

Stimulation of muscle anabolism by resistance exercise and ingestion of leucine plus protein

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Abstract: Leucine is known to stimulate muscle protein synthesis and anabolism. However, evidence for the efficacy of additional leucine to enhance the response of muscle anabolism to resistance exercise and protein ingestion is unclear. Thus, we investigated the response of net muscle protein balance to ingestion of additional leucine with protein in association with resistance exercise. Two groups of untrained subjects performed an intense bout of leg resistance exercise following ingestion of 1 of 2 drinks: flavored water (PL); or 16.6 g of whey protein + 3.4 g of leucine (W+L). Arteriovenous amino acid balance across the leg was measured to assess the anabolic response of muscle in each group. Arterial amino acid concentrations increased in response to ingestion of W+L. Amino acid concentrations peaked between 60 and 120 min after ingestion, and then declined to baseline values. Valine concentration decreased to levels significantly lower than baseline. Net balance of leucine, threonine, and phenylalanine did not change following PL ingestion, but increased and remained elevated above baseline for 90–120 min following W+L ingestion. Leucine (138 ± 37 and -23 ± 23 mg), phenylalanine (58 ± 28 and -38 ± 14 mg), and threonine (138 ± 37 and -23 ± 23 mg) uptake was greater for W+L than for PL over the 5.5 h following drink ingestion. Our results indicate that the whey protein plus leucine in healthy young volunteers results in an anabolic response in muscle that is not greater than the previously reported response to whey protein alone.

Key words: arteriovenous balance, net muscle protein synthesis, muscle biopsies, branched-chain amino acids, area under the curve.

Résumé : Contexte. La leucine, on le sait, stimule la synthèse des protéines du muscle et l'anabolisme. Néanmoins, on ne connaît pas vraiment l'effet de l'addition de leucine à un apport de protéines sur l'amélioration de l'anabolisme musculaire en réponse à des exercices de force. But de l'étude. Le but de cette étude est d'analyser l'effet d'un supplément de leucine à un apport de protéines sur le bilan musculaire protéique net consécutif à la pratique d'exercices de force. Méthodologie expérimentale. Deux groupes de sujets non entraînés participent à une séance d'exercices de force des membres inférieurs après avoir consommé une des deux boissons suivantes : de l'eau aromatisée, PL ou 16,6 g de protéines lactosériques additionnées de 3,4 g de leucine, W + L. On mesure la différence artério-veineuse des concentrations d'acides aminés au niveau du membre inférieur pour évaluer la réponse anabolique des muscles dans chaque groupe de sujets. Résultats. La concentration artérielle d'acides aminés augmente après la consommation de W + L. La concentration atteint un sommet entre la 60^e et la 120^e minute suivant la consommation puis chute jusqu'à la valeur de repos. La concentration de valine baisse à un niveau significativement inférieur à la valeur de repos. Le bilan net de leucine, de thréonine et de phénylalanine ne varie pas après la consommation de PL, mais augmente et reste au-dessus des valeurs de repos durant les 90 à 120 min consécutives à la consommation de W + L. Dans les 5,5 h suivant la consommation des boissons, la captation de leucine (138 ± 37 et -23 ± 23 mg), de phénylalanine (58 ± 28 et -38 ± 14 mg) et de thréonine (138 ± 37 et -23 ± 23 mg) est plus importante après la consommation de W + L qu'après la consommation de PL, respectivement. Conclusion. D'après nos observations, l'addition de leucine à une boisson de protéines lactosériques ne suscite pas chez de jeunes volontaires en bonne santé une réponse anabolique supérieure à ce qui est rapporté concernant la consommation seule de protéines lactosériques.

Mots-clés : différence artério-veineuse, bilan musculaire protéique net, biopsies musculaires, acides aminés à chaîne ramifiée, surface sous la courbe.

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Introduction

The balance between muscle protein synthesis and breakdown is the metabolic basis for changes in muscle proteins, which ultimately result in adaptations to exercise training, including muscle hypertrophy. Gains in protein result only from positive net muscle protein balance over a given time period. Positive net muscle protein balance may result from an increase in muscle protein synthesis and (or) a decrease in muscle protein breakdown.

Exercise and nutrition profoundly influence net muscle protein balance. Resistance exercise increases muscle protein synthesis and breakdown, resulting in improved balance (Biolo et al. 1995*b*; Phillips et al. 1997). However, positive net muscle protein balance does not occur in the absence of ingestion of a source of amino acids (Biolo et al. 1995*b*; Phillips et al. 1997). There is an additive effect of amino acid ingestion, in the form of free amino acids, intact proteins or proteins in food, and resistance exercise (Borsheim et al. 2002; Elliot et al. 2006; Miller et al. 2003; Tipton et al. 1999, 2001, 2003, 2004, 2007; Wilkinson et al. 2007). Hyperaminoacidemia and resistance exercise stimulate muscle protein synthesis, resulting in positive net muscle protein balance (Borsheim et al. 2002; Elliot et al. 2006; Miller et al. 2003; Tipton et al. 1999, 2001, 2003, 2004, 2007; Wilkinson et al. 2007). The bulk of the response of positive net muscle protein balance to exercise and amino acids stems from increased muscle protein synthesis, rather than decreased breakdown (Biolo et al. 1997; Borsheim et al. 2002; Tipton et al. 1999).

It is clear that ingestion of essential amino acids is sufficient to stimulate muscle protein synthesis and positive net muscle protein balance following exercise (Borsheim et al. 2002; Tipton et al. 1999, 2001). Protein synthesis results not only from provision of substrate for synthetic processes, but also from stimulation of signaling pathways in muscle cells. The initiation of messenger (m)RNA translation appears to be a key regulatory process. Of the essential amino acids, leucine has been identified as the most potent for stimulation of intracellular signaling in rats (Anthony et al. 1999, 2001; Kimball and Jefferson 2001). Thus, leucine is thought to be critical for stimulation of positive net muscle protein balance for muscle hypertrophy.

Recent studies have focused on the role of leucine for stimulation of muscle anabolism in humans. In elderly humans, excess leucine combined with protein has been shown to further stimulate muscle protein synthesis and positive net muscle protein balance (Katsanos et al. 2006; Rieu et al. 2006). Following exercise in the young and elderly, however, the evidence for efficacy of additional leucine is less clear (Koopman et al. 2005, 2008). Whereas Koopman et al. (2005) demonstrated that muscle protein synthesis was greater following the addition of free leucine coingested with protein and carbohydrate than following carbohydrate alone, it was not greater than protein plus carbohydrate. Similarly, muscle protein synthesis was not greater when leucine was added to carbohydrate plus protein in elderly men following exercise (Koopman et al. 2008). In these studies, ingestion of these nutrients was in small doses every 30 min after exercise for 6 h. Whole body protein balance was enhanced by the addition of leucine (Koopman et al.

2005, 2008), but there was no measurement of net protein balance in muscle. The response of muscle protein synthesis to prolonged, modest changes in amino acid concentrations, as occurs after ingestion of intact protein or during continuous infusion of amino acids (Biolo et al. 1997; Bohe et al. 2001), is quite different than the response to a rapid increase in the concentrations of amino acids, as result from a bolus ingestion (Borsheim et al. 2002; Tipton et al. 2004, 2007). Following bolus ingestion, amino acid concentrations and net balance rise quickly, then decline to basal levels (Borsheim et al. 2002; Tipton et al. 2004, 2007).

Previously, we demonstrated that the anabolic response was greater when free amino acids were ingested prior to, rather than following, exercise (Tipton et al. 2001). However, the response was similar when whey proteins were ingested before and after exercise (Tipton et al. 2007). Thus, it seems that the response of muscle anabolism may differ if amino acids are provided as intact proteins or as free amino acids. It is possible that stimulation of signaling pathways by the high leucine content of the essential amino acids prior to exercise is a key aspect of the difference in results between these studies (Tipton et al. 2001, 2007). Thus, ingestion of leucine combined with protein before exercise may be optimal for stimulation of positive net muscle protein balance.

The aim of this study was to determine if increased leucine would stimulate postexercise net muscle protein balance in healthy young volunteers when ingested with whey protein prior to exercise. We hypothesized that ingestion of a bolus of whey protein with additional leucine before resistance exercise would maximally stimulate positive net muscle protein balance during recovery. Furthermore, this response was compared with the response of muscle to whey proteins alone in a previous study with an identical protocol (Tipton et al. 2007).

Materials and methods

Subjects

Subjects were healthy young males and females who had not participated in regular resistance training for at least 5 years prior to participation in this study. Subjects were randomly assigned to 1 of 2 groups, each receiving a 300 mL bolus of solution immediately before a heavy leg resistance exercise bout. Subjects ingested either water plus artificial sweetener and flavoring (PL) or water plus 16.6 g of whey proteins and 3.4 g of leucine (W+L). Subject characteristics are summarized in Table 1.

Pretesting

Medical screening

The study design, purpose, and possible risks were explained to the subjects, and written consent was obtained. The Institutional Review Board and the General Clinical Research Center (GCRC) of The University of Texas Medical Branch at Galveston approved the study. Prior to participation in the experiments, each subject had a complete series of medical screening tests for the purpose of disclosing any pre-existing medical or physical conditions that would preclude participation in the study.

Table 1. Characteristics of subjects in 2 groups consuming 1 of 2 drinks: placebo (PL) or whey protein plus additional leucine (W+L).

Characteristics	PL	W+L
<i>n</i> (males–females)	8 (5/3)	7 (6/1)
Age (y)	29.8±2.8	25.3±1.6
Mass (kg)	79.2±3.9	91.0±6.1
Height (m)	1.71±0.03	1.80±0.03
BMI (kg·m ⁻²)	26.9±1.0	28.1±1.6
1RM (kg)	91.9±10.4	133.9±4.1
Leg volume (L)	9.7±0.3	11.2±0.7

Note: Values are means ± SE. BMI, body mass index; 1RM, 1 repetition maximum for leg extension exercise.

Exercise test

At least 5 days prior to the research protocol, a 1-repetition maximum (1RM) for leg extension exercise was determined for each subject. The 1RM was defined as the maximum weight that could be lifted and held for a 1-s count.

Experimental protocol

General

This experimental protocol was designed to quantify the response of net muscle protein balance, as represented by amino acid balance across the leg in 2 groups of subjects following ingestion of a bolus of W+L or PL. For each group, sampling began prior to bolus ingestion and continued for 335 min following ingestion of the bolus. Thus, amino acid uptake is quantified for ~5.5 h from the drink ingestion for each group.

Study protocol

Subjects were admitted to the GCRC the night before each study, given a standard dinner and then allowed only water until the start of the study the following morning. The general protocol for each group is identical to that of previous studies (Elliot et al. 2006; Tipton et al. 2004, 2007). At approximately 0545 hours on each study day, an 18-gauge polyethylene catheter was inserted into a vein on the forearm for blood sampling. Additionally, a 3 Fr, 8 cm polyethylene catheter (Cook, Inc., Bloomington, Ind.) was inserted into the femoral vein and femoral artery under local anesthesia. Both femoral catheters were used for blood sampling, while the femoral arterial catheter was also used for indocyanine green (ICG) infusion for determination of leg blood flow. Systemic concentration of ICG was measured from a peripheral vein. Patency of all catheters was maintained by saline infusion. At ~0600 hours (time = -120 min), background blood samples for insulin, amino acid, and ICG concentration were taken.

Muscle biopsies from the vastus lateralis were taken to measure intracellular amino acid concentrations. The muscle biopsies were taken immediately before drink ingestion and at 90 and 300 min following drink ingestion. Each biopsy was taken, under local anesthetic, from the lateral portion of the vastus lateralis, approximately 10–15 cm above the knee. A 5-mm Bergstrom biopsy needle was used to sample ~50 mg of mixed muscle tissue. The sample was quickly rinsed, blotted, divided into 2 or 3 pieces, frozen in liquid

nitrogen, and stored at -80 °C for future processing for intracellular amino acid concentration analysis. Biopsies were taken at least 1 cm apart in an attempt to minimize the impact of local inflammation from previous biopsy samples. The second biopsy was taken proximal to the first, and the third was taken distal to the first.

Blood flow was measured in periods chosen to best characterize the leg blood flow during and following exercise and during protein ingestion (Elliot et al. 2006; Tipton et al. 2004, 2007). A continuous ICG infusion rate (0.5 mg·min⁻¹) was initiated 10 min prior to each blood flow measurement period. If it was necessary to interrupt the ICG infusion for arterial sampling, ICG was allowed to flow uninterrupted for at least 5–6 min prior to subsequent sampling for blood flow. Leg volume was estimated using an anthropometric approach (Biolo et al. 1995a).

Three arteriovenous samples were taken immediately after cessation of the ICG infusion, and 15, 10, and 5 min prior to drink ingestion (beginning of exercise bout), for the predrink and pre-exercise baseline, 20 min after drink ingestion (20 min into the resistance exercise bout; after set 6), 35 min following drink ingestion at the end of exercise, and 50, 65, 82, 85, 88, 105, 115, 125, 140, 155, 185, 215, 245, 275, and 305 min after drink ingestion (beginning of exercise bout) to determine the amino acid concentrations for determination of net muscle protein balance. These samples correspond to -40, -15, 0, 15, 30, 47, 50, 53, 70, 80, 90, 105, 120, 150, 180, 210, 240, 270, and 300 min following exercise. Arterial blood samples for insulin analysis also were collected periodically throughout the protocol (i.e., predrink, and 20, 50, 65, 88, 125, 140, 155, 185, 215, 275, and 335 min following drink ingestion). Blood samples were analyzed for phenylalanine, leucine, and threonine concentrations, using the internal standard method and gas chromatography-mass spectrometry (GCMS) analysis, as in previous studies (Biolo et al. 1997; Elliot et al. 2006; Tipton et al. 2004, 2007).

For each trial, the exercise routine was initiated immediately after drink ingestion and lasted ~35 min. It consisted of 10 sets of 8 repetitions of leg extensions at 80% of 1RM. Each set was completed in approximately 30–45 s, with a 2 min rest between sets. If a subject could not complete the required repetitions for any given set, the resistance was lowered, so that all repetitions were completed. This routine has been used previously to increase blood flow and muscle protein metabolism (Elliot et al. 2006; Tipton et al. 2003, 2004, 2007).

Analysis of samples

Blood

Concentrations of free amino acids (phenylalanine, leucine, and threonine) in arterial and venous whole blood were determined by GCMS (Hewlett Packard 5973, Palo Alto, Calif.), using an internal standard solution, as described elsewhere (Biolo et al. 1997; Elliot et al. 2006; Tipton et al. 2004). Since the tube weight and the amount of blood was known, the blood amino acid concentration could 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 spectrophotometrically.

metrically by measuring the ICG concentration in serum from the femoral vein and the peripheral vein, as described elsewhere (Biolo et al. 1997; Elliot et al. 2006; Tipton et al. 2004). Leg plasma flow was calculated from steady-state values of dye concentration and converted to whole blood flow using the hematocrit (Biolo et al. 1997; Elliot et al. 2006; Tipton et al. 2004). Plasma samples were also analyzed for amino acid concentrations by high-performance liquid chromatography (Waters Alliance HPLC System 2690, Milford, Mass.), as described elsewhere (Borsheim et al. 2008). Serum insulin levels were determined by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, Calif.). Intra-assay coefficient of variation was <10%.

Muscle

Muscle biopsy tissue samples were analyzed for free intracellular amino acid concentrations, as described elsewhere (Biolo et al. 1997; Elliot et al. 2006; Tipton et al. 2004, 2007). Upon thawing, ~20–25 mg of tissue was weighed and protein precipitated with 0.5 mL of 10% perchloroacetic acid. Tissue was then homogenized and centrifuged, and the supernatant was collected. This procedure was repeated 2 more times, and the pooled supernatant (~1.3 mL) was processed, as were the blood samples (described in the section on blood, above). Muscle free amino acid concentration was measured with the internal standard method, with corrections for the contribution of extracellular fluid, as described in the section on blood, above, and in previous studies (Biolo et al. 1997; Elliot et al. 2006; Tipton et al. 2004).

Calculations

Net muscle protein balance

Net muscle protein balance (NB) was calculated as

$$NB = (Ca - Cv) \times BF$$

where Ca is the arterial amino acid concentration in whole blood, Cv is the venous amino acid concentration in whole blood, and BF is the leg blood flow.

Amino acid exchange across the leg

Total amino acid exchange (mg) was calculated for phenylalanine, leucine, and threonine from the area under the curve (AUC) of the net balance over the time following ingestion of the drink, using GraphPad Prism software (GraphPad Software; LaJolla, Calif.) (Elliot et al. 2006; Tipton et al. 2003, 2004, 2007). Positive values represent net uptake (anabolism) and negative values represent net release (catabolism).

AUC was calculated for 335 min from the drink ingestion for all subjects, using GraphPad Prism software. The baseline was the predrink resting sample, and AUC was calculated for the entire sampling time (i.e., during exercise plus recovery from exercise). This approach has been used previously to determine net amino acid uptake during recovery from exercise with various nutritional interventions (Elliot et al. 2006; Tipton et al. 2001, 2003, 2004, 2007).

Insulin and arterial amino acid concentrations

AUC was calculated for the insulin values, with the baseline value from the sample immediately prior to drink ingestion.

Calculation of AUC for insulin was stopped when the insulin value returned to baseline (~120 min following exercise).

AUC for arterial plasma concentrations from high-performance liquid chromatography analysis for each of the 6 selected amino acids were calculated for all subjects. The baseline was the predrink resting sample, and AUC was calculated for the entire sampling time (i.e., during exercise plus recovery from exercise).

Data presentation and statistical analysis

Data are presented as means \pm standard error. Amino acid arterial concentrations and net balances are presented across time. Amino acid exchange (i.e., AUC for net balance across the leg) is presented for each group. Muscle intracellular amino acid concentrations are presented for each biopsy sample for each group.

For the primary endpoint, AUC of net amino acid balance, as well as insulin AUC and the AUC of the arterial concentration for the 6 essential amino acids, were compared with 1-way analysis of variance (ANOVA). Significance was set at $p < 0.05$. When significance was indicated by the ANOVA, differences between group values were discerned using Tukey's post hoc test.

For the secondary endpoints, muscle intracellular concentration, arterial amino acid concentration, blood flow, and net balance over time were analyzed with 2-way repeated-measures ANOVA, with time as the within factor and group (PL or W+L) as the between factor. Given a significant group \times time interaction, Bonferroni's post hoc test was used to delineate differences between groups at individual time points. A 1-way ANOVA and Dunnett's post hoc procedure were run on each group to determine differences between each time point within a group. For muscle intracellular concentrations, whenever ANOVA revealed a significant difference, Tukey's post hoc procedure was used to locate the pair-wise differences.

Results

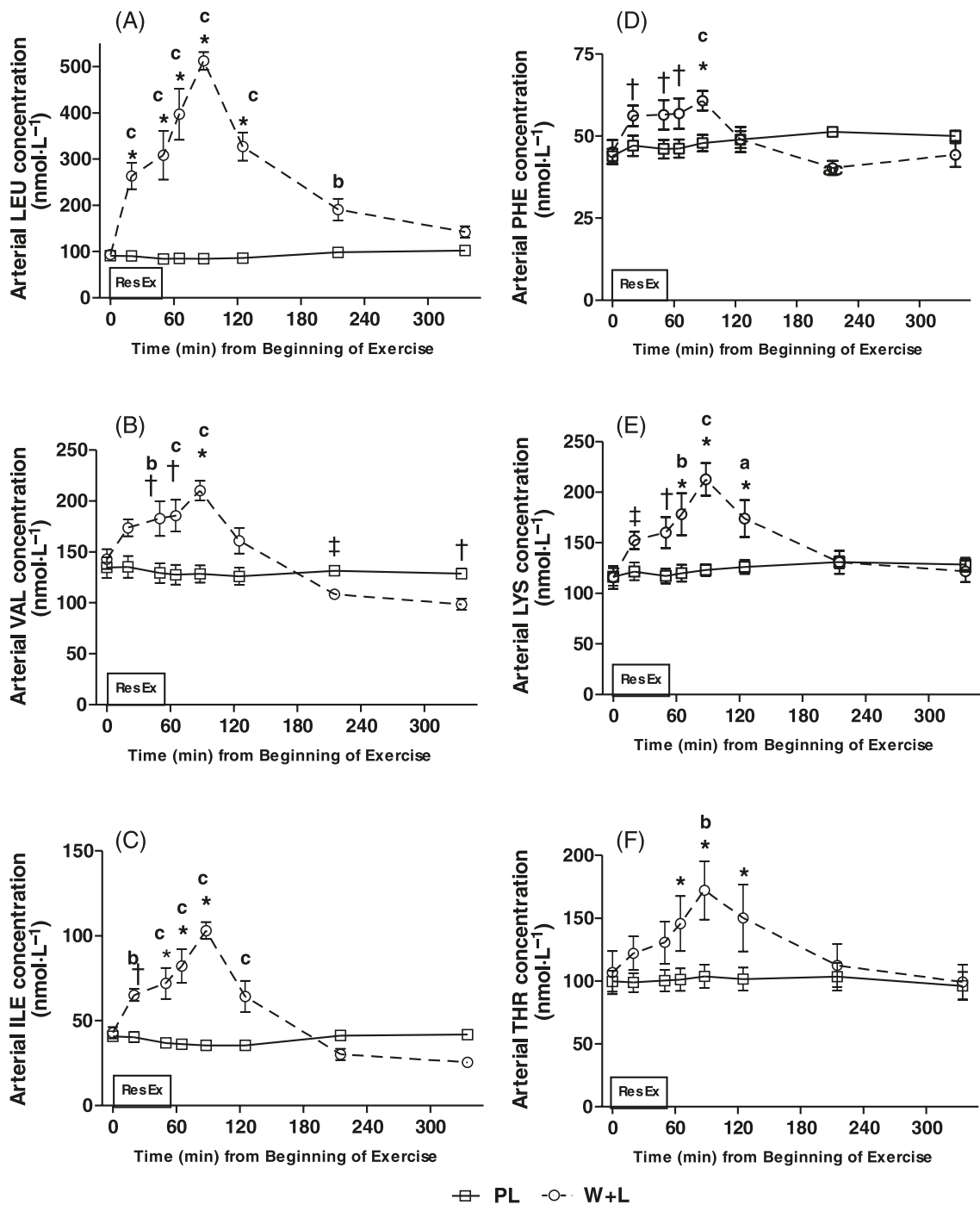
Serum insulin AUC and leg blood flow

W+L ($1944 \pm 386 \mu\text{U}\cdot\text{mL}^{-1} \times 120 \text{ min}$) AUC for insulin was significantly greater ($p = 0.05$) than PL AUC ($179 \pm 386 \mu\text{U}\cdot\text{mL}^{-1} \times 120 \text{ min}$). There were no significant differences in leg blood flow between PL and W+L over the entire study period (data not shown).

Blood amino acid concentrations

Arterial concentrations of 6 essential amino acids for both groups are illustrated in Fig. 1. Amino acid concentrations did not change following PL ingestion, but increased immediately in response to ingestion of W+L. Arterial amino acid concentrations were significantly ($p < 0.05$) increased over baseline, up to 125 min after beginning exercise for W+L. Amino acid concentrations peaked 88 min after beginning exercise. For W+L, mean values of valine, isoleucine, phenylalanine, lysine, and threonine returned to baseline level or below by 125 min from drink ingestion. Valine concentrations were significantly less than baseline at 215 and 335 min following drink ingestion. Leucine concentrations

Fig. 1. Arterial amino acid concentrations during and following exercise with ingestion of placebo (PL) or 16.6 g of whey proteins plus 3.4 g of leucine (W+L). (A) arterial leucine concentration; (B) arterial isoleucine concentrations; (C) arterial valine concentrations; (D) arterial phenylalanine concentrations; (E) arterial lysine concentrations; (F) arterial threonine concentrations. *, W+L significantly different from baseline, $p < 0.001$; †, W+L significantly different from baseline, $p < 0.01$; ‡, W+L significantly different from baseline, $p < 0.05$. a, W+L significantly different from PL, $p < 0.05$; b, W+L significantly different from PL, $p < 0.01$; c, W+L significantly different from PL, $p < 0.001$. ResEx, resistance exercise bout.



for W+L remained significantly above those for PL for 215 min following drink ingestion.

AUC of leucine ($p < 0.001$), isoleucine, threonine, and lysine ($p < 0.05$) was greater for W+L than for PL (Table 2).

Net amino acid balance

Net amino acid balances over time are summarized in

Fig. 2. For PL, amino acid balances remained unchanged throughout the protocol. During W+L, phenylalanine balance seemed to be biphasic following drink ingestion. During exercise, phenylalanine balance increased immediately after drink ingestion, but declined back to baseline levels at the end of exercise. Phenylalanine balance peaked a second time at 65 min following drink ingestion (30 min after ces-

Table 2. Area under the curve of arterial amino acid concentrations ($\text{nmol} \cdot \text{mL}^{-1} \times 5.5 \text{ h}$) for PL and W+L groups.

Amino acid	PL	W+L
Leucine	663 \pm 1203	55530 \pm 4346*
Valine	-1681 \pm 1682	-638 \pm 1687
Isoleucine	-566 \pm 604	2790 \pm 979†
Phenylalanine	1762 \pm 564	912 \pm 688
Lysine	3297 \pm 2216	11666 \pm 1657†
Threonine	460 \pm 1920	6678 \pm 767†

Note: Values are means \pm SE.

*Significantly different from PL, $p < 0.0001$.

†Significantly different from PL, $p < 0.01$.

sation of exercise), returned to baseline levels at ~ 100 min following ingestion, and remained at baseline levels throughout the remainder of the sampling period for W+L. W+L phenylalanine balance was greater than that for PL ($p = 0.001$).

Net leucine balance increased immediately after ingestion and remained greater than baseline for 90 min during W+L. W+L leucine balance was greater than that for PL for the first 90 min following ingestion.

Like phenylalanine balance, there was a biphasic pattern to threonine balance during W+L. Threonine balance increased immediately following drink ingestion, declined to baseline levels at the end of exercise, only to increase again after exercise ended. Threonine balance remained elevated over baseline levels until 90 min following drink ingestion for W+L. W+L threonine balance was greater than that for PL at 20 and 45–90 min following drink ingestion.

Amino acid exchange (AUC)

Exchange during PL was numerically negative for all 3 amino acids, but none were significantly different from 0 (Fig. 3). Exchange of all 3 amino acids was greater than PL for W+L.

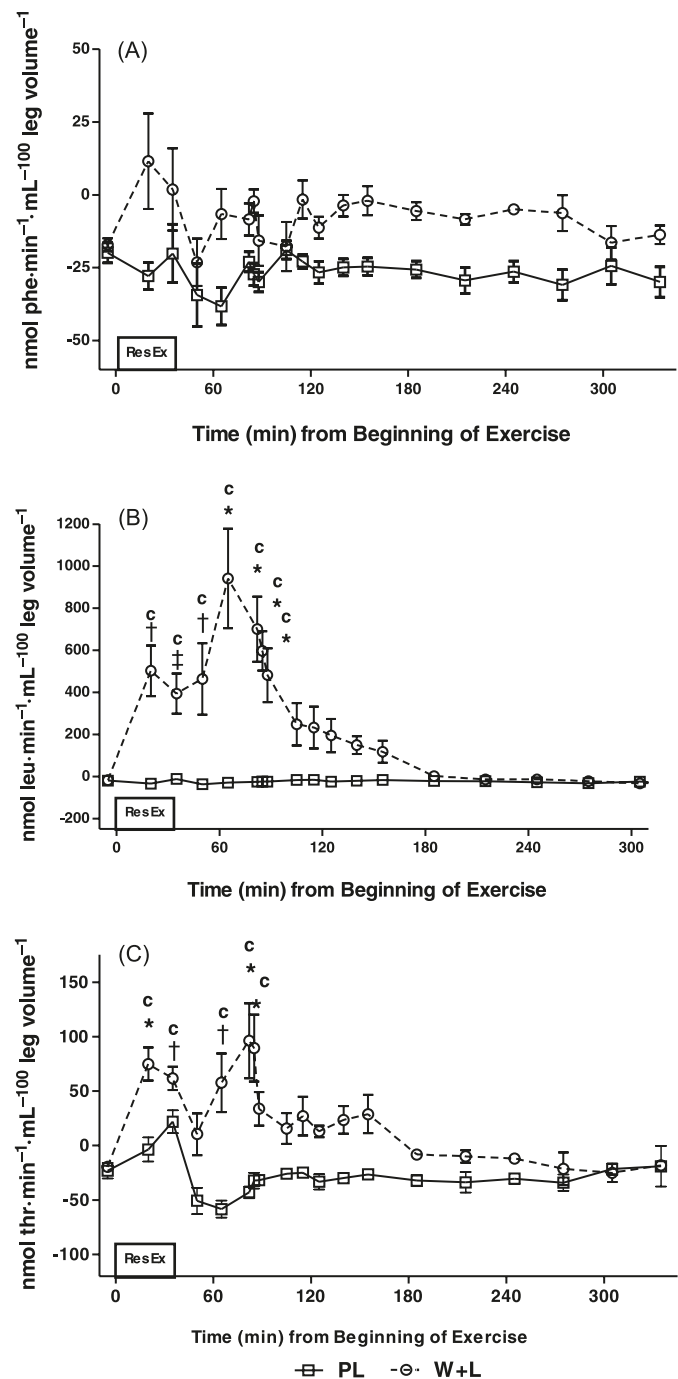
Muscle intracellular amino acid concentrations

There were no statistically significant changes in phenylalanine or threonine muscle intracellular concentrations at any of the sampled time points (Fig. 4). Muscle leucine concentration was greater than at baseline for W+L at 90 min following drink ingestion, but declined back to baseline levels by 300 min.

Discussion

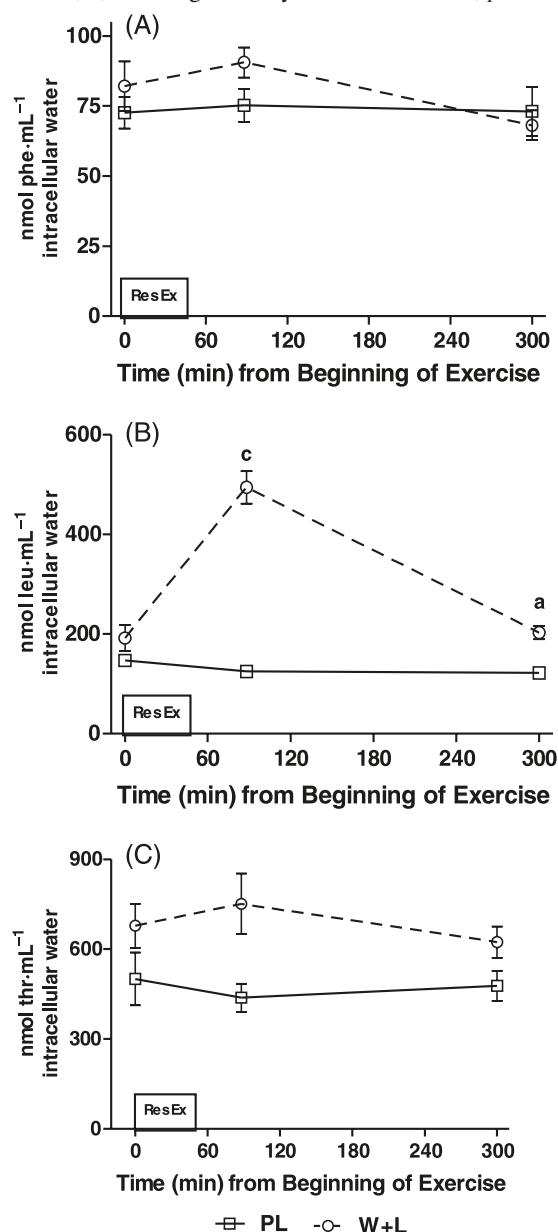
This study was designed to determine if the anabolic response of muscle to whey protein ingestion would be enhanced by increasing the proportion of leucine. This study is the first to investigate the response of net muscle protein balance following resistance exercise to protein plus leucine ingestion in humans. We found that positive net muscle protein balance, as determined by amino acid uptake across the leg, was stimulated by ingestion of a mixture of whey protein with a higher proportion of leucine immediately prior to resistance exercise. However, the anabolic response to ingestion of 16.6 g of whey protein plus 3.4 g of leucine in this study was not superior to the response to 20 g of whey

Fig. 2. Net amino acid balance across the legs of volunteers during and following exercise with ingestion of placebo (PL) or 16.6 g of whey proteins plus 3.4 g of leucine (W+L). *, W+L significantly different from baseline, $p < 0.001$; †, W+L significantly different from baseline, $p < 0.01$; ‡, W+L significantly different from baseline, $p < 0.05$; c, W+L significantly different from PL, $p < 0.001$. phe, phenylalanine; leu, leucine; ResEx, resistance exercise bout; thr, threonine.



proteins reported in a previous study (Tipton et al. 2007). Thus, the overall anabolic response was not improved by increasing the amount of leucine ingested with whey proteins, at least when the drinks were isonitrogenous and isoenergetic.

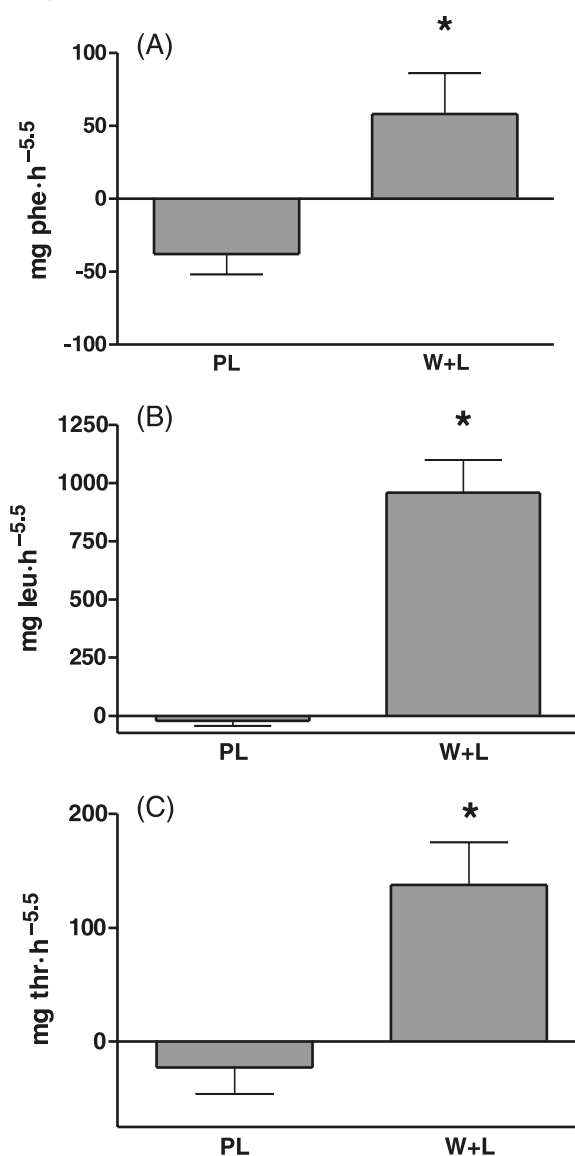
Fig. 3. Muscle intracellular amino acid concentrations immediately prior to drink ingestion, ~90 min following drink ingestion (~55 min following exercise) and 300 min following drink ingestion. Drinks were placebo (PL) 20 g or 16.6 g of whey proteins plus 3.4 g of leucine (W+L). a, W+L significantly different from PL, $p < 0.05$; c, W+L significantly different from PL, $p < 0.001$.



It is clear that increased amino acid availability from ingestion of amino acids and protein results in increased muscle protein synthesis and positive net muscle protein balance following exercise (Borsheim et al. 2002; Tipton et al. 1999, 2004; Tipton and Sharp 2005). Leucine has long been recognized as an important anabolic agent in muscle (for reviews, see Matthews 2005; Rennie et al. 2006). Early studies in resting humans associated leucine with amelioration of muscle protein breakdown (Louard et al. 1990; Nair et al. 1992b). However, it is not clear that adding leucine to protein will enhance the anabolic response to exercise.

More recently, several studies in rats demonstrated that

Fig. 4. Exchange of amino acids across the leg (area under the curve for net balance) for 335 min following ingestion either placebo (PL) or 16.6 g of whey proteins plus 3.4 g of leucine (W+L) immediately prior to resistance exercise. *, Significantly different from PL.



leucine administration increased muscle protein synthesis at rest and following exercise mediated through stimulation of translation initiation (Anthony et al. 1999, 2000a; Bolster et al. 2004b). These studies have provided a rationale for claims that leucine supplementation is critical for exercise-induced muscle hypertrophy. The applicability to our results is, however, questionable.

Aside from potential species differences, the rats in those studies performed strenuous treadmill running (Anthony et al. 1999, 2000b; Gautsch et al. 1998), rather than resistance exercise. As opposed to resistance exercise in rats (Hernandez et al. 2000; Kubica et al. 2005) and humans (Biolo et al. 1995b; Phillips et al. 1997), muscle protein synthesis is inhibited by the exercise model used by Anthony and colleagues (Anthony et al. 1999, 2000b; Gautsch et al. 1998). Therefore, the impact of leucine was to restore muscle pro-

tein synthesis to basal levels. Since resistance exercise results in an anabolic response in humans (i.e., an increase in muscle protein synthesis), resulting in an improvement in net muscle protein balance (Biolo et al. 1995*b*; Phillips et al. 1997), it is likely that the impact of leucine is quite different during recovery from exercise, particularly resistance exercise, in humans. However, very few studies have investigated the response of muscle metabolism to resistance exercise with ingestion of leucine or coingestion of leucine and protein in humans.

The influence of leucine on muscle protein anabolism following exercise in humans has only recently begun to be investigated. It has long been recognized that essential amino acid ingestion following resistance exercise increases muscle protein synthesis and results in positive net muscle protein balance (Borsheim et al. 2002; Rasmussen et al. 2000; Tipton et al. 1999, 2001). The influence of essential amino acids is attributed to provision of amino acids as a substrate for protein synthesis, as well as stimulation of intracellular signaling of translation initiation by the leucine (Tipton and Sharp 2005). Dreyer et al. (2008) recently reported that ingestion of essential amino acids in free form plus additional leucine increased intracellular signaling and muscle protein synthesis, compared with exercise alone. However, no comparison was made to amino acids without, or with less, leucine. Thus, the influence of leucine per se could not be determined (Dreyer et al. 2008).

Why protein ingestion before or after resistance exercise stimulates positive net muscle protein balance in young volunteers (Tipton et al. 2004, 2007). Compared with a previous study, using an identical study protocol (Tipton et al. 2007), our results suggest that additional leucine does not stimulate net muscle protein balance over that of whey protein ingested with resistance exercise. The uptakes of threonine and phenylalanine in our study when whey protein plus leucine was ingested (138 ± 37 and 58 ± 28 , respectively) and in a previous study (Tipton et al. 2007) when whey protein alone was ingested were nearly identical (140 ± 28 and 62 ± 22 , respectively). Taken together, these data suggest that the addition of leucine to whey protein does not enhance the anabolic response of muscle following resistance exercise.

Using a similar design to our study, Koopman et al. (2005) recently examined the response of whole body and muscle protein metabolism to resistance exercise followed by ingestion of carbohydrate, carbohydrate and protein, and carbohydrate and protein plus additional leucine. Coingestion of protein with carbohydrate resulted in a ~34%, non-statistically significant, increase in muscle protein synthesis over carbohydrate alone. Addition of leucine to the protein and carbohydrate elevated muscle protein synthesis significantly, by ~58%, over carbohydrate alone, but there was no significant increase over carbohydrate plus protein without leucine (Koopman et al. 2005). These data may be used to suggest that ingestion of large amounts of additional leucine may further increase muscle protein synthesis over protein and carbohydrate ingestion (Manninen 2006), but the increase does not seem to be particularly dramatic — especially considering the large dose of leucine involved — given that the elevation is not statistically greater than when protein is ingested. Similarly, in elderly men, coingestion of

leucine with protein and carbohydrate did not further elevate muscle protein synthesis following light exercise, compared with carbohydrate plus protein without leucine (Koopman et al. 2008). It may be that the response of elderly muscle to leucine is different from that of muscle in young adults (Katsanos et al. 2006), or that the intensity of the exercise was not sufficient to engender a response similar to the previous study (Koopman et al. 2005).

No measurement of muscle protein breakdown or muscle protein balance was made in these studies (Koopman et al. 2005, 2006), so a direct comparison to our results cannot be made. Nonetheless, since it is likely that the response of net muscle protein balance is primarily a result of increased synthesis (Biolo et al. 1997; Borsheim et al. 2002; Tipton et al. 1999), it is possible to assume that the difference in muscle protein synthesis represents differences in positive net muscle protein balance; these results (Koopman et al. 2005) may be considered contradictory to our data. Certainly, the response of whole body protein balance suggests that addition of leucine increased the overall anabolic response (Koopman et al. 2005). There are methodological differences between studies, which may account for any differences between results. Whereas our subjects ingested a bolus of protein with additional leucine, Koopman et al. (2005) administered their supplements in small doses throughout the study period, resulting in not only a different pattern of administration, but also a much larger amount of leucine ingested. Their subjects ingested ~9 g of leucine hourly during the postexercise period (Koopman et al. 2005). Thus, the total amount of leucine ingested was ~16 times that ingested by our subjects. Perhaps more importantly, the protein was ingested constantly throughout the protocol in their study, thus, amino acid availability remained elevated for 5–6 h (Koopman et al. 2005); however, it had returned to baseline by 2 h following exercise in our study. Finally, our subjects did not receive carbohydrate in addition to protein and leucine, we administered the bolus immediately prior to exercise, as opposed to after the exercise, and the exercise bouts were different. It is not clear which, if any, of these methodological differences between studies accounted for any differences in the anabolic response. However, the ingestion of a single bolus of a supplement is more likely than frequent small boluses to be practiced by most exercisers.

Others have used more indirect methods to assess the importance of leucine for the anabolic response to exercise. Karlsson and colleagues (2004) examined the response of translation initiation signaling factors, specifically aspects of the mTOR signaling pathway, to ingestion of branched-chain amino acids following exercise. Ingestion of branched-chain amino acids, including a large amount of leucine (~3.3 g), dramatically increased site-specific phosphorylation (i.e., activation of p70s6 kinase in muscle following resistance exercise, compared with exercise alone) (Karlsson et al. 2004). Presumably, this difference would result in differences in muscle protein synthesis (Bolster et al. 2004*a*; Hernandez et al. 2000) and, thus, increased net muscle protein balance. However, no measurement of synthesis was made in that study (Karlsson et al. 2004), and increased phosphorylation of p70s6 kinase does not necessarily correspond to increased protein synthesis in re-

sponse to leucine in skeletal muscle (Escobar et al. 2005, 2006, 2007). Since we did not measure activation of signaling pathways in our study, our results offer no evidence that translation initiation signaling or muscle protein synthesis was increased further by the extra leucine.

However, the lack of an enhanced anabolic response to leucine, despite any potential increase in translation signaling in our study, may be due to the impact of high levels of leucine on blood amino acid levels. Increased levels of leucine decrease availability of other amino acids in pigs, rats, and humans (Escobar et al. 2005, 2006, 2007; Hagenfeldt and Wahren 1980; Nair et al. 1992a). The extra leucine particularly impacts the other branched-chain amino acids, likely due to increased activation of branched-chain keto acid dehydrogenase activity and, thus, increased oxidation (Block et al. 1985). In our study, compared with the results of a previous study (Tipton et al. 2007), arterial concentrations of the essential amino acids, including isoleucine and valine, were lower when additional leucine was ingested with whey protein (Fig. 1). Similar results are reported by Koopman et al. (Koopman et al. 2005, 2008). Since changes in arterial amino acid concentrations regulate muscle protein synthesis (Bohe et al. 2003; Kobayashi et al. 2003; Rennie et al. 2002; Tipton et al. 1999; Wolfe and Miller 1999; Wolfe 2000) and any increase in signaling would result in a need for increased substrate for protein synthesis, the drop may offset any further increase in the anabolic response due to stimulation of translation initiation signaling by the leucine. Since the subjects in W+L group ingested ~17% less protein than the subjects in a previous study (Tipton et al. 2007), it might be expected that amino acid levels would be lower. However, the differences in amino acid levels between W+L and previous results seem to be greater than can be accounted for by the difference in protein ingestion. In fact, when directly compared, the AUC of threonine, isoleucine, and valine were actually lower when leucine was ingested than when whey protein alone was ingested (Tipton et al. 2007), and were no different from placebo (Table 2). These data provide evidence that decreased availability of essential amino acids may have limited the increase in muscle protein synthesis due to stimulation of signaling pathways by the additional leucine.

Alternatively, it may be that the decrease in amino acid levels with additional leucine ingestion is due to increased muscle protein synthesis and, therefore, increased amino acid uptake. However, our data do not support this notion. Whereas we did not directly measure muscle protein synthesis, uptake of phenylalanine and threonine by the leg was virtually identical for the leucine group in this study and the group that ingested only whey protein in a previous study (Tipton et al. 2007). Therefore, increased synthesis and, thus, uptake is unlikely to explain the decreased amino acid concentrations.

It is interesting to note that leucine supplementation seems to be more effective when the muscle is catabolic. Intracellular signaling in response to feeding is impaired in elderly muscle, resulting in a reduced response of muscle protein synthesis (Cuthbertson et al. 2005; Katsanos et al. 2006). However, high amounts of leucine seemingly overcome this deficiency (Cuthbertson et al. 2005; Katsanos et al. 2006; Rieu et al. 2006). Results from studies in old rat

muscle are consistent with this interpretation (Dardevet et al. 2000, 2002; Rieu et al. 2003). Similarly, in the rat models used to examine the impact of leucine, signaling deficits occur following exercise that are ameliorated by leucine supplementation, leading to a restoration of muscle protein synthesis rates (Anthony et al. 1999; Gautsch et al. 1998). However, in young muscle, or following exercise in the young or elderly, when muscle anabolism is stimulated, the efficacy of the leucine is absent (Koopman et al. 2007), or at least is less clear (Koopman et al. 2005). Our data seem to support this contention. Interestingly, this concept has yet to be systematically investigated. No study has examined the impact of leucine alone, or even as part of branched-chain amino acids, on muscle protein synthesis or net balance following resistance exercise in young or elderly volunteers.

In conclusion, our results demonstrate that ingestion of whey protein plus extra leucine stimulates positive net muscle protein balance to a similar level, compared with whey protein alone (Tipton et al. 2007). Thus, these data demonstrate that increasing the amount of leucine ingested with whey protein prior to exercise does not increase the response of positive net muscle protein balance, at least in an isonitrogenous, isoenergetic manner. It is likely that the anabolic pathways are fully stimulated by the amino acid content of the whey protein and (or) the exercise stimulus, and that additional leucine decreases amino acid availability; thus, additional leucine offers no further advantage in these young healthy volunteers. The response may be different in catabolic situations, and this notion should be investigated in future studies.

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