exercising human muscle in response to amino acid ingestion were 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 recovery period monitored here. Other possible explanations for the similar effect of BCAA on resting and exercising muscle could be 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 placebo vs. 1.2-fold in the BCAA condition) and exercising muscle (2.4-fold vs. 1.5-fold). Although we did not assume statistical differences from baseline (Pre-Ex), the 2.4-fold elevation (individual values ranging from 1.6 to 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 et al. (20) demonstrated a 1.5-fold increase in the level of Rheb mRNA above baseline following combined resistance exercise and amino acid ingestion, but they did not examine the effect of exercise alone. Rheb is a low-molecular-weight GTPase located immediately upstream of mTOR and is 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). This interaction is rapidly inhibited in cultured cells during withdrawal of leucine from the incubation medium (38); however, future studies are needed to clarify whether a change 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 REDD1 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 REDD1 and -2 is in agreement with the findings of Drummond et al. (20). Moreover, our findings suggest that exercise rather than amino acids is responsible for the lower expression observed here.

In conclusion, BCAA supplementation reduced 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 levels of phenylalanine and tyrosine, as well as the sum of EAA (BCAA excluded) in both resting and exercising muscle during the 3-h recovery period was more pronounced with BCAA supplementation. These observations, together with the BCAA-induced enlargement in p70S6k phosphorylation, provide additional support for the view that BCAA has an anabolic effect on human skeletal muscle, an effect that appears to be similar in resting and exercising human muscle.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.B. and W.A. analyzed data; M.B., W.A., and E.B. interpreted results of experiments; M.B. prepared figures; M.B. drafted manuscript; M.B., W.A., and E.B. edited and revised manuscript; M.B., W.A., and E.B. approved final version of manuscript; W.A. and E.B. conception and design of research; W.A. and E.B. performed experiments.

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