LEUCINE AND CITRULLINE IN HUMAN MUSCLE PROTEIN SYNTHESIS

NUTRITIONAL AND CONTRACTILE REGULATION OF HUMAN MUSCLE PROTEIN SYNTHESIS: ROLE OF LEUCINE AND CITRULLINE

By

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ABSTRACT

Amino acids are key nutritional stimuli that are both substrate for muscle protein synthesis (MPS), and signaling molecules that regulate the translational machinery. There is a dose-dependent relationship between protein intake and MPS that differs between young and elderly subjects. The current thesis contains results from three separate studies that were conducted to examine to potential to enhance smaller doses of protein, known to be suboptimal in their capacity to stimulate MPS, through supplementation with specific amino acids, namely leucine and citrulline.

The first two studies examined the potential to enhance the muscle protein synthetic capacity of a smaller, suboptimal dose of whey protein with leucine. In study one, we concluded that leucine supplementation of a suboptimal dose of protein could render it as effective at enhancing rates of MPS as ~four times as much protein (25 g) under resting conditions, but not following resistance exercise. In study two, we examined the potential of leucine and branched-chain amino acids to enhance the MPS response of a suboptimal dose of protein within the context of mixed macronutrient ingestion. We concluded that supplementation with a relatively high dose of leucine could render it as effective at enhancing MPS rates as ~four times as much protein (25 g) under both resting and post-exercise conditions.

In study three, we examined the potential of citrulline supplementation to enhance blood flow, microvascular circulation, and MPS in response to a suboptimal dose of whey protein in elderly subjects. We concluded that supplementation of a suboptimal dose of protein with citrulline did not augment bulk blood flow or muscle microvascular

circulation. The major findings from the works presented in this thesis is that smaller doses of protein that normally elicit a suboptimal increase in MPS can be made more anabolic when supplemented with specific amino acids.

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FORMAT AND ORGANIZATION OF THIS THESIS

This thesis was prepared in the "sandwich" thesis format as outlined in the McMaster University School of Graduate Studies Thesis Preparation Guide. This thesis is comprised of a general introduction, three original research papers (Chapters 2-4) and a general conclusion. Chapter 2 and 3 have been accepted for publication, while Chapter 4 is in preparation for submission. The candidate is the first author of each chapter.

CONTRIBUTION TO PAPERS WITH MULTIPLE AUTHORS

CHAPTER 2

Publication

Churchward-Venne TA, Burd NA, Mitchell CJ, West DW, Philp A, Marcotte GR, Baker SK, Baar K, Phillips SM. Supplementation of a suboptimal protein dose with leucine or essential amino acids: effects on myofibrillar protein synthesis at rest and following resistance exercise in men. J Physiol. 2012; 590:2751-65.

Contributions

TA Churchward-Venne and SM Phillips planned the study. TA Churchward-Venne obtained ethics board approval. TA Churchward-Venne, CJ Mitchell, DWD West, SK Baker, and SM Phillips collected the data. SM Phillips supervised the study and obtained muscle biopsies. TA Churchward-Venne and T Rerecich performed intracellular amino acid analysis. TA Churchward-Venne and T Rerecich performed blood metabolite analysis. TA Churchward-Venne, NA Burd, T Rerecich performed analyses for measurement of myofibrillar protein synthesis. TA Churchward-Venne, A Philp, GR Marcotte, and K Baar performed mRNA analysis. TA Churchward-Venne performed Western blot analysis. TA Churchward-Venne drafted the manuscript. All authors contributed to revising intellectual content of the manuscript and approved the final version.

CHAPTER 3

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Churchward-Venne TA, Breen L, Di Donato DM, Hector AJ, Mitchell CJ, Moore DR, Stellingwerff T, Breuille D, Offord EA, Baker SK, and Phillips SM. Leucine supplementation of a low protein mixed macronutrient beverage enhances myofibrillar protein synthesis in young men: a double blind randomised trial. Am J Clin Nutr. 2013 Nov 27. [Epub ahead of print].

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CHAPTER 4

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction:

Skeletal muscle, beyond its obvious role in locomotion, plays a key role in whole body protein metabolism as it serves as the principle reservoir for amino acids. These amino acids, when released from skeletal muscle, support protein synthesis in other tissues and organs throughout the body and serve as precursors for hepatic gluconeogenesis. Like all body proteins, skeletal muscle proteins are subject to 'turnover', which describes the dynamic processes of continual synthesis (i.e. movement of free amino acids into proteins) and degradation (release of peptide-bound amino acids into the free metabolic pool) of proteins. When there is a balance between rates of muscle protein synthesis and breakdown, skeletal muscle mass is maintained (1).

1.2 Net Protein Balance

A positive net protein balance occurs when proteins are synthesized at a rate exceeding their removal (i.e. following a protein-rich meal) and results in net muscle protein accretion. Conversely, a negative net protein balance occurs when the rate of muscle protein breakdown exceeds the rate at which muscle protein is being synthesized (i.e. during periods of fasting) and results in net protein loss (1). Under normal conditions, feeding-induced gains in muscle protein serve to balance the loss of muscle protein that occurs during the postabsorptive state, thereby facilitating renewal of the structural and functional components of muscle and maintenance of a stable muscle mass (1). In addition to food intake, the muscle contraction associated with loading (i.e., resistance

exercise) serves as a strong stimulus for the synthesis of new muscle protein (2-4). However, in the absence of a source of amino acids, net muscle protein balance after resistance exercise remains negative due to a concomitant rise in the rate of muscle protein breakdown (3). When amino acid (5, 6) provision occurs after resistance exercise, rates of muscle protein synthesis surpass those of muscle protein breakdown, resulting in a net positive protein balance and protein accretion (5). In healthy individuals, feeding-induced changes in muscle protein synthesis are ~3-5 times greater over the course of a given day than measurable changes in muscle protein breakdown, demonstrating that muscle protein synthesis is highly responsive, is the locus of regulation, and represents the primary process determining changes in net muscle protein balance (5, 4). As such, a positive net muscle protein balance and increases in muscle protein content are highly dependent on changes in muscle protein synthesis which provides the foundation for increased skeletal muscle mass following prolonged periods of resistance exercise training.

Although it is well recognized that the protein/amino acid component of a mixed meal is primarily responsible for the postprandial stimulation of muscle protein synthesis (7), what is not clear is the stimulatory capacity of individual amino acids and their contribution to the observed response of muscle protein synthesis following ingestion of a complete protein. The experiments that form this thesis have focused on two amino acids, leucine and citrulline, and have sought to gain a more comprehensive understanding of their role in the regulation of human skeletal muscle protein synthesis. The essential amino acid leucine has garnered considerable attention due to its ability to activate

translation initiation and stimulate muscle protein synthesis in animals (8, 9). The non-essential amino acid citrulline has also been reported to stimulate muscle protein synthesis rates comparable to those observed following leucine intake when administered to rats (10), and is a key amino acid precursor for nitric oxide synthesis (11). To address the ability of leucine and citrulline to regulate human muscle protein synthesis, we designed studies involving the supplementation of these amino acids to lower doses of dietary protein that have previously been demonstrated to be "suboptimal" for the stimulation of muscle protein synthesis. In addition, in the studies herein we utilized a unilateral model of knee-extensor based resistance exercise to examine the effect(s) of these amino acids in combination with a loading stimulus on skeletal muscle protein synthesis. Thus, within a single experiment we were able to make measurements in: the postabsorptive state, and in the postprandial state in both a resting (non-exercised) condition, and following the contractile stimulation of resistance exercise. The strength of this model is that all measurements taken under different conditions (i.e. postabsorptive, postprandial at rest, and postprandial post-exercise) come from the same individual. Between-group comparisons were restricted to treatments (i.e., differing amino acid mixtures), but were powered according to the least significant difference to detect differences between parameters of interest.

1.3 Stable isotope tracers to assess human muscle protein synthesis

The studies conducted as part of this thesis have utilized stable isotope tracer methodologies to obtain dynamic measures of muscle protein synthesis (12). Stable isotopes are non-radioactive heavier atoms of a particualr element (i.e. carbon), and thus

can be distinguished from their more common elemental counterparts via their mass difference. It is this characteristic that renders them useful as metabolic tracers, since the incorporation of isotope labelled substances into biological samples can be determined using various forms of mass spectrometry. In this thesis a primary mass spectrometric methodology was isotope ratio mass spectrometry (IRMS). Rates of human muscle protein synthesis were first measured by Halliday and colleagues in 1975 using ^{15}N labelled lysine (13), which is a labile label since it can be lost to urea formation. The studies outlined in this thesis have utilized the primed, continuous intravenous infusion technique (14), using L-[*ring*-$^{13}C_6$]phenylalanine coupled with skeletal muscle biopsy sampling to obtain a direct measure of the fractional synthetic rate (FSR; % $\bullet$ h^{-1}) of skeletal muscle protein. The fractional synthetic rate refers to the fraction of the protein-bound pool (i.e. muscle) that is synthesized per unit of time (i.e. hours). Successful implementation of the technique requires a steady-state of tracer labelling or "enrichment" in the chosen precursor pool, be it aminoacyl tRNA, plasma, or the intracellular free amino acid pool, coupled with serial skeletal muscle biopsy sampling to determine the change in protein-bound enrichment in a muscle sample over a given period of time. It is important to highlight that skeletal muscle is composed of a variety of different types of proteins that serve different physiological functions. For example the contractile proteins actin and myosin, referred to as myofibrillar proteins, as well as mitochondrial proteins can be isolated from a biopsy sample allowing for the determination of tracer enrichment and therefore the FSR within that specific protein fraction (15). The studies outlined in this thesis have made measures of myofibrillar FSR,

the most abundant protein fraction in skeletal muscle representing ~50-60% of the total skeletal muscle protein pool (16). Myofibrillar proteins also represent the functional contractile protein pool and are therefore subject to important adaptations in response to the contractile activity associated with exercise.

1.4 Translational control of muscle protein synthesis

The molecular mechanisms regulating nutrient and exercise-mediated changes in muscle protein synthesis have been linked to increases in mRNA translation. Many of the cell-signaling pathways that are activated in response to these stimuli converge on the protein known as the mammalian (or more recently mechanistic) target of rapamycin (mTORC1), a serine-threonine protein kinase that is important in the regulation of cellular growth (17). Specifically, the mTORC1 pathway has been identified as a key regulator of exercise (18) and nutrient-mediated (19) increases in human muscle protein synthesis. The translation of mRNA into a polypeptide is conventionally described as consisting of three stages: initiation, elongation, and termination. Translation initiation consists of bringing the mRNA to the ribosome and involves a multitude of eukaryotic initiation factors that are involved in the assembly of the translation-ready ribosome. The elongation phase involves the addition of amino acids to the growing polypeptide chain and requires the presence of eukaryotic elongation factors. At the end of elongation, the newly synthesized polypeptide is released from the translational machinery when the termination codon is reached, thus completing the process of protein translation. The current body of research suggests that amino acids and loading-type exercise increase

muscle protein synthesis primarily through increasing the rate of translation initiation (17).

1.5 Nutrient mediated regulation of human muscle protein synthesis

The effect of nutrition on human muscle protein turnover was first described by Rennie and colleagues in 1982 when they observed a doubling of the rate of muscle protein synthesis in response to ingestion of mixed macronutrients when assessed using stable isotope tracer techniques (12). A few years later, the same group showed that the stimulatory effect of mixed nutrients on muscle protein synthesis could largely be obtained by infusing amino acids alone (7). Since these initial observations, it has been demonstrated that only the essential amino acids possess the capacity to stimulate muscle protein synthesis in humans; typical non-essential amino acids apparently lack this ability (20, 21). There is a saturable dose-dependent relationship between amino acid/protein intake and muscle protein synthesis (22, 23), that is altered with ageing (24, 25) and the protein source ingested (26). Cuthbertson and colleagues (22) examined the dose-dependent relationship between amino acid intake and myofibrillar protein synthesis in young and elderly participants and demonstrated that at rest, 10 g of essential amino acids was sufficient to maximally stimulate myofibrillar protein synthesis (22). This dose of amino acids is very close to what is found in ~20-25 g of high quality protein, a dose of protein that appears sufficient to maximally stimulate muscle protein synthesis following an acute bout of resistance exercise in young men (discussed below) (23). Feeding-mediated changes in muscle protein synthesis are quite transient, lasting ~ 3 hours (27), and peaking at ~1.5 hours (28) before returning to postabsorptive rates. Interestingly,

muscle protein synthesis returns to basal rates despite the presence of continued postprandial amino acid availability (29, 28) and elevated signaling through the mTORC1 pathway (28). Thus, skeletal muscle becomes refractory to stimulation despite continued amino acid availability, phenomenon termed the "muscle full" effect (29, 28). Although the mechanisms responsible for this effect are currently unclear, the phenomenon suggests that protein distribution between feedings may be important to maximally stimulate muscle protein accretion over the course of the day (30).

Although several different sources of dietary protein including whey (27, 31, 32), egg (23), soy (33, 31), casein (31), and beef (34) possess the capacity to stimulate muscle protein synthesis, whey protein appears particularly efficacious among different sources of dietary protein at stimulating increased rates of muscle protein synthesis (31, 35). Whey protein has also been shown to be superior at promoting resistance exercise-mediated gains in skeletal muscle mass (36) when compared against other sources of dietary protein such as soy (31, 26) or casein (31, 35). The mechanism(s) responsible for the exercise-mediated differences between proteins are not entirely clear but may relate to important differences in the amino acid profile and/or amino acid digestibility due to differences in the digestion/absorption kinetics of the proteins (37). As an acid soluble protein, whey is associated with a very rapid, large, but transient increase in postprandial amino acid availability (38), a characteristic that has been shown to enhance muscle protein synthesis and cell-signaling through mTORC1 after resistance exercise (39). Whey protein is also relatively enriched in the branched-chain amino acid leucine, having a content of ~11-13% (depending on the protein fraction of whey), whereas other high

quality proteins (defined by their Protein Digestibility Corrected Amino Score of 1.0) such as isolated soy and micellar casein are about 6% and 8% leucine by content, respectively. Leucine appears unique among amino acids in its capacity to regulate translation initiation of muscle protein synthesis (8, 9). All of the studies outlined in this thesis have utilized whey protein as the base protein source when examining nutrient and combined nutrient-exercise effects on muscle protein synthesis.

1.6 Amino acids and the molecular regulation of muscle protein synthesis

Amino acids stimulate muscle protein synthesis, in part, through activation of mTORC1 (22, 40). Activation of proteins is generally measured as phosphorylation of residues (i.e. Ser, Tyr, Thr) that have been shown, or at least are thought to be, rate determining for activation of the protein in question. Most proteins are kinases that through phosphorylation, have an effect on a terminal kinase that results in activation/deactivation of a particular pathway. Once activated, mTORC1 can increase the phosphorylation of downstream protein targets involved in translational initiation of protein synthesis such as p70S6k and 4E-BP1 (41). Of the essential amino acids, leucine appears to possess a unique capacity to enhance signalling through the mTORC1 pathway (40) and can stimulate increased rates of human muscle protein synthesis when provided independent of other amino acids (20, 42). Fujita and colleagues reported increased phosphorylation of Akt, mTOR, p70S6k, and 4E-BP1 and reduced phosphorylation of eEF2 (involved in the elongation phase of protein translation) in conjunction with enhanced rates (~100%) of muscle protein synthesis following a leucine-enriched essential amino acid and carbohydrate treatment in young men (43). In addition, the same

research group (19) has demonstrated that rapamycin administration in humans blunts the essential amino acid-mediated increase in human muscle protein synthesis, highlighting an essential role of mTORC1 in the amino acid mediated regulation of muscle protein synthesis.

Great strides have been made in recent years in an attempt to unravel the mechanism(s) behind how amino acids activate mTORC1 (44, 45). The ability of amino acids to activate mTORC1 may be mediated via hVps34 (46, 47), and/or mitogen-activated protein kinase kinase kinase kinase (MAP4K) 3, which is activated in response to amino acids and results in the phosphorylation of p70S6k in an mTORC1-dependent manner (48). In addition, recent work has demonstrated that the amino acid mediated activation of mTORC1 involves its translocation to the lysosome within the cell where its direct upstream activator, Rheb, resides (49). The recruitment of mTORC1 to the lysosome appears to involve Rag GTPases (44), while subsequent docking and anchoring of mTORC1 to the lysosome requires proteins referred to as “ragulators”(45). Leucyl tRNA synthetase has also recently been identified as having a role in the amino acid mediated activation of mTORC1 via its localization at the lysosome (50). In response to increases in intracellular leucine concentrations, leucyl tRNA appears to translocate to the lysosome and bind Rag GTPase, thereby activating mTORC1 (50). These findings suggest that amino acid transport into the intracellular environment is an important component in the amino acid mediated activation of mTORC1.

In addition to their role in regulating amino acid availability via transport across the sarcolemma, certain amino acid transporters may act as “transceptors” (51) serving to

monitor changes in both intra- and extracellular amino acid availability and relay information to the cell interior (52). Recent investigations in humans have demonstrated that amino acid transporter mRNA abundance (i.e., LAT1/SLC7A5, SNAT2/SLC38A2, PAT1/SLC36A1) and protein expression (LAT1/SLC7A5, SNAT2/SLC38A2) are increased in response to essential amino acid ingestion (53, 32), and may play a role in the amino acid-stimulated activation of mTORC1 (51).

1.7 Combined resistance exercise-amino acid mediated regulation of human muscle protein synthesis

Skeletal muscle is a dynamic tissue that possesses a remarkable ability to adapt when exposed to the contractile and metabolic demands of exercise; however, the physiological adaptations that occur in response to exercise vary depending on the nature of the stimuli encountered (54), and the genetic makeup of the individual (55). It is becoming clear that skeletal muscle somehow senses and distinguishes the signals associated with different forms of exercise (i.e. contraction intensity, duration, volume, mode) and adapts in a way that is specific to the nature of the stimulus imposed. For example, chronic performance of resistance-type exercise is associated with adaptations that include muscle hypertrophy and greater force producing capacity (i.e. strength), adaptations that must involve both acute and chronic alterations in muscle protein turnover.

The contractile stimulation associated with resistance exercise serves as a strong stimulus to increase muscle protein synthesis (2), an effect that is modulated by contraction intensity (56) and volume (57, 58). Chesley and colleagues (2) were the first

to measure human muscle protein synthesis following an acute bout of resistance exercise. Using a parallel group design they showed that muscle protein synthesis was increased when assessed at 4 h and 24 h post-exercise (2); later work has extended these findings by demonstrating that a single bout of resistance exercise in the untrained state increases the rate of muscle protein synthesis for up to 48 hours (4). In addition to acting as a strong stimulus to promote muscle protein synthesis, resistance exercise also results in an increase in the rate of muscle protein breakdown such that net muscle protein balance after resistance exercise, while still negative, is less so than that in the resting state (3). Amino acid provision following resistance exercise acts to enhance the rate of muscle protein synthesis, but also suppress the rate of muscle protein breakdown, thereby stimulating a positive net protein balance and net muscle protein accretion during post-exercise recovery (5).

Although the protein/amino acid mediated increase in muscle protein synthesis is quite transient lasting ~3 hours (29, 27, 28), resistance exercise increases the rate of muscle protein synthesis for up to 48 hours, at least in the untrained state (4). To date, several studies have demonstrated that protein or amino acid intake after resistance exercise acts to further enhance muscle protein synthesis (5, 6). For example, Dreyer and colleagues reported a ~41% increase in muscle protein synthesis above basal after resistance exercise in the absence of any nutritional intervention, however, the increase was ~145% when a leucine rich essential amino acid/carbohydrate treatment was ingested after exercise (59). Moore and colleagues (23) were the first to examine the dose-response relationship between protein intake and muscle protein synthesis after resistance exercise.

Ingestion of 20 g of high quality egg protein isolate post-exercise produced greater rates of muscle protein synthesis than ingestion of either doses of 5 g or 10 g protein, but was not significantly different than the response seen with ingestion of 40 g of protein in young men (23). These findings are consistent with the dose-response relationship between essential amino acid ingestion and the increase in myofibrillar protein synthesis reported during resting conditions (22) and suggest that ~20-25 g high quality protein (~0.25 g protein/kg or ~8-10 g essential amino acids) after resistance exercise acutely 'saturates' the process of muscle protein synthesis. Amino acids supplied in excess of this quantity are not used for the synthesis of new muscle protein but are instead diverted toward oxidation (23).

Several studies have reported the impact of the temporal pattern of nutrient provision relative to exercise on muscle protein metabolism (60-62). Although muscle protein synthesis has been reported to be augmented with pre-exercise (60) and during exercise (61, 63) amino acid provision, the current consensus is that protein-amino acid ingestion immediately after exercise is more efficacious in promoting muscle protein accretion during the post-exercise period than consuming these nutrients immediately before or during exercise (64). This conclusion may relate to muscle protein synthesis, an energy consuming process, being a low priority during resistance exercise due to ATP being directed towards fueling contractile activity (65). The studies outlined in this thesis have utilized immediate post-exercise protein-amino acid provision to investigate the postprandial response of muscle protein synthesis.

1.8 Resistance exercise and the molecular regulation of muscle protein synthesis

The molecular mechanisms that regulate increased rates of muscle protein synthesis in response to mechanical stimuli have been linked to increases in mRNA translation (66). Data from rodent and cell models have identified mTORC1 as a key protein regulating mRNA translation and muscle protein synthesis in response to increased loading (67, 68). For a more in-depth review on the molecular regulation of mRNA translation via nutrition and exercise see (17). It is now well established that an acute bout of resistance exercise stimulates increased rates of muscle protein synthesis in association with enhanced signalling through mTORC1 in human muscle (69, 70, 18, 57, 71). Drummond and colleagues (18) demonstrated the essential role of mTORC1 in the contraction-mediated stimulation of muscle protein synthesis by demonstrating that administration of rapamycin, an inhibitor of mTORC1, abolished the increase in muscle protein synthesis and reduced p70S6k, rpS6, and eEF2 phosphorylation during the early post-exercise recovery period in young men (18). Precisely how resistance exercise stimulates mTORC1 activation is not clear, although a phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) independent mechanism involving mechanical activation of phospholipase D1 and the production of phosphatidic acid, has been suggested (72). In addition, amino acid transporter mRNA and protein expression (i.e., LAT1/SLC7A5, SNAT2/SLC38A2, PAT1/SLC36A1) has been reported to increase following an acute bout of resistance exercise and may exert a role in the activation of mTORC1 after resistance exercise (73).

1.9 Role of leucine in the regulation of muscle protein synthesis

Essential amino acids are primarily responsible for the amino acid mediated stimulation of muscle protein synthesis (74, 75), whereas typical non-essential amino acids appear ineffective in this regard (21, 75). The branched-chain amino acid (BCAA) leucine appears unique among the essential amino acids as a key regulator of translation initiation of muscle protein synthesis (9, 76). For example, the independent provision of leucine can stimulate an increase in muscle protein synthesis through activation of the mTORC1-p70S6k pathway in animals (9, 77). In addition, work in cell culture utilizing C2C12 cells has demonstrated that leucine is the most potent among the essential amino acids in its ability to increase the phosphorylation of protein targets of the mTORC1 pathway (40). It is because of this unique capacity that leucine has been suggested as a direct modulator of muscle protein synthesis, in addition to its more obvious role as amino acid substrate. Given that leucine appears to play a key role in the amino acid mediated regulation of muscle protein synthesis, recent research has focused on utilizing leucine in nutritional interventions to modulate muscle protein synthesis and/or augment muscle mass in humans. Tipton and colleagues (78) examined the effect of free leucine (3.4 g) added to whey protein (16.6 g) during recovery after resistance exercise and reported no difference in rates of muscle protein synthesis with the addition of free leucine compared to 20 g whey protein. However, these data are not surprising given the findings of Moore and colleagues (23) who reported that 20 g protein was sufficient to maximally stimulate muscle protein synthesis after resistance exercise. Addition of leucine to a near saturating quantity of high quality protein may not be expected to further

stimulate muscle protein synthesis in young subjects. However, recent work has firmly established that leucine, ingested independent of other amino acids, robustly stimulates human muscle protein synthesis (42), corroborating results from over 20 years ago when intravenously administered leucine was demonstrated to stimulate human muscle protein synthesis (20). Of interest is that metabolites of leucine such as β-hydroxy-β-methylbutyrate (ß-HMB) are also capable of stimulating human muscle protein synthesis, although the effect appears less robust than observed for leucine (42). Therefore, although mixtures of amino acids and complete proteins can stimulate muscle protein synthesis [for review see (79)], certain individual amino acids (i.e. leucine, phenylalanine, and threonine) also possess this capacity (20, 21, 42) (**Figure 1**). What is not clear is whether a full complement of amino acid substrate is necessary to sustain elevated rates of muscle protein synthesis. Studies in animals have shown that the independent provision of leucine leads to a decline in circulating essential amino acid concentrations and reduces the duration of the amino acid-mediated increase in muscle protein synthesis (80); however, when this decrease is prevented and basal amino acid concentrations are maintained, the amino acid mediated stimulation of muscle protein synthesis is sustained for a longer period of time (81).

2.0 Age-related alterations in human muscle protein synthesis

Ageing is associated with sarcopenia, a geriatric condition characterized by a decline in skeletal muscle mass (myopenia) and strength (dynapenia) (82). Sarcopenia results in a diminished capacity to perform the physical activities associated with daily

living and increases the risk of falls and fractures, thereby reducing quality of life, and ultimately increasing risk for morbidity and mortality. Clinically, sarcopenia has been

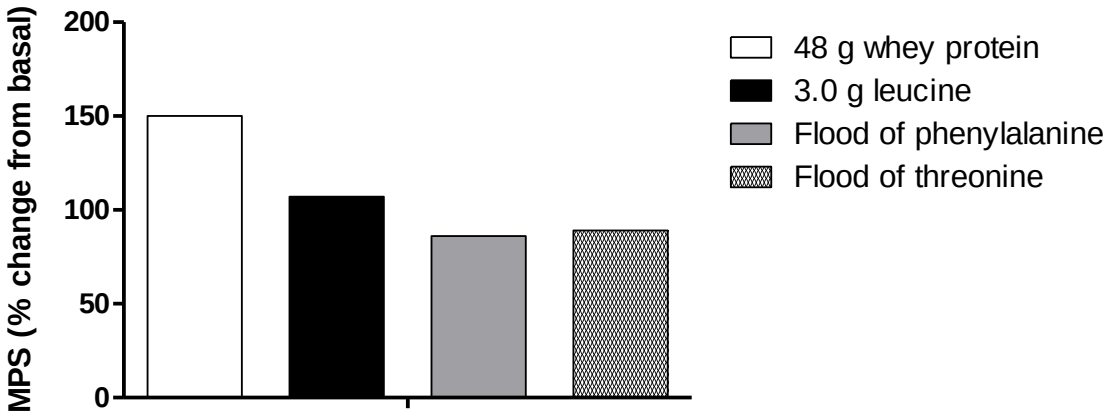

Figure 1. Change in muscle protein synthesis (% change from basal) in response to 48 g whey protein (28), 3.0 g leucine (42), and intravenous flooding doses of phenylalanine and threonine (21). Note that the tracer incorporation times are different between studies which will impact the calculated muscle protein synthesis rates.

defined using muscle mass that is greater than two standard deviations below the mean measured in young adults of the same sex and ethnicity (83). The loss of skeletal muscle mass must ultimately stem from an imbalance between protein synthesis and breakdown that favours a net negative protein balance and net tissue loss. The possible age-related derangements in protein turnover are thus declines in muscle protein synthesis, increments in muscle protein breakdown, or a combination of the two. In support of a role for decrements in muscle protein synthesis, a common observation is that elderly individuals show an attenuated response of muscle protein synthesis than their younger counterparts to the normally robust stimulatory effects of amino acid/protein intake (84,

85, 25, 26) and resistance exercise (56, 86). Additionally, the elderly are less sensitive to the anti-proteolytic effects of insulin than their younger counterparts and may therefore have a lower net protein balance with meal feeding due to a lesser suppression of proteolysis (87).

Yang and colleagues (25) compared the effects of graded doses of whey protein intake (10, 20, 40 g) on muscle protein synthesis rates under non-exercised resting conditions, and following an acute bout of resistance exercise in elderly men. It was demonstrated that ingestion of 40 g of whey protein stimulated muscle protein synthesis rates more than ingestion of 20 g following resistance exercise, and also tended to do so under resting conditions (25). Thus, when compared to the young, in whom 20 g protein is sufficient to induce a maximal stimulation of muscle protein synthesis (23), the elderly require relatively larger quantities of protein after resistance exercise (40 g) to robustly stimulate muscle protein synthesis, and appear less sensitive to lower doses of protein. Although the mechanism(s) underlying this age-related 'anabolic resistance' are not fully understood, age-associated impairments in vascular endothelial function may be involved (88, 89). For example, although amino acid provision and hyperinsulinemia increase blood flow (84), muscle micovascular perfusion (90), and muscle protein synthesis in the young, these responses are blunted in the elderly (84, 89). This age-related impairment in endothelial function may, in part, be due to reduced nitric oxide (NO) availability (91), leading to diminished NO-mediated capillary recruitment and impaired metabolic regulation. In support of this possibility, administration of the NO donor sodium nitroprusside has been shown to restore the stimulation of muscle protein synthesis by

insulin in older adults, under fasting conditions (88). In addition, when amino acids are provided in conjunction with sodium nitroprusside, skeletal muscle protein synthesis is stimulated in older individuals to rates comparable to those observed in young subjects (92). Given these findings, interventions designed to increase NO availability may hold promise as a means to restore age-related impairments in vascular hemodynamics and subsequently, muscle protein synthesis in the elderly.

2.1 Citrulline as a pharmaconutrient to overcome age-related anabolic resistance

Citrulline is a non-protein amino acid, capable of affecting nitric oxide synthesis via stimulation of de-novo arginine production (11). The synthesis of NO requires arginine and the enzyme NO synthase (NOS) of which there are three isoforms: neuronal NOS, endothelial NOS, and inducible NOS (iNOS). Previous studies have shown that citrulline supplementation induces a greater increase in NO synthesis than that observed following arginine supplementation, demonstrating that citrulline is a more effective NO precursor than arginine (11). Dietary arginine bioavailability is only ~60% (93) since arginine is converted to ornithine in the liver through the action of arginase I, whereas citrulline escapes hepatic clearance and is not a substrate for arginase I. In addition to its role as a precursor for NO, citrulline has been shown to independently stimulate increased rates of muscle protein synthesis in rodents (94, 10). These changes in muscle protein synthesis occur concomitantly with increased phosphorylation of components of the mTOR signaling pathway (10). Therefore, citrulline may stimulate increased rates of muscle protein synthesis in the elderly through indirect mechanisms involving NO mediated increases in tissue perfusion and amino acid availability, and/or via a direct

effect on the translational machinery by increasing the activity of the mTORC1/p70S6k pathway.

2.2 Specific objective of the studies

The primary objective of the studies comprising this thesis was to examine the potential to enhance the rates of muscle protein synthesis in response to ingestion of "suboptimal" quantities of protein (i.e., less than 20-25 g in young adults and 40 g in older adults) through the addition of specific amino acids (leucine - studies 1 and 2; and citrulline - study 3), thought to be important in the amino acid-mediated regulation of muscle protein synthesis. Therefore we measured myofibrillar protein synthesis (studies 1-3), intramuscular cell signaling molecule phosphorylation (studies 1-3), and blood flow (study 3) in the postabsorptive state, and in the postprandial state at rest and following acute resistance exercise. The chapters that follow describe in detail the studies that were undertaken to address the objectives outlined above.

2.2.1 Suboptimal protein supplemented with leucine or a mixture of essential amino acids

The objective of Study 1 (Chapter 2) was to examine the effects of supplementing a dose of protein (6.25 g), containing an amount of amino acids previously demonstrated to be "suboptimal" for the stimulation of muscle protein synthesis (22, 23), with leucine or a mixture of essential amino acids devoid of leucine on muscle protein synthesis rates under resting and post-exercise conditions. We compared these protein/amino acid treatments to a dose of protein (25 g), containing a sufficient quantity of essential amino acids to maximally stimulate muscle protein synthesis rates under both resting (22) and

post-exercise conditions (23). It was hypothesized that if leucine was in fact the principle amino acid directing muscle protein synthesis, as appears to be the case in animals (95), then it might be possible to enhance the muscle protein synthetic effects of a suboptimal dose of protein simply by supplementing it with leucine, to match the leucine content of a maximally effective dose of protein (~3.0 g) (23). However, we were also aware of data demonstrating that other essential amino acids (i.e. phenylalanine and valine) are stimulatory for human muscle protein synthesis when infused at high doses (21), so were interested to assess the impact of supplementing a suboptimal dose of protein with a complete mixture of essential amino acids on muscle protein synthesis rates. To gain information on potential underlying mechanisms, we incorporated Western blot analysis to determine the phosphorylation of select protein targets within the mTORC1 pathway, and measured the mRNA abundance of select amino acid transporters (i.e. LAT-1, PAT-1).

2.2.2 Leucine & BCAA supplementation of a low protein mixed-macronutrient beverage

Study 2 (Chapter 3) was a double-blind randomized clinical trial designed to assess the efficacy of supplementing a low-protein mixed macronutrient beverage, containing a quantity of essential amino acids previously demonstrated to be suboptimal in maximally stimulating muscle protein synthesis rates with feeding (22) and resistance exercise (23), with different doses of leucine and BCAAs on muscle protein synthesis rates. As part of a parallel group design, subjects were randomly assigned to a positive control containing 25 g whey protein (W25 = 3.0 g of leucine), a negative control

containing 6.25 g whey protein (W6 = 0.75 g of leucine) or treatments consisting of 6.25 g of whey protein supplemented with the following: a lower dose of leucine (W6+Low-Leu = 3.0 g of leucine); a higher dose of leucine (W6+High-Leu = 5.0 g of leucine), or a higher dose of leucine plus isoleucine, and valine (W6+BCAA = 5.0 g of leucine). Skeletal muscle protein synthesis and the phosphorylation status of protein targets of the mTORC1 pathway were examined under postabsorptive conditions and in the postprandial state under rested and post-exercise conditions. It was hypothesized that W25, W6+Low-Leu, W6+BCAA, and W6+High-Leu would stimulate greater postprandial myofibrillar protein synthesis rates than W6 under resting conditions with no differences between treatments. During post-exercise recovery, it was hypothesized that W6+BCAA, W25, and W6+High-Leu would elicit increases in myofibrillar protein synthesis that were equivalent, but greater than W6+Low-Leu and W6 due to a sustained elevation of myofibrillar protein synthesis at the later time-points examined.

2.2.3 Citrulline vs. non-essential amino acids to promote muscle protein synthesis in the elderly

Study 3 (Chapter 4) was designed to examine the potential of citrulline supplementation (10 g), as compared to a mixture of non-essential amino acids (10 g), to enhance the muscle protein synthetic effects of a smaller dose of whey protein (15 g) containing an amount of essential amino acids previously reported to be suboptimal for stimulating muscle protein synthesis rates in the elderly (24, 25). It was hypothesized that citrulline would enhance arginine availability (96), the precursor for nitric oxide synthesis in a reaction catalyzed by endothelial nitric oxide synthase (eNOS). Given that arterial

blood flow (84) and muscle microvascular perfusion (89) are impaired in the elderly in response to combined hyperinsulinemia and amino acid provision, supplementation of a suboptimal quantity of protein with citrulline was hypothesized to enhance femoral artery blood flow and muscle microvascular perfusion, concomitant with enhanced delivery of amino acid substrates and increased rates of myofibrillar protein synthesis and signalling through select protein targets of the mTORC1 pathway. As a positive control, we compared the response of myofibrillar protein synthesis, femoral artery blood flow, muscle microvascular perfusion, and anabolic cell signalling through mTORC1 to a dose of protein (45 g) able to elicit a more robust stimulation of muscle protein synthesis (24, 25).

References:

[1] Rennie, M. J., Wackerhage, H., Spangenburg, E. E., Booth, F. W. (2004) Control of the size of the human muscle mass. *Annu Rev Physiol* **66**, 799-828.

[2] Chesley, A., MacDougall, J. D., Tarnopolsky, M. A., Atkinson, S. A., Smith, K. (1992) Changes in human muscle protein synthesis after resistance exercise. *J Appl Physiol* **73**, 1383-1388.

[3] Biolo, G., Maggi, S. P., Williams, B. D., Tipton, K. D., Wolfe, R. R. (1995) Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans. *Am J Physiol* **268**, E514-520.

[4] Phillips, S. M., Tipton, K. D., Aarsland, A., Wolf, S. E., Wolfe, R. R. (1997) Mixed muscle protein synthesis and breakdown after resistance exercise in humans. *Am J Physiol* **273**, E99-107.

[5] Biolo, G., Tipton, K. D., Klein, S., Wolfe, R. R. (1997) An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein. *Am J Physiol* **273**, E122-129.

[6] Tipton, K. D., Ferrando, A. A., Phillips, S. M., Doyle, D., Jr., Wolfe, R. R. (1999) Postexercise net protein synthesis in human muscle from orally administered amino acids. *Am J Physiol* **276**, E628-634.

[7] Bennet, W. M., Connacher, A. A., Scrimgeour, C. M., Smith, K., Rennie, M. J. (1989) Increase in anterior tibialis muscle protein synthesis in healthy man during mixed amino acid infusion: studies of incorporation of [1-13C]leucine. *Clin Sci (Lond)* **76**, 447-454.

[8] Anthony, J. C., Anthony, T. G., Kimball, S. R., Vary, T. C., Jefferson, L. S. (2000) Orally administered leucine stimulates protein synthesis in skeletal muscle of postabsorptive rats in association with increased eIF4F formation. *J Nutr* **130**, 139-145.

[9] Anthony, J. C., Yoshizawa, F., Anthony, T. G., Vary, T. C., Jefferson, L. S., et al. (2000) Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. *J Nutr* **130**, 2413-2419.

[10] Le Plenier, S., Walrand, S., Noirt, R., Cynober, L., Moinard, C. (2012) Effects of leucine and citrulline versus non-essential amino acids on muscle protein synthesis in fasted rat: a common activation pathway? *Amino Acids* **43**, 1171-1178.

[11] El-Hattab, A. W., Hsu, J. W., Emrick, L. T., Wong, L. J., Craigen, W. J., et al. (2012) Restoration of impaired nitric oxide production in MELAS syndrome with citrulline and arginine supplementation. *Mol Genet Metab* **105**, 607-614.

[12] Rennie, M. J., Edwards, R. H., Halliday, D., Matthews, D. E., Wolman, S. L., et al. (1982) Muscle protein synthesis measured by stable isotope techniques in man: the effects of feeding and fasting. *Clin Sci (Lond)* **63**, 519-523.

[13] Halliday, D., McKeran, R. O. (1975) Measurement of muscle protein synthetic rate from serial muscle biopsies and total body protein turnover in man by continuous intravenous infusion of L-(alpha-15N)lysine. *Clin Sci Mol Med* **49**, 581-590.

[14] Rennie, M. J., Smith, K., Watt, P. W. (1994) Measurement of human tissue protein synthesis: an optimal approach. *Am J Physiol* **266**, E298-307.

[15] Rooyackers, O. E., Balagopal, P., Nair, K. S. (1997) Measurement of synthesis rates of specific muscle proteins using needle biopsy samples. *Muscle Nerve Suppl* **5**, S93-96.

[16] Hasten, D. L., Pak-Loduca, J., Obert, K. A., Yarasheski, K. E. (2000) Resistance exercise acutely increases MHC and mixed muscle protein synthesis rates in 78-84 and 23-32 yr olds. *Am J Physiol Endocrinol Metab* **278**, E620-626.

[17] Kimball, S. R., Jefferson, L. S. (2010) Control of translation initiation through integration of signals generated by hormones, nutrients, and exercise. *J Biol Chem* **285**, 29027-29032.

[18] Drummond, M. J., Fry, C. S., Glynn, E. L., Dreyer, H. C., Dhanani, S., et al. (2009) Rapamycin administration in humans blocks the contraction-induced increase in skeletal muscle protein synthesis. *J Physiol* **587**, 1535-1546.

[19] Dickinson, J. M., Fry, C. S., Drummond, M. J., Gundermann, D. M., Walker, D. K., et al. (2011) Mammalian target of rapamycin complex 1 activation is required for the stimulation of human skeletal muscle protein synthesis by essential amino acids. *J Nutr* **141**, 856-862.

[20] Smith, K., Barua, J. M., Watt, P. W., Scrimgeour, C. M., Rennie, M. J. (1992) Flooding with L-[1-13C]leucine stimulates human muscle protein incorporation of continuously infused L-[1-13C]valine. *Am J Physiol* **262**, E372-376.

[21] Smith, K., Reynolds, N., Downie, S., Patel, A., Rennie, M. J. (1998) Effects of flooding amino acids on incorporation of labeled amino acids into human muscle protein. *Am J Physiol* **275**, E73-78.

[22] Cuthbertson, D., Smith, K., Babraj, J., Leese, G., Waddell, T., et al. (2005) Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. *FASEB J* **19**, 422-424.

[23] Moore, D. R., Robinson, M. J., Fry, J. L., Tang, J. E., Glover, E. I., et al. (2009) Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *Am J Clin Nutr* **89**, 161-168.

[24] Pennings, B., Groen, B. B., de Lange, A., Gijsen, A. P., Zorenc, A. H., et al. (2012) Amino acid absorption and subsequent muscle protein accretion following graded intakes of whey protein in elderly men. *Am J Physiol Endocrinol Metab*.

[25] Yang, Y., Breen, L., Burd, N. A., Hector, A. J., Churchward-Venne, T. A., et al. (2012) Resistance exercise enhances myofibrillar protein synthesis with graded intakes of whey protein in older men. *Br J Nutr* 1-9.

[26] Yang, Y., Churchward-Venne, T. A., Burd, N. A., Breen, L., Tarnopolsky, M. A., et al. (2012) Myofibrillar protein synthesis following ingestion of soy protein isolate at rest and after resistance exercise in elderly men. *Nutr Metab (Lond)* **9**, 57.

[27] Moore, D. R., Tang, J. E., Burd, N. A., Rerecich, T., Tarnopolsky, M. A., et al. (2009) Differential stimulation of myofibrillar and sarcoplasmic protein synthesis with protein ingestion at rest and after resistance exercise. *J Physiol* **587**, 897-904.

[28] Atherton, P. J., Etheridge, T., Watt, P. W., Wilkinson, D., Selby, A., et al. (2010) Muscle full effect after oral protein: time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling. *Am J Clin Nutr* **92**, 1080-1088.

[29] Bohe, J., Low, J. F., Wolfe, R. R., Rennie, M. J. (2001) Latency and duration of stimulation of human muscle protein synthesis during continuous infusion of amino acids. *J Physiol* **532**, 575-579.

[30] Areta, J. L., Burke, L. M., Ross, M. L., Camera, D. M., West, D. W., et al. (2013) Timing and distribution of protein ingestion during prolonged recovery from resistance exercise alters myofibrillar protein synthesis. *J Physiol*.

[31] Tang, J. E., Moore, D. R., Kujbida, G. W., Tarnopolsky, M. A., Phillips, S. M. (2009) Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men. *J Appl Physiol* **107**, 987-992.

[32] Churchward-Venne, T. A., Burd, N. A., Mitchell, C. J., West, D. W., Philp, A., et al. (2012) Supplementation of a suboptimal protein dose with leucine or essential amino acids: effects on myofibrillar protein synthesis at rest and following resistance exercise in men. *J Physiol*.

[33] Wilkinson, S. B., Tarnopolsky, M. A., Macdonald, M. J., Macdonald, J. R., Armstrong, D., et al. (2007) Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage. *Am J Clin Nutr* **85**, 1031-1040.

[34] Robinson, M. J., Burd, N. A., Breen, L., Rerecich, T., Yang, Y., et al. (2013) Dose-dependent responses of myofibrillar protein synthesis with beef ingestion are enhanced with resistance exercise in middle-aged men. *Appl Physiol Nutr Metab* **38**, 120-125.

[35] Pennings, B., Boirie, Y., Senden, J. M., Gijsen, A. P., Kuipers, H., et al. (2011) Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate in older men. *Am J Clin Nutr* **93**, 997-1005.

[36] Volek, J. S., Volk, B. M., Gomez, A. L., Kunces, L. J., Kupchak, B. R., et al. (2013) Whey protein supplementation during resistance training augments lean body mass. *J Am Coll Nutr* **32**, 122-135.

[37] Phillips, S. M., Tang, J. E., Moore, D. R. (2009) The role of milk- and soy-based protein in support of muscle protein synthesis and muscle protein accretion in young and elderly persons. *J Am Coll Nutr* **28**, 343-354.

[38] Boirie, Y., Dangin, M., Gachon, P., Vasson, M. P., Maubois, J. L., et al. (1997) Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc Natl Acad Sci U S A* **94**, 14930-14935.

[39] West, D. W., Burd, N. A., Coffey, V. G., Baker, S. K., Burke, L. M., et al. (2011) Rapid aminoacidemia enhances myofibrillar protein synthesis and anabolic intramuscular signaling responses after resistance exercise. *Am J Clin Nutr* **94**, 795-803.

[40] Atherton, P. J., Smith, K., Etheridge, T., Rankin, D., Rennie, M. J. (2010) Distinct anabolic signalling responses to amino acids in C2C12 skeletal muscle cells. *Amino Acids* **38**, 1533-1539.

[41] Dibble, C. C., Manning, B. D. (2013) Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. *Nat Cell Biol* **15**, 555-564.

[42] Wilkinson, D. J., Hossain, T., Hill, D. S., Phillips, B. E., Crossland, H., et al. (2013) Effects of leucine and its metabolite beta-hydroxy-beta-methylbutyrate on human skeletal muscle protein metabolism. *J Physiol* **591**, 2911-2923.

[43] Fujita, S., Dreyer, H. C., Drummond, M. J., Glynn, E. L., Cadenas, J. G., et al. (2007) Nutrient signalling in the regulation of human muscle protein synthesis. *J Physiol* **582**, 813-823.

[44] Sancak, Y., Peterson, T. R., Shaul, Y. D., Lindquist, R. A., Thoreen, C. C., et al. (2008) The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* **320**, 1496-1501.

[45] Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A. L., Nada, S., et al. (2010) Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell* **141**, 290-303.

[46] Nobukuni, T., Joaquin, M., Roccio, M., Dann, S. G., Kim, S. Y., et al. (2005) Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc Natl Acad Sci U S A* **102**, 14238-14243.

[47] Gulati, P., Gaspers, L. D., Dann, S. G., Joaquin, M., Nobukuni, T., et al. (2008) Amino acids activate mTOR complex 1 via Ca2+/CaM signaling to hVps34. *Cell Metab* **7**, 456-465.

[48] Findlay, G. M., Yan, L., Procter, J., Mieulet, V., Lamb, R. F. (2007) A MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling. *Biochem J* **403**, 13-20.

[49] Sengupta, S., Peterson, T. R., Sabatini, D. M. (2010) Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. *Mol Cell* **40**, 310-322.

[50] Han, J. M., Jeong, S. J., Park, M. C., Kim, G., Kwon, N. H., et al. (2012) Leucyl-tRNA synthetase is an intracellular leucine sensor for the mTORC1-signaling pathway. *Cell* **149**, 410-424.

[51] Hundal, H. S., Taylor, P. M. (2009) Amino acid transceptors: gate keepers of nutrient exchange and regulators of nutrient signaling. *Am J Physiol Endocrinol Metab* **296**, E603-613.

[52] Dickinson, J. M., Rasmussen, B. B. (2013) Amino acid transporters in the regulation of human skeletal muscle protein metabolism. *Curr Opin Clin Nutr Metab Care* **16**, 638-644.

[53] Drummond, M. J., Glynn, E. L., Fry, C. S., Timmerman, K. L., Volpi, E., et al. (2010) An increase in essential amino acid availability upregulates amino acid transporter expression in human skeletal muscle. *Am J Physiol Endocrinol Metab* **298**, E1011-1018.

[54] Egan, B., Zierath, J. R. (2013) Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metab* **17**, 162-184.

[55] Timmons, J. A. (2011) Variability in training-induced skeletal muscle adaptation. *J Appl Physiol (1985)* **110**, 846-853.

[56] Kumar, V., Selby, A., Rankin, D., Patel, R., Atherton, P., et al. (2009) Age-related differences in the dose-response relationship of muscle protein synthesis to resistance exercise in young and old men. *J Physiol* **587**, 211-217.

[57] Burd, N. A., Holwerda, A. M., Selby, K. C., West, D. W., Staples, A. W., et al. (2010) Resistance exercise volume affects myofibrillar protein synthesis and anabolic signalling molecule phosphorylation in young men. *J Physiol* **588**, 3119-3130.

[58] Burd, N. A., West, D. W., Staples, A. W., Atherton, P. J., Baker, J. M., et al. (2010) Low-load high volume resistance exercise stimulates muscle protein synthesis more than high-load low volume resistance exercise in young men. *PLoS One* **5**, e12033.

[59] Dreyer, H. C., Drummond, M. J., Pennings, B., Fujita, S., Glynn, E. L., et al. (2008) Leucine-enriched essential amino acid and carbohydrate ingestion following resistance exercise enhances mTOR signaling and protein synthesis in human muscle. *Am J Physiol Endocrinol Metab* **294**, E392-400.

[60] Tipton, K. D., Rasmussen, B. B., Miller, S. L., Wolf, S. E., Owens-Stovall, S. K., et al. (2001) Timing of amino acid-carbohydrate ingestion alters anabolic response of muscle to resistance exercise. *Am J Physiol Endocrinol Metab* **281**, E197-206.

[61] Beelen, M., Koopman, R., Gijsen, A. P., Vandereyt, H., Kies, A. K., et al. (2008) Protein coingestion stimulates muscle protein synthesis during resistance-type exercise. *Am J Physiol Endocrinol Metab* **295**, E70-77.

[62] Fujita, S., Dreyer, H. C., Drummond, M. J., Glynn, E. L., Volpi, E., et al. (2009) Essential amino acid and carbohydrate ingestion before resistance exercise does not enhance postexercise muscle protein synthesis. *J Appl Physiol* **106**, 1730-1739.

[63] Beelen, M., Tieland, M., Gijsen, A. P., Vandereyt, H., Kies, A. K., et al. (2008) Coingestion of carbohydrate and protein hydrolysate stimulates muscle protein synthesis during exercise in young men, with no further increase during subsequent overnight recovery. *J Nutr* **138**, 2198-2204.

[64] Drummond, M. J., Dreyer, H. C., Fry, C. S., Glynn, E. L., Rasmussen, B. B. (2009) Nutritional and contractile regulation of human skeletal muscle protein synthesis and mTORC1 signaling. *J Appl Physiol (1985)* **106**, 1374-1384.

[65] Atherton, P. J., Rennie, M. J. (2009) It's no go for protein when it's all go. *J Physiol* **587**, 1373-1374.

[66] Kimball, S. R., Farrell, P. A., Jefferson, L. S. (2002) Invited Review: Role of insulin in translational control of protein synthesis in skeletal muscle by amino acids or exercise. *J Appl Physiol (1985)* **93**, 1168-1180.

[67] Baar, K., Esser, K. (1999) Phosphorylation of p70(S6k) correlates with increased skeletal muscle mass following resistance exercise. *Am J Physiol* **276**, C120-127.

[68] Bodine, S. C., Stitt, T. N., Gonzalez, M., Kline, W. O., Stover, G. L., et al. (2001) Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol* **3**, 1014-1019.

[69] Dreyer, H. C., Fujita, S., Cadenas, J. G., Chinkes, D. L., Volpi, E., et al. (2006) Resistance exercise increases AMPK activity and reduces 4E-BP1 phosphorylation and protein synthesis in human skeletal muscle. *J Physiol* **576**, 613-624.

[70] Glover, E. I., Oates, B. R., Tang, J. E., Moore, D. R., Tarnopolsky, M. A., et al. (2008) Resistance exercise decreases eIF2Bepsilon phosphorylation and potentiates the feeding-induced stimulation of p70S6K1 and rpS6 in young men. *Am J Physiol Regul Integr Comp Physiol* **295**, R604-610.

[71] Dreyer, H. C., Fujita, S., Glynn, E. L., Drummond, M. J., Volpi, E., et al. (2010) Resistance exercise increases leg muscle protein synthesis and mTOR signalling independent of sex. *Acta Physiol (Oxf)* **199**, 71-81.

[72] O'Neil, T. K., Duffy, L. R., Frey, J. W., Hornberger, T. A. (2009) The role of phosphoinositide 3-kinase and phosphatidic acid in the regulation of mammalian target of rapamycin following eccentric contractions. *J Physiol* **587**, 3691-3701.

[73] Drummond, M. J., Fry, C. S., Glynn, E. L., Timmerman, K. L., Dickinson, J. M., et al. (2011) Skeletal muscle amino acid transporter expression is increased in young and older adults following resistance exercise. *J Appl Physiol* **111**, 135-142.

[74] Tipton, K. D., Gurkin, B. E., Matin, S., Wolfe, R. R. (1999) Nonessential amino acids are not necessary to stimulate net muscle protein synthesis in healthy volunteers. *J Nutr Biochem* **10**, 89-95.

[75] Volpi, E., Kobayashi, H., Sheffield-Moore, M., Mittendorfer, B., Wolfe, R. R. (2003) Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults. *Am J Clin Nutr* **78**, 250-258.

[76] Crozier, S. J., Kimball, S. R., Emmert, S. W., Anthony, J. C., Jefferson, L. S. (2005) Oral leucine administration stimulates protein synthesis in rat skeletal muscle. *J Nutr* **135**, 376-382.

[77] Escobar, J., Frank, J. W., Suryawan, A., Nguyen, H. V., Kimball, S. R., et al. (2006) Regulation of cardiac and skeletal muscle protein synthesis by individual branched-chain amino acids in neonatal pigs. *Am J Physiol Endocrinol Metab* **290**, E612-621.

[78] Tipton, K. D., Elliott, T. A., Ferrando, A. A., Aarsland, A. A., Wolfe, R. R. (2009) Stimulation of muscle anabolism by resistance exercise and ingestion of leucine plus protein. *Appl Physiol Nutr Metab* **34**, 151-161.

[79] Churchward-Venne, T. A., Burd, N. A., Phillips, S. M., Research Group, E. M. (2012) Nutritional regulation of muscle protein synthesis with resistance exercise: strategies to enhance anabolism. *Nutr Metab (Lond)* **9**, 40.

[80] Escobar, J., Frank, J. W., Suryawan, A., Nguyen, H. V., Kimball, S. R., et al. (2005) Physiological rise in plasma leucine stimulates muscle protein synthesis in neonatal pigs by enhancing translation initiation factor activation. *Am J Physiol Endocrinol Metab* **288**, E914-921.

[81] Escobar, J., Frank, J. W., Suryawan, A., Nguyen, H. V., Davis, T. A. (2007) Amino acid availability and age affect the leucine stimulation of protein synthesis and eIF4F formation in muscle. *Am J Physiol Endocrinol Metab* **293**, E1615-1621.

[82] Rosenberg, I. H. (1989) Summary comments. *Am J Clin Nutr* **50**, 1231-1233.

[83] Baumgartner, R. N., Koehler, K. M., Gallagher, D., Romero, L., Heymsfield, S. B., et al. (1998) Epidemiology of sarcopenia among the elderly in New Mexico. *Am J Epidemiol* **147**, 755-763.

[84] Volpi, E., Mittendorfer, B., Rasmussen, B. B., Wolfe, R. R. (2000) The response of muscle protein anabolism to combined hyperaminoacidemia and glucose-induced hyperinsulinemia is impaired in the elderly. *J Clin Endocrinol Metab* **85**, 4481-4490.

[85] Katsanos, C. S., Kobayashi, H., Sheffield-Moore, M., Aarsland, A., Wolfe, R. R. (2005) Aging is associated with diminished accretion of muscle proteins after the ingestion of a small bolus of essential amino acids. *Am J Clin Nutr* **82**, 1065-1073.

[86] Kumar, V., Atherton, P. J., Selby, A., Rankin, D., Williams, J., et al. (2012) Muscle protein synthetic responses to exercise: effects of age, volume, and intensity. *J Gerontol A Biol Sci Med Sci* **67**, 1170-1177.

[87] Wilkes, E. A., Selby, A. L., Atherton, P. J., Patel, R., Rankin, D., et al. (2009) Blunting of insulin inhibition of proteolysis in legs of older subjects may contribute to age-related sarcopenia. *Am J Clin Nutr* **90**, 1343-1350.

[88] Timmerman, K. L., Lee, J. L., Fujita, S., Dhanani, S., Dreyer, H. C., et al. (2010) Pharmacological vasodilation improves insulin-stimulated muscle protein anabolism but not glucose utilization in older adults. *Diabetes* **59**, 2764-2771.

[89] Timmerman, K. L., Dhanani, S., Glynn, E. L., Fry, C. S., Drummond, M. J., et al. (2012) A moderate acute increase in physical activity enhances nutritive flow and the muscle protein anabolic response to mixed nutrient intake in older adults. *Am J Clin Nutr* **95**, 1403-1412.

[90] Vincent, M. A., Clerk, L. H., Lindner, J. R., Price, W. J., Jahn, L. A., et al. (2006) Mixed meal and light exercise each recruit muscle capillaries in healthy humans. *Am J Physiol Endocrinol Metab* **290**, E1191-1197.

[91] Taddei, S., Virdis, A., Ghiadoni, L., Salvetti, G., Bernini, G., et al. (2001) Age-related reduction of NO availability and oxidative stress in humans. *Hypertension* **38**, 274-279.

[92] Dillon, E. L., Casperson, S. L., Durham, W. J., Randolph, K. M., Urban, R. J., et al. (2011) Muscle protein metabolism responds similarly to exogenous amino acids in healthy younger and older adults during NO-induced hyperemia. *Am J Physiol Regul Integr Comp Physiol* **301**, R1408-1417.

[93] Wu, G., Meininger, C. J., Knabe, D. A., Bazer, F. W., Rhoads, J. M. (2000) Arginine nutrition in development, health and disease. *Curr Opin Clin Nutr Metab Care* **3**, 59-66.

[94] Osowska, S., Duchemann, T., Walrand, S., Paillard, A., Boirie, Y., et al. (2006) Citrulline modulates muscle protein metabolism in old malnourished rats. *Am J Physiol Endocrinol Metab* **291**, E582-586.

[95] Norton, L. E., Layman, D. K., Bunpo, P., Anthony, T. G., Brana, D. V., et al. (2009) The leucine content of a complete meal directs peak activation but not duration of skeletal muscle protein synthesis and mammalian target of rapamycin signaling in rats. *J Nutr* **139**, 1103-1109.

[96] Curis, E., Nicolis, I., Moinard, C., Osowska, S., Zerrouk, N., et al. (2005) Almost all about citrulline in mammals. *Amino Acids* **29**, 177-205.

CHAPTER 2

TITLE: Supplementation of a suboptimal protein dose with leucine or essential amino acids: effects on myofibrillar protein synthesis at rest and following resistance exercise in men

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Supplementation of a suboptimal protein dose with leucine or essential amino acids: effects on myofibrillar protein synthesis at rest and following resistance exercise in men

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Key points summary:

- Essential amino acids (EAA) stimulate increased rates of myofibrillar protein synthesis (MPS).
- Leucine is a key regulator of MPS in rodents, however its importance relative to the other EAA is not clear.
- About 20g of protein maximally stimulates MPS after resistance exercise in young men, but we do not know if smaller doses can be made better by adding certain amino acids.
- We report that a suboptimal dose of whey protein (6.25g) supplemented with either leucine or a mixture of EAAs without leucine stimulates MPS similar to 25g of whey protein under resting conditions; however, only 25g of whey sustains exercise-induced rates of MPS.
- Adding leucine or a mixture of EAA without leucine to a suboptimal dose of whey is as effective as 25g whey at stimulating fed rates of MPS, however 25g of whey is better suited to increase resistance exercise-induced muscle anabolism.

Word count – 149

ABSTRACT

Leucine is a nutrient regulator of muscle protein synthesis by activating mTOR and possibly other proteins in this pathway. The purpose of this study was to examine the role of leucine in the regulation of human myofibrillar protein synthesis (MPS). Twenty-four males completed an acute bout of unilateral resistance exercise prior to consuming either: a dose (25 g) of whey protein (WHEY); 6.25 g whey protein with total leucine equivalent to WHEY (LEU); or 6.25 g whey protein with total essential amino acids (EAA) equivalent to WHEY for all EAA except leucine (EAA-LEU). Measures of MPS, signalling through mTOR, and amino acid transporter (AAT) mRNA abundance were made while fasted (FAST), and following feeding under rested (FED) and post-exercise (EX-FED) conditions. Leucinemia was equivalent between WHEY and LEU and elevated compared to EAA-LEU ($P = 0.001$). MPS was increased above FAST at 1-3h post-exercise in both FED ($P < 0.001$) and EX-FED ($P < 0.001$) conditions with no treatment effect. At 3-5h, only WHEY remained significantly elevated above FAST in EX-FED (WHEY 184% vs. LEU 55% and EAA-LEU 35%; $P = 0.036$). AAT mRNA abundance was increased above FAST after feeding and exercise with no effect of leucinemia. In summary, a low dose of whey protein supplemented with leucine or all other essential amino acids was as effective as a complete protein (WHEY) in stimulating postprandial MPS; however only WHEY was able to sustain increased rates of MPS post-exercise and may therefore be most suited to increase exercise-induced muscle protein accretion.

Abbreviations used: 4E-BP1, eukaryotic initiation factor 4E binding protein 1; AAT, amino acid transporter; Akt, protein kinase B; ATF4, activating transcription factor 4; AUC, area-under-the-curve; BCAA, branch-chain amino acid; CD98, glycoprotein CD98; ERK 1/2, extracellular signal-regulated kinase 1/2; EAA, essential amino acid; EAA-LEU, nutritional treatment consisting of 6.25 g whey protein supplemented with a mixture of essential amino acids but no leucine; EX-FED, response to combined feeding and resistance exercise; FAST, rested fasted condition; FED, response to feeding; FSR, fractional synthetic rate; GCN2, general control nonrepressed; LAT1, L-type amino acid transporter type 1; LEU, nutritional treatment consisting of 6.25 g whey protein supplemented with leucine; MPS, myofibrillar protein synthesis; mTOR, mammalian target of rapamycin; p38 MAPK, p38 mitogen activated protein kinase; p70S6k, 70 kDa ribosomal protein S6 kinase 1; PAT1, proton-coupled amino acid transporter type 1; WHEY, nutritional treatment consisting of 25 g whey protein.

INTRODUCTION

Ingestion or infusion of amino acids stimulates an increase in skeletal muscle protein synthesis (Bennet *et al.*, 1989; Bohe *et al.*, 2001; Bohe *et al.*, 2003; Atherton *et al.*, 2010a), an effect that is enhanced by prior resistance exercise (Tipton *et al.*, 1999a; Wilkinson *et al.*, 2007; Moore *et al.*, 2009a; Moore *et al.*, 2009b; Tang *et al.*, 2009; West *et al.*, 2009). The essential amino acids (EAA) are primarily responsible for this stimulation of muscle protein synthesis, with no apparent requirement for the non-essential amino acids (Smith *et al.*, 1998; Tipton *et al.*, 1999b; Borsheim *et al.*, 2002; Volpi *et al.*, 2003). Several animal studies have demonstrated that leucine independently stimulates muscle protein synthesis by activating components of the mammalian target of rapamycin (mTOR) signalling cascade (Anthony *et al.*, 2000a; Anthony *et al.*, 2000b; Anthony *et al.*, 2002; Bolster *et al.*, 2004; Crozier *et al.*, 2005). This activation appears critical for both the contraction (Drummond *et al.*, 2009), and EAA-mediated (Dickinson *et al.*, 2011) increase in muscle protein synthesis. Thus, leucine has been investigated as a pharmaconutrient with the potential to promote increases in muscle protein synthesis (Koopman *et al.*, 2005; Katsanos *et al.*, 2006; Koopman *et al.*, 2006; Rieu *et al.*, 2006; Koopman *et al.*, 2008; Tipton *et al.*, 2009; Glynn *et al.*, 2010) and lean tissue mass (Verhoeven *et al.*, 2009; Leenders *et al.*, 2011). Nonetheless, while some studies indicate a role for leucine in the regulation of human muscle protein synthesis (Smith *et al.*, 1992; Katsanos *et al.*, 2006; Rieu *et al.*, 2006), other studies have not found an enhanced rate of muscle protein synthesis following leucine infusion (Nair *et al.*, 1992), after increasing the amount of leucine within a mixed EAA solution (Glynn *et al.*, 2010), or by the

addition of free leucine to a protein containing supplement (Koopman *et al.*, 2008; Tipton *et al.*, 2009).

There is a dose-dependent relationship between amino acid (Bohe *et al.*, 2003; Cuthbertson *et al.*, 2005) and protein (Moore *et al.*, 2009a) provision and muscle protein synthesis. We previously reported that ~20 g of isolated egg protein (containing ~8.6 g EAA and ~1.7 g leucine) stimulated muscle protein synthesis after resistance exercise above that observed with both 5 g and 10 g of protein but was not further stimulated with ingestion of 40g of protein indicating that 20g of egg protein is saturating for muscle protein synthesis after resistance exercise (Moore *et al.*, 2009a). These data are consistent with previous reports of a dose-dependent relationship between EAA ingestion and myofibrillar protein synthesis (MPS) up to a maximal stimulation at ~10 g EAA [containing ~2.1g leucine; (Cuthbertson *et al.*, 2005)]. These dose-response data may provide insight into why other studies (Koopman *et al.*, 2008; Tipton *et al.*, 2009; Glynn *et al.*, 2010) did not report a benefit of additional leucine on muscle protein synthesis when a sufficient amount of EAA and/or leucine is provided.

Given what we know about the ingested protein dose-response of muscle protein synthesis (Bohe *et al.*, 2003; Cuthbertson *et al.*, 2005; Moore *et al.*, 2009a), the aim of the present investigation was to examine the effects of supplementing a ‘sub-optimal’ dose of whey protein (6.25 g whey containing ~0.75 g of leucine) with additional leucine (LEU), or a mixture of EAA with no leucine (EAA-LEU) on MPS at rest and following acute resistance exercise compared to a dose (25 g containing ~3.0 g of leucine) of whey protein (WHEY) which is sufficient to induce a maximal stimulation of muscle protein

synthesis after resistance exercise (Moore *et al.*, 2009a). The sub-optimal protein dose (6.25 g) was chosen to represent ¼ of the 25 g dose in the WHEY treatment. We hypothesized that LEU would result in a stimulation of MPS equivalent to WHEY in both feeding (FED) and combined feeding and resistance exercise (EX-FED) conditions. Alternatively, we hypothesized that EAA-LEU would result in an increase in MPS in both the FED and EX-FED conditions, but the response would be significantly less than both LEU and WHEY due to the lower leucine content. In an attempt to gain insight into the mechanistic underpinnings of the response of MPS, we also examined changes in the phosphorylation status of protein targets of the Akt-mTOR pathway and in the mRNA abundance of select amino acid transporters (AAT) that have recently been shown to be regulated by EAA (Drummond *et al.*, 2010) and resistance exercise (Drummond *et al.*, 2011).

METHODS

Participants and Ethical Approval. Twenty-four recreationally active, young adult male participants (22±0.6 years; 1.80±0.02 m; 76.4±2.0 kg; BMI 24.3±0.6 kg•m^{-2}) voluntarily agreed to participate in the study. Participants were deemed healthy based on responses to a routine health screening questionnaire. Each participant was informed of the purpose of the study, the associated experimental procedures, and any potential risks prior to providing written consent. The study was approved by the Hamilton Health Sciences Research Ethics Board and conformed to the standards for the use of human subjects in research as outlined in the most recent update of the *Declaration of Helsinki*. The study

also conformed to the standards established by the Canadian Tri-Council Policy on the ethical use of human subjects (2010)

Experimental Design. Approximately 1-2 weeks prior to participating in the experimental infusion trial, study participants underwent unilateral strength testing of the knee-extensor muscles. Participants performed a 10 repetition maximum (10-RM) test of both standard seated knee-extension (Atlantis Precision Series C-105) and seated leg press (Maxam Strength, Hamilton, Ontario, Canada) exercise with their dominant leg. In addition, each participant underwent a whole-body dual-energy X-ray absorptiometry scan (QDR-4500A; Hologic; software version 12.31) to measure body composition. The study participants physical characteristics are shown in **Table 1**. Participants were assigned to one of three post-exercise nutritional treatment groups (described below) that were counter-balanced for bodyweight.

Table 1. Participants characteristics

	WHEY	LEU	EAA-LEU
Age, y	22.1 (0.8)	21.5 (1.1)	22.5 (1.3)
Height, m	1.8 (0.02)	1.8 (0.02)	1.8 (0.02)
Weight, kg	77.3 (3.9)	76.5 (3.9)	75.4 (2.7)
BMI, kg/m^2	25.0 (1.2)	24.2 (1.2)	23.8 (0.7)
Fat-free mass, kg	63.2 (2.9)	64.6 (3.8)	63.4 (2.4)
Bodyfat, %	17.9 (2.2)	16.1 (2.4)	16.5 (1.2)

Values are mean ± SEM (n = 8 per treatment group).

Study participants were provided with a pre-packaged standardized diet that was consumed the day prior to the experimental infusion trial. Diets were designed to provide sufficient energy to maintain energy balance as determined by the Harris-Benedict

equation and were adjusted using a moderate activity factor (1.4-1.6) to account for participants reported physical activity patterns. The macronutrient distribution was 55% carbohydrates, 30% lipids, and 15% protein. The study participants were told to refrain from physical exercise for 72 h prior to the experimental infusion trial and to consume their evening meal no later than 2200 h.

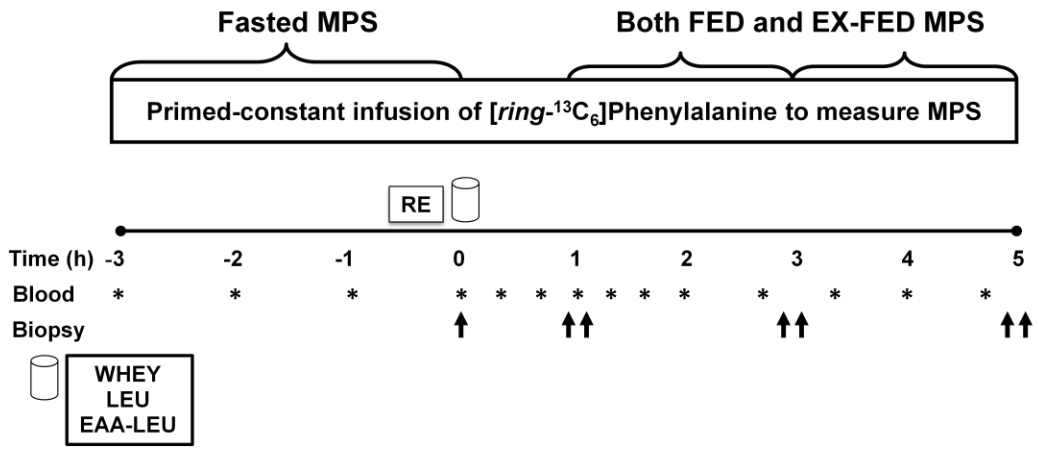


Figure 1. Schematic of the experimental protocol. Study participants consumed either EAA-LEU, LEU, or WHEY (see Methods) in single-blinded fashion (n = 8 per treatment group) immediately following resistance exercise. Exercise consisted of 4 sets each of unilateral seated knee extension and leg press. Asterisk indicates blood sample; single upward arrow indicates unilateral biopsy; double upward arrow indicates bilateral biopsy.

Infusion Protocol. Participants reported to the lab at ~0600 the morning of the experimental infusion trial in an overnight postabsorptive state. A catheter was inserted into an antecubital vein and a baseline blood sample was taken before initiating a 0.9% saline drip to keep the catheter patent to allow for repeated arterialized blood sampling over the course of the experimental trial. Arterialized blood samples (Copeland *et al.*,

1992) were obtained repeatedly over the course of the infusion trial by wrapping a heating blanket around the forearm. Blood samples were collected into 4 ml heparinized evacuated tubes and chilled on ice. A second catheter was placed in the antecubital vein of the opposite arm before initiating a primed continuous infusion (0.05 μmol•kg^{-1}•min^{-1}; 2.0 μmol•kg^{-}1 prime) of [*ring*-$^{13}C_6$] phenylalanine (Cambridge Isotope Laboratories, Woburn, MA). The infusate was passed through a 0.2-μm filter before entering the participant's bloodstream. Our research group has recently validated a method (Burd *et al.*, 2011) in which the resting (fasted) fractional synthetic rate (FSR) of MPS is calculated based on the ^{13}C enrichment of a pre-infusion baseline blood sample obtained from tracer-naïve participants, and a single biopsy taken following a period of tracer incorporation (Miller *et al.*, 2005; Mittendorfer *et al.*, 2005; Tang *et al.*, 2009; West *et al.*, 2009; Burd *et al.*, 2010b; Tang *et al.*, 2011). This method assumes that the ^{13}C enrichment of a mixed plasma protein fraction reflects the ^{13}C enrichment of muscle protein (Heys *et al.*, 1990). Thus, the baseline rate of MPS was calculated using a pre-infusion baseline blood sample and a single resting skeletal muscle biopsy sample obtained ~2.5 h after the onset of the primed constant infusion. Participants then performed an acute bout of unilateral resistance exercise consisting of 4 sets of 10-12 repetitions of both seated knee-extension (Atlantis Precision Series C-105) and leg-press (Maxam Fitness, Hamilton, Ontario, Canada) exercise at ~95% of their previously determined 10-RM with an inter-set rest-interval of 2 minutes. Immediately following completion of the resistance exercise, participants were administered 1 of 3 post-exercise nutrient treatments orally in a single-blinded fashion and bilateral biopsy samples were obtained at 1, 3, and 5 h post-

exercise recovery from a FED) and EX-FED leg. Muscle biopsies were obtained from the *vastus lateralis* muscle using a 5 mm Bergström needle modified for manual suction under 2% xylocaine local anaesthesia. Biopsy samples were immediately freed from visible blood, fat, and connective tissue, and immediately frozen in liquid nitrogen for further analysis as previously described (West *et al.*, 2009; Burd *et al.*, 2010a). Each biopsy sample was obtained from a separate incision ~ 4-5cm apart. Each participant underwent a total of 7 skeletal muscle biopsies; 4 from the rested leg, and 3 from the exercised leg. Specific details of the infusion protocol are outlined in **Figure 1**.

Drink Composition. Study participants were administered protein/amino acid based nutrient solutions in a blinded manner. The amino acid/protein composition of each of the 3 nutrient treatments is outlined in **Table 2**. Briefly, the 3 nutrient treatments were as follows: WHEY, which consisted of: 25g whey protein isolate (total leucine = 3.0 g); LEU: 6.25 g whey protein isolate supplemented with free-form leucine (total leucine = 3.0 g); EAA-LEU: 6.25 g whey protein isolate supplemented with free-form EAA but without added leucine (total EAA = to WHEY for each individual EAA except leucine which was 0.75 g). The whey protein isolate (biPro, Davisco Foods, Le Sueur, MN) was independently tested (Telmark, Matawan, NJ) in triplicate for content analysis. The free-form essential amino acids used were as follows: L-leucine, L-isoleucine, L-valine, L-histidine, L-phenylalanine, L(+)-lysine, L-threonine, and L-methionine (Sigma Life Science; Sigma-Aldrich, St. Louis MO). All nutrient solutions were prepared with 300 mL of water (see Table 2). To minimize disturbances in isotopic equilibrium following amino acid ingestion, nutrient solutions were enriched to 4% with tracer according to a

phenylalanine content of 3.5% in whey protein. Our research group has recently shown this method to be valid for maintaining isotopic steady state in both the plasma free and muscle intracellular free precursor pools after protein ingestion and resistance exercise (Burd *et al.*, 2011)

Table 2. Total and essential amino acid content of the nutritional treatments

	Nutritional Treatment		
	WHEY	**LEU**	**EAA-LEU**
Alanine, g	1.15	0.29	0.29
Arginine, g	0.53	0.13	0.13
Aspartic Acid, g	2.80	0.70	0.70
Cystine, g	0.78	0.19	0.19
Glutamic Acid, g	4.10	1.03	1.03
Glycine, g	0.43	0.11	0.11
Proline, g	1.05	0.26	0.26
Serine, g	0.63	0.16	0.16
Tyrosine, g	0.88	0.22	0.22
Tryptophan, g	0.68	0.17	0.17
Histidine, g*	0.55	0.14	0.55
Isoleucine, g*	1.35	0.34	1.35
Leucine, g*	3.00	3.00	0.75
Lysine, g*	2.70	0.68	2.70
Methionine, g*	0.58	0.14	0.58
Phenylalanine, g*	0.88	0.22	0.88
Threonine, g*	1.10	0.28	1.10
Valine, g*	1.38	0.34	1.38
Total, g	24.57	8.40	12.55
ΣEAA, g	11.54	5.14	9.29
ΣNEAA, g	13.03	3.26	3.26

* Content included as an essential amino acid (EAA). Non-essential amino acid (NEAA)

Analytical Methods. Blood glucose was measured using a blood glucose meter (OneTouch Ultra 2, Lifescan Inc., Milpitas, CA, USA). Blood amino acid concentrations

were analyzed by high performance liquid chromatography (HPLC) as described previously (Wilkinson *et al.*, 2007). Plasma L-[*ring*-$^{13}C_6$] phenylalanine enrichment was determined as previously described (Glover *et al.*, 2008). Plasma insulin concentration was measured using a commercially available immunoassay kit (ALPCO Diagnostics, Salem, NH, USA).

Muscle samples (~40-50 mg) were homogenized on ice in buffer (10 μl mg^{-1} 25mM Tris 0.5% v/v Triton X-100 and protease/phosphatase inhibitor cocktail tablets (Complete Protease Inhibitor Mini-Tabs, Roche, Indianapolis, IN, USA; PhosSTOP, Roche Applied Science, Mannhein, Germany)). Samples were then centrifuged at 15,000 *g* for 10 minutes 4°C. The supernatant was removed and protein concentrations were determined via the Bradford Assay. The pellet containing the myofibrillar proteins was stored at -80° C until future processing. Working samples of equal concentration were prepared in Laemmli buffer (Laemmli, 1970). Equal amounts (20 µg) of protein were loaded onto 10% or 15% SDS-polyacrylamide gels for separation by electrophoresis. Proteins were then transferred to a polyvinylidene fluoride membrane, blocked (5% skim milk) and incubated overnight at 4°C in primary antibody: phospho-Akt^{Ser473} (1:1000, Cell Signalling Technology, #9271) phospho-mTOR^{Ser2448} (1:1000, Cell Signalling Technology, #2971) phospho-p70S6k^{Thr389} (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz,CA, USA; #11759), phospho-4E-BP1$^{Thr37/46}$ (1:1000, Cell Signalling Technology, #9459), phospho-Erk1/2 $^{Tyr202/204}$ (1:1000, Cell Signalling Technology, #9101), and phospho-p38$^{Thr180/Tyr182}$ (1:1000, Cell Signalling Technology, #9215). Membranes were then washed and incubated in secondary antibody (1 h at room temperature) before

detection with chemiluminescence (SuperSignalWest Dura Extended Duration Substrate, ThermoScientific, #34075) on a FluorChem SP Imaging system (Alpha Innotech, Santa Clara, CA, USA). Phosphorylation status was expressed relative to α-tubulin abundance (1:2000, Sigma-Alderich, St. Louis, MO, USA #T6074) and is presented for each protein as a fold-change from rested fasted conditions (FAST). Images were quantified by spot densitometry using ImageJ software (National Institute of Health, USA).

RNA was isolated from muscle using the phenol/chloroform method as previously described (Philp *et al.*, 2010). RNA was quantified using an Epoch Multi-Volume Spectrophotometer (BioTek, Winooski, VT) at 260 and 280 nm. Firststrand cDNA was synthesized on a Thermo Hybaid cycler (Thermo Scientific) from 1 µg of RNA using the reverse transcription system (Promega, Hampshire, UK) according to the manufacturer's instructions.

Quantitative real-time PCR was performed to measure relative mRNA expression using an Eppendorf Light Cycler PCR machine, SYBR Green PCR plus reagents (Sigma Aldrich), and previously published primers for LAT1, CD98, PAT1, GCN2, and ATF4 (Drummond *et al.*, 2010; Drummond *et al.*, 2011). 10µl PCR reactions were assayed in triplicate on a 96-well heat-sealed PCR plate (Thermo Fisher Scientific). Each reaction contained 5 µl of SYBR Green *Taq*, 1 µl of forward and reverse primers, and 3 µl of cDNA (1:10 dilution). Target gene expression was calculated relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed normalized to basal (FAST) values. Absolute CT for GAPDH was unchanged by any of the treatments (data not shown).

Muscle biopsy samples were processed as previously described (Moore *et al.*, 2009b). Briefly, to determine the intracellular enrichment, ~20-25 mg of muscle was homogenized in 0.6 M perchloric acid/L. Free amino acids in the resulting supernatant fluid were then passed over an ion-exchange resin (Dowex 50WX8-200 resin Sigma-Aldrich Ltd) and converted to their heptafluorobutyric derivatives for analysis via gas chromatography–mass spectrometry (models 6890 GC and 5973 MS; Hewlett-Packard) by monitoring ions 316 and 322 after electron ionization. To determine muscle free intracellular amino acid concentrations, samples were processed as previously described (Wilkinson *et al.*, 2007). Briefly, muscle samples were derivatized and analyzed by HPLC (HPLC: Waters model 2695; column: Waters Nova-Pak C_{18}, 4 µm; detector: Waters 474 scanning fluorescence detector). This method achieved separation of 19 of the 20 physiologic amino acids, with the exception of tryptophan (not included in the analysis). To determine myofibrillar protein-bound enrichments, a separate piece (~40-50 mg) of muscle was homogenized in a standard buffer containing protease and phosphatase inhibitors as described above under 'Immunoblotting'. The supernatant fluid was collected for Western blot analysis as described above, and the pellet was further processed to extract myofibrillar proteins as previously described (Moore *et al.*, 2009b). The resulting myofibrillar 'enriched' protein pellet was hydrolyzed in 6 M HCL at 110° overnight. Subsequently, the free amino acids were purified using ion-exchange chromatography and converted to their N-acetyl-n-propyl ester derivatives for analysis by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS: Hewlett Packard 6890; IRMS model Delta Plus XP, Thermo Finnagan, Waltham, MA USA).

Calculations. The fractional synthetic rate (FSR) of MPS was calculated using the standard precursor-product equation:

$$FSR = [(E_{2b} - E_{1b}) / (E_{IC} \times t)] \times 100$$

Where E_b is the enrichment of bound (myofibrillar) protein, E_{IC} is the average enrichment of the intracellular free amino acid precursor pool of two muscle biopsies, and t is the tracer incorporation time in h. The utilization of "tracer naïve" subjects allowed us to use a pre-infusion blood sample (i.e., a mixed plasma protein fraction) as the baseline enrichment (E_{1b}) for calculation of resting (i.e. fasted) FSR (Miller *et al.*, 2005; Mittendorfer *et al.*, 2005; Tang *et al.*, 2009). This approach is based on the fact that the 'natural' ^{13}C enrichment ($\delta^{13}C_{PDB}$) in blood is the same as that of muscle protein; an assumption recently confirmed by our research group (West *et al.*, 2009) and others (Heys *et al.*, 1990).

Statistics. Anthropometric measures and strength tests were compared using a one-factor (treatment) ANOVA. Blood amino acids (leucine, BCAA, EAA, total amino acids), plasma insulin, and blood glucose were analyzed using a two-factor (treatment × time) repeated measures ANOVA. Blood leucine AUC was analyzed using a one-factor (treatment) ANOVA. Plasma enrichments were analyzed using a two-factor (treatment × time) repeated measures ANOVA and linear regression. Intracellular precursor pool enrichments were analyzed using a two-factor (treatment × time) repeated measures ANOVA for each condition (i.e. FED and EX-FED), a two-factor ANOVA (treatment × condition) at each time point (1, 3, and 5h), and linear regression. Intracellular amino acids, protein phosphorylation, mRNA expression, and myofibrillar FSR were analyzed

using a two-factor (treatment × time) repeated measures ANOVA for each condition and a two-factor ANOVA (treatment × condition) at each time point. Protein phosphorylation and mRNA abundance are expressed as fold-change from FAST. A Tukey post-hoc analysis was performed whenever a significant F ratio was found to isolate specific differences. Statistical analyses were performed using SigmaStat 3.1 software (Systat Software Inc., Point Richmond, CA). Values are expressed as means ± standard error of the mean (SEM), and means were considered to be statistically different for P values < 0.05.

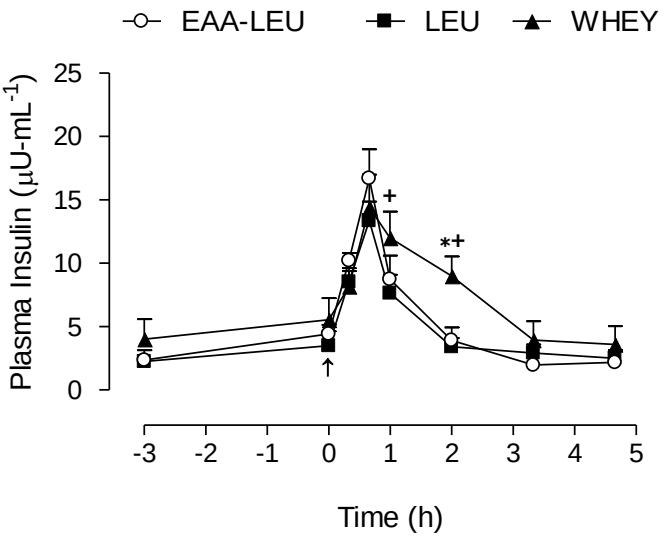
RESULTS

Participant characteristics. Participant characteristics are shown in Table 1. There were no differences between treatment groups for any anthropometric variable measured.

Exercise variables. There were no differences between treatment groups for participant's unilateral 10-RM test when measured for seated leg-press (P = 0.68) or knee extension exercise (P = 0.78). Further, the exercise volume, defined as the product of exercise load (kg) and repetitions (i.e. load × repetitions) was not different per set between treatment groups for either seated leg press (P= 0.78) or knee extension exercise (P = 0.78; data not shown).

Blood glucose, plasma insulin, and blood amino acid concentrations. Baseline blood glucose averaged 5.3±0.1 mmol/L in each treatment group, and did not differ between treatment groups (P = 0.81). Plasma insulin concentration peaked at 40 minutes post treatment administration in all treatment groups before declining. However, insulin concentration following WHEY remained elevated above LEU at 1 h, and both LEU and

EAA-LEU at 2 h post-treatment (see **Supplemental Figure 1** under "Supplemental data" in the online issue).



Supplemental Figure 1. Mean (±SEM) plasma insulin concentration (µU-mL^{-1}) following EAA-LEU, LEU, and WHEY treatments. Upward arrow indicates time of treatment administration. *****Significantly greater than EAA-LEU ($P < 0.05$); **+**Significantly greater than LEU ($P < 0.05$).

Blood leucine concentrations showed a large but transient increase following LEU as compared to WHEY, with WHEY demonstrating a more moderate but sustained increase (**Figure 2A**). In brief, LEU was significantly increased above WHEY at 40 and 60 minutes, while WHEY was elevated above LEU at 80, 100, and 120 minutes post treatment administration. Despite these differences, area under the leucine curve was not different between LEU and WHEY; however, both treatments were significantly greater than EAA-LEU ($P = 0.001$ **Figure 2A inset**). Blood BCAA increased after treatment

administration, peaking at ~1-h for LEU and EAA-LEU. WHEY was significantly increased above LEU and EAA-LEU from 80-120 minutes, and LEU at 160 minutes after ingestion (**Figure 2B**). Blood EAA (including leucine) showed a similar interaction (treatment x time) effect ($P < 0.001$) with WHEY being elevated above LEU and EAA-LEU from 80-120 minutes and LEU at 160 minutes post treatment administration (**Figure 2C**). Blood total amino acid showed a significant interaction ($P < 0.001$) effect such that WHEY was significantly increased above LEU and EAA-LEU from 80-120 minutes post treatment administration (**Figure 2D**).

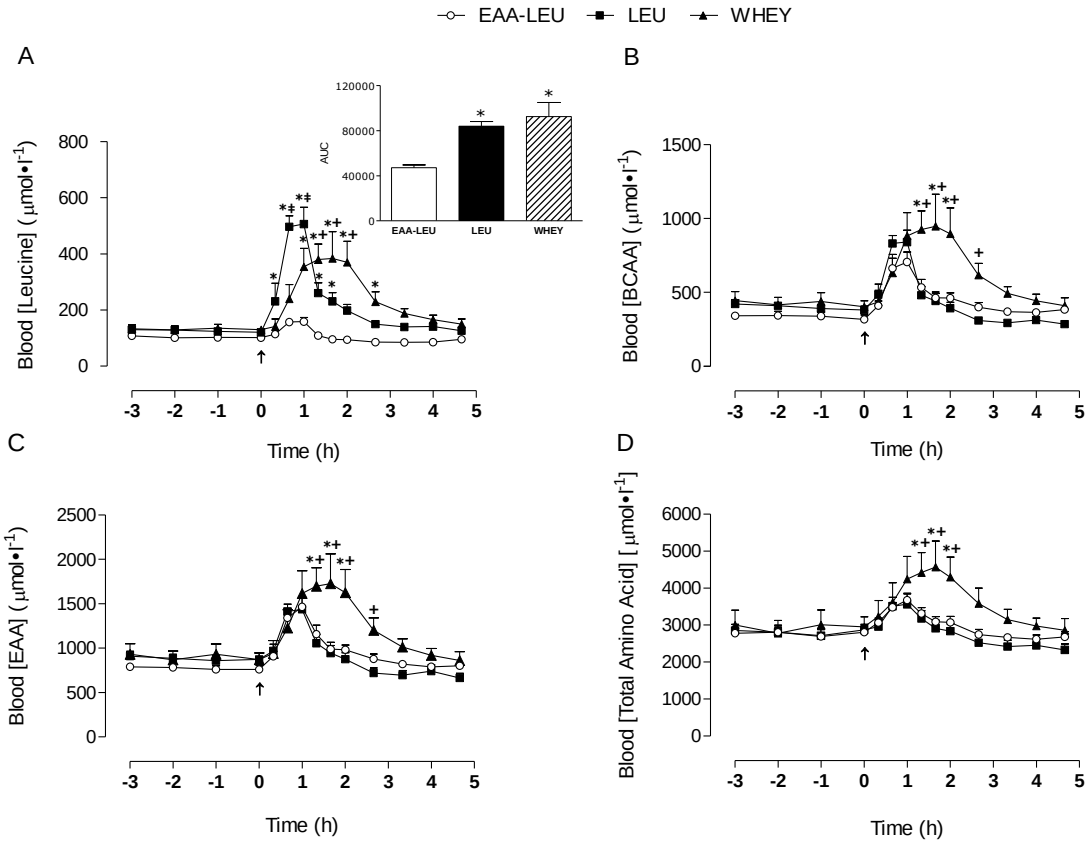
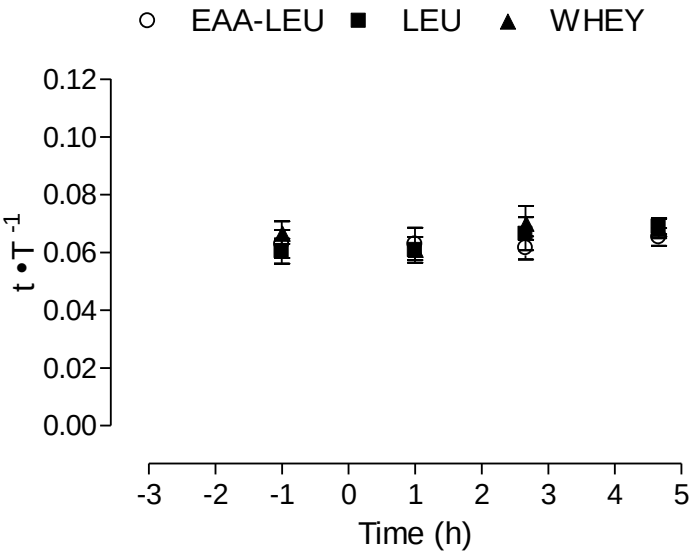


Figure 2. Mean (± SEM) blood concentrations (μmol•L^{-1}) of leucine (A), branched chain amino acids (BCAA) (B), essential amino acids (EAA) (C), and total amino acids (D) following EAA-LEU, LEU, and WHEY treatments. Inset shows the area under the curve (AUC). Upward arrow indicates time of treatment administration. *****Significantly greater than EAA-LEU ($P < 0.05$); +Significantly greater than LEU ($P < 0.05$); ‡Significantly greater than WHEY ($P < 0.05$).

Plasma and intracellular free phenylalanine enrichments. Plasma free phenylalanine enrichments were not different between treatments ($P = 0.66$) and were stable across time (P = 0.34). The slope of plasma free phenylalanine enrichment by time was also not different from zero in any treatment group (EAA-LEU *P* 0.95; LEU *P* 0.11; WHEY *P* 0.40) (see **Supplemental Figure 2** under "Supplemental data" in the online issue).

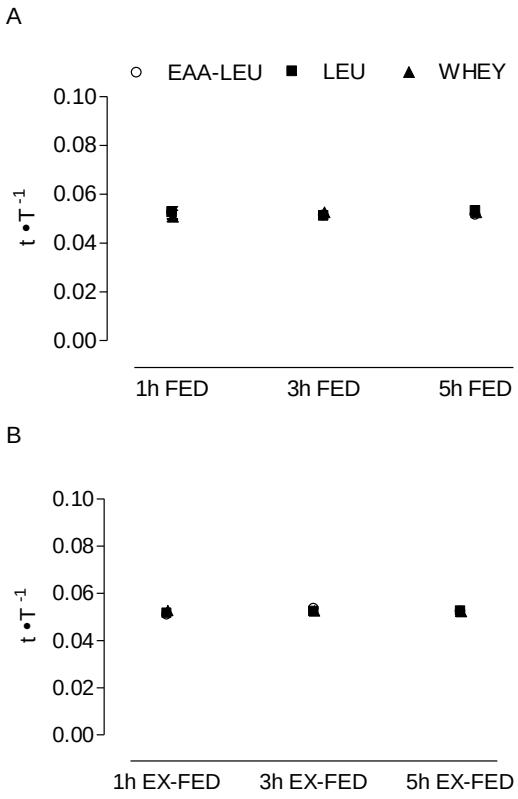


Supplemental Figure 2. Mean (± SEM) plasma free phenylalanine enrichments (tracer-to-tracee ratio $t \bullet T^{-1}$) over time. Data were analyzed using a 2-factor (treatment × time) repeated measures ANOVA and linear regression. There were no differences between treatments ($P = 0.66$) or across time ($P = 0.34$). The slope of plasma free phenylalanine enrichment by time was not different from zero for any treatment group (EAA-LEU $P = 0.95$; LEU $P = 0.11$; WHEY $P = 0.40$).

Similarly, intracellular free phenylalanine enrichments were not different between treatments and were stable across time in both FED (time, $P = 0.92$; treatment, $P = 0.90$) and EX-FED (time, $P = 0.30$; treatment $P = 0.88$) conditions when measured at 1, 3, and 5h post-exercise. Further there were no differences between conditions at 1h ($P = 0.90$), 3h ($P = 0.42$), or 5 ($P = 0.98$). The slope of intracellular free phenylalanine enrichment by time in both FED and EX-FED conditions was also not different from zero in any treatment group, indicating measurements were made at an isotopic plateau (EAA-LEU FED $P = 0.77$, EX-FED $P = 0.41$; LEU FED $P = 0.84$, EX-FED $P = 0.68$; WHEY FED $P = 0.56$, EX-FED $P = 0.84$; see **Supplemental Figure 3** under "Supplemental data" in the online issue).

Myofibrillar protein synthesis. FED rates of MPS were increased above FAST when measured 1-3h post-exercise recovery ($P < 0.001$; EAA-LEU = 0.063 ± 0.008; LEU = 0.068 ± 0.006; WHEY = 0.061 ± 0.009). By 3-5h post-exercise recovery, FED rates of MPS were increased above FAST over 1-3h post-exercise recovery ($P = 0.001$; EAA-LEU = 0.069 ± 0.012; LEU = 0.068 ± 0.014; WHEY = 0.064 ± 0.007). However, the rates of MPS remained increased above FAST at 3-5h exercise recovery only after

ingestion of WHEY versus LEU and EAA-LEU (EAA-LEU = 0.050 ± 0.005; LEU = 0.048 ± 0.012; WHEY = 0.088 ± 0.010; **Figure 3B**).



Supplemental Figure 3. Mean (± SEM) intracellular free phenylalanine enrichments (tracer-to-tracee ratio – $t \bullet T^{-1}$) in both FED (A) and EX-FED (B) conditions. Conditions (i.e. FED and EX-FED) were analyzed separately using a 2-factor (treatment × time) repeated measures ANOVA (FED: time $P = 0.92$; treatment, $P = 0.90$. EX-FED: time $P = 0.30$; treatment $P = 0.88$). Condition effects were analyzed separately at 1 ($P = 0.90$), 3 ($P = 0.42$), and 5h ($P = 0.98$) post-exercise recovery using a 2-factor (treatment × condition) ANOVA. Data were also analyzed using linear regression for the difference of the linear regression slope from zero (EAA-LEU FED $P = 0.77$, EX-FED $P = 0.41$; LEU FED $P = 0.84$, EX-FED $P = 0.68$; WHEY FED $P = 0.56$, EX-FED $P = 0.84$).

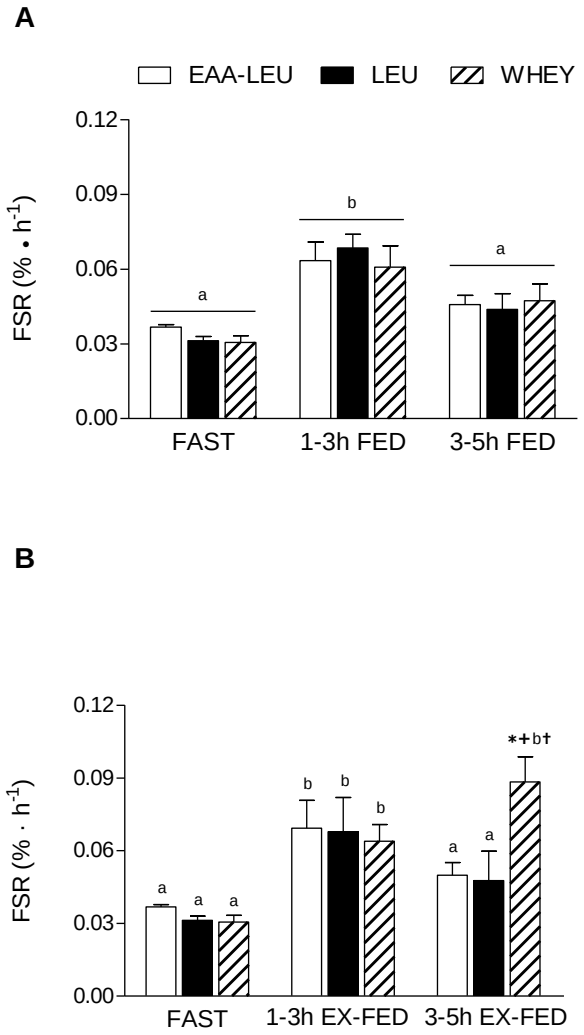


Figure 3. Mean (± SEM) fractional synthetic rate (FSR) (%•h^{-1}) calculated during FAST, and over both early (1-3h), and late (3-5h) time periods of post-exercise recovery in both FED (A) and EX-FED (B) conditions after EAA-LEU, LEU, and WHEY treatments. Times with different letters are significantly different from each other within that treatment and condition. *****Significantly greater than EAA-LEU within that time and condition ($P < 0.05$); +Significantly greater than LEU within that time and condition ($P < 0.05$); †Significantly greater than FED condition at that time-point ($P < 0.05$).

Intracellular amino acids. Intracellular leucine concentration was increased above FAST at 1h post-exercise recovery in LEU and WHEY; however the increase in LEU was significantly greater than both WHEY and EAA-LEU (**Figure 4A**). A similar response was observed in the EX-FED condition ($P < 0.001$); however, intracellular leucine concentrations in EAA-LEU decreased below basal levels at 3- and 5h post-exercise recovery and were significantly lower than both LEU and WHEY at these time-points (**Figure 4B**). Intracellular BCAA in the FED condition with EAA-LEU showed concentrations that were increased above LEU at 3h, and both LEU and WHEY at 5h post-exercise recovery (**Figure 4C**). Similar results were observed in the EX-FED treatment whereby EAA-LEU showed concentrations that were increased above LEU and WHEY at both 3- and 5h post-exercise recovery ($P < 0.001$) (**Figure 4D**). Intracellular EAA in the FED condition showed a main effect ($P < 0.001$) for treatment, with concentrations in EAA-LEU being greater than both LEU and WHEY (**Figure 4E**). In the EX-FED condition, a main effect of time ($P < 0.001$) demonstrated an increase above FAST at 1h followed by a decrease below FAST at 5h post-exercise recovery, such that at 5h, the intracellular EAA concentration in FED was greater than EX-FED ($P = 0.009$) (**Figure 4F**).

Amino acid transporter mRNA expression. The mRNA expression of ATF4 was increased above FAST in both FED ($P < 0.001$) and EX-FED ($P < 0.001$) conditions at 1, 3, and 5h post-exercise recovery (main effect for Time) with no differences between treatment groups (**Supplemental Material Figure 4A-B**). GCN2 mRNA expression

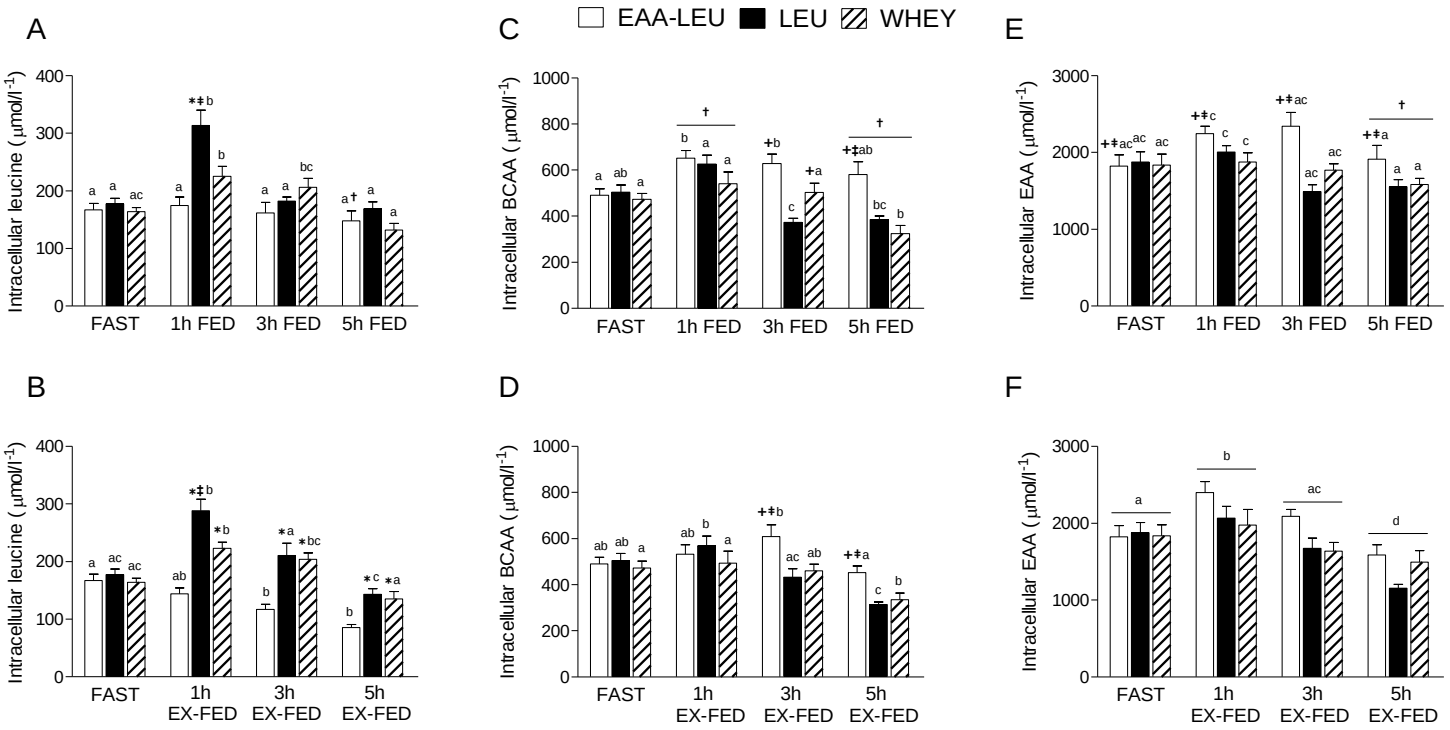
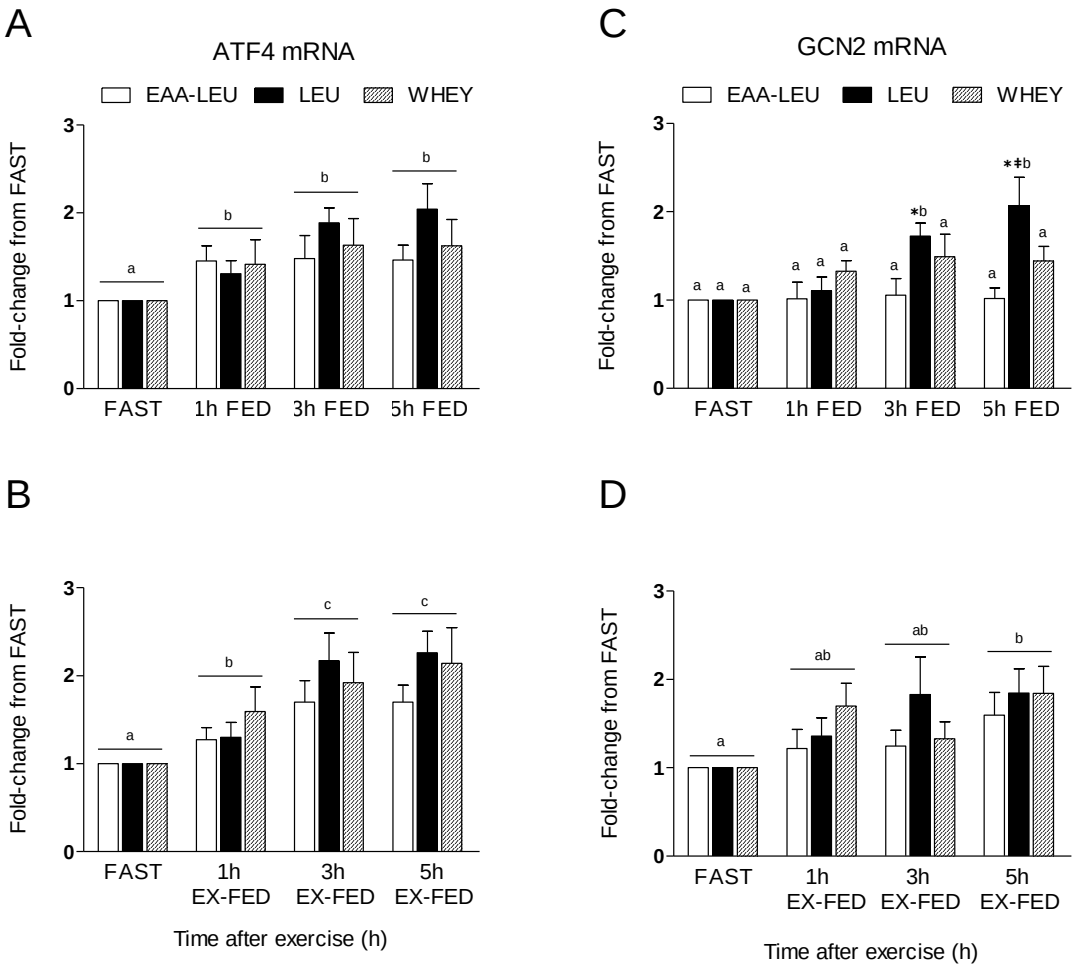

A
B
C
D
E
F
EAA-LEU
LEU
WHEY
Intracellular leucine (µmol/l-1)
Intracellular BCAA (µmol/l-1)
Intracellular EAA (µmol/l-1)
FAST
1h FED
3h FED
5h FED
1h EX-FED
3h EX-FED
5h EX-FED

Figure 4. Mean (± SEM) intracellular concentrations (μmol/l^{-1}) of leucine (A and B), branched chain amino acids (BCAA) (C and D), and essential amino acids (EAA) (E and F) measured during FAST and at 1, 3, and 5 post-exercise recovery in both FED and EX-FED conditions following EAA-LEU, LEU, and WHEY treatments. Times with different letters are significantly different from each other within that treatment and condition. *****Significantly greater than EAA-LEU within that time and condition (P < 0.05); +Significantly greater than LEU within that time and condition (P < 0.05); ‡Significantly greater than WHEY within that time and condition (P < 0.05); †Significantly greater than EX-FED condition at that time-point (P < 0.05).

demonstrated a significant (P = 0.004) interaction effect in the FED condition whereby gene expression was increased to a greater extent in LEU vs. EAA-LEU at 3h and both EAA-LEU and WHEY at 5h post-exercise recovery (**Supplemental Material Figure 4C**). In the EX-FED condition, there was a main effect for time (P = 0.003) (**Supplemental Material Figure 4D**). There were no differences between treatments in the mRNA expression of CD98 (SLC3A2) in either FED or EX-FED conditions, however the increase at 5h post-exercise recovery was significantly greater in EX-FED vs. FED (P = 0.003) (**Figure 5A-B**). Similarly, there were no treatment effects for the mRNA expression of LAT1 (SLC7A5) in either FED or EX-FED conditions, however the increase at 5h post-exercise was greater in EX-FED vs. FED (P = 0.025) (**Figure 5C-D**). Lastly, there was a main effect (P 0.031) for treatment when examining changes in the mRNA expression of PAT1 (SLC36A1) in the EX-FED condition whereby WHEY was significantly greater (P = 0.031) than EAA-LEU (**Figure 5E-F**).

Muscle signalling. Protein kinase B (p-Akt^{Ser473}) was increased at 1h in both FED (P < 0.001) and EX-FED (P = 0.001) conditions with no effect of treatment (**Figure 6A and**

6B). Similarly, p-mTOR^{Ser2448}was significantly elevated at 3h in FED (P = 0.037) (**Figure 6C**), and 1, 3, and 5h in EX-FED (P < 0.001) (**Figure 6D**). Phosphorylation of p70S6k$^{Thr\ 389}$ showed a significant interaction in both FED (P = 0.008) and EX-FED (P = 0.013) conditions. In FED, LEU and WHEY were significantly elevated above EAA-LEU at 3h and 5h (**Figure 6E**), while in EX-FED, both LEU and WHEY were increased above EAA-LEU at 3h, while LEU was increased above EAA-LEU at 5h post-exercise recovery (**Figure 6F**). Phosphorylation of p-4E-BP1$^{Thr\ 37/46}$ in FED was increased above FAST at 1 and 3h post-exercise recovery (P < 0.001) but returned to basal by 5h (**Supplemental Material Figure 5A**). However, in EX-FED phosphorylation was increased at 1, 3, and 5h, (P < 0.001) such that at 5h the increase in EX-FED was greater than FED (P = 0.001) (**Supplemental Material Figure 5B**). Lastly, p-extracellular regulated kinase 1/2$^{Thr202/Tyr204}$ (**Supplemental Material Figure 5C-D**) and p-p38$^{Thr180/Tyr182}$ (**Supplemental Material Figure 5E-F**) MAPK were unchanged in the FED condition but showed time dependent increases in the EX-FED condition (ERK, P = 0.001; p38, P < 0.001) with no effect of treatment. As such, ERK 1/2 was significantly increased in EX-FED vs. FED at 1, 3, and 5h, while p38 was higher in EX-FED vs. FED at 1h post-exercise recovery. See **Supplemental Figure 6** under "Supplemental data" in the online issue for representative blot images for each protein target.



Supplemental Figure 4. Mean (± SEM) mRNA expression of ATF4 (A and B) and GCN2 (C and D) (expressed as fold-difference from FAST) at 1, 3, and 5 post-exercise recovery in both FED and EX-FED conditions following EAA-LEU, LEU, and WHEY treatments. Times with different letters are significantly different from each other within that treatment and condition. *****Significantly greater than EAA-LEU within that time and condition ($P < 0.05$); ‡Significantly greater than WHEY within that time and condition ($P < 0.05$).

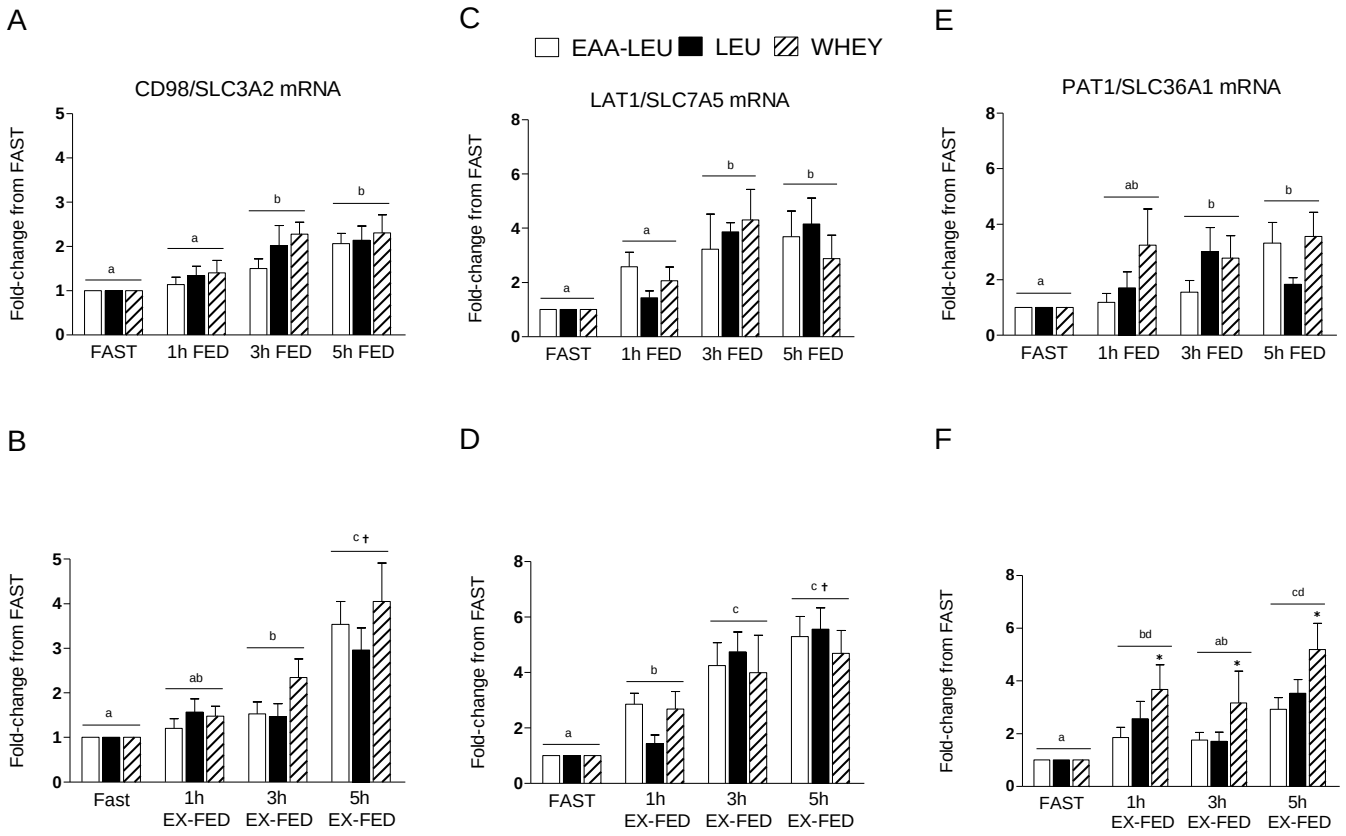


Figure 5. Mean (± SEM) mRNA expression of CD98 (SLC3A2) (A and B), LAT1 (SLC7A5) (C and D), and PAT1 (SLC36A1) (E and F) (expressed as fold-difference from FAST) at 1, 3, and 5 post-exercise recovery in both FED and EX-FED conditions following EAA-LEU, LEU, and WHEY treatments. Times with different letters are significantly different from eachother within that treatment and condition. *****Significantly greater than EAA-LEU within that time and condition (P<0.05); †Significantly greater than EX-FED condition at that time-point ($P < 0.05$).

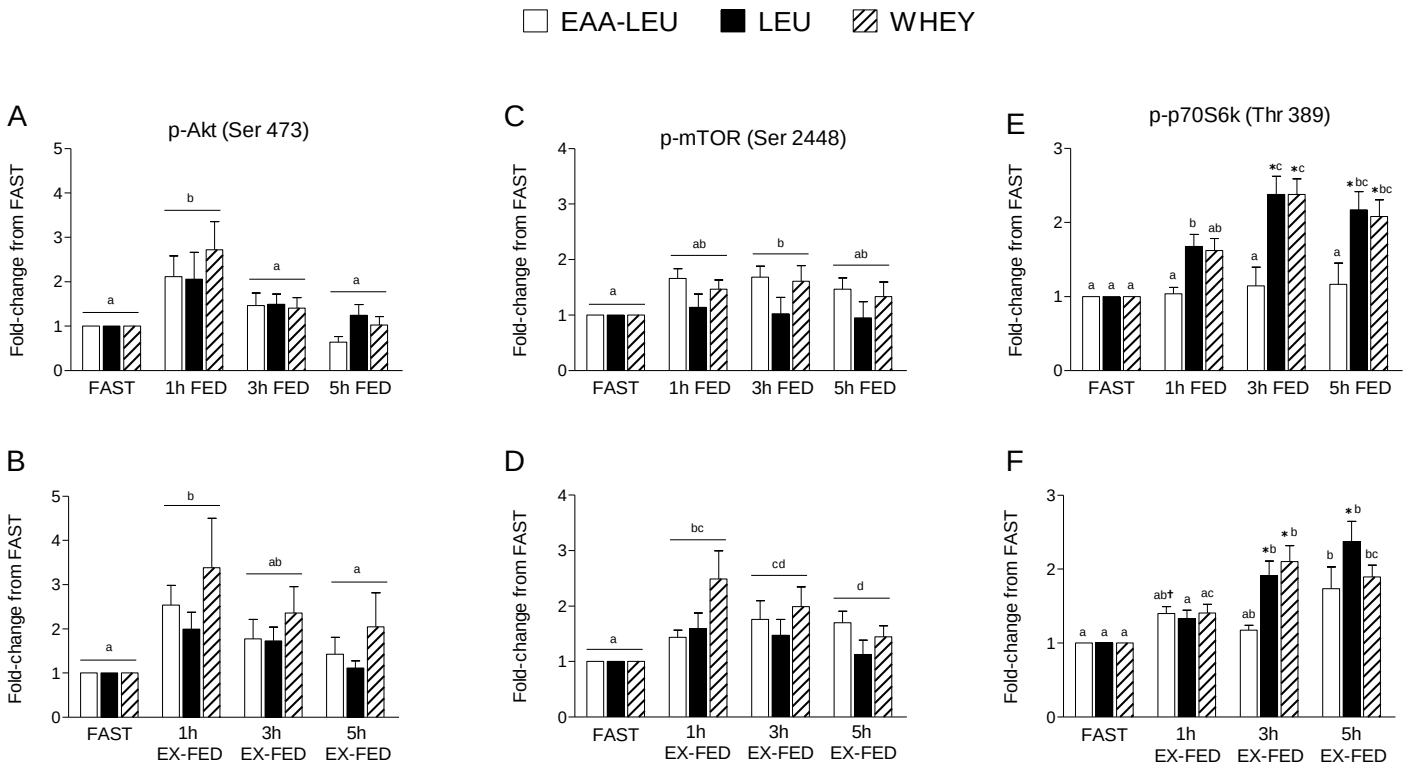
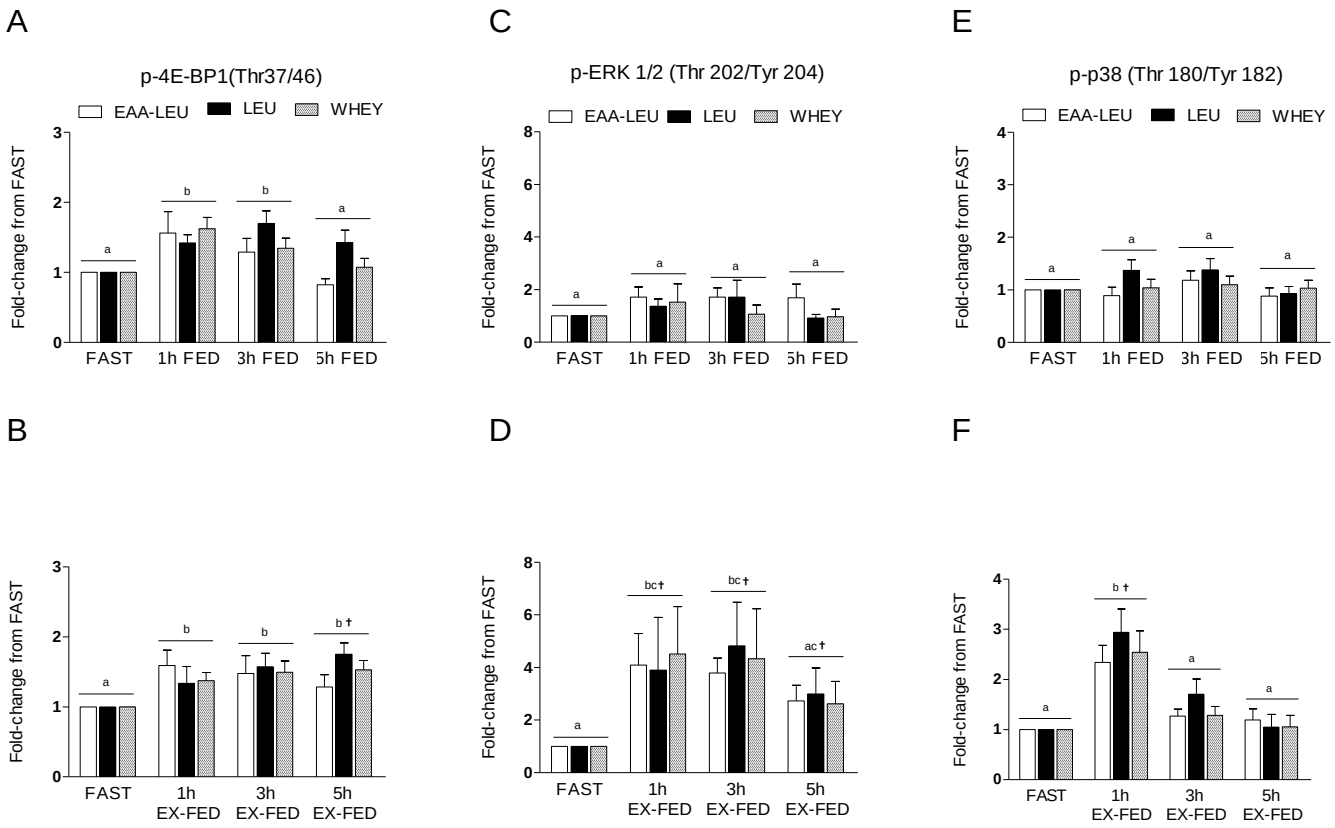
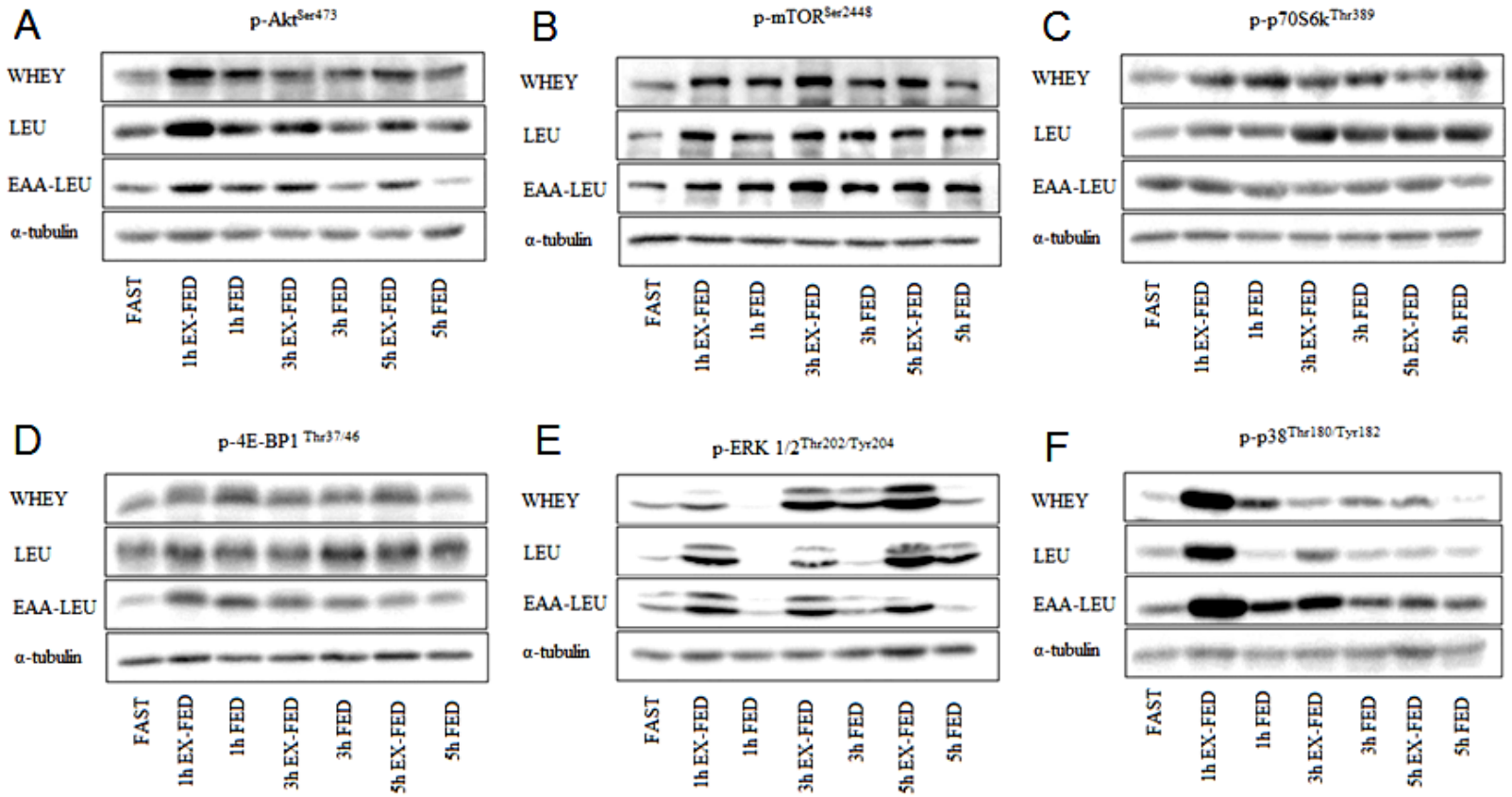


Figure 6. Mean (± SEM) phosphorylation status of Akt ^{Ser473} (A and B), mTOR^{Ser2448} (C and D), and p70S6k^{Thr389} (E and F) (expressed as fold-difference from FAST) at 1, 3, and 5 post-exercise recovery in both FED (top panel) and EX-FED (bottom panel) conditions following EAA-LEU, LEU, and WHEY treatments. Times with different letters are significantly different from eachother within that treatment and condition. *****Significantly greater than EAA-LEU within that time and condition ($P < 0.05$); +Significantly greater than LEU within that time and condition ($P < 0.05$); ‡Significantly greater than WHEY within that time and condition ($P < 0.05$).



Supplemental Figure 5. Mean (± SEM) phosphorylation status of 4E-BP1$^{Thr\ 37/46}$ (A and B), ERK 1/2$^{Thr202/Tyr204}$ (C and D) and p38$^{Thr180/Tyr182}$ (E and F) (expressed as fold-difference from FAST) at 1, 3, and 5 post-exercise recovery in both FED and EX-FED conditions following EAA-LEU, LEU, and WHEY treatments. Times with different letters are significantly different from eachother within that treatment and condition. †Significantly greater than EX-FED condition at that time-point; all $P < 0.05$.



Supplemental Figure 6. Representative blot images for p-Akt ^{Ser473}, p-mTOR^{Ser2448}, p-p70S6k^{Thr389}, p-4E-BP1$^{Thr\ 37/46}$, p-ERK 1/2$^{Thr202/Tyr204}$, p-p38$^{Thr180/Tyr182}$, and α-tubulin at FAST, 1h EX-FED, 1h FED, 3h EX-FED; 3h FED, 5h EX-FED, and 5h FED following EAA-LEU, LEU, and WHEY treatments. Image contrast adjusted to improve clarity.

DISCUSSION

In this study we report that a dose of whey protein, previously shown to be less than maximally effective for stimulating muscle protein synthesis after resistance exercise (Moore *et al.*, 2009a) supplemented with leucine (LEU) resulted in an early (1-3h post-exercise recovery) increase in both FED and EX-FED rates of MPS equal to that seen following ingestion of 25 g of whey protein (WHEY). Contrary to our hypothesis, supplementation of a low dose of whey protein with a mixture of EAA devoid of leucine (EAA-LEU treatment) also resulted in a robust early stimulation of MPS that was not different than that achieved after LEU and WHEY. However, despite similar early responses of MPS, EX-FED rates over 3-5h were only sustained following WHEY, whereas EX-FED rates of MPS in both LEU and EAA-LEU had decreased to values not significantly different from FAST. Interestingly, these differences between 3-5h occurred despite blood amino acid concentrations that had returned to basal levels in all treatments. In the absence of exercise we did not see difference in the rates of MPS between treatments indicating that signalling events as well as amino acid supply were all more than adequate to stimulate a full and robust response that rose and fell within the 4 h incorporation time period, as we (Moore *et al.*, 2009b) and others (Atherton *et al.*, 2010a), have shown previously.

Both *in-vitro* (Buse & Reid, 1975) and *in-vivo* (Anthony *et al.*, 1999; Anthony *et al.*, 2000a; Anthony *et al.*, 2000b; Crozier *et al.*, 2005; Escobar *et al.*, 2005, 2006) evidence from animals supports a role for leucine as a nutrient regulator of muscle protein synthesis, capable of phosphorylating proteins involved in mRNA translation initiation,

primarily through the mTOR signalling pathway including 4E-BP1, p70S6k, and rpS6. (Anthony *et al.*, 2000b; Suryawan *et al.*, 2008). It is currently unclear whether MPS is regulated by changes in extracellular (Bohe *et al.*, 2003), or intracellular (Biolo *et al.*, 1995) EAA and/or leucine availability. In rodents, the leucine content of a meal, and the subsequent postprandial leucinemia direct the peak activation of muscle protein synthesis such that feeding proteins containing a higher proportion of leucine results in a greater plasma leucine concentration and subsequently, a greater increase in muscle protein synthesis (Norton *et al.*, 2009). Our current findings do not support the notion that the postprandial stimulation of MPS is directly proportional only to the rise in blood leucine (Rennie *et al.*, 2006; Norton *et al.*, 2009) under rested or post-exercise conditions in young men. Specifically, we observed pronounced differences in blood leucine concentration (Figure 2A) that were apparently of little consequence to either the FED or EX-FED MPS response when measured over 1-3h post-exercise (Figure 3A and 3B). Thus, in humans, peak activation of MPS does not appear to be driven by leucinemia. Potentially, amino acid transport across the sarcolemma (Hundal & Taylor, 2009) and intracellular amino acid availability (Biolo *et al.*, 1995) may be important in the regulation of MPS.

Previous reports have demonstrated that ~10 g of EAA is sufficient to maximally stimulate MPS under both resting and post-exercise conditions in young healthy subjects (Cuthbertson *et al.*, 2005; Moore *et al.*, 2009a). We observed that LEU resulted in an early (1-3h post-exercise) stimulation of MPS equal to that of WHEY, despite containing only ~45% of the total EAA content (11.5 g vs. 5.1 g). This suggests that leucine can

potently stimulate MPS; however, we observed a similar rise in MPS in the EAA-LEU treatment as that seen with LEU and WHEY despite containing only ~25% of the leucine of LEU and WHEY (WHEY = 3.0 g; LEU = 3.0 g; vs. EAA-LEU = 0.75 g leucine). Thus, we speculate that in young healthy individuals, the leucine content provided by ~6.25 g of whey protein (~0.75 g) appears to be sufficient to activate and induce a maximal stimulation of MPS provided adequate amounts of the other EAA are provided (i.e., amounts equivalent to ~25g whey protein or ~8.5 g EAA). Alternatively, there may be other EAA, in addition to leucine, that can stimulate MPS. For example, valine, phenylalanine, and threonine have been shown to increase human muscle protein synthesis when administered as a flooding dose (Smith *et al.*, 1998). Further, the effect of each individual EAA on mTORC1 signalling in C_2C_{12} myotubes showed that EAA in addition to leucine can enhance both p70S6k and rpS6 phosphorylation (Atherton *et al.*, 2010b), suggesting that other EAA in addition to leucine can activate protein synthetic signalling pathways.

We reported that a sustained elevation of MPS occurs when resistance exercise is followed by the immediate provision of 25 g of whey protein (Moore *et al.*, 2009b; West *et al.*, 2011) despite aminoacidemia equivalent to basal levels. In agreement with these findings, WHEY was able to sustain the EX-FED response over 3-5h post-exercise recovery in the present study while MPS in both LEU and EAA-LEU had declined to resting values. These results suggest that the ability of amino acids to sustain the contraction mediated increase in MPS is not solely dependent on leucine availability as leucine AUC was matched between LEU and WHEY. However, WHEY was associated

with a protracted aminoacidemia as compared to LEU and EAA-LEU (Figure 2A-D), which may have acted as a signal to extend the EX-FED response of MPS. Alternatively, while non-essential amino acids (NEAA) are not necessary to 'turn on' MPS and/or direct the magnitude of the response (Smith *et al.*, 1998; Tipton *et al.*, 1999b; Borsheim *et al.*, 2002; Volpi *et al.*, 2003), there were large differences in the amount of total NEAA provided in each treatment (WHEY = 13.0 g; LEU = 3.3 g; EAA = 3.3 g). Hence, it is conceivable that NEAA may be required to sustain elevated rates of MPS under conditions of a higher 'anabolic drive' stimulated by resistance exercise compared to feeding alone. Under such conditions, more NEAA may be required to serve as substrates necessary for the synthesis of new muscle proteins or other functions; further studies are necessary to examine this hypothesis.

The precise mechanism(s) underpinning the observed changes in MPS following resistance exercise and amino acid intake appear to involve activation of the Akt/mTOR signalling cascade (Anthony *et al.*, 2000b; Cuthbertson *et al.*, 2005; Atherton *et al.*, 2010a; Dickinson *et al.*, 2011). We observed an increase in the phosphorylation status of Akt ^{Ser473} at 1h post-exercise recovery, an upstream regulator of mTOR. Consistent with this finding, we also observed a significant increase in the phosphorylation status of mTOR^{Ser2448}, that was evident earlier and was sustained for longer in the EX-FED vs. FED condition (Figure 6C-D), and both p70S6k^{Thr389} and 4E-BP1$^{Thr\ 37/46}$; downstream targets of mTOR involved in translation initiation. Notably, however, while the phosphorylation status of p70S6k^{Thr389} was markedly increased above fasted conditions following both LEU and WHEY, no changes were observed following EAA-LEU, except

at 5h EX-FED when MPS was no longer significantly elevated (Figure 6E-F). These findings suggest that leucine is a potent regulator of p70S6k ^{Thr389} signalling (Atherton *et al.*, 2010b; Glynn *et al.*, 2010), and corroborate previous findings demonstrating that single point-in-time changes in signalling molecule phosphorylation do not always reflect changes in dynamic measures of protein synthesis (Greenhaff *et al.*, 2008; Glynn *et al.*, 2010).

In an attempt to further elucidate how protein/amino acids and resistance exercise interact to affect MPS we measured the mRNA abundance of select skeletal muscle amino acid transporters (AAT) and members of the general amino acid control pathway including general control nonrepressed (GCN2) and activating transcription factor (ATF4). The transcription factor ATF4 has been reported to upregulate AAT (Harding *et al.*, 2003), and can itself be upregulated in response to GCN2 activation (Ameri & Harris, 2008) and anabolic stimuli such as amino acid and insulin sufficiency (Adams, 2007; Malmberg & Adams, 2008). In agreement, select AAT have recently been shown to be upregulated in human muscle in response to EAA intake (Drummond *et al.*, 2010) and resistance exercise (Drummond *et al.*, 2011). We observed a large increase in gene expression for the AAT LAT1 (SLC7A5), PAT1 (SLC36A1), and CD98 (SLC3A2) consistent with previous reports (Drummond *et al.*, 2010; Drummond *et al.*, 2011), as well as time dependent increases in ATF4 Thus, the increases in amino acid and insulin availability may have acted as a signal to increase ATF4 expression, allowing for the subsequent upregulation of AAT expression. Our findings further demonstrate that changes in the mRNA expression of these transporters are not dependent upon the level of

leucine intake after resistance exercise, and also that combined feeding and exercise appear to prolong the increase in gene expression compared to feeding alone (Figure 5A-B and C-D). We did not measure changes in protein content of these transporters following feeding and resistance exercise, and it remains possible that the nutritional treatments may have demonstrated a differential response at the protein level. Further research is needed to elucidate the functional and physiological significance of changes in these transporters following EAA and resistance exercise.

In conclusion, our model allowed us to address the specific role of total meal leucine content versus that of EAA found in a dose of protein that maximally stimulates MPS after resistance exercise. We report that both LEU and EAA-LEU were as effective as WHEY at stimulating both FED and EX-FED rates of MPS over 1-3h post-exercise recovery. These findings demonstrate that while leucine is potent in its ability to stimulate MPS, only a relatively small amount (0.75 g) is required to achieve a maximal stimulation of MPS when other EAA are provided in larger quantities (~8.5 g). However, only WHEY, containing both EAA and NEAA amino acids, was able to sustain the elevated rates of MPS 3-5h after resistance exercise and therefore may be a better choice to support resistance exercise induced anabolism. The increase in the phosphorylation of $p70S6k^{Thr389}$ following treatment administration was associated with leucine intake (i.e. increased in WHEY and LEU) but not MPS. We conclude that supplementing a suboptimal dose of whey protein (6.25 g) with leucine, or a mixture of EAA without leucine, is an effective strategy to stimulate rates of postprandial MPS comparable to the response elicited following ingestion of 25 g of whey protein, and suggest that only a

small amount (~0.75 g) of leucine is required to stimulate MPS in young healthy individuals when ample amounts of other EAA are provided. These findings may have important implications for individuals unable to tolerate a full protein meal.

REFERENCES

(2010). Canadian Institutes of Health Research, Natural Sciences and Engineering Research Council of Canada, and Social Sciences and Humanities Research Council of Canada, Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans. .

Adams CM. (2007). Role of the transcription factor ATF4 in the anabolic actions of insulin and the anti-anabolic actions of glucocorticoids. *The Journal of biological chemistry* **282,** 16744-16753.

Ameri K & Harris AL. (2008). Activating transcription factor 4. *The international journal of biochemistry & cell biology* **40,** 14-21.

Anthony JC, Anthony TG, Kimball SR, Vary TC & Jefferson LS. (2000a). Orally administered leucine stimulates protein synthesis in skeletal muscle of postabsorptive rats in association with increased eIF4F formation. *The Journal of nutrition* **130,** 139-145.

Anthony JC, Anthony TG & Layman DK. (1999). Leucine supplementation enhances skeletal muscle recovery in rats following exercise. *The Journal of nutrition* **129,** 1102-1106.

Anthony JC, Reiter AK, Anthony TG, Crozier SJ, Lang CH, MacLean DA, Kimball SR & Jefferson LS. (2002). Orally administered leucine enhances protein synthesis in skeletal muscle of diabetic rats in the absence of increases in 4E-BP1 or S6K1 phosphorylation. *Diabetes* **51,** 928-936.

Anthony JC, Yoshizawa F, Anthony TG, Vary TC, Jefferson LS & Kimball SR. (2000b). Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. *The Journal of nutrition* **130,** 2413-2419.

Atherton PJ, Etheridge T, Watt PW, Wilkinson D, Selby A, Rankin D, Smith K & Rennie MJ. (2010a). Muscle full effect after oral protein: time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling. *The American journal of clinical nutrition* **92,** 1080-1088.

Atherton PJ, Smith K, Etheridge T, Rankin D & Rennie MJ. (2010b). Distinct anabolic signalling responses to amino acids in C2C12 skeletal muscle cells. *Amino acids* **38,** 1533-1539.

Bennet WM, Connacher AA, Scrimgeour CM, Smith K & Rennie MJ. (1989). Increase in anterior tibialis muscle protein synthesis in healthy man during mixed amino acid infusion: studies of incorporation of [1-13C]leucine. *Clin Sci (Lond)* **76,** 447-454.

Biolo G, Maggi SP, Williams BD, Tipton KD & Wolfe RR. (1995). Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans. *The American journal of physiology* **268,** E514-520.

Bohe J, Low A, Wolfe RR & Rennie MJ. (2003). Human muscle protein synthesis is modulated by extracellular, not intramuscular amino acid availability: a dose-response study. *The Journal of physiology* **552,** 315-324.

Bohe J, Low JF, Wolfe RR & Rennie MJ. (2001). Latency and duration of stimulation of human muscle protein synthesis during continuous infusion of amino acids. *The Journal of physiology* **532,** 575-579.

Bolster DR, Vary TC, Kimball SR & Jefferson LS. (2004). Leucine regulates translation initiation in rat skeletal muscle via enhanced eIF4G phosphorylation. *The Journal of nutrition* **134,** 1704-1710.

Borsheim E, Tipton KD, Wolf SE & Wolfe RR. (2002). Essential amino acids and muscle protein recovery from resistance exercise. *American journal of physiology Endocrinology and metabolism* **283,** E648-657.

Burd NA, Holwerda AM, Selby KC, West DW, Staples AW, Cain NE, Cashaback JG, Potvin JR, Baker SK & Phillips SM. (2010a). Resistance exercise volume affects myofibrillar protein synthesis and anabolic signalling molecule phosphorylation in young men. *The Journal of physiology* **588,** 3119-3130.

Burd NA, West DW, Rerecich T, Prior T, Baker SK & Phillips SM. (2011). Validation of a single biopsy approach and bolus protein feeding to determine myofibrillar protein synthesis in stable isotope tracer studies in humans. *Nutrition & metabolism* **8,** 15.

Burd NA, West DW, Staples AW, Atherton PJ, Baker JM, Moore DR, Holwerda AM, Parise G, Rennie MJ, Baker SK & Phillips SM. (2010b). Low-load high volume resistance exercise stimulates muscle protein synthesis more than high-load low volume resistance exercise in young men. *PloS one* **5,** e12033.

Buse MG & Reid SS. (1975). Leucine. A possible regulator of protein turnover in muscle. *The Journal of clinical investigation* **56,** 1250-1261.

Copeland KC, Kenney FA & Nair KS. (1992). Heated dorsal hand vein sampling for metabolic studies: a reappraisal. *The American journal of physiology* **263,** E1010-1014.

Crozier SJ, Kimball SR, Emmert SW, Anthony JC & Jefferson LS. (2005). Oral leucine administration stimulates protein synthesis in rat skeletal muscle. *The Journal of nutrition* **135,** 376-382.

Cuthbertson D, Smith K, Babraj J, Leese G, Waddell T, Atherton P, Wackerhage H, Taylor PM & Rennie MJ. (2005). Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **19,** 422-424.

Dickinson JM, Fry CS, Drummond MJ, Gundermann DM, Walker DK, Glynn EL, Timmerman KL, Dhanani S, Volpi E & Rasmussen BB. (2011). Mammalian target of rapamycin complex 1 activation is required for the stimulation of human skeletal muscle protein synthesis by essential amino acids. *The Journal of nutrition* **141,** 856-862.

Drummond MJ, Fry CS, Glynn EL, Dreyer HC, Dhanani S, Timmerman KL, Volpi E & Rasmussen BB. (2009). Rapamycin administration in humans blocks the contraction-induced increase in skeletal muscle protein synthesis. *The Journal of physiology* **587,** 1535-1546.

Drummond MJ, Fry CS, Glynn EL, Timmerman KL, Dickinson JM, Walker DK, Gundermann DM, Volpi E & Rasmussen BB. (2011). Skeletal muscle amino acid transporter expression is increased in young and older adults following resistance exercise. *J Appl Physiol* **111,** 135-142.

Drummond MJ, Glynn EL, Fry CS, Timmerman KL, Volpi E & Rasmussen BB. (2010). An increase in essential amino acid availability upregulates amino acid transporter expression in human skeletal muscle. *American journal of physiology Endocrinology and metabolism* **298,** E1011-1018.

Escobar J, Frank JW, Suryawan A, Nguyen HV, Kimball SR, Jefferson LS & Davis TA. (2005). Physiological rise in plasma leucine stimulates muscle protein synthesis in neonatal pigs by enhancing translation initiation factor activation. *American journal of physiology Endocrinology and metabolism* **288,** E914-921.

Escobar J, Frank JW, Suryawan A, Nguyen HV, Kimball SR, Jefferson LS & Davis TA. (2006). Regulation of cardiac and skeletal muscle protein synthesis by individual branched-chain amino acids in neonatal pigs. *American journal of physiology Endocrinology and metabolism* **290,** E612-621.

Glover EI, Oates BR, Tang JE, Moore DR, Tarnopolsky MA & Phillips SM. (2008). Resistance exercise decreases eIF2Bepsilon phosphorylation and potentiates the feeding-induced stimulation of p70S6K1 and rpS6 in young men. *American journal of physiology Regulatory, integrative and comparative physiology* **295,** R604-610.

Glynn EL, Fry CS, Drummond MJ, Timmerman KL, Dhanani S, Volpi E & Rasmussen BB. (2010). Excess leucine intake enhances muscle anabolic signaling but not net protein anabolism in young men and women. *The Journal of nutrition* **140,** 1970-1976.

Greenhaff PL, Karagounis LG, Peirce N, Simpson EJ, Hazell M, Layfield R, Wackerhage H, Smith K, Atherton P, Selby A & Rennie MJ. (2008). Disassociation between the effects of amino acids and insulin on signaling, ubiquitin ligases, and protein turnover in human muscle. *American journal of physiology Endocrinology and metabolism* **295,** E595-604.

Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, Stojdl DF, Bell JC, Hettmann T, Leiden JM & Ron D. (2003). An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Molecular cell* **11,** 619-633.

Heys SD, McNurlan MA, Park KG, Milne E & Garlick PJ. (1990). Baseline measurements for stable isotope studies: an alternative to biopsy. *Biomedical & environmental mass spectrometry* **19,** 176-178.

Hundal HS & Taylor PM. (2009). Amino acid transceptors: gate keepers of nutrient exchange and regulators of nutrient signaling. *American journal of physiology Endocrinology and metabolism* **296,** E603-613.

Katsanos CS, Kobayashi H, Sheffield-Moore M, Aarsland A & Wolfe RR. (2006). A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly. *American journal of physiology Endocrinology and metabolism* **291,** E381-387.

Koopman R, Verdijk L, Manders RJ, Gijsen AP, Gorselink M, Pijpers E, Wagenmakers AJ & van Loon LJ. (2006). Co-ingestion of protein and leucine stimulates muscle protein synthesis rates to the same extent in young and elderly lean men. *The American journal of clinical nutrition* **84,** 623-632.

Koopman R, Verdijk LB, Beelen M, Gorselink M, Kruseman AN, Wagenmakers AJ, Kuipers H & van Loon LJ. (2008). Co-ingestion of leucine with protein does not

further augment post-exercise muscle protein synthesis rates in elderly men. *The British journal of nutrition* **99,** 571-580.

Koopman R, Wagenmakers AJ, Manders RJ, Zorenc AH, Senden JM, Gorselink M, Keizer HA & van Loon LJ. (2005). Combined ingestion of protein and free leucine with carbohydrate increases postexercise muscle protein synthesis in vivo in male subjects. *American journal of physiology Endocrinology and metabolism* **288,** E645-653.

Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680-685.

Leenders M, Verdijk LB, van der Hoeven L, van Kranenburg J, Hartgens F, Wodzig WK, Saris WH & van Loon LJ. (2011). Prolonged leucine supplementation does not augment muscle mass or affect glycemic control in elderly type 2 diabetic men. *The Journal of nutrition* **141,** 1070-1076.

Malmberg SE & Adams CM. (2008). Insulin signaling and the general amino acid control response. Two distinct pathways to amino acid synthesis and uptake. *The Journal of biological chemistry* **283,** 19229-19234.

Miller BF, Olesen JL, Hansen M, Dossing S, Crameri RM, Welling RJ, Langberg H, Flyvbjerg A, Kjaer M, Babraj JA, Smith K & Rennie MJ. (2005). Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise. *The Journal of physiology* **567,** 1021-1033.

Mittendorfer B, Andersen JL, Plomgaard P, Saltin B, Babraj JA, Smith K & Rennie MJ. (2005). Protein synthesis rates in human muscles: neither anatomical location nor fibre-type composition are major determinants. *The Journal of physiology* **563,** 203-211.

Moore DR, Robinson MJ, Fry JL, Tang JE, Glover EI, Wilkinson SB, Prior T, Tarnopolsky MA & Phillips SM. (2009a). Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *The American journal of clinical nutrition* **89,** 161-168.

Moore DR, Tang JE, Burd NA, Rerecich T, Tarnopolsky MA & Phillips SM. (2009b). Differential stimulation of myofibrillar and sarcoplasmic protein synthesis with protein ingestion at rest and after resistance exercise. *The Journal of physiology* **587,** 897-904.

Nair KS, Schwartz RG & Welle S. (1992). Leucine as a regulator of whole body and skeletal muscle protein metabolism in humans. *The American journal of physiology* **263,** E928-934.

Norton LE, Layman DK, Bunpo P, Anthony TG, Brana DV & Garlick PJ. (2009). The leucine content of a complete meal directs peak activation but not duration of skeletal muscle protein synthesis and mammalian target of rapamycin signaling in rats. *The Journal of nutrition* **139,** 1103-1109.

Philp A, Perez-Schindler J, Green C, Hamilton DL & Baar K. (2010). Pyruvate suppresses PGC1alpha expression and substrate utilization despite increased respiratory chain content in C2C12 myotubes. *American journal of physiology Cell physiology* **299,** C240-250.

Rennie MJ, Bohe J, Smith K, Wackerhage H & Greenhaff P. (2006). Branched-chain amino acids as fuels and anabolic signals in human muscle. *The Journal of nutrition* **136,** 264S-268S.

Rieu I, Balage M, Sornet C, Giraudet C, Pujos E, Grizard J, Mosoni L & Dardevet D. (2006). Leucine supplementation improves muscle protein synthesis in elderly men independently of hyperaminoacidaemia. *The Journal of physiology* **575,** 305-315.

Smith K, Barua JM, Watt PW, Scrimgeour CM & Rennie MJ. (1992). Flooding with L-[1-13C]leucine stimulates human muscle protein incorporation of continuously infused L-[1-13C]valine. *The American journal of physiology* **262,** E372-376.

Smith K, Reynolds N, Downie S, Patel A & Rennie MJ. (1998). Effects of flooding amino acids on incorporation of labeled amino acids into human muscle protein. *The American journal of physiology* **275,** E73-78.

Suryawan A, Jeyapalan AS, Orellana RA, Wilson FA, Nguyen HV & Davis TA. (2008). Leucine stimulates protein synthesis in skeletal muscle of neonatal pigs by enhancing mTORC1 activation. *American journal of physiology Endocrinology and metabolism* **295,** E868-875.

Tang JE, Lysecki PJ, Manolakos JJ, MacDonald MJ, Tarnopolsky MA & Phillips SM. (2011). Bolus arginine supplementation affects neither muscle blood flow nor muscle protein synthesis in young men at rest or after resistance exercise. *The Journal of nutrition* **141,** 195-200.

Tang JE, Moore DR, Kujbida GW, Tarnopolsky MA & Phillips SM. (2009). Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein

synthesis at rest and following resistance exercise in young men. *J Appl Physiol* **107,** 987-992.

Tipton KD, Elliott TA, Ferrando AA, Aarsland AA & Wolfe RR. (2009). Stimulation of muscle anabolism by resistance exercise and ingestion of leucine plus protein. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme* **34,** 151-161.

Tipton KD, Ferrando AA, Phillips SM, Doyle D, Jr. & Wolfe RR. (1999a). Postexercise net protein synthesis in human muscle from orally administered amino acids. *The American journal of physiology* **276,** E628-634.

Tipton KD, Gurkin BE, Matin S & Wolfe RR. (1999b). Nonessential amino acids are not necessary to stimulate net muscle protein synthesis in healthy volunteers. *The Journal of nutritional biochemistry* **10,** 89-95.

Verhoeven S, Vanschoonbeek K, Verdijk LB, Koopman R, Wodzig WK, Dendale P & van Loon LJ. (2009). Long-term leucine supplementation does not increase muscle mass or strength in healthy elderly men. *The American journal of clinical nutrition* **89,** 1468-1475.

Volpi E, Kobayashi H, Sheffield-Moore M, Mittendorfer B & Wolfe RR. (2003). Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults. *The American journal of clinical nutrition* **78,** 250-258.

West DW, Burd NA, Coffey VG, Baker SK, Burke LM, Hawley JA, Moore DR, Stellingwerff T & Phillips SM. (2011). Rapid aminoacidemia enhances myofibrillar protein synthesis and anabolic intramuscular signaling responses after resistance exercise. *The American journal of clinical nutrition* **94,** 795-803.

West DW, Kujbida GW, Moore DR, Atherton P, Burd NA, Padzik JP, De Lisio M, Tang JE, Parise G, Rennie MJ, Baker SK & Phillips SM. (2009). Resistance exercise-induced increases in putative anabolic hormones do not enhance muscle protein synthesis or intracellular signalling in young men. *The Journal of physiology* **587,** 5239-5247.

Wilkinson SB, Tarnopolsky MA, Macdonald MJ, Macdonald JR, Armstrong D & Phillips SM. (2007). Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage. *The American journal of clinical nutrition* **85,** 1031-1040.

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CHAPTER 3

TITLE: Leucine supplementation of a low protein mixed macronutrient beverage enhances myofibrillar protein synthesis in young men: a double blind randomised trial.

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Leucine supplementation of a low protein mixed macronutrient beverage enhances myofibrillar protein synthesis in young men: a double blind randomised trial.

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Running head: Leucine and myofibrillar protein synthesis

List of abbreviations: 4E-BP1, eukaryotic initiation factor 4E binding protein 1; Akt, protein kinase B; BCAA, branched-chain amino acid; eEF2, eukaryotic elongation factor 2; EX-FED, response to combined feeding and resistance exercise; FED, response to feeding; FSR, fractional synthetic rate; MPS, myofibrillar protein synthesis; mTOR, mechanistic target of rapamycin; rpS6, ribosomal protein s 6.

This trial is registered at clinicaltrials.gov as: NCT 1530646

Abstract

Background: Leucine (Leu) is a key amino acid involved in the regulation of skeletal muscle protein synthesis. **Objective:** To assess the effect of supplementing a lower-protein mixed macronutrient beverage with varying doses of Leu or a mixture of branched chain amino acids (BCAA) on myofibrillar protein synthesis (MPS) at rest and after exercise. **Design:** In a parallel group design, forty adult males (21 ± 1 y) completed uni-lateral knee-extensor resistance exercise prior to ingesting either: 25g whey (W25, 3.0g Leu), 6.25g whey (W6, 0.75g Leu), 6.25g whey supplemented with Leu (W6+Low-Leu, 3.0g Leu), 6.25g whey supplemented with Leu (W6+High-Leu, 5.0g Leu), or: 6.25 g whey supplemented with Leu, isoleucine, and valine (W6+BCAA, 5.0g Leu). A primed continuous infusion of L-[*ring*-$^{13}C_6$] phenylalanine with serial muscle biopsies was used to measure MPS under baseline fasted and postprandial conditions in both a rested (FED) and exercised (EX-FED) leg. **Results:** Area under the blood leucine curve was greatest for W6+High-Leu vs. W6 and W6+Low-Leu (P<0.001). In the postprandial period, rates of MPS were increased above baseline over 0-1.5 h in all treatments. Over 1.5-4.5 h, MPS remained increased above baseline following all treatments, but was greatest following W25 (~267%) and W6+High-Leu (~220%) (P=0.002). **Conclusions:** A low-protein (6.25g) mixed macronutrient beverage can be as effective as a high protein dose (25g) at stimulating increased MPS rates when supplemented with a high (total leucine = 5.0g) amount of leucine. These results have important implications for formulation of protein beverages designed to enhance muscle anabolism.

INTRODUCTION

Provision of a complete mixture of amino acids increases muscle protein synthesis (MPS) rates (1) through activation of the target of rapamycin complex-1 (2). This effect on MPS is primarily due to essential amino acids (EAA), as non-essential amino acids do not stimulate MPS (3-5). The relationship between amino acid (6) and protein (7, 8) intake and MPS is dose-dependent and saturable. Of the EAA, the branched chain amino acid (BCAA) leucine is a key determinant of the postprandial stimulation of MPS following protein intake in rodents (9). In-vivo animal studies have shown that the independent administration of leucine, but not isoleucine or valine, can stimulate MPS rates (10, 11), to the same extent as a complete mixtures of EAA or complete protein (12, 13). Some research in humans has focused on the efficacy of leucine supplementation to promote increases in MPS (14-17) and augment skeletal muscle mass (18, 19). While some studies have demonstrated increased rates of MPS with leucine administration (20-22), others have not (17, 23-25). However, provision of leucine can result in reduced circulating concentrations of isoleucine and valine (18, 26, 27), which could lower MPS (28); thus, the inclusion of all BCAA as opposed to leucine alone may be efficacious. Addition of BCAA, and in particular leucine, to stimulate MPS in a less than optimally effective dose of protein may represent an effective strategy to increase MPS following feeding or under the influence of the markedly anabolic stimulus of resistive exercise.

The aim of the present study was to assess the potential to enhance the effect of a dose of protein containing a quantity of EAA previously demonstrated to be suboptimal in maximally stimulating MPS rates with feeding (6) and following exercise (7), on MPS

rates when ingested as part of a mixed macronutrient beverage. Subjects were randomly assigned to a positive control (25 g of whey: W25 = 3.0 g of leucine), a negative control (6.25 g of whey: W6 = 0.75 g of leucine) or treatments consisting of 6.25 g of whey supplemented with: a lower dose of leucine (W6+Low-Leu = 3.0 g of leucine); a higher dose of leucine (W6+High-Leu = 5.0 g of leucine), or a higher dose of leucine plus isoleucine, and valine (W6+BCAA = 5.0 g of leucine). Rates of MPS and phosphorylation status of protein targets of the Akt-mTORC-1 pathway were examined under postabsorptive conditions and in the postprandial state under rested and post-exercise conditions. We examined a temporally early (0-1.5 h) and late (1.5-4.5 h) postprandial period during both resting and post-exercise recovery conditions since leucine has been suggested to direct the peak activation, but not duration of MPS (9). We hypothesized that W6+Low-Leu, W6+BCAA, W25, and W6+High-Leu would stimulate greater postprandial MPS rates than W6 under resting conditions with no differences between treatments. During post-exercise recovery, we hypothesized that W6+BCAA, W25, and W6+High-Leu would elicit increases in MPS that were equivalent, but greater than W6+Low-Leu and W6 due to maintenance of the MPS response over the later time period examined.

SUBJECTS AND METHODS

Study participants. Forty young adult males between 18-35 years of age were recruited via advertisements posted on the McMaster University campus to participate in the study. The characteristics of the study participants are presented in **Table 1**. In a double blind manner, participants were randomly assigned to one of five parallel treatment groups (*n* =

Table 1. Participant characteristics

	W6	W6+Low-Leu	W25	W6+BCAA	W6+High-Leu
Age, y	20.5 (1.1)	20.4 (0.6)	20.9 (0.6)	20.8 (0.8)	19.5 (0.1)
Height, m	1.80 (0.03)	1.76 (0.02)	1.76 (0.02)	1.83 (0.03)	1.76 (0.03)
Weight, kg	79.4 (3.5)	77.7 (3.3)	78.1 (2.8)	81.3 (3.8)	79.4 (3.4)
BMI, kg/m^2	24.5 (0.7)	25.0 (1.0)	25.2 (1.0)	24.3 (0.8)	25.7 (1.2)
Fat-free mass, kg	66.6 (2.4)	64.2 (2.3)	66.0 (2.8)	70.4 (2.9)	64.8 (2.7)
Fat-mass, kg	12.9 (1.3)	13.6 (1.6)	12.1 (0.8)	10.9 (1.1)	14.6 (1.2)
1-RM Strength, kg	64.5 (1.7)	71.3 (4.2)	65.1 (3.2)	67.3 (3.6)	59.4 (3.5)

Values are mean ± SEM (n = 8 per treatment group). W6 (6.25 g whey protein); W6+Low-Leu (6.25 g whey protein supplemented with leucine to 3.0 g total leucine); W25 (25 g whey protein); W6+BCAA (6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine); W6+High-Leu (6.25 g whey protein supplemented with leucine to 5.0 g total leucine).

8 per group) in a block design balanced for bodyweight. The randomization technique was performed using the minimization technique implemented by the Nestle computer program Trialbalance. An individual at McMaster, not directly involved with the study, was responsible for randomization and treatment preparation. Five codes were generated and their corresponding group assignments were stored in five separate code-break envelopes, held and sealed until the completion of all data analysis. After entering a participant's bodyweight in the Trialbalance system, an individual subject code was generated corresponding to treatment group allocation which was known only to the individual who was responsible for randomization and treatment preparation. Only the individual participant's code was placed in the treatment container. None of the study participants reported engaging in a structured program of resistance exercise within the last year, but reported being recreationally active ~2-3 times per week. Participants were deemed healthy based on responses to a routine health screening questionnaire. Each

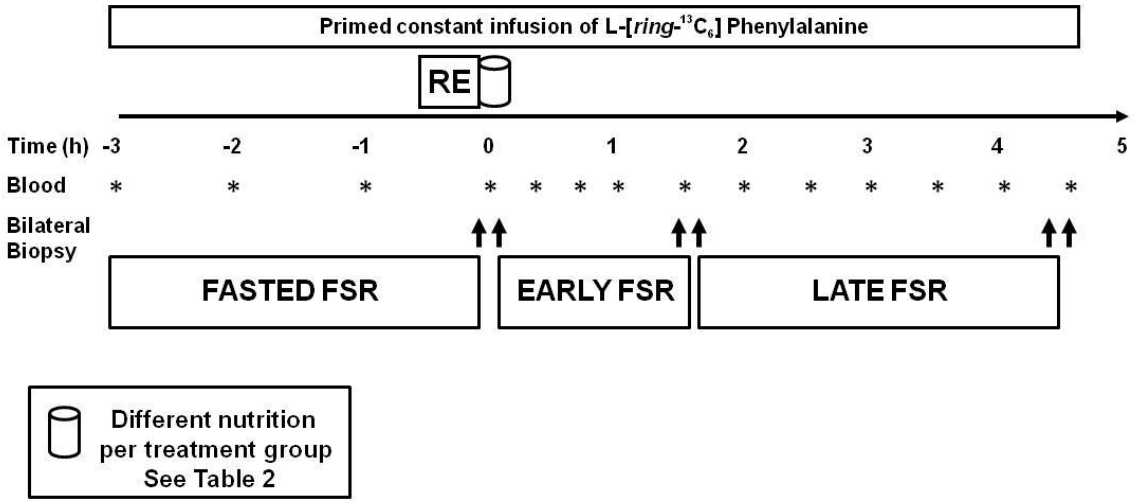
participant was informed of the purpose of the study, experimental procedures, and potential risks prior to providing written consent. The study was approved by the Hamilton Health Sciences Research Ethics Board and conformed to the standards for the use of human subjects in research as outlined in the most recent update of the *Declaration of Helsinki*. The study also conformed to standards established by the Canadian Tri-Council Policy on the ethical use of human subjects (29). The study took place at the Ivor Wynne Centre, Department of Kinesiology, McMaster University, Ontario Canada, from 2011-2012. The primary outcome measure was a change in myofibrillar FSR ($\% \bullet h^{-1}$) as assessed by the incorporation of $^{13}C_6$ labelled phenylalanine into myofibrillar proteins.

Pretesting. Approximately one week prior to the experimental infusion trial, study participants underwent unilateral strength testing of the knee-extensor muscles. Participants performed a series of graded knee-extensions to determine their single repetition maximum strength (1-RM) with their self-reported dominant leg using a seated knee-extension device (Atlantis Precision Series C-105, Quebec, Canada). In addition, each participant underwent a whole-body dual-energy X-ray absorptiometry scan (QDR-4500A; Hologic; software version 12.31, Bedford, MA) to measure body composition (Table 1). Participants were provided with pre-packaged standardized diets that were consumed during the two days immediately preceding the experimental infusion trial. Diets were designed to provide sufficient energy to maintain energy balance as determined by the Harris-Benedict equation and were adjusted using a moderate activity factor (1.4-1.6) to account for participants self-reported physical activity patterns. The macronutrient distribution of the diets was 55% carbohydrate, 30% fat, and 15% protein.

Participants were instructed to consume all food and beverages provided and avoid consumption of food and beverages (other than water) not provided as part of the standardized diet. Participants were instructed to abstain from strenuous physical exercise for 72 h prior to the experimental infusion trial and to consume their evening meal no later than 20 00 h the evening prior to the trial.

Experiment. Participants reported to the lab at ~0600 the morning of the experimental infusion trial following an overnight fast. A catheter was inserted into an antecubital vein and a baseline blood sample was taken before initiating a 0.9% saline drip to keep the catheter patent to allow for repeated arterialized blood sampling. Arterialized blood samples (30) were obtained repeatedly during the infusion trial (**Online Supplemental Material Figure 1**) by wrapping a heating blanket around the forearm. Blood samples were collected into 4 ml heparinized evacuated tubes and chilled on ice. A second catheter was inserted into the antecubital vein of the opposite arm prior to initiating a primed continuous infusion (0.05 μmol•kg^{-1}•min^{-1}; 2.0 μmol•kg^{-}1 prime) of [*ring*-$^{13}C_6$] phenylalanine (Cambridge Isotope Laboratories, Woburn, MA). The infusate was passed through a 0.2-μm filter before entering the blood. The baseline (fasted) fractional synthetic rate (FSR) was calculated based on the ^{13}C enrichment of mixed plasma proteins obtained from the pre-infusion blood sample and skeletal muscle biopsy following ~3 hours of tracer incorporation (31, 32). Participants performed an acute bout of unilateral seated knee-extension resistance exercise (Atlantis Precision Series C-105, Quebec, Canada) consisting of 8 sets of 10-12 repetitions at ~80% of their previously

determined 1-RM with an inter-set rest-interval of 2 minutes. Immediately following completion of the



Online Supplemental Material Figure 1. Schematic of the experimental protocol. Participants were block randomized to one of five possible treatment groups (see Table 2) in a double-blind fashion (n = 8 per treatment group). Treatments were administered immediately following the first set of bilateral skeletal muscle biopsies after unilateral resistance exercise (RE), consisting of 8 sets of 8-10 repetitions of seated knee extension with a 2 min inter-set rest interval. Asterisk indicates blood sample; double upward arrow indicates bilateral biopsy.

resistance exercise, participants underwent bilateral biopsies from both the rested and exercised leg and immediately ingested their designated nutrient treatment (**Table 2**). Bilateral biopsy samples were then obtained at 1.5 h and 4.5 h following treatment administration from a rested fed (FED) and exercise-fed (EX-FED) leg. Muscle biopsies were obtained from the *vastus lateralis* muscle using a 5 mm Bergström needle custom adapted for manual suction under 2% xylocaine local anaesthesia. The tissue samples were freed from visible blood, fat, and connective tissue, and immediately frozen in liquid nitrogen for further analysis as previously described (33, 34). Each biopsy sample was

obtained from a separate incision ~2-3 cm apart. Each participant underwent a total of 6 skeletal muscle biopsies; 3 from each leg. Details of the infusion protocol are outlined in Online Supplemental Material Figure 1.

Table 2. Amino acid, protein, CHO, and fat content of the nutritional treatments

	Nutritional Treatment Group				
	W6	W6+Low-Leu	W25	W6+BCAA	W6+High-Leu
Endogenous AA					
Alanine, g	0.29	0.29	1.15	0.29	0.29
Arginine, g	0.13	0.13	0.53	0.13	0.13
Aspartic Acid, g	0.70	0.70	2.80	0.70	0.70
Cystine, g	0.19	0.19	0.78	0.19	0.19
Glutamic Acid, g	1.03	1.03	4.10	1.03	1.03
Glycine, g	0.11	0.11	0.43	0.11	0.11
Histidine, g	0.14	0.14	0.55	0.14	0.14
Isoleucine, g	0.34	0.34	1.35	0.34	0.34
Leucine, g	0.75	0.75	3.00	0.75	0.75
Lysine, g	0.68	0.68	2.70	0.68	0.68
Methionine, g	0.14	0.14	0.58	0.14	0.14
Phenylalanine, g	0.22	0.22	0.88	0.22	0.22
Proline, g	0.26	0.26	1.05	0.26	0.26
Serine, g	0.16	0.16	0.63	0.16	0.16
Threonine, g	0.28	0.28	1.10	0.28	0.28
Tryptophan, g	0.17	0.17	0.68	0.17	0.17
Tyrosine, g	0.22	0.22	0.88	0.22	0.22
Valine, g	0.35	0.35	1.38	0.35	0.35
Added AA					
Alanine, g	3.18	2.05	0.00	0.03	1.05
Glycine, g	3.17	2.05	0.00	0.03	1.05
Leucine, g	0.00	2.25	0.00	4.25	4.25
Isoleucine, g	0.00	0.00	0.00	1.01	0.00
Valine, g	0.00	0.00	0.00	1.03	0.00
Added CHO, g	35.0	35.0	22.60	35.0	35.0
Added Fat, g	5.68	5.68	5.68	5.68	5.68
Totals					
Whey Protein, g	6.15	6.15	24.57	6.15	6.15
EAA, g	2.89	5.14	11.54	9.18	7.14
NEAA, g	9.61	7.36	13.03	3.32	5.36
Total Protein, g	12.5	12.5	24.57	12.5	12.5
Leucine, g	0.75	3.00	3.00	5.00	5.00
Isoleucine, g	0.34	0.34	1.35	1.35	0.34
Valine, g	0.35	0.35	1.38	1.38	0.35
BCAA, g	1.43	3.68	5.73	7.73	5.68
CHO, g	35.0	35.0	22.90	35.0	35.0
Fat, g	5.68	5.68	5.68	5.68	5.68
Kcal	241	241	241	241	241

Beverage composition. Study participants were administered the nutrient treatments orally in a double-blinded manner immediately following resistance exercise. All treatments were provided in coloured plastic containers. Treatments were similar in colour, smell, and taste since their main constituents were the same, only provided in different quantities depending on the treatment. The macronutrient and amino acid composition of each of the 5 treatments is outlined in Table 2. The W6+Low-Leu, W6+BCAA, W6+High-Leu, and W6 treatments were iso-nitrogenous, iso-energetic, and macronutrient-matched while the positive control (W25) contained a reduced amount of carbohydrate and more protein to be energy-matched to the other treatments. The whey protein isolate (biPro, Davisco Foods, Le Sueur, MN) was independently tested (Telmark, Matawan, NJ) in triplicate for content analysis. The free-form amino acids used were as follows: L-leucine, L-isoleucine, L-valine, L-alanine, L-glycine (Sigma Life Science; Sigma-Aldrich, St. Louis MO). The CHO source was sucrose while the fat source was hydrogenated coconut oil (Nestle Coffee Mate™, Nestec Limited, Lausanne, Switzerland). All nutrient treatments were prepared in 300 mL of water (see Table 2). To minimize disturbances in isotopic equilibrium following amino acid ingestion, beverages were enriched to 4% with L-[*ring*-$^{13}C_6$] phenylalanine based on a phenylalanine content of 3.5% in the whey protein.

Analytical methods. Blood glucose was measured using a blood glucose meter (OneTouch Ultra 2, Lifescan Inc., Milpitas, CA). Blood amino acid concentrations were analyzed via high performance liquid chromatography as described previously (35). Plasma L-[*ring*-$^{13}C_6$] phenylalanine enrichment was determined as previously described

(36). Plasma insulin concentration was measured using a commercially available immunoassay kit (ALPCO Diagnostics, Salem, NH).

Muscle samples (~40-50 mg) were homogenized on ice in buffer (10 μl mg^{-1} 25mM Tris 0.5% v/v Triton X-100 and protease/phosphatase inhibitor cocktail tablets (Complete Protease Inhibitor Mini-Tabs, Roche, Indianapolis, IN; PhosSTOP, Roche Applied Science, Mannhein, Germany)). Samples were then centrifuged at 15,000 *g* for 10 minutes 4°C. The supernatant was removed and protein concentrations were determined via BCA protein assay (Thermo Scientific, Rockford, IL). The pellet containing the myofibrillar proteins was stored at -80° C for future processing. Working samples of equal concentration were prepared in Laemmli buffer (37). Equal amounts (20 µg) of protein were loaded onto 10% or gradient precast gels (BIO-RAD Mini-PROTEAN TGX Gels, Bio-Rad Laboratories, Hercules, CA) for separation by electrophoresis. Proteins were then transferred to a polyvinylidene fluoride membrane, blocked (5% skim milk) and incubated overnight at 4°C in primary antibody: phospho-Akt^{Ser473} (1:1000, Cell Signalling Technology, #4058), phospho-mTOR^{Ser2448} (1:1000, Cell Signalling Technology, #2971), phospho-p70 S6 Kinase^{Thr389} (1:1000, Cell Signalling Technology, #9234), phospho-4E-BP1$^{Thr37/46}$ (1:1000, Cell Signalling Technology, #2855), phospho eEF2^{Thr56} (1:1000, Cell Signalling Technology, #2331), phospho-S6 Ribosomal protein (1:2000, Cell Signalling Technology, #2215). Membranes were then washed and incubated in secondary antibody (1 h at room temperature) before detection with chemiluminescence (SuperSignalWest Dura Extended Duration Substrate, ThermoScientific, #34075) on a FluorChem SP Imaging system (Alpha Innotech, Santa

Clara, CA). Phosphorylation status was expressed relative to α-tubulin (1:2000, Cell Signalling Technology, #2125) and is presented for each protein as fold-change from baseline (fasted) conditions. Images were quantified by spot densitometry using ImageJ software (US National Institutes of Health).

Muscle biopsy samples were processed as previously described (38). To determine the intracellular $^{13}C_6$ phenylalanine enrichment, ~20-25 mg of muscle was homogenized in 0.6 M perchloric acid/L. Free amino acids in the resulting supernatant fluid were then passed over an ion-exchange resin (Dowex 50WX8-200 resin Sigma-Aldrich Ltd) and converted to their heptafluorobutyric derivatives for analysis via gas chromatography–mass spectrometry (models 6890 GC and 5973 MS; Hewlett-Packard, Palo Alto, CA) by monitoring ions 316 and 322 after electron ionization. To determine muscle free intracellular amino acid concentrations, samples were processed as previously described (35). Briefly, muscle samples were derivatized and analyzed by HPLC (HPLC: Waters model 2695; column: Waters Nova-Pak C_{18}, 4 µm; detector: Waters 474 scanning fluorescence detector, Milford, MA). To determine myofibrillar protein-bound enrichments, a separate piece (~40-50 mg) of muscle was homogenized in a standard buffer containing protease and phosphatase inhibitors as described above. The supernatant fluid was collected for Western blot analysis and the pellet was further processed to extract myofibrillar proteins as previously described (38). The resulting myofibrillar 'enriched' protein pellet was hydrolyzed in 6 M HCL at 110° overnight. Subsequently, the free amino acids were purified using ion-exchange chromatography and converted to their N-acetyl-n-propyl ester derivatives for analysis by gas chromatography

combustion isotope ratio mass spectrometry (GC-C-IRMS: Hewlett Packard 6890; IRMS model Delta Plus XP, Thermo Finnagan, Waltham, MA).

Calculations. The fractional synthetic rate (FSR) of myofibrillar protein was calculated using the standard precursor-product equation:

$$\text{FSR} = [(E_{2b} - E_{1b}) / (E_{IC} \times t)] \times 100$$

Where E_b is the enrichment of bound (myofibrillar) protein, E_{IC} is the average enrichment of the intracellular free amino acid precursor pool of two muscle biopsies, and t is the tracer incorporation time in h. The utilization of "tracer naïve" subjects allowed us to use a pre-infusion blood sample (i.e., a mixed plasma protein fraction) as the baseline enrichment (E_{1b}) for calculation of baseline (fasted) FSR (31, 39, 40); an approach validated by our research group (33) and others (41).

Statistics. Strength tests and dietary run-in variables were compared using a one-factor (treatment) ANOVA. Blood glucose and plasma insulin were analyzed using a two-factor (treatment × time) repeated measures ANOVA. Data for area under the curve above baseline (AUC_{pos}), maximum concentration (Cmax), time of maximum concentration (Tmax), and area under the curve below baseline (AUC_{neg}) were analyzed using a one-factor (treatment) ANOVA. Plasma enrichments were analyzed using a two-factor (treatment × time) repeated measures ANOVA and linear regression. Intracellular precursor pool enrichments were analyzed using a three-factor (treatment × time × condition) mixed model ANOVA and linear regression. Intracellular amino acids (leucine, isoleucine, valine, and sum of the EAA), protein phosphorylation, and

myofibrillar FSR were analyzed using a three-factor (treatment × time × condition) mixed model ANOVA. Protein phosphorylation is expressed as fold-change from baseline (fasted). A Tukey post-hoc analysis was performed whenever a significant F ratio was found to isolate specific differences. Statistical analyses were performed using SPSS software package (version 16). For data that did not pass normality, the values were transformed using the square root, reciprocal, or natural log of the value. Statistical analysis was performed on the transformed data but mean ± standard error of the mean (SEM) of non-transformed data are presented in graphic or tabular form for clarity. Means were considered to be statistically significant for P values < 0.05. The study was powered based on previous work from our group (7) showing that 20 g protein stimulates significantly greater MPS rates than 5 g protein during the initial 4 hours post-exercise with no further increase in MPS when 40 g protein was ingested. Therefore, W6+Low-Leu was chosen to show a significantly greater average post-exercise myofibrillar FSR than W6. W6+Low-Leu was chosen over W6+BCAA and W6+High-Leu, as this treatment was hypothesized to be less likely to demonstrate efficacy. Therefore, the trial was designed to show a relevant effect in myofibrillar FSR of 0.033% $\bullet$ h^{-1}. The standard deviation was assumed to be 0.022% $\bullet$ h^{-1} based on our previous observations (7). In order to show this effect as significant with a two-sided statistical test, with an experiment wise false positive rate of 5% and a power of 80%, n = 8 per group were needed.

RESULTS

Participant characteristics. Participant characteristics are shown in Table 1. Each treatment group consisted of n = 8 randomly assigned participants, all of whom received

their intended treatment and whose results were analysed for the primary and secondary outcomes.

Exercise variables. There were no significant differences between treatment groups for 1-RM (Table 1), or the product of load (kg) x volume (# of repetitions) for exercise performed during the experiment (data not shown; all $P > 0.05$).

Dietary run-in. Participants received ~1.2 g protein/kg bodyweight/day during the standardized diet with no significant differences between treatment groups. There were no differences between treatment groups for total energy, protein, carbohydrate, or fat (data not shown) (all $P > 0.05$).

Blood glucose, plasma insulin, and blood amino acid concentrations. Blood glucose concentration showed a rapid but transient increase, being elevated above baseline at 20 and 40 minutes following treatment administration (main effect for time, $P = <0.001$). In addition, blood glucose concentration was greater in W6 than W25 (W6: 5.50 ± 0.33 vs. W25: 4.95 ± 0.20 mM; main effect for treatment, $P = 0.019$).

Plasma insulin concentration increased rapidly following treatment administration showing a main effect for time ($P < 0.001$; **Figure 1)**. Area under the insulin curve (AUC inset; Figure 1) following treatment administration was not different between treatment groups ($P = 0.497$).

Concentration over time for blood leucine, isoleucine, valine, and sum of the essential amino acids are shown in panel A-D respectively in **Figure 2**. No statistical analysis was performed on the concentration over time data. Area under the curve above baseline (AUC_{pos}), maximum concentration (Cmax), time of maximum concentration (Tmax), and

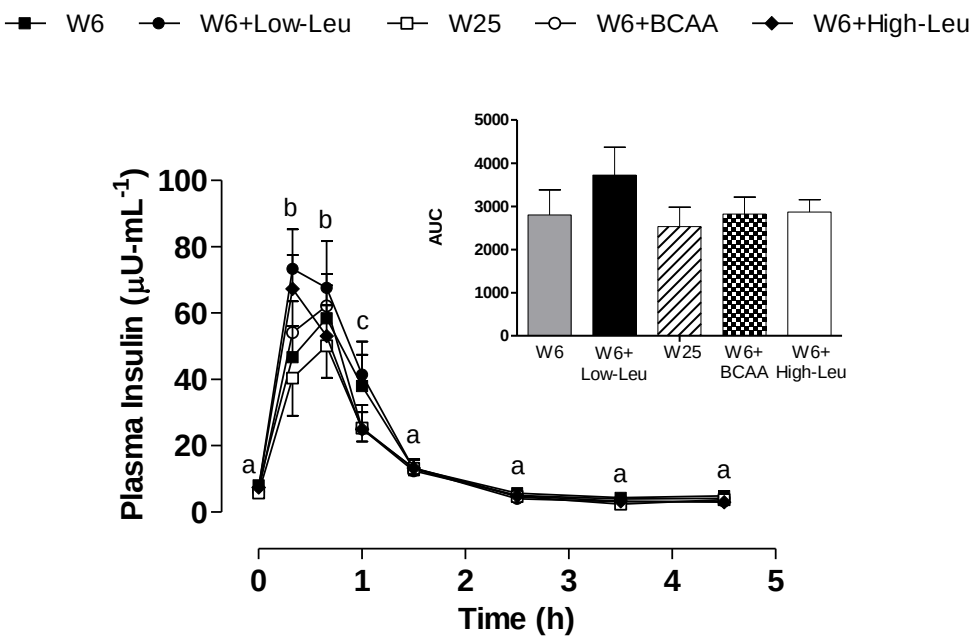


Figure 1. Values are mean ± SEM (n = 8 per treatment group). Plasma insulin concentration (μmol•L^{-1}) following treatment administration. Inset shows the AUC. Time course data were analyzed using a 2-factor (treatment × time) repeated measures ANOVA with Tukey's post hoc test (main effect for time, $P < 0.001$; treatment × time interaction, $P = 0.26$). Times with different letters are significantly different from each other. AUC (inset) was analyzed using a 1-factor (treatment) ANOVA with Tukey's post hoc test ($P = 0.497$). W6 (6.25 g whey protein); W6+Low-Leu (6.25 g whey protein supplemented with leucine to 3.0 g total leucine); W25 (25 g whey protein); W6+BCAA (6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine); W6+High-Leu (6.25 g whey protein supplemented with leucine to 5.0 g total leucine).

area under the curve below baseline (AUC_{neg}) were analyzed for blood leucine, isoleucine, valine, and ΣEAA and are presented in **Table 3**. Both AUC_{pos} and Cmax for blood leucine were greatest following W6+High-Leu being significantly different from W6+Low-Leu, W6, and W25. For both isoleucine and valine, AUC_{neg} was reduced following W6+BCAA and W25, being significantly different from W6+High-Leu. Time

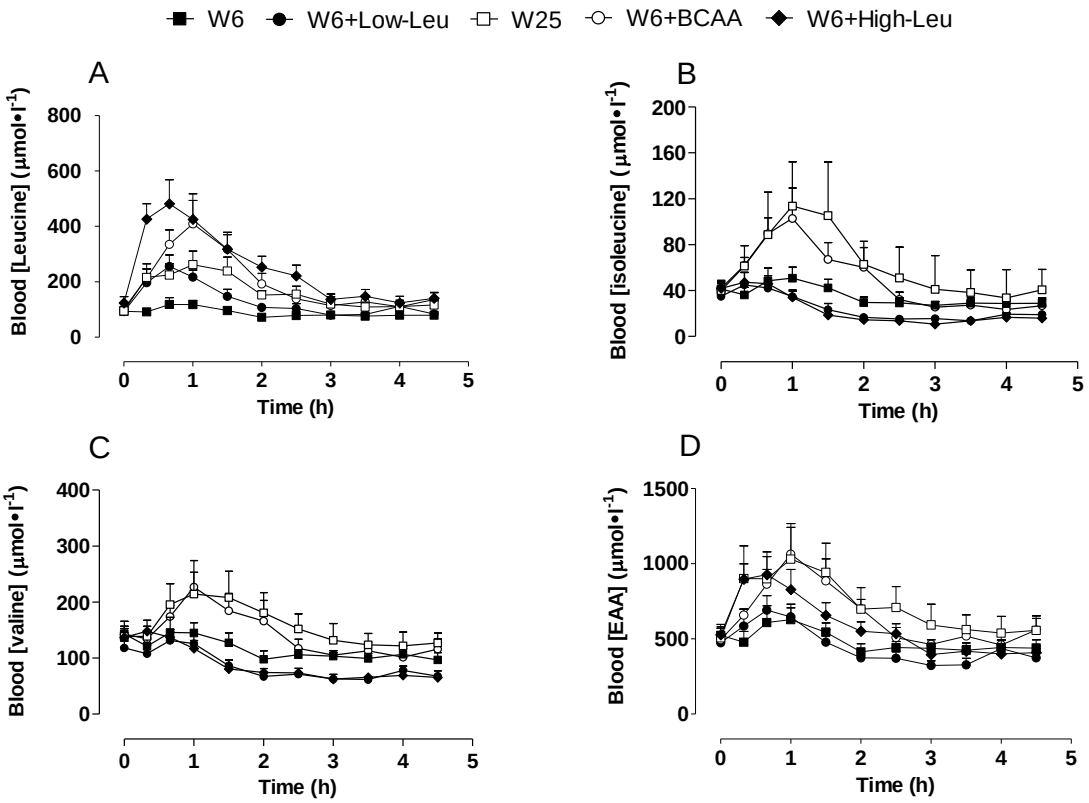


Figure 2. Values are mean ± SEM (*n* = 8 per treatment group). Blood concentrations (µmol•L^{-1}) of leucine (A), isoleucine (B) valine (C) and ΣEAA (D) following treatment administration. No statistical analysis was performed on the time-course data. W6 (6.25 g whey protein); W6+Low-Leu (6.25 g whey protein supplemented with leucine to 3.0 g total leucine); W25 (25 g whey protein); W6+BCAA (6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine); W6+High-Leu (6.25 g whey protein supplemented with leucine to 5.0 g total leucine).

of maximum concentration (Tmax) for leucine, isoleucine, valine, and ΣEAA tended to occur latest for W25 and most rapidly for W6+High-Leu (Table 3).

Table 3. Variables of blood leucine, isoleucine, valine, and ΣEAA following treatment administration

	W6	W6+Low-Leu	W25	W6+BCAA	W6+High-Leu	*P*
leucine						
AUC^{pos}	3223 ± 1465	12234 ± 1629‡	19252 ± 3393‡	27517 ± 4493‡*	35278 ± 6016‡*	< 0.001
C_{max} (μmol•l^{-1})	145 ± 18	295 ± 40	309 ± 51	459 ± 75‡	554 ± 74$^{‡*\#}$	< 0.001
T_{max} (min)	49 ± 8	43 ± 5	75 ± 14	51 ± 7	41 ± 9	0.084
AUC_{neg}	-4654 ± 1906$^{\#}$	-1510 ± 903	-196 ± 157	-991 ± 469	-565 ± 282	0.025
isoleucine						
AUC^{pos}	1145 ± 764	432 ± 142	6692 ± 1766$^{‡*+}$	4225 ± 883$^{‡*+}$	344 ± 157	< 0.001
C_{max} (μmol•l^{-1})	61 ± 9	55 ± 10	131 ± 29$^{‡*+}$	122 ± 22^{*+}	55 ± 8	< 0.001
T_{max} (min)	57 ± 9	40 ± 5	70 ± 8^{*+}	45 ± 6	35 ± 6	0.008
AUC_{neg}	-2919 ± 721	-3503 ± 581$^{\#}$	-643 ± 261	-2073 ± 445	-5618 ± 1039$^{†\#}$	< 0.001
valine						
AUC^{pos}	2189 ± 1741	489 ± 165	7752 ± 2516$^{‡*+}$	5347 ± 1373$^{‡*+}$	665 ± 337	< 0.001
C_{max} (μmol•l^{-1})	178 ± 21	150 ± 14	229 ± 44	246 ± 40*	162 ± 16	0.035
T_{max} (min)	53 ± 10	40 ± 5	74 ± 10^{+}	58 ± 10	33 ± 5	0.015
AUC_{neg}	-9690 ± 2451	-9457 ± 1689	-2174 ± 486	-5247 ± 2163	-13988 ± 2012$^{\#†}$	0.001
ΣEAA						
AUC^{pos}	11739 ± 6425	11841 ± 2310	62722 ± 18780‡*	48937 ± 11917‡*	31047 ± 7267	< 0.001
C_{max} (μmol•l^{-1})	742 ± 85	801 ± 95	1175 ± 188	1183 ± 175‡	1088 ± 90	0.008
T_{max} (min)	53 ± 10	40 ± 5	83 ± 17	56 ± 7	39 ± 9	0.054
AUC_{neg}	-22492 ± 9420	-18734 ± 4681	-3028 ± 1282*	-6135 ± 2122	-16198 ± 5315	0.013

Values are mean ± SEM (n = 8 per treatment group). Blood concentrations ($\mu mol \bullet L^{-1}$) of leucine, isoleucine, valine, and sum (Σ) of the essential amino acids (EAA) following treatment administration. Area under the curve above baseline (AUC^{pos}), maximum concentration (Cmax), time of maximum concentration (Tmax), and area under the curve below baseline (AUC_{neg}) were each analyzed for blood leucine, isoleucine, valine, and ΣEAA using a 1 factor (treatment) ANOVA with Tukey's post hoc test. ‡ Significantly different from W6; * significantly different from W6+Low-Leu; # significantly different from W25; † significantly different from W6+BCAA; + significantly different from W6+High-Leu. W6 (6.25 g whey protein); W6+Low-Leu (6.25 g whey protein supplemented with leucine to 3.0 g total leucine); W25 (25 g whey protein); W6+BCAA (6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine); W6+High-Leu (6.25 g whey protein supplemented with leucine to 5.0 g total leucine).

Intracellular leucine, isoleucine, valine, and ΣEAA.

Intracellular concentrations of leucine, isoleucine, valine and ΣEAA are shown in **Table 4**. Intracellular leucine demonstrated a time × treatment interaction (P = 0.031), increasing at 1.5 h post-treatment for all treatment groups except W6, but returning to values not different from baseline by 4.5 h. Intracellular isoleucine demonstrated a time × condition interaction (P = 0.012) increasing above baseline at 1.5 h in the FED condition only. Intracellular valine demonstrated a main effect for time (P = 0.006) falling below baseline fasted concentrations at 4.5 h. There were no time (P = 0.691), treatment (P = 0.661), or condition (P = 0.707) effects for ΣEAA.

Plasma and intracellular free phenylalanine enrichments.

Intracellular free phenylalanine enrichments were not different across time (P = 0.337), between conditions (P = 0.128), or between treatment groups (P = 0.746). Further, there were no interaction effects for any of the factors (time × treatment, P = 0.941; condition ×

Table 4. Intracellular concentrations (μM) of leucine, isoleucine, valine, and ΣEAA following treatment administration

	W6	W6+Low-Leu	W25	W6+BCAA	W6+High-Leu	*P*
leucine						
Fasted	214 ± 11[a]	209 ± 15[a]	212 ± 16[a]	229 ± 17[a]	222 ± 18[a]	
1.5 h FED	215 ± 18[a]	248 ± 16[b]	274 ± 32[b]	313 ± 24[‡b]	344 ± 16[‡*#b]	
4.5 h FED	210 ± 8[a]	221 ± 14[a]	248 ± 8[‡a]	232 ± 12[a]	263 ± 27[‡*a]	treatment × time = 0.031
1.5 h EX-FED	222 ± 19[a]	267 ± 12[b]	255 ± 23[b]	299 ± 36[‡b]	351 ± 17[‡*#b]	
4.5 h EX-FED	188 ± 14[a]	205 ± 20[a]	224 ± 17[‡a]	225 ± 15[a]	249 ± 31[‡*a]	
isoleucine						
Fasted[a]	216 ± 27	254 ± 33	322 ± 41	296 ± 38	290 ± 37	
1.5 h FED[b]	219 ± 32	294 ± 65	365 ± 47	389 ± 67	312 ± 46	
4.5 h FED[a]	208 ± 36	276 ± 64	333 ± 39	297 ± 52	261 ± 50	time × condition = 0.012
1.5 h EX-FED[a]	218 ± 44	299 ± 64	340 ± 38	321 ± 44	284 ± 56	
4.5 h EX-FED[a]	215 ± 57	311 ± 76	330 ± 30	348 ± 71	302 ± 65	
valine						
Fasted[a]	232 ± 23	280 ± 26	259 ± 24	261 ± 16	288 ± 25	
1.5 h FED[a]	252 ± 27	224 ± 9	280 ± 29	262 ± 15	308 ± 22	
4.5 h FED[b]	222 ± 16	216 ± 14	295 ± 6	240 ± 17	195 ± 16	time = 0.006
1.5 h EX-FED[a]	246 ± 21	250 ± 13	274 ± 30	248 ± 21	255 ± 33	
4.5 h EX-FED[b]	208 ± 27	212 ± 16	264 ± 26	246 ± 25	232 ± 33	
ΣEAA						
Fasted	2966 ± 185	2730 ± 157	2718 ± 217	3142 ± 273	3016 ± 229	
1.5 h FED	3336 ± 366	2837 ± 201	3118 ± 288	2946 ± 134	3212 ± 269	time = 0.69
4.5 h FED	3301 ± 256	2801 ± 176	2925 ± 136	2741 ± 115	2818 ± 350	treatment = 0.66
1.5 h EX-FED	3190 ± 171	3110 ± 251	2684 ± 212	2732 ± 277	2626 ± 149	condition, = 0.71
4.5 h EX-FED	3163 ± 277	2657 ± 273	3198 ± 290	3066 ± 181	3261 ± 359	

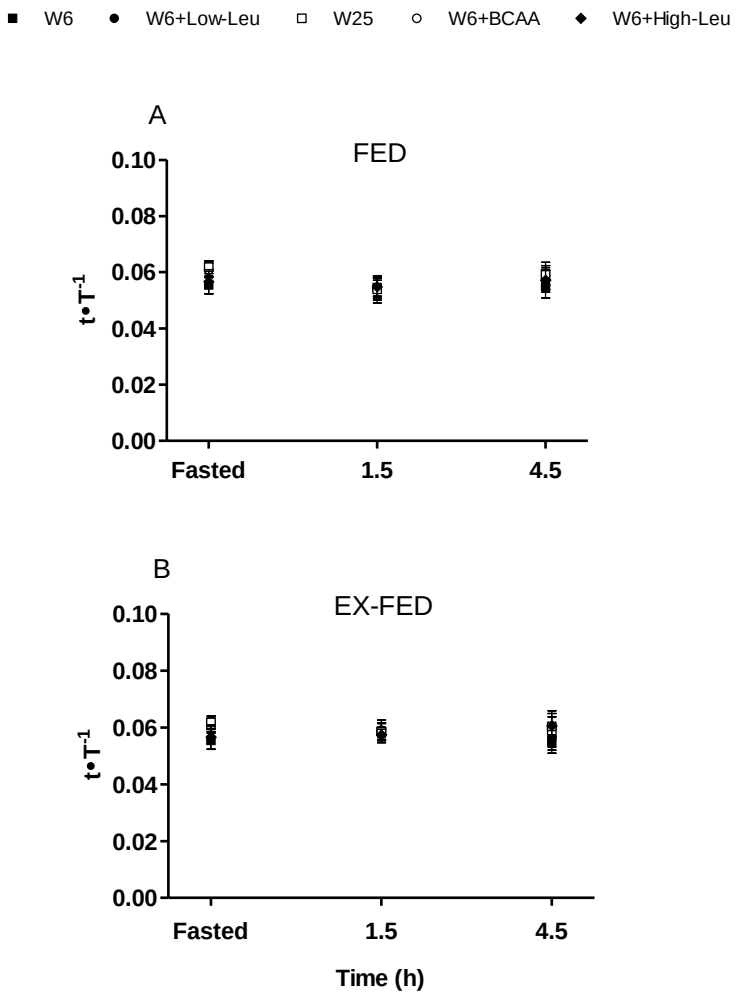
Values are mean ± SEM (n = 8 per treatment group). Intracellular concentrations ($\mu mol \bullet L^{-1}$) of leucine, isoleucine, valine, and sum (Σ) of the essential amino acids (EAA) following treatment administration. Data for leucine, isoleucine, valine, and ΣEAA were each analyzed using a 3-factor (treatment × time × condition) mixed-model ANOVA with Tukey's post hoc test. Times with different letters within treatment columns are significantly different from each other within that treatment. Times with different letters within the time/condition column are significantly different from each other. ‡ Significantly different from W6; * significantly different from W6+Low-Leu; # significantly different from W25. W6 (6.25 g whey protein); W6+Low-Leu (6.25 g whey protein supplemented with leucine to 3.0 g total leucine); W25 (25 g whey protein); W6+BCAA (6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine); W6+High-Leu (6.25 g whey protein supplemented with leucine to 5.0 g total leucine).

treatment, P = 0.992; time × condition, P = 0.117; time × condition × treatment, P = 0.975). The slope of the intracellular free phenylalanine enrichment was not different from zero for any of the treatment groups in either FED or EX-FED condition (see **Online Supplemental Material Figure 2)**.

Plasma free phenylalanine enrichments did not differ between treatment groups (P = 0.917) or across time (P = 0.58). The slope of the plasma free phenylalanine enrichment was not different from zero for any treatment group (see **Online Supplemental Material Figure 3)**.

Myofibrillar protein synthesis.

Myofibrillar FSR rates are shown in panels A-D respectively in **Figure 3**. Panels A and B show myofibrillar FSR ($\% \bullet h^{-1}$) during the early 0-1.5 h and late 1.5-4.5 h response, while panels C and D show the aggregate 0-4.5 h response under both FED and EX-FED



Online Supplemental Material Figure 2. Values are mean ± SEM (*n* = 8 per treatment group). Intracellular free phenylalanine enrichments (tracer-to-tracee ratio - $t \bullet T^{-1}$) from biopsies obtained at time 0 (Fasted), 1.5 h, and 4.5 h in both FED (A) and EX-FED (B) conditions. Data analyzed using a 3-factor (treatment × time × condition) mixed-model ANOVA with Tukey's post hoc test (time, *P* = 0.337; conditions, *P* = 0.128; treatment, *P* = 0.746; time × treatment, *P* = 0.941; condition × treatment, *P* = 0.992; time × condition, *P* = 0.117; time × condition × treatment, *P* = 0.975). Linear regression was used to examine the slope of intracellular free phenylalanine enrichment × time for each treatment in both FED (W6: *P* = 0.911; W6+Low-Leu: *P* = 0.642; W25: *P* = 0.136; W6+BCAA: *P* = 0.507; W6+High-Leu: *P* = 0.914) and EX-FED (W6: *P* = 0.934; W6+Low-Leu: *P* = 0.244; W25: *P* = 0.422; W6+BCAA: *P* = 0.777; W6+High-Leu: *P* =

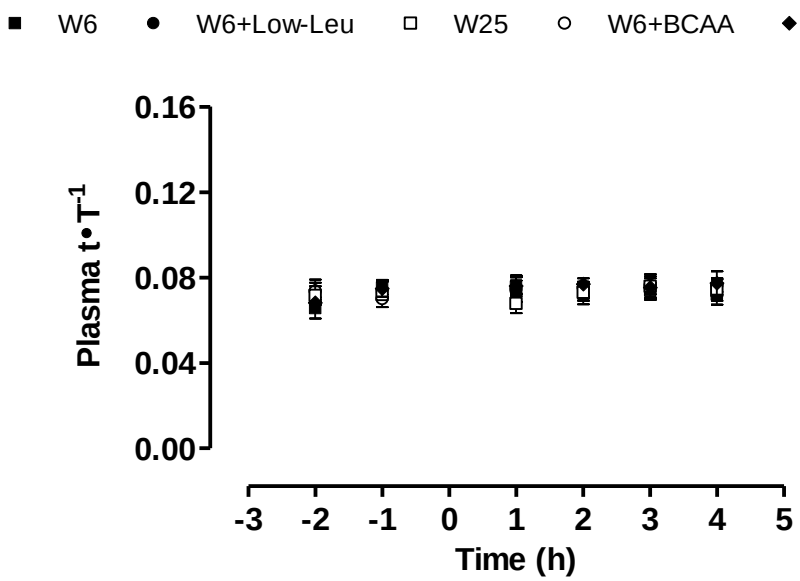
0.438) conditions. W6 (6.25 g whey protein); W6+Low-Leu (6.25 g whey protein supplemented with leucine to 3.0 g total leucine); W25 (25 g whey protein); W6+BCAA (6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine); W6+High-Leu (6.25 g whey protein supplemented with leucine to 5.0 g total leucine).

conditions. Over the 0-1.5 h and 1.5-4.5 h period, myofibrillar FSR demonstrated a treatment × time interaction ($P = 0.002$) whereby FSR was increased compared with baseline (fasted) in all treatment groups when measured over 0-1.5 h. Over 1.5-4.5 h post-exercise, FSR remained increased compared to baseline in all treatment groups; however, FSR in W25 and W6+High-Leu was greater than W6+Low-Leu, W6+BCAA, and W6. Similarly, the aggregate myofibrillar FSR response over 0-4.5 h demonstrated a treatment × time interaction ($P = 0.005$) whereby FSR was increased compared with baseline in all treatment groups during the 0-4.5 h postprandial period, however FSR was greater in W25 and W6+High-Leu than W6+Low-Leu, W6+BCAA, and W6. There were no significant differences between FED and EX-FED conditions when examined over the 0-1.5 h and 1.5-4.5 h period ($P = 0.483$) or over the aggregate 0-4.5 h period ($P = 0.419$).

Muscle anabolic signalling

Changes in the phosphorylation status of signaling proteins involved in the regulation of mRNA translation initiation and elongation are shown in **Table 5**. Protein kinase B (p-Akt^{Ser473}) showed a treatment × time × condition interaction ($P = 0.025$). p-mTOR^{Ser2448} showed a treatment × time interaction ($P = 0.041$) whereby at 1.5 h, p-mTOR^{Ser2448} was

increased following W6+Low-Leu, W25, and W6+High-Leu. At 4.5 h, p-mTOR^{Ser2448} remained increased above baseline only following W6+High-Leu. p-p70S6k$^{Thr\ 389}$ showed



Online Supplemental Material Figure 3. Values are mean ± SEM (n = 8 per treatment group). Plasma free phenylalanine enrichments (tracer-to-tracee ratio - $t \bullet T^{-1}$) over time. Time course data were analyzed using a 2-factor (treatment × time) repeated measures ANOVA with Tukey's post hoc test (treatment, P = 0.917; time, P = 0.58). Linear regression was used to examine the slope of plasma free phenylalanine enrichment × time for each treatment (W6: P = 0.455; W6+Low-Leu: P = 0.858; W25: P = 0.424; W6+BCAA: P = 0.357; W6+High-Leu: P = 0.156). W6 (6.25 g whey protein); W6+Low-Leu (6.25 g whey protein supplemented with leucine to 3.0 g total leucine); W25 (25 g whey protein); W6+BCAA (6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine); W6+High-Leu (6.25 g whey protein supplemented with leucine to 5.0 g total leucine).

no effect of time ($P = 0.377$), treatment ($P = 0.353$), or condition ($P = 0.062$) at the times examined. p-4E-BP1$^{Thr\ 37/46}$ showed a condition × time interaction ($P = 0.044$) whereby both conditions (FED and EX-FED) were increased above baseline at 1.5 h, while at 4.5 h, p-4E-BP1$^{Thr\ 37/46}$ was significantly greater in the EX-FED vs. FED condition. For p-rpS6$^{Ser\ 240/244}$ there was a condition × time interaction ($P < 0.001$) whereby both conditions (FED and EX-FED) were increased above baseline fasted at both 1.5 h and 4.5 h; however the increase in the EX-FED condition at 1.5 h was greater than the FED condition. p-eEF2$^{Thr\ 56}$ showed no effect of time ($P = 0.197$), treatment ($P = 0.384$), or condition ($P = 0.091$) at the times examined. Representative blot images are shown as **Online Supplemental Material Figure 4.**

DISCUSSION

Our results demonstrate that the addition of a higher dose of leucine to a smaller amount of protein (6.25 g) within a mixed macronutrient beverage enhanced MPS to same level as that seen with four times as much whey protein (25 g). Muscle protein synthesis is increased in response to exercise and protein feeding in healthy individuals (42), and is the primary variable determining diurnal changes in net muscle protein balance (43). It has been demonstrated that MPS is stimulated in a protein/EAA dose-dependent manner up to ~10 g of EAA at rest (6), and ~20 g of protein (~8.6 g EAA) after resistance exercise (7). Whey protein was utilized as the base protein source in this study because it is a high quality protein source that robustly stimulates postprandial MPS rates (for review see (44)). However, protein is typically co-ingested with CHO and fat during meals, which may alter the kinetics of gut amino acid absorption (45). Thus, in this trial

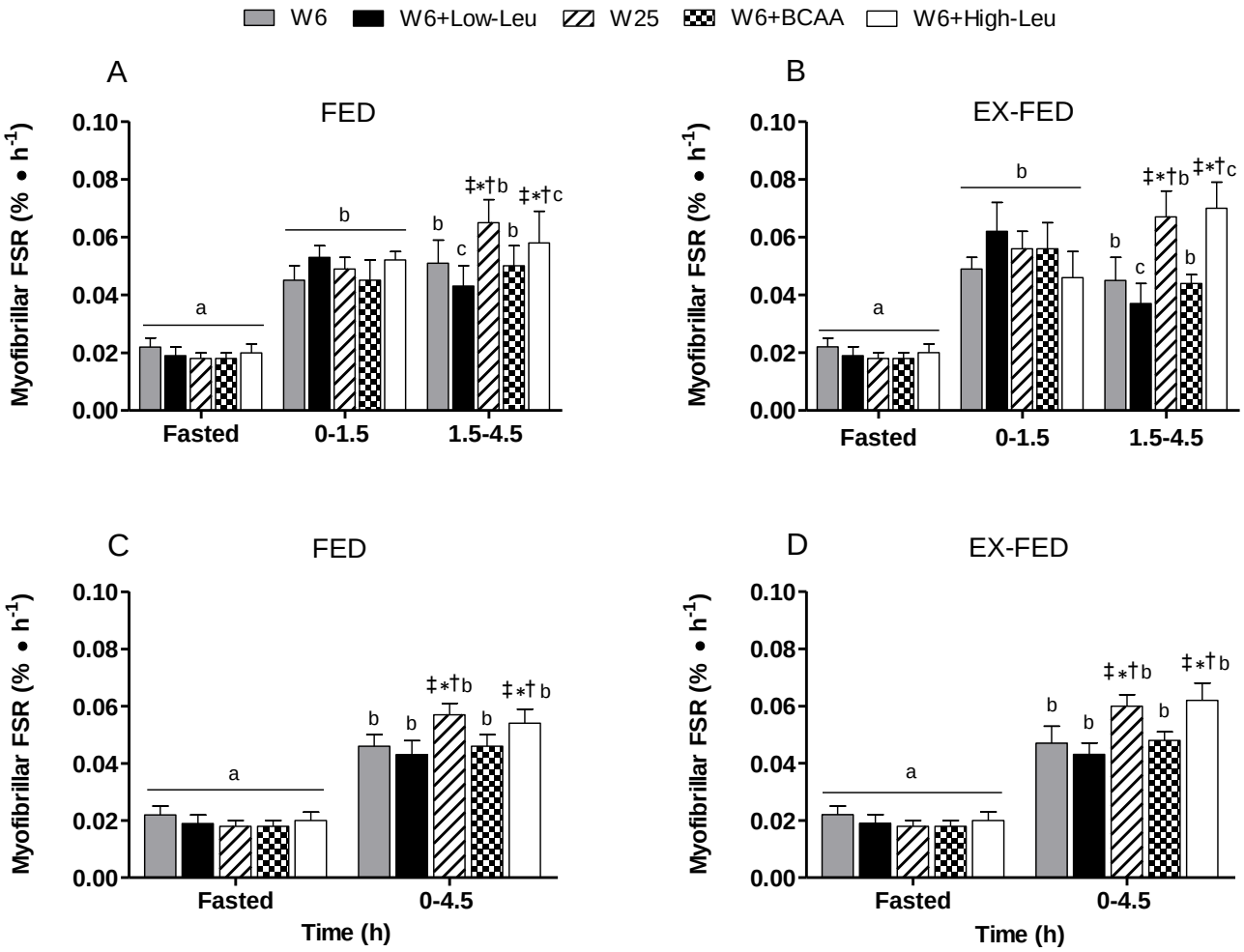

W6
W6+Low-Leu
W25
W6+BCAA
W6+High-Leu
A
FED
B
EX-FED
C
FED
D
EX-FED
Myofibrillar FSR (% • h-1)
0.10
0.08
0.06
0.04
0.02
0.00
Fasted
0-1.5
1.5-4.5
0-4.5
Time (h)
a
b
c
‡*†b
‡*†c

Figure 3. Values are mean ± SEM (n = 8 per treatment group). Myofibrillar fractional synthetic rate (FSR) (%•h^{-1}) calculated during baseline (fasted) conditions, over both early (0-1.5 h), and late (1.5-4.5 h) time periods (Panels A-B), and over the aggregate 0-4.5 h period post-exercise recovery period (Panels C-D) in both FED and EX-FED conditions after treatment administration. Data were analyzed using a 3-factor (treatment × time × condition) mixed-model ANOVA with Tukey's post hoc test for analysis of the 0-1.5 h and 1.5-4.5 h response (treatment × time interaction, P = 0.002; treatment × time × condition, P = 0.799), and the aggregate 0-4.5 h response (treatment × time interaction, P = 0.005; treatment × time × condition, P = 0.942). Times with different letters are significantly different from each other within that treatment. ‡ Significantly different from W6 within that time; *****significantly different from W6+Low-Leu within that time; † significantly different from W6+BCAA within that time. W6 (6.25 g whey protein); W6+Low-Leu (6.25 g whey protein supplemented with leucine to 3.0 g total leucine); W25 (25 g whey protein); W6+BCAA (6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine); W6+High-Leu (6.25 g whey protein supplemented with leucine to 5.0 g total leucine).

we tested the efficacy of mixed macronutrient beverages with varying doses of whey protein and amino acids to stimulate MPS.

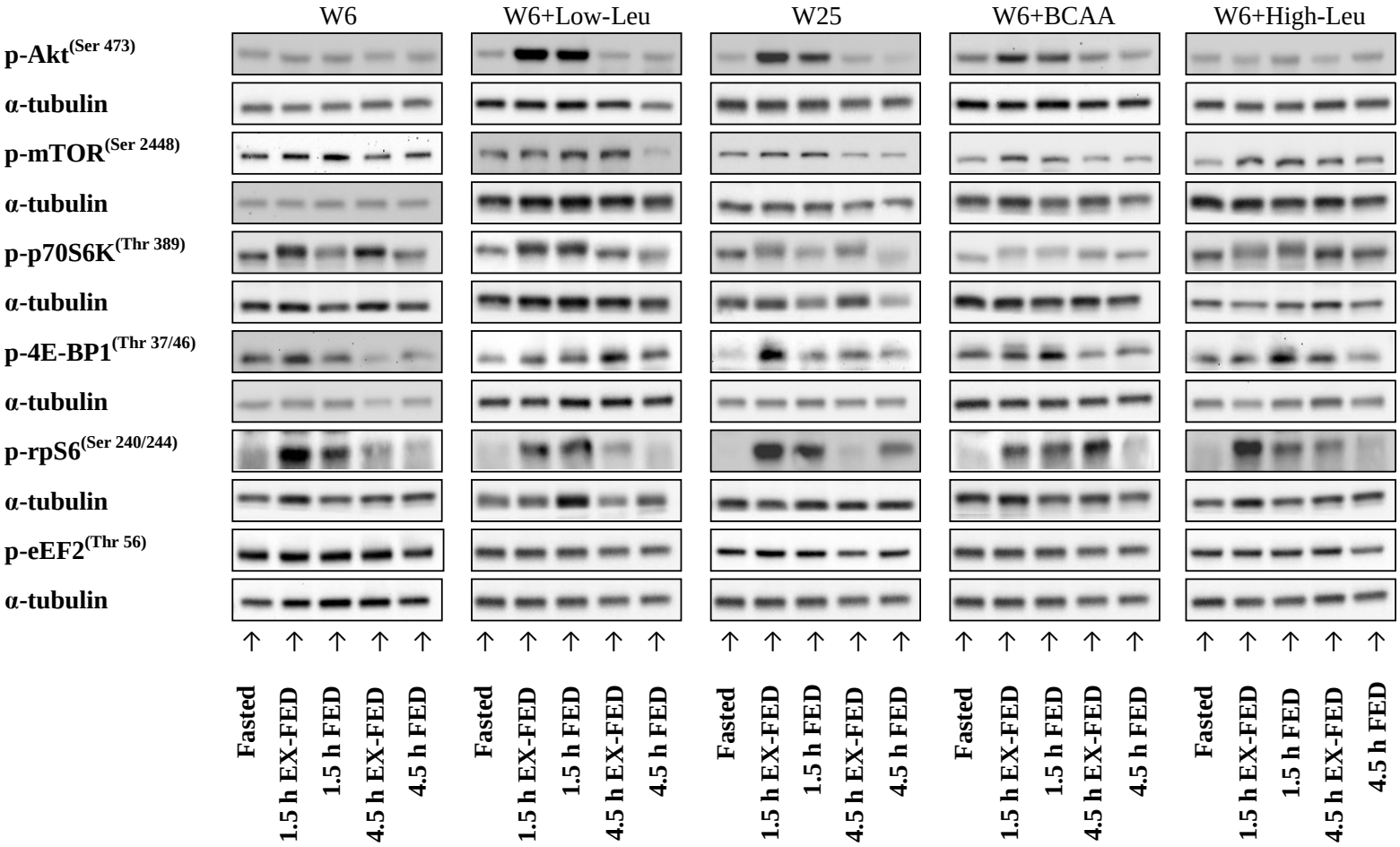
Consistent with our previous results utilizing protein feeding alone (7), we found that a low dose of protein (W6) was suboptimal for stimulating maximal MPS rates compared to four times as much whey protein (W25) even within a mixed macronutrient beverage over the aggregate 0-4.5 h postprandial period. Interestingly, supplementing this low protein dose with a high proportion of leucine (W6+High-Leu) stimulated MPS to an equivalent magnitude and duration as that stimulated following ingestion of an energy-matched mixed macronutrient beverage containing 25 g of whey protein (W25). Previous work has shown that 25 g of whey protein is a dose of protein and EAA

Table 5. Western-blot analysis of protein synthesis-associated signalling proteins following treatment administration

	W6	W6+Low-Leu	W25	W6+BCAA	W6+High-Leu	*P*
	Fold difference from baseline					
p-Akt$^{(Ser\ 473)}$						
1.5 h FED	1.61 ± 0.30	2.89 ± 0.69^{‡†a}	2.09 ± 0.38^{a}	1.52 ± 0.25	1.76 ± 0.18	
4.5 h FED	0.70 ± 0.17	1.67 ± 0.40$^{‡†+\$}$	1.01 ± 0.24	0.71 ± 0.16	0.74 ± 0.09	treatment × time × condition = 0.025
1.5 h EX-FED	1.45 ± 0.18	4.23 ± 1.40$^{‡+\$a}$	3.69 ± 0.64$^{‡\$a}$	2.47 ± 0.24	2.03 ± 0.46	
4.5 h EX-FED	1.38 ± 0.41$^{\$}$	1.07 ± 0.10	1.50 ± 0.30	1.20 ± 0.16	1.17 ± 0.21	
p-mTOR$^{(Ser\ 2448)}$						
1.5 h FED	1.05 ± 0.15	1.59 ± 0.49^{a}	1.52 ± 0.29^{a}	1.35 ± 0.15	1.89 ± 0.31^{‡†a}	
4.5 h FED	0.62 ± 0.15	0.86 ± 0.19	1.25 ± 0.22	0.89 ± 0.15	1.63 ± 0.26^{‡†a}	
1.5 h EX-FED	1.23 ± 0.16	1.45 ± 0.29^{a}	1.60 ± 0.09^{a}	1.45 ± 0.14	2.14 ± 0.36^{‡†a}	treatment × time = 0.041
4.5 h EX-FED	0.87 ± 0.11	1.41 ± 0.39	1.10 ± 0.23	1.11 ± 0.14	1.71 ± 0.25^{‡†a}	
p-p70S6K1$^{(Thr\ 389)}$						
1.5 h FED	1.04 ± 0.10	1.13 ± 0.09	1.02 ± 0.07	0.98 ± 0.08	1.12 ± 0.13	
4.5 h FED	0.97 ± 0.13	1.02 ± 0.11	0.97 ± 0.13	0.86 ± 0.12	1.14 ± 0.14	time = 0.38
1.5 h EX-FED	1.15 ± 0.11	1.13 ± 0.09	1.06 ± 0.06	0.92 ± 0.10	1.07 ± 0.11	treatment = 0.35
4.5 h EX-FED	1.01 ± 0.12	1.06 ± 0.08	1.02 ± 0.10	0.93 ± 0.09	1.33 ± 0.09	condition = 0.06
p-4E-BP1$^{(Thr\ 37/46)}$						
1.5 h FED	1.02 ± 0.12	1.19 ± 0.12	1.25 ± 0.18	1.20 ± 0.12	1.58 ± 0.24	
4.5 h FED	0.73 ± 0.07	0.95 ± 0.15	0.86 ± 0.17	1.04 ± 0.19	0.85 ± 0.14	condition × time = 0.044 (1.5h > baseline)
1.5 h EX-FED	1.05 ± 0.17	1.28 ± 0.16	1.77 ± 0.48	1.38 ± 0.20	1.59 ± 0.38	(1.5h EX-FED > FED)
4.5 h EX-FED	1.02 ± 0.18	1.05 ± 0.18	1.20 ± 0.29	1.12 ± 0.10	1.45 ± 0.22	
p-rpS6$^{(Ser\ 240/244)}$						
1.5 h FED	1.72 ± 0.41	2.46 ± 0.93	1.93 ± 0.32	2.66 ± 0.65	2.45 ± 0.56	
4.5 h FED	1.31 ± 0.22	2.31 ± 0.59	2.09 ± 0.42	1.63 ± 0.32	1.12 ± 0.30	condition × time < 0.001 (all times > baseline)
1.5 h EX-FED	3.25 ± 1.08	3.34 ± 0.93	4.45 ± 1.63	3.67 ± 1.27	4.49 ± 1.48	(1.5h EX-FED > FED)
4.5 h EX-FED	1.47 ± 0.42	2.86 ± 1.26	1.38 ± 0.15	2.41 ± 0.65	2.44 ± 0.59	
p-eEF2$^{(Thr\ 56)}$						
1.5 h FED	0.95 ± 0.06	0.96 ± 0.05	1.02 ± 0.06	1.11 ± 0.09	1.08 ± 0.04	
4.5 h FED	0.93 ± 0.07	1.08 ± 0.13	1.12 ± 0.12	0.99 ± 0.07	1.00 ± 0.06	time = 0.197
1.5 h EX-FED	0.92 ± 0.03	1.15 ± 0.08	1.12 ± 0.10	1.09 ± 0.05	1.13 ± 0.04	treatment = 0.384
4.5 h EX-FED	0.88 ± 0.06	1.11 ± 0.10	1.03 ± 0.07	1.07 ± 0.11	1.05 ± 0.04	condition = 0.091

Values are mean ± SEM (n = 8 per treatment group). Phosphorylation status of Akt ^{Ser473}, mTOR ^{Ser2448}, p70S6k ^{Thr389}, 4E-BP1 $^{Thr37/46}$, rpS6 $^{Ser240/244}$, eEF2 ^{Thr56}, expressed as fold-difference from baseline (fasted), at 1.5 h and 4.5 h post-exercise recovery in both FED and EX-FED conditions following treatment administration. Data for Akt ^{Ser473}, mTOR ^{Ser2448}, p70S6k ^{Thr389}, 4E-BP1 $^{Thr37/46}$, rpS6 $^{Ser240/244}$, eEF2 ^{Thr56} were analyzed using a 3-factor (treatment × time × condition) mixed-model ANOVA with Tukey's post hoc test. A superscript letter "a" indicates a difference from baseline. ‡ Significantly different from W6; # significantly different from W25; † significantly different from W6+BCAA; + significantly different from W6+High-Leu; $ significantly different from opposite condition at the same time-point. W6 (6.25 g whey protein); W6+Low-Leu (6.25 g whey protein supplemented with leucine to 3.0 g total leucine); W25 (25 g whey protein); W6+BCAA (6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine); W6+High-Leu (6.25 g whey protein supplemented with leucine to 5.0 g total leucine).

sufficient to induce a maximal stimulation of MPS rates at rest (6) and following resistance exercise (7). Of interest, there were no differences among treatments in MPS during the 0-1.5 h postprandial period; however, W6+High-Leu and W25 stimulated greater MPS over the 1.5-4.5 h and aggregate 0-4.5 h period than each of the other treatments (Figure 3). The somewhat surprising lack of difference in MPS rates among treatments during the early post-exercise/feeding period occurred despite markedly divergent blood and intramuscular leucine, isoleucine, valine, and EAA concentrations (Figure 2 and Tables 3 and 4). Presumably, this lack of difference early after feeding (i.e. up to 1.5 h) suggests that amino acid availability and/or nutrient signals (leucine) that serve to trigger MPS were equivalent in all conditions. In contrast, in the latter portion of the protocol, only in the W6+High-Leu treatment showed an MPS response that was


W6
W6+Low-Leu
W25
W6+BCAA
W6+High-Leu
p-Akt(Ser 473)
α-tubulin
p-mTOR(Ser 2448)
α-tubulin
p-p70S6K(Thr 389)
α-tubulin
p-4E-BP1(Thr 37/46)
α-tubulin
p-rpS6(Ser 240/244)
α-tubulin
p-eEF2(Thr 56)
α-tubulin
Fasted
1.5 h EX-FED
1.5 h FED
4.5 h EX-FED
4.5 h FED

Online Supplemental Material Figure 4. Representative western blot images for p-Akt^{Ser473}, p-mTOR^{Ser2448}, p-p70S6k^{Thr389}, p-4E-BP1$^{Thr\ 37/46}$, p-rpS6$^{Ser240/244}$, p-eEF2^{Thr56}, and α-tubulin during Fasted, and 1.5 h EX-FED, 1.5 h FED, 4.5 h EX-FED; and 4.5 h FED following nutrient treatment administration. W6 (6.25 g whey protein); W6+Low-Leu (6.25 g whey protein supplemented with leucine to 3.0 g total leucine); W25 (25 g whey protein); W6+BCAA (6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine); W6+High-Leu (6.25 g whey protein supplemented with leucine to 5.0 g total leucine).

equivalent to that of W25, despite containing only one quarter of the whey protein dose and ~62% of the EAA content. That the W6+High-Leu was effective may relate to the fact that this treatment elicited the greatest blood leucine AUC^{pos} (Table 3), the greatest intracellular leucine concentration when assessed at 1.5 h (Table 4), and led to a sustained increase in the phosphorylation of mTOR$^{(ser2448)}$ at 4.5 h (Table 5). We also observed an increase in the phosphorylation of targets downstream of mTOR$^{(ser2448)}$ including 4E-BP1$^{(Thr37/46)}$ and rpS6$^{(Ser\ 240/244)}$ although there were no statistically significant treatment effects (Table 5). We powered our study to detect relevant differences in myofibrillar FSR (% $\bullet$ h^{-1}), thus we may have lacked the statistical power to detect important differences in intracellular signalling molecule phosphorylation. In partial agreement with our observation of the MPS response following the W6+High-Leu treatment, previous studies have shown that a high proportion of leucine (3.5 vs. 1.8 g) within a 10 g EAA solution resulted in greater intramuscular cell signalling and a more prolonged mixed MPS response (24).

Contrary to our hypothesis, the W6+BCAA treatment resulted in MPS rates that were less robust compared to W6+High-Leu and W25. These differences occurred despite the fact that supplemental isoleucine and valine attenuated the decline in the concentrations of these amino acids in the blood as compared to that observed following W6+High-Leu (Table 3); however, intracellular concentrations of isoleucine and valine were not statistically different between these treatments (Table 4). Additionally, W6+BCAA was associated with a lower intracellular leucine concentration at 1.5 h, a lower mean leucine AUC^{pos}, a lower leucine Cmax, and a greater Tmax when compared to W6+High-Leu (Table 3). We have shown that a rapid aminoacidemia following protein feeding stimulates greater MPS rates following resistance exercise than a slow protracted aminoacidemia (46). Therefore, we speculate that the greater Tmax for leucine, isoleucine, valine and ΣEAA following W6+BCAA as compared to W6+High-Leu may partially explain the observed differences in MPS rates. Given that BCAA share a common intestinal transporter, the differences in the amino acid appearance profiles between W6+BCAA and W6+High-Leu likely represents antagonism between the BCAA for uptake from the gut, which is congruent with data showing that isoleucine and valine compete with and can impede leucine absorption (47). The same could be true for the trans-sarcolemmal BCAA transport since the BCAA share the same transporter at that site (48).

We have previously shown that a suboptimal protein dose (6.25 g whey) supplemented with leucine (total leucine = 3.0 g) or a complete mixture of EAA devoid of leucine (total leucine = 0.75 g) can stimulate postprandial MPS rates equivalent to that

stimulated following ingestion of 25 g whey protein (total leucine = 3.0 g) under resting but not post-resistance exercise conditions (14). Similarly, in this study W6+Low-Leu (total leucine = 3.0 g) and W6 (total leucine = 0.75 g) were as effective as W25 (total leucine = 3.0 g) at stimulating MPS rates when assessed during the early 0-1.5 h, but not the later 1.5-4.5 h period. We found no difference between FED vs. EX-FED rates of MPS, likely because of our choice of tissue sampling times. Our current results extend those of our previous work (14) by demonstrating that within the context of a mixed macronutrient beverage, a suboptimal protein dose (6.25 g) supplemented with a higher proportion of leucine (5.0 g total) is as effective at stimulating increased MPS rates as a dose of protein (25 g) able to induce a maximal stimulation of MPS rates following resistance exercise (7) and a dose of EAA that maximally stimulates MPS at rest (6).

A novel aspect of our current study was that the protein and amino acids were co-ingested with CHO and fat. In our previous work (14) in which protein and free amino acids were ingested in isolation, supplementing 6.25 g of whey to contain 3.0 g of leucine induced peak blood amino acid concentrations of ~550.0 μM, whereas in this study, the same protein dose supplemented up to 5.0 g leucine was necessary to achieve similar peak blood leucine concentrations when co-ingested with CHO and fat as part of a mixed macronutrient beverage. Thus, as has been reported previously (45, 49), co-ingestion of protein with additional macronutrients attenuates the postprandial rise in blood amino acid concentrations.

While several studies have assessed the effects of protein-CHO co-ingestion on MPS rates (49, 50), few studies have examined the MPS response following physiological

(i.e. single bolus) co-ingestion of protein, CHO, and fat (51). Although the addition of CHO to protein does not further stimulate increased MPS rates when adequate protein is provided (49, 50), it is not clear whether insulin can enhance MPS rates following intake of a suboptimal protein dose in the young. In the present study we observed robust increases in MPS rates in the W6 treatment, consisting of only 6.25 g whey protein but co-ingested with 35.0 g CHO. Whether the MPS response to W6 was enhanced by addition of CHO or whether only 0.75 g of leucine serves as a sufficient nutrient signal to stimulate early increases in MPS rates in young healthy individuals (14) requires further investigation. In conclusion, our results demonstrate that when a 'suboptimal' dose of protein (6.25 g), is supplemented with a relatively high dose of leucine (W6+High-Leu), rates of MPS are equivalent in both magnitude and duration to those observed following ingestion of an energy-matched beverage containing a saturating for MPS, 25 g dose of whey protein (7). These findings demonstrate that within the context of mixed macronutrient intake, suboptimal protein doses can be made more effective in stimulating MPS through the addition of a high proportion of free leucine. This may be of importance in the development of nutritional formulations designed to promote skeletal muscle anabolism which may be of particular significance to individuals in whom total protein intake is restricted or inadequate.

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Author's contributions to manuscript: SMP, TS, DRM, DB, EAO designed the study; TAC-V, LB, DMDD, CJM, TS, SKB, and SMP conducted the research; TAC-V, AJH, and SMP analyzed data; TAC-V and SMP wrote the manuscript; all authors assisted in editing and having meaningful input into the manuscript; TAC-V and SMP had primary responsibility for final content. All authors read and approved the final manuscript. DRM and TS were and DB and EAO are employees of Nestec S.A., which is a subsidiary of Nestlé Ltd. and provides professional assistance, research, and consulting services for food, dietary, dietetic, and pharmaceutical products of interest to Nestlé Ltd; the authors declare no financial conflicts of interest. TAC-V, LB, DMDD, AJH, CJM, SKB, and SMP have no conflicts of interest to declare.

REFERENCES

1. Bennet WM, Connacher AA, Scrimgeour CM, Smith K, Rennie MJ. Increase in anterior tibialis muscle protein synthesis in healthy man during mixed amino acid infusion: studies of incorporation of [1-13C]leucine. Clin Sci (Lond) 1989;76(4):447-54.

2. Dickinson JM, Fry CS, Drummond MJ, Gundermann DM, Walker DK, Glynn EL, Timmerman KL, Dhanani S, Volpi E, Rasmussen BB. Mammalian target of rapamycin complex 1 activation is required for the stimulation of human skeletal muscle protein synthesis by essential amino acids. The Journal of nutrition 2011;141(5):856-62. doi: 10.3945/jn.111.139485.

3. Smith K, Reynolds N, Downie S, Patel A, Rennie MJ. Effects of flooding amino acids on incorporation of labeled amino acids into human muscle protein. The American journal of physiology 1998;275(1 Pt 1):E73-8.

4. Tipton KD, Gurkin BE, Matin S, Wolfe RR. Nonessential amino acids are not necessary to stimulate net muscle protein synthesis in healthy volunteers. The Journal of nutritional biochemistry 1999;10(2):89-95.

5. Volpi E, Kobayashi H, Sheffield-Moore M, Mittendorfer B, Wolfe RR. Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults. The American journal of clinical nutrition 2003;78(2):250-8.

6. Cuthbertson D, Smith K, Babraj J, Leese G, Waddell T, Atherton P, Wackerhage H, Taylor PM, Rennie MJ. Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2005;19(3):422-4. doi: 10.1096/fj.04-2640fje.

7. Moore DR, Robinson MJ, Fry JL, Tang JE, Glover EI, Wilkinson SB, Prior T, Tarnopolsky MA, Phillips SM. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. The American journal of clinical nutrition 2009;89(1):161-8. doi: 10.3945/ajcn.2008.26401.

8. Yang Y, Breen L, Burd NA, Hector AJ, Churchward-Venne TA, Josse AR, Tarnopolsky MA, Phillips SM. Resistance exercise enhances myofibrillar protein synthesis with graded intakes of whey protein in older men. The British journal of nutrition 2012:1-9. doi: 10.1017/S0007114511007422.

9. Norton LE, Layman DK, Bunpo P, Anthony TG, Brana DV, Garlick PJ. The leucine content of a complete meal directs peak activation but not duration of skeletal muscle protein synthesis and mammalian target of rapamycin signaling in rats. The Journal of nutrition 2009;139(6):1103-9. doi: 10.3945/jn.108.103853.

10. Anthony JC, Yoshizawa F, Anthony TG, Vary TC, Jefferson LS, Kimball SR. Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. The Journal of nutrition 2000;130(10):2413-9.

11. Escobar J, Frank JW, Suryawan A, Nguyen HV, Kimball SR, Jefferson LS, Davis TA. Regulation of cardiac and skeletal muscle protein synthesis by individual branched-chain amino acids in neonatal pigs. American journal of physiology Endocrinology and metabolism 2006;290(4):E612-21. doi: 10.1152/ajpendo.00402.2005.

12. Crozier SJ, Kimball SR, Emmert SW, Anthony JC, Jefferson LS. Oral leucine administration stimulates protein synthesis in rat skeletal muscle. The Journal of nutrition 2005;135(3):376-82.

13. Anthony JC, Lang CH, Crozier SJ, Anthony TG, MacLean DA, Kimball SR, Jefferson LS. Contribution of insulin to the translational control of protein synthesis in skeletal muscle by leucine. American journal of physiology Endocrinology and metabolism 2002;282(5):E1092-101. doi: 10.1152/ajpendo.00208.2001.

14. Churchward-Venne TA, Burd NA, Mitchell CJ, West DW, Philp A, Marcotte GR, Baker SK, Baar K, Phillips SM. Supplementation of a suboptimal protein dose with leucine or essential amino acids: effects on myofibrillar protein synthesis at rest and following resistance exercise in men. The Journal of physiology 2012. doi: 10.1113/jphysiol.2012.228833.

15. Koopman R, Verdijk L, Manders RJ, Gijsen AP, Gorselink M, Pijpers E, Wagenmakers AJ, van Loon LJ. Co-ingestion of protein and leucine stimulates muscle protein synthesis rates to the same extent in young and elderly lean men. The American journal of clinical nutrition 2006;84(3):623-32.

16. Koopman R, Wagenmakers AJ, Manders RJ, Zorenc AH, Senden JM, Gorselink M, Keizer HA, van Loon LJ. Combined ingestion of protein and free leucine with carbohydrate increases postexercise muscle protein synthesis in vivo in male subjects. American journal of physiology Endocrinology and metabolism 2005;288(4):E645-53. doi: 10.1152/ajpendo.00413.2004.

17. Tipton KD, Elliott TA, Ferrando AA, Aarsland AA, Wolfe RR. Stimulation of muscle anabolism by resistance exercise and ingestion of leucine plus protein. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme 2009;34(2):151-61. doi: 10.1139/H09-006.

18. Verhoeven S, Vanschoonbeek K, Verdijk LB, Koopman R, Wodzig WK, Dendale P, van Loon LJ. Long-term leucine supplementation does not increase muscle mass or strength in healthy elderly men. The American journal of clinical nutrition 2009;89(5):1468-75. doi: 10.3945/ajcn.2008.26668.

19. Leenders M, Verdijk LB, van der Hoeven L, van Kranenburg J, Hartgens F, Wodzig WK, Saris WH, van Loon LJ. Prolonged leucine supplementation does not augment muscle mass or affect glycemic control in elderly type 2 diabetic men. The Journal of nutrition 2011;141(6):1070-6. doi: 10.3945/jn.111.138495.

20. Smith K, Barua JM, Watt PW, Scrimgeour CM, Rennie MJ. Flooding with L-[1-13C]leucine stimulates human muscle protein incorporation of continuously infused L-[1-13C]valine. The American journal of physiology 1992;262(3 Pt 1):E372-6.

21. Rieu I, Balage M, Sornet C, Giraudet C, Pujos E, Grizard J, Mosoni L, Dardevet D. Leucine supplementation improves muscle protein synthesis in elderly men independently of hyperaminoacidaemia. The Journal of physiology 2006;575(Pt 1):305-15. doi: 10.1113/jphysiol.2006.110742.

22. Katsanos CS, Kobayashi H, Sheffield-Moore M, Aarsland A, Wolfe RR. A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly. American journal of physiology Endocrinology and metabolism 2006;291(2):E381-7. doi: 10.1152/ajpendo.00488.2005.

23. Nair KS, Schwartz RG, Welle S. Leucine as a regulator of whole body and skeletal muscle protein metabolism in humans. The American journal of physiology 1992;263(5 Pt 1):E928-34.

24. Glynn EL, Fry CS, Drummond MJ, Timmerman KL, Dhanani S, Volpi E, Rasmussen BB. Excess leucine intake enhances muscle anabolic signaling but not net protein anabolism in young men and women. The Journal of nutrition 2010;140(11):1970-6. doi: 10.3945/jn.110.127647.

25. Koopman R, Verdijk LB, Beelen M, Gorselink M, Kruseman AN, Wagenmakers AJ, Kuipers H, van Loon LJ. Co-ingestion of leucine with protein does not further

augment post-exercise muscle protein synthesis rates in elderly men. The British journal of nutrition 2008;99(3):571-80. doi: 10.1017/S0007114507812013.

26. Swendseid ME, Villalobos J, Figueroa WS, Drenick EJ. The effects of test doses of leucine, isoleucine or valine on plasma amino acid levels. The unique effect of leucine. The American journal of clinical nutrition 1965;17(5):317-21.

27. Hagenfeldt L, Eriksson S, Wahren J. Influence of leucine on arterial concentrations and regional exchange of amino acids in healthy subjects. Clin Sci (Lond) 1980;59(3):173-81.

28. Escobar J, Frank JW, Suryawan A, Nguyen HV, Davis TA. Amino acid availability and age affect the leucine stimulation of protein synthesis and eIF4F formation in muscle. American journal of physiology Endocrinology and metabolism 2007;293(6):E1615-21. doi: 10.1152/ajpendo.00302.2007.

29. Canadian Institutes of Health Research, Natural Sciences and Engineering Research Council of Canada, and Social Sciences and Humanities Research Council of Canada, Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans. . 2010.

30. Copeland KC, Kenney FA, Nair KS. Heated dorsal hand vein sampling for metabolic studies: a reappraisal. The American journal of physiology 1992;263(5 Pt 1):E1010-4.

31. Tang JE, Moore DR, Kujbida GW, Tarnopolsky MA, Phillips SM. Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men. J Appl Physiol 2009;107(3):987-92. doi: 10.1152/japplphysiol.00076.2009.

32. Burd NA, West DW, Staples AW, Atherton PJ, Baker JM, Moore DR, Holwerda AM, Parise G, Rennie MJ, Baker SK, Phillips SM. Low-load high volume resistance exercise stimulates muscle protein synthesis more than high-load low volume resistance exercise in young men. PloS one 2010;5(8):e12033. doi: 10.1371/journal.pone.0012033.

33. West DW, Kujbida GW, Moore DR, Atherton P, Burd NA, Padzik JP, De Lisio M, Tang JE, Parise G, Rennie MJ, Baker SK, Phillips SM. Resistance exercise-induced increases in putative anabolic hormones do not enhance muscle protein synthesis or intracellular signalling in young men. The Journal of physiology 2009;587(Pt 21):5239-47. doi: 10.1113/jphysiol.2009.177220.

34. Burd NA, Holwerda AM, Selby KC, West DW, Staples AW, Cain NE, Cashaback JG, Potvin JR, Baker SK, Phillips SM. Resistance exercise volume affects myofibrillar protein synthesis and anabolic signalling molecule phosphorylation in young men. The Journal of physiology 2010;588(Pt 16):3119-30. doi: 10.1113/jphysiol.2010.192856.

35. Wilkinson SB, Tarnopolsky MA, Macdonald MJ, Macdonald JR, Armstrong D, Phillips SM. Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage. The American journal of clinical nutrition 2007;85(4):1031-40.

36. Glover EI, Oates BR, Tang JE, Moore DR, Tarnopolsky MA, Phillips SM. Resistance exercise decreases eIF2Bepsilon phosphorylation and potentiates the feeding-induced stimulation of p70S6K1 and rpS6 in young men. American journal of physiology Regulatory, integrative and comparative physiology 2008;295(2):R604-10. doi: 10.1152/ajpregu.00097.2008.

37. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227(5259):680-5.

38. Moore DR, Tang JE, Burd NA, Rerecich T, Tarnopolsky MA, Phillips SM. Differential stimulation of myofibrillar and sarcoplasmic protein synthesis with protein ingestion at rest and after resistance exercise. The Journal of physiology 2009;587(Pt 4):897-904. doi: 10.1113/jphysiol.2008.164087.

39. Mittendorfer B, Andersen JL, Plomgaard P, Saltin B, Babraj JA, Smith K, Rennie MJ. Protein synthesis rates in human muscles: neither anatomical location nor fibre-type composition are major determinants. The Journal of physiology 2005;563(Pt 1):203-11. doi: 10.1113/jphysiol.2004.077180.

40. Miller BF, Olesen JL, Hansen M, Dossing S, Crameri RM, Welling RJ, Langberg H, Flyvbjerg A, Kjaer M, Babraj JA, Smith K, Rennie MJ. Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise. The Journal of physiology 2005;567(Pt 3):1021-33. doi: 10.1113/jphysiol.2005.093690.

41. Heys SD, McNurlan MA, Park KG, Milne E, Garlick PJ. Baseline measurements for stable isotope studies: an alternative to biopsy. Biomedical & environmental mass spectrometry 1990;19(3):176-8.

42. Rennie MJ, Wackerhage H, Spangenburg EE, Booth FW. Control of the size of the human muscle mass. Annual review of physiology 2004;66:799-828. doi: 10.1146/annurev.physiol.66.052102.134444.

43. Glynn EL, Fry CS, Drummond MJ, Dreyer HC, Dhanani S, Volpi E, Rasmussen BB. Muscle protein breakdown has a minor role in the protein anabolic response to essential amino acid and carbohydrate intake following resistance exercise. American journal of physiology Regulatory, integrative and comparative physiology 2010;299(2):R533-40. doi: 10.1152/ajpregu.00077.2010.

44. Churchward-Venne TA, Burd NA, Phillips SM, Research Group EM. Nutritional regulation of muscle protein synthesis with resistance exercise: strategies to enhance anabolism. Nutrition & metabolism 2012;9(1):40. doi: 10.1186/1743-7075-9-40.

45. Burke LM, Winter JA, Cameron-Smith D, Enslen M, Farnfield M, Decombaz J. Effect of intake of different dietary protein sources on plasma amino Acid profiles at rest and after exercise. International journal of sport nutrition and exercise metabolism 2012;22(6):452-62.

46. West DW, Burd NA, Coffey VG, Baker SK, Burke LM, Hawley JA, Moore DR, Stellingwerff T, Phillips SM. Rapid aminoacidemia enhances myofibrillar protein synthesis and anabolic intramuscular signaling responses after resistance exercise. The American journal of clinical nutrition 2011;94(3):795-803. doi: 10.3945/ajcn.111.013722.

47. Szmelcman S, Guggenheim K. Interference between leucine, isoleucine and valine during intestinal absorption. The Biochemical journal 1966;100(1):7-11.

48. Hyde R, Taylor PM, Hundal HS. Amino acid transporters: roles in amino acid sensing and signalling in animal cells. The Biochemical journal 2003;373(Pt 1):1-18.

49. Staples AW, Burd NA, West DW, Currie KD, Atherton PJ, Moore DR, Rennie MJ, Macdonald MJ, Baker SK, Phillips SM. Carbohydrate does not augment exercise-induced protein accretion versus protein alone. Medicine and science in sports and exercise 2011;43(7):1154-61. doi: 10.1249/MSS.0b013e31820751cb.

50. Koopman R, Beelen M, Stellingwerff T, Pennings B, Saris WH, Kies AK, Kuipers H, van Loon LJ. Coingestion of carbohydrate with protein does not further augment postexercise muscle protein synthesis. American journal of physiology Endocrinology and metabolism 2007;293(3):E833-42. doi: 10.1152/ajpendo.00135.2007.

51. Elliot TA, Cree MG, Sanford AP, Wolfe RR, Tipton KD. Milk ingestion stimulates net muscle protein synthesis following resistance exercise. Medicine and science in sports and exercise 2006;38(4):667-74. doi: 10.1249/01.mss.0000210190.64458.25.

CHAPTER 4

TITLE: Role of citrulline in the regulation of myofibrillar protein synthesis and muscle microvascular perfusion in elderly men under resting and post-exercise conditions

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Role of citrulline in the regulation of myofibrillar protein synthesis and muscle microvascular perfusion in elderly men under resting and post-exercise conditions

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List of abbreviations: 4E-BP1, eukaryotic initiation factor 4E binding protein 1; Akt, protein kinase B; BCAA, branched-chain amino acid; EAA, essential amino acid; eEF2, eukaryotic elongation factor 2; EX-FED, response to combined feeding and resistance exercise; FED, response to feeding; FSR, fractional synthetic rate; MPS, myofibrillar protein synthesis; mTOR, mechanistic target of rapamycin; NEAA, nonessential amino acid; rps6, ribosomal protein s 6; WHEY, treatment consisting of 45 g whey protein; WHEY+CIT, treatment consisting of 15 g whey protein + 10 g citrulline; WHEY+NEAA, treatment consisting of 15 g whey protein + 10 g of a mixture of nonessential amino acids.

ABSTRACT

Ageing is associated with a relative diminution of the stimulatory effect of aminoacidemia on muscle protein synthesis; termed anabolic resistance. Anabolic resistance may involve reductions in feeding-induced blood flow and/or muscle microvascular perfusion, which may limit amino acid delivery. Citrulline is a non-protein amino acid that serves as a precursor for arginine, which is a substrate for the synthesis of the vasodilator nitric oxide. We aimed to examine the effect of citrulline co-ingestion with whey protein on femoral arterial blood flow, muscle microvascular perfusion, myofibrillar protein synthesis (MPS), and signalling through mTORC1. In a parallel group design, twenty-one elderly males completed an acute bout of resistance exercise prior to ingestion of: 45 g whey protein (WHEY), 15 g whey protein with 10 g of citrulline (WHEY+CIT), or 15 g whey protein with 10 g of non-essential amino acids (WHEY+NEAA). A primed continuous infusion of L-[*ring*-$^{13}C_6$] phenylalanine with serial muscle biopsies was used to measure MPS and signaling through the Akt-mTORC1 pathway, while contrast enhanced ultrasound was used to measure changes in microvascular circulation under baseline (fasted) and postprandial conditions in both a rested (FED) and exercised leg (EX-FED). Argininemia was significantly greater in WHEY+CIT compared to WHEY and WHEY+NEAA from 30-300 min post-exercise ($P < 0.001$), but there were differences between treatments in plasma nitrate/nitrite, femoral artery blood flow, or microvascular circulation (all $P > 0.05$). The phosphorylation of p70S6k^{Thr389} was greater in WHEY compared to WHEY+NEAA, but not WHEY+CIT. We conclude the citrulline co-ingestion with whey protein increases plasma arginine

availability but not markers of nitric oxide metabolism, blood flow, or microvascular circulation in elderly men under resting or post-exercise conditions.

INTRODUCTION

The loss of skeletal muscle mass (myopenia) and strength (dynapenia) with advancing age, collectively termed sarcopenia, is a major cause of physical frailty in older adults (1). Declines in muscle mass are likely due to alterations in muscle protein turnover that favour a net-negative protein balance [muscle protein breakdown > muscle protein synthesis (MPS)] (2). Amino acid availability and physical exercise are potent anabolic stimuli for adult muscle since they stimulate the synthesis of new muscle proteins. However ageing is associated with a reduced sensitivity of muscle protein synthesis in response to amino acid provision (3) and resistance exercise (4), a phenomenon termed 'anabolic resistance' [for review see (5)]. For example, ingestion of relatively small doses of protein stimulate increased MPS rates in the young (6, 7), but they fail to do so in the elderly (8-12). Although the mechanisms underlying anabolic resistance are not fully understood, age-related impairments in vascular function may be involved (13, 14).

Aminoacidemia and insulinemia increase MPS (3), arterial blood flow (3), and muscle microvascular perfusion (15) in the young, however, these responses are blunted in the elderly (3, 14). This age-related impairment in endothelial function may, in part, be due to reduced nitric oxide (NO) synthesis (16) and, hence, a diminished NO-mediated vasodilatory response to meal-induced hyperinsulinemia (17). Consequently, there may be a reduction in amino acid delivery to tissues, which could diminish a meal-induced rise in MPS in the elderly. As a critical element of insulin-mediated vasodilation (17), NO is synthesized in endothelial cells via the transformation of arginine into citrulline in a reaction catalyzed by the enzyme endothelial nitric oxide synthase (eNOS).

Citrulline supplementation increases NO synthesis more effectively than arginine (18) which undergoes substantial intestinal and hepatic metabolism to ornithine and urea by arginase (19). Whereas ~40% of ingested arginine is removed by the splanchnic tissues during first pass clearance (20), citrulline is not metabolized in the intestine or liver (21). In addition, citrulline may act as a direct nutrient signaling molecule (22). For example, citrulline stimulates increased rates of MPS in elderly malnourished rats (23, 24), an effect that may be mTORC1 dependent (24). Therefore, citrulline may be a useful pharmaconutrient to counteract age-related anabolic resistance of MPS through NO mediated increases in blood flow and muscle microvascular perfusion and/or via acting as a direct nutrient signaling molecule capable of activating mTORC1 signaling and MPS.

The aim of the present study was to assess the effect of supplementation of a relatively low dose of protein (15 g whey), previously shown to be suboptimal for maximal stimulation of MPS in the elderly (9-11), with citrulline (WHEY+CIT) versus the same dose of whey protein supplemented with a mixture of non-essential amino acids (WHEY+NEAA). The primary outcome measures were MPS, femoral arterial blood flow, muscle microvascular perfusion, and phosphorylation of select protein targets within the mTORC1 pathway in both the postabsorptive state, and in the postprandial state (FED) at rest and following an acute bout of resistance exercise (EX-FED) in elderly men. As a positive control, we compared these treatments to a dose of protein (WHEY; 45 g whey) we (10-12) and others (9) have shown to stimulate greater MPS rates than 15 g whey protein in the elderly. We hypothesized that WHEY+CIT would stimulate MPS rates comparable to that observed following WHEY despite containing only 33% of the

whey protein due to improved muscle microvascular perfusion. In contrast, we hypothesized that WHEY+NEAA would result in a reduced response of microvascular perfusion and MPS as compared to both WHEY and WHEY+CIT since typical non-essential amino acids do not stimulate increased MPS rates (25, 26).

SUBJECTS AND METHODS

Study participants. Twenty-one independently living healthy elderly adult males between 65-80 years of age inclusive were recruited to participate in the study via advertisements posted on the McMaster University campus and in local newspapers. The characteristics of the study participants are presented in **Table 1**. In a blinded manner, participants were randomly assigned to one of three parallel treatment groups (n = 7 per group) balanced for bodyweight. Participants were light-to-moderately active, non-smokers, non-diabetic, and considered generally healthy based on responses to a routine health screening questionnaire. Participants taking medications controlling blood pressure were allowed in the study. Each participant was informed of the purpose of the study, experimental procedures, and potential risks prior to providing written consent. The study was approved by the Hamilton Health Sciences Research Ethics Board and conformed to the standards for the use of human subjects in research as outlined in the most recent update of the *Declaration of Helsinki*. The study also conformed to standards established by the Canadian Tri-Council Policy on the ethical use of human subjects (27).

Table 1. Participants Characteristics

	WHEY	WHEY+CIT	WHEY+NEAA
Age, y	72.4 ± 1.9	73.7 ± 1.3	71.9 ± 2.0
Height, m	1.7 ± 0.02	1.7 ± 0.02	1.7 ± 0.03
Weight, kg	82.1 ± 3.6	79.3 ± 3.0	78.9 ± 3.8
Fat-free mass, kg	61.8 ± 1.7	60.7 ± 1.3	60.5 ± 2.2
Body-fat, %	24.3 ±1.6	24.5 ± 1.8	23.0 ± 1.8
BMI, kg/m^2	26.8 ± 1.0	28.0 ± 0.8	26.7 ± 0.7
10-RM strength, kg	31.8 ± 2.4	34.4 ± 3.7	30.7 ± 3.0
Fasting blood glucose, mM	5.5 ± 0.3	5.4 ± 0.3	5.7 ± 0.1
Fasting plasma insulin, $\mu U\text{-}mL^{-1}$	3.8 ± 1.0	3.1 ± 0.5	4.5 ± 0.4

Values are mean ± SEM (n = 7 per treatment group). WHEY (45 g whey protein); WHEY+CIT (15 g whey protein supplemented with 10 g citrulline); WHEY+NEAA (15 g whey protein supplemented with 10 g non-essential amino acids).

Pretesting. Approximately one week prior to the experimental infusion trial, study participants underwent unilateral strength testing for the knee-extensors. Participants performed a series of graded-load knee-extensions to determine their ten-repetition maximum strength (10-RM) with their self-reported dominant leg using a seated knee-extension device (Atlantis C-605 unilateral leg extension Quebec, Canada). In addition, each participant underwent a whole-body dual-energy X-ray absorptiometry scan (QDR-4500A; Hologic; software version 12.31, Bedford, MA) to measure body composition (Table 1). Participants were instructed to abstain from strenuous physical exercise for 72 h prior to the experimental infusion trial and to consume their evening meal no later than 20 00 h the evening prior to the trial.

Physical Activity. Participants were required to wear a pedometer (Accusplit, Livermore, CA) for three days immediately prior to the experimental infusion trial to monitor their

daily step count. Participants were instructed to begin using the pedometer upon waking and to remove it immediately prior to sleep after recording their daily step-count.

Dietary Intake. Participants were required to complete diet records for three days immediately prior to the experimental infusion trial to provide an estimate of their habitual macronutrient intake as analyzed using a commercially available software program (NutriBase v.11.0, CyberSoft Inc, AR, USA). Reference lists for portion size estimates were provided to the study participants, who were instructed to record all food or drink consumed in a diet log during the 3-day period.

Experimental Protocol. Participants reported to the lab via automobile or public transportation at ~0600 the morning of the experimental infusion trial following an overnight fast. A catheter was inserted into an antecubital vein and a baseline blood sample was taken before initiating a 0.9% saline drip to keep the catheter patent to allow for repeated arterialized blood sampling. Arterialized blood samples (28) were obtained repeatedly during the infusion trial (**Figure 1**) by wrapping a heating blanket around the forearm. Blood samples were collected into 4 ml EDTA evacuated tubes and placed on ice. A second catheter was inserted into the antecubital vein of the opposite arm prior to initiating a primed continuous infusion (0.05 μmol•kg^{-1}•min^{-1}; 2.0 μmol•kg^{-}1 prime) of [*ring*-$^{13}C_6$] phenylalanine (Cambridge Isotope Laboratories, Woburn, MA). The infusate was passed through a 0.2-μm filter before entering the blood. Subjects rested for ~1.5 hours in a supine position at which point resting heart rate and blood pressure were measured with an automated sphygmomanometer (Dinamap Pro 100, Critikon LCC,

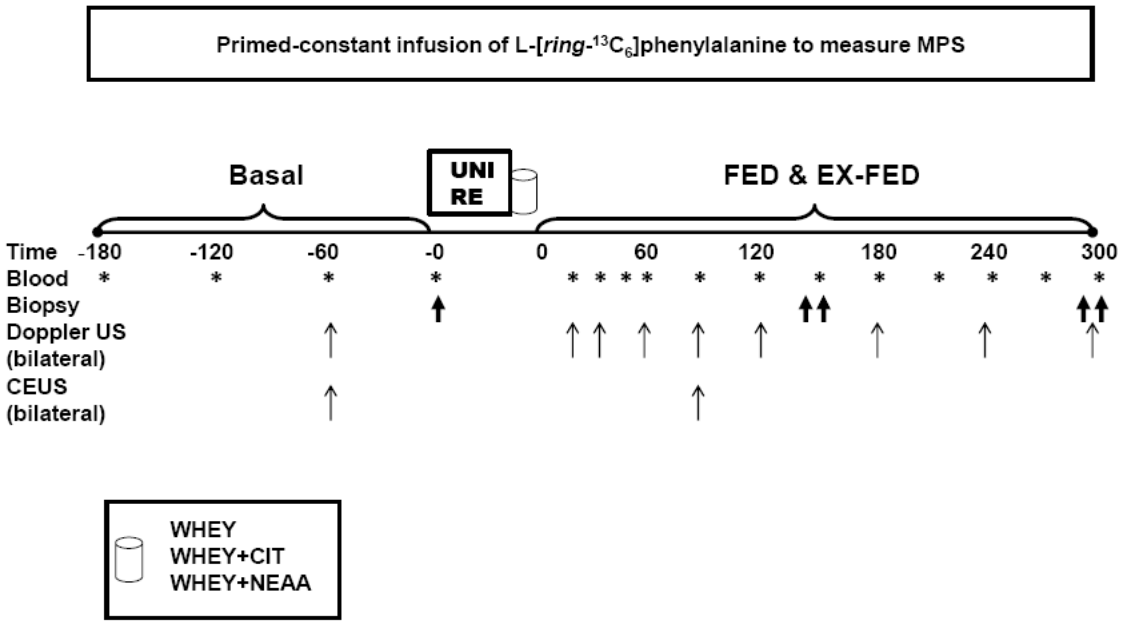


Figure 1. Schematic of the experimental protocol. Participants were block randomized to one of three possible treatment groups in a blinded fashion (n = 7 per treatment group). Prior to resistance exercise (RE) and treatment administration, each subject underwent baseline measures of femoral arterial blood flow, and microvascular circulation within the *vastus lateralis* prior to undergoing a unilateral biopsy. Treatments were administered immediately following unilateral RE, consisting of 6 sets of 8-10 repetitions of seated knee extension with a 2 min inter-set rest interval. Asterisk indicates blood sample; bold upward arrow indicates a biopsy sample; thin upward arrow indicates measure of bulk blood flow (bilateral) and microvascular circulation (bilateral).

Tampa, FL), immediately prior to assessment of blood flow through the right and left common femoral artery using a Doppler Vivid q ultrasound system (GE Medical Systems, Horton, Norway) to obtain basal measures of bulk flow to each leg. Immediately following heart rate, blood pressure, and femoral flow measures, perflutren lipid microspheres (Definity; Lantheus Medical Imaging) were injected into the antecubital

vein to examine parameters of microvascular circulation within each *vastus lateralis* muscle simultaneously by contrast enhanced ultrasound (CEUS) imaging. After ~3 hours of tracer incorporation, participants underwent a single skeletal muscle biopsy. Muscle biopsies were obtained from the *vastus lateralis* muscle using a 5 mm Bergström needle custom adapted for manual suction under 2% xylocaine local anaesthesia. The tissue samples were freed from visible blood, fat, and connective tissue, and immediately frozen in liquid nitrogen for further analysis as previously described (29, 30).

The baseline (Fasted) fractional synthetic rate (FSR) was calculated based on the ^{13}C enrichment of mixed plasma proteins obtained from the pre-infusion blood sample and skeletal muscle biopsy following ~3 hours of tracer incorporation (31, 32). Following the first biopsy, participants performed an acute bout of unilateral seated knee-extension resistance exercise (Atlantis C-605 unilateral leg extension Quebec, Canada) consisting of 6 sets of 8-10 repetitions at ~80% of their previously determined 10-RM with an inter-set rest-interval of 2 minutes. Following completion of the resistance exercise, participants immediately ingested their designated nutrient treatment in a blinded manner (**Table 2**) and returned to bed where they rested in a supine position for the remainder of the protocol. Heart rate, blood pressure, and blood flow through the right and left common femoral artery was then assessed at 15, 30, 60, 90, 120, 180, 240, and 300 minutes post-exercise, while CEUS-derived measures of microvascular circulation were obtained at 90 minutes following exercise and treatment administration. Bilateral biopsy samples were obtained from the *vastus lateralis* muscle at 150 min and 300 min following exercise and nutrition. Each biopsy sample was obtained from a separate incision ~2-3 cm apart. The

unilateral exercise model employed allowed us to obtain measures of bulk blood flow, microvascular circulation, MPS, and protein phosphorylation status under resting baseline conditions, and in the postprandial state from a rested fed (FED) and exercise-fed (EX-FED) leg. Details of the infusion protocol are outlined in Figure 1.

Table 2. Protein and amino acid composition of the nutritional treatments.

	Nutritional Treatments		
	WHEY	WHEY+CIT	WHEY+NEAA
Endogenous AA			
Alanine, g	2.07	0.69	0.69
Arginine, g	0.95	0.32	0.32
Aspartic Acid, g	5.04	1.68	1.68
Cystine, g	1.40	0.47	0.47
Glutamic Acid, g	7.38	2.46	2.46
Glycine, g	0.77	0.26	0.26
Histidine, g*	0.99	0.33	0.33
Isoleucine, g*	2.43	0.81	0.81
Leucine, g*	5.40	1.80	1.80
Lysine, g*	4.86	1.62	1.62
Methionine, g*	1.04	0.35	0.35
Phenylalanine, g*	1.58	0.53	0.53
Proline, g	1.89	0.63	0.63
Serine, g	1.13	0.38	0.38
Threonine, g*	1.98	0.66	0.66
Tryptophan, g	1.22	0.41	0.41
Tyrosine, g	1.48	0.53	0.53
Valine, g*	2.48	0.83	0.83
Added AA			
Alanine, g	-	-	0.95
Arginine, g	-	-	0.43
Aspartic Acid, g	-	-	2.31
Citrulline, g	-	10.0	-
Cystine, g	-	-	0.64
Glutamic Acid, g	-	-	3.38
Glycine, g	-	-	0.35
Proline, g	-	-	0.87
Serine, g	-	-	0.51
Tryptophan, g	-	-	0.56
Totals			
Whey Protein, g	45.00	15.00	15.00
Citrulline, g	0.00	10.0	0.00
Leucine, g*	5.40	1.80	1.80
ΣBCAA, g*	10.31	3.44	3.44
ΣEAA, g*	20.75	6.92	6.92
ΣNEAA, g	23.40	17.80	17.80
Total Amino Acids, g	44.15	24.72	24.72

*Essential amino acid

Femoral artery blood flow. At each time-point examined, the diameter of the common femoral artery, ~2-3 cm proximal to the bifurcation to the superficial and profundus segments, was determined from in both legs (FED and EX-FED) using the Vivid q BT10 (General Electric Healthcare, Milwaukee, WI, USA) echocardiography console and linear array transducer (12L-RS) operating in Duplex mode. B-mode images of the common femoral artery were acquired at a frequency of 13 MHz, and a frame rate of 10 frames per second for 30 seconds at each time point. Consistent positioning of the probes between measures within a limb for each participant was ensured using anatomical landmarks. Artery diameters were determined offline using electronic callipers (Echopac Version 110.0.2; GE Medical Systems) on the end-systolic and end-diastolic frames. The arterial diameter value at each time-point represents the average of 9 sequential end-systolic and end-diastolic measurements. Simultaneously, blood velocity signals were obtained using pulsed wave Doppler (4 MHz) with the sample gate encompassing the entire cross-section of the vessel and the signal was adjusted for insonation angle (33). Automated peak velocity tracings were generated offline using the internal analysis tools (Echopac Version 110.0.2; GE Medical Systems) during each 30 s data collection windows and the average peak velocity was subsequently generated. The mean blood velocity (MBV) was calculated to be 50% of the peak blood velocity according to the assumption of laminar flow in the straight section of the common femoral artery where the pulsed wave velocity signal was obtained.

Microvascular Flow. Microvascular circulation was measured bilaterally and simultaneously within the *vastus lateralis* muscle under baseline resting conditions and in

the postprandial period under both FED and EX-FED conditions using CEUS while the participant lay supine. The temperature in the testing room was consistent and held at 22º C. Participants were instructed to remain as still as possible during the measurement. Real time contrast imaging was used to record the acoustic intensity curves generated from the resonating microbubbles in an area of interest in the skeletal muscle and subsequently determine the microbubble appearance characteristics following the bolus injection of the agent using curve fitting techniques. Perflutren lipid microspheres (Definity; Lantheus Medical Imaging) were activated by a vial mixer (Vialmix^{TM}, Lantheus Medical Imaging) at 4,500 oscillations/min for 45 s, at which point the activated microbubbles were drawn into a sterile syringe. Prior to microbubble administration, both ultrasound transducers were placed in custom-made clamp stands that were attached to the examination bed and positioned to permit visualization of *vastus lateralis* muscle tissue in each leg and reduce movement artefact. Participants then received an intravenous bolus injection of 10 µl/kg of the activated contrast agent, followed immediately by a 10 mL saline 'flush' per manufacturer's instructions. At each data collection time point, data acquisition was initiated simultaneously in both ultrasound machines and coincided with the bolus agent injection. Microbubble appearance was assessed in the *vastus lateralis* muscle of both legs ~3-5 cm proximal to the site of muscle biopsy acquisition, simultaneously. Contrast ultrasound measurements were recorded using 2 identical Vivid q BT10 (General Electric Healthcare, Milwaukee, WI, USA) echocardiography consoles equipped with Echopac (Version 110.0.2; GE Medical Systems) software for storing and analysing the data and M4S-RS matrix phased array transducers transmitting at 1.6 MHz and receiving at

3.3MHz in left ventricular contrast mode. The ultrasound depth was set at 5.0 cm, the power was 12dB the mechanical index was 0.20 and the acquisition rate was 28Hz. These settings were identical between ultrasound machines and for all data acquisition. Recording of a 180 second video clip in contrast mode permitted later offline analysis of the CEUS-generated time-intensity curves within the region of interest. Data was stored using the EchoPAC software package and analysed using Q-Analysis (Echopac Version 110.0.2; GE Medical Systems). The background subtracted video intensity was measured from a region of interest (ROI) within the *vastus lateralis* muscle. The ROI was visually identified as containing no arterioles or venules (through the lack of consistent contrast microbubble appearance). The y-axis was set to display the intensity of the signal in dB and acoustic intensity data was determined for each region of interest. The average intensity within a ROI was calculated in linear units and displayed as a function of time, generating a time-intensity curve.

For analysis of the CEUS data, acoustic intensity data were first smoothed using a local regression filter (robust locally weighted scatter-plot smoothing) (34) with a window span of 10. Smoothed data were then curve fitted (GraphPad Prism; Version 5) using a gamma-variate function (35). The resulting curves were imported into a computer software program (LabChart 7; ADInstruments Inc., Colorado Springs, CO, USA), where various curve parameters relating to microvascular hemodynamics were measured including peak intensity, area-under-the curve (AUC), wash-in time, and full-width half maximum (FWHM). A Perfusion Index was calculated as the quotient of AUC and FWHM (35).

Beverage composition. Study participants consumed their nutrient treatments in a blinded manner immediately following resistance exercise. The amino acid composition of each of the 3 treatments is outlined in Table 2. The whey protein isolate (biPro, Davisco Foods, Le Sueur, MN) was independently tested (Telmark, Matawan, NJ) in triplicate for content analysis. The free-form amino acids used were as follows: L-citrulline, L-alanine, L-arginine, L-asparginine, L-cysteine, L-glutamine, L-glycine, L-proline, L-serine, L-tryptophan (Sigma Life Science; Sigma-Aldrich, St. Louis MO). All nutrient treatments were prepared in 300 mL of water (see Table 2). To minimize disturbances in isotopic equilibrium following amino acid ingestion, beverages were enriched to 4% with L-[*ring*-$^{13}C_6$] phenylalanine based on a measured phenylalanine content of 3.5% in the whey protein.

Analytical methods. Blood glucose was measured using a blood glucose meter (OneTouch Ultra 2, Lifescan Inc., Milpitas, CA). Plasma insulin concentration was measured using a commercially available immunoassay kit (ALPCO Diagnostics, Salem, NH). Plasma amino acid concentrations (except for citrulline and arginine) were analyzed via GC/MS using the Phenomenex EZ: faast^{TM} (Torrance, CA, USA) amino acid analysis kit as per the manufacturer's instructions. Plasma citrulline and arginine concentrations were analyzed via high performance liquid chromatography as described previously (36). Plasma L-[*ring*-$^{13}C_6$] phenylalanine enrichment was determined as previously described (37). Analysis of plasma nitrite and nitrate was determined as previously described (38) using a dedicated ENO-20 HPLC system (EiCom Corp., San Diego, CA, USA). Standard curves were performed using known molar concentrations of both nitrite and nitrate.

Muscle samples (~40-50 mg) were homogenized on ice in buffer (10 μl mg^{-1} 25mM Tris 0.5% v/v Triton X-100 and protease/phosphatase inhibitor cocktail tablets (Complete Protease Inhibitor Mini-Tabs, Roche, Indianapolis, IN; PhosSTOP, Roche Applied Science, Mannhein, Germany)). Samples were then centrifuged at 15,000 *g* for 10 minutes 4°C. The supernatant was removed and protein concentrations were determined via BCA protein assay (Thermo Scientific, Rockford, IL). The pellet containing the myofibrillar proteins was stored at -80° C for future processing. Working samples of equal concentration were prepared in Laemmli buffer (39). Equal amounts (20 µg) of protein were loaded onto 10% or gradient precast gels (BIO-RAD Mini-PROTEAN TGX Gels, Bio-Rad Laboratories, Hercules, CA) for separation by electrophoresis. Proteins were then transferred to a polyvinylidene fluoride membrane, blocked (5% skim milk) and incubated overnight at 4°C in primary antibody: phospho-Akt^{Ser473} (1:1000, Cell Signalling Technology, #4058), phospho-mTOR^{Ser2448} (1:1000, Cell Signalling Technology, #2971), phospho-AMPK^{Thr172} (1:1000, Cell Signalling Technology, #2531), phospho-p70 S6 Kinase^{Thr389} (1:1000, Cell Signalling Technology, #9234), phospho-4E-BP1$^{Thr37/46}$ (1:1000, Cell Signalling Technology, #2855), phospho-S6 Ribosomal protein (1:2000, Cell Signalling Technology, #2215), and phospho eEF2^{Thr56} (1:1000, Cell Signalling Technology, #2331). Membranes were then washed and incubated in secondary antibody (1 h at room temperature) before detection with chemiluminescence (SuperSignalWest Dura Extended Duration Substrate, ThermoScientific, #34075) on a FluorChem SP Imaging system (Alpha Innotech, Santa Clara, CA). Phosphorylation status was expressed relative to α-tubulin (1:2000, Cell

Signalling Technology, #2125) and is presented for each protein as fold-change from basal fasted conditions. Images were quantified by spot densitometry using ImageJ software (US National Institutes of Health).

Muscle biopsy samples were processed as previously described (40). To determine the intracellular $^{13}C_6$ phenylalanine enrichment, ~20-25 mg of muscle was homogenized in 0.6 M perchloric acid/L. Free amino acids in the resulting supernatant fluid were then passed over an ion-exchange resin (Dowex 50WX8-200 resin Sigma-Aldrich Ltd) and converted to their heptafluorobutyric derivatives for analysis via gas chromatography–mass spectrometry (models 6890 GC and 5973 MS; Hewlett-Packard, Palo Alto, CA) by monitoring ions 316 and 322 after electron ionization. To determine myofibrillar protein-bound enrichments, a separate piece (~40-50 mg) of muscle was homogenized in a standard buffer containing protease and phosphatase inhibitors as described above. The supernatant fluid was collected for Western blot analysis as described above, and the pellet was further processed to extract myofibrillar proteins as previously described (40). The resulting myofibrillar 'enriched' protein pellet was hydrolyzed in 0.5 M HCL at 110° for 24 hours. Subsequently, the free amino acids were purified using ion-exchange chromatography and converted to their N-acetyl-n-propyl ester derivatives for analysis by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS: Hewlett Packard 6890; IRMS model Delta Plus XP, Thermo Finnagan, Waltham, MA).

Calculations. Mean limb flood flow (*Q*) was calculated from mean velocity and area using the formula:

$$Q\ (\text{mL}\bullet\text{min}^{-1}) = \text{MBV}\ \pi \bullet r^2 \bullet\ 60$$

Where MBV is mean blood velocity ($\text{cm}\bullet\text{s}^{-1}$), r is the mean radius of the artery (cm) during the cardiac cycle, and 60 is a constant ($\text{s}\bullet\text{min}^{-1}$) to convert the calculated flow from millilitres per second to millilitres per minute. The mean radius of the artery was calculated from the diameter assuming the artery was circular ($r = d/2$). The mean diameter was calculated from the maximum diameter of the artery during systole (d_{max}, cm) and minimum of the artery during diastole (d_{min}, cm) weighted to the percentage of time spent at each diameter in the cardiac cycle: $d = (1/3)d_{max} + (2/3)d_{min}$.

Contrast enhanced ultrasound generated time-intensity curves were fitted to a gamma-variate function (35):

$$\text{SI}(t) = \text{SI}_{\text{peak}} \times[(t/\text{TTP})^{\beta\times\text{TTP}}\ e^{-\beta\times(t-\text{TTP})}]$$

Where SI_{peak} is the peak intensity value, TTP is the time to peak and β is the coefficient of the wash-in slope.

The fractional synthetic rate (FSR) of myofibrillar protein was calculated using the standard precursor-product equation:

$$\text{FSR} = [(E_{2b} - E_{1b}) / (E_{IC} \times t)] \times 100$$

where E_b is the enrichment of bound (myofibrillar) protein, E_{IC} is the average enrichment of the intracellular free amino acid precursor pool of two muscle biopsies, and t is the tracer incorporation time in h. The utilization of "tracer naïve" subjects allowed us to use

a pre-infusion blood sample (i.e., a mixed plasma protein fraction) as the baseline enrichment (E_{1b}) for calculation of resting (i.e. Fasted) FSR (41, 42, 31); an approach validated by our research group (29) and others (43).

Statistics. Anthropometric measures, strength tests, daily step-count, and dietary intake parameters were compared using a one-factor (treatment) ANOVA. Blood glucose, plasma insulin, plasma amino acids (leucine, ΣBCAA, ΣEAA, ΣNEAA, citrulline, arginine, and ornithine), and plasma nitrite-nitrate were analyzed using a two-factor (treatment × time) repeated measures ANOVA. Systolic and diastolic blood pressure, mean arterial pressure (MAP), and heart rate were analyzed using a two-factor (treatment × time) repeated measures ANOVA. Plasma enrichments were analyzed using a two-factor (treatment × time) repeated measures ANOVA and linear regression. Intracellular precursor pool enrichments, femoral artery blood flow, parameters of microvascular circulation from time-intensity curve analysis, myofibrillar FSR, and protein phosphorylation were analyzed using a three-factor (treatment × time × condition) mixed model ANOVA. Protein phosphorylation is expressed as fold-change from Fasted. A Tukey post-hoc analysis was performed whenever a significant F ratio was found to isolate specific differences. Statistical analyses were performed using SPSS software package (version 16). Values are expressed as means ± standard error of the mean (SEM). Means were considered to be statistically different for P values < 0.05.

RESULTS

Participant characteristics. Participant characteristics are shown in Table 1. Each treatment group consisted of $n = 7$ randomly assigned participants. There were no

differences between treatment groups for any of the anthropometric or descriptive variables examined (all $P > 0.05$).

Exercise variables and physical activity. There were no differences between treatment groups for unilateral 10-RM (Table 1), or the product of load (kg) x volume (# of repetitions) for exercise performed during the experiment (data not shown; all $P > 0.05$). Daily step-count over the three days prior to the experimental trial averaged 6269 ± 566, 5980 ± 1091, and 6189 ± 873 for WHEY, WHEY+CIT, and WHEY+NEAA groups respectively with no differences between groups ($P > 0.05$).

Dietary intake. Participants self-reported dietary intake averaged over the three days immediately prior to the experimental infusion trial is shown in **Supplemental Material Table 1**. Participants consumed ~1.0 g protein/kg bodyweight/day with no differences between treatment groups. There were no differences between treatment groups for total energy, or the percent energy contribution from protein, carbohydrate, or fat (all $P > 0.05$).

Blood glucose, plasma insulin, and plasma leucine, ΣBCAA, ΣEAA, and ΣNEAA. Blood glucose concentration demonstrated a main effect for time ($P < 0.001$), being transiently elevated above baseline values at 30 min post-treatment.

Supplemental Table 1. Participants Dietary Intake

	WHEY	WHEY+CIT	WHEY+NEAA
Total energy (kcal)	1900 ± 101	1969 ± 176	1863 ± 93
Protein ($g \cdot kg^{-1}d^{-1}$)	0.96 ± 0.06	0.96 ± 0.06	1.03 ± 0.07
% Carbohydrate	47.9 ± 2.9	52.9 ± 2.3	50.7 ± 1.8
% Protein	16.8 ± 1.5	15.8 ± 1.1	17.3 ± 1.0
% Fat	35.3 ± 2.5	31.3 ± 2.8	32.0 ± 1.8

Dietary intake calculated over 3 days immediately prior to the experimental infusion trial. Values are means ± SEM.

Online Supplemental Material Table 1. Values are mean ± SEM (*n* = 7 per treatment group). Average self-reported dietary intake calculated over 3 days immediately prior to the experimental infusion trial. Total energy (kcal), total protein ($g \cdot kg^{-1}d^{-1}$), and the percent energy contribution from carbohydrate, protein, and fat are shown. WHEY (45 g whey protein); WHEY+CIT (15 g whey protein supplemented with 10 g citrulline); WHEY+NEAA (15 g whey protein supplemented with 10 g non-essential amino acids).

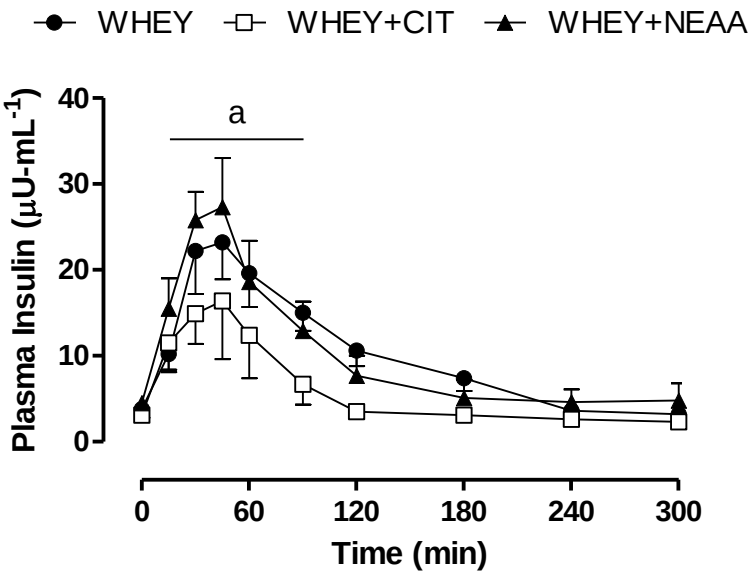


Figure 2. Values are mean ± SEM (n = 7 per treatment group). Plasma insulin concentration ($\mu mol \bullet L^{-1}$) following treatment administration. Data were analyzed using a 2-factor (treatment × time) repeated measures ANOVA with Tukey's post hoc test (main effect for time, $P < 0.001$). The letter "a" indicates a significant difference from baseline (time 0). WHEY (45 g whey protein); WHEY+CIT (15 g whey protein supplemented with 10 g citrulline); WHEY+NEAA (15 g whey protein supplemented with 10 g non-essential amino acids).

Plasma insulin concentration demonstrated a main effect for time ($P < 0.001$), increasing above baseline from 15-90 minutes post-treatment administration (**Figure 2)**.

The concentration of plasma leucine, ΣBCAA, ΣEAA, ΣNEAA over time is shown in **Figure 3** panels A-D. Plasma leucine showed a treatment × time interaction (P <0.001) with WHEY being greater than WHEY+CIT at 30 minutes, and both WHEY+CIT and WHEY+NEAA from 45-300 minutes (Panel A). In a similar fashion, plasma ΣBCAA showed a treatment × time interaction (P <0.001) with WHEY being greater than WHEY+CIT at 30 minutes, and both WHEY+CIT and WHEY+NEAA from 45-300 minutes (Panel B). Plasma ΣEAA (leucine, isoleucine, valine, threonine, methionine, phenylalanine, lysine, histidine) showed a treatment × time interaction (P <0.001) with WHEY being greater than WHEY+CIT at 45 and 60 minutes, and both WHEY+CIT and WHEY+NEAA from 90-240 minutes (Panel C). Lastly, plasma ΣNEAA (alanine, glycine, serine, proline, asparginine, glutamic acid, glutamine, tyrosine, tryptophan) demonstrated a treatment × time interaction (P <0.001) with WHEY+NEAA being greater than WHEY and WHEY+CIT from 30-90 minutes (Panel D).

Plasma citrulline, arginine, and ornithine. The concentration of plasma citrulline, arginine, and ornithine are shown in **Figure 4** panels A-C. Plasma citrulline increased rapidly following ingestion demonstrating treatment × time interaction (P <0.001) with the increase following WHEY+CIT being greater than that for WHEY and WHEY+NEAA from 15-300 minutes (Panel A). Plasma arginine demonstrated a treatment × time interaction (P <0.001) with the increase following WHEY+CIT being greater than that for WHEY and WHEY+NEAA from 30-300 minutes (Panel B). Plasma ornithine demonstrated a treatment × time interaction (P <0.001) with the increase in plasma ornithine following WHEY+CIT being greater than that for WHEY and WHEY+NEAA from 90-300 minutes (Panel C).

Plasma nitrate and nitrite. The concentration of plasma nitrate and nitrite are shown in **Figure 5** panels A-B. Plasma nitrate demonstrated a main effect for time (P < 0.001) decreasing below baseline concentrations from 240-300 minutes post-exercise. Similarly, plasma nitrite showed a main effect for time (P = 0.003) being increased above baseline at 180 minutes and trending to remain elevated at 240 min (P = 0.059). The ratio of plasma nitrite/nitrate (data not shown) also demonstrated a main effect for time (P < 0.001) being increased above baseline values from 120-300 minutes post-exercise.

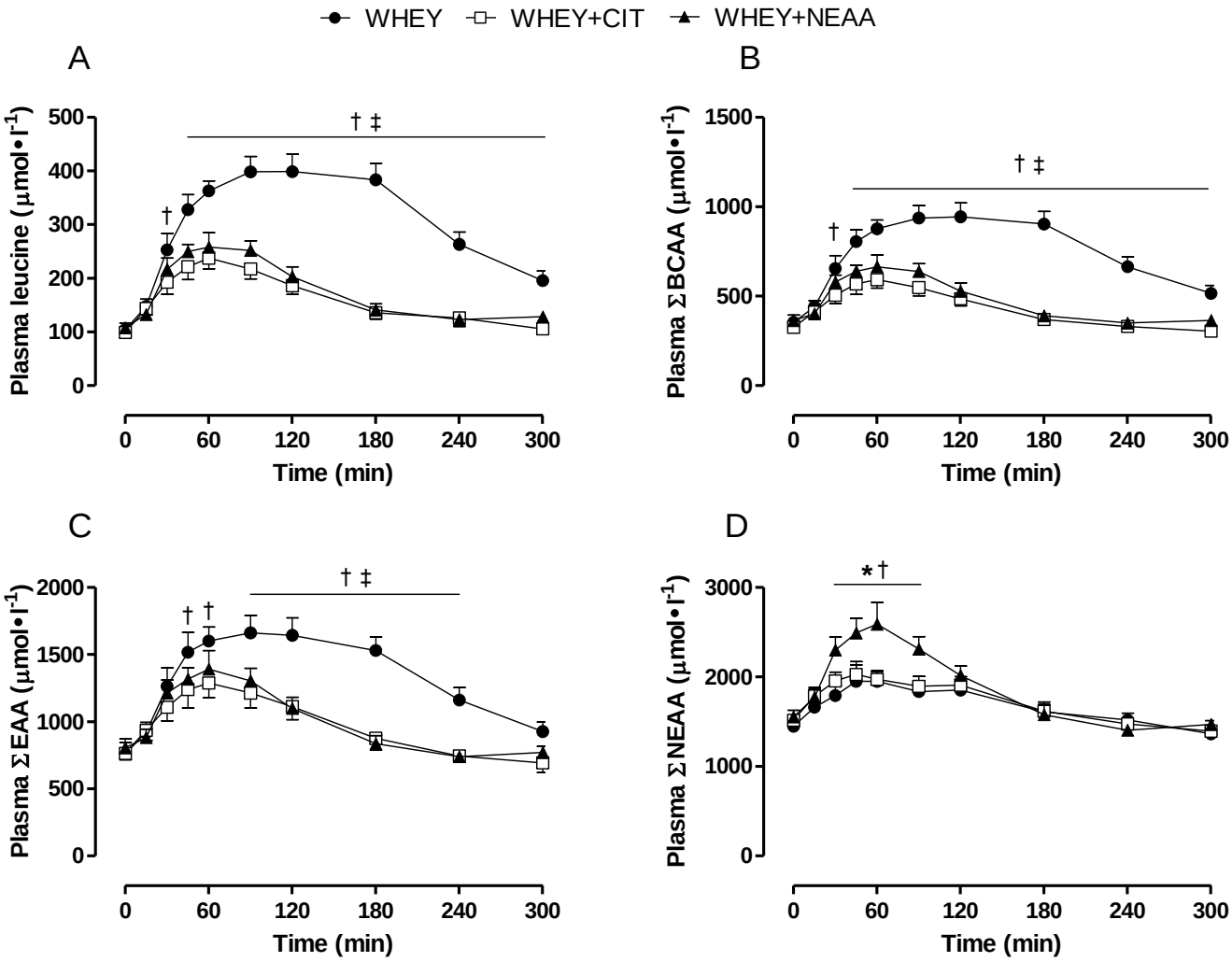

WHEY
WHEY+CIT
WHEY+NEAA
A
Plasma leucine (µmol•l-1)
Time (min)
B
Plasma ΣBCAA (µmol•l-1)
Time (min)
C
Plasma ΣEAA (µmol•l-1)
Time (min)
D
Plasma ΣNEAA (µmol•l-1)
Time (min)

Figure 3. Values are mean ± SEM (n = 7 per treatment group). Plasma concentrations ($\mu mol \bullet L^{-1}$) of leucine (A), ΣBCAA (B), ΣEAA (C), and ΣNEAA (D) following treatment administration. Data were analyzed using a 2-factor (treatment × time) repeated measures ANOVA with Tukey's post hoc test (treatment × time interaction, $P < 0.001$). * significantly different from WHEY; † significantly different from WHEY+CIT; ‡ significantly different from WHEY+NEAA. Time dependent differences from baseline (time 0) within each treatment are not shown. WHEY (45 g whey protein); WHEY+CIT (15 g whey protein supplemented with 10 g citrulline); WHEY+NEAA (15 g whey protein supplemented with 10 g non-essential amino acids).

Heart rate and blood pressure. Systolic blood pressure (mmHg), diastolic blood pressure (mmHg), mean arterial pressure (mmHg), and heart rate (beats-min) are shown in **Table 3**. Systolic blood pressure demonstrated a main effect for time ($P < 0.001$) being increased at 300 min vs. 0 min (basal conditions). In a similar fashion, diastolic blood pressure demonstrated a main effect for time ($P < 0.001$). Mean arterial pressure (mmHg) over the course of the infusion trial showed a main effect for time ($P < 0.001$), decreasing below pre-exercise baseline values at 30 minutes post-exercise. Heart rate (bpm) demonstrated a main effect for time ($P < 0.001$) being increased above baseline following exercise at all time points assessed during the infusion trial.

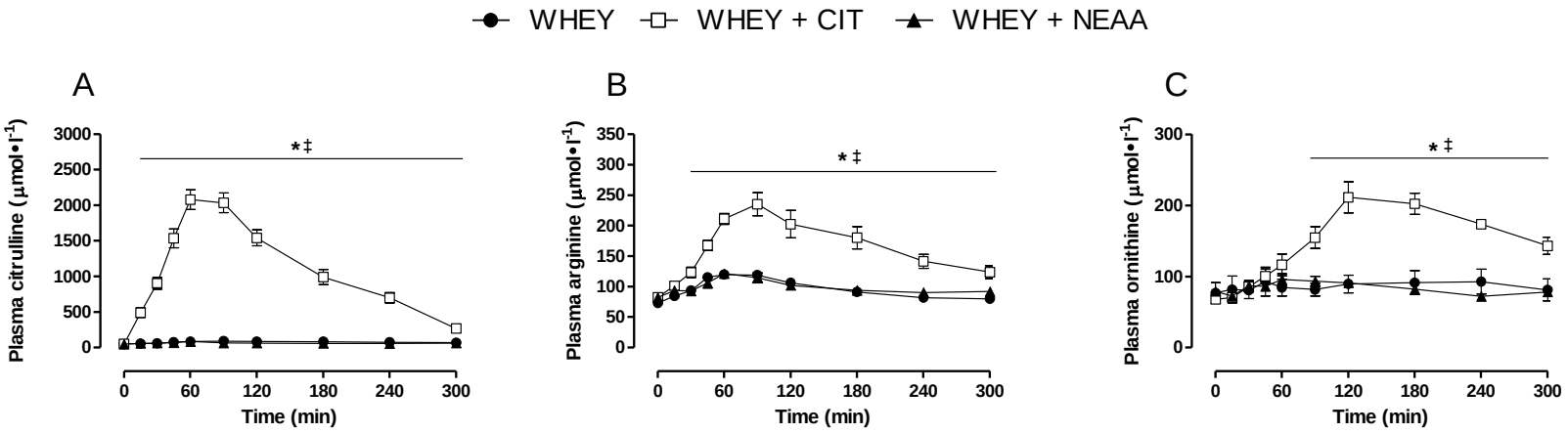


Figure 4. Values are mean ± SEM (n = 7 per treatment group). Plasma concentrations (µmol•L^{-1}) of citrulline (A), arginine (B), and ornithine (C), following treatment administration. Data were analyzed using a 2-factor (treatment × time) repeated measures ANOVA with Tukey's post hoc test (treatment × time interaction, $P < 0.001$). * significantly different from WHEY; † significantly different from WHEY+CIT; ‡ significantly different from WHEY+NEAA. Time dependent differences from baseline (time 0) within each treatment are not shown. WHEY (45 g whey protein); WHEY+CIT (15 g whey protein supplemented with 10 g citrulline); WHEY+NEAA (15 g whey protein supplemented with 10 g non-essential amino acids).

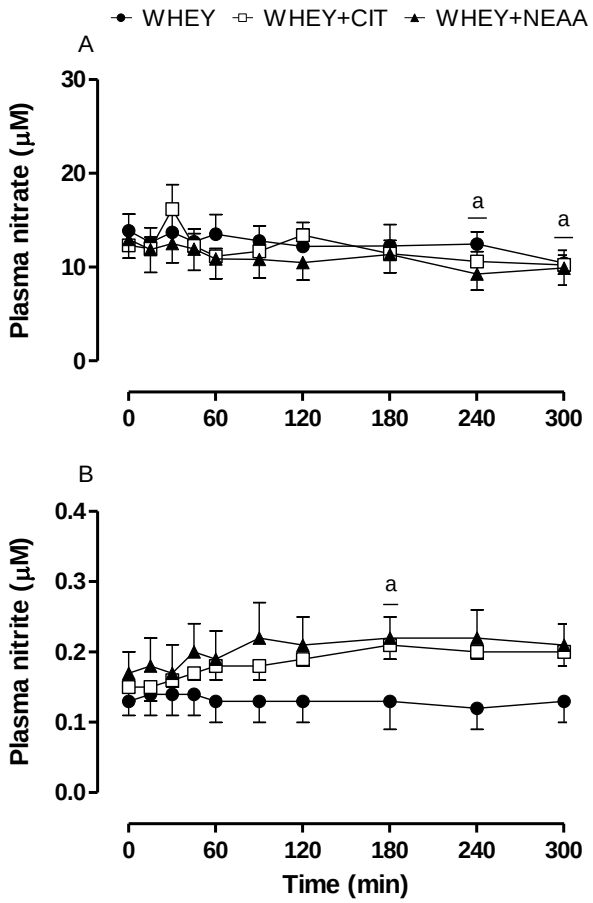


Figure 5. Values are mean ± SEM (n = 7 per treatment group). Plasma concentrations (μmol•L^{-1}) of nitrate (A), and nitrite (B) following treatment administration. Data were analyzed using a 2-factor (treatment × time) repeated measures ANOVA with Tukey's post hoc test (Nitrate: main effect for time, $P < 0.001$; Nitrite: main effect for time, $P = 0.003$). The letter "a" indicates a significant difference from baseline (time 0). WHEY (45 g whey protein); WHEY+CIT (15 g whey protein supplemented with 10 g citrulline); WHEY+NEAA (15 g whey protein supplemented with 10 g non-essential amino acids).

Table 3. Systolic and diastolic blood pressure, mean arterial pressure, and heart rate during the experimental infusion trial

	Time (min)									
	0	15	30	60	90	120	180	240	300	*P*
Systolic BP									a	
WHEY	152 ± 7	146 ± 6	143 ± 7	143 ± 6	144 ± 6	147 ± 7	146 ± 7	154 ± 7	161 ± 8	main effect for
WHEY+CIT	138 ± 6	139 ± 7	133 ± 6	138 ± 5	138 ± 6	140 ± 6	141 ± 6	144 ± 7	143 ± 7	time,
WHEY+NEAA	150 ± 5	153 ± 8	144 ± 7	142 ± 7	139 ± 7	144 ± 6	149 ± 6	147 ± 1	161 ± 7	*P* < 0.001
Diastolic BP										
WHEY	83 ± 2	81 ± 3	77 ± 2	79 ± 3	80 ± 3	80 ± 2	82 ± 2	85 ± 2	86 ± 3	main effect for
WHEY+CIT	79 ± 3	76 ± 3	74 ± 2	76 ± 3	73 ± 2	77 ± 3	78 ± 3	79 ± 3	78 ± 3	time,
WHEY+NEAA	85 ± 4	86 ± 4	80 ± 4	78 ± 3	79 ± 3	84 ± 4	86 ± 4	82 ± 2	90 ± 6	*P* < 0.001
MAP			a							
WHEY	106 ± 2	103 ± 3	99 ± 3	100 ± 3	101 ± 3	102 ± 3	103 ± 3	108 ± 3	111 ± 4	main effect for
WHEY+CIT	98 ± 4	97 ± 4	94 ± 3	97 ± 3	95 ± 3	98 ± 4	99 ± 4	101 ± 4	100 ± 4	time,
WHEY+NEAA	106 ± 4	108 ± 5	101 ± 5	99 ± 4	99 ± 4	104 ± 5	107 ± 4	104 ± 1	114 ± 6	*P* < 0.001
HR						a				
WHEY	59 ± 2	63 ± 2	63 ± 2	64 ± 2	63 ± 2	64 ± 2	64 ± 2	67 ± 1	65 ± 1	main effect for
WHEY+CIT	58 ± 1	64 ± 3	64 ± 4	64 ± 3	63 ± 2	62 ± 2	63 ± 3	64 ± 4	65 ± 3	time,
WHEY+NEAA	62 ± 2	63 ± 2	64 ± 3	64 ± 2	65 ± 2	63 ± 2	63 ± 2	65 ± 2	65 ± 2	*P* < 0.001

Values are mean ± SEM (*n* = 7 per treatment group). Systolic blood pressure (mmHg), diastolic blood pressure (mmHg), mean arterial pressure (mmHg), and heart rate (beats per minute) during the experimental trial. Data were each analyzed using a 2-factor (treatment × time) repeated measures ANOVA with Tukey's post hoc test. The letter "a" indicates a difference from time 0 (baseline). WHEY (45 g whey protein); WHEY+CIT (15 g whey protein supplemented with 10 g citrulline); WHEY+NEAA (15 g whey protein supplemented with 10 g non-essential amino acids).

Femoral artery blood flow. Femoral artery blood flow in both FED and EX-FED conditions is shown in **Figure 6.** There was a significant condition × time interaction (P <0.001), whereby blood flow in the EX-FED condition was increased above basal (time 0) and significantly different from the FED condition at 15, 30, and 60 min post-exercise, whereas there was no change from baseline in the FED condition.

Muscle microvascular perfusion. Analysis of CEUS-derived time-intensity curve parameters are shown in Table 4. All time-intensity curve parameters are calculated from the fitted curve and not the raw image data. Peak intensity (arbitrary intensity units (AIU)) demonstrated a main effect for time (P = 0.001), being increased above the basal (Fasted) time-period at 90 min. A similar response was observed for the Area Under the Curve (AUC) (P = 0.036), and the Perfusion Index (P = 0.001). There were no statistical effects for the Wash-in Time or Time for Full-Width Half-Maximum (FWHM) (Table 4).

Plasma and intracellular free phenylalanine enrichments. Plasma free phenylalanine enrichments did not differ between treatment groups (P = 0.56) or across time (P = 0.84). The slope of the plasma free phenylalanine enrichment was not different from zero for any treatment group (see **Online Supplemental Material Figure 1)**. Intracellular free phenylalanine enrichments did not differ between treatment groups (P = 0.97), across time (P = 0.74), or between conditions (P = 0.73). The slope of the intracellular free phenylalanine enrichment was not different from zero for any of the treatment groups in either FED or EX-FED condition (see **Online Supplemental Material Figure 2)**.

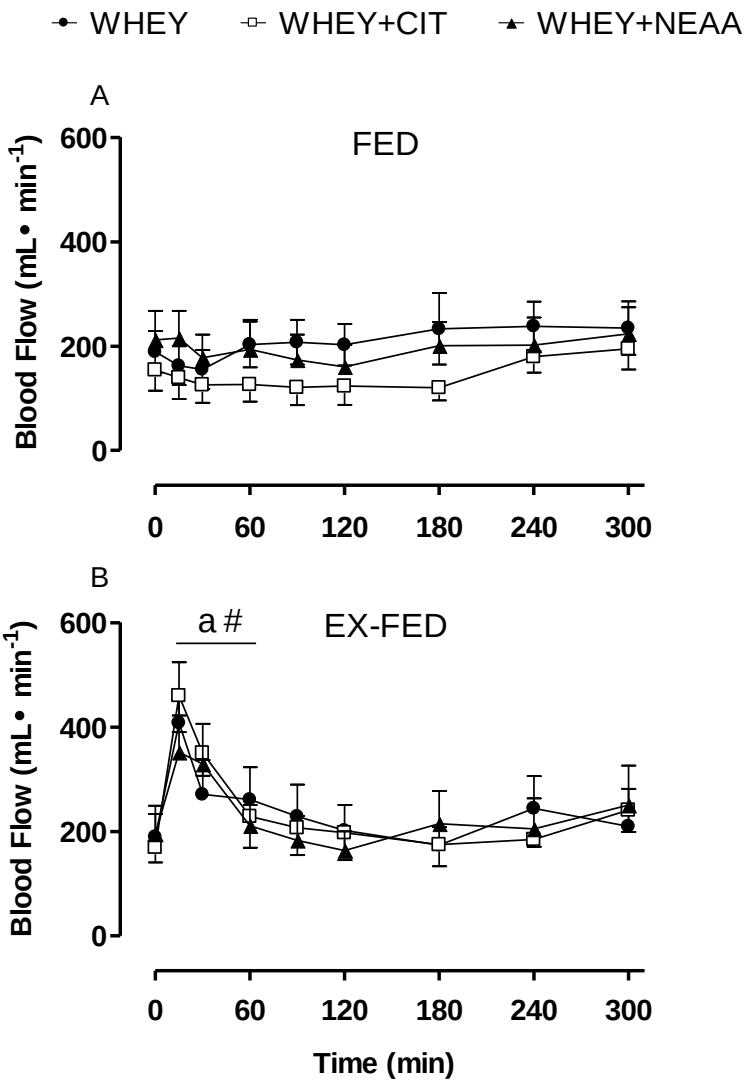
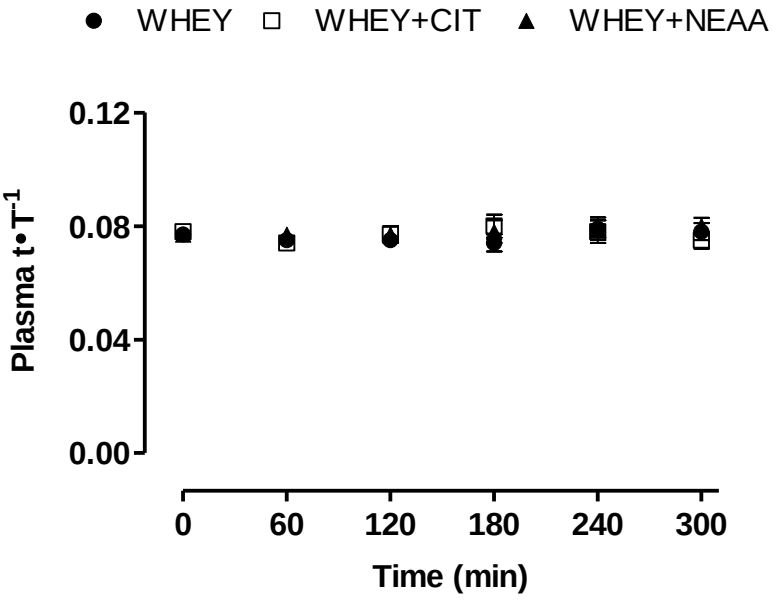


Figure 6. Values are mean ± SEM (n = 7 per treatment group). Femoral artery blood flow (mL• min^{-1}) under both FED (A) and EX-FED (B) conditions following treatment administration. Data were analyzed using a 3-factor (treatment × time × condition) mixed ANOVA with Tukey's post hoc test (time × condition interaction, $P < 0.001$). The letter "a" indicates a significant difference from baseline (time 0). # significantly different from opposite condition at the same time-point. WHEY (45 g whey protein); WHEY+CIT (15 g whey protein supplemented with 10 g citrulline); WHEY+NEAA (15 g whey protein supplemented with 10 g non-essential amino acids).

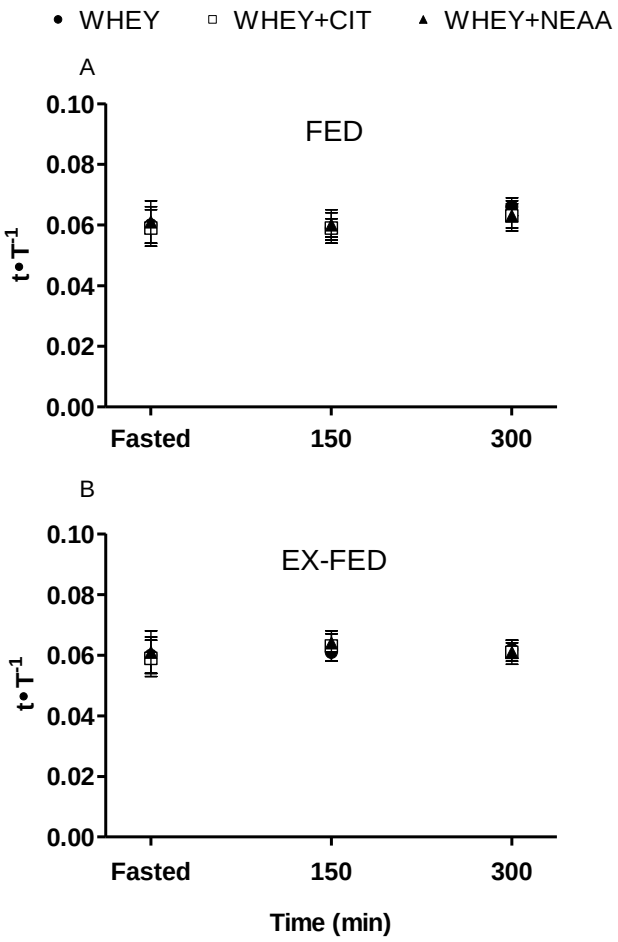
Table 4. Parameters of contrast enhanced ultrasound derived time-intensity curve analysis

	WHEY	WHEY+CIT	WHEY+NEAA	*P*
Peak intensity (AIU)				
Fasted EX	1.00 ± 0.32	1.04 ± 0.19	1.15 ± 0.16	
Fasted REST	0.89 ± 0.18	0.99 ± 0.16	0.92 ± 0.17	main effect for time (90 min >
90 min EX-FED	1.97 ± 0.41	1.71 ± 0.40	1.57 ± 0.31	Fasted), $P = 0.001$
90 min FED	1.69 ± 0.24	1.82 ± 0.47	1.30 ± 0.23	
AUC (AIU x sec)				
Fasted EX	111.89 ± 33.94	130.02 ± 27.89	108.57 ± 22.31	
Fasted REST	124.70 ± 37.09	108.75 ± 26.54	108.22 ± 20.67	main effect for time (90 min >
90 min EX-FED	241.65 ± 55.26	187.05 ± 28.97	137.11 ± 22.39	Fasted), $P = 0.036$
90 min FED	133.45 ± 13.79	154.14 ± 32.59	153.57 ± 37.99	
Wash-in time (sec)				
Fasted EX	42.62 ± 10.68	47.17 ± 4.05	33.89 ± 6.69	
Fasted REST	44.97 ± 7.24	43.15 ± 4.23	33.89 ± 6.09	time, $P = 0.99$
90 min EX-FED	37.45 ± 4.67	39.98 ± 5.00	40.61 ± 11.89	treatment, $P = 0.74$
90 min FED	35.24 ± 6.67	44.30 ± 4.85	47.82 ± 5.93	condition, $P = 0.73$
Time for FWHM (sec)				
Fasted EX	107.06 ± 22.11	111.89 ± 8.74	88.26 ± 16.23	
Fasted REST	124.17 ± 26.20	97.50 ± 11.05	123.14 ± 28.66	time, $P = 0.60$
90 min EX-FED	111.14 ± 9.38	104.98 ± 18.24	91.93 ± 14.20	treatment, $P = 0.93$
90 min FED	89.60 ± 17.48	113.41 ± 24.99	109.72 ± 12.53	condition, $P = 0.51$
Perfusion index				
Fasted EX	1.08 ± 0.35	1.12 ± 0.20	1.24 ± 0.17	
Fasted REST	0.92 ± 0.19	1.06 ± 0.17	0.97 ± 0.19	main effect for time (90 min >
90 min EX-FED	2.14 ± 0.46	2.10 ± 0.51	1.70 ± 0.34	Fasted), $P = 0.001$
90 min FED	1.83 ± 0.26	1.93 ± 0.52	1.37 ± 0.24	

Values are mean ± SEM (*n*: 6 WHEY; 7 WHEY+CIT; 6 WHEY+NEAA). Parameters of contrast enhanced ultrasound derived time-intensity curve analysis examined under basal Fasted conditions in each leg (EX and REST), and at 90 min following exercise and treatment administration in both EX-FED and FED conditions. Data for Peak Intensity, Area Under the Curve (AUC), Wash-in Time, Time for Full-Width Half-Maximum (FWHM), and Perfusion Index were analyzed using a 3-factor (treatment × time × condition) mixed-model ANOVA with Tukey's post hoc test. WHEY (45 g whey protein); WHEY+CIT (15 g whey protein supplemented with 10 g citrulline); WHEY+NEAA (15 g whey protein supplemented with 10 g non-essential amino acids).



Online Supplemental Material Figure 1. Values are mean ± SEM (*n* = 7 per treatment group). Plasma free phenylalanine enrichments (tracer-to-tracee ratio - $t \bullet T^{-1}$) over time. Time course data were analyzed using a 2-factor (treatment × time) repeated measures ANOVA with Tukey's post hoc test (treatment, *P* = 0.56; time, *P* = 0.84; time × treatment, P = 0.94). Linear regression was used to examine the slope of plasma free phenylalanine enrichment × time for each treatment (WHEY: *P* = 0.37; WHEY+CIT: *P* = 0.93; WHEY+NEAA: *P* = 0.32). WHEY (45 g whey protein); WHEY+CIT (15 g whey protein supplemented with 10 g citrulline); WHEY+NEAA (15 g whey protein supplemented with 10 g non-essential amino acids).



Online Supplemental Material Figure 2. Values are mean ± SEM (*n* = 7 per treatment group). Intracellular free phenylalanine enrichments (tracer-to-tracee ratio - $t \bullet T^{-1}$) from biopsies obtained at time 0 (Fasted), 150 min, and 300 min in both FED (A) and EX-FED (B) conditions. Data were analyzed using a 3-factor (treatment × time × condition) mixed-model ANOVA with Tukey's post hoc test (time, *P* = 0.74; condition, *P* = 0.73; treatment, *P* = 0.97; time × treatment, *P* = 0.99; condition × treatment, *P* = 0.82; time × condition, *P* = 0.09; time × condition × treatment, *P* = 0.99). Linear regression was used to examine the slope of intracellular free phenylalanine enrichment × time for each treatment in both FED (WHEY: *P* = 0.73; WHEY+CIT: *P* = 0.55; WHEY+NEAA: *P* = 0.84) and EX-FED (WHEY: *P* = 0.70; WHEY+CIT: *P* = 0.70; WHEY+NEAA: *P* = 0.99) conditions. WHEY (45 g whey protein); WHEY+CIT (15 g whey protein supplemented with 10 g citrulline); WHEY+NEAA (15 g whey protein supplemented with 10 g non-essential amino acids).

Muscle anabolic signalling. Changes in the phosphorylation status of signaling proteins involved in the regulation of mRNA translation initiation and elongation are shown in **Table 5.** Protein kinase B (p-Akt^{Ser473}) showed both a time × treatment ($P = 0.004$) and time × condition ($P = 0.01$) interaction. p-mTOR^{Ser2448} demonstrated a main effect for time ($P = 0.003$) and was increased above basal (rest) at both 150 min h and 300 min. pAMPK^{Thr172} showed no differences between treatments ($P = 0.80$), across time ($P = 0.71$), or between conditions ($P = 0.48$). p-p70S6k$^{Thr\ 389}$ showed main effects for time ($P < 0.001$), treatment ($P = 0.02$), and condition ($P = 0.008$); there was a trend for a time × condition interaction ($P = 0.057$). p-4E-BP1$^{Thr\ 37/46}$ showed a time × condition interaction ($P = 0.004$) whereby both conditions (FED and EX-FED) were increased above Rest at 150 min, while at 300 min, p-4E-BP1$^{Thr\ 37/46}$ phosphorylation remained elevated compared to baseline only in the EX-FED condition. p-rpS6$^{Ser\ 240/244}$ showed a time × condition × treatment interaction ($P = 0.016$). p-eEF2$^{Thr\ 56}$ showed a main effect of time ($P < 0.001$), and was increased above baseline at both 150 min and 300 min. Representative blot images are shown as supplemental material (**Online Supplemental Material Figure 3).**

DISCUSSION

The current study examined the impact of supplementing a dose of whey protein (15 g), previously demonstrated to be suboptimal for the stimulation of MPS under resting (9), and post-exercise (10) conditions in elderly men, with dose-matched citrulline

Table 5. Western-blot analysis of protein synthesis-associated signalling proteins following treatment administration

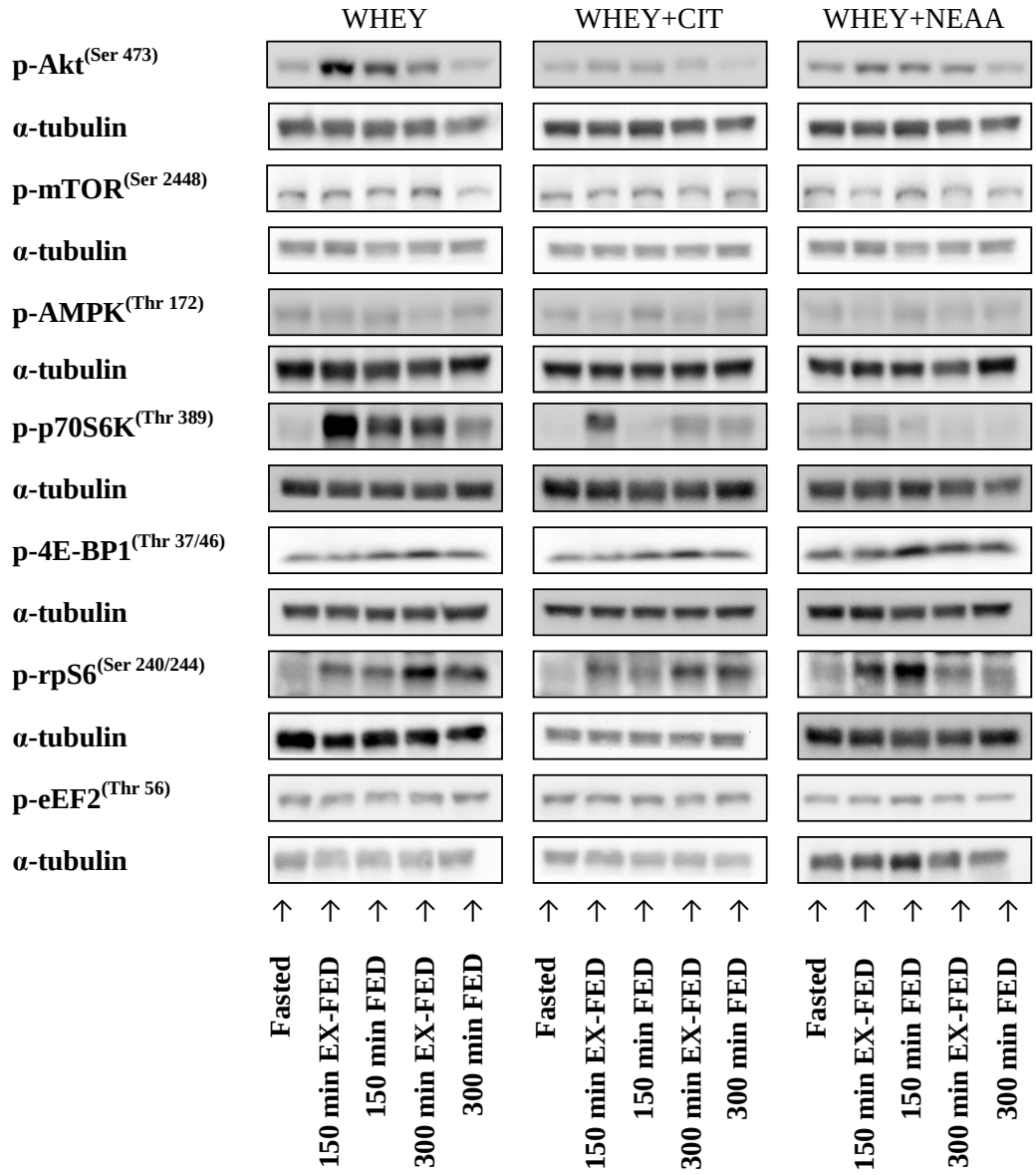
	WHEY	WHEY+CIT	WHEY+NEAA	
	Fold difference from Fasted			
p-Akt$^{(Ser\ 473)}$				*P*
150 min EX-FED	2.55 ± 0.50^{a†‡}	1.31 ± 0.14	1.67 ± 0.23	time × treatment, $P = 0.004$ time × condition (300 EX-FED > FED), $P = 0.01$
150 min FED	2.51 ± 0.43^{a†‡}	1.37 ± 0.25	1.20 ± 0.14	
300 min EX-FED	1.49 ± 0.15^{a†‡}	1.07 ± 0.10	1.29 ± 0.19	
300 min FED	1.19 ± 0.15^{a†‡}	0.72 ± 0.11	0.68 ± 0.08	
p-mTOR$^{(Ser\ 2448)}$				
150 min EX-FED	1.35 ± 0.10	1.32 ± 0.24	1.20 ± 0.15	main effect for time (150, 300 > Fasted), $P = 0.003$
150 min FED	1.31 ± 0.37	1.29 ± 0.20	1.24 ± 0.10	
300 min EX-FED	1.24 ± 0.12	1.32 ± 0.15	1.26 ± 0.15	
300 min FED	1.38 ± 0.23	1.24 ± 0.21	0.79 ± 0.08	
p-AMPK$^{(Thr\ 172)}$				
150 min EX-FED	0.95 ± 0.07	0.89 ± 0.09	0.97 ± 0.12	time, $P = 0.71$ treatment, $P = 0.80$ condition, $P = 0.48$
150 min FED	0.91 ± 0.08	1.07 ± 0.10	1.10 ± 0.09	
300 min EX-FED	0.91 ± 0.11	0.98 ± 0.08	1.02 ± 0.08	
300 min FED	0.96 ± 0.11	0.95 ± 0.13	0.95 ± 0.12	
p-p70S6K1$^{(Thr\ 389)}$				
150 min EX-FED	5.30 ± 0.96‡	5.05 ± 1.14	3.73 ± 0.95	main effect for time (150, 300 > Fasted), $P < 0.001$; treatment $P = 0.02$; condition (EX-FED > FED), $P = 0.008$
150 min FED	5.37 ± 1.38‡	1.81 ± 0.33	1.74 ± 0.40	
300 min EX-FED	4.66 ± 1.09‡	3.20 ± 0.80	2.35 ± 0.51	
300 min FED	3.10 ± 0.55‡	2.84 ± 0.73	1.57 ± 0.69	
p-4E-BP1$^{(Thr\ 37/46)}$				
150 min EX-FED	1.54 ± 0.10	1.79 ± 0.23	1.42 ± 0.16	time × condition (300 EX-FED > FED), $P = 0.004$
150 min FED	1.73 ± 0.33	2.19 ± 0.39	1.94 ± 0.60	
300 min EX-FED	1.34 ± 0.22	1.78 ± 0.56	1.73 ± 0.52	
300 min FED	1.01 ± 0.27	1.56 ± 0.38	1.12 ± 0.31	
p-rpS6$^{(Ser\ 240/244)}$				
150 min EX-FED	1.77 ± 0.18^{a}	2.11 ± 0.21$^{a\ddagger\#}$	1.51 ± 0.16^{a}	time × treatment × condition, $P = 0.021$
150 min FED	1.39 ± 0.18^{a}	1.15 ± 0.14	1.43 ± 0.19^{a}	
300 min EX-FED	1.92 ± 0.35^{a}	1.62 ± 0.15	2.12 ± 0.43$^{a\#}$	
300 min FED	1.65 ± 0.19^{a}	1.48 ± 0.16^{a}	1.28 ± 0.23	
p-eEF2$^{(Thr\ 56)}$				
150 min EX-FED	1.09 ± 0.04	1.11 ± 0.07	0.99 ± 0.03	main effect for time (150, 300 > Fasted), $P < 0.001$
150 min FED	1.11 ± 0.05	1.13 ± 0.05	1.12 ± 0.07	
300 min EX-FED	1.14 ± 0.04	1.13 ± 0.08	1.13 ± 0.09	
300 min FED	1.13 ± 0.03	1.10 ± 0.07	1.16 ± 0.07	

Values are mean ± SEM ($n = 7$ per treatment group). Phosphorylation status of Akt$^{(Ser473)}$, mTOR$^{(Ser2448)}$, AMPK$^{(Thr172)}$, p70S6k $^{(Thr389)}$, 4E-BP1$^{(Thr37/46)}$, rpS6$^{(Ser240/244)}$, eEF2$^{(Thr56)}$, expressed as fold-difference from baseline (i.e. the Fasted time-point), at 150 min and 300 min post-exercise recovery in both FED and EX-FED conditions following treatment administration. Data for Akt$^{(Ser473)}$, mTOR$^{(Ser2448)}$, AMPK$^{(Thr172)}$, p70S6k $^{(Thr389)}$, 4E-BP1$^{(Thr37/46)}$, rpS6$^{(Ser240/244)}$, and eEF2$^{(Thr56)}$, were analyzed using a 3-factor (treatment × time × condition) mixed-model ANOVA with Tukey's post hoc test. A superscript letter

"a" indicates a difference from baseline. † significantly different from WHEY+CIT; ‡ significantly different from WHEY+NEAA; # significantly different from opposite condition at the same time-point. WHEY (45 g whey protein); WHEY+CIT (15 g whey protein supplemented with 10 g citrulline); WHEY+NEAA (15 g whey protein supplemented with 10 g non-essential amino acids).

(WHEY+CIT) or a mixture of non-essential amino acids (WHEY+NEAA), on femorial arterial blood flow, microvascular circulation, MPS, and signaling through the Akt-mTORC1 pathway in skeletal muscle. We compared these treatments to a dose of whey protein (45 g), previously demonstrated to elicit a more optimal stimulation of MPS in the elderly (9, 10). Contrary to our hypothesis, our results demonstrate that although supplementation with citrulline (10 g) to a smaller "suboptimal dose of whey protein" (15 g whey protein) substantially increased plasma arginine availability, this was not associated with increases in plasma nitrate-nitrite, femoral artery blood flow, or changes in muscle microvascular circulation as compared to the same dose of whey protein (15 g whey protein) supplemented with a complete mixture of non-essential amino acids (10 g), or three times the quantity of whey protein (45 g whey protein) when examined under both resting and post-exercise conditions in healthy elderly men.

Ageing is associated with the development of vascular endothelial dysfunction (44), a key characteristic of which is impaired endothelial-dependent vasodilation (45). Nitric oxide (NO), produced from the amino acid arginine in an enzymatic reaction catalyzed by eNOS, is an endothelial derived vasodilator that decreases with advancing age (16). In line with these observations, previous studies have demonstrated that the elderly exhibit reduced peripheral (i.e. limb arterial) blood flow as compared to their



Online Supplemental Material Figure 3. Representative western blot images for p-Akt^{Ser473}, p-mTOR^{Ser2448}, p-AMPK^{Thr172}, p-p70S6k^{Thr389}, p-4E-BP1$^{Thr\ 37/46}$, p-rpS6$^{Ser240/244}$, p-eEF2^{Thr56}, and α-tubulin during Fasted, and 150 min EX-FED, 150 min FED, 300 min EX-FED; and 300 min FED following nutrient treatment administration. WHEY (45 g whey protein); WHEY+CIT (15 g whey protein supplemented with 10 g citrulline); WHEY+NEAA (15 g whey protein supplemented with 10 g non-essential amino acids).

younger counterparts when examined at rest (i.e. in the postabsorptive state) (46), and in response to vasodilatory stimuli such as food intake (47, 48) and exercise (49, 46, 48). In addition to reductions in peripheral blood flow, ageing has also been associated with impairments in microvascular perfusion in response to insulin (50, 13), mixed nutrients (14), and exercise (51). These age-related declines in blood flow and microvascular perfusion may contribute to declines in insulin sensitivity and the muscle protein anabolic response to feeding and exercise (3, 15, 52, 14) since changes in blood flow regulate the delivery of insulin and amino acids in skeletal muscle (17, 13). In support of this notion, when blood flow and microvascular perfusion are enhanced either pharmacologically (13, 53), or through exercise (14), muscle protein anabolism is enhanced. Clearly, strategies to enhance age-related declines in blood flow responses to feeding and exercise may be useful to offset anabolic resistance.

Citrulline serves as a precursor for L-arginine synthesis via the enzymes argininosuccinate synthase and argininosuccinate lyase (54), and has been reported to enhance NO synthesis more effectively than dose-matched consumption of arginine (18). A large proportion of ingested arginine is subject to first-pass splanchnic extraction where it is degraded in the intestine to yield ornithine and proline (55), while in the liver arginine serves as substrate for urea synthesis (19). Citrulline, on the other hand is not metabolized in the intestine or liver (21) and therefore has a much higher systemic availability. Consistent with findings from previous studies using citrulline (56, 57, 18), we found that WHEY+CIT resulted in greater arginine availability from 30-300 minutes

post-treatment vs. WHEY and WHEY+NEAA (Figure 4, Panel B). A large change in arginine was not expected following WHEY or WHEY+NEAA due to the fact that the arginine content of whey protein is relatively low (~2%). We utilized a 10 g dose of citrulline based on data from Moinard and colleagues (57) who suggested a 10 g dose of citrulline as the most appropriate for use in clinical practice based on their observation (57) of saturation of arginine synthesis with greater doses of citrulline (i.e. 15 g). Interestingly, and contrary to our hypotheses, the hyperargininemia following WHEY+CIT was not associated with an increases in plasma nitrate or nitrite concentrations (Figure 5), enhanced femoral artery blood flow (Figure 6), or changes in microvascular circulation (Table 4) as compared to the other treatments. El-Hattab and colleagues (18) demonstrated substantial increases in NO synthesis following citrulline administration (as assessed via stable isotope tracer methodology) without a measurable change in plasma nitrate/nitrite levels (18). As such, the lack of increase in plasma nitrate and nitrite may not mean that citrulline failed to alter NO synthesis. Regardless, even if citrulline had enhanced NO availability to a greater extent than WHEY and WHEY+NEAA, it did not augment femoral artery blood flow or microvascular circulatory variables at the timepoints we measured. We observed time-dependent increases in CEUS-derived time-intensity curve variables including peak intensity, AUC, and the calculated perfusion index; however, no changes were observed in the wash-in time or time for FWHM (Table 4). Peak intensity and AUC are measures that relate to local microvascular blood volume (35, 58), while the wash-in time and time for FWHM are measures that relate to microvascular blood flow (35, 58). Therefore, the increase in

peak intensity and AUC concomitant with a greater increase in the perfusion index (calculated as the quotient of AUC and time for FWHM) is consistent with previous studies demonstrating changes in microvascular perfusion and blood volume, but not microvascular blood flow velocity after exercise (51) and the NO donor SNP (53). This observation is consistent with microvascular recruitment resulting in a greater endothelial surface area, thereby resulting in maintenance or a reduction in blood flow velocity due to travel through an increased capillary network.

Insulin is well recognized for its capacity to stimulate blood flow and recruitment of the microvasculature (for review see (59)). Early postprandial increases in insulin were apparent from 15-90 following treatment administration however, there were no significant differences between groups (Figure 2). One possibility is that this increase in plasma insulin following treatment administration served as a sufficient stimulus to promote microvascular recruitment, thereby overriding any potential impact of citrulline generated arginine on NO mediated vasodilation of the microvasculature. However, we feel this is unlikely given the relatively low postprandial insulin response (~25 $\mu U\text{-}mL^{-1}$) and growing recognition that the inability of the elderly to mount a robust anabolic response following nutrient intake relates to a reduced sensitivity of skeletal muscle to the vasodilatory effects of insulin (50, 13), and requires non-physiological doses of insulin to be remedied (60).

Alternatively, microvascular circulation was only assessed at a single time-point (90 minutes) in the postprandial period in both FED and EX-FED conditions; therefore we may have missed important treatment dependent differences that may have occurred at

other times during the infusion protocol. There are currently limits on the quantity of Definity® contrast agent that can be administered to individuals; we chose to make our measures of microvascular circulation at 90 min following treatment administration since blood arginine had previously been reported to peak at this time following a 10 g bolus of citrulline (57). A more frequent assessment of muscle microvascular circulation is necessary to more definitively assess the impact of citrulline on parameters of microvascular circulation. Finally, an important consideration relates to assessing the impact of citrulline when co-ingested with a low dose of protein, as opposed to examining the independent action of citrulline. In vitro data has demonstrated that the amino acid glutamine can antagonize the beneficial effects of citrulline on NO production (61), thus whether our strategy of adding citrulline to 15 g whey protein (containing ~2.5 g glutamine) reduced the potential of citrulline to enhance NO remains a possibility.

Previous studies in animals have shown that citrulline is as effective as leucine at stimulating muscle protein synthesis (24) although it does not appear to enhance signaling through mTORC1 and its downstream targets p70S6k$^{(Thr389)}$, rpS6$^{(Ser235/236-240/244)}$, or 4E-BP1$^{(Ser65)}$ to the same extent as leucine (24). Similarly, in the current study we noted subtle differences in p70S6k$^{(Thr389)}$ and rpS6$^{(Ser240/244)}$ following WHEY+CIT as compared to WHEY+NEAA as reported previously in animals (24). However, we found that WHEY (containing 45 g of whey protein) resulted in the greatest phosphorylation of Akt$^{(Ser473)}$ and p70S6k$^{(Thr389)}$ signaling response (Table 5). This is perhaps not surprising given that the role of leucine as a nutrient signaling molecule regulating translation initiation of MPS (62, 63). Citrulline, in a manner similar to leucine, has been suggested

to act as a signaling molecule with the capacity to stimulate muscle protein synthesis in a manner independent of its capacity to stimulate arginine synthesis (22). In support of this, an in-vitro study using isolated muscles from malnourished rats reported increases in muscle protein synthesis and anabolic signaling when incubated in citrulline, suggesting that citrulline itself and not arginine is responsible for stimulating protein synthesis.

In conclusion, our results demonstrate the citrulline supplementation (10 g) of a smaller "suboptimal dose of whey protein" (15 g whey protein) can substantially increase plasma arginine availability, however, this was not associated with increases in plasma nitrate-nitrite, femoral artery blood flow, or changes in muscle microvascular circulation as compared to the same dose of whey protein (15 g whey protein) supplemented with a complete mixture of non-essential amino acids (10 g), or three times the quantity of whey protein (45 g whey protein) when examined under both resting and post-exercise conditions in healthy elderly men.

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Author's contributions to manuscript: TAC-V and SMP designed the study; TAC-V, MJM, LMC, CJM, SKB, and SMP conducted the research; TAC-V, MJM, LMC, NB, TP, and SMP analyzed data; TAC-V wrote the manuscript; all authors assisted in editing and having meaningful input into the manuscript; TAC-V and SMP had primary responsibility for final content. All authors read and approved the final manuscript. TAC-V, MJM, LMC, NB, TP, SKB, and SMP have no conflicts of interest to declare.

References

[1] Doherty, T. J. (2003) Invited review: Aging and sarcopenia. *J Appl Physiol* **95**, 1717-1727.

[2] Rennie, M. J., Wackerhage, H., Spangenburg, E. E., Booth, F. W. (2004) Control of the size of the human muscle mass. *Annu Rev Physiol* **66**, 799-828.

[3] Volpi, E., Mittendorfer, B., Rasmussen, B. B., Wolfe, R. R. (2000) The response of muscle protein anabolism to combined hyperaminoacidemia and glucose-induced hyperinsulinemia is impaired in the elderly. *J Clin Endocrinol Metab* **85**, 4481-4490.

[4] Kumar, V., Selby, A., Rankin, D., Patel, R., Atherton, P., et al. (2009) Age-related differences in the dose-response relationship of muscle protein synthesis to resistance exercise in young and old men. *J Physiol* **587**, 211-217.

[5] Breen, L., Phillips, S. M. (2011) Skeletal muscle protein metabolism in the elderly: Interventions to counteract the 'anabolic resistance' of ageing. *Nutr Metab (Lond)* **8**, 68.

[6] Tang, J. E., Manolakos, J. J., Kujbida, G. W., Lysecki, P. J., Moore, D. R., et al. (2007) Minimal whey protein with carbohydrate stimulates muscle protein synthesis following resistance exercise in trained young men. *Appl Physiol Nutr Metab* **32**, 1132-1138.

[7] Moore, D. R., Robinson, M. J., Fry, J. L., Tang, J. E., Glover, E. I., et al. (2009) Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *Am J Clin Nutr* **89**, 161-168.

[8] Katsanos, C. S., Kobayashi, H., Sheffield-Moore, M., Aarsland, A., Wolfe, R. R. (2005) Aging is associated with diminished accretion of muscle proteins after the ingestion of a small bolus of essential amino acids. *Am J Clin Nutr* **82**, 1065-1073.

[9] Pennings, B., Groen, B. B., de Lange, A., Gijsen, A. P., Zorenc, A. H., et al. (2012) Amino acid absorption and subsequent muscle protein accretion following graded intakes of whey protein in elderly men. *Am J Physiol Endocrinol Metab*.

[10] Yang, Y., Breen, L., Burd, N. A., Hector, A. J., Churchward-Venne, T. A., et al. (2012) Resistance exercise enhances myofibrillar protein synthesis with graded intakes of whey protein in older men. *Br J Nutr* 1-9.

[11] Yang, Y., Churchward-Venne, T. A., Burd, N. A., Breen, L., Tarnopolsky, M. A., et al. (2012) Myofibrillar protein synthesis following ingestion of soy protein isolate at rest and after resistance exercise in elderly men. *Nutr Metab (Lond)* **9**, 57.

[12] Robinson, M. J., Burd, N. A., Breen, L., Rerecich, T., Yang, Y., et al. (2013) Dose-dependent responses of myofibrillar protein synthesis with beef ingestion are enhanced with resistance exercise in middle-aged men. *Appl Physiol Nutr Metab* **38**, 120-125.

[13] Timmerman, K. L., Lee, J. L., Fujita, S., Dhanani, S., Dreyer, H. C., et al. (2010) Pharmacological vasodilation improves insulin-stimulated muscle protein anabolism but not glucose utilization in older adults. *Diabetes* **59**, 2764-2771.

[14] Timmerman, K. L., Dhanani, S., Glynn, E. L., Fry, C. S., Drummond, M. J., et al. (2012) A moderate acute increase in physical activity enhances nutritive flow and the muscle protein anabolic response to mixed nutrient intake in older adults. *Am J Clin Nutr* **95**, 1403-1412.

[15] Vincent, M. A., Clerk, L. H., Lindner, J. R., Price, W. J., Jahn, L. A., et al. (2006) Mixed meal and light exercise each recruit muscle capillaries in healthy humans. *Am J Physiol Endocrinol Metab* **290**, E1191-1197.

[16] Taddei, S., Virdis, A., Ghiadoni, L., Salvetti, G., Bernini, G., et al. (2001) Age-related reduction of NO availability and oxidative stress in humans. *Hypertension* **38**, 274-279.

[17] Clark, M. G., Wallis, M. G., Barrett, E. J., Vincent, M. A., Richards, S. M., et al. (2003) Blood flow and muscle metabolism: a focus on insulin action. *Am J Physiol Endocrinol Metab* **284**, E241-258.

[18] El-Hattab, A. W., Hsu, J. W., Emrick, L. T., Wong, L. J., Craigen, W. J., et al. (2012) Restoration of impaired nitric oxide production in MELAS syndrome with citrulline and arginine supplementation. *Mol Genet Metab* **105**, 607-614.

[19] Morris, S. M., Jr. (1992) Regulation of enzymes of urea and arginine synthesis. *Annu Rev Nutr* **12**, 81-101.

[20] Castillo, L., Chapman, T. E., Yu, Y. M., Ajami, A., Burke, J. F., et al. (1993) Dietary arginine uptake by the splanchnic region in adult humans. *Am J Physiol* **265**, E532-539.

[21] Wu, G., Meininger, C. J., Knabe, D. A., Bazer, F. W., Rhoads, J. M. (2000) Arginine nutrition in development, health and disease. *Curr Opin Clin Nutr Metab Care* **3**, 59-66.

[22] Cynober, L., Moinard, C., De Bandt, J. P. (2010) The 2009 ESPEN Sir David Cuthbertson. Citrulline: a new major signaling molecule or just another player in the pharmaconutrition game? *Clin Nutr* **29**, 545-551.

[23] Osowska, S., Duchemann, T., Walrand, S., Paillard, A., Boirie, Y., et al. (2006) Citrulline modulates muscle protein metabolism in old malnourished rats. *Am J Physiol Endocrinol Metab* **291**, E582-586.

[24] Le Plenier, S., Walrand, S., Noirt, R., Cynober, L., Moinard, C. (2012) Effects of leucine and citrulline versus non-essential amino acids on muscle protein synthesis in fasted rat: a common activation pathway? *Amino Acids* **43**, 1171-1178.

[25] Smith, K., Reynolds, N., Downie, S., Patel, A., Rennie, M. J. (1998) Effects of flooding amino acids on incorporation of labeled amino acids into human muscle protein. *Am J Physiol* **275**, E73-78.

[26] Volpi, E., Kobayashi, H., Sheffield-Moore, M., Mittendorfer, B., Wolfe, R. R. (2003) Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults. *Am J Clin Nutr* **78**, 250-258.

[27] (2010) Canadian Institutes of Health Research, Natural Sciences and Engineering Research Council of Canada, and Social Sciences and Humanities Research Council of Canada, Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans. .

[28] Copeland, K. C., Kenney, F. A., Nair, K. S. (1992) Heated dorsal hand vein sampling for metabolic studies: a reappraisal. *Am J Physiol* **263**, E1010-1014.

[29] West, D. W., Kujbida, G. W., Moore, D. R., Atherton, P., Burd, N. A., et al. (2009) Resistance exercise-induced increases in putative anabolic hormones do not enhance muscle protein synthesis or intracellular signalling in young men. *J Physiol* **587**, 5239-5247.

[30] Burd, N. A., Holwerda, A. M., Selby, K. C., West, D. W., Staples, A. W., et al. (2010) Resistance exercise volume affects myofibrillar protein synthesis and anabolic signalling molecule phosphorylation in young men. *J Physiol* **588**, 3119-3130.

[31] Tang, J. E., Moore, D. R., Kujbida, G. W., Tarnopolsky, M. A., Phillips, S. M. (2009) Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men. *J Appl Physiol* **107**, 987-992.

[32] Burd, N. A., West, D. W., Staples, A. W., Atherton, P. J., Baker, J. M., et al. (2010) Low-load high volume resistance exercise stimulates muscle protein synthesis more than high-load low volume resistance exercise in young men. *PLoS One* **5**, e12033.

[33] Gill, R. W. (1985) Measurement of blood flow by ultrasound: accuracy and sources of error. *Ultrasound Med Biol* **11**, 625-641.

[34] Strouthos, C., Lampaskis, M., Sboros, V., McNeilly, A., Averkiou, M. (2010) Indicator dilution models for the quantification of microvascular blood flow with bolus administration of ultrasound contrast agents. *IEEE Trans Ultrason Ferroelectr Freq Control* **57**, 1296-1310.

[35] Greis, C. (2011) Quantitative evaluation of microvascular blood flow by contrast-enhanced ultrasound (CEUS). *Clin Hemorheol Microcirc* **49**, 137-149.

[36] Wilkinson, S. B., Tarnopolsky, M. A., Macdonald, M. J., Macdonald, J. R., Armstrong, D., et al. (2007) Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage. *Am J Clin Nutr* **85**, 1031-1040.

[37] Glover, E. I., Oates, B. R., Tang, J. E., Moore, D. R., Tarnopolsky, M. A., et al. (2008) Resistance exercise decreases eIF2Bepsilon phosphorylation and potentiates the feeding-induced stimulation of p70S6K1 and rpS6 in young men. *Am J Physiol Regul Integr Comp Physiol* **295**, R604-610.

[38] Bryan, N. S., Grisham, M. B. (2007) Methods to detect nitric oxide and its metabolites in biological samples. *Free Radic Biol Med* **43**, 645-657.

[39] Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

[40] Moore, D. R., Tang, J. E., Burd, N. A., Rerecich, T., Tarnopolsky, M. A., et al. (2009) Differential stimulation of myofibrillar and sarcoplasmic protein synthesis with protein ingestion at rest and after resistance exercise. *J Physiol* **587**, 897-904.

[41] Miller, B. F., Olesen, J. L., Hansen, M., Dossing, S., Crameri, R. M., et al. (2005) Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise. *J Physiol* **567**, 1021-1033.

[42] Mittendorfer, B., Andersen, J. L., Plomgaard, P., Saltin, B., Babraj, J. A., et al. (2005) Protein synthesis rates in human muscles: neither anatomical location nor fibre-type composition are major determinants. *J Physiol* **563**, 203-211.

[43] Heys, S. D., McNurlan, M. A., Park, K. G., Milne, E., Garlick, P. J. (1990) Baseline measurements for stable isotope studies: an alternative to biopsy. *Biomed Environ Mass Spectrom* **19**, 176-178.

[44] Toda, N. (2012) Age-related changes in endothelial function and blood flow regulation. *Pharmacol Ther* **133**, 159-176.

[45] Gerhard, M., Roddy, M. A., Creager, S. J., Creager, M. A. (1996) Aging progressively impairs endothelium-dependent vasodilation in forearm resistance vessels of humans. *Hypertension* **27**, 849-853.

[46] Donato, A. J., Uberoi, A., Wray, D. W., Nishiyama, S., Lawrenson, L., et al. (2006) Differential effects of aging on limb blood flow in humans. *Am J Physiol Heart Circ Physiol* **290**, H272-278.

[47] Skilton, M. R., Lai, N. T., Griffiths, K. A., Molyneaux, L. M., Yue, D. K., et al. (2005) Meal-related increases in vascular reactivity are impaired in older and diabetic adults: insights into roles of aging and insulin in vascular flow. *Am J Physiol Heart Circ Physiol* **288**, H1404-1410.

[48] Phillips, B., Williams, J., Atherton, P., Smith, K., Hildebrandt, W., et al. (2012) Resistance exercise training improves age-related declines in leg vascular conductance and rejuvenates acute leg blood flow responses to feeding and exercise. *J Appl Physiol (1985)* **112**, 347-353.

[49] Poole, J. G., Lawrenson, L., Kim, J., Brown, C., Richardson, R. S. (2003) Vascular and metabolic response to cycle exercise in sedentary humans: effect of age. *Am J Physiol Heart Circ Physiol* **284**, H1251-1259.

[50] Timmerman, K. L., Lee, J. L., Dreyer, H. C., Dhanani, S., Glynn, E. L., et al. (2010) Insulin stimulates human skeletal muscle protein synthesis via an indirect mechanism involving endothelial-dependent vasodilation and mammalian target of rapamycin complex 1 signaling. *J Clin Endocrinol Metab* **95**, 3848-3857.

[51] Durham, W. J., Casperson, S. L., Dillon, E. L., Keske, M. A., Paddon-Jones, D., et al. (2010) Age-related anabolic resistance after endurance-type exercise in healthy humans. *FASEB J* **24**, 4117-4127.

[52] Clark, M. G. (2008) Impaired microvascular perfusion: a consequence of vascular dysfunction and a potential cause of insulin resistance in muscle. *Am J Physiol Endocrinol Metab* **295**, E732-750.

[53] Dillon, E. L., Casperson, S. L., Durham, W. J., Randolph, K. M., Urban, R. J., et al. (2011) Muscle protein metabolism responds similarly to exogenous amino acids in healthy younger and older adults during NO-induced hyperemia. *Am J Physiol Regul Integr Comp Physiol* **301**, R1408-1417.

[54] Curis, E., Nicolis, I., Moinard, C., Osowska, S., Zerrouk, N., et al. (2005) Almost all about citrulline in mammals. *Amino Acids* **29**, 177-205.

[55] Wu, G. (1998) Intestinal mucosal amino acid catabolism. *J Nutr* **128**, 1249-1252.

[56] Rouge, C., Des Robert, C., Robins, A., Le Bacquer, O., Volteau, C., et al. (2007) Manipulation of citrulline availability in humans. *Am J Physiol Gastrointest Liver Physiol* **293**, G1061-1067.

[57] Moinard, C., Nicolis, I., Neveux, N., Darquy, S., Benazeth, S., et al. (2008) Dose-ranging effects of citrulline administration on plasma amino acids and hormonal patterns in healthy subjects: the Citrudose pharmacokinetic study. *Br J Nutr* **99**, 855-862.

[58] Dietrich, C. F., Averkiou, M. A., Correas, J. M., Lassau, N., Leen, E., et al. (2012) An EFSUMB introduction into Dynamic Contrast-Enhanced Ultrasound (DCE-US) for quantification of tumour perfusion. *Ultraschall Med* **33**, 344-351.

[59] Barrett, E. J., Eggleston, E. M., Inyard, A. C., Wang, H., Li, G., et al. (2009) The vascular actions of insulin control its delivery to muscle and regulate the rate-limiting step in skeletal muscle insulin action. *Diabetologia* **52**, 752-764.

[60] Fujita, S., Glynn, E. L., Timmerman, K. L., Rasmussen, B. B., Volpi, E. (2009) Supraphysiological hyperinsulinaemia is necessary to stimulate skeletal muscle protein anabolism in older adults: evidence of a true age-related insulin resistance of muscle protein metabolism. *Diabetologia* **52**, 1889-1898.

[61] Bryk, J., Ochoa, J. B., Correia, M. I., Munera-Seeley, V., Popovic, P. J. (2008) Effect of citrulline and glutamine on nitric oxide production in RAW 264.7 cells in an arginine-depleted environment. *JPEN J Parenter Enteral Nutr* **32**, 377-383.

[62] Anthony, J. C., Yoshizawa, F., Anthony, T. G., Vary, T. C., Jefferson, L. S., et al. (2000) Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. *J Nutr* **130**, 2413-2419.

[63] Crozier, S. J., Kimball, S. R., Emmert, S. W., Anthony, J. C., Jefferson, L. S. (2005) Oral leucine administration stimulates protein synthesis in rat skeletal muscle. *J Nutr* **135**, 376-382.

CHAPTER 5

CONCLUSION & DISCUSSION

The two most powerful non-pharmacological factors that regulate muscle protein metabolism are nutrition and exercise (1-3). Amino acids alone can stimulate muscle protein synthesis to nearly the same extent as a complete meal (4), highlighting their key role in the nutrient-mediated regulation of muscle protein synthesis. However, individual amino acids differ in their capacity to stimulate muscle protein synthesis (5) and activate "anabolic" cell signaling through mTORC1 (6), which may partially explain why sources of dietary protein that differ in their amino acid profile also vary in their capacity to stimulate muscle protein synthesis (7-9). Identifying amino acids that possess the capacity to stimulate muscle protein synthesis is of critical importance in the development of nutritional interventions designed to enhance skeletal muscle anabolism. The experiments outlined in the current thesis have focused on the role of the amino acids leucine (chapter 2 and 3) and citrulline (chapter 4) in the amino acid-mediated regulation of human muscle protein synthesis, both in the presence and absence of an acute resistance exercise stimulus. A wealth of in vitro (10, 11) and in-vivo animal data (12-15) supports leucine as a unique amino acid that can activate components of translation initiation and stimulate muscle protein synthesis. Citrulline is a non-essential amino acid that can stimulate nitric oxide production through enhancing arginine availability (16, 17), and has been shown to independently stimulate muscle protein synthesis in animal models (18-20). Despite a relatively large volume of information on the role of leucine in stimulating muscle protein synthesis in vitro, there is far less information available on the role of leucine in the

regulation of human muscle protein synthesis, particularly when coupled with resistance exercise. To date, there are no studies that have examined the role of citrulline as a nutrient regulator of human skeletal muscle protein synthesis at rest or following exercise. Studies examining the effect of leucine in the regulation of human muscle protein synthesis (chapter 2 and 3) were conducted in young healthy male subjects (18-35 y), while the role of citrulline was examined in a group of healthy older (65-80 y) male subjects. The aim of this chapter is to highlight the major findings from the studies outlined in chapters 2-4, discuss the implications and relevance of the results, and consider limitations of the work. Finally, suggestions for future avenues of research will be explored.

5.1 Study 1: Leucine vs. essential amino acid supplementation of a "suboptimal" protein dose: effects on myofibrillar protein synthesis and anabolic signaling

Smith and colleagues (21) provided some of the earliest work showing an anabolic effect of leucine on human skeletal muscle when they demonstrated that an intravenous flooding dose of leucine (~ 3.5 g total) resulted in a ~doubling of the rate of muscle protein synthesis in humans. However, flooding doses of other essential amino acids including valine, threonine, and phenylalanine were also reported to stimulate muscle protein synthesis (5), while non-essential amino acids (i.e. serine, alanine, arginine, and proline) could not (5). Therefore, Study 1 was designed to compare, in young subjects, the effects of supplementing a lower dose of protein (6.25 g), providing a quantity of essential amino acids shown to be suboptimal for the stimulation of muscle protein

synthesis at rest (22) and after exercise (23), with leucine or a mixture of essential amino acids (histidine, isoleucine, lysine, methionine, phenylalanine, threonine, valine) devoid of leucine, on myofibrillar protein synthesis rates. In studies 1-3, we included as a positive control treatment, a dose of protein shown to be maximally stimulatory for protein synthesis both at rest and post-exercise for younger (studies 1 and 2) and older (Study 3) subjects. Thus, in Study 1, the lower dose 'leucine spiked', and lower dose 'essential amino acid spiked' treatments were compared to a dose of protein (25 g), containing a quantity of essential amino acids sufficient to maximally stimulate muscle protein synthesis under resting (22) and post-exercise conditions (23). We hypothesized that if leucine was the (or a) key amino acid within a complete protein determining the amino acid mediated increase in muscle protein synthesis, then a smaller dose of protein supplemented with leucine may be as effective as a larger dose of protein (25 g) in stimulating myofibrillar protein synthesis. Alternatively, supplementing a low dose of protein with a complete mixture of essential amino acids but with no additional leucine (the 6.25 g protein dose contained ~0.75 g of leucine) would be less effective at stimulating myofibrillar protein synthesis due to a significantly lower leucine content (~0.75 g). It was demonstrated that under resting conditions (i.e. no resistance exercise), all treatments stimulated postprandial muscle protein synthesis roughly equivalently over the first 1-3 h, and returned basal postabsorptive values over 3-5 h. However, the response following an acute bout of resistance exercise was different in that although the early 1-3 h response of muscle protein synthesis was equivalent between treatments, only the treatment consisting of 25 g of whey protein was associated with a sustained elevation

in the rates of myofibrillar protein synthesis when assessed over 3-5 h. These findings demonstrate that supplementing a low (6.25 g) dose of protein with leucine or a mixture of essential amino acids without leucine can be as effective as ingesting 4 times the quantity of protein (25 g) at stimulating postprandial myofibrillar protein synthesis under resting conditions, but not following acute exercise.

Data in both humans (2) and animals (24) has suggested that postprandial hyperleucinemia serves as a key signal that directs the peak activation of muscle protein synthesis; however, the treatments in the current study resulted in a divergent response of blood leucine, yet there was an equivalent stimulation of muscle protein synthesis over 1-3 h. Thus, our results are in disagreement with the notion that the postprandial rise in blood leucine is the key determinant of the peak activation of muscle protein synthesis (2, 24), at least in young healthy subjects. Our findings suggest that if leucine is a unique regulator of muscle protein synthesis, a relatively small amount of leucine (0.75 g) may be a sufficient stimulus to at least "trigger" higher postprandial rates of muscle protein synthesis in young healthy subjects. In partial agreement with our hypothesis, reducing the concentration of leucine within a solution of mixed essential amino acids does not reduce the essential amino acid mediated stimulation of muscle protein synthesis in young subjects (25, 26). Therefore, although evidence supports a critical requirement for leucine in the amino acid mediated stimulation of muscle protein synthesis in the elderly (25, 27), this does not appear to be the case in younger subjects who appear to be far more 'leucine sensitive' than their older counterparts. Essential amino acids in addition to leucine (i.e. phenylalanine, valine, threonine) have been reported to possess the capacity to stimulate

human muscle protein synthesis (5). In addition, although leucine appears unique in its capacity to elicit phosphorylation of mTOR$^{(Ser2448)}$ and 4E-BP1$^{(Thr37/46)}$, and is the most potent in its capacity to enhance the phosphorylation of p70S6k$^{(Thr389)}$ and rpS6$^{(Ser235/236)}$, other essential amino acids can enhance signalling through p70S6k$^{(Thr389)}$ and rpS6 (6). In Study 1, the low leucine treatment (EAA-LEU) was supplemented with each individual essential amino acid except leucine to the level contained in 25 g whey protein. Therefore, supplementation with essential amino acids other than leucine may have contributed to the postprandial increase in myofibrillar protein synthesis over 1-3 h.

Although all treatments in Study 1 were effective at stimulating myofibrillar protein synthesis over the initial 1-3 h postprandial period, under both resting and post-exercise conditions, only the 25 g whey protein treatment was associated with a sustained elevation in myofibrillar protein synthesis over the later 3-5 h period; protein synthesis in both the leucine- and essential amino acid supplemented treatments declined to rates similar to the postabsorptive period. While the postprandial increase in muscle protein synthesis in response to a 'typical' protein containing meal is relatively transient, lasting ~2-3 h (28-30), resistance exercise mediated increases in muscle protein synthesis can last up to 48 h in untrained subjects (31). Given the relatively transient response of muscle protein synthesis to meal feeding, provision of a complete mixture of exogenous amino acid substrate may not be critical to maximally stimulate postprandial muscle protein synthesis under resting conditions. In support of this thesis, provision of the amino acid leucine stimulates muscle protein synthesis to a similar extent as feeding a complete protein when provided at rest (see (32)). Given that all amino acids are required for

polypeptide synthesis, the capacity of single amino acids to stimulate muscle protein synthesis must mean that amino acids are drawn from the aminoacyl tRNA pool. However, ingesting the full complement of amino acids may be important to sustain a more prolonged increase in muscle protein synthesis that occurs under the increased 'anabolic drive' of resistance exercise. Alternatively, there was a much more protracted response of blood leucine and the sum of essential amino acids following ingestion of 25 g whey protein which may have acted as a stimulus to sustain increased rates of myofibrillar protein synthesis (33) after resistance exercise. In addition to differences in the postprandial aminoacidemia, there were differences in p70S6k$^{(Thr389)}$ phosphorylation between treatments at 1 h, 3 h, and 5 h, but these changes in signaling did not match observed changes in myofibrillar protein synthesis. This finding supports results from previous studies showing that single point-in-time changes in signalling molecule phosphorylation are not always coincident with dynamic measures of muscle protein synthesis (34, 26, 35).

A potential limitation of Study 1 is that the nutritional treatments were not energy matched. Some evidence from animal studies suggests that cellular energy status is an important factor in determining the duration of postprandial muscle protein synthesis (36). Thus, since the 25 g whey protein treatment group had ~66% and ~50% more energy than the LEU and EAA-LEU groups respectively, it is difficult to know what impact, if any, this energy difference exerted on myofibrillar protein synthesis. Another factor worthy of consideration is the impact of free form crystalline amino acids when co-ingested with peptide-bound amino acids (i.e. protein). Free form amino acids are known

to be digested and absorbed more rapidly than peptide-bound amino acids (37) which could alter postprandial rates of protein synthesis (38-40). In addition, free form amino acids have been reported to be more susceptible to oxidative loss than peptide-bound amino acids (41), which might imply that a reduced proportion of amino acids would be available to support muscle protein synthesis when they are provided in free-form. Other research has demonstrated that whey protein stimulates greater rates of muscle protein synthesis than its constituent essential (42) and total amino acid content (43). Therefore, the results obtained using a combination of free-form and protein bound amino acids may be due to subtle differences in protein digestion/absorption and/or amino acid availability between free-form and peptide bound amino acids. Dardevet and colleagues (44) have suggested that the combination of free-form leucine and peptide-bound amino acid provision results in a "de-synchronization" in aminoacidemia whereby the free leucine signal appears rapidly, while the availability of other peptide-bound amino acids appears later due to delayed digestion and absorption. This lack of synchronization between the free leucine signal and peptide-bound amino acid availability, which they show would occur with ingestion of intact dietary proteins, has been suggested to explain why leucine supplementation is apparently ineffective at restoring muscle mass following atrophic stimuli in animals, whereas whey protein supplementation, which has a "synchronized" leucinemia and aminoacidemia due to all amino acids being peptide-bound, is effective at restoring muscle mass following atrophic stimuli (45).

The results from Study 1 show that leucine, or essential amino acid supplementation of a low dose (6.25 g) of whey protein can elicit a similar stimulation of

myofibrillar protein synthesis as that observed following ingestion of four times the quantity of whey protein (25 g) under resting conditions; however a 25 g dose of whey protein is better able to enhance myofibrillar protein synthesis during the post-exercise period. Since protein ingestion enhances the adaptive response to resistance exercise training (46), future longer-term studies are warranted to examine the potential for lower suboptimal doses of protein, with supplemental leucine, to enhance skeletal muscle mass when coupled with a program of resistance exercise training.

5.2 Study 2: Leucine and branched-chain amino acid supplementation of a low protein mixed macronutrient beverage: a double blind randomized trial

Study 2 built on the results from Study 1 by examining the effect of higher doses of leucine, and branched-chain amino acid (leucine, isoleucine, and valine) supplementation of a low dose (6.25 g) of protein ingested as part of a mixed macronutrient beverage on rates of myofibrillar protein synthesis under both resting and post-exercise conditions. Insulinemia in response to carbohydrate intake is generally considered to only be permissive, not stimulatory, for muscle protein synthesis (47) when amino acid availability is adequate (48). Nonetheless, it is not known whether increased carbohydrate-stimulated insulinemia might alter the effects of a suboptimal protein dose on muscle protein synthesis. Although previous research in animals has demonstrated that carbohydrate and leucine feeding can extend the duration of postprandial muscle protein synthesis (36), co-ingestion of protein with carbohydrate is associated with reduced postprandial blood amino acid concentrations (49, 50). Given this observation, higher

doses of leucine, in a mixed macronutrient beverage, may be required to increase plasma and intracellular leucine availability. As such, Study 2 was designed to investigate the addition of higher doses of leucine significantly greater (totaling 5.0 g) than those present in a maximally effective 20-25 g dose of high quality protein. In addition, to capture more of the "early" response of myofibrillar protein synthesis following treatment ingestion, we examined myofibrillar protein synthesis over 0-1.5 h and 1.5-4.5 h in the postprandial period under both resting and post-exercise conditions. In Study 1 we measured myofibrillar protein synthesis over 1-3 h and 3-5 h in the postprandial period, thus we may have missed an early divergent response in myofibrillar protein synthesis by waiting until 1 hour after treatment ingestion to begin our measurements. Thus, Study 2 was a double-blind randomized clinical trial investigating the effects of mixed macronutrient beverages containing a lower suboptimal dose of protein (6.25 g), that was one quarter of a maximally effective protein dose (25 g), supplemented with varying doses of leucine or branched chain amino acids. The amounts of leucine added provided a total leucine dose of 3.0 g, 5.0 g, and 5.0 g with additional isoleucine (1.35 g) and valine (1.34 g). As positive and negative controls, we compared the leucine and BCAA treatments to mixed macronutrient beverages containing 25 g protein and 6.25 g protein respectively, which were doses of protein containing a quantity of essential amino acids known to be sufficient (25 g protein) and suboptimal (6.25 g protein) for stimulating muscle protein synthesis in young subjects at rest and post-exercise (22, 23). In the resting condition, we hypothesized that all treatments would stimulate greater rates of myofibrillar protein synthesis than the negative control (6.25 g protein) but would not differ among each

other. During post-exercise recovery, we hypothesized that the high leucine (5.0 g) and BCAA treatments would be as effective as the positive control, but greater than the low leucine (3.0 g) treatment and negative control (0.75 g) due to a sustained increase in myofibrillar protein synthesis over the later 1.5-4.5 h period.

Our results demonstrated that within the context of mixed macronutrient ingestion, a suboptimal dose of protein (6.25 g) supplemented with a relatively large dose of leucine (5.0 g total) stimulated myofibrillar protein synthesis as effectively as 4 times as much protein (25 g). However, the same dose of protein (6.25 g) supplemented with a lower dose of leucine (3.0 g total) or a higher dose of leucine (to 5.0 g total) with additional isoleucine and valine (1.35 g and 1.34 g total respectively), was less effective. The greater rates of myofibrillar protein synthesis following a suboptimal dose of protein (6.25 g) supplemented with a relatively large dose of leucine (5.0 g total) were associated with a sustained elevation in the phosphorylation of $mTOR^{(Ser2448)}$ at 4.5 h. Leucine administration has previously been demonstrated to result in decreased concentrations of isoleucine, valine, and other amino acids in both the plasma and intracellular compartments (51-54). Several mechanisms could explain this observation including a leucine mediated stimulation of protein synthesis, a leucine mediated suppression of protein breakdown, changes in branched-chain amino acid transport resulting in altered amino acid uptake into circulation and possibly into the muscle, and increased branched chain amino acid oxidation (55). Previous work by Escobar and colleagues demonstrated that a relative hypoaminoacidemia following leucine provision was associated with a transient stimulation of muscle protein synthesis (56); however, if the

hypoaminoacidemia was prevented and amino acid levels maintained at postabsorptive concentrations via amino acid infusion, then the leucine mediated stimulation of muscle protein synthesis was sustained for a longer duration (57). We hypothesized that this decline in the branched-chain amino acids may be critical and that provision of these amino acids might be more effective than provision of leucine alone due to a more sustained elevation of myofibrillar protein synthesis over 1.5-4.5 h. In agreement with previous studies (51-53), we observed a greater negative area under the curve (concentration below basal postabsorptive values) for blood isoleucine and valine following both treatments in which 3.0 and 5.0 g of leucine was provided, although this was prevented when supplemental isoleucine and valine were consumed. Despite the decline in blood isoleucine and valine in the leucine 'spike' conditions, no treatment-dependent differences were observed in intracellular isoleucine or valine concentrations.

It is currently unclear whether changes in muscle protein synthesis are primary regulated by extracellular (58, 59) or intracellular (60, 61) amino acid availability. Kobayashi and colleagues (59) reported that a reduction in plasma amino acid availability to levels below normal postabsorptive concentrations (induced via haemodialysis) resulted in reduced rates of muscle protein synthesis in swine. Interestingly, the drop in protein synthesis paralleled the decline in plasma but not intracellular amino acids, which remained at relatively stable concentrations (59). Although protein synthesis could in theory be limited by amino acid availability, resulting in deficient charging of tRNA, charging of tRNA is not considered limiting for muscle protein synthesis under basal conditions (62). In addition, the K_m of aminoacyl tRNA synthetases are sufficiently low

that at most physiological concentrations of intracellular amino acids the enzyme is expected to be saturated (2). Therefore, although blood isoleucine and valine concentrations declined in both leucine supplemented treatments the fact there was no decline in their intracellular concentrations may explain why myofibrillar protein synthesis remained elevated over the later 1.5-4.5 h period.

Many of the limitations and points of consideration addressed in Study 1 are relevant when examining the result from Study 2. In Study 2 we, as in Study 1, utilized free-form amino acid supplementation of protein (peptide-bound). This once again raises questions as to the importance of differences in digestion and absorption between free and peptide-bound amino acids (37). Since protein digestion and absorption are known to be an important factor determining postprandial muscle protein synthesis (38-40), and the thesis that synchronized delivery of leucine is important for optimal stimulation of MPS, then mixtures of crystalline amino acids and intact protein may not be the best option. In addition, more of the leucine from the free leucine supplemented treatments may have been directed to oxidative loss than the peptide-bound leucine present in the positive control consisting of 25 g whey protein (41). The robust response following ingestion of 25 g whey protein may have related to more of the amino acids being available to support the synthesis of new muscle proteins. Therefore, as in Study 1, using a combination of free-form and protein bound amino acids may result in subtle differences between free and peptide-bound amino acids leading to amino acid oxidation (41), or differences in digestion/absorption (37, 45), creating a de-synchronization between the amino acid

signal and available substrate (44, 45). Another potential limitation of Study 2 relates to statistical power. The study was powered to detect differences between treatments in myofibrillar protein synthesis (FSR) between the positive and negative control groups, thus we may have been underpowered to detect important differences in other variables of interest including signalling molecule phosphorylation status. The time-points at which signalling molecule phosphorylation was examined were chosen secondary to our choice of biopsy sampling times for measures of myofibrillar protein synthesis. As a result, our time-course may not have been optimal to capture important differences in signalling molecule phosphorylation status that occur early after exercise (63).

Viewed as a whole, the results from Study 2 support a beneficial effect of leucine on myofibrillar protein synthesis although factors such as the dose of leucine administered and co-ingestion with other branched-chain amino acids are clearly important factors determining the capacity of leucine to enhance myofibrillar protein synthesis. Studies have demonstrated that metabolites of leucine including β-hydroxy-β-methylbutyrate (HMB) (32) and α-KIC (64) possess the capacity to stimulate muscle protein synthesis and future research will be required to define a possible synergistic contribution of these metabolites to the leucine mediated stimulation of muscle protein synthesis. From a practical perspective, it will also be important to assess outcomes such as changes in lean body mass and muscle function following long-term implementation smaller protein feedings supplemented with leucine.

5.3 Study 3: Citrulline versus non-essential amino acid supplementation of a low protein dose on myofibrillar protein synthesis and anabolic cell signaling in elderly men

Study 3 examined the impact of a lower dose of protein (15 g) supplemented with citrulline or a dose-matched mixture of non-essential amino acids on femoral artery blood flow, microvascular perfusion, myofibrillar protein synthesis, and signaling through the Akt-mTORC1 pathway in elderly men under both resting and post-exercise conditions. Previous work has demonstrated that 15 g of whey protein is suboptimal for the stimulation of muscle protein synthesis in the elderly under resting (65) and post-exercise conditions (66), with doses ranging from ~35-40 g demonstrating a greater response (65, 66). Citrulline is a non-protein amino acid (i.e. there is no codon for the addition of citrulline to growing peptide chains) that is a direct precursor for the synthesis of arginine that occurs in the kidneys via the enzymes argininosuccinate synthase and argininosuccinate lyase (67, 16). Synthesized arginine is then released into the renal vein and then to into systemic circulation (68). Arginine is the substrate for the enzyme nitric oxide synthase in the oxygen-dependent production of nitric oxide (NO); however, NO can also be produced through oxygen independent mechanisms that involve the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) and subsequent reduction to NO (69). As a ubiquitous signaling molecule, NO plays a critical role in endothelial function and contributes to the regulation of local and systemic vascular resistance and distribution of blood flow. The reason that we supplemented with citrulline as opposed to arginine is that, unlike arginine which undergoes substantial intestinal and hepatic metabolism to ornithine and urea by

arginase (70), citrulline is not metabolized in the intestine or liver, has a much higher (~10 fold) bioavailability in circulation than dose-matched arginine (71), and loss through urinary excretion is very low (< 5%) even at relatively high doses (71). In addition, arginine supplementation enhances arginase expression and activity thereby enhancing arginine catabolism (70), while citrulline does not increase arginase expression but actually reduces its activity (72). There is also previous research demonstrating that citrulline directly stimulates NO synthesis in humans more effectively than arginine (73). Thus, we hypothesized that ingestion of citrulline would enhance the provision of arginine to provide substrate for nitric oxide synthase and in doing so, enhance microvascular perfusion and concomitantly increase amino acid delivery, thereby stimulating increased rates of myofibrillar protein synthesis. We wished to test this thesis in elderly men for the main reason that they may have a reduced vasodilatory capacity in response to insulin (74, 75) and exercise (76). We compared citrulline ingestion with an equal dose of a mixture of non-essential amino acids (NEAA; alanine, arginine, aspartic acid, cystine, glutamine, glycine, proline, serine, and tryptophan), which lack the capacity to stimulate muscle protein synthesis (5, 77, 78). Citrulline and NEAA supplementation of a lower dose of protein (15 g) was compared to a 45 g dose of whey protein, which is likely a close to optimal dose of protein that we and others have previously shown to robustly stimulate muscle protein synthesis in the elderly (65, 66).

Previous work by Dillon and colleagues (79) demonstrated that amino acid provision in combination with the vasodilatory NO donor sodium nitroprusside (SNP), stimulated increased leg blood flow, muscle microvascular perfusion, and muscle protein

synthesis in elderly subjects such that their synthetic response was no different from that observed in young subjects. In the current study, citrulline supplementation increased plasma arginine availability, however, there were no treatment dependent differences in markers of NO metabolism including plasma nitrate and nitrite. El-Hattab and colleagues (73) demonstrated substantial increases in NO synthesis following citrulline administration (assessed using stable isotope tracer methodology) in the complete absence of increases in plasma nitrate/nitrite levels (73); thus, the lack of difference between our treatments in plasma nitrate and nitrite may not mean that citrulline failed to alter NO production. However, citrulline supplementation did not enhance femoral artery blood flow, or parameters of microvascular circulation under resting or post-exercise conditions as compared to treatments consisting of supplementation with a complete mixture of non-essential amino acids or a large 45 g bolus of whey protein.

Previous studies in animals have shown that citrulline is as effective as leucine in stimulating protein synthesis (19) and appears to enhance the phosphorylation of $p70S6k^{(Thr389)}$, $rpS6^{(Ser235/236-240/244)}$, or $4E\text{-}BP1^{(Ser65)}$ to the same extent as leucine (19). Similarly, in Study 3 we noted subtle differences in $p70S6k^{(Thr389)}$ and $rpS6^{(Ser240/244)}$ phosphorylation following citrulline administration as compared to non-essential amino acids, although a large 45 g bolus of whey protein generally resulted in the greatest signaling response. Citrulline may act as a unique signaling molecule (in a manner similar to leucine) with the capacity to stimulate muscle protein synthesis independent of increases in plasma arginine (17). In support of this thesis, a study using isolated muscles from malnourished rats reported increases in muscle protein synthesis and anabolic

signaling when incubated in citrulline, suggesting that citrulline itself and not arginine is responsible for stimulating protein synthesis. In fact, arginine provision does not stimulate human muscle protein synthesis (5, 80) or bulk blood flow under resting or post-exercise conditions (80). Therefore, citrulline itself, independent of arginine and/or increases in blood flow, may have induced enhanced signalling through the mTORC1 pathway.

We used a unilateral model of knee extensor resistance exercise to simultaneously derive measures of microvascular circulation at 90 minutes following treatment administration in both a resting and exercised leg. Recent work from Selkow and colleagues (81) reported that unilateral eccentric exercise of the plantar flexors (2 sets of 50 eccentric contractions) was associated with an increase in both microvascular blood volume and blood flow, with no change in blood flow velocity in the non-exercised contra-lateral limb when examined both immediately and 48 hours after exercise. Therefore, one possibility is that the unilateral knee-extensor exercise increased microvascular circulation in the contralateral limb, which might have masked, or at least reduced, the magnitude of an exercise versus non-exercised effect of the nutritional intervention. In addition, we only assessed microvascular circulation at a single time-point (90 minutes) during the post-exercise period, and as such, may have missed important treatment-dependent differences in microvascular circulation that occurred at other times. There are currently limits on the quantity of the Definity contrast agent (the only agent available for use in Canada) that can be administered to individuals. We elected to examine the response at 90 min since previous research reported that the Tmax

(time that maximum concentration is achieved) for blood arginine following ingestion of 10 g of citrulline occurred at ~90 minutes following ingestion (71). A more frequent assessment of muscle microvascular circulation would be necessary to more definitively assess the impact of citrulline on microvascular circulation. Another potential limitation relates to assessing the impact of citrulline when co-ingested with a low dose of protein, as opposed to examining the independent action of citrulline. In vitro data has demonstrated that the amino acid glutamine can antagonize the beneficial effects of citrulline on NO production (82), thus whether our strategy of adding citrulline to 15 g whey protein (containing ~2.5 g glutamine) reduced the potential of citrulline to enhance NO remains a possibility.

5.4 Overall conclusions

The overall theme of the studies that comprise this thesis has been to examine the potential to enhance the capacity of low, "suboptimal" doses of protein to stimulate myofibrillar protein synthesis through the addition of specific amino acids, including leucine and citrulline. This paradigm was examined using acute studies employing stable isotope tracer methodology in conjunction with a unilateral model of resistance exercise, thereby permitting examination of the independent effects of our given nutritional treatment under non-exercised resting conditions, and the combined effects of our nutritional treatment when coupled with the stimulus of resistance exercise. To gain some mechanistic insight into the observed changes in myofibrillar protein synthesis, Western blotting was performed in order to examine changes in the phosphorylation status of

select protein targets within the mTORC1 pathway. An important aspect of the studies conducted as part of this thesis was to identify amino acids that serve to regulate or amplify human muscle protein synthesis. From a practical perspective, supplementation of smaller "suboptimal" doses of protein is a concept that may be of particular relevance to the elderly since they require ingestion of higher doses of protein to optimize the stimulation of postprandial myofibrillar protein synthesis relative to their younger counterparts (**Figure 1**).

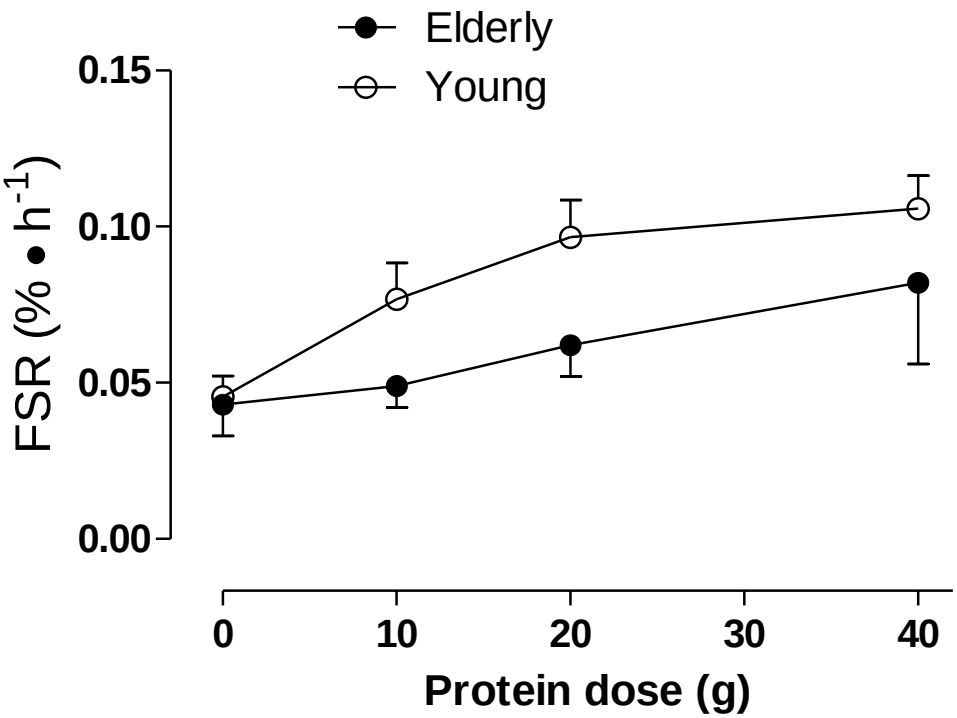

Figure 1. Comparison of protein dose response of myofibrillar protein synthesis per protein dose in young (23) and older subjects (66). Note that the proteins used were egg for young and whey for old which may favour an increased amplitude of response per g

of protein (due to leucine content) in the elderly. Also, values from (23) have been corrected from mixed to myofibrillar protein based on comparisons of standard differences observed in our lab.

For example, while muscle protein synthesis rates are maximally stimulated following ~20 g of dietary protein in the young (23), the elderly appear to require nearly double (~35-40 g) this amount of protein to achieve their peak muscle protein synthetic rate both at rest (65) and after resistance exercise (66). Food intake is generally reduced in the elderly (83) and consuming meals containing ~35-40 g of protein at every eating occasion may not be feasible. Therefore enhancing the capacity of smaller doses of protein to stimulate muscle protein synthesis through the addition of specific amino acids may be of great value to support increases in postprandial protein accretion. In addition, an important aspect of age related anabolic resistance is a relative insensitivity to the anabolic effects of smaller doses of dietary protein (84, 65, 66, 85, 86); rescuing the capacity of smaller doses of protein to stimulate muscle protein synthesis may be of considerable benefit to the elderly. Any benefits from leucine supplementation of smaller suboptimal doses of protein are likely to be enhanced when used within the context of exercise training. Resistance exercise is a strong stimulus to promote skeletal muscle hypertrophy, and protein supplementation has been sown to enhance the adaptive response to resistance exercise by promoting greater increases in both skeletal muscle mass and strength (87). To firmly establish the utility of leucine supplementation of smaller suboptimal doses of protein, long term investigations examining outcomes such as changes in skeletal muscle mass, strength, and function are necessary.

References

[1] Rennie, M. J., Wackerhage, H., Spangenburg, E. E., Booth, F. W. (2004) Control of the size of the human muscle mass. *Annu Rev Physiol* **66**, 799-828.

[2] Rennie, M. J., Bohe, J., Smith, K., Wackerhage, H., Greenhaff, P. (2006) Branched-chain amino acids as fuels and anabolic signals in human muscle. *J Nutr* **136**, 264S-268S.

[3] Atherton, P. J., Smith, K. (2012) Muscle protein synthesis in response to nutrition and exercise. *J Physiol* **590**, 1049-1057.

[4] Bennet, W. M., Connacher, A. A., Scrimgeour, C. M., Smith, K., Rennie, M. J. (1989) Increase in anterior tibialis muscle protein synthesis in healthy man during mixed amino acid infusion: studies of incorporation of [1-13C]leucine. *Clin Sci (Lond)* **76**, 447-454.

[5] Smith, K., Reynolds, N., Downie, S., Patel, A., Rennie, M. J. (1998) Effects of flooding amino acids on incorporation of labeled amino acids into human muscle protein. *Am J Physiol* **275**, E73-78.

[6] Atherton, P. J., Smith, K., Etheridge, T., Rankin, D., Rennie, M. J. (2010) Distinct anabolic signalling responses to amino acids in C2C12 skeletal muscle cells. *Amino Acids* **38**, 1533-1539.

[7] Wilkinson, S. B., Tarnopolsky, M. A., Macdonald, M. J., Macdonald, J. R., Armstrong, D., et al. (2007) Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage. *Am J Clin Nutr* **85**, 1031-1040.

[8] Tang, J. E., Moore, D. R., Kujbida, G. W., Tarnopolsky, M. A., Phillips, S. M. (2009) Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men. *J Appl Physiol* **107**, 987-992.

[9] Pennings, B., Boirie, Y., Senden, J. M., Gijsen, A. P., Kuipers, H., et al. (2011) Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate in older men. *Am J Clin Nutr* **93**, 997-1005.

[10] Buse, M. G., Reid, S. S. (1975) Leucine. A possible regulator of protein turnover in muscle. *J Clin Invest* **56**, 1250-1261.

[11] Fulks, R. M., Li, J. B., Goldberg, A. L. (1975) Effects of insulin, glucose, and amino acids on protein turnover in rat diaphragm. *J Biol Chem* **250**, 290-298.

[12] Anthony, J. C., Anthony, T. G., Kimball, S. R., Vary, T. C., Jefferson, L. S. (2000) Orally administered leucine stimulates protein synthesis in skeletal muscle of postabsorptive rats in association with increased eIF4F formation. *J Nutr* **130**, 139-145.

[13] Anthony, J. C., Yoshizawa, F., Anthony, T. G., Vary, T. C., Jefferson, L. S., et al. (2000) Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. *J Nutr* **130**, 2413-2419.

[14] Bolster, D. R., Vary, T. C., Kimball, S. R., Jefferson, L. S. (2004) Leucine regulates translation initiation in rat skeletal muscle via enhanced eIF4G phosphorylation. *J Nutr* **134**, 1704-1710.

[15] Crozier, S. J., Kimball, S. R., Emmert, S. W., Anthony, J. C., Jefferson, L. S. (2005) Oral leucine administration stimulates protein synthesis in rat skeletal muscle. *J Nutr* **135**, 376-382.

[16] Romero, M. J., Platt, D. H., Caldwell, R. B., Caldwell, R. W. (2006) Therapeutic use of citrulline in cardiovascular disease. *Cardiovasc Drug Rev* **24**, 275-290.

[17] Cynober, L., Moinard, C., De Bandt, J. P. (2010) The 2009 ESPEN Sir David Cuthbertson. Citrulline: a new major signaling molecule or just another player in the pharmaconutrition game? *Clin Nutr* **29**, 545-551.

[18] Osowska, S., Duchemann, T., Walrand, S., Paillard, A., Boirie, Y., et al. (2006) Citrulline modulates muscle protein metabolism in old malnourished rats. *Am J Physiol Endocrinol Metab* **291**, E582-586.

[19] Le Plenier, S., Walrand, S., Noirt, R., Cynober, L., Moinard, C. (2012) Effects of leucine and citrulline versus non-essential amino acids on muscle protein synthesis in fasted rat: a common activation pathway? *Amino Acids* **43**, 1171-1178.

[20] Ventura, G., Noirez, P., Breuille, D., Godin, J. P., Pinaud, S., et al. (2013) Effect of citrulline on muscle functions during moderate dietary restriction in healthy adult rats. *Amino Acids* **45**, 1123-1131.

[21] Smith, K., Barua, J. M., Watt, P. W., Scrimgeour, C. M., Rennie, M. J. (1992) Flooding with L-[1-13C]leucine stimulates human muscle protein incorporation of continuously infused L-[1-13C]valine. *Am J Physiol* **262**, E372-376.

[22] Cuthbertson, D., Smith, K., Babraj, J., Leese, G., Waddell, T., et al. (2005) Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. *FASEB J* **19**, 422-424.

[23] Moore, D. R., Robinson, M. J., Fry, J. L., Tang, J. E., Glover, E. I., et al. (2009) Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *Am J Clin Nutr* **89**, 161-168.

[24] Norton, L. E., Layman, D. K., Bunpo, P., Anthony, T. G., Brana, D. V., et al. (2009) The leucine content of a complete meal directs peak activation but not duration of skeletal muscle protein synthesis and mammalian target of rapamycin signaling in rats. *J Nutr* **139**, 1103-1109.

[25] Katsanos, C. S., Kobayashi, H., Sheffield-Moore, M., Aarsland, A., Wolfe, R. R. (2006) A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly. *Am J Physiol Endocrinol Metab* **291**, E381-387.

[26] Glynn, E. L., Fry, C. S., Drummond, M. J., Timmerman, K. L., Dhanani, S., et al. (2010) Excess leucine intake enhances muscle anabolic signaling but not net protein anabolism in young men and women. *J Nutr* **140**, 1970-1976.

[27] Rieu, I., Balage, M., Sornet, C., Giraudet, C., Pujos, E., et al. (2006) Leucine supplementation improves muscle protein synthesis in elderly men independently of hyperaminoacidaemia. *J Physiol* **575**, 305-315.

[28] Bohe, J., Low, J. F., Wolfe, R. R., Rennie, M. J. (2001) Latency and duration of stimulation of human muscle protein synthesis during continuous infusion of amino acids. *J Physiol* **532**, 575-579.

[29] Moore, D. R., Tang, J. E., Burd, N. A., Rerecich, T., Tarnopolsky, M. A., et al. (2009) Differential stimulation of myofibrillar and sarcoplasmic protein synthesis with protein ingestion at rest and after resistance exercise. *J Physiol* **587**, 897-904.

[30] Atherton, P. J., Etheridge, T., Watt, P. W., Wilkinson, D., Selby, A., et al. (2010) Muscle full effect after oral protein: time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling. *Am J Clin Nutr* **92**, 1080-1088.

[31] Phillips, S. M., Tipton, K. D., Aarsland, A., Wolf, S. E., Wolfe, R. R. (1997) Mixed muscle protein synthesis and breakdown after resistance exercise in humans. *Am J Physiol* **273**, E99-107.

[32] Wilkinson, D. J., Hossain, T., Hill, D. S., Phillips, B. E., Crossland, H., et al. (2013) Effects of leucine and its metabolite beta-hydroxy-beta-methylbutyrate on human skeletal muscle protein metabolism. *J Physiol* **591**, 2911-2923.

[33] Soop, M., Nehra, V., Henderson, G. C., Boirie, Y., Ford, G. C., et al. (2012) Coingestion of whey protein and casein in a mixed meal: demonstration of a more sustained anabolic effect of casein. *Am J Physiol Endocrinol Metab* **303**, E152-162.

[34] Greenhaff, P. L., Karagounis, L. G., Peirce, N., Simpson, E. J., Hazell, M., et al. (2008) Disassociation between the effects of amino acids and insulin on signaling, ubiquitin ligases, and protein turnover in human muscle. *Am J Physiol Endocrinol Metab* **295**, E595-604.

[35] Reidy, P. T., Walker, D. K., Dickinson, J. M., Gundermann, D. M., Drummond, M. J., et al. (2013) Protein blend ingestion following resistance exercise promotes human muscle protein synthesis. *J Nutr* **143**, 410-416.

[36] Wilson, G. J., Layman, D. K., Moulton, C. J., Norton, L. E., Anthony, T. G., et al. (2011) Leucine or carbohydrate supplementation reduces AMPK and eEF2 phosphorylation and extends postprandial muscle protein synthesis in rats. *Am J Physiol Endocrinol Metab* **301**, E1236-1242.

[37] Burd, N. A., West, D. W., Rerecich, T., Prior, T., Baker, S. K., et al. (2011) Validation of a single biopsy approach and bolus protein feeding to determine myofibrillar protein synthesis in stable isotope tracer studies in humans. *Nutr Metab (Lond)* **8**, 15.

[38] Boirie, Y., Dangin, M., Gachon, P., Vasson, M. P., Maubois, J. L., et al. (1997) Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc Natl Acad Sci U S A* **94**, 14930-14935.

[39] Dangin, M., Boirie, Y., Garcia-Rodenas, C., Gachon, P., Fauquant, J., et al. (2001) The digestion rate of protein is an independent regulating factor of postprandial protein retention. *Am J Physiol Endocrinol Metab* **280**, E340-348.

[40] Dangin, M., Boirie, Y., Guillet, C., Beaufrere, B. (2002) Influence of the protein digestion rate on protein turnover in young and elderly subjects. *J Nutr* **132**, 3228S-3233S.

[41] Nolles, J. A., Verreijen, A. M., Koopmanschap, R. E., Verstegen, M. W., Schreurs, V. V. (2009) Postprandial oxidative losses of free and protein-bound amino acids

in the diet: interactions and adaptation. *J Anim Physiol Anim Nutr (Berl)* **93**, 431-438.

[42] Katsanos, C. S., Chinkes, D. L., Paddon-Jones, D., Zhang, X. J., Aarsland, A., et al. (2008) Whey protein ingestion in elderly persons results in greater muscle protein accrual than ingestion of its constituent essential amino acid content. *Nutr Res* **28**, 651-658.

[43] Kanda, A., Nakayama, K., Fukasawa, T., Koga, J., Kanegae, M., et al. (2013) Post-exercise whey protein hydrolysate supplementation induces a greater increase in muscle protein synthesis than its constituent amino acid content. *Br J Nutr* **110**, 981-987.

[44] Dardevet, D., Remond, D., Peyron, M. A., Papet, I., Savary-Auzeloux, I., et al. (2012) Muscle wasting and resistance of muscle anabolism: the "anabolic threshold concept" for adapted nutritional strategies during sarcopenia. *ScientificWorldJournal* **2012**, 269531.

[45] Magne, H., Savary-Auzeloux, I., Migne, C., Peyron, M. A., Combaret, L., et al. (2012) Contrarily to whey and high protein diets, dietary free leucine supplementation cannot reverse the lack of recovery of muscle mass after prolonged immobilization during ageing. *J Physiol* **590**, 2035-2049.

[46] Cermak, N. M., Res, P. T., de Groot, L. C., Saris, W. H., van Loon, L. J. (2012) Protein supplementation augments the adaptive response of skeletal muscle to resistance-type exercise training: a meta-analysis. *Am J Clin Nutr*.

[47] Phillips, S. M. (2008) Insulin and muscle protein turnover in humans: stimulatory, permissive, inhibitory, or all of the above? *Am J Physiol Endocrinol Metab* **295**, E731.

[48] Staples, A. W., Burd, N. A., West, D. W., Currie, K. D., Atherton, P. J., et al. (2011) Carbohydrate does not augment exercise-induced protein accretion versus protein alone. *Med Sci Sports Exerc* **43**, 1154-1161.

[49] West, D. W., Burd, N. A., Tang, J. E., Moore, D. R., Staples, A. W., et al. (2010) Elevations in ostensibly anabolic hormones with resistance exercise enhance neither training-induced muscle hypertrophy nor strength of the elbow flexors. *J Appl Physiol* **108**, 60-67.

[50] Hamer, H. M., Wall, B. T., Kiskini, A., de Lange, A., Groen, B. B., et al. (2013) Carbohydrate co-ingestion with protein does not further augment post-prandial muscle protein accretion in older men. *Nutr Metab (Lond)* **10**, 15.

[51] Swendseid, M. E., Villalobos, J., Figueroa, W. S., Drenick, E. J. (1965) The effects of test doses of leucine, isoleucine or valine on plasma amino acid levels. The unique effect of leucine. *Am J Clin Nutr* **17**, 317-321.

[52] Hagenfeldt, L., Eriksson, S., Wahren, J. (1980) Influence of leucine on arterial concentrations and regional exchange of amino acids in healthy subjects. *Clin Sci (Lond)* **59**, 173-181.

[53] Eriksson, S., Hagenfeldt, L., Wahren, J. (1981) A comparison of the effects of intravenous infusion of individual branched-chain amino acids on blood amino acid levels in man. *Clin Sci (Lond)* **60**, 95-100.

[54] Alvestrand, A., Hagenfeldt, L., Merli, M., Oureshi, A., Eriksson, L. S. (1990) Influence of leucine infusion on intracellular amino acids in humans. *Eur J Clin Invest* **20**, 293-298.

[55] Harper, A. E., Miller, R. H., Block, K. P. (1984) Branched-chain amino acid metabolism. *Annu Rev Nutr* **4**, 409-454.

[56] Escobar, J., Frank, J. W., Suryawan, A., Nguyen, H. V., Kimball, S. R., et al. (2005) Physiological rise in plasma leucine stimulates muscle protein synthesis in neonatal pigs by enhancing translation initiation factor activation. *Am J Physiol Endocrinol Metab* **288**, E914-921.

[57] Escobar, J., Frank, J. W., Suryawan, A., Nguyen, H. V., Davis, T. A. (2007) Amino acid availability and age affect the leucine stimulation of protein synthesis and eIF4F formation in muscle. *Am J Physiol Endocrinol Metab* **293**, E1615-1621.

[58] Bohe, J., Low, A., Wolfe, R. R., Rennie, M. J. (2003) Human muscle protein synthesis is modulated by extracellular, not intramuscular amino acid availability: a dose-response study. *J Physiol* **552**, 315-324.

[59] Kobayashi, H., Borsheim, E., Anthony, T. G., Traber, D. L., Badalamenti, J., et al. (2003) Reduced amino acid availability inhibits muscle protein synthesis and decreases activity of initiation factor eIF2B. *Am J Physiol Endocrinol Metab* **284**, E488-498.

[60] Zoncu, R., Bar-Peled, L., Efeyan, A., Wang, S., Sancak, Y., et al. (2011) mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. *Science* **334**, 678-683.

[61] Han, J. M., Jeong, S. J., Park, M. C., Kim, G., Kwon, N. H., et al. (2012) Leucyl-tRNA synthetase is an intracellular leucine sensor for the mTORC1-signaling pathway. *Cell* **149**, 410-424.

[62] Ojamaa, K. M., Kimball, S. R., Jefferson, L. S. (1993) Aminoacylation of initiator methionyl-tRNA(i) under conditions inhibitory to initiation of protein synthesis. *Am J Physiol* **264**, E257-263.

[63] Camera, D. M., Edge, J., Short, M. J., Hawley, J. A., Coffey, V. G. (2010) Early time course of Akt phosphorylation after endurance and resistance exercise. *Med Sci Sports Exerc* **42**, 1843-1852.

[64] Escobar, J., Frank, J. W., Suryawan, A., Nguyen, H. V., Van Horn, C. G., et al. (2010) Leucine and alpha-ketoisocaproic acid, but not norleucine, stimulate skeletal muscle protein synthesis in neonatal pigs. *J Nutr* **140**, 1418-1424.

[65] Pennings, B., Groen, B. B., de Lange, A., Gijsen, A. P., Zorenc, A. H., et al. (2012) Amino acid absorption and subsequent muscle protein accretion following graded intakes of whey protein in elderly men. *Am J Physiol Endocrinol Metab*.

[66] Yang, Y., Breen, L., Burd, N. A., Hector, A. J., Churchward-Venne, T. A., et al. (2012) Resistance exercise enhances myofibrillar protein synthesis with graded intakes of whey protein in older men. *Br J Nutr* 1-9.

[67] Curis, E., Nicolis, I., Moinard, C., Osowska, S., Zerrouk, N., et al. (2005) Almost all about citrulline in mammals. *Amino Acids* **29**, 177-205.

[68] van de Poll, M. C., Soeters, P. B., Deutz, N. E., Fearon, K. C., Dejong, C. H. (2004) Renal metabolism of amino acids: its role in interorgan amino acid exchange. *Am J Clin Nutr* **79**, 185-197.

[69] Lundberg, J. O., Gladwin, M. T., Ahluwalia, A., Benjamin, N., Bryan, N. S., et al. (2009) Nitrate and nitrite in biology, nutrition and therapeutics. *Nat Chem Biol* **5**, 865-869.

[70] Morris, S. M., Jr. (1992) Regulation of enzymes of urea and arginine synthesis. *Annu Rev Nutr* **12**, 81-101.

[71] Moinard, C., Nicolis, I., Neveux, N., Darquy, S., Benazeth, S., et al. (2008) Dose-ranging effects of citrulline administration on plasma amino acids and hormonal patterns in healthy subjects: the Citrudose pharmacokinetic study. *Br J Nutr* **99**, 855-862.

[72] Shearer, J. D., Richards, J. R., Mills, C. D., Caldwell, M. D. (1997) Differential regulation of macrophage arginine metabolism: a proposed role in wound healing. *Am J Physiol* **272**, E181-190.

[73] El-Hattab, A. W., Hsu, J. W., Emrick, L. T., Wong, L. J., Craigen, W. J., et al. (2012) Restoration of impaired nitric oxide production in MELAS syndrome with citrulline and arginine supplementation. *Mol Genet Metab* **105**, 607-614.

[74] Timmerman, K. L., Lee, J. L., Dreyer, H. C., Dhanani, S., Glynn, E. L., et al. (2010) Insulin stimulates human skeletal muscle protein synthesis via an indirect mechanism involving endothelial-dependent vasodilation and mammalian target of rapamycin complex 1 signaling. *J Clin Endocrinol Metab* **95**, 3848-3857.

[75] Timmerman, K. L., Lee, J. L., Fujita, S., Dhanani, S., Dreyer, H. C., et al. (2010) Pharmacological vasodilation improves insulin-stimulated muscle protein anabolism but not glucose utilization in older adults. *Diabetes* **59**, 2764-2771.

[76] Durham, W. J., Casperson, S. L., Dillon, E. L., Keske, M. A., Paddon-Jones, D., et al. (2010) Age-related anabolic resistance after endurance-type exercise in healthy humans. *FASEB J* **24**, 4117-4127.

[77] Tipton, K. D., Gurkin, B. E., Matin, S., Wolfe, R. R. (1999) Nonessential amino acids are not necessary to stimulate net muscle protein synthesis in healthy volunteers. *J Nutr Biochem* **10**, 89-95.

[78] Volpi, E., Kobayashi, H., Sheffield-Moore, M., Mittendorfer, B., Wolfe, R. R. (2003) Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults. *Am J Clin Nutr* **78**, 250-258.

[79] Dillon, E. L., Casperson, S. L., Durham, W. J., Randolph, K. M., Urban, R. J., et al. (2011) Muscle protein metabolism responds similarly to exogenous amino acids in healthy younger and older adults during NO-induced hyperemia. *Am J Physiol Regul Integr Comp Physiol* **301**, R1408-1417.

[80] Tang, J. E., Lysecki, P. J., Manolakos, J. J., MacDonald, M. J., Tarnopolsky, M. A., et al. (2011) Bolus arginine supplementation affects neither muscle blood flow nor muscle protein synthesis in young men at rest or after resistance exercise. *J Nutr* **141**, 195-200.

[81] Selkow, N. M., Herman, D. C., liu, Z., Hertel, J., Hart, J. M., et al. (2013) Microvascular Perfusion Changes in the Contralateral Gastrocnemius Following Unilateral Eccentric Exercise. *J Nov Physiother* **3**, 163.

[82] Bryk, J., Ochoa, J. B., Correia, M. I., Munera-Seeley, V., Popovic, P. J. (2008) Effect of citrulline and glutamine on nitric oxide production in RAW 264.7 cells in an arginine-depleted environment. *JPEN J Parenter Enteral Nutr* **32**, 377-383.

[83] Tieland, M., Borgonjen-Van den Berg, K. J., van Loon, L. J., de Groot, L. C. (2012) Dietary protein intake in community-dwelling, frail, and institutionalized elderly people: scope for improvement. *Eur J Nutr* **51**, 173-179.

[84] Katsanos, C. S., Kobayashi, H., Sheffield-Moore, M., Aarsland, A., Wolfe, R. R. (2005) Aging is associated with diminished accretion of muscle proteins after the ingestion of a small bolus of essential amino acids. *Am J Clin Nutr* **82**, 1065-1073.

[85] Yang, Y., Churchward-Venne, T. A., Burd, N. A., Breen, L., Tarnopolsky, M. A., et al. (2012) Myofibrillar protein synthesis following ingestion of soy protein isolate at rest and after resistance exercise in elderly men. *Nutr Metab (Lond)* **9**, 57.

[86] Robinson, M. J., Burd, N. A., Breen, L., Rerecich, T., Yang, Y., et al. (2013) Dose-dependent responses of myofibrillar protein synthesis with beef ingestion are enhanced with resistance exercise in middle-aged men. *Appl Physiol Nutr Metab* **38**, 120-125.

[87] Cermak, N. M., Res, P. T., de Groot, L. C., Saris, W. H., van Loon, L. J. (2012) Protein supplementation augments the adaptive response of skeletal muscle to resistance-type exercise training: a meta-analysis. *Am J Clin Nutr* **96**, 1454-1464.