Leucine supplementation of a low-protein mixed macronutrient beverage enhances myofibrillar protein synthesis in young men: a double-blind, randomized trial[1–3]

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ABSTRACT

Background: Leucine is a key amino acid involved in the regulation of skeletal muscle protein synthesis.

Objective: We assessed the effect of the supplementation of a lower-protein mixed macronutrient beverage with varying doses of leucine or a mixture of branched chain amino acids (BCAAs) on myofibrillar protein synthesis (MPS) at rest and after exercise.

Design: In a parallel group design, 40 men (21 ± 1 y) completed unilateral knee-extensor resistance exercise before the ingestion of 25 g whey protein (W25) (3.0 g leucine), 6.25 g whey protein (W6) (0.75g leucine), 6.25 g whey protein supplemented with leucine to 3.0 g total leucine (W6+Low-Leu), 6.25 g whey protein supplemented with leucine to 5.0 g total leucine (W6+High-Leu), or 6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine. A primed continuous infusion of L-[*ring*-$^{13}C_6$] phenylalanine with serial muscle biopsies was used to measure MPS under baseline fasted and postprandial conditions in both a rested (response to feeding) and exercised (response to combined feeding and resistance exercise) leg.

Results: The area under the blood leucine curve was greatest for the W6+High-Leu group compared with the W6 and W6+Low-Leu groups ($P < 0.001$). In the postprandial period, rates of MPS were increased above baseline over 0–1.5 h in all treatments. Over 1.5–4.5 h, MPS remained increased above baseline after all treatments but was greatest after W25 (∼267%) and W6+High-Leu (∼220%) treatments ($P = 0.002$).

Conclusions: A low-protein (6.25 g) mixed macronutrient beverage can be as effective as a high-protein dose (25 g) at stimulating increased MPS rates when supplemented with a high (5.0 g total leucine) amount of leucine. These results have important implications for formulations of protein beverages designed to enhance muscle anabolism. This trial was registered at clinicaltrials.gov as NCT 1530646. *Am J Clin Nutr* 2014;99:276–86.

INTRODUCTION

The provision of a complete mixture of amino acids increases myofibrillar protein synthesis (MPS)[4] rates (1) through the activation of the target of rapamycin complex-1 (2). This effect on MPS is primarily attributable essential amino acids (EAAs) because nonessential amino acids do not stimulate MPS (3–5). The relation between amino acid (6) and protein (7, 8) intakes and MPS is dose dependent and saturable. Of EAAs, the branched-chain amino acid (BCAA) leucine is a key determinant of the postprandial stimulation of MPS after protein intake in rodents (9). In vivo animal studies have shown that the independent administration of leucine, but not isoleucine or valine, can stimulate MPS rates (10, 11) to the same extent as complete mixtures of EAA or complete protein (12, 13). Some research in humans has focused on the efficacy of leucine supplementation to promote increases in MPS (14–17) and augment skeletal muscle mass (18, 19). Although some studies have shown increased rates of MPS with leucine administration (20–22), other studies have not (17, 23–25). However, the provision of leucine can result in reduced circulating concentrations of isoleucine and valine (18, 26, 27), which could lower MPS (28); thus, the inclusion of all BCAAs as opposed to leucine alone may be efficacious. The addition of BCAAs, and in particular leucine, to stimulate MPS in a less than optimally effective dose of protein may represent an effective strategy to increase MPS after feeding or under the influence of the markedly anabolic stimulus of resistive exercise.

The aim of the current study was to assess the potential to enhance the effect of a dose of protein that contained a quantity of EAAs previously shown to be suboptimal in maximally

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[4] Abbreviations used: Akt, protein kinase B; AUC_{neg}, AUC below baseline; AUC_{pos}, AUC above baseline; BCAA, branched-chain amino acid; C_{max}, maximum concentration; EAA, essential amino acid; eEF2, eukaryotic elongation factor 2; EX-FED, response to combined feeding and resistance exercise; FED, response to feeding; FSR, fractional synthetic rate; MPS, myofibrillar protein synthesis; mTOR, mechanistic target of rapamycin; T_{max}, time of maximum concentration; W6, 6.25 g whey protein; W6+BCAAs, 6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine; W6+High-Leu, 6.25 g whey protein supplemented with leucine to 5.0 g total leucine; W6+Low-Leu, 6.25 g whey protein supplemented with leucine to 3.0 g total leucine; W25, 25 g whey protein; 1-RM, single-repetition maximum strength; 4E-BP1, eukaryotic initiation factor 4E binding protein 1.

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stimulating MPS rates with feeding (6) and after exercise (7) on MPS rates when ingested as part of a mixed macronutrient beverage. Subjects were randomly assigned to a positive control [25 g of whey protein (W25); 3.0 g leucine], a negative control [6.25 g whey protein (W6); 0.75 g leucine], or treatments that consisted of 6.25 g whey supplemented with a lower dose of leucine [6.25 g whey protein supplemented with leucine to 3.0 g total leucine (W6+Low-Leu)], a higher dose of leucine [6.25 g whey protein supplemented with leucine to 5.0 g total leucine (W6+High-Leu], or a higher dose of leucine plus isoleucine and valine [6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine (W6+BCAAs)]. Rates of MPS and the phosphorylation status of protein targets of the protein kinase B (Akt)–mechanistic target of rapamycin (mTOR) C-1 pathway were examined under postabsorptive conditions and in the postprandial state under rested and postexercise conditions. We examined a temporally early (0–1.5 h) and late (1.5–4.5 h) postprandial period during both resting and postexercise recovery conditions because leucine has been suggested to direct the peak activation but not the duration of MPS (9). We hypothesized that W6+Low-Leu, W6+BCAAs, W25, and W6+High-Leu would stimulate greater postprandial MPS rates than W6 would under resting conditions with no differences between treatments. During postexercise recovery, we hypothesized that W6+BCAAs, W25, and W6+High-Leu would elicit increases in MPS that were equivalent but greater than with W6+Low-Leu and W6 because of the maintenance of the MPS response over the later time period examined.

SUBJECTS AND METHODS

Study participants

Forty young men between 18–35 y of age were recruited via advertisements posted on the McMaster University campus to participate in the study. Characteristics of study participants are presented in **Table 1**. In a double-blind manner, participants were randomly assigned to 1 of 5 parallel treatment groups (n = 8/group) in a block design balanced for body weight. The randomization technique was performed by using the minimization technique implemented with the Trialbalance computer program (Nestlé). An individual at McMaster, who was not directly involved with the study, was responsible for random assignment and treatment preparation. Five codes were generated, and their corresponding group assignments were stored in 5 separate code-break envelopes that were held and sealed until the completion of all data analyses. After a participant's body weight was entered in the Trialbalance system, an individual subject code was generated that corresponded to the treatment-group allocation, which was known only to the individual who was responsible for random assignment and treatment preparation. Only the individual participant's code was placed in the treatment container. None of the study participants reported having engaged in a structured program of resistance exercise within the past year but reported being recreationally active ∼2–3 times/wk. Participants were deemed healthy on the basis of responses to a routine health-screening questionnaire. Each participant was informed of the purpose of the study, experimental procedures, and potential risks before providing written consent. The study was approved by the Hamilton Health Sciences Research Ethics Board and conformed to the standards for the use of human subjects in research as outlined in the most recent update of the Declaration of Helsinki. The study also conformed to standards established by the Canadian Tri-Council Policy on the ethical use of human subjects (29). The study took place at the Ivor Wynne Centre, Department of Kinesiology, McMaster University, Ontario, Canada, from 2011 to 2012. The primary outcome measure was a change in myofibrillar fractional synthetic rate (FSR) (%/h) as assessed by the incorporation of $^{13}C_6$-labeled phenylalanine into myofibrillar proteins.

Pretesting

Approximately 1 wk before the experimental infusion trial, study participants underwent unilateral strength testing of the knee-extensor muscles. Participants performed a series of graded knee-extensions to determine their single-repetition maximum strength (1-RM) with their self-reported dominant leg by using a seated knee-extension device (Atlantis Precision Series C-105). In addition, each participant underwent a whole-body dual-energy X-ray absorptiometry scan (QDR-4500A, software version 12.31; Hologic) to measure body composition (Table 1). Participants were provided with prepackaged standardized diets that were consumed during the 2 d immediately preceding the experimental infusion trial. Diets were designed to provide sufficient energy to maintain an energy balance as determined by the Harris-Benedict equation and were adjusted by using a moderate activity factor (1.4–1.6) to account for participants' self-reported physical activity patterns. The macronutrient distribution

TABLE 1
Participant characteristics[1]

	W6	W6+Low-Leu	W25	W6+BCAAs	W6+High-Leu
Age (y)	20.5 ± 1.1	20.4 ± 0.6	20.9 ± 0.6	20.8 ± 0.8	19.5 ± 0.1
Height (m)	1.80 ± 0.03	1.76 ± 0.02	1.76 ± 0.02	1.83 ± 0.03	1.76 ± 0.03
Weight (kg)	79.4 ± 3.5	77.7 ± 3.3	78.1 ± 2.8	81.3 ± 3.8	79.4 ± 3.4
BMI (kg/m^2)	24.5 ± 0.7	25.0 ± 1.0	25.2 ± 1.0	24.3 ± 0.8	25.7 ± 1.2
Fat-free mass (kg)	66.6 ± 2.4	64.2 ± 2.3	66.0 ± 2.8	70.4 ± 2.9	64.8 ± 2.7
Fat mass (kg)	12.9 ± 1.3	13.6 ± 1.6	12.1 ± 0.8	10.9 ± 1.1	14.6 ± 1.2
1-RM (kg)	64.5 ± 1.7	71.3 ± 4.2	65.1 ± 3.2	67.3 ± 3.6	59.4 ± 3.5

[1] All values are means ± SEMs. n = 8/treatment group. W6, 6.25 g whey protein; W6+BCAAs, 6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine; W6+High-Leu, 6.25 g whey protein supplemented with leucine to 5.0 g total leucine; W6+Low-Leu, 6.25 g whey protein supplemented with leucine to 3.0 g total leucine; W25, 25 g whey protein; 1-RM, single-repetition maximum strength.

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of diets was 55% carbohydrate, 30% fat, and 15% protein. Participants were instructed to consume all food and beverages provided and avoid the consumption of food and beverages (other than water) that were not provided as part of the standardized diet. Participants were instructed to abstain from strenuous physical exercise for 72 h before the experimental infusion trial and to consume their evening meal no later than 2000 the evening before the trial.

Experiment

Participants reported to the laboratory at ∼0600 on the morning of the experimental infusion trial after an overnight fast. A catheter was inserted into an antecubital vein, and a baseline blood sample was taken before the initiation of a 0.9% saline drip to keep the catheter patent to allow for repeated arterialized blood sampling. Arterialized blood samples (30) were obtained repeatedly during the infusion trial (*see* Online Supplemental Material Figure 1 under "Supplemental data" in the online issue) by wrapping a heating blanket around the forearm. Blood samples were collected into 4 mL heparinized evacuated tubes and chilled on ice. A second catheter was inserted into the antecubital vein of the opposite arm before the initiation of a primed continuous infusion (0.05 $\mu mol \cdot kg^{-1} \cdot min^{-1}$; 2.0 $\mu mol \cdot kg^{-1}$ prime) of [*ring*-$^{13}C_6$] phenylalanine (Cambridge Isotope Laboratories). The infusate was passed through a 0.2-μm filter before entering the blood. The baseline (fasted) FSR was calculated on the basis of the ^{13}C enrichment of mixed plasma proteins obtained from the preinfusion blood sample and skeletal muscle biopsy after ∼3 h tracer incorporation (31, 32). Participants performed an acute bout of unilateral seated knee-extension resistance exercise (Atlantis Precision Series C-105) that consisted of 8 sets of 10–12 repetitions at ∼80% of their previously determined 1-RM with an interset rest interval of 2 min. Immediately after completion of the resistance exercise, participants underwent bilateral biopsies from both rested and exercised legs and immediately ingested their designated nutrient treatment (**Table 2**). Bilateral biopsy samples were obtained at 1.5 and 4.5 h after treatment administration from a rested fed [response to feeding (FED)] and exercise-fed [response to combined feeding and resistance exercise (EX-FED)] leg. Muscle biopsies were obtained from the vastus lateralis muscle by using a 5 mm Bergström needle that was custom adapted for manual suction under 2% xylocaine local anesthesia. Tissue samples were freed from visible blood, fat, and connective tissue and immediately frozen in liquid nitrogen for additional analysis as previously described (33, 34). Each biopsy sample was obtained from a separate incision ∼2–3 cm apart. Each participant underwent a total of 6 skeletal muscle biopsies (3 biopsies from each leg). *See* Online Supplemental Material Figure 1 under "Supplemental data" in the online issue for an outline of details of the infusion protocol.

Beverage composition

Study participants were administered nutrient treatments orally in a double-blinded manner immediately after resistance exercise. All treatments were provided in colored plastic containers. Treatments were similar in color, smell, and taste because their main constituents were the same but provided in different quantities depending on the treatment. The macronutrient and amino acid composition of each of the 5 treatments is outlined in Table 2. The W6+Low-Leu, W6+BCAAs, W6+High-Leu, and W6 treatments were isonitrogenous, isoenergetic, and macronutrient-matched, whereas the positive control (W25) contained a reduced amount of carbohydrate and more protein to be energy-matched to the other treatments. The whey protein isolate (biPro; Davisco Foods) was independently tested (Telmark) in triplicate for content analysis. Free-form amino acids used were as follows: L-leucine, L-isoleucine, L-valine, L-alanine, and L-glycine (Sigma Life Science; Sigma-Aldrich). The carbohydrate source was

TABLE 2
AA, protein, carbohydrate, and fat contents of nutritional treatments[1]

	Nutritional treatment group				
	W6	W6+ Low-Leu	W25	W6+ BCAAs	W6+ High-Leu
Endogenous AAs (g)					
Alanine	0.29	0.29	1.15	0.29	0.29
Arginine	0.13	0.13	0.53	0.13	0.13
Aspartic acid	0.70	0.70	2.80	0.70	0.70
Cystine	0.19	0.19	0.78	0.19	0.19
Glutamic acid	1.03	1.03	4.10	1.03	1.03
Glycine	0.11	0.11	0.43	0.11	0.11
Histidine	0.14	0.14	0.55	0.14	0.14
Isoleucine	0.34	0.34	1.35	0.34	0.34
Leucine	0.75	0.75	3.00	0.75	0.75
Lysine	0.68	0.68	2.70	0.68	0.68
Methionine	0.14	0.14	0.58	0.14	0.14
Phenylalanine	0.22	0.22	0.88	0.22	0.22
Proline	0.26	0.26	1.05	0.26	0.26
Serine	0.16	0.16	0.63	0.16	0.16
Threonine	0.28	0.28	1.10	0.28	0.28
Tryptophan	0.17	0.17	0.68	0.17	0.17
Tyrosine	0.22	0.22	0.88	0.22	0.22
Valine	0.35	0.35	1.38	0.35	0.35
Added AAs (g)					
Alanine	3.18	2.05	0.00	0.03	1.05
Glycine	3.17	2.05	0.00	0.03	1.05
Leucine	0.00	2.25	0.00	4.25	4.25
Isoleucine	0.00	0.00	0.00	1.01	0.00
Valine	0.00	0.00	0.00	1.03	0.00
Added carbohydrate (g)	35.0	35.0	22.60	35.0	35.0
Added fat (g)	5.68	5.68	5.68	5.68	5.68
Totals					
Whey protein (g)	6.15	6.15	24.57	6.15	6.15
EAAs (g)	2.89	5.14	11.54	9.18	7.14
NEAAs (g)	9.61	7.36	13.03	3.32	5.36
Total protein (g)	12.5	12.5	24.57	12.5	12.5
Leucine (g)	0.75	3.00	3.00	5.00	5.00
Isoleucine (g)	0.34	0.34	1.35	1.35	0.34
Valine (g)	0.35	0.35	1.38	1.38	0.35
BCAAs (g)	1.43	3.68	5.73	7.73	5.68
Carbohydrate (g)	35.0	35.0	22.90	35.0	35.0
Fat (g)	5.68	5.68	5.68	5.68	5.68
kcal	241	241	241	241	241

[1] AA, amino acid; BCAA, branched-chain amino acid; EAA, essential amino acid; NEAA, nonessential amino acid; W6, 6.25 g whey protein; W6+BCAAs, 6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine; W6+High-Leu, 6.25 g whey protein supplemented with leucine to 5.0 g total leucine; W6+Low-Leu, 6.25 g whey protein supplemented with leucine to 3.0 g total leucine; W25, 25 g whey protein.

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sucrose, whereas the fat source was hydrogenated coconut oil (Nestlé Coffee Mate; Nestec Ltd). All nutrient treatments were prepared in 300 mL H_2O (Table 2). To minimize disturbances in the isotopic equilibrium after amino acid ingestion, beverages were enriched to 4% with L-[*ring*-$^{13}C_6$] phenylalanine on the basis of a phenylalanine content of 3.5% in the whey protein.

Analytic methods

Blood glucose was measured by using a blood glucose meter (OneTouch Ultra 2; Lifescan Inc). Blood amino acid concentrations were analyzed via HPLC as described previously (35). Plasma L-[*ring*-$^{13}C_6$] phenylalanine enrichment was determined as previously described (36). Plasma insulin concentrations were measured with the use of a commercially available immunoassay kit (ALPCO Diagnostics).

Muscle samples (~40–50 mg) were homogenized on ice in buffer [10 μL/mg 25 mM Tris 0.5% vol:vol Triton X-100 and protease/phosphatase-inhibitor cocktail tablets (Complete Protease Inhibitor Mini-Tabs, Roche; PhosSTOP, Roche Applied Science)]. Samples were centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatant fluid was removed, and protein concentrations were determined via a bicinchoninic acid protein assay (Thermo Scientific). The pellet that contained myofibrillar proteins was stored at −80°C for future processing. Working samples of equal concentrations were prepared in Laemmli buffer (37). Equal amounts (20 μg) of protein were loaded onto 10% or gradient precast gels (BIO-RAD Mini-PROTEAN TGX Gels; Bio-Rad Laboratories) for separation by electrophoresis. Proteins were transferred to a polyvinylidene fluoride membrane, blocked (5% skim milk), and incubated overnight at 4°C in primary antibody as follows: phospho-Akt^{Ser473} (1:1000, no. 4058; Cell Signaling Technology), phospho-mTOR^{Ser2448} (1:1000, no. 2971; Cell Signaling Technology), phospho-70 kDa ribosomal protein S6 kinase 1^{Thr389} (1:1000, no. 9234; Cell Signaling Technology), phospho-eukaryotic initiation factor 4E binding protein 1 (4E-BP1)$^{Thr37/46}$ (1:1000, no. 2855; Cell Signaling Technology), phospho-eukaryotic elongation factor 2 (eEF2)^{Thr56} (1:1000, no. 2331; Cell Signaling Technology), and phospho-S6 ribosomal protein (1:2000, no. 2215; Cell Signaling Technology). Membranes were washed and incubated in secondary antibody (1 h at room temperature) before detection with chemiluminescence (SuperSignalWest Dura Extended Duration Substrate, no. 34075; ThermoScientific) on a FluorChem SP Imaging system (Alpha Innotech). Phosphorylation status was expressed relative to α-tubulin (1:2000, no. 2125; Cell Signaling Technology) and is presented for each protein as the fold change from baseline (fasted) conditions. Images were quantified by spot densitometry with ImageJ software (version 1.45; NIH).

Muscle biopsy samples were processed as previously described (38). To determine the intracellular $^{13}C_6$ phenylalanine enrichment, ~20–25 mg muscle was homogenized in 0.6 M perchloric acid/L. Free amino acids in the resulting supernatant fluid were passed over an ion-exchange resin (Dowex 50WX8-200 resin; Sigma-Aldrich Ltd) and converted to their heptafluorobutyric derivatives for analysis via gas chromatography–mass spectrometry (models 6890 GC and 5973 MS; Hewlett-Packard) by monitoring ions 316 and 322 after electron ionization. To determine muscle free intracellular amino acid concentrations, samples were processed as previously described (35). Briefly, muscle samples were derivatized and analyzed by using HPLC (HPLC: Waters model 2695; column: Waters Nova-Pak C_{18}, 4 μm; detector: Waters 474 scanning fluorescence detector; Waters). To determine myofibrillar protein-bound enrichments, a separate piece (~40–50 mg) of muscle was homogenized in a standard buffer that contained protease and phosphatase inhibitors as described previously. The supernatant fluid was collected for Western blot analysis, and the pellet was further processed to extract myofibrillar proteins as previously described (38). The resulting myofibrillar enriched protein pellet was hydrolyzed in 6 M HCL at 110°C overnight. Subsequently, the free amino acids were purified by using ion-exchange chromatography and converted to their *N*-acetyl-*n*-propyl ester derivatives for analysis by using gas chromatography–combustion–isotope ratio mass spectrometry (GC-C-IRMS: Hewlett-Packard 6890; IRMS model: Delta Plus XP; Thermo Finnagan).

Calculations

The FSR of myofibrillar protein was calculated by using the standard precursor-product equation

$$FSR = [(E_{2b} - E_{1b}) \div (E_{IC} \times t)] \times 100 \qquad (1)$$

where E_b is the enrichment of bound (myofibrillar) protein, E_{IC} is the average enrichment of the intracellular free amino acid precursor pool of 2 muscle biopsies, and t is the tracer incorporation time in hours. The use of tracer-naïve subjects allowed us to use a preinfusion blood sample (ie, a mixed plasma protein fraction) as the baseline enrichment (E_{1b}) for the calculation of the baseline (fasted) FSR (31, 39, 40), which is an approach that has been validated by our research group (33) and other researchers (41).

Statistics

Strength tests and dietary run-in variables were compared by using a 1-factor (treatment) ANOVA. Blood glucose and plasma insulin were analyzed by using a 2-factor (treatment × time) repeated-measures ANOVA. Data for the AUC above baseline (AUC_{pos}), maximum concentration (C_{max},), time of maximum concentration (T_{max}), and AUC below baseline (AUC_{neg}) were analyzed by using a 1-factor (treatment) ANOVA. Plasma enrichments were analyzed by using a 2-factor (treatment × time) repeated-measures ANOVA and linear regression. Intracellular precursor pool enrichments were analyzed by using a 3-factor (treatment × time × condition) mixed-model ANOVA and linear regression. Intracellular amino acids (leucine, isoleucine, valine, and the sum of EAAs), protein phosphorylation, and myofibrillar FSR were analyzed by using a 3-factor (treatment × time × condition) mixed-model ANOVA. Protein phosphorylation is expressed as the fold change from baseline (fasted). Tukey's post hoc analysis was performed whenever a significant *F* ratio was shown to isolate specific differences. Statistical analyses were performed with a software package (SPSS version 16; SPSS Inc). For data that did not pass normality, values were transformed by using the square root, reciprocal, or ln of the value. The statistical analysis was performed on transformed data but means (±SEMs) of nontransformed data are presented in graphic or tabular form for clarity. Means were considered to be statistically significant for P values <0.05. The study was powered on the basis of previous work from our group (7) that

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showed that 20 g protein stimulates significantly greater MPS rates than 5 g protein does during the initial 4 h postexercise with no additional increase in MPS when 40 g protein was ingested. Therefore, W6+Low-Leu was chosen to show a significantly greater average postexercise myofibrillar FSR than that of W6. W6+Low-Leu was chosen over W6+BCAAs and W6+High-Leu because this treatment was hypothesized to be less likely to show efficacy. Therefore, the trial was designed to show a relevant effect in the myofibrillar FSR of 0.033%/h. The SD was assumed to be 0.022%/h on the basis of our previous observations (7). To show this effect as significant with a 2-sided statistical test with an experiment-wise false-positive rate of 5% and a power of 80%, a total of n = 8/group was needed.

RESULTS

Participant characteristics

Participant characteristics are shown in Table 1. Each treatment group consisted of 8 randomly assigned participants, all of whom received their intended treatment and whose results were analyzed for primary and secondary outcomes.

Exercise variables

There were no significant differences between treatment groups for 1-RM (Table 1) or the product of load (kg) × volume (no. of repetitions) for exercise performed during the experiment (data not shown; all $P > 0.05$).

Dietary run-in

Participants received ~1.2 g protein · kg body weight^{-1} · d^{-1} during the standardized diet with no significant differences between treatment groups. There were no differences between treatment groups for total energy, protein, carbohydrate, or fat (data not shown) (all $P > 0.05$).

Blood glucose, plasma insulin, and blood amino acid concentrations

Blood glucose concentration showed a rapid but transient increase and was elevated above baseline at 20 and 40 min after treatment administration (P-main effect for time < 0.001). In addition, blood glucose concentrations were greater in the W6 group than in the W25 group (5.50 ± 0.33 compared with 4.95 ± 0.20 mmol/L, respectively; P-main effect for treatment = 0.019).

Plasma insulin concentrations increased rapidly after treatment administration showing a main effect for time ($P < 0.001$; **Figure 1**). The area under the insulin curve (Figure 1, inset) after treatment administration was not different between treatment groups (P = 0.497).

Concentrations over time for blood leucine, isoleucine, valine, and the sum of the EAAs are shown in **Figure 2** (A–D, respectively). No statistical analysis was performed on the concentration over time data. The AUC_{pos}, C_{max}, T_{max}, and AUC_{neg} were analyzed for blood leucine, isoleucine, valine, and ΣEAAs and are presented in **Table 3**. Both the AUC_{pos} and C_{max} for blood leucine were greatest after the W6+High-Leu treatment and were significantly different from that with W6+Low-Leu and W6, while the C_{max} was also different from W25. For both isoleucine and valine, the AUC_{neg} was reduced after W6+BCAAs and W25 treatments and were significantly different from that after W6+High-Leu treatment. The T_{max} for leucine, isoleucine, valine, and ΣEAAs tended to occur latest in the W25 group and most rapidly for the W6+High-Leu group (Table 3).

Intracellular leucine, isoleucine, valine, and ΣEAAs

Intracellular concentrations of leucine, isoleucine, valine and ΣEAAs are shown in **Table 4**. Intracellular leucine showed a time × treatment interaction (P = 0.031), whereby it increased at 1.5 h posttreatment in all treatment groups except the W6 group but returned to values not different from baseline by 4.5 h. Intracellular isoleucine showed a time × condition interaction (P = 0.012) that

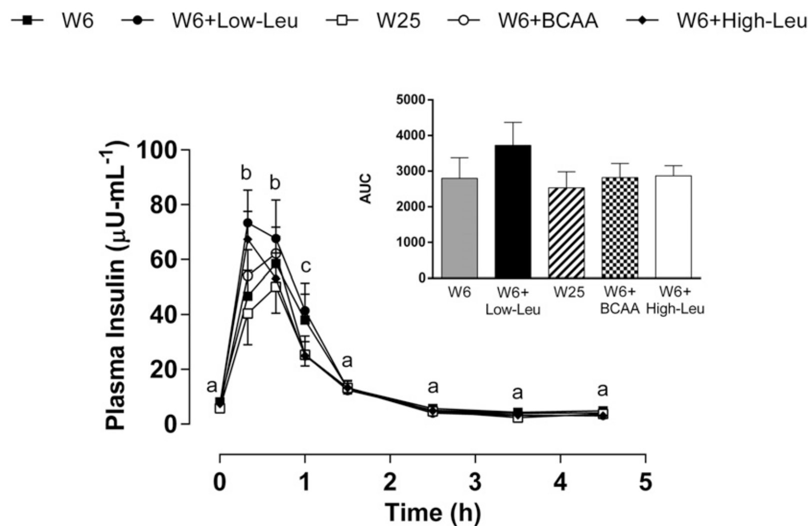

FIGURE 1. Mean (±SEM) plasma insulin concentrations (μmol/L) after treatment administration (n = 8/treatment group). Time-course data were analyzed by using a 2-factor (treatment × time) repeated-measures ANOVA with Tukey's post hoc test (P-main effect for time < 0.001; P-treatment × time interaction = 0.26). Times with different lowercase letters were significantly different from each other. Inset: The AUC was analyzed by using a 1-factor (treatment) ANOVA with Tukey's post hoc test (P = 0.497). W6, 6.25 g whey protein; W6+BCAA, 6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine; W6+High-Leu, 6.25 g whey protein supplemented with leucine to 5.0 g total leucine; W6+Low-Leu, 6.25 g whey protein supplemented with leucine to 3.0 g total leucine; W25, 25 g whey protein.

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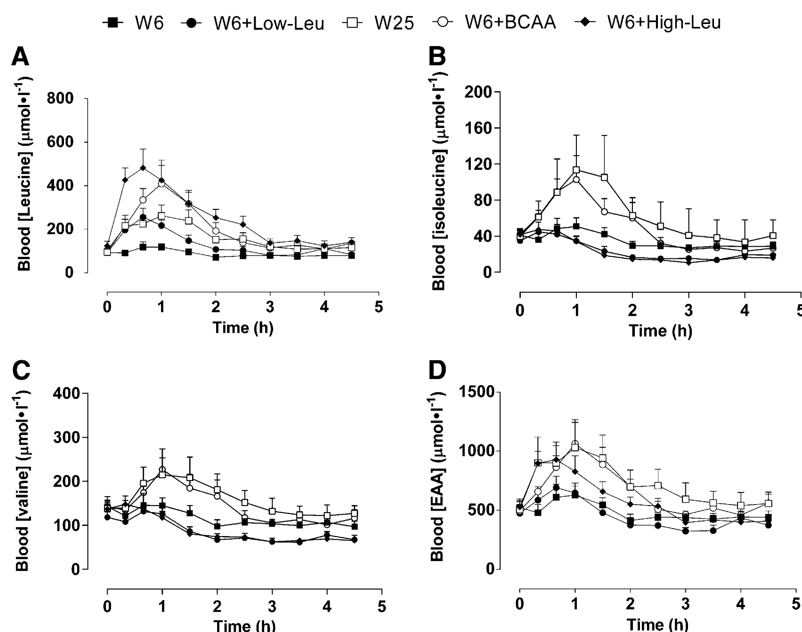

FIGURE 2. Mean (±SEM) blood concentrations (μmol/L) of leucine (A), isoleucine (B), valine (C), and ΣEAAs (D) after treatment administration (n = 8/treatment group). No statistical analysis was performed on time-course data. EAA, essential amino acid; W6, 6.25 g whey protein; W6+BCAA, 6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine; W6+High-Leu, 6.25 g whey protein supplemented with leucine to 5.0 g total leucine; W6+Low-Leu, 6.25 g whey protein supplemented with leucine to 3.0 g total leucine; W25, 25 g whey protein.

increased above baseline at 1.5 h in the FED condition only. Intracellular valine showed a main effect for time (P = 0.006) that fell below baseline fasted concentrations at 4.5 h. There were no time (P = 0.69), treatment (P = 0.66), or condition (P = 0.71) effects for ΣEAAs.

Plasma and intracellular free phenylalanine enrichments

Intracellular free phenylalanine enrichments were not different across time (P = 0.337), between conditions (P = 0.128), or between treatment groups (P = 0.746). In addition, there were no

TABLE 3
Variables of blood leucine, isoleucine, valine, and ΣEAAs after treatment administration[1]

	W6	W6+Low-Leu	W25	W6+BCAAs	W6+High-Leu	P
Leucine						
AUC_{pos}	3223 ± 1465	12,234 ± 1629‡	19,252 ± 3393‡	27,517 ± 4493‡,*	35,278 ± 6016‡,*	<0.001
C_{max} (μmol/L)	145 ± 18	295 ± 40	309 ± 51	459 ± 75‡	554 ± 74$^{\ddagger,*,\#}$	<0.001
T_{max} (min)	49 ± 8	43 ± 5	75 ± 14	51 ± 7	41 ± 9	0.084
AUC_{neg}	−4654 ± 1906$^{\#}$	−1510 ± 903	−196 ± 157	−991 ± 469	−565 ± 282	0.025
Isoleucine						
AUC_{pos}	1145 ± 764	432 ± 142	6692 ± 1766$^{\ddagger,*,+}$	4225 ± 883$^{\ddagger,*,+}$	344 ± 157	<0.001
C_{max} (μmol/L)	61 ± 9	55 ± 10	131 ± 29$^{\ddagger,*,+}$	122 ± 22$^{*,+}$	55 ± 8	<0.001
T_{max} (min)	57 ± 9	40 ± 5	70 ± 8$^{*,+}$	45 ± 6	35 ± 6	0.008
AUC_{neg}	−2919 ± 721	−3503 ± 581$^{\#}$	−643 ± 261	−2073 ± 445	−5618 ± 1039$^{\dagger,\#}$	<0.001
Valine						
AUC_{pos}	2189 ± 1741	489 ± 165	7752 ± 2516$^{\ddagger,*,+}$	5347 ± 1373$^{\ddagger,*,+}$	665 ± 337	<0.001
C_{max} (μmol/L)	178 ± 21	150 ± 14	229 ± 44	246 ± 40*	162 ± 16	0.035
T_{max} (min)	53 ± 10	40 ± 5	74 ± 10^{+}	58 ± 10	33 ± 5	0.015
AUC_{neg}	−9690 ± 2451	−9457 ± 1689	−2174 ± 486	−5247 ± 2163	−13,988 ± 2012$^{\#,\dagger}$	0.001
ΣEAAs						
AUC_{pos}	11,739 ± 6425	11,841 ± 2310	62,722 ± 18,780‡,*	48,937 ± 11,917‡,*	31,047 ± 7267	<0.001
C_{max} (μmol/L)	742 ± 85	801 ± 95	1175 ± 188	1183 ± 175‡	1088 ± 90	0.008
T_{max} (min)	53 ± 10	40 ± 5	83 ± 17	56 ± 7	39 ± 9	0.054
AUC_{neg}	−22,492 ± 9420	−18,734 ± 4681	−3028 ± 1282*	−6135 ± 2122	−16,198 ± 5315	0.013

[1] All values are means ± SEMs. n = 8/treatment group. Blood concentrations (μmol/L) of leucine, isoleucine, valine, and ΣEAAs after treatment administration are shown. The AUC_{pos}, C_{max}, T_{max}, and AUC_{neg} were each analyzed for blood leucine, isoleucine, valine, and ΣEAAs by using a 1-factor (treatment) ANOVA with Tukey's post hoc test. ‡Significantly different from W6; *significantly different from W6+Low-Leu; $^{\#}$significantly different from W25; †significantly different from W6+BCAAs; + significantly different from W6+High-Leu. AUC_{neg}, AUC below baseline; AUC_{pos}, AUC above baseline; C_{max}, maximum concentration; EAA, essential amino acid; T_{max}, time of maximum concentration; W6, 6.25 g whey protein; W6+BCAAs, 6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine; W6+High-Leu, 6.25 g whey protein supplemented with leucine to 5.0 g total leucine; W6+Low-Leu, 6.25 g whey protein supplemented with leucine to 3.0 g total leucine; W25, 25 g whey protein.

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TABLE 4
Intracellular concentrations (μmol/L) of leucine, isoleucine, valine, and ΣEAAs after treatment administration[1]

	W6	W6+Low-Leu	W25	W6+BCAAs	W6+High-Leu	*P*
Leucine						
Baseline	214 ± 11[a]	209 ± 15[a]	212 ± 16[a]	229 ± 17[a]	222 ± 18[a]	Treatment × time = 0.031
1.5-h FED	215 ± 18[a]	248 ± 16[b]	274 ± 32[b]	313 ± 24[‡,b]	344 ± 16[‡,*,#,b]	
4.5-h FED	210 ± 8[a]	221 ± 14[a]	248 ± 8[‡,a]	232 ± 12[a]	263 ± 27[‡,*,a]	
1.5-h EX-FED	222 ± 19[a]	267 ± 12[b]	255 ± 23[b]	299 ± 36[‡,b]	351 ± 17[‡,*,#,b]	
4.5-h EX-FED	188 ± 14[a]	205 ± 20[a]	224 ± 17[‡,a]	225 ± 15[a]	249 ± 31[‡,*,a]	
Isoleucine						
Baseline[a]	216 ± 27	254 ± 33	322 ± 41	296 ± 38	290 ± 37	Time × condition = 0.012
1.5-h FED[b]	219 ± 32	294 ± 65	365 ± 47	389 ± 67	312 ± 46	
4.5-h FED[a]	208 ± 36	276 ± 64	333 ± 39	297 ± 52	261 ± 50	
1.5-h EX-FED[a]	218 ± 44	299 ± 64	340 ± 38	321 ± 44	284 ± 56	
4.5-h EX-FED[a]	215 ± 57	311 ± 76	330 ± 30	348 ± 71	302 ± 65	
Valine						
Baseline[a]	232 ± 23	280 ± 26	259 ± 24	261 ± 16	288 ± 25	Time = 0.006
1.5-h FED[a]	252 ± 27	224 ± 9	280 ± 29	262 ± 15	308 ± 22	
4.5-h FED[b]	222 ± 16	216 ± 14	295 ± 6	240 ± 17	195 ± 16	
1.5-h EX-FED[a]	246 ± 21	250 ± 13	274 ± 30	248 ± 21	255 ± 33	
4.5-h EX-FED[b]	208 ± 27	212 ± 16	264 ± 26	246 ± 25	232 ± 33	
ΣEAAs						
Baseline	2966 ± 185	2730 ± 157	2718 ± 217	3142 ± 273	3016 ± 229	Time = 0.69
1.5-h FED	3336 ± 366	2837 ± 201	3118 ± 288	2946 ± 134	3212 ± 269	Treatment = 0.66
4.5-h FED	3301 ± 256	2801 ± 176	2925 ± 136	2741 ± 115	2818 ± 350	Condition = 0.71
1.5-h EX-FED	3190 ± 171	3110 ± 251	2684 ± 212	2732 ± 277	2626 ± 149	
4.5-h EX-FED	3163 ± 277	2657 ± 273	3198 ± 290	3066 ± 181	3261 ± 359	

[1] All values are means ± SEMs. *n* = 8/treatment group. Intracellular concentrations (μmol/L) of leucine, isoleucine, valine, and ΣEAAs after treatment administration. Data for leucine, isoleucine, valine, and ΣEAAs were each analyzed by using a 3-factor (treatment × time × condition) mixed-model ANOVA with Tukey's post hoc test. Times with different superscript letters within treatment columns were significantly different from each other within that treatment. Times with different superscript letters within the time/condition column were significantly different from each other. ‡Significantly different from W6; *significantly different from W6+Low-Leu; #significantly different from W25. EAA, essential amino acid; EX-FED, response to combined feeding and resistance exercise; FED, response to feeding; W6, 6.25 g whey protein; W6+BCAAs, 6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine; W6+High-Leu, 6.25 g whey protein supplemented with leucine to 5.0 g total leucine; W6+Low-Leu, 6.25 g whey protein supplemented with leucine to 3.0 g total leucine; W25, 25 g whey protein.

interaction effects for any of the factors (*P*-time × treatment= 0.941; *P*-condition × treatment = 0.992; *P*-time × condition = 0.117; *P*-time × condition × treatment = 0.975). The slope of the intracellular free phenylalanine enrichment was not different from zero for any of the treatment groups in either FED or EX-FED conditions (*see* Online Supplemental Material Figure 2 under "Supplemental data" in the online issue).

Plasma free phenylalanine enrichments did not differ between treatment groups (*P* = 0.917) or across time (*P* = 0.58). The slope of the plasma free phenylalanine enrichment was not different from zero for any treatment group (*see* Online Supplemental Material Figure 3 under "Supplemental data" in the online issue).

MPS

Myofibrillar FSR is shown in **Figure 3** (A–D, respectively). The myofibrillar FSR (%/h) during the early 0–1.5 h and late 1.5–4.5 h response is shown in Figure 3, A and B, whereas the aggregate 0–4.5 h response under both FED and EX-FED conditions is shown in Figure 3, C and D. Over the 0–1.5- and 1.5–4.5-h periods, the myofibrillar FSR showed a treatment × time interaction (*P* = 0.002), whereby the FSR was increased compared with at baseline (fasted) in all treatment groups when measured over 0–1.5 h. Over 1.5–4.5 h postexercise, the FSR remained increased compared with at baseline in all treatment groups; however, the FSR in W25 and W6+High-Leu groups was greater than in W6+Low-Leu, W6+BCAAs, and W6 groups. Similarly, the aggregate myofibrillar FSR response over 0–4.5 h showed a treatment × time interaction (*P* = 0.005), whereby the FSR was increased compared with at baseline in all treatment groups during the 0–4.5-h postprandial period; however, the FSR was greater in W25 and W6+High-Leu than W6+Low-Leu, W6+BCAAs, and W6 groups. There were no significant differences between FED and EX-FED conditions when examined over the 0–1.5- and 1.5–4.5-h periods (*P* = 0.483) or over the aggregate 0–4.5-h period (*P* = 0.419).

Muscle anabolic signaling

Changes in the phosphorylation status of signaling proteins involved in the regulation of messenger-RNA translation initiation and elongation are shown in **Table 5**. Akt (phospho-$\text{Akt}^{\text{Ser473}}$) showed a treatment × time × condition interaction (*P* = 0.025). Phospho-$\text{mTOR}^{\text{Ser2448}}$ showed a treatment × time interaction (*P* = 0.041), whereby at 1.5 h, phospho-$\text{mTOR}^{\text{Ser2448}}$ was increased after W6+Low-Leu, W25, and W6+High-Leu treatments. At 4.5 h, phospho-$\text{mTOR}^{\text{Ser2448}}$ remained increased above baseline (fasted) only after W6+High-Leu. Phospho-$\text{p70S6k}^{\text{Thr389}}$ showed no effect of time (*P* = 0.377), treatment (*P* = 0.353), or condition (*P* = 0.062) at the times examined. Phospho-$\text{4E-BP1}^{\text{Thr37/46}}$ showed a condition × time interaction

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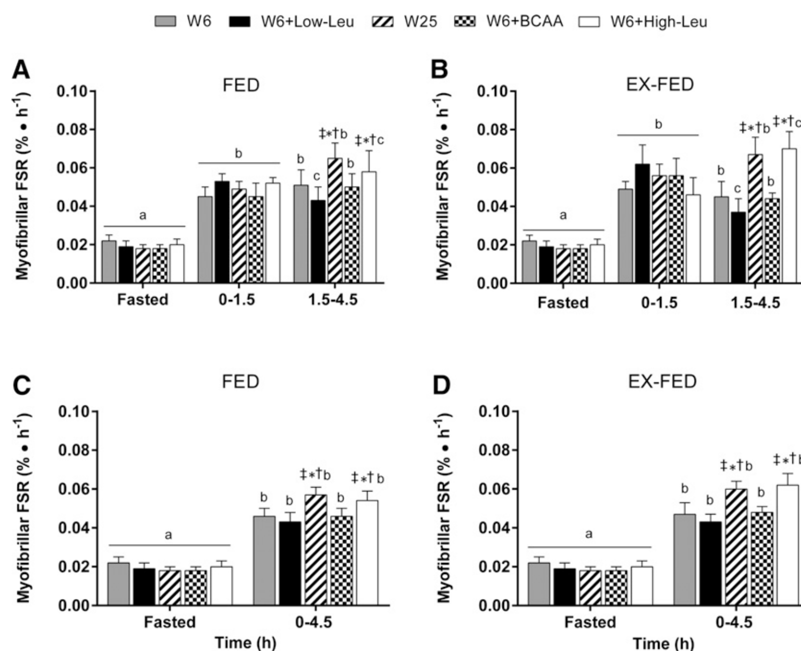

FIGURE 3. Values are means ± SEMs (n = 8/treatment group). Mean (±SEM) myofibrillar FSRs (%/h) calculated during baseline (fasted) conditions, over both early (0–1.5 h) and late (1.5–4.5 h) time periods (A and B), and over the aggregate 0–4.5-h postexercise recovery period (C and D) in both FED and EX-FED conditions after treatment administration. Data were analyzed by using a 3-factor (treatment × time × condition) mixed-model ANOVA with Tukey's post hoc test for analysis of the 0–1.5- and 1.5–4.5-h responses (P-treatment × time interaction = 0.002; P-treatment × time × condition = 0.799) and the aggregate 0–4-h response (P-treatment × time interaction = 0.005; P-treatment × time × condition = 0.942). Times with different lowercase letters were significantly different from each other within that treatment. [‡]Significantly different from W6 within that time; *significantly different from W6+Low-Leu within that time; [†]significantly different from W6+BCAA within that time. EX-FED, response to combined feeding and resistance exercise; FED, response to feeding; FSR, fractional synthetic rate; W6, 6.25 g whey protein; W6+BCAA, 6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine; W6+High-Leu, 6.25 g whey protein supplemented with leucine to 5.0 g total leucine; W6+Low-Leu, 6.25 g whey protein supplemented with leucine to 3.0 g total leucine; W25, 25 g whey protein.

(P = 0.044), whereby both conditions (FED and EX-FED) were increased above baseline (fasted) at 1.5 h. For phospho-ribosomal protein S6$^{Ser240/244}$, there was a condition × time interaction ($P <$ 0.001) whereby both conditions (FED and EX-FED) were increased above baseline fasted at both 1.5 and 4.5 h; however, the increase in the EX-FED condition at 1.5 h was greater than in the FED condition. Phospho-eEF2^{Thr56} showed no effect of time (P = 0.197), treatment (P = 0.384), or condition (P = 0.091) at the times examined. *See* Online Supplemental Material Figure 4 under "Supplemental data" in the online issue for representative blot images.

DISCUSSION

Our results showed that the addition of a higher dose of leucine to a smaller amount of protein (6.25 g) within a mixed macronutrient beverage enhanced MPS to the same level as that seen with 4 times as much whey protein (25 g). Muscle protein synthesis is increased in response to exercise and protein feeding in healthy individuals (42) and is the primary variable that determines diurnal changes in the net muscle-protein balance (43). It has been shown that MPS is stimulated in a protein-EAA dose-dependent manner up to ∼10 g EAAs at rest (6) and ∼20 g protein (∼8.6 g EAAs) after resistance exercise (7). Whey protein was used as the base protein source in this study because it is a high-quality protein source that robustly stimulates postprandial MPS rates (44). However, protein is typically co-ingested with carbohydrate and fat during meals, which may alter the kinetics of gut amino acid absorption (45). Thus, in this trial, we tested the efficacy of mixed macronutrient beverages with varying doses of whey protein and amino acids to stimulate MPS.

Consistent with our previous results when we used protein feeding alone (7), we showed that a low dose of protein (W6) was suboptimal for the stimulation of maximal MPS rates compared with 4 times as much whey protein (W25) even within a mixed macronutrient beverage over the aggregate 0–4.5-h postprandial period. The supplementation of this low-protein dose with a high proportion of leucine (W6+High-Leu) stimulated MPS to an equivalent magnitude and duration as that stimulated after ingestion of an energy-matched mixed macronutrient beverage that contained W25. Previous work has shown that W25 is a dose of protein and EAAs that is sufficient to induce a maximal stimulation of MPS rates at rest (6) and after resistance exercise (7). There were no differences in treatments in MPS during the 0–1.5-h postprandial period; however, W6+High-Leu and W25 stimulated greater MPS over the 1.5–4.5- and aggregate 0–4.5-h periods than did each of the other treatments (Figure 3). The somewhat surprising lack of difference in MPS rates in treatments during the early postexercise and postfeeding period occurred despite markedly divergent blood leucine, isoleucine, valine, and EAA concentrations (Figure 2, Table 3). Presumably, this lack of difference early after feeding (ie, ≤1.5 h) suggested that amino acid availability or nutrient signals (leucine) that served to trigger MPS were equivalent in all conditions. In contrast, in the latter portion of the protocol, only with the W6+High-Leu treatment was an MPS response shown that was equivalent to that with the W25 treatment, despite containing only one-quarter of the whey protein dose and ∼62% of the EAA content. That

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TABLE 5
Western-blot analysis of protein synthesis–associated signaling proteins after treatment administration[1]

	W6	W6+Low-Leu	W25	W6+BCAAs	W6+High-Leu	*P*
Phospho-Akt^{Ser473}						
1.5-h FED	1.61 ± 0.30	2.89 ± 0.69[‡,†,a]	2.09 ± 0.38[a]	1.52 ± 0.25	1.76 ± 0.18	Treatment × time × condition = 0.025
4.5-h FED	0.70 ± 0.17	1.67 ± 0.40[‡,†,+,$]	1.01 ± 0.24	0.71 ± 0.16	0.74 ± 0.09	
1.5-h EX-FED	1.45 ± 0.18	4.23 ± 1.40[‡,+,$,a]	3.69 ± 0.64[‡,$,a]	2.47 ± 0.24	2.03 ± 0.46	
4.5-h EX-FED	1.38 ± 0.41[$]	1.07 ± 0.10	1.50 ± 0.30	1.20 ± 0.16	1.17 ± 0.21	
Phospho-mTOR^{Ser2448}						
1.5-h FED	1.05 ± 0.15	1.59 ± 0.49[a]	1.52 ± 0.29[a]	1.35 ± 0.15	1.89 ± 0.31[‡,†,a]	Treatment × time = 0.041
4.5-h FED	0.62 ± 0.15	0.86 ± 0.19	1.25 ± 0.22	0.89 ± 0.15	1.63 ± 0.26[‡,†,a]	
1.5-h EX-FED	1.23 ± 0.16	1.45 ± 0.29[a]	1.60 ± 0.09[a]	1.45 ± 0.14	2.14 ± 0.36[‡,†,a]	
4.5-h EX-FED	0.87 ± 0.11	1.41 ± 0.39	1.10 ± 0.23	1.11 ± 0.14	1.71 ± 0.25[‡,†,a]	
Phospho-p70S6k1^{Thr389}						
1.5-h FED	1.04 ± 0.10	1.13 ± 0.09	1.02 ± 0.07	0.98 ± 0.08	1.12 ± 0.13	Time = 0.38
4.5-h FED	0.97 ± 0.13	1.02 ± 0.11	0.97 ± 0.13	0.86 ± 0.12	1.14 ± 0.14	Treatment = 0.35
1.5-h EX-FED	1.15 ± 0.11	1.13 ± 0.09	1.06 ± 0.06	0.92 ± 0.10	1.07 ± 0.11	Condition = 0.06
4.5-h EX-FED	1.01 ± 0.12	1.06 ± 0.08	1.02 ± 0.10	0.93 ± 0.09	1.33 ± 0.09	
Phospho-4E-BP1$^{Thr37/46}$						
1.5-h FED	1.02 ± 0.12	1.19 ± 0.12	1.25 ± 0.18	1.20 ± 0.12	1.58 ± 0.24	Condition × time = 0.044
4.5-h FED	0.73 ± 0.07	0.95 ± 0.15	0.86 ± 0.17	1.04 ± 0.19	0.85 ± 0.14	(1.5 h > baseline)
1.5-h EX-FED	1.05 ± 0.17	1.28 ± 0.16	1.77 ± 0.48	1.38 ± 0.20	1.59 ± 0.38	(1.5-h EX-FED > FED)
4.5-h EX-FED	1.02 ± 0.18	1.05 ± 0.18	1.20 ± 0.29	1.12 ± 0.10	1.45 ± 0.22	
Phospho-rpS6$^{Ser\ 240/244}$						
1.5-h FED	1.72 ± 0.41	2.46 ± 0.93	1.93 ± 0.32	2.66 ± 0.65	2.45 ± 0.56	Condition × time < 0.001
4.5-h FED	1.31 ± 0.22	2.31 ± 0.59	2.09 ± 0.42	1.63 ± 0.32	1.12 ± 0.30	(All times > baseline)
1.5-h EX-FED	3.25 ± 1.08	3.34 ± 0.93	4.45 ± 1.63	3.67 ± 1.27	4.49 ± 1.48	(1.5-h EX-FED > FED)
4.5-h EX-FED	1.47 ± 0.42	2.86 ± 1.26	1.38 ± 0.15	2.41 ± 0.65	2.44 ± 0.59	
Phospho-eEF2^{Thr56}						
1.5-h FED	0.95 ± 0.06	0.96 ± 0.05	1.02 ± 0.06	1.11 ± 0.09	1.08 ± 0.04	Time = 0.197
4.5-h FED	0.93 ± 0.07	1.08 ± 0.13	1.12 ± 0.12	0.99 ± 0.07	1.00 ± 0.06	Treatment = 0.384
1.5-h EX-FED	0.92 ± 0.03	1.15 ± 0.08	1.12 ± 0.10	1.09 ± 0.05	1.13 ± 0.04	Condition = 0.091
4.5-h EX-FED	0.88 ± 0.06	1.11 ± 0.10	1.03 ± 0.07	1.07 ± 0.11	1.05 ± 0.04	

[1] All values are fold difference from baseline and are means ± SEMs. *n* = 8/treatment group. The phosphorylation status of Akt^{Ser473}, mTOR^{Ser2448}, p70S6k^{Thr389}, 4E-BP1$^{Thr37/46}$, rpS6 $^{Ser240/244}$, and eEF2 ^{Thr56} is shown and expressed as the fold difference from baseline (fasted), at 1.5 h and 4.5 h postexercise recovery in both FED and EX-FED conditions after treatment administration. Data for Akt^{Ser473}, mTOR^{Ser2448}, p70S6k^{Thr389}, 4E-BP1$^{Thr37/46}$, rpS6$^{Ser240/244}$, and eEF2^{Thr56} were analyzed by using a 3-factor (treatment × time × condition) mixed-model ANOVA with Tukey's post hoc test. [a]Difference from baseline; [‡]significantly different from W6; [†]significantly different from W6+BCAAs; [+]significantly different from W6+High-Leu; [$]significantly different from the opposite condition at the same time point. Akt, protein kinase B; eEF2, eukaryotic elongation factor 2; EX-FED, response to combined feeding and resistance exercise; FED, response to feeding; mTOR, mechanistic target of rapamycin; p70S6k, 70 kDa ribosomal protein S6 kinase 1; rpS6, ribosomal protein S6; W6, 6.25 g whey protein; W6+BCAAs, 6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine; W6+High-Leu, 6.25 g whey protein supplemented with leucine to 5.0 g total leucine; W6+Low-Leu, 6.25 g whey protein supplemented with leucine to 3.0 g total leucine; W25, 25 g whey protein; 4E-BP1, eukaryotic initiation factor 4E binding protein 1.

the W6+High-Leu treatment was effective may relate to the fact that this treatment elicited the greatest blood leucine AUC_{pos} (Table 3), the greatest intracellular leucine concentration when assessed at 1.5 h (Table 4), and led to a sustained increase in the phosphorylation of mTOR^{Ser2448} at 4.5 h (Table 5). We also observed an increase in the phosphorylation of targets downstream of mTOR^{Ser2448} including 4E-BP1$^{Thr37/46}$ and ribosomal protein S6$^{Ser240/244}$ although there were no significant treatment effects (Table 5). We powered our study to detect relevant differences in the myofibrillar FSR (%/h), and thus, we may have lacked the statistical power to detect important differences in intracellular signaling molecule phosphorylation. In partial agreement with our observation of the MPS response after the W6+High-Leu treatment, previous studies have shown that a high proportion of leucine (3.5 compared with 1.8 g) within a 10-g EAA solution resulted in greater intramuscular cell signaling and a more prolonged mixed MPS response (24).

Contrary to our hypothesis, the W6+BCAAs treatment resulted in MPS rates that were less robust than with W6+High-Leu and W25 treatments. These differences occurred despite the fact that supplemental isoleucine and valine attenuated the decline in concentrations of these amino acids in the blood compared with that observed after the W6+High-Leu treatment (Table 3); however, intracellular concentrations of isoleucine and valine were not different between these treatments (Table 4). In addition, the W6+BCAAs treatment was associated with a lower intracellular leucine concentration at 1.5 h, a lower mean leucine AUC_{pos}, a lower leucine C_{max}, and a greater T_{max} than with the W6+High-Leu treatment (Table 3). We have shown that a rapid aminoacidemia after protein feeding stimulates greater MPS rates after resistance exercise than a slow protracted aminoacidemia (46). Therefore, we speculate that the greater T_{max} for leucine, isoleucine, valine and ΣEAAs after W6+BCAAs compared with W6+High-Leu may partially explain the observed differences in MPS rates. Because BCAAs share a common intestinal transporter, differences in amino acid appearance profiles between W6+BCAAs and W6+High-Leu treatments likely represents antagonism between BCAAs for uptake from the gut, which is

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congruent with data showing that isoleucine and valine compete with and can impede leucine absorption (47). The same effect could be true for the transsarcolemmal BCAA transport because BCAAs share the same transporter at that site (48).

We have previously shown that a suboptimal protein dose (6.25 g whey) supplemented with leucine (total leucine: 3.0 g) or a complete mixture of EAAs devoid of leucine (total leucine: 0.75 g) can stimulate postprandial MPS rates equivalent to that stimulated after the ingestion of 25 g whey protein (total leucine: 3.0 g) under resting but not postresistance exercise conditions (14). Similarly, in this study, W6+Low-Leu (total leucine: 3.0 g) and W6 (total leucine: 0.75 g) were as effective as W25 (total leucine: 3.0 g) at stimulating MPS rates when assessed during the early 0–1.5 h but not the later 1.5–4.5 h period. We showed no difference between FED compared with EX-FED rates of MPS, likely because of our choice of tissue sampling times. Our current results extend those of our previous work (14) by showing that, within the context of a mixed macronutrient beverage, a suboptimal protein dose (6.25 g) supplemented with a higher proportion of leucine (5.0 g total) was as effective at stimulating increased MPS rates as a dose of protein (25 g) able to induce a maximal stimulation of MPS rates after resistance exercise (7) and a dose of EAAs that maximally stimulates MPS at rest (6).

A novel aspect of our current study was that protein and amino acids were co-ingested with carbohydrate and fat. In our previous work (14), in which protein and free amino acids were ingested in isolation, the supplementation of 6.25 g whey to contain 3.0 g leucine induced peak blood amino acid concentrations ~550.0 μM, whereas in the current study, the same protein dose supplemented up to 5.0 g leucine was necessary to achieve similar peak blood leucine concentrations when co-ingested with carbohydrate and fat as part of a mixed macronutrient beverage. Thus, as has been reported previously (45, 49), the co-ingestion of protein with additional macronutrients attenuated the postprandial rise in blood amino acid concentrations.

Although several studies have assessed the effects of protein-carbohydrate co-ingestion on MPS rates (49, 50), few studies have examined the MPS response after a physiologic (ie single bolus) co-ingestion of protein, carbohydrate, and fat (51). Although the addition of carbohydrate to protein does not further stimulate increased MPS rates when adequate protein is provided (49, 50), it is not clear whether insulin can enhance MPS rates after the intake of a suboptimal protein dose in young individuals. In the current study, we observed robust increases in MPS rates in the W6 treatment that consisted of only 6.25 g whey protein but was co-ingested with 35.0 g carbohydrate. Whether the MPS response to W6 was enhanced by the addition of carbohydrate or whether only 0.75 g leucine serves as a sufficient nutrient signal to stimulate early increases in MPS rates in young, healthy individuals (14) requires additional investigation.

In conclusion, our results show that, when a suboptimal dose of protein (6.25 g) is supplemented with a relatively high dose of leucine (W6+High-Leu), rates of MPS are equivalent in both magnitude and duration to those observed after ingestion of an energy-matched beverage containing a saturating for MPS, 25-g dose of protein (W25) (7). These findings show that, within the context of mixed macronutrient intake, suboptimal protein doses can be made more effective in stimulating MPS through the addition of a high proportion of free leucine. This effect may be of importance in the development of nutritional formulations designed to promote skeletal muscle anabolism, which may be of particular significance to individuals in whom total protein intake is restricted or inadequate.

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