Soy-Dairy Protein Blend and Whey Protein Ingestion After Resistance Exercise Increases Amino Acid Transport and Transporter Expression in Human Skeletal Muscle

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Abstract

Increasing amino acid availability (via infusion or ingestion) at rest or post-exercise enhances amino acid transport into human skeletal muscle. It is unknown whether alterations in amino acid availability, from ingesting different dietary proteins, can enhance amino acid transport rates and amino acid transporter (AAT) mRNA expression. We hypothesized that the prolonged hyperaminoacidemia from ingesting a blend of proteins with different digestion rates post-exercise would enhance amino acid transport into muscle and AAT expression as compared to the ingestion of a rapidly digested protein. In a double-blind, randomized clinical trial we studied 16 young adults at rest and after acute resistance exercise coupled with post-exercise (1h) ingestion of either a (soy-dairy) protein blend or whey protein. Phenylalanine net balance and transport rate into skeletal muscle were measured using stable isotopic methods in combination with femoral A-V blood sampling and muscle biopsies obtained at rest, 3 and 5h post-exercise. Phenylalanine transport into muscle and mRNA expression of select amino acid transporters (LAT1/SLC7A5, CD98/SLC3A2 SNAT2/SLC38A2, PAT1/SLC36A1, CAT1/SLC7A1) increased to a similar extent in both groups (P<0.05). However, the ingestion of the protein blend resulted in a prolonged and positive net phenylalanine balance during post-exercise recovery as compared to whey protein (P<0.05). Post-exercise myofibrillar protein synthesis increased similarly between groups. We conclude that while both protein sources enhanced post-exercise AAT expression, transport into muscle and myofibrillar protein synthesis, post-exercise ingestion of a protein blend results in a slightly prolonged net amino acid balance across the leg as compared to whey protein.

Keywords: protein metabolism, protein anabolism, mTORC1, muscle protein synthesis, Leucine
We have recently demonstrated a prolonged post-exercise aminoacidemia, mixed muscle protein synthesis rate (MPS), and mTORC1 signaling response with post-exercise ingestion of a soy-dairy protein blend (52). Despite a significant increase in MPS with the protein blend at 3-5h post-exercise, there was no detectible difference (p=0.12) in mixed MPS between groups (whey versus blend). The purpose of the current study is to determine if different rates of digestion and subsequent prolonged changes in amino acid availability over time would create detectable differences in amino acid transport kinetics, mRNA expression, and myofibrillar protein synthesis during this later recovery period.

The combination of resistance exercise and increased amino acid availability is an effective and highly practical strategy for the promotion of skeletal muscle mass and strength (5, 37, 42, 66). Resistance exercise and essential amino acids (EAA) or protein exert separate and combined effects on skeletal muscle protein synthesis (MPS) and mammalian/mechanistic target of rapamycin complex 1 (mTORC1) signaling (4, 5, 10, 17, 21, 31, 48, 61). Interestingly, using stable isotopic methods, innovative studies demonstrated that resistance exercise in the fasted state and in combination with increased amino acid availability enhance the transport rate of amino acids from the circulation into the muscle cell (4-6).

Amino acid transporters facilitate amino acid flux across the muscle cell membrane to activate mTORC1 (16), which is thought to be essential in regulating muscle protein synthesis (3). Changes in amino acid availability stimulate the system A amino acid transporter SNAT2/SLC38A2, the cationic amino acid transporter 1 CAT1/SLC7A1 (44) and the system L amino acid transporter LAT1/solute-linked carrier (SLC)7A5 (which forms a heterodimer with CD98/SLC3A2) (23, 26, 68). LAT1/SLC7A5 and SNAT2/SLC38A2 function cooperatively to transport large neutral amino acids into the cell (26, 39) whereas proton-assisted transporters (PAT) such as PAT1/SLC36A, are thought to play a role in stimulating protein synthesis after amino acids such as leucine reach sufficient quantities in the cell to activate mTORC1 (34, 36).
More recently, our laboratory has demonstrated that human skeletal muscle amino acid transporter expression, transport rates, mTORC1 activation and MPS is stimulated by the separate (22, 23) and combined (8) effects of exercise and EAA supplementation. Protein ingestion is also an effective means to increase amino acid supply and to augment the muscle protein anabolic response to exercise (13, 30, 47, 48, 55, 59). However, proteins differ on the basis of digestion rate and composition of EAA, which together impact the metabolic fate (i.e., oxidation or incorporation into proteins) of the ingested protein source (49, 56, 64). Although many protein sources are considered to be of high quality, their varying amino acid composition may influence their amino acid transport in the gut (50) and also at the muscle membrane (57). Thus, protein ingestion represents a unique means to study amino acid transporter function in humans. This is an exciting area of investigation, yet only one study has examined human skeletal muscle amino acid transporter expression following resistance exercise and dietary protein ingestion (13). Although several studies have examined muscle protein net balance with consumption of dietary protein following resistance exercise (7, 25, 54, 58-60, 65), no study has examined how the ingestion of dietary protein after resistance exercise stimulates skeletal muscle amino acid transport rates during post-exercise recovery.

Amino acid transporters play a key role in muscle protein metabolism and activation of mTORC1 signaling by altering the delivery of substrate (amino acids) and/or by acting as a transporter/receptor (transceptor) of anabolic signaling (33, 38). Because of the sensitivity of skeletal muscle amino acid transporters to amino acid availability (23) we sought to examine if the prolonged hyperaminoacidemia associated with the ingestion of a blend of plant (25% soy) and dairy (50% casein; 25% whey) proteins (with varying digestion rates) would prolong the skeletal muscle net protein balance across the leg (an indicator of overall muscle protein anabolism) as compared to rapidly digested whey and whether this would influence amino acid transporter expression and amino acid transport into muscle. We hypothesized that the prolonged hyperaminoacidemia from ingesting a blend of proteins would reduce markers of
protein breakdown and enhance overall muscle protein anabolism, myofibrillar protein synthesis, amino acid transport into muscle, and amino acid transporter expression as compared to the ingestion of a rapidly digested protein.
Materials and Methods

Screening of participants.

Sixteen healthy, young subjects (age range: 19-30y) participated in this double-blind, randomized clinical trial. Subject characteristics can be found in Table 1. The subjects were a subset of volunteers that participated in a previous study (52); however, none of the data presented herein has been previously published. The participants were recruited through locally posted flyers, newspaper advertisements, and by word of mouth. The participants were healthy and recreationally active, but were not engaged in any regular exercise-training program (< 2 sessions high intensity aerobic or resistance exercise/week) at the time of enrollment. Screening of participants was performed on two separate days (>7 days apart) at the Institute for Translational Sciences-Clinical Research Center (ITS-CRC). The first screening day included 1 repetition maximum (1RM) strength testing, a clinical history, physical exam, and laboratory tests (complete blood count with differential, liver and kidney function tests, coagulation profile, fasting blood glucose, hepatitis B and C screening, HIV test, TSH, lipid profile, urinalysis, and drug screening). The second screening day included a second 1RM test and a dual-energy X-ray absorptiometry (DXA) scan (Hologic QDR 4500W, Bedford, MA) to measure lean and fat mass. A leg extension machine (Cybex-VR2, Medway, MA, USA) was used to establish a 1RM and the value was recorded as the highest weight lifted for a single repetition from the two testing days. All participants provided written informed consent before enrollment in the study. The study was approved by the Institutional Review Board of the University of Texas Medical Branch, and is in compliance with the Declaration of Helsinki as revised in 1983.

Study Design

Subjects were admitted to the UTMB ITS-CRC at ~1700h the day prior to the study. Subjects were instructed to refrain from exercise at least 72h before admission. The subjects
were given a standardized meal at 1900h prepared by the Bionutrition Division of the ITS-CRC with a macro-nutrient distribution of 20% protein, 60% carbohydrate, and 20% fat at 12 kcal/kg body weight. Subjects were provided water ad libitum. The subjects were randomized to ingest a soy-dairy protein blend (N=8) or whey protein (N=8) at 1h following a bout of high-intensity leg resistance exercise.

*Experimental Protocol*

All subjects underwent the stable isotope infusion protocol (Fig 1) at the same time of day (0600-1600h) on the day following admission. After an overnight fast (~10h), an 18 G polyethylene catheter was inserted into the antecubital vein, from which background blood draws for the measurement of phenylalanine concentration/enrichment and indocyanine green (ICG; Cardio-Green, Becton Dickinson and Co., Cockeysville, MD) concentration. This was followed by initiation of a primed, constant infusion (~10h) of L-[ring-\(^{13}\)C₆] phenylalanine (Sigma-Aldrich, St. Louis, MO, USA). The priming dose for the labeled phenylalanine was 2 \(\mu\)mol/kg and the infusion rate was 0.05 \(\mu\)mol/kg/min. A retrograde catheter was inserted (0700-0800h) into a hand vein on the contralateral arm and arterialized blood was extracted with the use of a heating pad prior to sampling. A catheter was inserted (0900-1000h) into the femoral artery and vein (retrograde) of one leg for blood sampling. The femoral arterial catheter was also used for the infusion of ICG. At ~1030h a continuous infusion of ICG dye (0.5 mg/min) was started in the femoral artery and was maintained for 7 min to measure leg blood flow in each sampling period. Plasma ICG concentration was measured in blood samples during the resting period and several times following protein ingestion (see below) from the femoral and wrist veins. At approximately 2 and 4h following initiation of the infusion muscle biopsies were taken from the lateral aspect of the *vastus lateralis* for the determination of resting (Rest) intracellular phenylalanine enrichment and concentration. All biopsies were collected with a 5mm Bergström biopsy needle under sterile procedure and local anesthesia (1% lidocaine). Following femoral
catheter placement and a series of blood draws the participants were moved to a leg extension
machine (Cybex-VR2, Medway, MA, USA) for high-intensity resistance exercise consisting of
eight sets of ten repetitions at 55% (set 1), 60% (set 2) 65% (set 3) and ~70% (sets 4-8) of the
participants previously determined 1RM with three min rest between sets. Exercise
characteristics can be found in Table 1. The nutritional supplements were ingested 1h following
exercise. Two additional muscle biopsies were collected 2 and 4h after protein ingestion
(corresponding to 3 and 5h after exercise) to represent Early and Late post-exercise periods
(Fig. 1). The measurements taken during the 1-2, 2-3, 3-4, 1-2.5, 2.5-4 and 1-4h post-ingestion
were averaged to represent the 2h, 3h, 4h, Early, Late and Entire periods, respectively (Fig 1).
The first, second, third and fourth muscle biopsies were sampled from two separate incisions on
the same leg, respectively. To minimize multiple sampling in a given area, skin incisions were
separated by ~7 cm while biopsies collected from the same incision were angled ~5 cm from
each other. This method has been previously utilized in our lab (18, 24, 27) and others (35, 48,
53). Muscle tissue was immediately blotted, frozen in liquid nitrogen and stored at -80°C until
analysis. Blood samples were collected before the infusion, during the resting and post-
exercise/post-ingestion time periods (Fig. 1) for the determination of blood enrichment (see
below) and amino acid concentration. The infusion study ended following the fourth muscle
biopsy and participants were then given a standard meal.

Protein Supplements

The protein beverages (Whey or Blend) were ingested at 1h post-exercise. The
beverages were dissolved in 300 ml of water and enriched (8%) with L-[ring-\textsuperscript{13}C\textsubscript{6}] phenylalanine
to in an attempt to maintain isotopic steady state in arterialized blood. The composition of the
beverages is similar to that we previously reported (52). To match leucine and EAA content
between the interventions, participants were given 0.305 or 0.337 g total protein·kg\textsuperscript{-1} lean mass
for Whey and Blend respectively. The amount of protein given in each group was based on the
8.6g of EAA in dietary (non-hydrolyzed) protein demonstrated to maximize the MPS response following resistance exercise (47). The Blend consisted of 20.1±0.9g total protein (providing 1.9±0.1g leucine, 1.0±0.1g phenylalanine, 1.3±0.02g valine and 9.0±0.4g EAA) composed of 50% protein from sodium caseinate, 25% protein from whey protein isolate and 25% protein from soy protein isolate. Whey consisted of 17.3±0.9g of protein (providing 1.9±0.1g leucine, 0.6±0.1g phenylalanine, 1.1±0.01g valine and 8.7±0.5g EAA) composed of 100% whey protein isolate.

Phenylalanine Amino Acid Concentration, ICG, Lactate, Glucose and Insulin

Concentrations of phenylalanine (femoral artery and vein) were measured in the blood using gas chromatography-mass spectrometry (GCMS) as previously described using an internal standard (19, 67). Plasma glucose and lactate concentration was measured using an automated glucose and lactate analyzer (YSI, Yellow Springs, OH) at rest, immediately post-exercise and 0, (at ingestion), 20, 40, 60, 80, 100, 120, 180 and 220 minutes post-ingestion. Serum concentrations of insulin were determined with an enzyme-linked immunosorbent assay (Millipore, St. Charles, MO) according to the manufacturer’s instructions at rest, immediately post-exercise and 0, 20, 40, 60, 80, 100 and 120 minutes post-ingestion. The serum ICG concentration to determine leg blood flow was measured spectrophotometrically (Beckman Coulter) at $\lambda = 805$ nm (41). The phenylalanine concentrations and blood flow measurements taken during the 1-2, 2-3, 3-4, 1-2.5, 2.5-4 and 1-4h post-ingestion were averaged to represent the 2h, 3h, 4h, Early, Late and Entire periods, respectively (Fig 1).

Amino Acid Parameters and Transport Rates

We calculated skeletal muscle amino acid transport rates from the enrichments and concentrations of phenylalanine in the femoral artery and vein and from the enrichment of
muscle tissue-free phenylalanine, using amino acid kinetics modeling as previously described (3, 29). Phenylalanine is used in this model because it is not oxidized by muscle, which allows for the calculation and measurement of amino acid net balance across the leg and MPS. The following amino acid parameters were measured:

- delivery to the leg, \( F_{in} = C_A \cdot BF \) (Eq 1)
- release from the leg, \( F_{out} = C_V \cdot BF \) (Eq 2)
- net balance across the leg, \( NB = (C_A - C_V) \cdot BF \) (Eq 3)
- transport into muscle, \( F_{M,A} = \left\{ \left[ \left( E_M - E_V \right) / \left( E_A - E_M \right) \cdot C_V \right] + C_A \right\} \cdot BF \) (Eq 4)
- transport from muscle, \( F_{V,M} = \left\{ \left[ \left( E_M - E_V \right) / \left( E_A - E_M \right) \cdot C_V \right] + C_V \right\} \cdot BF \) (Eq 5)

where, \( C_A \) and \( C_V \) are plasma phenylalanine concentrations in the femoral artery and vein, respectively; \( E_A, E_V, \) and \( E_M \) are phenylalanine enrichments (tracer/tracee ratio) in femoral arterial and venous plasma and in muscle, respectively; BF is leg blood flow. Data are presented per 100g leg lean mass. Similar values were obtained with correction by leg lean mass (from DXA) and leg volume (as demonstrated in (67)). Leg plasma flow was calculated from the steady state dye concentration values in the femoral and wrist vein as previously described (40, 41). Leg blood flow was calculated by correcting the plasma flow by the hematocrit.

Muscle samples were processed as previously described (67), and muscle free tissue phenylalanine enrichments and concentrations were determined by GCMS. The intracellular concentration of phenylalanine was then calculated from the tissue value, accounting for the ratio of intracellular to extracellular water (3).

**Myofibrillar and Nuclear Fraction Isolation**

About 30–50 mg of frozen muscle tissue was placed in buffer (18) and homogenized (1:9, w/v) and centrifuged at 3,400 \( \times g \) for 10 min at 4°C, followed by removal of the supernatant, which was used for western blotting for LAT1, SNAT2 and eEF2. The resulting
pellet was then suspended in isolation buffer (1 M sucrose, 1 M Tris/HCl, 1 M KCl, 0.5 M EDTA, pH 7.4) containing protease and phosphatase inhibitors and centrifuged for 10 min at 4°C and 700 × g. After 3 series of PBS buffer suspensions and centrifugations at 15,000 × g for 5 min at 4°C, the pellet was re-suspended and agitated on ice for 2x20 min and in a 4°C sonication bath in high salt buffer (1:4, w/v). The slurry was centrifuged at 15,000 × g for 10 min at 4°C and the supernatant was taken as the nuclear extract which was assayed for protein concentration with the BCA protein assay (Pierce, Rockford, IL) and used for western blotting for activating transcription factor 4 (ATF4). The nuclear isolation was verified by examination of cytoplasmic and nuclear protein fractions run on the same gel and probed for antibodies specific to Histone H3 (for nuclear) and Hexokinase (for cytoplasmic).

The resulting pellet was fully suspended in double distilled water and centrifuged at 15,000 × g for 5 min at 4°C. To precipitate the myofibrillar proteins, 1 ml of 0.3M NaOH was added to re-suspend the pellet and this heated at 50°C for 30 min with frequent vortexing. After centrifugation at 10,000 × g for 5 min at 4°C, the supernatant was collected and an additional 1 ml of 0.3M NaOH was added to re-suspend the pellet and this heated at 37°C for 10 min with frequent vortexing. After centrifugation at 10,000 × g for 5 min at 4°C, the supernatant was collected and the collagen pellet was discarded. Precipitate was created by addition of 1 ml PCA to the collected supernatant and pelleted at 805 × g for 10 min at 4°C. This pellet was washed 2x with 70% ethanol and then hydrolysed overnight in 1.5 ml 6M HCL.

**Western blot analysis**

Western blot analysis was conducted as described previously (22). Immunoblot data were normalized to an internal loading control, which was loaded on all gels for comparison across blots, and data are adjusted to represent fold change from basal. Antibodies utilized were LAT1/SLC7A5 (ab85226, Abcam, Cambridge, MA), SNAT2/SLC38A2 (Santa Cruz Biotechnologies, Santa Cruz, CA), ATF4 (Santa Cruz Biotechnologies, Santa Cruz, CA),
Histone 3H (Cell Signaling), phospho-eEF2 (Thr-56) (Cell Signaling), total-eEF2 (Cell Signaling) and monoclonal alpha-tubulin (Sigma-Aldrich, St Louis, MO). LAT1/SLC7A5 and SNAT2/SLC38A2 were normalized to alpha-tubulin and ATF4 was normalized to Histone H3 to account for differences in loading.

**Myofibrillar Protein Synthesis**

Bound proteins from the myofibrillar fraction and muscle intracellular free amino acids were extracted from biopsy samples as described above. GCMS (GCMS, 6890 Plus CG, 5973N MSD, 7683 autosampler, Agilent Technologies, Palo Alto, CA) measurements were made to determine bound tracer enrichments for L-[ring-13C6] phenylalanine as previously described (67). Using the external standard curve approach (11), muscle myofibrillar protein-bound phenylalanine enrichment was analyzed by GCMS after protein hydrolysis and amino acid extraction (18, 63). We calculated myofibrillar protein synthesis as fractional synthesis rate (FSR) by measuring the incorporation rate of the phenylalanine tracer into the proteins ($\Delta$ protein bound enrichment over time) and using the precursor-product model to calculate the synthesis rate:

$$FSR = \frac{\Delta E_p}{t} \frac{1}{\left[ \frac{E_M(1) + E_M(2)}{2} \right]} \times 60 \times 100$$

where $\Delta E_p$ is the increment in protein-bound phenylalanine enrichment between two sequential biopsies, $t$ is the time between the two sequential biopsies, and $E_M(1) + E_M(2)$ are the phenylalanine enrichments in the free intracellular pool in the two sequential biopsies. Due to lack of tissue we were only able to calculate resting FSR with (N=4) in each group. Data are expressed as percent per h (%/ h).

**RNA extraction and Semiquantitative real-time PCR**

RNA isolation, cDNA synthesis, and real-time qPCR were performed as we have previously described (23). Total RNA was isolated by homogenizing 10-20 mg tissue with a
hand-held homogenizing dispenser (T10 Basic Ultra Turrax, IKA, Wilmington, NC) in 1 ml of Tri reagent. The RNA was separated into an aqueous phase using 0.2 ml of chloroform and subsequently precipitated from the aqueous phase using 0.5 ml of isopropanol. RNA was washed with 1 ml of 75% ethanol, air-dried, and suspended in a known amount of nuclease-free water. RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and RNA was DNase-treated using a commercially available kit (DNA-free, Ambion, Austin, TX). A total of 1 μg of RNA was reverse transcribed into cDNA according to the directions provided by the manufacturer (iScript, BioRad, Hercules, CA). Real-time qPCR was carried out with an iQ5 Multicolor Real Time PCR cycler (BioRad). cDNA was analyzed with SYBR green fluorescence (iQ SYBR green supermix; BioRad). Primer sequences for genes of interest (LAT1/SLC7A5, CD98/SLC3A2 SNAT2/SLC38A2, PAT1/SLC36A1, CAT1/SLC7A1) have been previously published (23). β2-Microglobulin was utilized as a normalization/housekeeping gene. Relative fold changes were determined from the Ct values using the $2^{-ΔΔCt}$ method (43).

**Statistics**

All outcomes were assessed using standard ANOVA and ANCOVA models. With baseline as a covariate, an ANCOVA model for each outcome was used to determine possible differences between groups at each time point. To test marginal outcomes and differences across time points, a repeated measures ANOVA model was used in which a random-intercept model was used to account for subject-to-subject variability. Pairwise comparisons were calculated and tested using standard post-hoc contrast methods. All pairwise comparisons were done using contrasts in the ANOVA model, with Tukey testing for post-hoc adjustment. Assumptions of normality and homogeneity of variance were tested, and transformations were used as necessary to make all tests reliable. All calculations were done in SAS, version 9.3.

**Results**
Subject and Exercise Characteristics

The subjects were effectively randomized as their baseline and exercise characteristics (Table 1) were not different (p > 0.05).

Plasma glucose in the femoral artery and vein (data not shown) increased from Rest only immediately post-exercise and were not different (p > 0.05) between groups. Plasma lactate in the femoral artery and vein (data not shown) increased from Rest for the first hour post-exercise and was not different (p > 0.05) between groups. Serum insulin (Table 2) was not different (p > 0.05) between groups and showed a time effect for an increase (p < 0.05) at 20, 40, 60 min post-ingestion compared to Rest. Compared to Rest, insulin was increased (p < 0.05) at 20 and 40 min post-ingestion in Blend, 20, 40, 60 min post-ingestion in Whey and decreased 140 min post-ingestion in Whey.

Arterial, Venous and Muscle Intracellular Phenylalanine Concentration

Phenylalanine arterial concentration increased in Blend at Early, 2h, 3h and Entire and in Whey at Early and 2h compared to Rest (p < 0.05). Phenylalanine venous concentration increased in Blend at Early, 2h, 3h and Entire and in Whey at Early and 2h compared to Rest (p < 0.05). Intracellular phenylalanine concentrations were similar between groups and there was an overall effect for a decreased concentration at Late compared to Rest (p < 0.05). There were no differences between groups for arterial, venous and muscle intracellular phenylalanine concentrations (Table 3).

Phenylalanine Enrichment

Arterial tracer-tracee ratio (TTR) was elevated across both groups at Late, 3h, 4h and Entire compared to at Rest (effect of time: p < 0.05; Table 4). This effect was driven largely through an increased arterial TTR in Whey at all post-ingestion time points compared to Rest (p < 0.05). Venous TTR was elevated across both groups at all post-exercise time points.
compared to Rest (effect of time: \( p < 0.05 \)). There was a group difference in venous TTR at 3h (\( p < 0.05 \)). Muscle TTR was increased across both groups at all post-exercise time points compared to Rest (effect of time: \( p < 0.05 \)) (Table 4). Overall these data only show minor perturbations in the steady state conditions at rest and post-exercise conditions, which permitted us to calculate amino acid transport into and out of leg muscle.

### Amino Acid Transport Rates, Net Balance and Transporter mRNA Expression

Blood flow was not different between groups or across time (\( p > 0.05 \); Table 3). Phenylalanine Net Balance across the entire leg became positive at Early and 2h and was less negative at 3h in the Blend compared to Rest (\( p < 0.05 \)). With the period analysis (Table 3), there was no change in Net Balance with Whey. In the point analysis (Fig. 2), the Net Balance became less negative at 0 min in Whey and was positive at only 20 and 40 min post-ingestion as compared to Rest (\( p < 0.05 \)). With the Blend, the Net Balance became positive at 20, 40, 60, 80, 100 and 120 min post-ingestion compared to Rest (\( p < 0.05 \)). This positive net balance caused an overall time effect at 20, 40, 60, 80, 100 and 120 min post-ingestion compared to Rest (\( p < 0.05 \)) in the Blend. There was a group difference and a more positive Net Balance in Blend than Whey at 60 and 120 min post-ingestion and a group difference and a more positive Net Balance in Whey than Blend at 20 min post-ingestion (\( p < 0.05 \)). Phenylalanine delivery to the leg was not different between the groups and there was an overall time effect at Early (\( p = 0.058 \)), Late, 3h, 4h and Entire compared to Rest (\( p < 0.05 \)) (data not shown). Phenylalanine release from the leg was not different between the groups and there was an overall time effect at Late, 3h (\( p = 0.054 \)), 4h and Entire (\( p = 0.053 \)) compared to Rest (\( p < 0.05 \)) (data not shown).

For both groups combined, inward transport of phenylalanine into leg muscle increased at Early, Late, 2h and 3h compared to Rest (effect of time: \( p < 0.05 \)). Phenylalanine inward transport increased in Blend during Early, 3h and Entire and in Whey during Early, 2h and Entire compared to Rest (\( p < 0.05 \)). There were no group differences (\( p > 0.05 \)). For Blend, outward
transport of phenylalanine increased at Early (p = 0.050), Late, 2h and 3h (p = 0.056) compared to Rest (effect of time: p < 0.05). Phenylalanine outward transport increased in Blend during the Early, 3h and Entire and in Whey during Early, 2h and Entire compared to Rest (p < 0.05). There were no group differences (Table 5, Fig 3 A-B).

CD98/SLC3A2, PAT1/SLC36A1 and CAT1/SLC7A1 mRNA expression were elevated at 2 and 4h post-ingestion as compared to Rest for both groups (p < 0.05). LAT1/SLC7A5 mRNA expression was elevated (p < 0.05) at 2 and 4h post-ingestion as compared to Rest for Blend and only at 2h for Whey. However, there was a trend (p = 0.06) for LAT1/SLC7A5 mRNA expression to be elevated 4h post-ingestion as compared to Rest in Whey. SNAT2/SLC38A2 mRNA expression was elevated at 2h post-ingestion as compared to Rest for both groups (p < 0.05). CAT1/SLC7A1 mRNA expression was greater at 4h than at 2h post-ingestion for both groups (p < 0.05). With Whey only PAT1/SLC36A1 mRNA expression was greater at 4h than at 2h post-ingestion (p < 0.05; Fig 4 A-E). LAT1 and SNAT2 protein expression was not different (p > 0.05) from Rest at any time point or between groups (Fig 5). Nuclear ATF4 protein expression (a known regulator of amino acid transporter expression) was not different (p > 0.05) between groups and was only elevated (p < 0.05) from Rest in the Blend at 2h post-ingestion (Fig 5). Representative immunoblots for protein expression data are shown in Fig 5.

Myofibrillar Protein Synthesis and Markers of Protein Turnover

We have previously shown that both Blend and Whey increase mixed muscle protein synthesis and mTORC1 signaling to a similar extent following resistance exercise (52). However, to confirm that no group differences occurred during post-exercise recovery we compared post-exercise myofibrillar protein synthesis rates between Blend and Whey, and whether other markers of protein synthesis (eEF2 phosphorylation) and breakdown (MAFbx and MuRF-1 mRNA) differed between groups. Phosphorylated eEF2 was not different (p > 0.05) between groups, but was reduced (p < 0.05) at 2 and 4h post-ingestion with the Blend, but only
at 2h with Whey (Fig 5). Resting myofibrillar protein synthesis was not different (p = 0.662) between Whey (0.035 ± 0.011 %/h) and Blend (0.0413 ± 0.008 %/h) so we pooled the resting data. Post-exercise myofibrillar protein synthesis increased above resting values in both groups (p<0.05) and was not different (p = 0.333) between Whey (0.093 ± 0.007 %/h) and Blend (0.081 ± 0.009 %/h) (Fig 6). mRNA expression of MuRF-1 was increased at 2h in Blend and 4h post-ingestion in both Whey and Blend compared to Rest (p < 0.05). There were no group differences for either MAFbx or MuRF-1 mRNA expression (Fig 7 A-B). mRNA expression of MAFbx was unaltered compared to Rest in both groups (p < 0.05).
Discussion

In the current study, we utilized arterial and venous femoral catheterization of the leg and vastus lateralis muscle biopsies during infusion of a stable isotopically labeled amino acid tracer to comprehensively assess several measures of skeletal muscle amino acid transport and muscle protein anabolism in the post-exercise recovery period following the ingestion of a soy-dairy protein blend (Blend) or whey (Whey). Importantly, we examined how post-exercise protein ingestion impacts the immediate post-exercise recovery transport kinetics and also the adaptive response to expand the amino acid transporter machinery (mRNA expression). We report two novel findings: 1) increased post-exercise phenylalanine net balance (i.e., an indicator of overall muscle protein anabolism) across the leg was prolonged with Blend ingestion during the acute post-exercise recovery phase (0-2h post-ingestion) as compared to Whey; and 2) dietary protein ingestion of Blend and Whey increased post-exercise amino acid (phenylalanine) transport into muscle and mRNA expression of amino acid transporters associated with the regulation of mTORC1 signaling and muscle protein synthesis.

Similar to studies with resistance exercise and/or amino acids (4, 5, 9, 51, 61, 62), we observed increased amino acid flux across the muscle cell membrane with the post-exercise ingestion of dietary protein. As predicted, the prolonged aminoacidemia in the Blend delayed the amino acid flux to its highest point, 2-3hr post-ingestion, whereas in Whey it was highest at 1-2 hour post-ingestion. However, both groups experienced a similar increase when the values where averaged over the 1-2.5h and 1-4h periods, which is probably why we did not detect differences between groups in MPS. This transport data and the slight differences in mTORC1 signaling (52) suggest that although the end result (MPS) could be the same, the mechanism to stimulate MPS may be different. The magnitude of amino acid transport rate was less with dietary protein compared to previous studies using crystalline amino acids (5, 9, 51, 61, 62).

Interestingly, Biolo et al. were not able to detect an increase in phenylalanine transport at a similar time following resistance exercise in the fasted state (4), however, they did demonstrate
increased transport of lysine, leucine and alanine. In a follow-up study (5), these investigators
provided an infusion of amino acids in a similar post-exercise recovery period and significantly
increased amino acid concentrations, particularly phenylalanine, to twice the amount
demonstrated in this study with ingestion of dietary protein. This suggests that phenylalanine
transport is an effective means to assess the enhanced post-exercise protein anabolic response
of exogenous amino acids. Further support for this concept, by the same researchers,
demonstrated that insulin infusion alone following resistance exercise was insufficient to
stimulate phenylalanine transport (6). Although, insulin is thought to independently stimulate
amino acid transport (1, 46), these reports suggest that post-exercise insulin action on muscle
protein synthesis and amino acid transport requires excess amino availability in human skeletal
muscle (2, 19). Even with these differences in magnitude of the response, we arrive at a similar
conclusion - that the increased amino acid availability (from dietary protein) following exercise is
likely driving the increased phenylalanine transport in this model.

Changes in amino acid availability stimulate SNAT2/SLC38A2, CAT1/SLC7A1
LAT1/SLC7A5 and CD98/SLC3A2 (23, 26, 44, 68). We found an increase in mRNA expression,
from rest, of select amino acid transporters, (LAT1/SLC7A5, CD98/SLC3A2, SNAT2/SLC38A2,
PAT1/SLC36A1 and CAT1/SLC7A1), concomitant with increased mTORC1 signaling and MPS
at 3 and 5h of recovery from resistance exercise coupled with whey or protein blend ingestion
1h post-exercise. As compared to the previously examined fasted state post-exercise response
in young adults (22), we see increases in SNAT2/SLC38A2, PAT1/SLC36A1, CD98/SLC3A2
and CAT1/SLC7A1 at 3h post-exercise (2h post-ingestion) with Whey and Blend. This further
supports the sensitivity of these amino acid transporters to amino acid availability and their
possible role in promoting MPS. By 5h post-exercise (4h post-ingestion) we demonstrated
similar values to the fasted study (22) indicating that the prolonged amino acid availability and
mTORC1 signaling in the blend or the strong initial anabolic signal from whey did not cause
further stimulation via this mechanism. We have previously reported that a combination of
essential amino acids and exercise (15) stimulate increased expression of similar amino acid transporters (LAT1/SLC7A5, SNAT2/SLC38A2), but not increased CD98/SLC3A2 and PAT1/SLC36A1 mRNA expression as we show in this study. This may be a factor of a difference in the level (20g EAA vs. ~9g) and type (EAA only vs. EAA and NEAA) of amino acid availability. CAT1/SLC7A1 expression tends to be greater at 3h post-exercise with protein ingestion compared to fasting recovery (22) or 20g EAA (15), which could be due to the NEAA in the ingested protein.

A recent study showed that 25g of whey protein ingestion following resistance exercise increased skeletal muscle amino acid transporter expression above rest at similar time points (13) compared to our study, however, the fold changes reported in that study were approximately double what we found in our study. Given the sensitivity of these transporter mechanisms to amino acid availability and muscle contraction (4, 5, 15, 20) this may be a reflection of the different dose of protein (25g Whey from (13) vs 20.1g Whey vs 17.3g Blend) or the overall content of leucine (3g from (13) vs 1.9g) ingested. Preliminary data from our laboratory suggest that when subjects ingest 10 grams of EAA with low (1.8g) vs high (3.5g) amounts of leucine the high leucine group exhibited greater stimulation of skeletal muscle amino acid transporter expression (unpublished observations). Interestingly, LAT1/SLC7A5 expression appears to be ~1-2 fold higher 3h post-exercise (2h post-ingestion) when 20g leucine-enriched EAA are ingested following resistance exercise (15) compared to fasted conditions (22) or here with protein ingestion suggesting the higher leucine content may be driving this response. Thus it may be that leucine content of a protein source is a key regulator of amino acid transporter expression. In addition, it is likely that increases in amino acid transporter protein expression occurred beyond the 5h post-exercise time point as observed in our previous resistance exercise study (23). Amino acid transporter mRNA expression and amino acid transport kinetics are loosely linked outcomes during the short time frame of our acute study. We propose the changes in mRNA expression and eventual increases in protein
expression are likely to have an impact when the muscle is exposed to a subsequent increase in amino acid availability (i.e., the next meal). More research in this area is needed as very little is known regarding the kinetics and functional relevance of amino acid transporters in human muscle biology.

The molecular mechanisms driving the increase in amino acid transporter expression are poorly understood. It has been suggested, from data collected in cell culture studies, that the nuclear transcription factor, AFT4, regulates gene expression of select amino acid transporters (1, 46) in conditions of amino acid deprivation (1), overabundance (45) and presence of insulin (1, 45). However, this relationship is not as pronounced in human skeletal muscle under physiological conditions of crystalline amino acid ingestion (23) or following resistance exercise in the fasted state (22). Here we demonstrate nuclear ATF4 to slightly increase 2h post-ingestion of the Blend, which may play a role in promoting the increase in amino acid transporter gene expression. We did not see this same response in Whey, which may be a factor of the biopsy sampling time. Further evidence is needed to determine the role of ATF4 or other transcription factors (e.g. GCN2) in regulating amino acid transporter expression in human skeletal muscle in response to muscle contraction or amino acid availability.

As with previous studies (25, 54, 58-60), we also demonstrated that whey protein exhibits a rapid increase in amino acid net balance that is short-lived, returning to resting values around the first hour following post-exercise ingestion. As a novel feature, in this study, we demonstrated that the Blend had a less rapid rise in net balance across the leg, but was able to prolong a positive net balance to 2h post-ingestion. Additionally, the net balance in the Blend was greater than Whey at 60 and 120 min post-ingestion. This difference between groups could reflect a transient increase in the intracellular AA pool, potentially be due to a greater reduction in breakdown, in the blend, during 1-2h post-ingestion, which we unfortunately could not accurately assess due to the confounding influences of recent exercise and amino acid flux.
perturbations. This prolonged net balance is likely due to the intermediate digestion of soy and the prolonged digestion of casein. This prolonged hyperaminoacidemia is not just specific to phenylalanine (which had a slightly higher content in the blend), but valine as we have previously reported (52). This suggests that a similar effect on amino acid net balance could be occurring with other amino acids besides phenylalanine. As external support of our net balance data, milk (25, 65) or the slowly digested casein (54) can also prolong net balance up to 2h post-ingestion. A previous study demonstrated that a blend of fast (whey) and slowly (casein) digested proteins provided as fat-free milk had a prolonged post-exercise net balance as compared to a single protein provided as soymilk (65). This provides further evidence that combining proteins with varying digestion rates can sustain the post-exercise net protein balance.

We also examined two key markers of muscle protein breakdown – the E3 ligases MuRF-1 and MAFbx. We found similar expression patterns for both atrogenes with Blend or Whey ingestion 3 and 5h following resistance exercise. Although MuRF-1 was up regulated in both groups, the expression level was ~ 2 fold less than what we have reported following resistance exercise in the fasted state (28). As mentioned earlier, in reference to early net balance differences, any potential difference in breakdown between the groups may have occurred sometime before 3h post-exercise (2h post-ingestion). Thus after this time, the mRNA data suggest that the additional amino acid supply and/or equivalent insulin stimulus in both Blend and Whey were effective in reducing markers associated with post-exercise muscle protein breakdown which may also be an important part in the overall muscle protein turnover response to exercise combined with post-exercise protein intake.

Similar to our previous report with mixed muscle protein synthesis (52) we found no difference in post-exercise myofibrillar protein synthesis between Whey and Blend. As we have previously suggested (52), a blend of proteins with different digestion rates and prolonged aminoacidemia may have a different cellular response, but a similar effect (MPS) compared to a
bolus of whey protein when matched for leucine. In the protein blend (soy, whey and casein) it seems likely that there is an initial anabolic signal generated with initial whey digestion, albeit weaker than only a whey bolus, that is prolonged with stimulation from slower released soy and casein. These post-exercise rates of myofibrillar protein synthesis are comparable to those reported elsewhere for dietary protein ingestion following resistance exercise (37). Given the divergent results regarding muscle protein anabolism between net phenylalanine balance across the leg and vastus lateralis protein synthesis, it is important to note key differences in these methods. 1) The temporal differences in myofibrillar protein synthesis were assessed during the later period of recovery, 2-4h post-ingestion at a time when net balance was similar between groups; 2) Net balance assesses uptake of phenylalanine in all the muscles of the leg irrespective of the potential protein(s) being synthesized, which experience different or in some instances no activation with exercise, whereas the precursor product assessment of myofibrillar protein synthesis is only specific to the activity of that protein fraction in the vastus lateralis. Some of the mechanisms for the post-exercise muscle protein anabolism with protein blend ingestion are likely increased translation initiation, as we have reported (52), but also increased translation elongation as suggested by the decreases in eEF2 phosphorylation demonstrated in this study. These data offer further support for the hypothesis that a blend of protein with different digestion rates and prolonged aminoacidemia may have a different cellular response, but a similar effect (MPS) to that of rapidly digested whey.

When researchers supply a dose of protein well above the leucine threshold the amount of leucine probably has little additional effect on rates of MPS, which have already been maximized (47). On the other hand, because we did not oversupply protein (~20g protein; 9g EAA), we believe that matching the leucine content is essential in our investigation as leucine content plays a very important role in regulating MPS. Two recent studies have elegantly demonstrated that the leucine content in a supplement is a primary stimulator of MPS, especially when the total protein or content of other amino acids is low (12, 14).
the proteins according to leucine content we ended up with a difference in total protein and calories between the ingested proteins. The difference in total protein ingested (<3g) was very minimal and is mostly composed of NEAA, which does not stimulate muscle protein synthesis. We have demonstrated that adding 120 kcals does not further stimulate muscle protein anabolism when sufficient EAA are provided (32). Thus, the 10-20 kcal difference in total energy (in this study) is unlikely to have influenced the response.

Limitations to the study are as follows; 1) We did not assess the kinetics of other amino acids following resistance exercise, which could be variable (4). 2) Due to the challenges of maintaining an isotopic steady state with multiple perturbations in kinetic parameters following the combination of exercise and dietary protein ingestion, we could only assess a later (2-5 h) post-exercise period, not the immediate post-exercise period (0-2 h) without violating the assumptions of our stable isotopic model for calculating amino acid transport rates. As such, this only allowed us to accurately calculate the transport model parameters of inward and outward transport. However, the measurement of the rate of amino acid transport into leg muscle is the focus of our study and a novel means to investigate the effects of dietary protein.

In summary, we found that the increase in post-exercise phenylalanine net balance across the leg (an indicator of muscle protein anabolism) was prolonged with ingestion of a protein blend compared to whey protein. We also report that ingesting a protein blend or whey protein enhances the rate of amino acid transport into muscle, increases select amino acid transporter (LAT1/SLC7A5, CD98/SLC3A2, SNAT2/SLC38A2, PAT1/SLC36A1, CAT1/SLC7A1) mRNA expression, and increases post-exercise myofibrillar protein synthesis. These results provide further support for the efficacy of ingesting a protein blend to increase and prolong post-exercise muscle protein anabolism. Further research is necessary to determine the efficacy of protein blend supplementation on muscle growth and strength during chronic resistance exercise training.
We would like to thank our subjects, nurses and staff at the ITS-CRC for their assistance in screening, admitting and assisting with the subjects during data collection. Also we extend our thanks to Dr. Denis Gore for assistance with placement of central lines, Shaheen Dhanani for assistance with recruitment, and also Ming Zheng and Shelley Medina for technical assistance. This study was supported by grants from Solae LLC, NIH T32-HD07539, P30 AG024832 and NIH R01 AR049877. Current affiliation for D.K. Walker is: Center for Translational Research in Aging & Longevity, Texas A&M University, College Station, TX 77843, U.S.A. Current affiliation for M.J. Drummond is: Department of Physical Therapy, University of Utah, Salt Lake City, Utah, 84108 U.S.A. Current affiliation for J.M. Dickinson is: Department of Exercise and Wellness, Arizona State University, Phoenix, Arizona, 85004 U.S.A. Current affiliation for K.L. Timmerman is: Department of Kinesiology and Health, Miami University, Oxford, OH 45056 U.S.A. Current affiliation for D.M Gundermann is: School of Veterinary Medicine, University of Wisconsin, Madison, WI 53706 U.S.A.

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This trial was registered at clinicaltrials.gov as NCT01358305.

Authors' contributions

PTR participated in the design of the study, recruitment and screening of subjects, collected the data, analyzed the data, performed the statistical analysis, and wrote and edited the manuscript. DKW, JMD, KLT and MJD participated in the design of the study, collected the data and edited the manuscript. DMG collected the data and edited the manuscript. KJ performed the statistical
analysis and edited the manuscript. EV participated in study design and coordination and edited the manuscript. BBR conceived of the study, participated in its design and coordination and helped to draft the manuscript. MCB and RM edited the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that this study was funded by Solae, LLC, now DuPont Nutrition & Health
References


45. Luo JQ, Chen DW, and Yu B. Upregulation of amino acid transporter expression induced by L-leucine availability in L6 myotubes is associated with ATF4 signaling through mTORC1-dependent mechanism. *Nutrition* 29: 284-290, 2013.


Table and Figure Legends

Table 1
Subject and exercise characteristics of participants randomized to receive Whey (N=8) or Blend (N=8) at 1h post-exercise. Mean ± SE.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Whey (N=8)</th>
<th>Blend (N=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>25 ± 3</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>6/2</td>
<td>5/3</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>25 ± 3</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>Resting Blood Pressure</td>
<td>120/80</td>
<td>122/80</td>
</tr>
</tbody>
</table>

Table 2
Serum insulin (pmol·L⁻¹) at Rest, immediately post-exercise and post-ingestion (min) for subjects given Whey (N=8) and a Blend (N=8) at 1h post-exercise. Mean ± SE. *p < 0.05 vs Rest.

<table>
<thead>
<tr>
<th>Time</th>
<th>Whey (N=8)</th>
<th>Blend (N=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>5 ± 2</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>1min</td>
<td>6 ± 2</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>3min</td>
<td>7 ± 2</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>5min</td>
<td>8 ± 2</td>
<td>9 ± 2</td>
</tr>
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</table>

Table 3
Femoral artery and vein blood and intracellular muscle phenylalanine concentration (nmol·ml⁻¹) leg blood flow (ml·min⁻¹·100 g leg muscle⁻¹) and phenylalanine net balance (nmol·min⁻¹·100 g lean leg mass⁻¹) across the leg at rest and post-ingestion for subjects given Whey (N=8) and a Blend (N=8) at 1h post-exercise. Values are Mean ± SE. Comparisons are: Rest vs. Early, Late, Entire, 2h, 3h & 4h; Early vs Late; 2h vs 3h vs 4h. *p < 0.05 vs Rest; †p < 0.05 main effect of time; ‡p < 0.05 Early vs Late; §p < 0.05 2h vs 3h; ‖p < 0.05 2h vs 4h; ‡‡p < 0.05 3h vs 4h.

<table>
<thead>
<tr>
<th>Time</th>
<th>Whey (N=8)</th>
<th>Blend (N=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>10 ± 2</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Early</td>
<td>12 ± 2</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>Late</td>
<td>14 ± 2</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Entire</td>
<td>16 ± 2</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>2h</td>
<td>18 ± 2</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>3h</td>
<td>20 ± 2</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>4h</td>
<td>22 ± 2</td>
<td>23 ± 2</td>
</tr>
</tbody>
</table>

Table 4
Phenylalanine enrichments as tracer to tracee ratio (%) at rest and post-ingestion for subjects given Whey (N=8) and a Blend (N=8) at 1h post-exercise. Values are Mean ± SE. Comparisons are: Rest vs. Early, Late, Entire, 2h, 3h & 4h. *p < 0.05 vs Rest; †p < 0.05 group effect at time point; ‡p < 0.05 main effect of time.

<table>
<thead>
<tr>
<th>Time</th>
<th>Whey (N=8)</th>
<th>Blend (N=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>5 ± 2</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Early</td>
<td>6 ± 2</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Late</td>
<td>7 ± 2</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Entire</td>
<td>8 ± 2</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>2h</td>
<td>9 ± 2</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>3h</td>
<td>10 ± 2</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>4h</td>
<td>11 ± 2</td>
<td>12 ± 2</td>
</tr>
</tbody>
</table>

Table 5
Phenylalanine transport rates (nmol·min⁻¹·100 g lean leg mass⁻¹) across the muscle membrane at rest and post-ingestion for subjects given Whey (N=8) and a Blend (N=8) at 1h post-exercise. Values are Mean ± SE. Comparisons are: Rest vs. Early, Late, Entire, 2h, 3h & 4h. *p < 0.05 vs Rest.

<table>
<thead>
<tr>
<th>Time</th>
<th>Whey (N=8)</th>
<th>Blend (N=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>2 ± 0.5</td>
<td>3 ± 0.5</td>
</tr>
<tr>
<td>Early</td>
<td>3 ± 0.5</td>
<td>4 ± 0.5</td>
</tr>
<tr>
<td>Late</td>
<td>4 ± 0.5</td>
<td>5 ± 0.5</td>
</tr>
<tr>
<td>Entire</td>
<td>5 ± 0.5</td>
<td>6 ± 0.5</td>
</tr>
<tr>
<td>2h</td>
<td>6 ± 0.5</td>
<td>7 ± 0.5</td>
</tr>
<tr>
<td>3h</td>
<td>7 ± 0.5</td>
<td>8 ± 0.5</td>
</tr>
<tr>
<td>4h</td>
<td>8 ± 0.5</td>
<td>9 ± 0.5</td>
</tr>
</tbody>
</table>
Figure 1
Study Design

Figure 2
Phenylalanine net balance at Rest and during 4h post-ingestion for subjects given Whey (N=8) and a Blend (N=8) at 1h post-exercise. Mean ± SE: *Whey vs. rest, p < 0.05; #Blend vs. rest, p < 0.05; Blend vs. Whey, †p < 0.05.

Figure 3
Phenylalanine inward (A) and outward (B) transport averages during Rest, Early, Late and Entire periods for subjects given Whey (N=8) and a Blend (N=8) at 1h post-exercise. Mean ± SE: †effect of time, p < 0.05; *different from rest, p < 0.05.

Figure 4
mRNA expression of LAT1/SLC7A5(A), CD98/SLC3A2(B), SNAT2/SLC38A2(C), PAT1/SLC36A1(D) and CAT1/SLC7A1(E) during Rest, Early, and Late periods for subjects given Whey (N=8) and a Blend (N=8) at 1h post-exercise. Mean ± SE: *different from rest, p < 0.05; a2h vs 4h, p < 0.05.

Figure 5
Fold change from rest of protein expression of LAT1, SNAT2, ATF4 and eEF2 phosphorylation in the hours post-ingestion for subjects given Whey (N=8) and a Blend (N=8) at 1h post-exercise. Representative immunoblots of protein expression are shown for samples at rest and in the hours post-ingestion for subjects given Whey and a Blend at 1h post-exercise. All samples were loaded in duplicate. Representative blots for the groups were found on separate blots, yet all samples were derived at the same time and processed in parallel. Mean ± SE. *p < 0.05 vs Rest. ATF4 is presented as N=7 in each group.

Figure 6
Skeletal muscle myofibrillar fractional synthetic rate in the vastus lateralis at rest (pooled from Whey (N=4) and a Blend (N=4) and during the post-exercise recovery period for subjects given Whey (N=8) and a Blend (N=8) at 1h post-exercise. Mean ± SE.

Figure 7
mRNA expression of MURF-1 (A) and MAFbx (B) during Rest, Early, and Late periods for subjects given Whey (N=8) and a Blend (N=8) at 1h post-exercise. Mean ± SE: *different from rest, p < 0.05.
## Table 1 Characteristics

### Subject

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Age</th>
<th>BMI (kg·m⁻²)</th>
<th>% Fat</th>
<th>Lean Mass (kg)</th>
<th>Leg Volume (L)</th>
<th>Leg mass (kg)</th>
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<tbody>
<tr>
<td>Blend</td>
<td>8</td>
<td>22.3 ± 1.0</td>
<td>26.6 ± 0.8</td>
<td>23.9 ± 1.4</td>
<td>59.5 ± 2.5</td>
<td>10.9 ± 0.5</td>
<td>11.3 ± 0.5</td>
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<tr>
<td>Whey</td>
<td>8</td>
<td>23.6 ± 1.0</td>
<td>25.0 ± 1.3</td>
<td>25.1 ± 2.7</td>
<td>56.6 ± 3.0</td>
<td>10.4 ± 0.5</td>
<td>10.8 ± 0.6</td>
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### Exercise

<table>
<thead>
<tr>
<th></th>
<th>1RM (kg)</th>
<th>Total weight lifted (kg)</th>
<th>% -1RM Mean</th>
<th>Time (min)</th>
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<tbody>
<tr>
<td>Blend</td>
<td>124 ± 7</td>
<td>6265 ± 353</td>
<td>65 ± 1</td>
<td>26 ± 1</td>
</tr>
<tr>
<td>Whey</td>
<td>126 ± 11</td>
<td>6302 ± 527</td>
<td>63 ± 1</td>
<td>27 ± 2</td>
</tr>
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</table>
**Table 2. Serum insulin**

<table>
<thead>
<tr>
<th></th>
<th>Rest</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>100</th>
<th>140</th>
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<tbody>
<tr>
<td>Blend</td>
<td>29.1 ± 4.6</td>
<td>38.9 ± 11.5</td>
<td>66.1 ± 17.4*</td>
<td>77.5 ± 10.1*</td>
<td>48.9 ± 6.8*</td>
<td>31.4 ± 4.4</td>
<td>27.2 ± 3.7</td>
<td>19.6 ± 2.8</td>
</tr>
<tr>
<td>Whey</td>
<td>24.6 ± 2.3</td>
<td>30.0 ± 4.9</td>
<td>62.4 ± 11.0*</td>
<td>70.8 ± 16.8*</td>
<td>51.9 ± 9.2*</td>
<td>36.6 ± 6.8</td>
<td>23.9 ± 3.1</td>
<td>17.3 ± 2.7*</td>
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## Table 3. Phenylalanine concentrations, leg blood flow and phenylalanine net balance

<table>
<thead>
<tr>
<th>Time Post-Ingestion</th>
<th>1-2.5h</th>
<th>2.5-4h</th>
<th>1-2h</th>
<th>2-3h</th>
<th>3-4h</th>
<th>1-4h</th>
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<tbody>
<tr>
<td></td>
<td>Rest</td>
<td>Early</td>
<td>Late</td>
<td>Entire</td>
<td>Entire</td>
<td>Entire</td>
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<tr>
<td><strong>Arterial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blend</td>
<td>57.2 ± 1.3</td>
<td>70.8 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60.0 ± 2.7</td>
<td>74.4 ± 2.4&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>63.8 ± 2.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>58.5 ± 3.0</td>
</tr>
<tr>
<td>Whey</td>
<td>59.0 ± 2.6</td>
<td>64.0 ± 3.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.6 ± 2.9</td>
<td>68.1 ± 3.0&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>57.5 ± 2.8</td>
<td>56.0 ± 3.1</td>
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<tr>
<td>Blend</td>
<td>62.6 ± 1.7</td>
<td>71.2 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63.0 ± 2.0</td>
<td>72.7 ± 2.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>67.2 ± 1.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>61.3 ± 2.4</td>
</tr>
<tr>
<td>Whey</td>
<td>63.0 ± 2.5</td>
<td>66.3 ± 3.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60.0 ± 2.7</td>
<td>70.0 ± 3.3&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>60.8 ± 2.9</td>
<td>59.0 ± 2.7</td>
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<tr>
<td><strong>Blood Flow</strong></td>
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<tr>
<td>Blend</td>
<td>2.41 ± 0.22</td>
<td>3.47 ± 0.98</td>
<td>3.23 ± 0.49</td>
<td>3.57 ± 1.23</td>
<td>3.26 ± 0.59</td>
<td>3.27 ± 0.46</td>
</tr>
<tr>
<td>Whey</td>
<td>2.66 ± 0.41</td>
<td>3.23 ± 0.52</td>
<td>3.79 ± 0.55</td>
<td>3.11 ± 0.45</td>
<td>3.65 ± 0.64</td>
<td>3.75 ± 0.57</td>
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<td><strong>Net Balance</strong></td>
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<tr>
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<td>-12.3 ± 1.5</td>
<td>2.3 ± 4.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-8.0 ± 2.4</td>
<td>9.3 ± 5.7&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>-12.0 ± 4.3</td>
<td>-3.5 ± 4.4</td>
<td>-12.7 ± 4.5</td>
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<tr>
<td>Blend</td>
<td>68.7 ± 2.8</td>
<td>77.0 ± 4.9</td>
<td>61.5 ± 3.0&lt;sup&gt;†&lt;/sup&gt;</td>
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<td>69.3 ± 3.2</td>
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<td>74.6 ± 9.7</td>
<td>60.4 ± 4.7&lt;sup&gt;†&lt;/sup&gt;</td>
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<td>67.5 ± 6.3</td>
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Table 4. Phenylalanine enrichments

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<th>1-2.5h</th>
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<td>Early</td>
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<td><strong>Femoral Artery</strong></td>
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<tr>
<td>Blend</td>
<td>7.92 ± 0.10</td>
<td>7.80 ± 0.19</td>
<td>8.02 ± 0.21</td>
<td>7.77 ± 0.16</td>
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<td>7.99 ± 0.23</td>
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<td>Whey</td>
<td>7.74 ± 0.15</td>
<td>8.30 ± 0.28*</td>
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<td>8.18 ± 0.26*</td>
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<td>Blend†</td>
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<td>6.62 ± 0.11</td>
<td>6.75 ± 0.18</td>
<td>6.50 ± 0.18</td>
<td>6.68 ± 0.12</td>
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<tr>
<td>Whey†</td>
<td>5.97 ± 0.20</td>
<td>7.00 ± 0.15a</td>
<td>7.09 ± 0.21</td>
<td>6.92 ± 0.16</td>
<td>7.14 ± 0.21#</td>
<td>7.06 ± 0.20</td>
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<tr>
<td>Blend†</td>
<td>4.50 ± 0.22</td>
<td>5.95 ± 0.14*</td>
<td>5.98 ± 0.07*</td>
<td>5.93 ± 0.11*</td>
<td>5.95 ± 0.06*</td>
<td>6.02 ± 0.11*</td>
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<tr>
<td>Whey†</td>
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<td>6.18 ± 0.18*</td>
<td>5.95 ± 0.28*</td>
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<tr>
<td><strong>Inward Transport</strong></td>
<td>Blend</td>
<td>83.0 ± 12.1</td>
<td>115.2 ± 17.5</td>
<td>178.4 ± 53.5*</td>
<td>124.1 ± 23.2</td>
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<td>Whey</td>
<td>82.1 ± 12.9</td>
<td>124.8 ± 14.2*</td>
<td>104.6 ± 18.4</td>
<td>117.8 ± 30.1</td>
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<tr>
<td><strong>Outward Transport</strong></td>
<td>Blend</td>
<td>92.3 ± 13.1</td>
<td>105.9 ± 15.5</td>
<td>187.0 ± 52.7*</td>
<td>132.2 ± 23.8</td>
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<tr>
<td></td>
<td>Whey</td>
<td>92.7 ± 14.0</td>
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<td>129.4 ± 29.3</td>
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A

Inward Transport

nmol min⁻¹ 100 mg leg⁻¹

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<th>Entire</th>
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<tbody>
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</table>

Post-Exercise Period

†

* p<0.05

B

Outward Transport

nmol min⁻¹ 100 mg leg⁻¹

<table>
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<tr>
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<th>Late</th>
<th>Entire</th>
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<tbody>
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<tr>
<td>Whey</td>
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</table>

Post-Exercise Period

†

* p<0.05
A

Fold Change from Rest

MuRF-1 mRNA

Time Period Post-Ingestion

Rest 2h 4h

Blend Whey

* *

B

Fold Change from Rest

MAFbx mRNA

Time Period Post-Ingestion

Rest 2h 4h

Blend Whey

* *