Increased net muscle protein balance in response to simultaneous and separate ingestion of carbohydrate and essential amino acids following resistance exercise.

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Abstract

Relative to essential amino acids (EAA), carbohydrate (CHO) ingestion stimulates a delayed response of net muscle protein balance (NBAL). We investigated if staggered ingestion of CHO and EAA would superimpose the response of NBAL following resistance exercise, thus resulting in maximal anabolic stimulation. Eight recreationally-trained subjects completed two trials, combined (COMB; drink one- CHO plus EAA, drink two- placebo) and separated (SEP; drink one- CHO, drink two- EAA) postexercise ingestion of CHO and EAA. Drink one was administered 1 h following an acute exercise bout and was followed by drink two 1 h later. A primed, continuous infusion of L-[ring-$^{13}C_6$]-phenylalanine was combined with femoral arteriovenous sampling and muscle biopsies for the determination of muscle protein kinetics. Arterial amino acid concentrations increased following ingestion of EAA in both conditions. No difference between conditions was observed for phenylalanine delivery to the leg (COMB: 167±23 μmol/min/100mL leg vol*6 h; SEP: 167±21 μmol/min/100mL leg vol*6 h, P>0.05). In the 1st hour following ingestion of the drink containing EAA, phenylalanine uptake was 50% greater for SEP than COMB. However, phenylalanine uptake was similar for COMB (110±19 mg) and SEP (117±24 mg) over the 6 h period. These data suggest that whereas separation of CHO and EAA ingestion following exercise may have a transient physiological impact on NBAL, this response is not reflected over a longer period. Thus, separation of CHO and EAA ingestion is unnecessary to optimize post-exercise muscle protein metabolism.

Key words: Nutrient Timing, Resistance Exercise Recovery, Muscle Protein Balance, Muscle Protein Synthesis.

Introduction

Nutritional strategies aimed at maximizing the anabolic response of muscle to resistance exercise interest individuals who would benefit from muscle growth (Rennie and Tipton 2000; Tipton and Ferrando 2008; Wolfe 2002; Wolfe 2006). The provision of nutrients in close proximity to exercise is required to switch net muscle protein balance (NBAL) - the metabolic basis for changes in muscle mass - from negative to positive (Biolo et al. 1997; Tipton et al. 1999). Multiple factors, including nutrient timing (Tipton et al. 2001; Tipton et al. 2007), modulate the acute post-exercise response of muscle protein metabolism to nutrition. For the maximal stimulation of NBAL, careful timing of nutrient provision in relation to exercise should be considered (Churchward-Venne et al. 2012; Tipton and Witard, 2007). Hence, timing of nutrient intake in relation to exercise and the provision of other nutrients may be important for maximising NBAL.

Essential amino acids (EAA) (Borsheim et al. 2002; Borsheim et al. 2004a; Tipton et al. 1999) and, to a lesser extent, carbohydrates (CHO) (Borsheim et al. 2004b Miller et al. 2003;) influence the response of NBAL following resistance exercise. The independent ingestion of EAA after resistance exercise results in a rapid and profound improvement in NBAL, primarily by stimulation of muscle protein synthesis (MPS) (Borsheim et al. 2002; Drummond et al. 2008a; Miller et al. 2003; Tipton et al. 1999). Less pronounced is the impact of post exercise CHO ingestion on muscle protein metabolism (Borsheim et al. 2004a; Borsheim et al. 2004b; Miller et al. 2003). Whereas CHO alone has little, if any, impact on MPS following exercise (Borsheim et al. 2004b; Miller et al. 2003) post exercise NBAL is improved (Borsheim et al., 2004b), primarily due to insulin-mediated attenuation of muscle protein breakdown (MPB) following exercise (Biolo et al. 1999; Glynn et al. 2010a).

Relative to amino acids, not only is the magnitude of the response of NBAL to CHO less (Miller et al. 2003), but the onset of improved NBAL with CHO ingestion is delayed (Borsheim et al. 2004b) during postexercise recovery. Despite rapid increases in arterial plasma insulin concentrations following CHO ingestion, there is no improvement in NBAL during the first hour after CHO intake (Borsheim et al. 2004b; Miller et al. 2003). However, NBAL is increased during the second and third hours after CHO intake (Borsheim et al. 2004b; Miller et al. 2003). Hence, peak NBAL post exercise does not appear to coincide with its peak insulin concentration; rather the action of insulin on NBAL is delayed (Borsheim et al. 2004b). Thus, taken together with the immediate response of NBAL to EAA ingestion (Borsheim et al. 2002; Borsheim et al. 2004a; Miller et al. 2003; Tipton et al. 2001) a nutritional strategy that coordinates the differing time-related responses of NBAL to CHO and EAA may result in an additive effect that may prove beneficial for maximizing NBAL.

The co-ingestion of CHO with a source of EAA postexercise is known to increase NBAL (Miller et al., 2003; Borsheim et al., 2004a; Glynn et al., 2010a). All past studies that have examined the impact on NBAL of co-ingesting CHO and amino acids have administered the nutrients simultaneously (Borsheim et al. 2004a; Koopman et al. 2007; Miller et al. 2003). Given that the response of NBAL to insulin from CHO ingestion appears to be delayed (Borsheim et al. 2004b; Miller et al. 2003), we propose that postponing the intake of EAA relative to CHO could amplify the response of NBAL during exercise recovery. That is, provision of EAA such that the peak responses of CHO and EAA are superimposed should enhance the overall response of NBAL following exercise. Since Borsheim et al. demonstrated NBAL does not begin to increase until ~1 h after CHO ingestion (Borsheim et al. 2004b), we chose to administer a drink containing 15 g of EAA 1 h after a drink containing 50 g of CHO. The chosen doses of CHO and EAA were intended to provide a robust response of NBAL based on previous work that demonstrated stimulation of

NBAL by ingestion of 50 g of CHO (Miller et al. 2003) and a maximal stimulation of MPS with 10 g of EAA (Cuthbertson et al. 2005). Thus, the primary aim of the present study was to investigate whether the timing of EAA ingestion in relation to CHO ingestion impacts the response of amino acid uptake, representative of NBAL, to resistance exercise. We hypothesized that the staggered post exercise ingestion of CHO and EAA would elicit a greater anabolic response of muscle during exercise recovery compared with the simultaneous post exercise ingestion of CHO and EAA.

Materials and Methods

Subjects

Eight (5 males, 3 females) recreationally-active volunteers (age: 29.8 ± 2.5 yr; BMI: 25.3 ± 4.4 kg / m^2; leg volume: 10.0 ± 0.8 L) were recruited to participate in this study. Individuals who participated in regular exercise 1-3 times per week, but abstained from regular resistance training or competitive sports were eligible to participate. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Institutional Review Board and the General Clinical Research Center (GCRC) of the University of Texas Medical Branch, Galveston. All subjects completed a series of medical screening tests for the purpose of disclosing any pre-existing medical or physical conditions that would preclude participation in the study. The study design, purpose, and possible risks were explained to each subject before written consent was obtained. Participants were reminded of their right to withdraw from the study at any time without provision of reason.

Study overview

This experimental protocol was designed to quantify the response of NBAL, as represented by phenylalanine balance across the leg following ingestion of EAA and CHO during recovery from an

intense resistance exercise bout. Each participant completed two trials within a single-blinded, randomized study design (Figure 1). The response of NBAL was determined following exercise when the ingestion of CHO and EAA were combined (COMB) or separated (SEP). In COMB, participants consumed a drink containing CHO and EAA 1 h following exercise followed by a placebo drink 1 h later. In SEP, a drink containing only EAA was ingested 1 h following consumption of a drink containing CHO only. Trials were separated by at least 1 wk and no more than 2 mos. Since previous studies showed no differences in the anabolic response to EAA ingestion at 1 and 3h following exercise (Rasmussen et al. 2000), any differences detected between treatments in the present study could be attributed to the composition of the ingested solutions.

Pretesting

Anthropometric measures

Leg volume was estimated using an anthropometric approach as previously described (DEMPSTER et al., 1964).

One repetition maximum (1RM) exercise test

At least five days prior to the study protocol, a 1RM test of bilateral leg strength on leg extension was determined for each subject as previously described (Mayhew et al. 1995). The mean 1RM value achieved was 92.1±11.9 kg.

Experimental protocol

Subjects were admitted to the GCRC the night before each infusion study, given a standardized meal and then allowed only water (ad libitum) until the commencement of the study the following morning. At ~05:45, an 18-gauge, polyethylene catheter was inserted into a vein on the forearm to allow blood sampling. Additionally, a polyethylene catheter (Cook, Inc., Bloomington, IN) was inserted into the femoral vein and femoral artery under local anaesthesia. Both femoral catheters were used for blood sampling. In addition, the femoral arterial catheter was used for indocyanine

green (ICG) infusion for determination of leg blood flow as per Tipton et al. (2003) (Tipton et al., 2003). Systemic concentration of ICG was measured from a peripheral vein. Patency of all catheters was maintained by saline infusion. A blood sample was taken prior to an intense, leg resistance exercise bout and NBAL was determined over a 6 h period following ingestion of the first drink. A primed (2 µmol·kg^{-1}) L-[ring-$^{13}C_6$]-phenylalanine tracer (infusion rate: 0.05 µmol·kg^{-1}·min^{-1}) was continuously infused from time point -180 min for 9 h (until time point 360 min).

Exercise protocol

The exercise bout consisted of 10 sets of 8 repetitions of leg extensions at 80% 1RM, interspersed by a 2 min rest interval between sets, which was completed in ~25 min. We have previously utilized this routine to increase blood flow and muscle protein metabolism (8, 9).

Drink composition and timing schedule

Subjects consumed two drinks in each trial, drink 1 at 1 h post exercise and drink 2 at 2 h post exercise. In SEP, drink 1 contained 50g of sucrose and drink 2 contained 15g of EAA. In COMB, drink 1 contained 50g of sucrose plus 15g of EAA and drink 2 was a placebo drink, comprising of an artificial sweetener. The amino acid content of the EAA drink was based on the composition of muscle protein (in percent wt:wt): His, 10.9; Iso, 10.1; Leu, 18.6; Lys, 15.5; Meth, 3.1; Phe, 15.5 (including 3.8% L-[ring-$^{13}C_6$]-phenylalanine; Thr, 14.7; Val, 11.5). All drinks were dissolved in 500 mL of water.

Blood sampling

At ~06:00 (- 180 min), background blood samples for amino acid enrichment and ICG concentration were taken. Thereafter, arteriovenous (A-V) samples were collected at -12 and -8 min for the post exercise/pre-drink period, and at 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 140, 160, 180, 210, 240, 270, 300, 330 and 360 min for the measurement of the amino acid enrichments and concentrations.

Muscle biopsies

Four biopsies per trial were collected from separate incisions during the post exercise period; biopsy one (B1) - immediately pre drink 1, biopsy two (B2) - 1 h post drink 1 and immediately pre drink 2, biopsy three (B3) – 2 h post drink 1 and 1 h post drink 2, biopsy four (B4) – 6 h post drink 1 and 5 h post drink 2. Muscle biopsies were taken from the lateral portion of the vastus lateralis muscle ~10-15 cm above the knee using a 5 mm Bergstrom biopsy needle (Depuy, Warsaw, IN). Under local anesthetic (1% lidocaine), a sample of ~50-100 mg of muscle tissue was extracted from the vastus lateralis. The sample was rinsed quickly, blotted and divided into two or three pieces before being frozen in liquid nitrogen and stored at -80°C for future processing.

Blood flow

Leg blood flow was determined using ICG dilution, as previously described (Biolo et al. 1995; Phillips et al. 1997). Briefly, a continuous ICG infusion (IR=0.5 mg•min^{-1}) was initiated at -25 min and was maintained until -10 min for the measurement of leg blood flow prior to drink ingestion. Thereafter, blood flow was determined for five different measurement periods (19-29 min, 59-69 min, 165-180 min, 255-270 min and 340-355 min) designed to characterize changes in leg blood flow following exercise and drink ingestion. Leg plasma flow was calculated from steady-state values of dye concentration and converted to blood flow using hematocrit values (Biolo et al. 1997; Elliot et al. 2006; Tipton et al. 2004).

Analyses

Blood

The enrichment of phenylalanine in whole blood was measured by Gas Chromatography-Mass Spectrometry (GC-MS; model 5989B, Hewlett-Packard, Palo Alto, CA) (see Table S1). Briefly, 500 µL of the sulfosalicylic extract was passed over a cation exchange column (Dowex AG 50W-8X, 100-200 mesh H+ form; Bio-Rad Laboratories, Richmond, CA) and dried under vacuum using

a Speed Vac (Savant Instruments, Farmingdale, NY). The amino acids were converted to their tert-butyldimethylsilyl (t-BDMS) derivative. Isotopic enrichments were calculated as a tracer-to-tracee ratio (t/T).

Concentrations of phenylalanine and leucine were determined using an internal standard solution and GCMS analysis, as previously described (Biolo et al. 1995). The internal standards used were [U-$^{13}C_9$-^{15}N] phenylalanine (50 µmol/L) and L-[$^{13}C_6$]leucine (115 µmol/L), added in a ratio of ~100 µL/mL of blood. Because the tube weight and the amount of blood were known, the blood amino acid concentration could also be determined from the internal standard enrichments measured by GCMS, based on the amount of blood and internal standard added. Leg blood flow was determined by spectrophotometrically measuring the ICG concentration in serum from the femoral vein and the peripheral vein as described previously (Biolo et al. 1997; Elliot et al. 2006). Leg plasma flow was calculated from steady-state values of dye concentration, and converted to blood flow using the hematocrit (2,3). Serum insulin levels were determined by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA). Intraassay coefficient of variation (CV) was 10%. Plasma glucose concentrations were determined by the glucose oxidase method using a glucose auto-analyzer (Beckman Instruments, Brea, CA).

Muscle tissue

Biopsies (~30 mg) were analyzed for mixed protein-bound and free intracellular amino acid enrichment (see Table S2), as previously described (Biolo et al. 1995; Phillips et al. 1997). Briefly, tissue was weighed and protein precipitated with 0.5 mL of 10% perchloric acid. The tissue was then homogenized and centrifuged, and the supernatant (intracellular) was collected. This procedure was repeated two more times, and the pooled supernatant (~1.3 mL) was processed as described in Blood. Intracellular enrichment was determined by correction for extracellular fluid on the basis of the chloride method (Bergstrom et al. 1974). Intracellular amino acid concentrations were measured

with the internal standard method, with corrections for the contribution of extracellular fluid and for overlapping spectra, as described in Blood.

The remaining pellet of muscle tissue was used to determine the enrichment of protein-bound L-[ring-$^{13}C_6$]phenylalanine using GC-MS (model 5973; Hewlett-Packard) with a splitless injection and positive electron-impact ionization, as previously described (Phillips et al., 1997; Tipton et al., 1999). Briefly, the pellet was further washed before being placed overnight in an oven at 50°C. The dried pellet was hydrolyzed at 110°C for 24 h with 6 N hydrochloric acid before being passed over a cation exchange column (Dowex AG 50W-8X, 100-200 mesh H+ form; Bio-Rad Laboratories, Richmond, CA), dried by a Speed Vac, and derivatized with t-BDMS, as described for Blood. Mass-to-charge ratios 237 and 240 were monitored. Enrichment from the protein-bound samples was determined with a linear standard curve of known m + 6-to-m + 3 ratios and corrected back to the absolute change in m + 6 enrichment over selected incorporation periods.

Calculations

Glucose uptake

Glucose uptake was calculated at each time point following drink 1 as the A-V difference in glucose concentration multiplied by the mean blood flow over a specified period of time. Thus:

$$\text{Glucose uptake} = (C_a - C_v) \times BF$$

where C_a = arterial glucose concentration, C_v = venous glucose concentration, and BF = leg blood flow.

Phenylalanine and leucine delivery to the leg

Phenylalanine and leucine delivery to the leg were calculated at each time point as the femoral arterial concentration multiplied by the mean blood flow over a specified period of time. Thus;

$$\text{Amino acid delivery} = C_a \times BF$$

where C_a = arterial amino acid concentration and BF = leg blood flow. Phenylalanine and leucine delivery to the leg also were calculated as area under the curve (AUC) over a 6 h period following ingestion of drink one in each drink condition.

Net muscle protein balance (NBAL)

Because phenylalanine is not oxidized in muscle, phenylalanine net balance across the exercised leg was chosen to represent NBAL. NBAL was calculated at each time point from the difference between the femoral arterial and venous phenylalanine concentrations multiplied by the mean blood flow over a specified time period. Thus;

$$NBAL = (C_a - C_v) \times BF$$

where C_a = arterial phenylalanine concentration, C_v = venous phenylalanine concentration and BF = leg blood flow.

The primary endpoint of this study is the comparison of the NBAL over a 6 h recovery period in COMB and SEP. NBAL (mg) was calculated from the area under the curve (AUC) of NBAL over the entire 6 h period following ingestion of drink 1 in both drink conditions. AUC of NBAL also was calculated for 1 and 3 h periods following ingestion of the EAA-containing drink (i.e., drink 1for COMB, drink 2 for SEP) to provide information about the immediate physiological response to EAA ingestion. Baseline was set as the value prior to the drink in the corresponding condition for both measurement periods. Positive values represented net uptake (anabolism) and negative values represented net release (catabolism).

Fractional Synthesis Rate (FSR)

The fractional synthesis rate (FSR) of mixed muscle protein was determined by the rate of incorporation of L-[ring-$^{13}C_6$]phenylalanine over time using the free intracellular muscle L-[ring-$^{13}C_6$]phenylalanine enrichment as precursor as shown below:

$$FSR\ (\%\cdot h^{-1}) = (E_{M2} - E_{M1})/(E_P \cdot t) \times 60 \times 100$$

where E_{M1} and E_{M2} = the enrichments of the protein-bound L-[ring-$^{13}C_6$]phenylalanine at the start and end of the chosen sampling period, respectively, E_P = the average intracellular L-[ring-$^{13}C_6$]phenylalanine enrichment over the incorporation period and t = time in min. FSR was determined for the first hour after EAA ingestion and the entire six hour period of assessment.

Data presentation and statistical analysis

Due to technical difficulties, muscle intracellular phenylalanine and leucine concentrations are shown for five subjects. All other data are presented as means ± SE (n = 8) and statistical analyses were performed using Statistical Package for Social Sciences (SPSS) 15.0 for Windows (SPSS Inc., Chicago, IL). Significance was set at $P < 0.05$.

Primary endpoints

NBAL and FSR were compared between COMB and SEP using a two-tailed Paired Student's t-test. These comparisons between conditions were made for two time periods each - the first h after ingestion of the EAA-containing drink (drink one in COMB and drink two in SEP) and the entire 6 h period (5 h after drink two).

Secondary endpoints

Serum insulin concentrations, glucose uptake, plasma and muscle intracellular phenylalanine and leucine concentrations, phenylalanine delivery to the leg and phenylalanine NBAL across the leg were analyzed using two-way (drink and time) analysis of variance (ANOVA) with repeated measures (time-point). Where a significant main effect of drink condition, time or drink condition × time interaction was detected, a least significance difference (LSD) post hoc test was performed to locate the paired-wise differences. Phenylalanine and leucine delivery to the leg over the 6 h period

following ingestion of drink 1 expressed as AUC were compared between COMB and SEP using a two-tailed Paired Student's t-test.

Results

Insulin and glucose concentrations and glucose uptake

The response of arterial insulin concentrations during COMB and SEP were virtually identical (Figure 2) peaking at ~65 μU/mL at 20 min following ingestion of either the CHO only or CHO + EAA drink (drink 1 in both conditions) and returning to baseline by 160 min after ingestion of drink 1 in both COMB and SEP. In both COMB and SEP, a marked increase in glucose concentration and glucose uptake relative to baseline following CHO ingestion ($P < 0.05$) was followed by a return to baseline 120 min later, with no differences detected between conditions ($P > 0.05$, data not shown).

INSERT FIGURE 2 HERE

Blood amino acid concentrations

Arterial phenylalanine and leucine concentrations for COMB and SEP are shown in Fig. 3. Leucine concentration declined by ~30% 40-50 min following ingestion of CHO alone compared with baseline ($P < 0.05$). Mean phenylalanine concentration also decreased following ingestion of CHO alone (drink 1, SEP), however this decline failed to reach statistical significance ($P > 0.05$). Phenylalanine and leucine concentrations increased immediately in response to ingestion of the EAA-containing drink (i.e., drink 1 for COMB and drink 2 for SEP) and peaked 30 min following EAA ingestion for both. Phenylalanine and leucine concentrations remained increased above baseline for 180 min following the CHO + EAA drink one and 100 min following the EAA drink two in COMB and SEP, respectively ($P < 0.05$).

INSERT FIGURE 3 HERE

Muscle intracellular amino acid concentrations

Intracellular concentrations of phenylalanine and leucine (Table 1) were similar between COMB and SEP prior to drink ingestion (Biopsy 1) ($P > 0.05$). Increases in intracellular phenylalanine and leucine concentrations were observed following ingestion of the EAA-containing drink (Biopsy 2 in COMB and Biopsy 3 in SEP) ($P < 0.05$). Phenylalanine concentrations were higher for COMB compared with SEP 1 h post drink 1 (Biopsy 2) (~110%, $P < 0.05$) and 2 h post drink 1 (Biopsy 3) (~58%, $P < 0.05$). The >2 fold higher mean intracellular leucine concentration in COMB vs. SEP 1 h post drink 1 (Biopsy 2) failed to reach statistical significance ($P > 0.05$). No difference in leucine concentration was observed 2 h (biopsy 3) or 6 h (biopsy 4) following drink 1.

INSERT TABLE 1 HERE

Blood flow and amino acid delivery to the leg

Figure 4 shows blood flow for each time period in COMB and SEP. A main effect of time was observed whereby blood flow was lower 4, 5 and 6 h post drink ingestion vs. pre drink ($P < 0.05$). No difference in blood flow was observed between drink conditions ($P > 0.05$).

INSERT FIGURE 4 HERE

Phenylalanine and leucine delivery to the leg expressed over time is shown in Fig. 5. The general pattern of delivery was similar for both amino acids. Amino acid delivery decreased from post exercise values following ingestion of CHO alone (drink 1, SEP), however this decline failed to reach statistical significance ($P > 0.05$) A marked increase in amino acid delivery to the leg was observed in both COMB and SEP following ingestion of the EAA-containing drink (drink 1 in COMB, drink 2 in SEP). Amino acid delivery to the leg was increased above baseline from 1-2 h post drink 1 in COMB ($P < 0.05$). For SEP, amino acid delivery increased above baseline from 30-150 min following EAA (drink 2) ingestion ($P < 0.05$). No difference between test-drink conditions was observed for AUC of phenylalanine (COMB: 167 ± 23 µmol/min/100mL leg vol*6 h; SEP: 167 ± 21 µmol/min/100mL leg vol*6 h, $P > 0.05$) or leucine (COMB: 301 ± 33 µmol/min/100mL leg

343 vol*6 h; SEP: 290 ± 36 μmol/min/100mL leg vol*6 h, P > 0.05) delivery to the leg when expressed
344 as AUC over a 6 h period following ingestion of drink 1.

345 INSERT FIGURE 5 HERE

346 **NBAL across the leg**

347 NBAL over time only is presented in Fig. 6. A biphasic response of NBAL in response to post
348 exercise drink ingestion was observed for COMB. NBAL switched from negative to positive values
349 immediately following ingestion of CHO + EAA-containing drink 1 for COMB: mean NBAL
350 values peaked at 20 min, but declined markedly by ~60 min post drink 1. NBAL peaked a second
351 time 70-80 min following drink 1 (=10 and 20 min following drink 2), returned to baseline levels
352 from 120 min and remained at baseline for the remainder of the sampling period. NBAL remained
353 negative throughout the entire 60 min period following ingestion of CHO-containing drink 1 in
354 SEP. Immediately following ingestion of the EAA-containing drink 2, NBAL increased markedly;
355 mean NBAL values peaked at ~40 min post drink 2 (100 min post drink 1). Phenylalanine NBAL
356 returned to baseline values 120 following drink 2 (180 min following drink 1) and remained at
357 baseline thereafter.

358 INSERT FIGURE 6 HERE

359 Fig. 7 displays net uptake of phenylalanine, i.e. AUC of NBAL, over 1h, 3h and 6h (overall) post
360 drink periods for COMB and SEP trials. Net uptake of phenylalanine determined 1 h following
361 ingestion of the EAA-containing drink (i.e., drink 1 in COMB and drink 2 in SEP) was ~50%
362 higher in SEP vs. COMB (P < 0.05). Net uptake of phenylalanine over the 3 h and entire 6 h period
363 following ingestion of drink 1 was not different between COMB and SEP (P > 0.05).

364 INSERT FIGURE 7 HERE

365 **Mixed muscle protein fractional synthetic rate**

Mixed muscle protein FSR, determined over a one hour incorporation period following ingestion of EAA-containing drink (i.e., drink 1 in COMB and drink 2 in SEP), was not different between COMB (0.110 ± 0.050 % / h) and SEP (0.109 ± 0.022 % / h, $P > 0.05$). FSR, determined over the total 6 h incorporation period, was similar between COMB (0.086 ± 0.007 % / h) and SEP (0.089 ± 0.009 % / h, $P > 0.05$).

Discussion

The present study was novel in comparing the response of NBAL to the combined (COMB) vs. separated (SEP) timed ingestion of EAA in relation to CHO after resistance exercise. EAA ingested either simultaneously with CHO or delayed by 1h following CHO ingestion resulted in positive NBAL. NBAL during the 1st hour following ingestion of EAA was ~50% greater when CHO was ingested 1h prior than when both nutrients were ingested concurrently. However, this difference in NBAL was not evident when the response was determined over longer time periods, i.e., for 3h and 6h following EAA ingestion. Thus, any physiological increase in NBAL due to delayed ingestion of EAA relative to CHO following resistance exercise seems to be transient and thus unlikely to be important from a practical standpoint.

The increase in postexercise NBAL when EAA ingestion is delayed relative to CHO likely is a result of superimposing the response of NBAL to each nutrient. It is well established that there is a robust and immediate response of NBAL to EAA ingestion following – and prior to – resistance exercise (Borsheim et al. 2002; Drummond et al. 2008a; Tipton et al. 1999; Tipton et al., 2001). Whereas, there is a response of NBAL to hyperinsulinemia (Borsheim et al. 2004b) from ingestion of CHO alone following resistance exercise, the magnitude of the response is less than that for EAA (Borsheim et al. 2004b; Miller et al. 2003). Our results support the results of studies that demonstrated greater NBAL when EAA and CHO are ingested concurrently compared to CHO

alone (Borsheim et al. 2004a; Miller et al. 2003). However, our current results also extend the prior results by demonstrating that the response to temporal separation of the ingestion of these nutrients increases NBAL by ~50%, at least initially. This greater response of NBAL in the first hour after ingestion of EAA in SEP than COMB is likely the result of the immediate response to EAA superimposed with the delayed response to CHO.

Our data do not allow us to determine the metabolic determinants of the increased NBAL in both SEP and COMB following EAA ingestion. However, previous studies clearly indicate that the increase in NBAL from EAA ingestion primarily is due to increased MPS (Borsheim et al. 2002; Glynn et al. 2010b; Tipton et al. 2001). Our FSR data seem to support this contention. Whereas we have no measurement of basal MPS in the present study, the mixed muscle FSR determined 1 and 6h after EAA ingestion are ~2X previously reported basal mixed FSR values (Biolo et al. 1995; Biolo et al. 1997; Dreyer et al. 2006; Drummond et al. 2008a) and are consistent with mixed FSR reported in response to resistance exercise and hyperaminoacidemia in other studies (Biolo et al. 1997; Burke et al. 2012; Dreyer et al. 2008). Thus, our data suggest that MPS is increased and is a major contributing factor to the increased NBAL with EAA ingestion following resistance exercise. Increased MPS, in turn, seems to be associated with the increased delivery of amino acids to the muscle. Previous work suggests that the increased delivery of amino acids to the muscle and the subsequent increased transport of amino acids into the muscle may be a critical factor for stimulation of MPS and NBAL (Biolo et al. 1995; Biolo et al. 1997; Tipton et al. 1999; Tipton et al. 2001). Whereas there was a substantial increase in amino acid delivery to the muscle with EAA ingestion (Figure 4), we found no overall differences in amino acid delivery to the leg between drink conditions. Thus, the difference in NBAL in the first hour after EAA ingestion must be due to factors other than delivery of amino acids to the muscle. These delivery data are supported by the FSR data for the first hour after EAA ingestion. We noted no differences between trials suggesting

that increased MPS does not explain the differences in NBAL between COMB and SEP. Previous work has shown CHO ingestion improves NBAL via an insulin-mediated attenuation of the increase in MPB following resistance exercise (Borsheim et al. 2004b; Miller et al. 2003). Whereas it is clear that hyperinsulinemia from CHO ingestion does not impact post-exercise MPS (Koopman et al. 2007; Staples et al. 2011), a decrease in MPB may contribute to the differences in NBAL in the first hour after ingestion of the EAA in each condition. That is, the delay in the response of NBAL with CHO ingestion seems to be due primarily to a delayed response of MPB to the CHO ingestion (Borsheim et al. 2004b; Miller et al. 2003). Thus, whereas increased NBAL seems likely to be attributable to an increase in MPS in response to EAA ingestion, the delayed response of MPB to CHO may contribute to the initial difference in NBAL with separate ingestion of CHO and EAA compared to simultaneous ingestion of the nutrients.

This study was designed to determine if the physiological response to a temporal separation of CHO and EAA ingestion was different than that to a simultaneous ingestion of CHO and EAA following resistance exercise. However, it is possible that our results may be limited to the timing and amount of ingested nutrients. It is possible that the results simply reflect differences due to timing of EAA ingestion in relation to the resistance exercise bout rather than in relation to CHO. We cannot dismiss the possibility that ingestion of EAA 2h following exercise could be more effective for stimulation of NBAL than 1h following exercise. However, previous data show no difference in the response of NBAL between 1h and 3h (Rasmussen et al. 2000). Thus, it seems unlikely that the difference between trials in net uptake in the first hour following ingestion of EAA is attributable to the timing of EAA ingestion in relation to the exercise bout. Moreover, our results may be limited to the timing of ingested nutrients chosen for this study. Previously, the peak response of NBAL seemed to be ~2-3h following CHO ingestion (Borsheim et al. 2004b). In the present study, the EAA was ingested 1h after CHO and the peak response of NBAL occurred during

438 the first hour after EAA ingestion. Thus, it is possible that the optimal confluence of the NBAL
439 response to the two nutrients may have not been achieved. Nevertheless, the subsequent efflux of
440 amino acids (Figure 6) suggests that uptake of more amino acids would not have increased net
441 muscle protein synthesis.

442 The amount of CHO and EAA ingested also may have an important role in the observed
443 responses of muscle protein metabolism following exercise. We chose amounts of CHO and EAA
444 that are above that reported to provide maximal responses (Borsheim et al. 2004b; Cuthbertson et
445 al. 2005). Thus, our results may be limited to the amounts of nutrients aimed to engender a maximal
446 response of NBAL. We (Borsheim et al. 2002; Tipton et al. 1999) and others (Dreyer et al. 2008;
447 Drummond et al., 2008b) have demonstrated that as little as 3-6g of EAA ingested following
448 exercise stimulate NBAL. However, the response to smaller amounts may not be maximal
449 (Cuthbertson et al. 2005). Thus, it is possible that further stimulation of NBAL by prior CHO
450 ingestion could be more effective with a submaximal response to EAA. Future studies should
451 investigate factors that could interact with the timing of the nutrients to stimulate postexercise
452 NBAL.

453 On the other hand, it could be argued that assessing the physiological impact of EAA
454 ingestion only in the first hour after ingestion may bias, or at least limit, the interpretation of the
455 results. It is interesting that, despite a clear 50% increase in NBAL with delayed ingestion of EAA
456 relative to CHO during the first hour after EAA ingestion, there is no difference when NBAL is
457 considered over longer time periods, i.e. 3h after EAA ingestion or the entire 6h period. Differences
458 in interpretation of the results may be due to methodological considerations. Ingestion of large
459 amounts of EAA results in rapid stimulation of uptake of amino acids (Borsheim et al. 2002; Miller
460 et al. 2003; Tipton et al., 2001). However, not all amino acids taken up by the muscle are
461 necessarily incorporated into muscle proteins. This lack of incorporation likely explains the fact that

the uptake calculated for the first hour after EAA ingestion is different for COMB and SEP, but this difference does not reflect the response over the longer time periods (Figure 7). It is likely that some of the amino acids initially transported into the muscle are not incorporated into proteins and there is a subsequent efflux of these amino acids. The NBAL observed in the last few hours of the measurement is lower than the baseline values (Figure 6), suggesting that amino acids are released during this time period and not incorporated into muscle proteins. This notion is supported by our measured FSR data. There was no difference in FSR in the first hour following EAA ingestion regardless of whether CHO are ingested simultaneously (COMB) or one hour prior to EAA ingestion (SEP). Thus, the initial difference in NBAL between COMB and SEP may not reflect a difference in the incorporation of amino acids taken up by the muscle into muscle proteins due to increased MPS. Instead, net uptake over longer periods (3h and 6h – Figure 7) may better reflect the actual physiological anabolic response to the ingestion pattern.

It should be noted that another methodological issue also might have influenced the response of NBAL. Interestingly, a biphasic response of NBAL to drink ingestion was observed in COMB, but not SEP. NBAL peaked 20 min after ingestion of the CHO+EAA-containing drink 1 and then a second time ~20 min after ingestion of the water placebo drink 2. The likely driver of this response of NBAL was the pattern of arterial amino acid concentration that followed a similar biphasic pattern. This biphasic phenomenon has been observed in a previous study that administered a first drink containing EAA+CHO followed by a second water placebo drink (Tipton et al. 2001). Thus, this biphasic pattern seems to be an artifact of the placebo ingestion.

The reason for the second peak in arterial amino acid concentration and NBAL is not obvious, but it seems unlikely that it significantly impacts the conclusions. One possibility may be that the 500 mL water placebo drink 2 served to increase the rate of gastric emptying of amino acids into the gut. It is well known that gastric emptying is increased with a ingestion of high fluid

volume (Costill and Saltin 1974). Thus, it is feasible that amino acids still remained in the stomach after the ingestion of drink 1 and the introduction of drink 2 served to increase gastric emptying leading to increased absorption of amino acids and a second peak in the appearance of amino acids into the circulation. Since there was no additional drink consumed after the EAA-containing drink in SEP, arterial concentrations and NBAL begin to decline as gastric-emptying and absorption slow and amino acids are taken up by tissues, including the liver. This notion is supported by the fact that arterial amino acid concentrations and delivery are still elevated above baseline at the end of the 6h period suggesting amino acids continue to be absorbed from the gut. Hence, it is possible that NBAL during SEP may have been greater if a large volume of fluid was ingested at some point after the drink 2 in SEP. This biphasic response of NBAL in COMB may have been an artifact of study design that led to a different response of NBAL for COMB. This changed pattern is unlikely to influence NBAL calculated over the entire 6h since all amino acids likely would have appeared into the circulation by this time, albeit at a slower rate. Thus, the physiological relevance of the similarity in NBAL between trials is uncertain.

To conclude, our results suggest that delaying the ingestion of EAA by 1h after CHO has a physiological impact on the postexercise response of NBAL, however this transient effect may not be sufficient to sustain an improved NBAL over a longer period. The seemingly disparate responses over the first hour after EAA ingestion compared to the six hours after exercise may be due to methodological considerations. Regardless, from a practical perspective, separating the ingestion of CHO and EAA could be considered unlikely to be an important component of a nutritional strategy aimed at maximizing the anabolic response of muscle to resistance exercise. Instead, a more simple approach of ingesting CHO and EAA together is sufficient to engender an improved net muscle protein balance.

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Author contributions

K.D.T., A.A.F and R.R.W. contributed to the conception and the design of the experiment.

O.C.W., T.L.C. and K.D.T. contributed to collection, analysis, and interpretation of data.

O.C.W., A.A.F., R.R.W. and K.D.T contributed to drafting or revising the content of the manuscript.

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Figure Captions

Fig. 1: Schematic representation of the study protocol. AV = arteriovenous; Ex = exercise; BF = blood flow. COMB = drink regimen that combined postexercise ingestion of CHO and EAA. SEP = drink regimen that separated postexercise ingestion of CHO and EAA. D1 = drink one (CHO+EAA or CHO only for COMB and SEP, respectively), D2 = drink two (Placebo or EAA only for COMB and SEP, respectively).

Fig. 2: Arterial insulin concentrations (μU / mL) before and following combined (COMB) or separate (SEP) ingestion of carbohydrate and essential amino acids. Drink 1 = CHO+EAA or CHO only for COMB and SEP, respectively and Drink 2 = Placebo or EAA only for COMB and SEP, respectively. [a]significantly different from -12 min values in COMB. [b]significantly different from -12 min values in SEP. Data are means ± SE (n=8).

Fig. 3: Arterial phenylalanine (a) and leucine (b) concentrations (nmol/mL) before and following combined (COMB) or separate (SEP) ingestion of carbohydrate and essential amino acids. Drink 1 = CHO+EAA or CHO only for COMB and SEP, respectively and Drink 2 = Placebo or EAA only for COMB and SEP, respectively. [a]significantly different from -12 min values in COMB. [b]significantly different from -12 min values in SEP. [c]significant difference between SEP and COMB at corresponding time point ($P < 0.05$). Data are means ± SE (n=8).

Fig. 4: Blood flow measurements before and following combined (COMB, solid bars) or separate (SEP, open bars) ingestion of carbohydrate and essential amino acids. Post Ex = post exercise period, PD h1 = post drink hour 1, PD h2 = post drink hour 2, PD h3 = post drink hour 3, PD h5 =

post drink hour 5. PD h6 – post drink hour 6. #significantly different (both COMB and SEP taken together) from Post Ex.

Fig. 5: Phenylalanine (a) and leucine (b) delivery to the leg before and following combined (COMB) or separate (SEP) ingestion of carbohydrate and essential amino acids Drink 1 = CHO+EAA or CHO only for COMB and SEP, respectively and Drink 2 = Placebo or EAA only for COMB and SEP, respectively. [a]significantly different from baseline values in COMB ($P < 0.05$). [b]significantly different from baseline values in SEP. [c]significant difference between SEP and COMB at corresponding time point ($P < 0.05$). Data are means ± SE (n=8).

Fig. 6: Phenylalanine net balance across the leg before and following combined (COMB) or separate (SEP) ingestion of carbohydrate and essential amino acids. Drink 1 = CHO+EAA or CHO only for COMB and SEP, respectively and Drink 2 = Placebo or EAA only for COMB and SEP, respectively. . [a]significantly different from -12 min values in COMB. [b]significantly different from -12 min values in SEP. [c]significant difference between SEP and COMB at corresponding time point ($P < 0.05$). Data are means ± SE (n=8).

Fig. 7: Net phenylalanine exchange across the leg over 1 h (a), 3 h (b) & 6 h (c) following ingestion of drink one for separate (SEP) and combined (COMB) ingestion of CHO and essential amino acids trials. *significant difference between SEP and COMB ($P < 0.05$). Data are means ± SE (n=8).

Table 1: Mean muscle intracellular amino acid concentrations in COMB and SEP.

	Phenylalanine				**Leucine**			
	COMB		**SEP**		**COMB**		**SEP**	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Biopsy 1	88	7	79	4	114	11	118	9
Biopsy 2	197[a]	29	94[c]	17	204[a]	34	95	5
Biopsy 3	182[a]	24	115[b,c]	13	184[a]	39	181[b]	17
Biopsy 4	106	12	89	8	142	19	144	17

Values are means (n=5, 3 males and 2 females) ± SE, expressed as nmol/mL IC water. COMB=value when drink 1 consisted of carbohydrate and essential amino acids and drink 2 consisted of placebo. SEP=value when drink 1 consisted of carbohydrate and drink two consisted of essential amino acids. Biopsy 1 = biopsy collected immediately post drink one, Biopsy 2 = biopsy collected 1 h post drink one, Biopsy 3 = biopsy collected 2 h post drink one, 1 h post drink two, Biopsy four = biopsy collected 6 h following drink one, 5 h following drink two. [a]significantly different from Biopsy 1 in COMB, [b]significantly different from Biopsy 1 in SEP, [c]significantly different from COMB at corresponding time point.