

Addition of Carbohydrate or Alanine to an Essential Amino Acid Mixture Does Not Enhance Human Skeletal Muscle Protein Anabolism^{1–3}

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Abstract

In humans, essential amino acids (EAAs) stimulate muscle protein synthesis (MPS) with no effect on muscle protein breakdown (MPB). Insulin can stimulate MPS, and carbohydrates (CHOs) and insulin decrease MPB. Net protein balance (NB; indicator of overall anabolism) is greatest when MPS is maximized and MPB is minimized. To determine whether adding CHO or a gluconeogenic amino acid to EAAs would improve NB compared with EAA alone, young men and women ($n = 21$) ingested 10 g EAA alone, with 30 g sucrose (EAA+CHO), or with 30 g alanine (EAA+ALA). The fractional synthetic rate and phenylalanine kinetics (MPS, MPB, NB) were assessed by stable isotopic methods on muscle biopsies at baseline and 60 and 180 min following nutrient ingestion. Insulin increased 30 min postingestion in all groups and remained elevated in the EAA+CHO and EAA+ALA groups for 60 and 120 min, respectively. The fractional synthetic rate increased from baseline at 60 min in all groups ($P < 0.05$; EAA = 0.053 ± 0.018 to $0.090 \pm 0.039\% \cdot h^{-1}$; EAA+ALA = 0.051 ± 0.005 to $0.087 \pm 0.015\% \cdot h^{-1}$; EAA+CHO = 0.049 ± 0.006 to $0.115 \pm 0.024\% \cdot h^{-1}$). MPS and NB peaked at 30 min in the EAA and EAA+CHO groups but at 60 min in the EAA+ALA group and NB was elevated above baseline longer in the EAA+ALA group than in the EAA group ($P < 0.05$). Although responses were more robust in the EAA+CHO group and prolonged in the EAA+ALA group, AUCs were similar among all groups for fractional synthetic rate, MPS, MPB, and NB. Because the overall muscle protein anabolic response was not improved in either the EAA+ALA or EAA+CHO group compared with EAA, we conclude that protein nutritional interventions to enhance muscle protein anabolism do not require such additional energy. *J. Nutr.* 143: 307–314, 2013.

Introduction

The muscle protein anabolic response to a mixed meal or mixed amino acid ingestion is driven by essential amino acids (EAAs)⁹ and attributable to large increases in muscle protein

synthesis (MPS) with little to no change in the rate of muscle protein breakdown (MPB) (1–4). Conversely, elevated insulin concentrations following carbohydrate (CHO) ingestion may decrease MPB (5–9), though decreases in MPB following insulin infusion are not always observed (10–12). There are conflicting reports in the literature regarding the effect of insulin on MPS; however, it is clear that infusing insulin directly into the femoral artery does increase MPS in human subjects (5,7,10–17). Nonetheless, protein accretion occurs when the net protein balance (NB = MPS – MPB) becomes positive. Therefore, nutrient combinations that maximally stimulate synthesis while minimizing breakdown should result in a greater muscle protein anabolic response than those that affect only one component of the equation. Ingestion of 10 g EAA has been shown to elicit a robust muscle protein synthetic response at rest in human skeletal muscle (18). However, it remains unclear whether the addition of non-EAA energy to 10 g EAA will result in an improved anabolic response to nutrients at rest, either by potentiating the effect of EAA on MPS or decreasing MPB.

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³ Supplemental Figures 1–3 and Tables 1 and 2 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

⁹ Abbreviations used: ALA, alanine; BF, blood flow; CHO, carbohydrate; EAA, essential amino acid; EAA+ALA, 10 g EAA + 30 g ALA; EAA+CHO, 10 g EAA + 30 g sucrose; ICG, indocyanine green; ISTD, internal standard; LLM, leg lean mass; MPB, muscle protein breakdown; MPS, muscle protein synthesis; NB, net protein balance.

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Several cellular signaling pathways activated in response to increased energy availability, EAAs, and insulin are linked by common intermediates. Therefore, it is mechanistically plausible that CHOs or increased energy availability could lead to changes in MPS and MPB. Stimulation of MPS by EAAs occurs in part by signaling through the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway (19–23). Activation of mTOR and downstream effectors ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) lead to enhanced translation initiation (24,25). Akt/PKB (protein kinase B) is involved in the insulin signaling cascade and can activate mTOR through direct phosphorylation (26,27). Insulin may also affect MPB via Akt regulation of certain atrogenes (28–31). Thus, additional energy provided with EAAs may act through cell-signaling pathways to enhance mTOR activity and/or decrease the activity of catabolic pathways.

Additional energy could improve the overall net muscle protein anabolic response to EAAs by regulating signaling pathways responsive to energy availability, insulin, and/or amino acids. Therefore, the purpose of this study was to examine the effect of additional energy on the protein anabolic response to 10 g EAA. Specifically, we compared solutions containing 10 g EAA alone or 10 g EAA + 30 g sucrose (EAA+CHO). To further distinguish the effects of pure CHO from other energy, such as that from a gluconeogenic amino acid, we investigated the addition of alanine (ALA) to EAA [10 g EAA + 30 g ALA (EAA+ALA)], which was isocaloric to EAA+CHO. Non-EAAs such as ALA do not significantly contribute to the stimulation of MPS following amino acid ingestion in animals or humans (2,4,32), and the gluconeogenic properties of ALA make it a readily available, albeit slow-releasing, energy source. Despite the isocaloric nature of the EAA+CHO and EAA+ALA solutions, we expected EAA+CHO to induce a greater insulin response, leading to decreased MPB compared with EAA or EAA+ALA. Therefore, we hypothesized that only the addition of CHO to 10 g EAA would improve muscle NB and that this would be primarily driven by decreases in MPB rather than increases in MPS.

Materials and Methods

Participants. We studied 21 young participants (9 men, 12 women; age 30 ± 1 y) who reported not being currently engaged in any regular exercise training during the screening interview. Subjects were randomized to 1 of 3 groups: EAA, EAA+CHO, or EAA+ALA. A subset of data was previously reported for the EAA group (33). However, due to presentation of the data herein, only data for phenylalanine concentration and partial data for arterial plasma glucose and insulin were previously reported (33). Screenings of participants were performed as previously described (33). Volunteers were asked to refrain from performing vigorous physical activity for 48 h prior to participating in the study. All participants gave informed written consent before participating in the study, which was approved by the Institutional Review Board of the University of Texas Medical Branch (which is in compliance with the Declaration of Helsinki as revised in 1983). A DXA scan (Hologic QDR 4500W) was performed to measure body composition and lean mass. The participants' physical characteristics are summarized in Table 1 and did not differ between groups.

Experimental design. Figure 1 shows the study design, the details of which were previously reported (33,34). Briefly, all participants were studied following an overnight fast under basal conditions and at the same time of day (between 0700 and 1600 h). After drawing a background blood sample, a primed continuous infusion of L-[$^{13}\text{C}_6$] phenylalanine (priming dose = $2 \mu\text{mol} \cdot \text{kg}^{-1}$, infusion rate = $0.05 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) was begun and maintained at a constant rate until the end of the

TABLE 1 Participant characteristics¹

Characteristic	EAA	EAA+ALA	EAA+CHO
<i>n</i> (men, women)	3, 4	3, 4	3, 4
Age, y	32 ± 2	28 ± 1	29 ± 1
Height, cm	170 ± 5	167 ± 5	172 ± 5
Weight, kg	73 ± 3	72 ± 6	71 ± 6
BMI, $\text{kg} \cdot \text{m}^{-2}$	25.5 ± 1.1	25.5 ± 1.1	23.9 ± 1.1
Lean body mass, kg	53 ± 4	47 ± 5	49 ± 6
Body fat, %	24.8 ± 3.5	30.5 ± 3.5	26.9 ± 4.7
Leg fat mass, ² kg	3.5 ± 0.5	3.6 ± 0.3	3.5 ± 0.6
LLM, kg	8.6 ± 0.8	8.5 ± 1.1	8.7 ± 1.2
Trunk fat mass, kg	8.0 ± 1.2	10.0 ± 1.7	7.8 ± 1.3
Trunk lean mass, kg	26.1 ± 1.7	21.1 ± 2.0	23.0 ± 2.8

¹ Values are mean \pm SEM. EAA, 10 g essential amino acids; EAA+ALA, essential amino acids + 30 g alanine; EAA+CHO, 10 g essential amino acids + 30 g carbohydrate as sucrose; LLM, leg lean mass.

² Leg fat and lean mass are reported for catheterized and biopsied leg.

experiment (Isotec, Sigma-Aldrich). Two and one-half to 3 h after starting the tracer infusion, the first muscle biopsy was obtained from the lateral portion of the vastus lateralis using a sterile procedure and local anesthesia (1% lidocaine). Two hours after the first biopsy and initiating the baseline period, infusion of indocyanine green (ICG; Akorn) was begun in the femoral artery ($0.5 \text{ mg} \cdot \text{min}^{-1}$) and maintained for 10 min. The sequence was repeated for a total of 4 sets of blood draws ~ 15 min apart. Following the fourth set of blood draws, a second muscle biopsy was obtained, concluding the baseline period. After the second baseline biopsy, participants ingested a nutrient solution according to group assignment: EAA, EAA+CHO, or EAA+ALA (compositions below). Muscle biopsies were then sampled at 60 and 180 min following ingestion of the nutrient solution. ICG infusion and blood sampling were performed during each hour as described above.

Nutrient solution composition. All solutions (EAA, EAA+ALA, EAA+CHO) contained the same 10 g EAAs mixed in a noncaloric, noncaffeinated, carbonated beverage (500 mL) as previously reported (33,34): L-histidine (1.1 g), L-isoleucine (1.0 g), L-leucine (1.85 g), L-lysine (1.55 g), L-methionine (0.3 g), L-phenylalanine (1.55 g), L-threonine (1.45 g), and L-valine (1.2 g) (Sigma-Aldrich). In addition, the EAA+CHO solution contained 30 g sucrose and the isocaloric EAA+ALA solution contained 30 g ALA (Sigma-Aldrich).

Determination of blood flow, plasma glucose, and insulin concentrations. The serum ICG concentration to determine leg blood flow (BF) was spectrophotometrically measured (Beckman Coulter) at $\lambda = 805 \text{ nm}$ (35). To normalize for lean mass in all calculations, BF is expressed per 100 g leg lean mass (LLM). Methods for plasma glucose, insulin, and glucose uptake measures were previously published (33).

Amino acid enrichments and concentrations. Enrichment of blood phenylalanine was determined on *tert*-butyldimethylsilyl derivatives using L-[^{15}N]phenylalanine as internal standard (ISTD) and by GC-MS (6890 Plus GC, 5973N MSD/DS, 7683 autosampler, Agilent Technologies) to calculate phenylalanine concentrations as previously described (36): $C_A = [(C_{\text{ISTD}} \cdot (\text{ISTD}/\text{mL blood})/E_{\text{ISTD(A)}}) \cdot (1 + E_A)]$, where C_A is the amino acid concentration in the femoral artery, C_{ISTD} is the concentration of the ISTD used, $E_{\text{ISTD(A)}}$ is the enrichment of the ISTD in the femoral artery, and E_A is the free tracer enrichment (tracer:tracee ratio) in the femoral artery. The same equation was used to calculate the phenylalanine concentration in the femoral vein by replacing (A) with (V). Muscle tissue samples were ground and intracellular free amino acids and muscle proteins extracted as previously described (36). Intracellular free enrichments of phenylalanine were determined by GC-MS using L-[^{15}N]phenylalanine as an ISTD and protein-bound phenylalanine enrichments were measured by GC-MS (36).

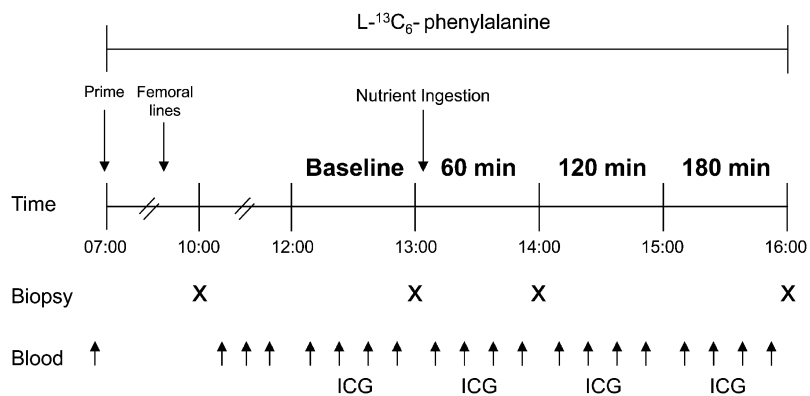


FIGURE 1 Study design. A primed, constant infusion of L-¹³C₆-phenylalanine was begun at 0700 h followed by placement of arterial and venous femoral catheters. The study consisted of a basal period (baseline) followed by nutrient ingestion (EAA, EAA+ALA, or EAA+CHO) and 3 additional 60-min periods. In each period, ICG was infused to measure BF, blood samples were collected every 15 min, and muscle biopsies from the vastus lateralis were obtained at baseline (2) and 60 and 180 min following nutrient ingestion. Arrow indicates blood draw. BF, blood flow; EAA, essential amino acids; EAA+ALA, 10 g essential amino acids + 30 g alanine; EAA+CHO, 10 g essential amino acids + 30 g carbohydrate as sucrose; ICG, indocyanine green; X, muscle biopsy.

Calculations. Phenylalanine kinetics were calculated using both the 2-pool and 3-pool models of leg muscle amino acid kinetics and have been described in detail elsewhere (13,33,37,38). AUCs were calculated across time for each subject on raw values and then averaged by group (SigmaPlot v. 11.0; Systat Software).

Immunoblot analysis. Details of homogenization and immunoblotting techniques can be found elsewhere (39). Briefly, equal amounts of total protein (determined spectrophotometrically) were loaded into each lane and the samples separated by SDS-PAGE (Criterion, Bio-Rad). Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) then incubated in primary (overnight) and secondary (1 h) antibodies. Chemiluminescent solution (ECL plus, Amersham BioSciences) was applied to each blot for 5 min. OD measurements (ChemiDoc, Bio-Rad) were taken and densitometric analysis performed using Quantity One 4.5.2 software (Bio-Rad). Membranes containing phospho-detected proteins were stripped of primary and secondary antibodies using Restore Western blot Stripping Buffer (Pierce Biotechnology) and then reprobed for total protein. Total protein for each phospho-protein was determined for each blot and did not change over the course of the experiment from baseline, except for total mTOR at 60 min in EAA+ALA. Data are presented as phosphorylation status relative to total protein, normalized to baseline (fold change).

Antibodies. The primary phospho-antibodies were purchased from Cell Signaling: Akt (Ser⁴⁷³), mTOR (Ser²⁴⁴⁸), 4E-BP1 (Thr^{37/46}), and S6K1 (Thr³⁸⁹) at 1:500. Total antibodies purchased from Cell Signaling were Akt, mTOR, S6K1, and 4E-BP1 at 1:1000. Anti-rabbit IgG HRP-conjugated secondary antibody was purchased from Amersham Bioscience (1:2000).

Statistical methods. Values are expressed as mean ± SEM. The majority of comparisons were performed on raw data using 2-way repeated-measures ANOVA (SigmaPlot v. 11.0; Systat Software Inc.), the effects being group (EAA, EAA+CHO, EAA+ALA) and time (depending on parameter: baseline and 30, 60, 120, and 180 min). Post hoc testing was performed using the Bonferroni *t* test for multiple comparisons compared with a control (baseline for time and EAA for group). AUC comparisons were performed using 1-way ANOVA and Bonferroni *t* test for all pairwise comparisons. If a test of normality or equal variance failed, simple transformations (i.e., \sqrt{x} , \ln , x^{-1}) were performed to attain equal variance and/or normalize histograms of the data. For all analyses, significance was set at $P \leq 0.05$ and P -trend < 0.1.

Results

BF, plasma insulin, glucose, and glucose uptake. BF significantly increased from baseline at 60 min in the EAA+CHO group but quickly returned to basal levels (Fig. 2A). On the other hand, BF in the EAA and EAA+ALA groups was unchanged compared with baseline. Arterial plasma insulin

concentrations significantly increased in all groups at 30 min compared with baseline, but to a greater extent in the EAA+ALA and EAA+CHO groups than in the EAA group (Fig. 2B). Insulin concentrations remained elevated at 60 min in the EAA+ALA and EAA+CHO groups but not in the EAA group (Fig. 2B). At 120 min, insulin remained elevated in the EAA+ALA group but not in the EAA+CHO group (Fig. 2B). There were no significant differences compared with baseline or EAA at 180 min (Fig. 2B). The arterial plasma glucose concentrations in the EAA+CHO group were elevated compared with baseline and the EAA group at 30 and 60 min postnutrient ingestion, with no other within- or between-group (compared with EAA only) differences (Fig. 2C). Similarly, glucose uptake was elevated compared with baseline and EAA only in the EAA+CHO group at 30 min (Fig. 2D).

Phenylalanine enrichments, concentration, delivery, transport, and intracellular availability. Data for Figures 3 and 4 are normalized to LLM. The arterial phenylalanine concentration increased compared with baseline in all groups at 30, 60, 120, and 180 min postingestion but was significantly lower in the EAA+ALA than in the EAA group at 60 min (Fig. 3A). Phenylalanine delivery to the leg (F_{in}) increased compared with baseline at all time points in the EAA group, all but 180 min in the EAA+CHO group, and all but 30 min in the EAA+ALA group, with no group differences (Fig. 3B). Muscle inward transport ($F_{m,a}$) followed a similar pattern, except EAAs did not remain significantly elevated at 180 min (Fig. 3C). The intracellular availability of phenylalanine increased compared with baseline in the EAA+CHO group at 30, 60, and 120 min but only at 180 min in the EAA+ALA group; in the EAA group, it was elevated at 30 and 60 min (Fig. 3D). There were no group differences in intracellular availability. Data for values relative to leg volume (per 100 mL leg) are shown in Supplemental Figures 1 and 2. Data for blood and muscle bound and intracellular phenylalanine are presented in Supplemental Table 1 and Supplemental Figure 3, respectively.

Muscle protein turnover. The 2-pool model measure of MPS (R_d , per 100 g LLM) increased over baseline in all groups at 30 and 60 min with no group differences ($P < 0.05$; data not shown). The 3-pool measure of MPS ($F_{o,m}$, per 100 g LLM) increased from baseline at 30 min in all groups but did not reach significance at 30 min in the EAA+ALA group ($P = 0.055$) (Fig. 4A). All groups were significantly elevated over baseline at 60 min and returned toward basal levels by 120 min (Fig. 4A). At 180 min, $F_{o,m}$ in the EAA+CHO group had significantly dropped

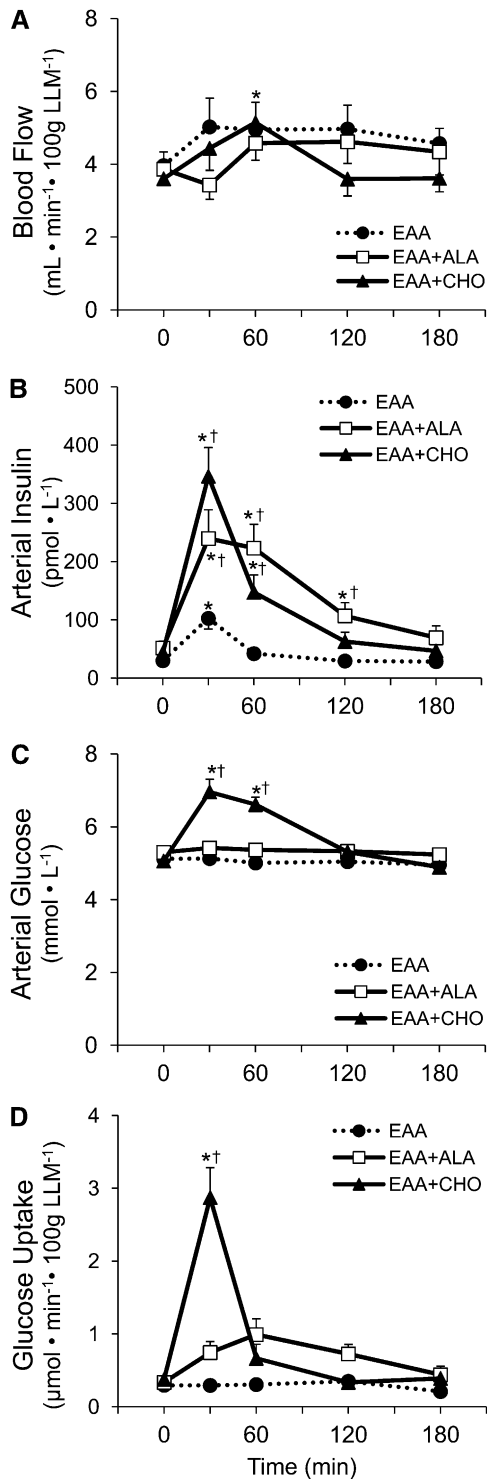


FIGURE 2 Leg BF (A), arterial insulin (B), and glucose concentrations (C) and leg glucose uptake (D) in young men and women following ingestion of EAA alone or with ALA or CHO. Values are mean \pm SEM, $n = 7$ /group. *Within-group difference from baseline, $P < 0.05$; †different from EAA, $P < 0.05$. BF, blood flow; EAA, essential amino acids; EAA+ALA, 10 g essential amino acids + 30 g alanine; EAA+CHO, 10 g essential amino acids + 30 g carbohydrate as sucrose.

below baseline. There were no group differences in $F_{O,m}$. Both 2- and 3-pool model indicators of MPB (R_a , $F_{m,o}$) showed significant main effects for time, but post hoc tests did not reveal any significant changes from baseline in any group (Fig. 4B).

Although no individual differences were identified with post hoc tests, MPB visually appeared to decrease at 2 h compared with baseline only in the EAA+CHO group and simple t tests revealed differences (EAA+CHO 2 h vs. baseline: $R_a = 51 \pm 8$ vs. 69 ± 11 ,

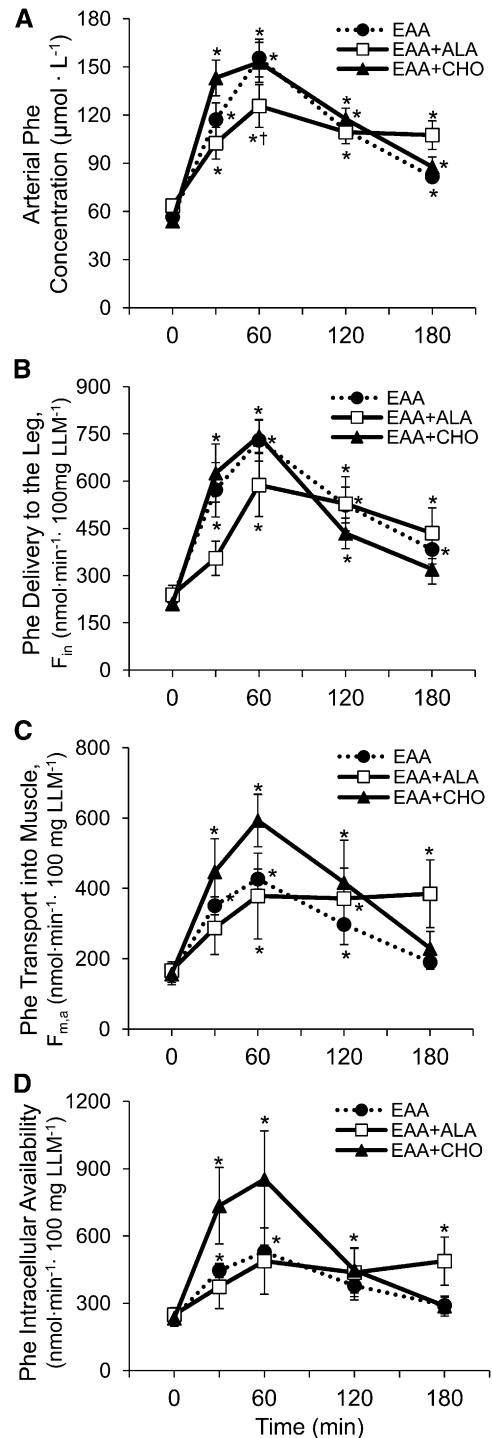


FIGURE 3 Phenylalanine concentration in the artery (A), delivery to the leg (F_{in}) (B), transport into muscle ($F_{m,a}$) (C), and intracellular availability (D) following nutrient ingestion in young men and women. Values are mean \pm SEM, $n = 7$ /group, except EAA+ALA and EAA+CHO $F_{m,a}$ and intracellular availability $n = 6$. *Within-group difference from baseline, $P < 0.05$; †different from EAA, $P < 0.05$. EAA, essential amino acids; EAA+ALA, 10 g essential amino acids + 30 g alanine; EAA+CHO, 10 g essential amino acids + 30 g carbohydrate as sucrose.

t test $P = 0.027$; $F_{m,o} = 58 \pm 12$ vs. 77 ± 10 $\text{nmol} \cdot \text{min}^{-1} \cdot 100$ g LLM^{-1} , t test $P = 0.047$). NB increased from baseline in all groups at 30 and 60 min (Fig. 4C). Only EAA+ALA remained elevated over baseline at 120 min and was significantly higher than the EAA group at 120 and 180 min (Fig. 4C).

Fractional synthetic rate. Mixed muscle protein fractional synthetic rate was significantly elevated 60 min following nutrient ingestion in all groups (Fig. 5A). Figure 5B shows the range of

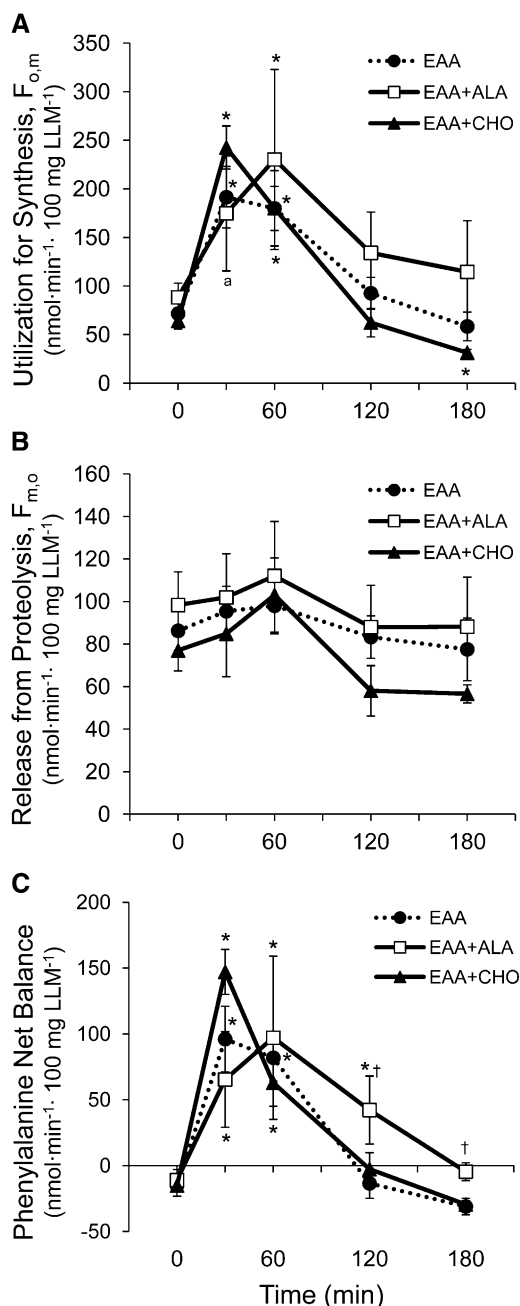


FIGURE 4 Phenylalanine utilization for protein synthesis ($F_{o,m}$) (A), release from proteolysis ($F_{m,o}$) (B), and net balance (C) following nutrient ingestion in young men and women. Values are mean \pm SEM, $n = 7$ /group, except EAA+ALA and EAA+CHO $F_{o,m}$ and $F_{m,o}$ $n = 6$. *Within-group difference from baseline, $P < 0.05$; †different from EAA, $P < 0.05$. ‡Different from EAA, $P = 0.06$. EAA, essential amino acids; EAA+ALA, 10 g essential amino acids + 30 g alanine; EAA+CHO, 10 g essential amino acids + 30 g carbohydrate as sucrose.

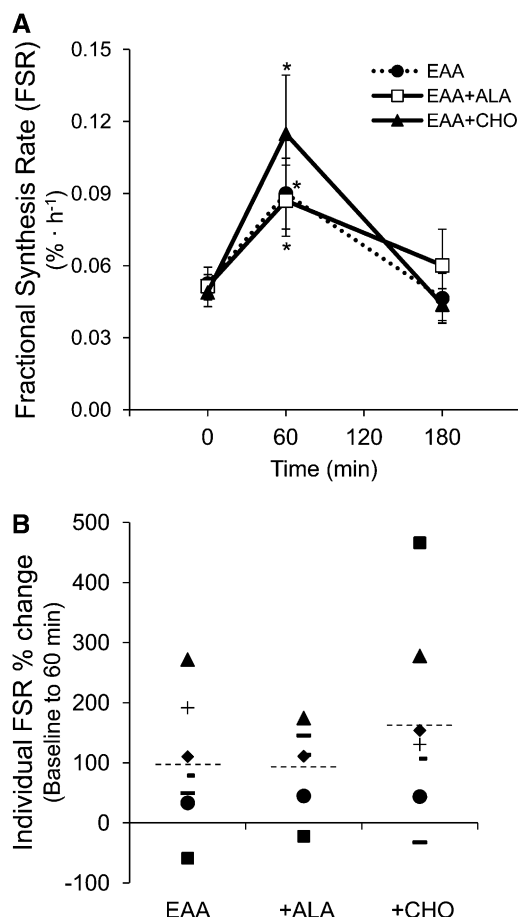


FIGURE 5 Fractional synthetic rate postnutrient ingestion (A) and individual participant responses 60 min following nutrient ingestion (B) in young men and women. Values are mean \pm SEM (A) and raw values (B). (A) $n = 7$ /group (missing 1 data point, EAA+CHO 180 min; missing 1 data point, EAA+ALA 60 and 180 min). *Within-group difference from baseline, $P < 0.05$. (B) EAA and EAA+CHO, $n = 7$; EAA+ALA $n = 6$. Dotted line indicates group mean. EAA, essential amino acids; EAA+ALA, 10 g essential amino acids + 30 g alanine; EAA+CHO, 10 g essential amino acids + 30 g carbohydrate as sucrose.

individual fractional synthetic rate responses 60 min following nutrient ingestion of EAA, EAA+ALA, or EAA+CHO.

AUC. AUC data for Figures 2–4 are presented in Supplemental Table 2. The EAA AUCs for arterial plasma glucose concentration, glucose uptake, and insulin were significantly lower than those of EAA+ALA and EAA+CHO. There were no other differences in AUC for any other parameter.

Cell signaling. Western blot analysis results are presented in Table 2. Phosphorylation of Akt (Ser⁴⁷³) relative to total protein increased from baseline at 60 min only in the EAA+CHO group and was significantly lower than baseline at 180 min in the EAA group. Phosphorylation of mTOR (Ser²⁴⁴⁸) relative to total protein increased from baseline at 60 min in all groups but remained elevated at 180 min in only the EAA+ALA and EAA+CHO groups. S6K1 phosphorylation (Thr³⁸⁹) relative to total protein significantly increased at 60 min in all groups. In the EAA+CHO group, phospho-4E-BP1 (Thr^{37/46}) relative to total protein slightly increased over baseline at 60 min ($P = 0.08$) but did not reach significance in any group.

TABLE 2 Western-blot analyses of synthesis-associated signaling proteins following nutrient ingestion in young men and women¹

	Time post-ingestion					
	60 min			180 min		
	EAA	EAA+ALA	EAA+CHO	EAA	EAA+ALA	EAA+CHO
	<i>fold of baseline</i>					
Akt (Ser ⁴⁷³)	1.0 ± 0.2	1.2 ± 0.1	1.9 ± 0.4*	0.6 ± 0.1*	0.7 ± 0.2	1.0 ± 0.3
mTOR (Ser ²⁴⁴⁸)	3.4 ± 1.2*	3.3 ± 1.3*	5.0 ± 2.3*	1.7 ± 0.5	2.1 ± 0.4*	4.0 ± 1.7*
S6K1 (Thr ³⁸⁹)	17.1 ± 4.7*	13.0 ± 4.6*	14.6 ± 2.8*	5.3 ± 1.3	7.2 ± 3.3	5.8 ± 1.6
4EBP-1 (Thr ^{37/46})	1.0 ± 0.1	1.1 ± 0.2	1.6 ± 0.1 ^a	1.4 ± 0.3	1.4 ± 0.3	1.0 ± 0.2

¹ Values are mean ± SEM, *n* = 7/group. *Within-group difference from baseline, *P* < 0.05; ^a*P* = 0.08. EAA, essential amino acids; EAA+ALA, 10 g EAA + 30 g alanine; EAA+CHO, 10 g EAA + 30 g carbohydrate as sucrose.

Discussion

The current study was designed to examine the effect of additional energy on the protein anabolic response to 10 g EAA. Specifically, we examined the effect of adding 30 g CHO or the gluconeogenic amino acid ALA to 10 g EAA (EAA+CHO and EAA+ALA, respectively). We hypothesized that only EAA+CHO ingestion would significantly increase circulating insulin concentrations, in turn decreasing MPB and improving the net muscle protein anabolic response to EAA alone. The primary and novel findings from this study are that: 1) MPB was not significantly lowered in the EAA+CHO or EAA+ALA group despite substantially increased circulating insulin concentrations in both groups; and 2) despite differences in the magnitude and duration of several outcome measures at individual time points, the net protein anabolic response with added energy was similar to that of EAA alone (33). Therefore, we conclude that the addition of energy or insulin is not necessary to cause an additive increase in the muscle protein anabolic response to EAA when a sufficient amount of EAA is provided.

Compared with EAA alone, EAA+CHO induced large and rapid increases in plasma insulin, glucose, and glucose uptake. The pattern of insulin release and glucose uptake in the EAA+ALA group was less robust but prolonged compared with the EAA+CHO group, though all groups clearly demonstrated an acute insulin response to the nutrients. The prolonged insulin response in the EAA+ALA group may be due to a greater second-phase insulin response. The second phase of insulin release occurs 120–180 min following the first phase and is an ATP-dependent process during which insulin granules in reserve pools are mobilized to the readily releasable pool (40,41). In the postabsorptive state, the majority of carbons from ingested ALA will be used to make glucose via hepatic gluconeogenesis (42), and therefore it is possible that the delayed appearance of glucose was driving the prolonged insulin response in the EAA+ALA group. However, to adequately determine whether this occurred in our subjects would require the use of tracer techniques to measure gluconeogenesis rates to confirm if gluconeogenesis contributes to the response in EAA+ALA.

Greenhaff et al. (43) showed decreases in MPB at insulin concentrations ≥ 200 pmol · L⁻¹ (~30 μU · mL⁻¹). The authors also illustrated that a ceiling is reached between ~200 and 500 pmol · L⁻¹, whereby increasing insulin concentrations do not further decrease MPB (43). Therefore, a “threshold” insulin concentration must be reached to significantly lower MPB. Ingestion of EAA+ALA or EAA+CHO increased insulin >200 pmol · L⁻¹, yet MPB did not change. Therefore, in addition to a concentration threshold, a time threshold may exist such that the threshold concentration must be maintained for a minimum

amount of time in order to significantly affect MPB. This may clarify cases in the literature where decreases in MPB are not observed for relatively high concentrations of insulin if the duration of the increase was short. It would also suggest an advantage for certain study designs to observe an effect, such as constant infusion/clamp studies over bolus delivery methods. Based on the results presented herein, we propose the time threshold for oral nutrient ingestion is >60 min, though future studies should be conducted to further investigate this hypothesis.

As we did not detect any changes in MPB, we decided to focus on whether activation of the mTORC1 signaling pathway was associated with the changes we found in MPS for each group. Phosphorylation of mTOR increased in all groups at 60 min but remained elevated in only the EAA+ALA and EAA+CHO groups by 180 min, consistent with MPS data. Downstream of mTOR, S6K1 phosphorylation increased at 60 min and was similar among groups, whereas phosphorylation of 4E-BP1 tended to increase at 60 min in only the EAA+CHO group. Despite elevated insulin concentrations in all groups at 30 min, glucose uptake significantly increased above baseline in only the EAA+CHO group. This may explain why we observed a significant increase in Akt phosphorylation in only the EAA+CHO group. However, the phosphorylation response of Akt occurs rapidly and it is therefore possible that we did not capture the maximal Akt response due to timing of muscle biopsies. Despite enhanced mTORC1 signaling in the EAA+CHO group and to a lesser extent in the EAA+ALA compared with EAA group, the overall 3-h net anabolic response (NB AUC) was similar among the 3 groups, possibly indicating that 10 g EAA elicits a maximal mTORC1 signaling response. In other words, similar to the ceiling effect that occurs with insulin and MPB, at a certain level of activation of mTORC1 signaling, additional increases do not further improve the muscle protein anabolic response (18,44).

Our finding that adding CHO to an EAA mixture does not enhance muscle protein anabolism is consistent with several human exercise studies. Koopman et al. (45), Staples et al. (46), and Miller et al. (47) found that when nutrients were ingested following resistance exercise, the addition of CHO to a protein solution did not result in an increase in MPS compared with protein intake alone. Additionally, Beelen et al. (48) also found no added benefit to the addition of CHO to protein during continuous endurance exercise. To our knowledge, this is the first study to provide evidence that energy added to an EAA mixture ingested orally as a single bolus at rest also does not alter MPB and NB.

The finding that additional calories are not needed to maximize MPS and muscle protein anabolism is an important result that can be applied to designing evidence-based nutritional

interventions to promote muscle mass while minimizing caloric intake and weight gain. Furthermore, it is often difficult for hospitalized patients to consume a sufficient amount of protein to counteract muscle loss from bed rest and the development of a low-calorie, highly potent nutrient mixture that maximizes MPS should be useful in maintaining muscle mass in such clinical situations. However, the concept of anabolic resistance to amino acids and insulin in certain clinical populations complicates the direct translation of our findings in young, healthy individuals (17,49). Future studies should examine whether adding energy to sufficient EAAs improves the protein anabolic response at rest in clinical scenarios such as bed rest and in aging.

In summary, the study design used allowed us to compare the protein anabolic effects of adding energy as CHO or ALA to 10 g EAA alone. The overall muscle protein anabolic response was not improved by the addition of energy to EAAs, and this is the first study to our knowledge to report such a finding of bolus oral nutrient ingestion at rest without additional confounding variables such as exercise. We conclude that EAA or protein nutritional interventions to enhance muscle protein anabolism do not require additional energy from CHO or gluconeogenic non-EAAs such as ALA, and the use of low-calorie but highly efficient EAA mixtures may be useful in promoting muscle growth and/or counteracting muscle loss in health and disease.

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