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Activation of mTORC1 by leucine is potentiated by branched-chain amino acids and even more so by essential amino acids following resistance exercise

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Moberg M, Apró W, Ekblom B, van Hall G, Holmberg H-C, Blomstrand E. Activation of mTORC1 by leucine is potentiated by branched-chain amino acids and even more so by essential amino acids following resistance exercise. *Am J Physiol Cell Physiol* 310: C874–C884, 2016. First published April 6, 2016; doi:10.1152/ajpcell.00374.2015.—Protein synthesis is stimulated by resistance exercise and intake of amino acids, in particular leucine. Moreover, activation of mammalian target of rapamycin complex 1 (mTORC1) signaling by leucine is potentiated by the presence of other essential amino acids (EAA). However, the contribution of the branched-chain amino acids (BCAA) to this effect is yet unknown. Here we compare the stimulatory role of leucine, BCAA, and EAA ingestion on anabolic signaling following exercise. Accordingly, eight trained volunteers completed four sessions of resistance exercise during which they ingested either placebo, leucine, BCAA, or EAA (including the BCAA) in random order. Muscle biopsies were taken at rest, immediately after exercise, and following 90 and 180 min of recovery. Following 90 min of recovery the activity of S6 kinase 1 (S6K1) was greater than at rest in all four trials (Placebo<Leucine<BCAA<EAA; $P < 0.05$ time × supplement), with a ninefold increase in the EAA trial. At this same time point, phosphorylation of eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) at $Thr^{37/46}$ was unaffected by supplementation, while that of Thr^{46} alone exhibited a pattern similar to that of S6K1, being 18% higher with EAA than BCAA. However, after 180 min of recovery this difference between EAA and BCAA had disappeared, although with both these supplements the increases were still higher than with leucine (40%, $P < 0.05$) and placebo (100%, $P < 0.05$). In summary, EAA ingestion appears to stimulate translation initiation more effectively than the other supplements, although the results also suggest that this effect is primarily attributable to the BCAA.

4E-BP1; S6K1; BCAA; skeletal muscle

TO GAIN SKELETAL MUSCLE MASS, the rate of muscle protein synthesis must exceed the rate of breakdown. Protein synthesis can be stimulated by resistance exercise in combination with amino acids, and both factors mediate this effect by activation of the mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway (35). The activation of mTORC1 leads to phosphorylation of well-characterized downstream targets and subsequent enhancement of the capacity for mRNA translation (13). If mTORC1 signaling is inhibited in vivo by rapamycin, stimulation of protein synthesis by amino acids in both animal (2, 47) and human skeletal muscle (18) is attenuated, highlighting the important role of mTORC1 in this context.

The current view is that stimulation of protein synthesis by a mixture of amino acids is due to the essential amino acids (EAA) present (12, 48), primarily leucine (38). To date, several lines of evidence indicate that leucine alone can potently stimulate mTORC1 signaling and protein synthesis in vivo, both in animal (16, 19) and human skeletal muscle (28, 45, 51). Although leucine does have distinctive anabolic properties, there are data to suggest that, by itself, this amino acid cannot account for the entire anabolic stimulus by a mixture of amino acids. For example, we have recently shown that, in connection with resistance exercise, intake of EAA stimulates the activity of 70-kDa ribosomal protein S6 kinase 1 (S6K1) in human skeletal muscle to a greater extent than leucine alone (4).

Early findings on isolated cells indicate that the stimulation of S6K1 by a complete mixture of amino acids is mediated largely by the branched-chain amino acids (BCAA) in the mixture (41). Moreover, oral supplementation with the three BCAA potently stimulates mTORC1 signaling in human skeletal muscle following resistance exercise (3, 34). In addition, isoleucine alone enhances the phosphorylation of both S6K1 and the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) in rat skeletal muscle (2), with the former effect shown to involve sensing of isoleucine by leucyl-tRNA synthetase (30). Despite these observations, overall experimental support for the individual potency of isoleucine and valine in this context is limited, and it therefore seems plausible that these amino acids act synergistically with leucine. Preliminary data from our laboratory indicate that oral ingestion of BCAA results in more pronounced activation of mTORC1 by resistance exercise than leucine alone.

Accordingly, our objective here was to determine whether a mixture of BCAA stimulates mTORC1 signaling and protein synthesis to a greater extent than leucine alone. To this end, protein phosphorylation of components in the mTORC1 pathway, S6K1 kinase activity, the interaction between 4E-BP1 and eIF4E, and muscle protein fractional synthetic rate (FSR) were examined in trained subjects supplied with either EAA, the three BCAA, or leucine alone in connection with resistance exercise. Our hypothesis was that the BCAA and EAA would

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stimulate mTORC1 signaling and FSR to a similar degree and to a greater extent than leucine alone.

METHODS

Subjects. Eight healthy male volunteers participated in the study. To meet the set criteria for enrollment, they had a resistance training experience of at least a year and they were able to lift at least four times their body weight in leg press. The subjects' characteristics were as follows; the mean (±SE) age 27 ± 2 yr, body weight 84 ± 3 kg, height 181 ± 3 cm, and maximal leg strength 430 ± 13 kg. After being fully informed of the purpose of the study and associated risks, all subjects provided written consent to participate. The study protocol was approved by the Regional Ethical Review Board in Stockholm and performed in accordance with the principles outlined in the Declaration of Helsinki.

Pretests. Prior to initiation of the experimental protocol, each subject participated in four preliminary test sessions. On the first occasion, each subject performed a bilateral one-repetition maximum (1RM) test in the leg press machine (243 Leg Press 45°, Gymleco, Stockholm, Sweden). Thus, following warmup with leg presses at low load, the 1RM was assessed by gradually increasing the load until the subject could no longer perform a single repetition (90–180° knee angle). All attempts were separated by at least 5 min of rest. To minimize training effects during the actual experiments, the subsequent three pretests involved familiarization in the form of performance of the entire protocol by each subject (see below). These pretests were scheduled 1 wk apart with the final test being performed 7–10 days prior to the first trial.

Experimental trials. The subjects were instructed to refrain from any type of vigorous physical activity for 2 days prior to each trial and to document their food intake for the 2 days prior to the first trial and then repeated that same intake prior to each of the remaining trials. On trial days, subjects reported to the laboratory at 06:00 AM after fasting since 09:00 PM the evening before. Upon arrival, subjects were instructed to take a supine position and had a 17G Teflon catheter inserted into the antecubital vein of each arm, one to be used for repeated blood sampling and the other for infusion of the stable isotope. After a baseline blood sample was collected, a primed constant infusion of L-[ring-$^{13}C_6$]-phenylalanine (prime 2 μmol/kg; 0.05 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, Cambridge Isotope Laboratories, Danvers, MA) was initiated and continued thereafter for the duration of the experiment (~6 h). The subjects then rested for 2 h under isotope infusion to enable tracer equilibration and quantification of muscle protein fractional synthetic rate (FSR) at rest based on baseline enrichment of plasma protein (33) and a single biopsy. After 2 h an initial baseline biopsy was taken from the vastus lateralis muscle of one leg with a Weil-Blakesley conchotome (AB Wisex, Mölndal, Sweden) under local anesthesia (32). In addition, during these initial 2 h of rest and tracer infusion, a blood sample was collected into EDTA-tubes once every 30 min.

After completion of this baseline sampling, the subjects were seated in the leg press machine and started the exercise protocol. The first three warmup sets consisted of 10 repetitions at 0, 30, and 60% of their individual 1RM. Thereafter, the subjects performed 10 sets of heavy resistance exercise to fatigue, starting at 85% of their 1RM and gradually reducing the load so that they could perform at least 8, but no more than 12 repetitions to fatigue. All sets were separated by 3 min of rest while seated in the leg press. The first trial set the criteria for the following trials so that the subjects would perform the same individual number of repetitions and workload with matching time under tension during resistance exercise in the subsequent trials.

Immediately following completion of the final set, subjects returned to a supine position and a second muscle biopsy was taken, with additional biopsies following 90 and 180 min of recovery. The biopsies were taken from alternating legs (i.e., 1st; right, 2nd; left, 3rd; right, 4th; left) all from a new incision ~2 cm proximal to the previous one. In the subsequent trial the leg for the initial biopsy was alternated and the biopsies were all collected 2 cm medial to the previous ones. In the third and fourth trials, the biopsies were collected in the same manner but 2 cm proximal to the previous ones. These tissue samples were immediately blotted free of blood and frozen in liquid nitrogen for storage at −80°C until analysis. Blood samples were collected immediately prior to and after the warmup sets and after completion of the fourth, seventh, and tenth sets, as well as 15, 30, 60, 90, 120, 150, and 180 min after completion of exercise and maintained on ice for 1 min. Following centrifugation at 10,000 *g* for 3 min, the plasma obtained was transferred to new Eppendorf tubes and placed in liquid nitrogen for storage at −80°C until analysis.

In a double-blind and counterbalanced order, each subject consumed one of four drinks containing flavored water (Placebo), leucine alone (Leucine), all three branch-chained amino acids (BCAA), or essential amino acids including the BCAA (EAA) during each trial. The EAA supplementation (290 mg/kg) consisted of eight essential amino acids (Ajinomoto, Kanagawa, Japan); 13.6% L-histidine, 9.5% L-isoleucine, 17.1% L-leucine, 17.8% L-lysine, 2.9% L-methionine, 14.3% L-phenylalanine, 13.6% L-threonine, and 11.4% L-valine. The BCAA supplement (110 mg/kg) contained 25% L-isoleucine, 45% L-leucine, and 30% L-valine. Leucine alone was given at a dose of 50 mg/kg, so that the amount of leucine in all of the amino acid supplements was identical. In addition to amino acids, all drinks contained salts and artificial sweetener and were lemon-flavored. These drinks (150 ml each) were consumed immediately before and after the warmup sets and following the fourth and eighth sets and after 15, 30, 60, 90, and 120 min of recovery, giving a total volume of 1.35 liters. A schematic overview of the experimental protocol is presented in Fig. 1.

Tissue processing. Muscle samples were lyophilized and then thoroughly dissected clean from blood and connective tissue under a light microscope (Carl Zeiss, Germany), leaving only very small intact bundles of fibers that were mixed extensively to obtain a highly

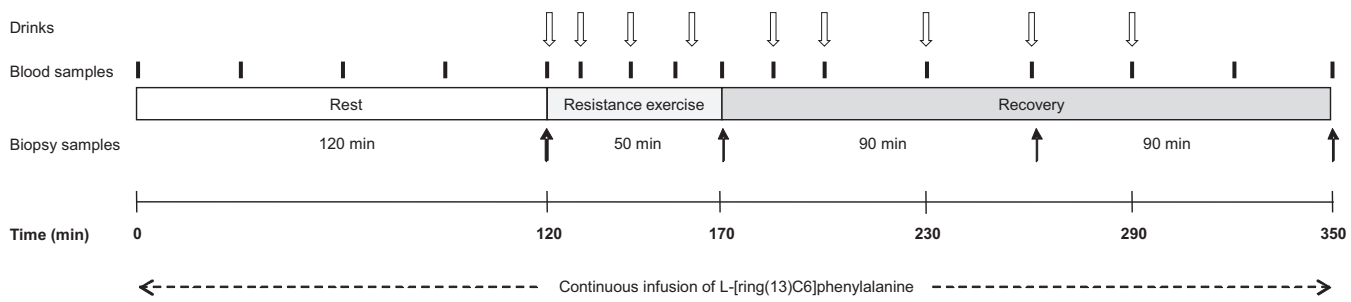

Fig. 1. A schematic overview of the experimental protocol. The black arrows indicate when muscle biopsies were taken, the small black bars indicate when blood was drawn, and the white arrows indicate when supplement was ingested.

homogenous sample free of contaminating tissue. Subsequent analyses were performed on aliquots of this sample preparation.

Plasma analysis. The plasma level of glucose was determined with a Biosen C Line (EKF Diagnostics, Cardiff, UK) and lactate was assayed spectrophotometrically as described by Bergmeyer (7). Plasma insulin levels were measured using an ELISA kit (Mercodia, Uppsala, Sweden) in accordance with the manufacturer's instructions.

Muscle levels of glycogen and free amino acids. The content of muscle glycogen was determined in 2 mg of lyophilized muscle according to the procedure of Leighton and coworkers (37). Levels of free amino acids in the muscle samples were determined using LC-MS/MS as described by Bornø and van Hall (11).

Homogenization of muscle tissue. To assess protein signaling, enzyme activities, protein-protein interactions and myofibrillar protein FSR muscle samples (~10 mg) were homogenized in ice-cold buffer (100 μl/mg dry wt) consisting of 2 mM HEPES (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1% Triton X-100, 1 mM Na_3VO_4, 2 mM dithiothreitol, 1% phosphatase inhibitor cocktail (Sigma P-2850) and 1% (vol/vol) Halt Protease Inhibitor Cocktail (Thermo Scientific, Rockford, IL) utilizing a BulletBlender (NextAdvance, Averill Park, NY) with 0.5 mm ZrO beads. The homogenates obtained were rotated for 30 min at 4°C and subsequently centrifuged at 10,000 *g* for 10 min at 4°C. The resulting supernatant was collected for immunoblotting and immunoprecipitation and the pellet was further processed for myofibrillar protein isotope enrichment (see below). The protein concentration in an aliquot of the supernatant (diluted 1:10 in distilled water) was determined using the Pierce 660 nm protein assay (Thermo Scientific), and the remaining supernatant was stored at −80°C until further analysis.

Immunoblotting. Muscle homogenates were diluted with 4 × Laemmli sample buffer (Bio-Rad, Richmond, CA) and homogenizing buffer to obtain a final protein concentration of 1.33 μg/μl. The diluted samples were then heated at 95°C for 5 min to denature the proteins present and samples were then stored at −20°C until being subjected to SDS-PAGE. For this purpose, 20 μg of protein from each sample was loaded onto 26-well Criterion TGX gradient gels (4–20% acrylamide; Bio-Rad) and electrophoresis was performed at 300V for 30 min on ice. Next, the gel was equilibrated in transfer buffer (25 mM Tris base, 192 mM glycine, and 10% methanol) for 30 min at 4°C after which proteins were transferred to polyvinylidine fluoride membranes (Bio-Rad) at a constant current of 300 mA for 3 h at 4°C. To confirm equal loading and transfer, the membranes were stained with MemCode Reversible Protein Stain Kit (Thermo Scientific) (1). For each target protein, all samples from each subject were loaded onto the same gel and all gels were run simultaneously.

After destaining, the membranes were blocked in Tris-buffered saline (TBS; 20 mM Tris base, 137 mM NaCl, pH 7.6) containing 5% nonfat dry milk for 1 h at room temperature. Thereafter, the membranes were incubated overnight with commercially available primary antibodies diluted in TBS supplemented with 0.1% Tween 20 containing 2.5% nonfat dry milk (TBS-TM). Following primary antibody incubation, membranes were washed with TBS-TM and incubated with secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. Next, the membranes were washed with TBS-TM (2 × 1 min, 3 × 10 min) and then subjected to four additional 5-min washes with TBS. Finally, the target proteins were visualized by applying Super Signal West Femto Chemiluminescent Substrate (Thermo Scientific) to the membranes, followed by detection on a Molecular Imager ChemiDoc XRS system and quantification of the resulting bands with the contour tool in the Quantity One software (version 4.6.3; Bio-Rad). Prior to blocking, the membranes originating from each gel were cut into strips containing each target protein and then assembled so that membranes for the entire sample set were exposed to the same blotting conditions. Following visualization, the membranes were stripped of the phosphospecific antibodies utilizing Restore Western Blot Stripping Buffer (Thermo Scientific) for 30 min at 37°C and thereafter washed and reprobed with primary antibodies that detected the total amount of each protein, as described above. In the case of Akt and 4E-BP1, total protein had to be analyzed on separate blots. The levels of all phosphoproteins were normalized to the total level of the corresponding protein.

Immunoprecipitation. To measure the kinase activity of S6K1 and the interaction between 4E-BP1 and eIF4E (eukaryotic initiation factor 4E), S6K1 and 4E-BP1 were first immunoprecipitated (IP) from muscle homogenates containing 500 and 350 μg of protein, respectively. For this purpose, the homogenates were incubated with either 5 μg rabbit anti-S6K1 antibody or 4 μg mouse anti-eIF4E antibody and an additional 500 μl of homogenization buffer (see above). The resulting protein-antibody complex was incubated overnight with 10 μl protein-G Sepharose beads (GE Healthcare, Uppsala, Sweden) or 15 μl protein-G magnetic beads (Thermo Scientific), respectively, at 4°C on a rotating platform.

Following this incubation, the beads with the immune-complexes were either spun down or trapped using a magnetic rack and washed twice in a high-salt (0.5 M NaCl) variant of the homogenization buffer. In the case of S6K1, the beads were subjected to a final wash in kinase-specific assay buffer prior to the assay (see below). The magnetic beads to which eIF4E was bound were suspended in 100 μl 1 × Laemmli sample buffer with 100 μM DTT and heated for 10 min at 70°C and then immunoblotted for eIF4E and 4E-BP1 as described above.

Kinase assay. After the wash in kinase-specific assay buffer (50 mM Tris·HCl pH 7.5, 0.03% BrijL23, 0.1% β-mercaptoethanol), the beads from each sample were suspended in 60 μl of this buffer and then divided into three 20-μl aliquots. Kinase-specific substrate was added to two of these and the third served as a blank without substrate. The assays were initiated by addition of 30 μl radiolabeled kinase-specific reaction mix once every 20 s and terminated at 20-s intervals by addition of 50 μl phosphoric acid (1% vol/vol) to each assay. The final reaction mix (50 μl) contained 100 μM ATP, 10 mM $MgCl_2$, ^{32}γ-ATP (specific activity: ~ 2.6×10^6 cpm/nmol) and 30 μM synthetic S6K1 substrate (KRRRLASLR) and incubation was carried out for 60 min at 30°C. After termination, the reaction mixtures were spotted onto squares of p81 Whatman filter paper (GE Healthcare), washed four times in phosphoric acid and once in acetone, allowed to dry, and finally immersed in scintillation fluid (FilterSafe, Zinsser Analytic, Frankfurt, Germany) and counted on a liquid scintillation counter (Beckman Coulter, Bromma, Sweden). The average values from the duplicate assays with substrate were corrected for background by subtraction of the blank (no substrate) and the values thus obtained expressed in $pmol \cdot min^{-1} \cdot mg^{-1}$.

Antibodies. For immunoblotting, primary antibodies against Akt (Ser^{473}, no. 9271), mTOR (Ser^{2448}, no. 2971; total, no. 2983), S6K1 (Thr^{389}, no. 9234; total no. 2708), 4E-BP1 ($Thr^{37/46}$, no. 2855; Ser^{65}, no. 9451; total, no. 9644) and eukaryotic elongation factor 2 (eEF2) (Thr^{56}, no. 2331; total, no. 2332), were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies recognizing 4E-BP1 Thr^{46} (no. sc-18090R) and total Akt (no. sc-1619) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

All primary antibodies were diluted 1:1,000 prior to use, except for antibodies against phospho-eEF2, total-4E-BP1, and 4E-BP1 Thr^{46}, which were diluted 1:2,000, 1:2,000, and 1:200, respectively. Secondary anti-rabbit (no. 7074) antibody (1:10,000) was purchased from Cell Signaling Technology and secondary anti-goat (no. ab7132; 1:10,000) antibody was purchased from Abcam.

For immunoprecipitation, antibodies against S6K1 (no. sc-230) and eIF4E (no. sc-271480) were purchased from Santa Cruz.

4EBP1 antibody validation. Evaluation of antibody specificity for phospho-4EBP1 (Thr^{46}; no. sc-18090R) was performed using blocking peptides for the Thr^{37} (no. sc-18080P) and Thr^{46} residues (no. sc-18090P) purchased from Santa Cruz. Each blocking peptide was combined with equal amounts of antibody and incubated overnight prior to dilution (1:1,000) in TBS-TM. The combined antibody and peptide solutions were then incubated with immunoblotted samples as

described above, and compared with an antibody solution (1:1,000) without peptides.

Incubation of the Thr^{46} antibody with the Thr^{46} peptide blocked the signal by close to 100% compared with antibody with no peptide. When incubated with the Thr^{37} peptide, the signal was blocked by ~40%. Thus, the specificity of the Thr^{46} antibody was determined to be ~60%.

Stable isotope enrichment. Enrichment of L-[ring-$^{13}C_6$]-phenylalanine was analyzed in both mixed muscle tissue and in the myofibrillar protein pool. The protein pellet obtained from extraction of the 10 mg of lyophilized muscle (see above) was washed once with 500 μl purified H_2O, dissolved in 1 ml homogenization buffer (see above), and then centrifuged at 1,600 *g* for 20 min at 4°C. The resulting supernatant was discarded and the pellet containing myofibrillar protein was lyophilized and stored at −80°C until further analysis. Immunoblotting confirmed that this pellet contained ~97% myofibrillar proteins (myosin/actin), ~1% sarcoplasmic proteins (GAPDH), and ~2% mitochondrial proteins (COX). To determine enrichment in mixed muscle protein, 5 mg dry muscle tissue was used.

The mixed muscle and the dried myofibrillar pellet were each combined with 100 μl of an internal standard (L-[ring-$^{13}C_9$]-phenylalanine, 5 μmol/l) and 100 μl of a standard solution of all amino acids after which the samples were pelleted and extracted twice with 500 μl 2% perchloric acid. To determine intracellular enrichment of free phenylalanine, the two extracts from the mixed muscle samples were combined and analyzed further using LC-MS/MS as described by Bornø and van Hall (11). The remaining pellet was washed twice with 70% ethanol, hydrolyzed overnight in 1 ml 6 M HCl at 110°C, dissolved in 500 μl of acetic acid (50%), and then passed through a cation exchange column. To determine enrichment of protein-bound phenylalanine, amino acids derived from the purified pellet were converted to their *N*-acetyl-*n*-propyl esters and analyzed by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS, Hewlett Packard 5890-Finnigan GC combustion III-Finnigan Deltaplus; Finnigan MAT, Bremen, Germany) as described previously (10).

Calculations for muscle fractional synthetic rate. Mixed muscle and myofibrillar protein fractional synthetic rate (FSR) were calculated employing the standard precursor-product method:

$$FSR = \Delta E_{p\ phe}/(E_{ic\ phe} \times t) \times 100$$

where $\Delta E_{p\ phe}$ is the difference in protein-bound phenylalanine enrichment between biopsies taken immediately after exercise and after 180 min of recovery or between the preinfusion plasma sample (33) and the first biopsy for calculation of FSR at rest; $E_{ic\ phe}$ is the intracellular phenylalanine enrichment in the biopsy collected 2 h after infusion for the calculation at rest and the average in the case of

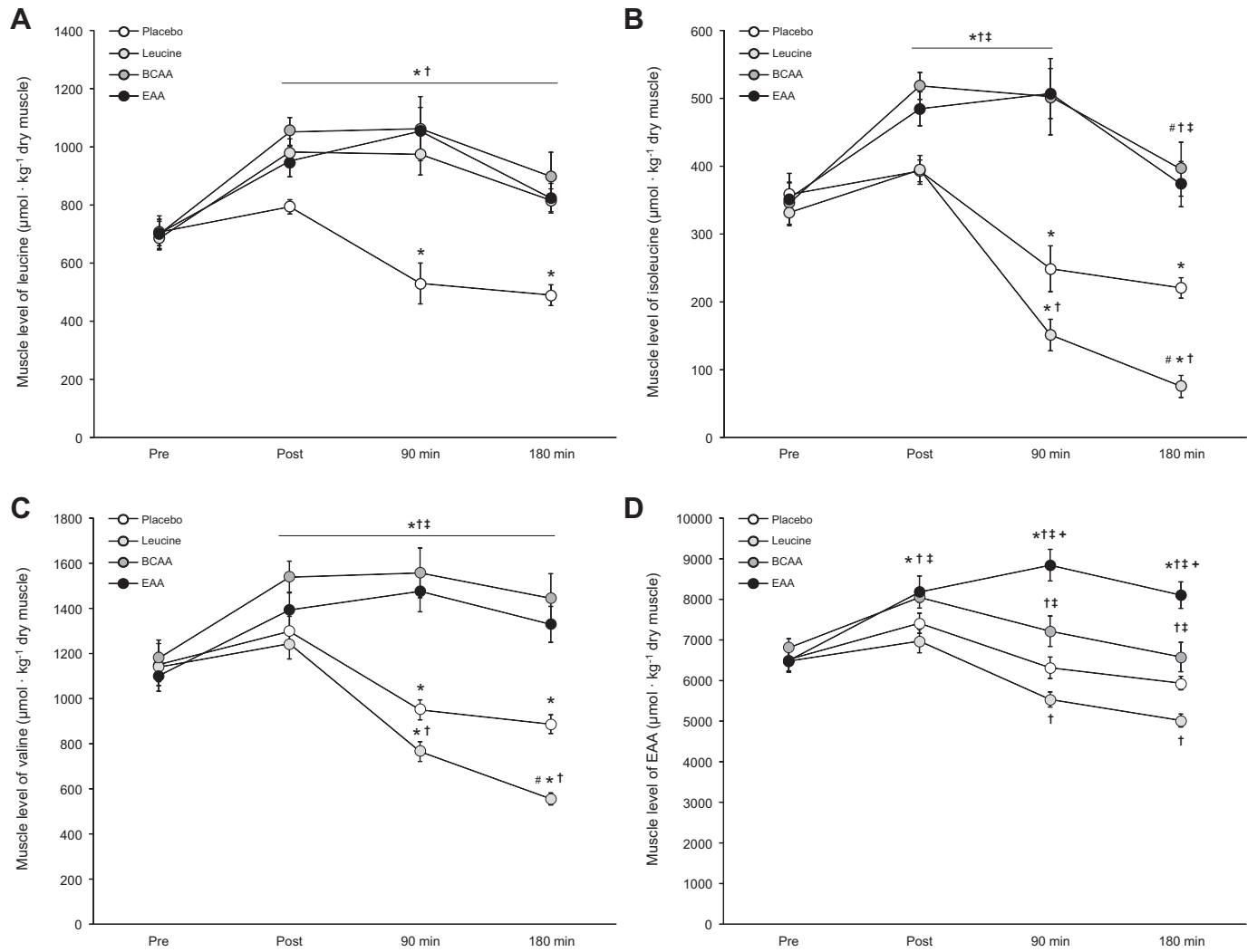

Fig. 2. Muscle levels of leucine (*A*), isoleucine (*B*), valine (*C*), and the sum of EAA in the biopsies taken at rest and following resistance exercise during the four trials (*D*). The values presented are means ± SE for 8 subjects. *$P < 0.05$ vs. rest, †$P < 0.05$ vs. Placebo, ‡$P < 0.05$ vs. Leucine, +$P < 0.05$ vs. BCAA, #$P < 0.05$ vs. 90 min after exercise.

recovery, and *t* is time between biopsies in hours multiplied by 100 to express FSR in percentage per hour (%/h).

Statistical analysis. Parametric statistical procedures were employed to calculate the means and standard errors of the mean (SE), which unless otherwise indicated are presented in the text (means ± SE). Fold changes were calculated relative to the average value at rest for all four trials. A two-way (time × supplement) repeated-measures ANOVA was applied to compare changes in intracellular signaling, kinase activity, protein-protein interactions, and levels of glycogen and amino acids. To evaluate changes in FSR and area under the curve (AUC), a one-way ANOVA was employed. Whenever a significant main or interaction effect was observed, a Fisher's LSD post hoc test was performed. All statistical analyses were performed with STATISTICA software (version 12.0; StatSoft, Tulsa, OK). $P < 0.05$ was considered statistically significant.

RESULTS

Exercise performance. Seven subjects performed the exact same number of repetitions at the same load in all four trials (a total of 103 ± 6 reps for 10 sets), whereas one subject performed one additional repetition in total in two of the trials. In addition, there were no interindividual differences in time under tension (a total of 343 ± 32 s). The heavy load was initiated at 85 ± 1% of each subject's 1RM, and the final set was performed at 65 ± 3% of each subject's 1RM.

Plasma concentrations of insulin, glucose, and lactate. The average preexercise insulin concentration for all four trials was 4.5 ± 0.5 mU/l, and the peak concentrations were attained 15–30 min after exercise in all trials (Placebo: 14 ± 2 mU/l, Leucine: 13 ± 2 mU/l, BCAA: 16 ± 2 mU/l, and EAA; 16 ± mU/l) with no significant differences between trials. However, the area under the curve (AUC) for insulin concentration was greater with EAA than Placebo or Leucine ($P < 0.05$).

As expected, the exercise resulted in a pronounced elevation in the level of lactate ($P < 0.05$) peaking at 10.6–12.1 mmol/l immediately following exercise in all trials, with no differences in the AUC between the interventions. The plasma glucose concentration fluctuated nonsignificantly between 4.0 and 5.6 mmol/l throughout the trials, and the AUC did not differ between trials.

Muscle glycogen. The baseline levels of muscle glycogen (averaging of 536 mmol/kg dry muscle) were similar in all trials. Following exercise, muscle glycogen content was low-

Table 1. *Muscle concentration of essential amino acids and tyrosine at rest and after resistance exercise in the four trials*

Amino Acid	Condition	Rest	Post	90 min	180 min
Histidine	Placebo	763 ± 46	819 ± 73	827 ± 59	846 ± 58
	Leucine	804 ± 73	726 ± 40	717 ± 50	821 ± 49
	BCAA	806 ± 40	850 ± 65	749 ± 66	763 ± 49
	EAA	795 ± 57	892 ± 49	922 ± 71	897 ± 69
Threonine	Placebo	1,929 ± 107	2,243 ± 60*	2,030 ± 104	1893 ± 86
	Leucine	1,849 ± 117	1,942 ± 70	1,765 ± 101	1636 ± 82*
	BCAA	1,884 ± 100	2,150 ± 124*	1,914 ± 118	1665 ± 95*
	EAA	1,883 ± 103	2,406 ± 147*†‡+	2,774 ± 113*†‡+	2,781 ± 143*†‡+
Methionine	Placebo	196 ± 20	229 ± 12	193 ± 24*	173 ± 12*
	Leucine	171 ± 9	215 ± 25	152 ± 10	110 ± 5*†
	BCAA	189 ± 15	219 ± 18	176 ± 22	127 ± 22*†
	EAA	192 ± 17	238 ± 14*	247 ± 19*†‡+	197 ± 12‡+
Valine	Placebo	1,151 ± 93	1,298 ± 67*	950 ± 44*	887 ± 42*
	Leucine	1,139 ± 65	1,243 ± 68	765 ± 44*†	555 ± 27*†#
	BCAA	1,179 ± 80	1,539 ± 71*†‡	1,558 ± 110*†‡	1,445 ± 109*†‡
	EAA	1,101 ± 68	1,393 ± 79*†‡	1,476 ± 91*†‡	1,329 ± 79*†‡
Isoleucine	Placebo	358 ± 31	393 ± 16	249 ± 34*	220 ± 15*
	Leucine	332 ± 19	395 ± 21	151 ± 23*†	75 ± 16*†#
	BCAA	345 ± 31	518 ± 20*†‡	502 ± 56*†‡	396 ± 40†‡#
	EAA	353 ± 23	485 ± 25*†‡	507 ± 37*†‡	374 ± 33†‡#
Leucine	Placebo	706 ± 56	794 ± 25	530 ± 70*	490 ± 36*
	Leucine	685 ± 39	982 ± 45*†	974 ± 71*†	816 ± 39*†
	BCAA	699 ± 51	1,051 ± 50*†	1,062 ± 110*†	898 ± 84*†
	EAA	702 ± 42	951 ± 54*†	1,054 ± 82*†	823 ± 51*†
Lysine	Placebo	1,192 ± 98	1,316 ± 170	1,243 ± 106‡+	1,243 ± 106‡+
	Leucine	1,199 ± 118	1,156 ± 102	944 ± 65	962 ± 38
	BCAA	1,281 ± 84	1,298 ± 78	958 ± 67	959 ± 94
	EAA	1,256 ± 131	1,454 ± 118	1,366 ± 133‡+	1,293 ± 156‡+
Tryptophan	Placebo	60 ± 3	83 ± 4*	58 ± 5	50 ± 3*
	Leucine	59 ± 3	77 ± 4*	44 ± 2*†	39 ± 3*†
	BCAA	62 ± 3	83 ± 4*	52 ± 5*†	39 ± 6*†
	EAA	61 ± 4	81 ± 3*	47 ± 3*†	31 ± 3*†(‡+)
Phenylalanine	Placebo	243 ± 21	279 ± 23	237 ± 30	209 ± 20
	Leucine	226 ± 14	261 ± 31	173 ± 13	147 ± 6*†
	BCAA	236 ± 16	268 ± 17	205 ± 21	155 ± 22*†
	EAA	235 ± 23	320 ± 30*†‡+	441 ± 36*†‡+	358 ± 25*†‡+
Tyrosine	Placebo	245 ± 23	260 ± 12	224 ± 23	190 ± 12*
	Leucine	212 ± 10	250 ± 20	163 ± 5*	122 ± 7*†
	BCAA	240 ± 10	256 ± 17	195 ± 22*	139 ± 22*†
	EAA	231 ± 16	265 ± 10†‡+	263 ± 21†‡+	206 ± 15‡+

The values in table are μmol/kg dry wt (means ± SE for 8 subjects). *$P < 0.05$ vs. rest; †$P < 0.05$ vs. Placebo; ‡$P < 0.05$ vs. Leucine; + $P < 0.05$ vs. BCAA; #$P < 0.05$ vs. 90 min of recovery.

ered significantly by 22–30%, again with no differences between interventions. During recovery, a minor, but statistically significant resynthesis of glycogen occurred (~7%) to a similar extent in all four trials.

Muscle concentrations of amino acids. The levels of leucine in muscle biopsies taken immediately after exercise were elevated in comparison to the value at pre-exercise (Pre) to a similar extent in all trials involving supplementation with amino acids (40–51%; $P < 0.05$ vs. Pre and Placebo, Fig. 2*A*), and these levels remained significantly higher throughout the entire recovery period. In the Placebo trial, the level of leucine 90 min after exercise was 25% lower than baseline ($P < 0.05$) and was still significantly lower at the end of the recovery period. In the BCAA and EAA trials, levels of isoleucine were elevated by 44–45% immediately and 90 min after exercise ($P < 0.05$ vs. Pre, Placebo and Leucine trials, Fig. 2*B*), returning to the baseline value by the end of recovery.

A pronounced reduction in isoleucine concentration was detected in the Placebo trial following 90 and 180 min of recovery (31% and 39%, respectively; $P < 0.05$ time × supplement), and even more so in the Leucine trial (55% and 78%, respectively; $P < 0.05$ time × supplement). Muscle levels of valine increased immediately after exercise in the BCAA and EAA trials (31% and 26%, respectively), and remained at a similar elevated level throughout recovery ($P < 0.05$ vs. Pre, Fig. 2*C*). Similar to what was observed for isoleucine, valine levels fell during recovery in the Placebo trial, and significantly more so in the Leucine trial, with an average reduction during recovery by 21% and 42%, respectively ($P < 0.05$ time × supplement). Muscle levels of the sum of EAA were increased to a similar extent immediately after exercise in the BCAA and EAA trials ($P < 0.05$ vs. Pre, Fig. 2*D*). With EAA supplementation these levels were maintained elevated the entire recovery period, whereas the levels were reduced to baseline values in the BCAA trial. The sum of EAA was unchanged at all time points in the Placebo trial, while being reduced by 15 and 23% after 90 and 180 min of recovery, respectively, in the Leucine trial. Muscle levels of the individual EAAs and tyrosine are documented in Table 1.

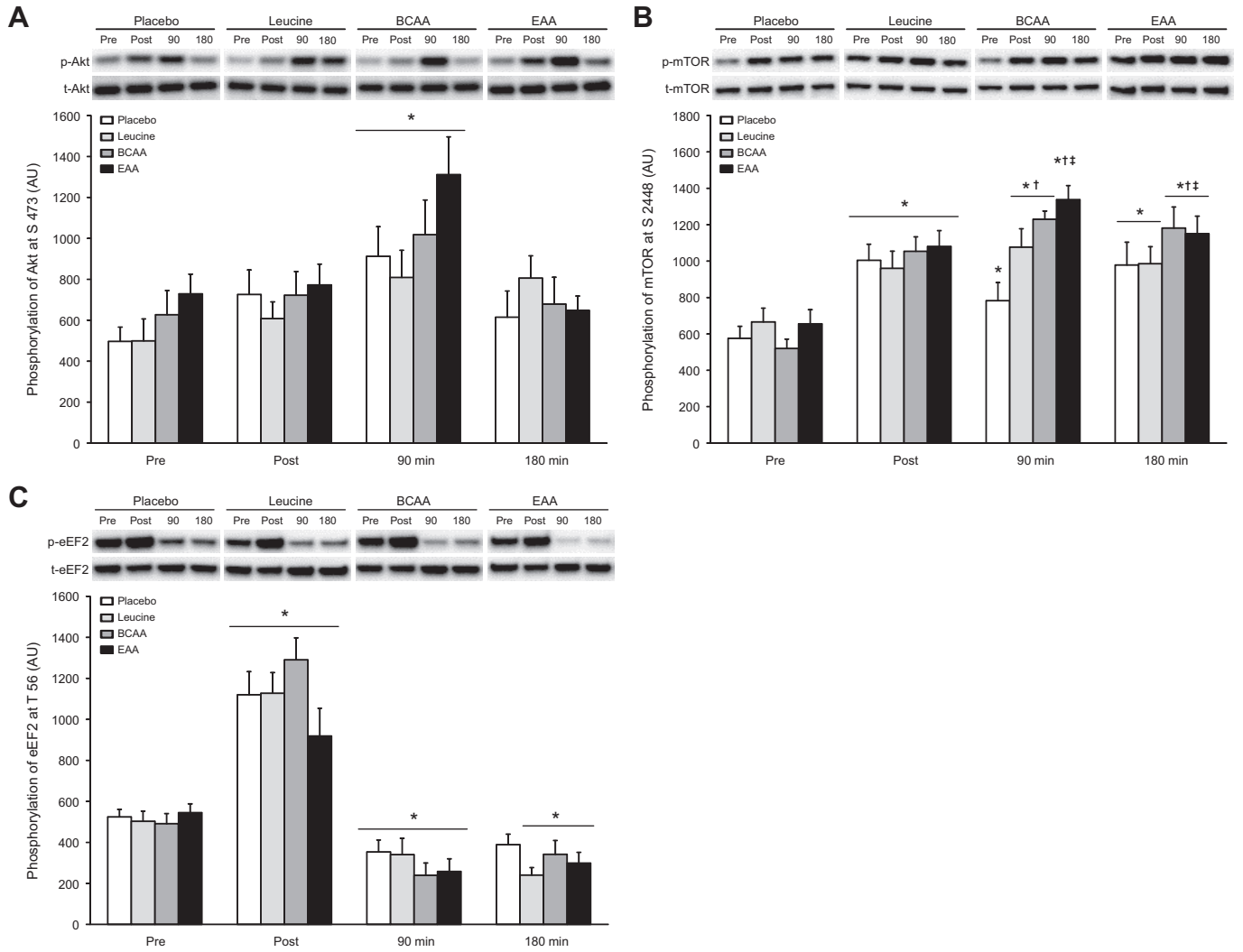

Fig. 3. Phosphorylation of Akt at Ser^{473} (*A*), mTOR at Ser^{2448} (*B*), and eEF2 at Thr^{56} (*C*). The values presented are means ± SE for 8 subjects. *$P < 0.05$ vs. rest, †$P < 0.05$ vs. Placebo, ‡$P < 0.05$ vs. Leucine, +$P < 0.05$ vs. BCAA. Above each graph representative immunoblots of the phosphorylated (*top*) and total protein (*bottom*) for one subject are shown (rearranged to match the order of trials in the graph).

Intracellular signaling and kinase activity. Following 90 min of recovery from exercise, the degree of phosphorylation of Akt at Ser^{473} was elevated to a similar extent in all four trials (31–73%; $P < 0.05$ vs. Pre, Fig. 3*A*), returning to baseline after 180 min of recovery. In addition, phosphorylation of mTOR at Ser^{2448} was enhanced by 58–78% ($P < 0.05$ vs. Pre, Fig. 3*B*) in all four trials immediately after exercise. Following 90 min of recovery, this phosphorylation remained elevated in the Placebo trial but had increased to a significantly higher level in the other trials (Leucine 37%, BCAA 57%, and EAA 71% vs. Placebo). In the final biopsy, mTOR phosphorylation was still significantly higher than the baseline value, more so in the BCAA and EAA trials than the Placebo and Leucine trials ($P < 0.05$). A pronounced elevation in the phosphorylation of eEF2 at Thr^{56} above the resting value (78–150%, $P < 0.05$) was observed immediately after exercise in all four trials. In contrast, after 90 min of recovery, Thr^{56} was hypophosphorylated in all four trials (reductions by: Placebo 33%, Leucine 33%, BCAA 51%, and EAA 52%, $P < 0.05$ vs. Pre, Fig. 3*C*), with no difference between supplements, and it was still hypophosphorylated to a similar extent at the end of recovery in all four trials.

The mean activity of S6K1 before exercise was 0.17 $pmol \cdot min^{-1} \cdot mg^{-1}$ protein for all trials. After 90 min of recovery, the activity was increased compared with that at baseline in all four trials ($P < 0.05$, Fig. 4) (Placebo: 0.40, Leucine: 0.74, BCAA: 0.94, and EAA: 1.47 $pmol \cdot min^{-1} \cdot mg^{-1}$; $P < 0.05$ time $\times$ supplement). Following 180 min of recovery, the activity was similar in the Placebo and Leucine trials (3-fold vs. Pre; $P < 0.05$), and 60–95% higher with BCAA and EAA than with the former two ($P < 0.05$). Lastly, S6K1 activity was well reflected by the S6K1 phosphorylation at the Thr^{389} residue (data not shown) as illustrated by the significant correlation between the two ($r = 0.70$; $P < 0.05$).

Immediately after exercise, phosphorylation of 4E-BP1 at residue $Thr^{37/46}$ and Thr^{46} was reduced by 35–61% in all trials ($P < 0.05$ vs. Pre, Fig. 5, *A* and *B*). These levels returned to baseline after 90 min of recovery in the Placebo and Leucine trials, while being significantly elevated in the BCAA and EAA trial in the case of Thr^{46} only (BCAA 28%, EAA 52%; $P < 0.05$ time $\times$ supplement). At the end of the recovery period this increase in the two latter trials remained, with no difference between them. Phosphorylation of the Ser^{65} residue of 4E-BP1 exhibited a pattern quite similar to that of Thr^{46}, but with no reduction immediately after exercise. After 90 min of recovery the increase above rest was 127% with BCAA supplementation and 214% with EAA ($P < 0.05$ time $\times$ supplement, Fig. 5*C*). At 180 min postexercise in these two trials, the extent of this phosphorylation was still higher than at rest and higher than with Placebo and Leucine ($P < 0.05$), but there was no longer any difference between BCAA and EAA.

eIF4E:4E-BP1 protein interaction. The amount of 4E-BP1 that immunoprecipitated together with eIF4E was elevated 17–31% immediately after exercise in all four trials ($P < 0.05$ vs. Pre, Fig. 5*D*). Following 90 min of recovery this amount was 25–33% less with Leucine, BCAA, and EAA than at baseline ($P < 0.05$ vs. Pre), but unaltered in the case of the Placebo. At the end of recovery, this amount was reduced relative to baseline in all four trials (Placebo: 19%, Leucine 22%, BCAA: 26%, and EAA: 37%; $P < 0.05$) and to a significantly greater extent in the EAA trial than in the Placebo and Leucine trials.

Fractional synthetic rate. The FSR values calculated varied widely from −0.065 to 0.157% per hour (Fig. 6). The myofibrillar protein enrichment displayed the same degree of variation and correlated well with mixed muscle protein enrichment ($r = 0.78$; $P < 0.05$). At rest during the first trial the FSR was 0.030 ± 0.007% per hour utilizing preinfusion plasma protein enrichment as a baseline. This value increased to 0.059 ± 0.005% per hour during the 3 h recovery in the first experiment for each subject ($P < 0.05$).

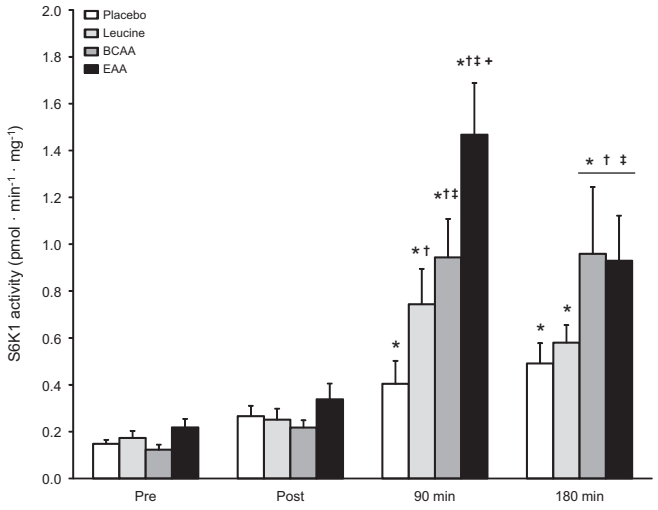

Fig. 4. Kinase activity of S6K1. The values presented are means ± SE for 8 subjects. *$P < 0.05$ vs. rest, †$P < 0.05$ vs. Placebo, ‡$P < 0.05$ vs. Leucine, +$P < 0.05$ vs. BCAA.

DISCUSSION

The novel finding of the current investigation was that ingestion of a mixture of EAA resulted in a greater activation of S6K1 kinase activity than ingestion of the BCAA, with the effect of leucine alone being less potent than either of these. Moreover, there was a more pronounced phosphorylation of 4E-BP1 in combination with a more extensive reduction of its interaction with eIF4E in the EAA trial. A secondary observation was that ingestion of amino acids appears to only enhance the exercise-induced phosphorylation of the Thr^{46} residue of 4EBP1, and not the commonly assessed combined $Thr^{37/46}$ residues.

We recently found that ingestion of a mixture of EAA stimulates S6K1 phosphorylation and activity to a greater extent than leucine alone after resistance exercise (4). We have also found that supplementation with all three BCAA potently activates mTORC1 signaling (3, 9, 34). Partially on the basis of those findings, we hypothesized here that the superior effect of EAA in our previous study (4) was mediated by the BCAA. In contrast to our hypothesis, ingestion of EAA stimulated S6K1 activity and 4E-BP1 phosphorylation to a greater extent than that of only BCAA, which albeit in line with our idea, were more potent than leucine alone. However, at the end of the recovery period there was no difference between the BCAA and EAA trials with regard to S6K1 activity and 4E-BP1 phosphorylation, although these levels were still higher than with leucine and placebo. The explanation for this is not

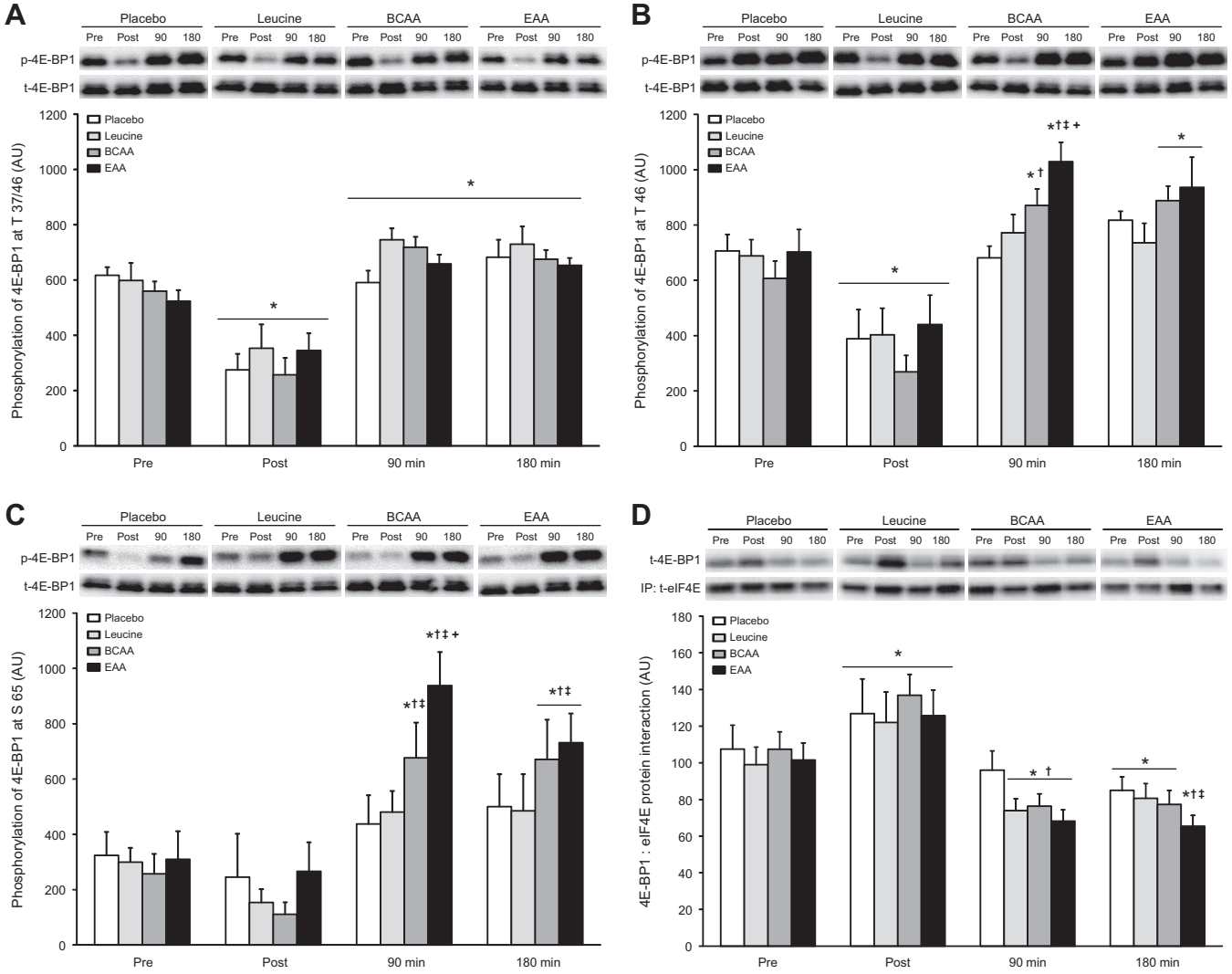

Fig. 5. Phosphorylation of 4E-BP1 at $Thr^{37/46}$ (*A*), Thr^{46} (*B*), Ser^{65} (*C*), and the amount of total 4E-BP1 that immunoprecipitated together with eIF4E (*D*). The values presented are means ± SE for 8 subjects. *$P < 0.05$ vs. rest, †$P < 0.05$ vs. Placebo, ‡$P < 0.05$ vs. Leucine, +$P < 0.05$ vs. BCAA. Above each graph representative immunoblots of both phosphorylated (*top*, expect for IP) and total protein (*bottom*) for one subject are shown (rearranged to match the order of trials in the graph).

readily apparent since muscle levels of EAA were maintained throughout recovery when these amino acids were supplied and there was no change in the level of BCAA from 90 to 180 min after exercise in the two trials. This indicates that the difference between leucine and BCAA in overall stimulation of mTORC1 is greater than between BCAA and EAA.

Importantly, the differences between the supplements with respect to stimulation of mTORC1 signaling were not due to differences in exercise performance or muscle level of leucine, which was similar at all time points during the Leucine, BCAA, and EAA trials. There are, however, a few potential explanations for these findings. These differences might reflect direct effects on mTORC1 signaling caused by EAA other than leucine, or a synergistic effect of these in combination with leucine. In addition, these could be indirect effects of the elevated levels of insulin caused by the supplementation.

Concerning the first of these potential mechanisms, some studies have shown that in cell cultures and animal muscle, the BCAA isoleucine and valine alone exert a low to modest impact on S6K1 phosphorylation and activity (2, 30, 31, 44), as well as enhancing 4E-BP1 phosphorylation (53), whereas others point to their inability in this regard (6, 20, 23). In this context, it is also interesting that Patti and coworkers (41) found that the BCAA as a group stimulates S6K1 activity to the same extent as a complete mixture of amino acids. Collectively, these data are in support of our finding that BCAA are superior to leucine alone in activating mTORC1.

With reference to the other EAA, a number of investigations in cell cultures show that individual EAA are capable of stimulating mTORC1 signaling (6, 31, 44, 53), although their individual effect is substantially less than that of leucine. While Fox and colleagues (23) concluded that individual EAA other than leucine are weak stimulators of 4E-BP1 phosphorylation, they exhibit clear synergistic effects in combination with leucine. No one has yet assessed the influence of individual EAA on mTORC1 signaling in human skeletal muscle, although Smith and coworkers (46) did demonstrate that a flooding dose of phenylalanine or threonine stimulates the rate of muscle

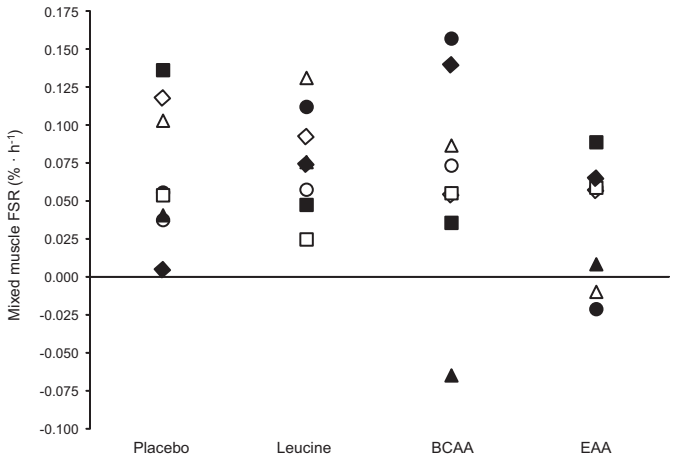

Fig. 6. Mixed muscle protein fractional synthetic rate during the 3-h recovery for all four trials. Each symbol represents an individual subject.

protein synthesis. Hence, it seems likely that some of the other EAA act in synergy with leucine and potentiate the effect of leucine on mTORC1 signaling.

With regard to the role of insulin in our observations here, Dennis et al. (17) reported that the activation of mTORC1 signaling in Rat2 fibroblasts carrying a constitutively active Rag-complex, which mimics maximal stimulation by amino acids, was further enhanced by insulin acting via the TSC-Rheb axis, a parallel signal to that of amino acids. This suggests that higher levels of insulin can mediate additional effects on mTORC1 under maximal amino acid stimulation. In the case of human muscle, elevating insulin levels from about 15 to 50 mU/l after EAA ingestion by adding carbohydrate to the supplement gave no additional stimulation of mTORC1 signaling (26). In contrast, Greenhaff and coworkers (27) demonstrated that in resting muscle under amino acid infusion, S6K1 phosphorylation responds to elevated insulin in the range of 5 to 167 mU/l, although this was not reflected in changes in muscle FSR. In the present study, the peak insulin concentration was similar in all trials (13 to 15 mU/l). These low levels of insulin and the comparable effects on Akt phosphorylation indicate that the potentiated mTORC1 signaling is not mediated by insulin, although it cannot be conclusively ruled out. This conclusion is in line with our previous observation that EAA stimulates S6K1 activity more potently than EAA without leucine, despite no differences in the levels of insulin or Akt phosphorylation (4).

Stimulation of protein synthesis by amino acids is dependent on mTORC1 signaling (2, 18) and enhanced S6K1 phosphorylation is associated with increases in protein synthesis (5, 36). Accordingly, the potent stimulation of S6K1 and 4E-BP1 in the EAA trial following exercise, which is indicative of a major stimulation of the rate limiting step in translation, would be expected to be reflected in increased rate of protein synthesis. Unfortunately, we were not able to accurately assess protein synthesis, as evidenced by the very large variations in FSR values in all four trials (Fig. 6). The reason for this is not readily apparent but is likely due to our use of multiple isotope infusions.

Nevertheless, while our findings provide support for the idea that EAA have the most potent effect on the protein synthetic machinery, we also provide new insights into the role of the various phosphorylation sites of the mTORC1 target 4EBP1. Of the seven phosphorylation sites identified on 4E-BP1, the ones considered most important for its function are Thr^{37}, Thr^{46}, Thr^{70}, and Ser^{65}, which are phosphorylated in that hierarchical order (25), where Thr^{37} and Thr^{46} phosphorylation appear to be permissive for the latter to occur (8, 29). The precise mechanism responsible for these different phosphorylations and their contribution to the release of eIF4E seems to be rather complex. While mTOR phosphorylates 4E-BP1 directly at Thr^{37} and Thr^{46} in vitro (13, 24), it has not yet been shown that mTOR itself phosphorylates Thr^{70} and Ser^{65}, even though these latter sites are clearly mTOR-dependent (8, 39, 50). Furthermore, it has been proposed that all three Thr residues are more or less important for resolving the interaction with eIF4E, while Ser^{65} is dispensable for this event (22, 25, 40) but is suggested to play a role in preventing reassociation (43). Our present data do not resolve this matter but are in general agreement with the latter model. First, the pattern of Thr^{46} phosphorylation is more closely associated with the interaction with eIF4E than that of Ser^{65}, e.g., the reduction in Thr^{46} immediately after exercise paralleled an enhanced interaction, which was not the case for Ser^{65}. Interestingly, the more pronounced phosphorylation of Ser^{65} in the EAA trial could explain the greater decrease in interaction at the end of recovery if this prevents rebinding of eIF4E. Surprisingly, our observations indicate that upon amino acid stimulation, mTORC1 appears to primarily phosphorylate the Thr^{46} residue of 4EBP1. This notion is supported by the finding that during recovery, Thr^{46} phosphorylation was elevated in a pattern which closely resembled that of S6K1 activity, with the largest increases seen in the BCAA and EAA trials. In contrast, there were no significant differences between trials with regard to the commonly assessed $Thr^{37/46}$ at any time point following exercise. This was unexpected, since in cells these two sites respond in a similar manner to mTORC1 stimulation (13, 21), but to our knowledge, the individual effect of feeding or exercise on these two sites has not been evaluated in human skeletal muscle. However, the results presented here should be interpreted with some caution, as the specificity of the Thr^{46} antibody was incomplete for this residue.

Muscle levels of leucine were similar in all trials with amino acid supplementation. While others have shown and argued that after amino acid ingestion, isoleucine and valine antagonize the uptake of leucine by the muscle (14, 15), our data do not support this notion. These differences could, however, relate to the pattern of administration, as Churchward-Venne and coworkers (14, 15) gave their BCAA- and leucine-enriched protein supplements as large boluses, while we administered our supplements, which were comparable in the amount of leucine but higher in BCAA content, as nine separate ingestions over the course of 170 min. Moreover, levels of isoleucine and valine decreased substantially during recovery in muscle in the Leucine trial. It would appear likely that this reflects an improved net balance of muscle protein and/or stimulation of BCAA metabolism by leucine ingestion (42, 49). The finding that muscle levels of valine and isoleucine were reduced to merely 48 and 22% of the resting level in the Leucine trial, respectively, might negatively influence the rate of protein synthesis over time due to substrate depletion (19, 52). Since muscle levels of the remaining EAA only were reduced by 16% during recovery, supplementation with the

BCAA would be of particular importance, since their levels were reduced by far the most and thus would be the most likely to become limiting.

In summary, oral supplementation with BCAA following resistance exercise stimulates mTORC1 signaling more potently than ingestion of leucine alone, but not as effectively as EAA. The more pronounced activation of S6K1 and 4E-BP1 following 90 min of recovery, together with the more sustained reduction in 4E-BP1:eIF4E interaction with EAA supplementation, indicate that a mixture of EAA stimulates translation initiation to a greater extent than BCAA. However, when considering a 180 min recovery period, the difference between the two supplements is reduced. In addition, the data we document here suggest that phosphorylation of the Thr^{46}, but not the Thr^{37} residue of 4E-BP1, is enhanced by amino acid stimulation. Furthermore, the former phosphorylation is more reflective of the 4E-BP1:eIF4E interaction than phosphorylation of Ser^{65}, which might instead be involved in preventing reassociation of these two proteins. The upstream mechanism underlying the potentiating effects of BCAA and, in particular, EAA on leucine-induced mTORC1 signaling requires further investigation to test the likelihood that the other EAA are directly involved.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

M.M., W.A., G.v.H., H.-C.H., and E.B. conception and design of research; M.M., W.A., B.E., and E.B. performed experiments; M.M., W.A., and G.v.H. analyzed data; M.M., G.v.H., H.-C.H., and E.B. interpreted results of experiments; M.M. prepared figures; M.M. drafted manuscript; M.M., W.A., B.E., G.v.H., H.-C.H., and E.B. approved final version of manuscript; W.A., B.E., G.v.H., H.-C.H., and E.B. edited and revised manuscript.

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