Endocrine responses and acute mTOR pathway phosphorylation to resistance exercise with leucine and whey

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ABSTRACT: Leucine ingestion reportedly activates the mTOR pathway in skeletal muscle, contributing to a hypertrophy response. The purpose of the study was to compare the post-resistance exercise effects of leucine and whey protein supplementation on endocrine responses and muscle mTOR pathway phosphorylation. On visit 1, subjects (X±SD; n=20; age=27.8±2.8yrs) provided baseline blood samples for analysis of cortisol, glucose and insulin; a muscle biopsy of the vastus lateralis muscle to assess mTOR signaling pathway phosphorylation; and were tested for maximum strength on the leg press and leg extension exercises. For visits 2 and 3, subjects were randomized in a double-blind crossover design to ingest either leucine and whey protein (10g+10g; supplement) or a non-caloric placebo. During these visits, 5 sets of 10 repetitions were performed on both exercises, immediately followed by ingestion of the supplement or placebo. Blood was sampled 30 min post-, and a muscle biopsy 45 min post-exercise. Western blots quantified total and phosphorylated proteins. Insulin increased ($\alpha<.05$) with supplementation with no change in glucose compared to placebo. Relative phosphorylation of AKT and rpS6 were greater with leucine and whey supplementation compared to placebo. Supplementation of leucine and whey protein immediately after heavy resistance exercise increases anabolic signaling in human skeletal muscle.

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INTRODUCTION

The goal of muscle hypertrophy can be attained through proper diet and appropriate exercise. Muscular contractions induced by moderate to intense resistance training have been shown to increase anabolic muscle signaling (P70s6k, mTOR), and when performed chronically, often leads to muscle hypertrophy [1]. With the use of dietary supplementation (protein) it is possible to increase the rate of muscle hypertrophy [30,31]. Branched chain amino acids (BCAA) supplementation has been shown to increase muscle protein anabolism [4], with leucine, a component of whey protein, being the most anabolic BCAA [35].

Leucine is one of the essential amino acids in the diet. It is capable, like all branched chain amino acids, of avoiding hepatic alterations due to the lack of L-branched chain aminotransferase in the liver [22]. Leucine enters muscle cells through the L type amino acid transporters [19,14], where it helps activate, via phosphorylation, mTOR by binding to nutrient sensitive molecules such as hVps34 and leucyl-tRNA synthetase [15]. Once this has occurred, mTOR then phosphorylates P70s6k and 4E-BP1. P70s6k in turn phosphorylates rpS6 which is a ribosomal protein specific to anabolic protein synthesis in myotubes. When 4E-BP1 is phosphorylated, it releases EIF4 which can interact with mRNA transcription. This activation in turn causes an anabolic response by activating RNA translation factors which leads to increased protein synthesis [21]. Increased activation of this pathway has been shown to lead to greater hypertrophy over time [1].

Leucine also stimulates insulin release from pancreatic beta cells by activating glutamate dehydrogenase (GDH) and AKT, which in turn lead to insulin release [33]. Leucine itself is associated with anabolic signaling in skeletal muscle [5] by interacting with IGF receptors that activate PI3K and thereby activates AKT in the muscle. Once AKT is activated it can also cause the phosphorylation of mTOR [26]. Furthermore, resistance training and leucine supplementation can possibly increase the release of insulin-like growth factor 1 (IGF1) in the body [12,24].

With resistance training alone there is a transient increase in muscle protein anabolism markers [30], but with protein supplementation (often whey) this activation is further increased [9, 17]. However, the amount of activation that occurs due to supplementation, specifically leucine, has not been well studied in humans, and there have been conflicting results on the amount of signaling that occurs. In general, it seems to have an additive effect, and it is relatively easy to saturate the signaling pathway via leucine supplementation [17]. In the rat model, saturation occurs at 0.675g of leucine per kg of bodyweight when applied as a bolus [34]. However, in humans, the exact amount has yet to be established, but suggestions for transient saturation are single doses of at least 1.8 grams, but this has not been fully investigated [13]. Furthermore, there is a lack of research showing a large training stimulus effect on muscle protein phosphorylation in conjunction with protein supplementation.

The purpose of this study was to analyze the effects of leucine-enriched whey protein supplementation on muscle anabolic signaling protein phosphorylation in healthy, resistance-trained males. Additionally, differences in insulin concentrations between leucine-enriched whey protein supplementation and a low-carbohydrate placebo was assessed.

MATERIALS AND METHODS

Subjects

Twenty healthy, recreationally resistance-trained men (2-10 hrs·wk^{-1}) who were not consuming any nutritional supplements or prescription drugs (mean±SD; age = 27.8±2.8 years, height = 1.78±0.07 m, weight = 81.3±11.0 kg) provided informed consent as approved by the institution's Human Subjects Committee to participate in this study.

Study Design

A randomized cross-over study design was conducted that involved three visits [13,16] and replicated previous research on muscle signaling pathways [17]. Visit 1 included baseline data collection, while visits 2 (3-7 d later) and 3 (5-9 days after visit 2) were experimental visits where either the supplement or placebo was randomly administered. The time of day for all visits was held constant (0900 – 1300 hrs) for each subject to avoid diurnal hormonal variations [31].

Baseline Session

Following a 12 hr overnight fast, both a blood sample and muscle biopsy were collected, followed by a 10 repetition maximum (10 RM) test on a 45° plate loaded leg press machine and a selectorized leg extension machine [2].

Experimental Sessions

For both sessions, subjects arrived fasted, confirmed abstention from physical training for the previous 48 hours, and performed 5 sets of 10 repetitions at their previously tested 10 RM for both the leg press and leg extension exercises, with 2-minute inter-set rest intervals. If a subject failed to achieve all 10 repetitions, the load was decreased by 4.5 kg for the following set. Immediately post-exercise, in randomized order, subjects ingested the supplement or placebo in 236.5 ml (8 ounces) of fluid. Blood samples were collected 30-45 minutes post-ingestion and muscle biopsies were taken 45-60 minutes post ingestion.

Supplement and placebo composition

The whey protein (10 g) plus leucine (10 g) supplement included 2 g of carbohydrates, and no other nutritive additives. The placebo supplement contained 4 g of carbohydrates and no other nutritive additives. Both of these compounds were independently tested for purity and composition validation (Covance Laboratories Inc., Madison, WI).

Blood Sampling and Analyses

Blood samples from an antecubital vein were collected in 10 mL vacutainers with appropriate additives (no additives [serum] or sodium fluoride/potassium oxalate). Serum aliquots were frozen at -80°C until assayed in duplicate with enzyme-linked immunosorbent assay (ELISA) kits for insulin, cortisol (Alpco Diagnostics, Salem, NH), and glucose (Arbor Assays, Ann Arbor, MI). Respective intra- and inter-assay variances were cortisol (CV=3.1% & 5.5%), insulin (CV=4.3% & 2.3%), and glucose (CV=4.7% & 4.6%).

Muscle Biopsies

Biopsies, using a Bergstrom needle (Bignel Surgical Inc., Essex, United Kingdom) with the double-chop technique [27] and suction [11], were obtained from the mid-belly of the vastus lateralis muscle, with subsequent biopsies 2-3 cm superior or inferior to the initial biopsy site. Samples were immediately frozen in liquid nitrogen (-159° C) and stored in a liquid nitrogen cooled storage tank until analyzed.

Muscle Protein Analysis

Portions of the muscle samples weighing 15.8±6.0 mg were extracted (10 μL·mg tissue^{-1}; 44 mL Tris HCl, 4 mL glycerol, 2.5 mL β-mercaptoethanol, 368 mM SDS), with protease and phosphatase inhibitors and PMSF (phenylmethanesulfonyl fluoride; Halt Inhibitor Cocktail, Thermo Scientific Inc., Rockford, IL) were added to the extraction buffer 1:100. Samples were homogenized for 30s x 30,000 RPM in glass test tubes, and aliquoted into two vials for each analysis. Prior to analysis, the vials were centrifuged at 16,000 g for 20 minutes at 4°C, with the supernatant collected for western blot analysis. Protein concentrations were assayed using a micro Lowry method with Peterson's modification (Sigma Aldrich, Saint Louis, MO). Proteins were separated via SDS-PAGE using 4-15% gradient gels at a constant current of 0.05 A, and then transferred to PVDF membranes.

Infra-red Labelled Secondary Antibodies

Primary monoclonal antibodies for both total and phosphorylated signaling proteins were applied for mTOR and AKT (R&D Systems, Minneapolis, MN), and for P70s6k, 4E-BP1, and rpS6 (Cell Signaling, Danvers, MA). Phospho-specific anti-rabbit secondary antibodies for mTOR (S2448) and AKT (S473, S474, S472), and anti-mouse (AKT) or anti-goat (mTOR) antibodies for total protein (Rockland, Gilbertsville PA) were used prior to imaging with an infra-red detection system (Li-Cor Biosciences Inc., Lincoln, NE). Both total and phosphorylated protein infrared light concentrations were quantified at the same time since the secondary antibodies for total (700 nm) and phosphorylated (800 nm) proteins were visible at different wavelengths.

Chemiluminescent Secondary Antibodies

Phospho-specific anti-rabbit primary antibodies for P70s6k (Thr 421, S424), rpS6 (S235, S236), and 4E-BP1 (S65) (Cell Signaling, Danvers, MA) were visualized using chemiluminescent-labelled secondary antibodies (Amersham ECL Prime Western Blot Detector, GE Healthcare and Life Sciences, Pittsburgh, PA), and imaged with a Fluorchem SP system (Protein Simple Inc. Santa Clara, CA). Blots were then stripped for 20 minutes (glycine 15g, SDS 1g, Tween 20 10mL, QS ultra-pure water 1L, pH 2.2) before being reimaged to verify removal of all secondary and primary antibodies. Blots were then reprobed for total content of P70s6k, rps6, and 4E-BP1 (Cell Signaling, Danvers, MA).

Western Blot Quantification

All samples for each subject were analyzed in the same blot, and all bands were quantified 3 times per individual with the mean value utilized for statistical analyses. The phosphorylated to total protein signal ratio was normalized to baseline for statistical comparisons. Due to insufficient samples for some subjects, analysis of protein was only performed on 17 subjects. Examples of representative protein luminescence and infrared concentration images are shown in each protein figure (Figures 1-5).

Dietary Records

Dietary intake was analyzed for the day prior to each visit using Diet Analysis Plus software, Version 10 (Cengage Learning, Inc. 2012). All analyses were performed by the same individual.

Statistical Analysis

Individual protein phosphorylation ratios (phosphorylated:total protein; arbitrary units) were normalized to a value of "1" for each subject's baseline measurement. Supplement and placebo condition ratios were reported relative to each subject's baseline ratio. All descriptive and performance data (X±SD) for the exercise sessions, as well as the ratios of phosphorylation to total protein of the signaling pathway proteins, and the blood variables were compared using one-factor repeated measures analyses of variance (ANOVAs) with least significant differences (LSD) post hoc analysis to determine differences (p<0.05).

RESULTS

Performance Data

Mean load on the leg press during exercise was 223.9±47.4 kg, with a decrease of 7.1% over the course of the 5 sets. Mean load

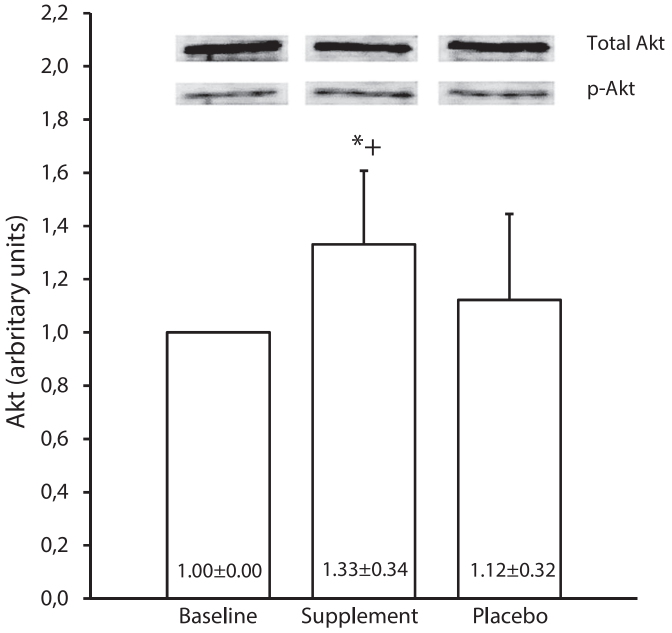

Fig. 1. Akt phosphorylated to total protein ratio normalized values to baseline session values (X±SD). There were significantly greater levels of phosphorylation for the supplement condition over both the placebo (+) and baseline visit (*) (p<.05).

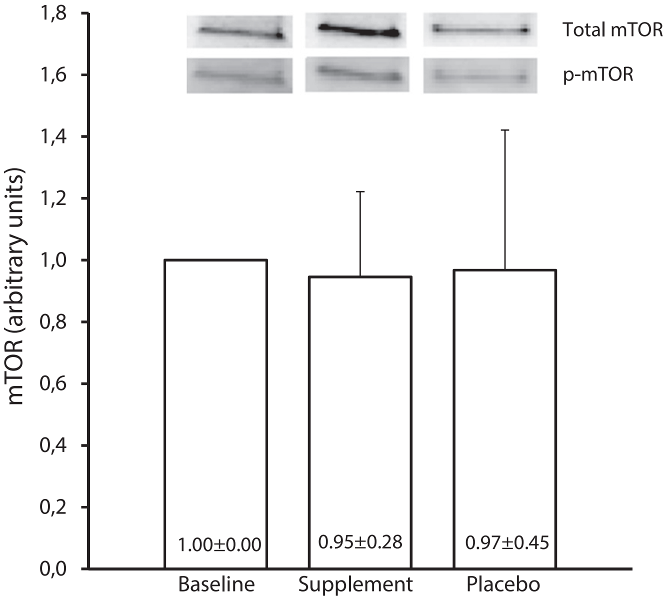

Fig. 2. mTOR phosphorylated to total protein ratio normalized values to baseline session values (X±SD). There was no significant change in levels of phosphorylation between any visit (p>.05).

on the leg extension machine was 67.5±9.6 kg with a decrease of 17% over the course of the 5 sets. Overall, this gave an average ratio body-weight-to-leg-press-weight of 2.76, and for the leg extension exercise this ratio was 0.83. The total amount of work for both lifting sessions, as measured by volume-load (repetitions x weight), was 10561±927 kg for the leg press and 2975±119 kg for the leg extension. There was no significant difference in weight lifted or relative fatigue for either testing condition for both exercises (*p*=0.99, 0.17 respectively). Relative fatigue was measured by the initial load on the movement compared to the final load performed in a given exercise.

Blood Analyses

Supplementation following resistance exercise increased insulin concentrations significantly over the other conditions (F [2, 18] = 24.139; *p*<0.001) (*p*<0.001 to baseline and placebo conditions), whereas the placebo condition exhibited no significant response over the baseline measurement (p=0.716). Neither glucose (F [2, 18] = 1.534, *p*=0.243) nor cortisol (F [2, 18] = 0.610, *p*=0.554) concentrations changed for the supplemented or placebo conditions (Table 1).

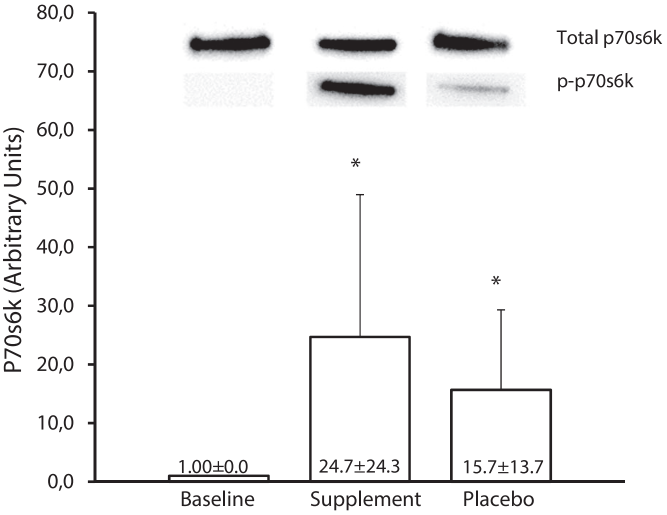

Fig. 3. p70s6k phosphorylated to total protein ratio normalized values to baseline session values (X±SD). Both the supplement and placebo conditions were significantly greater than the baseline session (*) (p<.05).

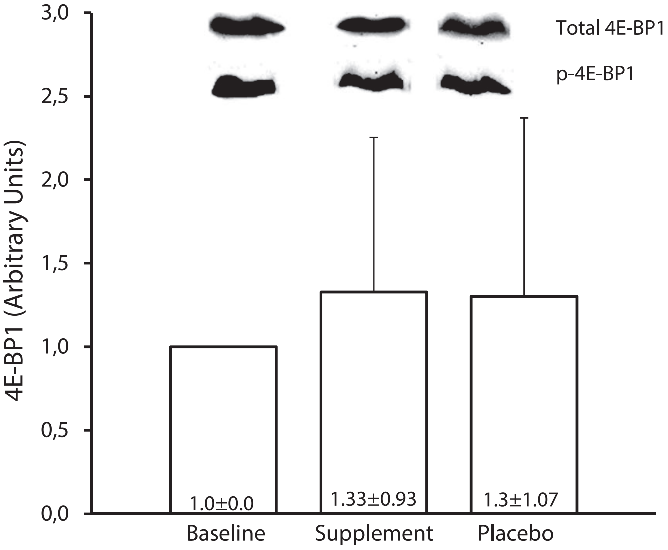

Fig. 4. 4E-BP1 phosphorylated to total protein ratio normalized values to baseline session values (X±SD). There was no significant change in levels of phosphorylation between any visit (p>.05).

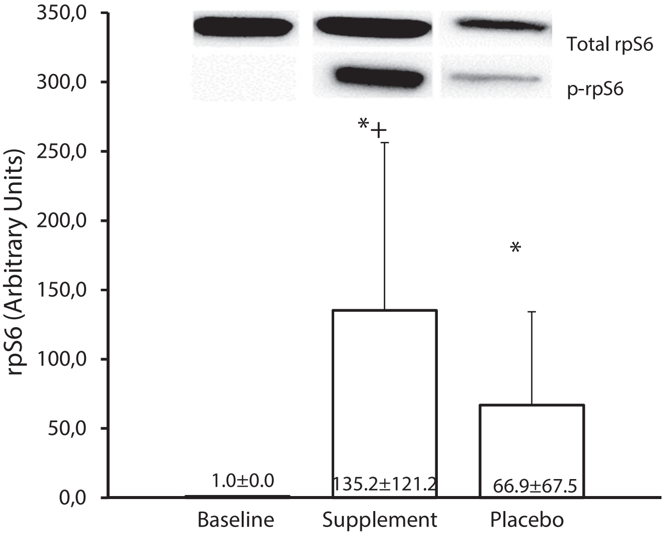

Fig. 5. rpS6 phosphorylated to total protein ratio normalized values to baseline session values (X±SD). Both the supplement and placebo conditions were significantly greater than the baseline session (*) (p<.05). The supplement session was significantly greater than the placebo condition (+) (p<.05).

Table 1. Concentrations for each blood variable (X±SD).

	Baseline	Supplement	Placebo
Insulin (μIU × ml^{-1})	15.4±17.3	85.6±69.9*	22.5±29.2
Cortisol (nmol × L^{-1})	471.7±176.0	495.8±221.7	517.3±201.7
Glucose (mg × dL^{-1})	2.6±1.0	2.6±0.7	2.9±0.7

* p<.05, different than baseline and placebo

Muscle Protein Analysis

Acute resistance exercise with leucine and whey supplementation increased AKT (F [2, 15] = 9.176, p=0.002) (p=0.003 from baseline and p=0.012 from placebo) and rpS6 (F [2, 15] =11.102, p=0.001) (p=0.001 from baseline and p=0.034 from placebo) phosphorylation level significantly over the other sample points (Figures 1 and 5). Phosphorylation of p70s6k (F [2, 15] = 10.343, p=0.002) increased similarly after resistance exercise for both the supplemented (p=0.003) and placebo conditions (p=0.001) (see Figure 3). Neither acute resistance exercise nor supplementation resulted in changes in phosphorylation of mTOR (F [2, 15] = 0.162, p=0.85) or 4E-BP1 (F [2, 15] = 1.522, p=0.232) (Figures 2 and 4).

Dietary Records

Dietary intake did not change at any time during the study (mean daily calories = 2516.9±852.1 $\mathrm{kcal \cdot d^{-1}}$; relative daily calories = 31.0±10.5 $\mathrm{kcal \cdot kg^{-1} \cdot d^{-1}}$; total protein = 120.1±41.9 $\mathrm{grams \cdot d^{-1}}$; relative daily protein intake = 20.2±7.3%.

DISCUSSION

This study indicates that following heavy resistance exercise, dietary supplementation with leucine-enriched whey protein enhanced anabolic signaling in muscle via mTOR signaling pathway and insulin. There was a large increase in circulating insulin concentrations after ingesting the supplement compared to both the resting and placebo conditions, similar to what has been previously reported [33]. Such an increase in insulin levels may be helpful for some populations due to insulin's anabolic effect on skeletal muscle [5]. Despite the increased insulin, no differences in blood glucose were observed, most likely due to the small amount of carbohydrate (4 grams) in the placebo and the lack of carbohydrate in the supplement.

There were no differences in cortisol concentrations, and observed values were typical of an individual after waking [18]. Even though there was considerable physical and physiological stress from the weight-training stimulus, there was no increase in cortisol from baseline. It is possible there was an anticipatory response during the baseline condition from anxiety for the biopsy and other tests to be performed [8], although the concentrations observed were within normal resting ranges. Since the mechanical work performed during both training sessions was nearly equal, similar cortisol responses would be expected. Cortisol reactivity is attenuated in well-trained compared to untrained individuals [6, 19], although McMillan et al. [19] used a greater volume of resistance exercise at approximately the same relative intensity. The present study used subjects that were resistance-trained, which may partly explain the lack of a cortisol response to the exercise stimulus. We do acknowledge that the post-exercise sampling schedule may not have captured the cortisol stress response, since cortisol levels decline after training bouts [18].

The majority of intramuscular signal activation over baseline was downstream from mTOR. This may be due to the short time course of response for the various steps in anabolic signaling after exercise [5], and the rapid uptake of ingested leucine into the blood stream [26,18,25,7]. Compared to baseline activity there was significant activation of a number of signaling proteins during both the supplement and placebo trials. Phosphorylation of AKT for the supplement condition was significantly greater than the placebo and baseline conditions, which may have been caused by the greater insulin concentrations following supplement ingestion compared to the placebo condition. This increased AKT phosphorylation could lead to more phosphorylation of mTOR which enhances muscle protein synthesis [14, 28]. Similar results have been previously reported with BCAA supplementation after physical exercise [4], although an increase in AKT phosphorylation independent of supplementation has also been reported [23]. Previously, this has been interpreted to be a lack of insulin response or supplement application even with moderate resistance training volumes [30] when compared to the research by Blomstrand et al [4].

Table 2. Significant changes in protein phosphorylation relative to baseline or placebo conditions.

	Supplement	Placebo
Insulin	↑↑	↔
AKT	↑	↔
mTOR	↔	↔
4E-BP1	↔	↔
P70s6k	↑	↑
rpS6	↑↑	↑

↑ = significant change relative to baseline
↑↑ = significant change relative to baseline and placebo
↔ = no change relative to baseline

There was no significant increase of phosphorylation of mTOR after either condition during the present study. There was considerable variation as indicated by the standard deviation for each time point in the study, but the means of the normalized activation did not significantly change. This is an interesting result since previous studies [3,4,23] have reported a positive effect for BCAA supplementation on mTOR activation. Our results could be influenced by the biopsy time point, since Blomstrand et al [4] performed the biopsy at one hour and two hours post-exercise where the greatest mTOR phosphorylation was observed at one hour with no change immediately after exercise.

For p70s6k, there was increased phosphorylation in the supplement and placebo conditions over the baseline, with similar responses for both conditions. This suggests that the acute exercise bout the subjects performed led to greater p70s6k activation, but with no greater effect due to supplementation, activation of this protein in a training bout has been related to increases in muscle size [28]. The result of the present study is different than other research [17], but the present exercise protocol did have a greater amount of resistance training volume that might have generated such a large amount of p70s6k phosphorylation that the difference the supplement made was not significant.

Ribosomal protein S6 (rpS6) showed the greatest amount of phosphorylation for both conditions, and a much greater amount of phosphorylation in the supplement condition compared to the placebo condition. This magnitude of phosphorylation suggested that there was an increase in mRNA synthesis. This is similar to the results reported by Karlsson et al, where after a lower volume of training than used in the present study, ingested BCAA resulted in greater rpS6 activation than either placebo or control visits [17]. Other research has shown an increase in activation directly after exercise with supplementation and for an hour thereafter [10].

Both the supplement and placebo conditions exhibited increases from baseline for phosphorylation of 4E-BP1. It should be noted that, as with mTOR, there was considerable variability in the results, perhaps contributing to the lack of statistical significance between the conditions. Previous research [15] has also shown activation of both rpS6 and 4E-BP1 at the same chronological point with leucine supplementation, while other research [10] has shown this same effect, but at two hours post-resistance exercise. It is possible that the timing of the biopsy was a confounding factor for determining 4E-BP1 activation. Regardless, Moore et al. [20] reported results that were similar to the present study, where there was an enhanced activation of rpS6 but not a significant increase in activation of 4E-BP1.

The results of the present study found moderate effects of leucine ingestion on phosphorylation for some proteins, but not others. This could perhaps be due to the slower time course of activation due to insulin activation of PI3K which in turn activated AKT, but not subsequently mTOR. The activation of p70s6k and rpS6 which are both further down the mTOR signaling pathway and are activated by mTOR suggests that mTOR had been activated prior to when the biopsy was taken in the present study. Since BCAAs are quickly digested and released into the blood stream [26], their effect could have contributed to a large and rapid anabolic response. Some studies have shown activation of mTOR at one hour and two hours post resistance training [3,4] (and other parts of the pathway [9,29]), and even at thirty minutes [16,23], with lower intensities or volumes of training [10,29].

CONCLUSIONS

The results of the present study demonstrated that dietary supplementation with whey and leucine in conjunction with a stressful resistance exercise session leads to an anabolic hormonal response and increased intramuscular protein signaling pathway phosphorylation for muscle hypertrophy (Table 2). The supplementation of leucine led to a much greater insulin response with no differences in cortisol or glucose levels. More research analyzing the time course of intramuscular protein phosphorylation when leucine supplementation is utilized needs to be performed. Finally, individuals seeking to increase the acute muscular hypertrophy response to resistance training may benefit from supplementing with leucine-enriched whey.

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Conflict of interests: the authors of this publication have no conflicts of interest to declare.

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