Leucine-enriched essential amino acid supplementation during moderate steady state exercise enhances postexercise muscle protein synthesis[1–5]

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ABSTRACT

Background: The effects of essential amino acid (EAA) supplementation during moderate steady state (ie, endurance) exercise on postexercise skeletal muscle metabolism are not well described, and the potential role of supplemental leucine on muscle protein synthesis (MPS) and associated molecular responses remains to be elucidated.

Objective: This randomized crossover study examined whether EAA supplementation with 2 different concentrations of leucine affected post–steady state exercise MPS, whole-body protein turnover, and mammalian target of rapamycin 1 (mTORC1) intracellular signaling.

Design: Eight adults completed 2 separate bouts of cycle ergometry [60 min, 60% $\dot{V}O_2$peak (peak oxygen uptake)]. Isonitrogenous (10 g EAA) drinks with different leucine contents [leucine-enriched (L)-EAA, 3.5 g leucine; EAA, 1.87 g leucine] were consumed during exercise. MPS and whole-body protein turnover were determined by using primed continuous infusions of [$^2H_5$]phenylalanine and [1-^{13}C]leucine. Multiplex and immunoblot analyses were used to quantify mTORC1 signaling.

Results: MPS was 33% greater ($P < 0.05$) after consumption of L-EAA (0.08 ± 0.01%/h) than after consumption of EAA (0.06 ± 0.01%/h). Whole-body protein breakdown and synthesis were lower ($P < 0.05$) and oxidation was greater ($P < 0.05$) after consumption of L-EAA than after consumption of EAA. Regardless of dietary treatment, multiplex analysis indicated that Akt and mammalian target of rapamycin phosphorylation were increased ($P < 0.05$) 30 min after exercise. Immunoblot analysis indicated that phosphorylation of ribosomal protein S6 and extracellular-signal regulated protein kinase increased ($P < 0.05$) and phosphorylation of eukaryotic elongation factor 2 decreased ($P < 0.05$) after exercise but was not affected by dietary treatment.

Conclusion: These findings suggest that increasing the concentration of leucine in an EAA supplement consumed during steady state exercise elicits a greater MPS response during recovery. This trial is registered at clinicaltrials.gov as NCT01366924. *Am J Clin Nutr* 2011;94:809–18.

INTRODUCTION

The effects of resistance exercise on skeletal muscle protein metabolism are well established (1–5). Resistance exercise, during which the muscle develops high contractile forces, stimulates a metabolic response after exercise that is primarily anabolic, with increased MPS[6] lasting for 48 h into recovery (4). Consumption of EAAs before, during, or immediately after exercise enhances the anabolic response by maximizing MPS and anabolic intracellular signaling through the mTORC1 pathway (6–11). However, the effects of endurance (ie, steady state) exercise may differ, given that the muscle contractile forces generated are lower and that endurance training does not typically result in muscle hypertrophy (12). Several studies have assessed MPS and mTORC1 intracellular signaling in response to endurance exercise alone (13–18) or coupled with feeding (15, 19–21). It is generally accepted that endurance exercise (fasted or fed) stimulates MPS, particularly mitochondrial protein synthesis, during recovery (11, 14, 15, 17, 20). However, not all studies support this theory, because some indicate little or no

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[6] Abbreviations used: APE, atom percent excess; BCAA, branched-chain amino acid; EAA, essential amino acid; ERK, extracellular-signal regulated protein kinase; L-EAA, leucine-enriched EAA; MPS, muscle protein synthesis; mTORC1, mammalian target of rapamycin complex 1; NOLD, nonoxidative leucine disposal; Ox, whole-body protein oxidation; $\dot{V}O_2$peak, peak oxygen uptake.

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effect of endurance exercise on MPS and anabolic intracellular signaling (13, 18, 21, 22).

The optimal dose of EAA necessary to maximize postexercise muscle anabolism remains undefined. Some reports indicate that MPS at rest and in recovery from resistance exercise reaches maximal stimulation after the consumption of 10 g EAA with an amino acid profile consistent with a 20-g serving of high-quality protein (23–25). Increasing the concentration of leucine within an optimal dose of EAA does not have an added stimulatory effect on resting and postresistance exercise MPS (26–28). In contrast with resistance exercise, sustained endurance exercise is mainly catabolic, yielding simultaneous reductions in MPS and plasma leucine concentrations during exercise, which may be attributed to the metabolic demand for BCAAs in exercising skeletal muscle (29). However, providing supplemental leucine during endurance exercise may enhance postexercise MPS by limiting reliance on endogenous protein stores. Only 2 studies have shown a benefit of protein supplementation throughout endurance exercise on postexercise whole-body protein utilization (30, 31). However, neither study assessed postexercise skeletal muscle protein metabolism nor has any study, to date, addressed whether the provision of supplemental leucine affects anabolic stimulus during recovery.

The current study was designed to assess the effect of consuming an optimal dose of EAA enriched with leucine throughout a moderate bout of endurance-type exercise on MPS, associated mTORC1 intracellular signaling, and whole-body protein turnover during recovery. We hypothesized that leucine supplementation during steady state exercise would spare endogenous leucine stores, thereby affecting MPS, mTORC1 intracellular signaling, and whole-body protein utilization during recovery.

SUBJECTS AND METHODS

Subjects

This study was approved by the Human Use Review Committee at the US Army Research Institute of Environmental Medicine, Natick, MA, and the Institutional Review Board at Tufts University. Human research volunteers provided informed voluntary consent and were medically cleared for participation. Investigators adhered to US Army Regulation 70–25 and US Army Medical Research and Material Command regulation 70–25 on the participation of volunteers in research.

The volunteers were free-living, active-duty military personnel who participated regularly in a combination of endurance (ie, running/marching) and resistance-type exercise (ie, calisthenics/free weights) 3–4 d/wk as part of their standard military physical training regimens. The volunteers were required to be weight stable (± 2 kg) for a minimum of 2 mo before initiating the study and recreationally fit as indicated by baseline-study screening (VO_2peak: 40–50 mL · kg^{-1} · min^{-1}). Prospective volunteers reporting metabolic or cardiovascular abnormalities, musculoskeletal injuries, or the use of medications known to influence protein metabolism were excluded from participation. Ten volunteers, 9 men and 1 woman, participated in this study. Due to analytic errors with the primary outcome variable (MPS), data for only 8 volunteers, 7 men and 1 woman, are included in the final report (**Table 1**).

TABLE 1
Baseline characteristics of the subjects[1]

Characteristic	Value
Age (y)	24 ± 2
Height (cm)	174 ± 3
Weight (kg)	76 ± 6
BMI (kg/m^2)	25 ± 2
Fat-free mass (kg)	58 ± 4
Body fat (%)	22 ± 2
VO_2peak (mL · kg^{-1} · min^{-1})	43 ± 1

[1] All values are means ± SEMs; n = 8 (7 men and 1 woman). Characteristics were determined by using common descriptive statistics. VO_2peak, peak oxygen consumption.

Experimental design

The study was designed to assess postexercise MPS, whole-body protein turnover, and anabolic intracellular signaling responses to EAA supplementation consumed during a moderate bout of steady state exercise. This 13 d randomized, crossover study included 2 identical assessments of protein turnover and intracellular signaling (days 8 and 13). The only variation on protocol days was the randomly assigned EAA supplement consumed during exercise, either an EAA or an L-EAA drink. To limit the potential for bias, the administration of the dietary treatments were balanced between study periods and blinded to the volunteers and study staff who performed data analysis, with the exception of the principal investigator and study coordinator who prepared and administered the treatments.

Diet and physical activity

The volunteers provided 3-d diet and activity records before study initiation to assess habitual dietary intake and physical activity. The volunteers were instructed to maintain a eucaloric diet throughout the study to maintain body weight; mean daily energy intake was 148.7 ± 15.2 kJ · kg^{-1} · d^{-1}. Body weight remained constant (prestudy: 76 ± 6 kg; day 13: 75 ± 6 kg) throughout the intervention, and dietary recalls confirmed compliance with the prescribed study diet. Macronutrient distribution was consistent with the study design; mean protein intake was 1.2 ± 0.1 g · kg^{-1} · d^{-1}, with the remaining energy derived from carbohydrate (55 ± 3%) and fat (32 ± 2%). Registered dietitians reviewed and analyzed 24 h diet recalls on days 2, 7, and 12 by using Food Processor SQL (version 10.0, 2006; ESHA Research). Volunteers were encouraged to maintain their usual training/physical activity for the duration of the study.

Baseline anthropometric measurements were performed by using standardized techniques and equipment. Height was measured in duplicate to the nearest 0.1 cm with an anthropometer (Item no. 101; Seritex Inc). After the subjects fasted overnight, body weight was measured by using a calibrated digital scale (WB-110A; Tanita) to the nearest 0.1 kg. Body composition was assessed at baseline by using dual-energy X-ray absorptiometry (DPX-IQ DXA; Lunar Corporation). VO_2peak was determined at baseline by analyzing expired gases during a progressive intensity cycle ergometer test with an open-circuit indirect respiratory system (TrueOne 2400 Metabolic Measurement System; ParvoMedics).

Infusion protocol

Infusion experiments to assess the primary outcome measures were completed on study days 8 and 13 between 0700 and 1230 after the subjects fasted overnight. The volunteers refrained from any physical activity (ie, resistance and endurance exercise) for 48 h before each infusion protocol and on study days 8 and 9 to minimize complications and the potential for carryover effects from one infusion experiment to the next. After catheters were placed into an antecubital vein for isotope infusion and into a heated dorsal hand vein for blood sampling, baseline blood and breath samples were obtained. A baseline muscle biopsy sample was obtained from the lateral portion of the vastus lateralis, as previously described (13). After the muscle biopsy was obtained (time 0), a bolus injection of [^{13}C]bicarbonate (2.35 μmol/kg) was administered to prime the bicarbonate pool, which was followed by a primed, constant infusions of L-[1-^{13}C]leucine (7.6 μmol/kg; 7.6 μmol $\cdot$ kg^{-1} $\cdot$ h^{-1}) and of L-[^{2}H$_5$]phenylalanine (2.8 μmol/kg; 0.07 μmol $\cdot$ kg^{-1} $\cdot$ min^{-1}). The L-[1-^{13}C]leucine infusion was infused until time point 195, whereas the L-[^{2}H$_5$]phenylalanine infusion was maintained until the completion of the protocol (**Figure 1**). Isotopes were commercially available (Cambridge Isotope Laboratories) and certified sterile and pyrogen free.

After resting for 30 min, the volunteers began a 60-min bout of moderate steady state (ie, endurance) exercise (60 $\pm$ 5% VO_2peak) on a cycle ergometer (Lode BV). Intensity was verified at 3 time points by using indirect calorimetry, and the workload was adjusted accordingly for accuracy. Throughout the exercise bout, the volunteers consumed equal volumes ($\approx$125 mL) of either the L-EAA or EAA supplement in 20-min intervals to minimize potential disturbances in isotopic steady state, beginning immediately after exercise initiation and ending at the completion of the exercise bout. Additional muscle biopsy samples were then obtained from the same incision 30 and 210 min after exercise. Muscle biopsy samples were obtained from the opposite leg during the second infusion experiment on study day 13 to limit possible residual effects from the initial trial. Visible blood and connective tissue were removed from the biopsy specimens, which were immediately frozen in liquid nitrogen. Blood samples were collected at 15-min intervals throughout recovery, and breath samples were collected until 105 min after exercise to measure isotopic enrichments, protein turnover, and insulin, glucose, and amino acid concentrations.

EAA drink composition

Isonitrogenous L-EAA and EAA drinks were dissolved in water (500 mL) with artificial sweetener. The experimental drink (L-EAA) provided 3.5 g leucine, whereas the control EAA supplement provided 1.87 g leucine. The percentage of leucine provided in the L-EAA and EAA drinks was based on earlier reports indicating changes in protein turnover and intracellular signaling when similar EAA supplements were consumed (7, 8, 26). L-EAA and EAA premixes were purchased commercially (Fortitech Inc), and amino acid profiles were confirmed by using chemical analysis (Covance Laboratories Inc). Small amounts of L-[1-^{13}C]leucine and L-[^{2}H$_5$]phenylalanine were added to each EAA drink to minimize possible disturbances in isotopic steady state (**Table 2**).

Determination of protein turnover

Muscle protein synthesis (fractional synthetic rate) of mixed skeletal muscle was calculated using the single pool precursor-product model to determine the rate of tracer incorporation from the muscle free amino acid intracellular pool into bound muscle protein between 30 min and 210 min after exercise (4).

Plasma [^{13}C]α-ketoisocaproate and breath $^{13}CO_2$ enrichments were determined by gas chromatography and isotope-ratio mass spectroscopy, respectively, by a commercial laboratory (Metabolic Solutions). Isotopic enrichment data from 5 time points after exercise were corrected for baseline enrichments and used to confirm isotopic steady state. Steady state conditions were

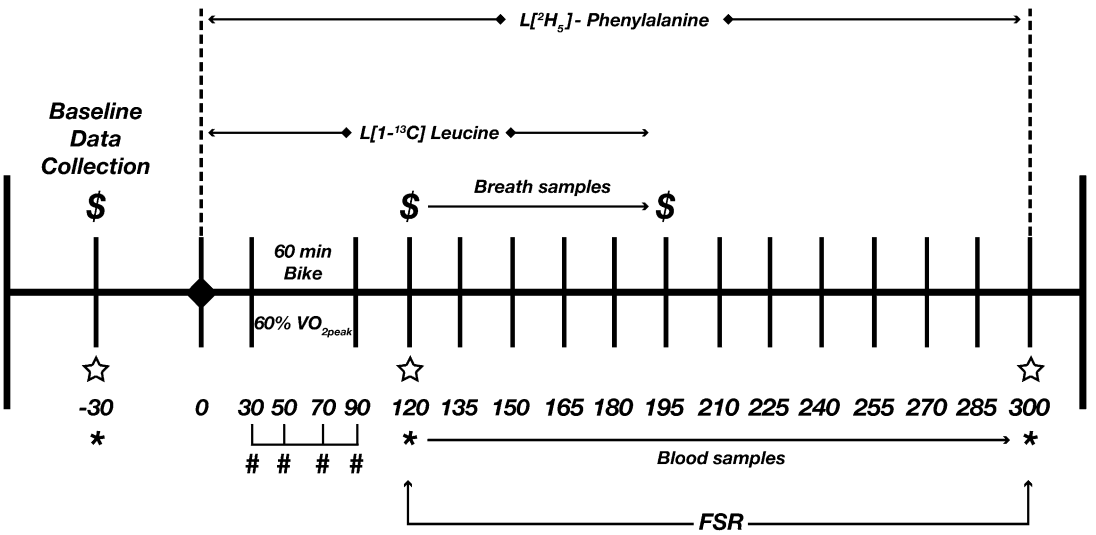

FIGURE 1. Schematic diagram of the infusion experiment. Muscle biopsy (☆), breath ($), and blood (⋆) samples and primed constant infusions (♦) of L-[^{2}H$_5$]phenylalanine and L-[1-^{13}C]leucine were used to determine muscle protein synthesis, mammalian target of rapamycin complex 1 intracellular signaling and whole-body protein turnover in recovery from a 60-min moderate bout of steady state exercise [cycle ergometry; 60 $\pm$ 5% peak oxygen consumption (VO_2peak)] during which volunteers consumed a leucine-enriched essential amino acid or essential amino acid drink (#). FSR, fractional synthetic rate.

TABLE 2
Amino acid composition of the leucine-enriched essential amino acid (L-EAA) and essential amino acid (EAA) supplements[1]

	L-EAA	EAA
	g/10 g	*g/10 g*
Histidine	0.80	1.08
Isoleucine	0.80	1.00
Leucine	3.50	1.87
Lysine	1.20	1.55
Methionine	0.30	0.32
Phenylalanine	1.40	1.55
Threonine	1.00	1.47
Valine	1.00	1.17
Total EAA	10.00	10.00
L-[$^2H_5$]Phenylalanine	0.10	0.11
L-[1-^{13}C]Leucine	0.21	0.11

[1] EAA profiles were confirmed by using chemical analysis (Covance Laboratories Inc). L-[$^2H_5$]Phenylalanine and L-[1-^{13}C]leucine were added to minimize disturbances in isotopic steady state.

assumed when the CV of the percentage enrichment and APE values at isotopic plateau were <10%. Data from the 5 time points were averaged for each subject, and group means were determined. Whole-body protein turnover (flux) was determined by using methods previously described by Campbell et al (32). Whole-body protein breakdown was calculated as the difference between flux (minus the tracer infusion rate) and leucine intake during the postprandial period. Accounting for the mass of the L-[1-^{13}C] leucine added to each dietary treatment, mean leucine intake during the postprandial period was 43.7 μmol $\cdot$ kg$^{-1}\cdot$ h^{-1} for the L-EAA trial and 23.3 μmol $\cdot$ kg$^{-1}\cdot$ h^{-1} for the EAA trial. Whole-body protein oxidation (Ox) was calculated from the $^{13}CO_2$ excretion rate by using the reciprocal pool model with a fractional bicarbonate retention factor of 0.83 (33). Whole-body protein synthesis (NOLD) was calculated as flux minus Ox. Net protein balance was calculated as the difference between total leucine intake (including the tracers) and oxidation. Whole-body protein turnover data are reported for only 6 (5 men, 1 woman) of the 8 volunteers with MPS data, because of technical errors with the L-[1-^{13}C]leucine infusate solutions.

Substrates and insulin analysis

Amino acid concentrations were determined in duplicate by using HPLC and *o*-phthaldialdehyde postcolumn derivatization (Agilent 1100 Series HPLC; Agilent Technologies). Glucose and insulin concentrations were measured in duplicate by using an advanced automated immunoassay instrument (Immulite 2000; Siemens Health Care Diagnostic).

Determination of mTORC1 intracellular signaling

Multiplexing and immunoblotting were used to quantify the phosphorylation status of proteins specific to the mTORC1 intracellular signaling pathway. Details of the muscle preparation and immunoblotting techniques were previously reported (13). Briefly, muscle samples were homogenized, incubated at 4°C for 15 min, and then centrifuged at 12,000 $\times$ *g* for 10 min at 4°C. Supernatant fluids were analyzed for protein by using the BCA Protein Assay (Thermo Scientific). Multiplexing was used to assess the phosphorylation of IRS-1 (Ser^{312}), Akt (Ser^{473}), TSC2 (Ser^{939}), mTOR (Ser^{2448}), GSKα (Ser^{21}), and GSKβ (Ser^{9}) by a commercial laboratory (Millipore Corporation). Phosphorylation data were expressed relative to total protein to correct for possible differences in the amount of protein loaded per well, and data are presented as fold changes compared with baseline (time 0) for the L-EAA and EAA trials.

For immunoblotting, equal amounts of total protein from the tissue homogenates originally prepared for multiplexing were subjected to SDS-PAGE by using precast Tris $\cdot$ HCl gels (Bio-Rad Laboratories) (13). Proteins were then transferred to polyvinylidene fluoride membranes followed by exposure to the appropriate phospho-specific primary antibodies. Corresponding total proteins were also immunoblotted to confirm equal loading of gels. Phospho-specific and total protein-specific primary antibodies for Akt (Ser^{473}), p70^{S6K} (Ser^{424}/Thr^{421}), p70^{S6K} (Thr^{389}), rpS6 (Ser$^{235/236}$), eEF2 (Thr^{56}), eIF4E (Ser^{209}), and extracellular-signal regulated protein kinase (ERK) 1/2 (Thr^{202}/Tyr^{204}) were produced in rabbits and purchased commercially (Cell Signaling Technology Inc). Labeling was performed by using anti-rabbit Ig conjugates with horseradish peroxidase (Cell Signaling). Signals were detected by using chemiluminescence (SuperSignal, West Pico Kit, Pierce Biotechnology) followed by exposure on imaging film (Kodak). Bands were quantified by using the ChemiGenius^2 Bioimaging system (Syngene). Phosphorylation data are expressed relative to total protein expression, and data are represented as fold changes compared with baseline (time 0) for the L-EAA and EAA trials.

Statistical analyses

Characteristics of the volunteers at baseline are described by using common descriptive statistics. A one-factor repeated-measures ANOVA was used to characterize dietary compliance during the 13-d intervention. Paired *t* tests were used, because of the within-subjects design (ie, dietary treatments), to assess the effects of L-EAA and EAA supplementation during exercise on postexercise MPS (primary outcome variable) as well as whole-body protein turnover. For all other purposes, data were analyzed by using repeated-measures ANOVA, with within-subjects factors for EAA dietary treatment (L-EAA and EAA) and time (infusion and biopsy time points). Mauchly's tests of sphericity were used to test for compound symmetry within the repeated-measures ANOVA. Huynh-Feldt and Greenhouse-Geisser corrections were applied if the assumption of sphericity was violated. In cases in which significant main effects (dietary treatment or time) or interactions were present, post hoc analyses were conducted by using Bonferroni adjustments to reduce the type I error rate. The α level for significance was set at $P < 0.05$. All data were analyzed by using SPSS (version 18.0, 2006; SPSS Inc) and expressed as means $\pm$ SEMs.

RESULTS

Phenylalanine and leucine enrichments

Individual fluctuations in plasma L-[$^2H_5$]phenylalanine did not occur, which suggests that steady state was achieved for the L-EAA (mean enrichment: 0.069 $\pm$ 0.002 tracer/tracee) and EAA (mean enrichment: 0.071 $\pm$ 0.002 tracer/tracee) infusion

trials (**Figure 2**A). Muscle intracellular L-[$^2H_5$]phenylalanine enrichment remained constant over time and was not different between trials (Figure 2B). Plasma [^{13}C]α-ketoisocaproate enrichment was at plateau and was similar for the L-EAA (mean enrichment: 6.1 ± 0.1 mole fraction % excess) and EAA (mean enrichment: 6.3 ± 0.3 mole fraction % excess) trials (Figure 2C). Mean $^{13}CO_2$ excretion was higher ($P < 0.05$) with L-EAA (0.044 ± 0.001 APE) than with EAA (0.027 ± 0.001 APE); however, it remained constant over time (Figure 2D).

Determination of protein turnover

Postexercise MPS was 33% higher ($P < 0.05$) with L-EAA (0.08 ± 0.01%/h) than with EAA (0.06 ± 0.01%/h) (**Figure 3**A). Flux was not different between trials; however, breakdown and NOLD were ~20% lower ($P < 0.05$) with L-EAA than with EAA. As such, postexercise leucine Ox for L-EAA (44 ± 5 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) was nearly 70% higher ($P < 0.05$) than that for EAA (26 ± 2 $\mu mol \cdot kg^{-1} \cdot h^{-1}$). Net protein balance was positive but not different ($P > 0.05$) between the L-EAA and EAA trials (Figure 3B).

Substrates and insulin analysis

A main effect of dietary treatment ($P < 0.05$) was noted for plasma leucine, because concentrations were ~31% higher for L-EAA (232 ± 15 μmol/L) than for EAA (176 ± 13 μmol/L). Plasma leucine showed a dietary treatment × time interaction ($P < 0.05$), because leucine concentrations were higher for L-EAA than for EAA at all time points after exercise (**Table 3**). In contrast, postexercise plasma isoleucine (57 ± 7 μmol/L) and valine (247 ± 17 μmol/L) concentrations were lower in those who consumed the L-EAA supplement than in those who consumed the EAA supplement (79 ± 8 and 278 ± 17 μmol/L, respectively). A dietary treatment × time interaction ($P < 0.05$) was observed for isoleucine and valine, because concentrations were higher with EAA than with L-EAA at all time points after exercise. Regardless of dietary treatment, mean plasma leucine, isoleucine, and valine were elevated ($P < 0.05$) after exercise and returned to baseline values 210 min into recovery. Total plasma EAA and combined BCAA were greater ($P < 0.05$) 30 and 45 min after exercise than at baseline; however, no differences were observed across dietary treatments. A main effect of time ($P < 0.05$) was observed for insulin and glucose (Table 3).

Multiplex determination of mTORC1 intracellular signaling

Independent of dietary treatment, phosphorylation of IRS-1 (Ser^{312}) was lower ($P < 0.05$) 210 min after than 30 min after exercise. However, IRS-1 (Ser^{312}) phosphorylation 210 min after exercise was lower with EAA than with L-EAA, as shown by a significant dietary treatment × time interaction ($P < 0.05$) (**Table 4**). A main effect of time ($P < 0.05$) was observed for Akt (Ser^{473}) and mTOR (Ser^{2448}), because phosphorylation was higher ($P < 0.05$) 30 min after exercise and returned to baseline 210 min after exercise. TSC2 (Ser^{939}) phosphorylation was influenced by time ($P < 0.05$), because levels were lower 210 min after exercise than at baseline or 30 min after exercise. Phosphorylation of Akt (Ser^{473}), TSC2 (Ser^{939}), and mTOR (Ser^{2448}) were not affected by dietary treatment. GSKα (Ser^{21}) and GSKβ (Ser^{9}) phosphorylation were not affected by dietary treatment or time.

Immunoblot determination of mTORC1 intracellular signaling

A main effect of time ($P < 0.05$) was noted for rpS6 (Ser$^{235/236}$), because phosphorylation was ~10 fold higher 30 and 210 min after exercise than at baseline (**Figure 4**). ERK1/2 (Thr^{202}/Tyr^{204}) was also influenced by time ($P < 0.05$); 2.6-fold higher phosphorylation was noted 210 min after exercise than at

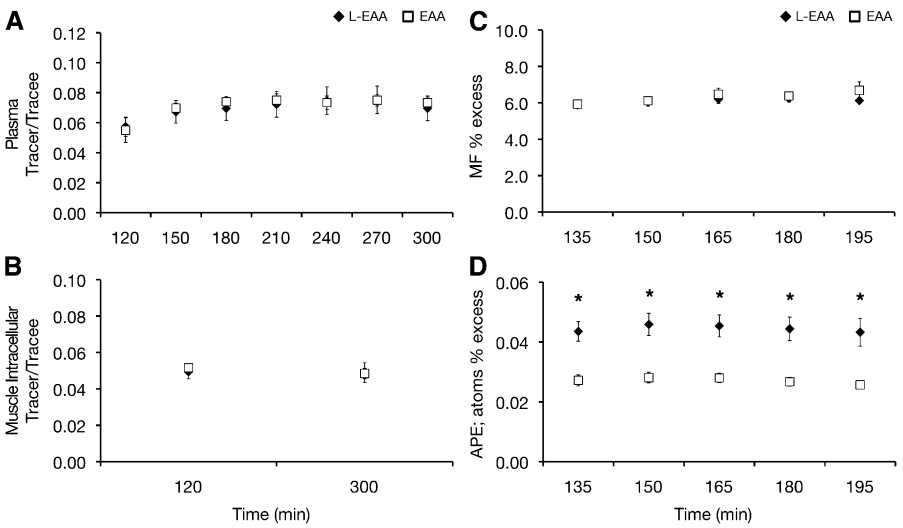

FIGURE 2. Mean (±SEM) plasma (A) and muscle intracellular (B) L-[$^2H_5$]-phenylalanine enrichments during recovery from a 60-min moderate bout of steady state exercise [cycle ergometry; 60 ± 5% peak oxygen consumption (VO_2peak)] after consumption of a leucine-enriched essential amino acid (L-EAA) or an essential amino acid (EAA) drink during exercise (n = 8). Postexercise mean (±SEM) 13C-α-ketoisocaproate plasma (C) and $^{13}CO_2$ breath (D) enrichments after volunteers consumed L-EAA and EAA drinks during exercise (n = 6). Isotopic steady state was analyzed by using repeated-measures ANOVA (within-subject factors: dietary treatment and time over the infusion trials) and was confirmed by a CV <10%. *Significantly different from EAA, $P < 0.05$. APE, atom percent excess; MF, mole fraction.

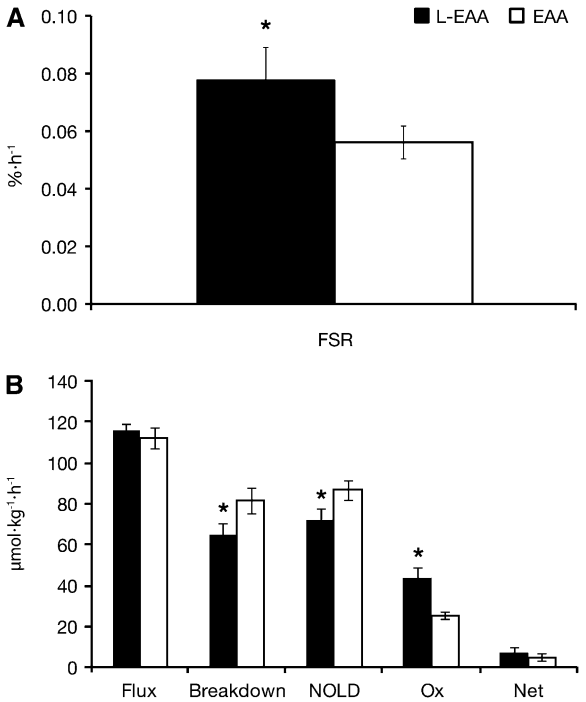

FIGURE 3. A: Mean (±SEM) muscle protein synthesis (fractional synthetic rate; FSR) in recovery from a 60-min moderate bout of steady state exercise [cycle ergometry; 60 ± 5% peak oxygen consumption (VO_2peak)] after consumption of a leucine-enriched essential amino acid (L-EAA) or an essential amino acid (EAA) drink during exercise (n = 8). B: Postexercise mean (±SEM) whole-body protein turnover (flux), breakdown synthesis (NOLD; nonoxidative leucine disposal), oxidation (Ox), and net balance (Net) after consumption of L-EAA and EAA drinks during exercise (n = 6). Paired t tests were used to determine differences between the L-EAA and EAA trials. *Significantly different from EAA, $P < 0.05$.

baseline. In contrast, eEF2 (Thr^{56}) phosphorylation was lower ($P < 0.05$) 30 and 210 min after exercise than at baseline. Similar to multiplex, Akt (Ser^{473}) phosphorylation appeared to be increased 30 min after exercise. Because of the inherent variability associated with immunoblot analysis, the change in Akt (Ser^{473}) phosphorylation was not statistically significant (P = 0.06). Although postexercise $p70^{S6K}$ (Ser^{424}/Thr^{421}) and (Thr^{389}) phosphorylation appeared numerically greater after exercise, no statistical differences were observed. Phosphorylation of rpS6 ($Ser^{235/236}$), ERK1/2 (Thr^{202}/Tyr^{204}), eEF2 (Thr^{56}), Akt (Ser^{473}), $p70^{S6K}$ (Ser^{424}/Thr^{421}), and $p70^{S6K}$ (Thr^{389}) were not influenced by dietary treatment. Phosphorylation of eIF4E (Ser^{209}) was not affected by dietary treatment or time.

DISCUSSION

The major finding from this applied human trial was that consumption of an EAA supplement enriched with leucine throughout moderate steady state exercise enhanced postexercise MPS by 33% when compared with an isonitrogenous EAA control. To the best of our knowledge, this was the first study to assess whether increasing leucine provision during endurance-type exercise by dietary supplementation enhances muscle protein anabolism in recovery. Until now, studies assessing the skeletal muscle anabolic response to increased leucine concentrations of an EAA- or protein-containing supplement have been performed at rest and after resistance exercise (7, 26, 28, 34, 35). In these studies, the stimulation of resting MPS was not enhanced by increasing the leucine concentration of EAA supplements to a level comparable with the L-EAA mixture used in the current investigation (26, 34). Similarly, others have shown that postresistance exercise net protein balance and MPS were increased in response to an EAA or protein supplement enriched with leucine (7, 28, 36). However, greater stimulation of MPS was observed only when compared with nonnitrogenous control subjects and not when compared with equal amounts of total EAA or protein, which suggested that added leucine is unnecessary for the stimulation of MPS when sufficient EAAs (or protein) are provided (28, 35, 36). Our findings indicate the opposite, whereby increasing the concentration of leucine in what may be considered an optimal dose of EAA elicits a greater stimulation of MPS after steady state exercise.

The MPS responses observed after L-EAA and EAA supplementation may be attributed in part to alterations in whole-body protein turnover. We suspect that increasing leucine availability during endurance-type exercise with L-EAA supplementation spared endogenous protein stores to a greater extent than did EAA alone, which contributed to the greater stimulation of MPS. Two studies have shown that consumption of dietary protein during endurance-type exercise spares endogenous protein (30, 31). Miller et al (31) found that consumption of 17 g protein during a 2-h run at 65% VO_{2max} suppressed whole-body proteolysis during recovery. Similar reductions in whole-body proteolysis were reported by Koopman et al (30), who provided 18 g protein every 30 min during a 6-h steady state exercise bout at 50% VO_{2max}. In the current study, whole-body proteolysis was lower with consumption of the L-EAA supplement, which suggests that the protein-sparing effect attributed to EAA ingestion during endurance-type exercise is enhanced by increasing leucine availability. Interestingly, whole-body protein synthesis was lower after supplementation with the L-EAA mixture, whereas MPS was higher with L-EAA than with EAA. The discrepant findings might partially be explained by the time frames during which synthesis was measured (NOLD; initial 105 min of recovery compared with MPS: entire 210 min of recovery), the relative contribution of MPS to whole-body protein synthesis, and by the increase in leucine oxidation observed throughout recovery. Miller et al (31) speculated that dietary amino acids consumed during exercise are preferentially oxidized, thereby limiting substrate availability for whole-body protein synthesis after exercise (37, 38). Our findings agree with that hypothesis and indicate that consumption of a small dose of EAA enriched with leucine during steady state exercise limits the dependence on endogenous protein and enhances MPS in recovery.

In the current study, observed differences in MPS may have been influenced by plasma amino acid availability. Although fluctuations in EAA and BCAA concentrations were similar between the L-EAA and EAA trials, plasma leucine concentrations were higher with the consumption of L-EAA than of EAA and remained elevated throughout recovery. Earlier reports have hypothesized that MPS is regulated by extracellular amino acid availability, because elevated MPS has been reported as a result of increased plasma amino acid concentrations after acute amino acid infusions (39–41) or ingestion (42). However, more recent publications suggest that intracellular amino acid

TABLE 3
Effects of leucine-enriched essential amino acid (L-EAA) and essential amino acid (EAA) supplementation during moderate steady state exercise on blood amino acid, insulin, and glucose concentrations in recovery[1]

	Baseline	30 min PE	45 min PE	90 min PE	150 min PE	210 min PE	Effect
Leucine (μmol/L)							
L-EAA	126 ± 8	381 ± 32[2]	339 ± 36[2]	218 ± 14[2]	174 ± 8[2]	156 ± 8[2]	D, T, D × T
EAA	125 ± 10	262 ± 22	231 ± 22	168 ± 16	142 ± 9	130 ± 6	
Isoleucine (μmol/L)							
L-EAA	64 ± 7	97 ± 12[2]	79 ± 12[2]	41 ± 5[2]	29 ± 4[2]	32 ± 42[2]	D, T, D × T
EAA	68 ± 7	131 ± 12	110 ± 13	69 ± 10	50 ± 5	47 ± 4	
Valine (μmol/L)							
L-EAA	243 ± 23	325 ± 25[2]	301 ± 22[2]	228 ± 15[2]	196 ± 12[2]	191 ± 12[2]	D, T, D × T
EAA	241 ± 19	368 ± 19	341 ± 24	270 ± 21	229 ± 14	217 ± 11	
BCAA (μmol/L)							
L-EAA	429 ± 35	791 ± 66	706 ± 67	481 ± 32	399 ± 22	377 ± 21	T
EAA	430 ± 33	743 ± 54	668 ± 57	501 ± 44	422 ± 25	344 ± 53	
EAA (μmol/L)							
L-EAA	889 ± 51	1499 ± 91	1364 ± 98	1000 ± 98	841 ± 37	802 ± 36	T
EAA	862 ± 59	1550 ± 82	1394 ± 101	1055 ± 83	874 ± 53	823 ± 35	
Insulin (pmol/L)							
L-EAA	25.8 ± 5.6	66.2 ± 22.1	46.0 ± 9.8	26.2 ± 5.5	21.0 ± 3.9	12.2 ± 4.3	T
EAA	25.0 ± 6.6	72.1 ± 20.4	46.6 ± 9.8	28.5 ± 7.9	18.9 ± 3.1	20.0 ± 4.4	
Glucose (mmol/L)							
L-EAA	4.9 ± 0.1	5.0 ± 0.3	4.9 ± 0.2	4.5 ± 0.0	4.7 ± 0.1	4.7 ± 0.1	T
EAA	5.0 ± 0.1	5.4 ± 0.3	4.9 ± 0.2	4.9 ± 0.1	4.8 ± 0.2	4.7 ± 0.1	

[1] Values are means ± SEM (n = 8). Blood amino acid, insulin, and glucose concentrations were measured during recovery, for 210 min after exercise (postexercise; PE), from a 60-min moderate bout of steady state exercise [cycle ergometry; 60 ± 5% VO_2peak (peak oxygen uptake)] after consumption of an L-EAA or EAA drink during exercise. Repeated-measures ANOVA (within-subject factors: dietary treatment and time over the infusion trials) with Bonferroni correction was used to determine the main effect of dietary treatment (D), the main effect of time (T), and the D × T interaction ($P < 0.05$). BCAA, branched-chain amino acids.

[2] Significantly different from EAA, $P < 0.05$.

transport and subsequent muscle amino acid concentrations are critical rate-limiting steps in amino acid metabolism that modulate MPS (43, 44). Intracellular amino acid transport at rest exhibits saturation-type kinetics, because higher plasma concentrations of leucine do not result in increased intracellular amino acid transport, leucine concentrations in muscle, and stimulation of MPS (26). However, prolonged endurance exercise combined with BCAA administration has been shown to accelerate transport of amino acids into muscle cells, resulting in higher intracellular amino acid concentrations after exercise (45, 46). It is important to note a limitation of the current study—the inability to determine muscle intracellular amino acid kinetics or availability. However, our findings do suggest that increasing plasma EAA concentrations, particularly leucine, combined with endurance-type exercise may upregulate muscle intracellular amino acid kinetics, resulting in greater MPS during recovery.

Some studies have shown that endurance exercise alone or combined with feeding stimulates mTORC1 intracellular signaling (16, 47–49), although other investigations have shown little or no effect of endurance exercise with or without nutrition

TABLE 4
Effects of leucine-enriched essential amino acid (L-EAA) and essential amino acid (EAA) supplementation during moderate steady state exercise on postexercise (PE) phosphorylation of muscle protein synthesis–associated intracellular signaling proteins by multiplex analysis[1]

	30 min PE		210 min PE		
	L-EAA	EAA	L-EAA	EAA	Effect
IRS-1 (Ser^{312})	1.15 ± 0.2	2.17 ± 0.6	0.90 ± 0.2[2]	0.40 ± 0.1	T, D × T
Akt (Ser^{473})	1.25 ± 0.2	1.29 ± 0.3	1.0 ± 0.3	0.52 ± 0.1	T
TSC2 (Ser^{939})	1.01 ± 0.2	0.98 ± 0.2	0.82 ± 0.1	0.44 ± 0.2	T
mTOR (Ser^{2448})	1.25 ± 0.2	1.67 ± 0.3	1.13 ± 0.2	0.56 ± 0.1	T
GSKα (Ser^{21})	0.93 ± 0.1	0.93 ± 0.1	0.97 ± 0.2	0.68 ± 0.1	
GSKβ (Ser^{9})	7.51 ± 5.9	2.19 ± 0.7	3.16 ± 1.7	1.71 ± 0.4	

[1] All values are means ± SEMs; n = 8. PE phosphorylation data are expressed relative to total protein and presented as fold changes compared with baseline (time 0) for the L-EAA and EAA trials. Akt, protein kinase B; GSK, glycogen synthase kinase; IRS-1, insulin receptor substrate-1; mTOR, mammalian target of rapamycin; TSC2, tuberous sclerosis complex 2. Repeated-measures ANOVA (within-subject factors: drink, L-EAA and EAA, and biopsy time points) with Bonferroni correction was used to determine the main effect of dietary treatment (D), the main effect of time (T), and the D × T interaction ($P < 0.05$).

[2] Significantly different from EAA at 210 min, $P < 0.05$.

	30 min PE		210 min PE		Representative blot[2]						
					Baseline		30 min PE		210 min PE		
	L-EAA	EAA	L-EAA	EAA	L	E	L	E	L	E	Effect
Akt (Ser^{473})	1.88 ± 0.53	4.10 ± 1.78	0.91 ± 0.13	0.76 ± 0.14							
$p70^{S6K}$ (Ser^{424}/Thr^{421})	11.42 ± 3.16	6.72 ± 1.92	9.25 ± 4.77	18.27 ± 8.31							
$p70^{S6K}$ (Thr^{389})	7.07 ± 3.67	9.20 ± 5.65	13.25 ± 9.03	7.75 ± 3.62							
rpS6 ($Ser^{235/236}$)	10.73 ± 3.13	9.94 ± 3.56	8.41 ± 1.51	8.86 ± 1.86							T
eEF2 (Thr^{56})	0.80 ± 0.06	0.89 ± 0.04	0.83 ± 0.06	0.87 ± 0.03							T
eIF4E (Ser^{209})	1.00 ± 0.16	1.31 ± 0.34	1.09 ±0.13	1.20 ± 0.24							
ERK 1/2 (Thr^{202}/Tyr^{204})	2.17 ± 0.41	3.07± 0.95	2.29 ± 0.51	2.99 ± 0.66							T

FIGURE 4. Effects of leucine-enriched essential amino acid (L-EAA; L) and essential amino acid (EAA; E) supplementation during moderate steady state exercise on postexercise (PE) phosphorylation of muscle protein synthesis–associated intracellular signaling proteins by using immunoblot analysis. Values are means ± SEMs ($n = 8$). PE phosphorylation data are expressed relative to total protein and are presented as fold changes compared with baseline (time 0) for the L-EAA and EAA trials. [2]Representative blots for phosphorylation (total protein expression was not different between dietary treatments or time; data not shown) at baseline (time 0), 30 min PE, and 210 min PE for the L and E trials. Repeated-measures ANOVA were used to determine the main effect of time (T) ($P < 0.05$). Akt, protein kinase B; eEF2, eukaryotic elongation factor 2; eIF4E, eukaryotic initiation factor 4E; ERK, extracellular-signal regulated protein kinase; $p70^{S6K}$, 70-kDa S6 kinase; rpS6, ribosomal protein S6.

on molecular mediators of MPS (13, 21, 22). The discrepant observations regarding the influence of endurance-type exercise and feeding on mTORC1 intracellular signaling may be attributed to between-study differences in exercise intensity and duration, in the training state of the study volunteers, and in the timing of the muscle biopsy collections during recovery (48, 50–52). In the current study, moderate steady state exercise coupled with EAA supplementation increased Akt, mTOR, rpS6, and ERK 1/2 and reduced eEF2 phosphorylation. The noted increase in ERK 1/2 phosphorylation is consistent with our previous report, which suggests that harvesting multiple biopsy samples from one incision site may elicit an inflammatory response that might influence intracellular signaling (13). Changes in Akt, mTOR, rpS6, and eEF2 phosphorylation are similar to those in other studies and are consistent with an upregulation of mRNA translation initiation, elongation, and increased MPS after exercise (1, 15, 16, 47–49). It is important to note that we were unable to detect significant differences in the phosphorylation of any critical mTORC1 intracellular signaling protein between the dietary treatment groups, despite the differences observed in MPS. Although enhanced intracellular signaling is not necessarily a definitive predictor of increased MPS (26, 53), our inability to identify the molecular mechanism accounting for the increase in MPS after L-EAA supplementation limits our interpretation of the study findings.

Similar to the recent publication by Glynn et al (26), the current study was designed to assess the influence of equal doses of EAA (10 g) with 2 different amounts of leucine (3.5 and 1.87 g) on human MPS. Importantly, our study provided an evaluation of the effects of EAA supplementation during steady state exercise on protein metabolism in recovery. To the best of our knowledge, this was the first study to report postexercise MPS and mTORC1 intracellular signaling and to show a greater anabolic response to EAA supplementation that increased the availability of leucine during a moderate bout of steady state exercise. We recognize that the additional leucine tracer added to the dietary treatments may have been confounding and led to an overestimation of the contribution of leucine intake to whole-body protein breakdown. Certainly, the timing and repeat doses (2.5 g EAA every 20 min for 1 h) of EAA would have had a minimal effect or no effect on steady state kinetics, because others have provided significantly more protein ($0.4\ g \cdot kg^{-1} \cdot h^{-1}$ over 3 h) without added leucine tracer and documented the maintenance of isotopic steady state (20). However, any effect of the added tracers would have been minimal, constant, and similar between dietary treatments given that the EAA mixtures were enriched equally. As such, the observed flux, Ox, and NOLD measurements were reliable indicators of steady state whole-body protein turnover (25, 31) and were consistent with our hypothesis that consuming EAA enriched with leucine during steady state exercise spares endogenous protein by providing a readily available source of leucine (and remaining BCAAs) to exercising skeletal muscle. Other limitations include the lack of stable-isotope data to model intracellular leucine kinetics, skeletal muscle proteolysis, and muscle amino acid concentrations. Furthermore, sample size may have reduced our ability to detect statistical effects for

secondary measures. Finally, a more thorough molecular analysis assessing leucine-sensitive signaling proteins (eg, hVps34 and MAP4K3) and proteolytic gene expression may assist in the elucidation of the mechanism conferring greater MPS after leucine supplementation.

In summary, consumption of a 10-g dose of EAA enriched with leucine during moderate endurance-type exercise stimulated increased MPS when compared with an isonitrogenous EAA supplement with an amino acid profile consistent with high-quality proteins. These data indicate that increasing leucine availability during steady state exercise promotes skeletal muscle protein anabolism and spares endogenous protein. The EAA supplement containing a higher concentration of leucine did not enhance mTORC1 intracellular signaling. Future studies are required to determine the molecular mechanisms associated with leucine on human MPS and whether the observed anabolic effects are influenced by nutrient timing or exercise intensity. Our findings indicate that increasing the leucine content of protein supplements provided for those populations susceptible to muscle loss, including proteolytic conditions—such as cachexia, sarcopenia, and calorie deprivation—may warrant further exploration.

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