ABSTRACT

The Effects of Branched-Chain Amino Acid and Leucine Ingestion on the ERK1/2 MAP Kinase Signal Transduction Pathway in Conjunction with an Acute Bout of Heavy Resistance Exercise

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Purpose: To determine if leucine or BCAA ingestion increases the activation of the ERK1/2 MAPK pathway greater than that which resistance exercise (RE) elicits alone; to determine a possible mechanism for a BCAA or leucine induced ERK1/2 MAPK pathway via IRS-1 and SHP-2. Methods: 30 males (22.5yrs; 81.1kg) were randomly assigned to 1 of 3 groups: Leucine (60mg/kg/bw), BCAA (120mg/kg/bw), or placebo. Subjects performed 4 sets of leg press and leg extension at 80% 1RM to failure. Supplementation was ingested at 3 time points: 30 minutes prior to RE, and immediately pre- and post-RE. Venous blood was sampled at baseline; 30min later, immediate post-exercise, 30min post-exercise; 2hrs post-exercise, and 6hrs post-exercise for serum glucose, insulin, GH, and IGF-1. Muscle biopsies were sampled at baseline, and 30min post, 2hr post, and 6hr post-exercise for MEK1, ERK1/2, IRS-1, and SHP-2. Skeletal muscle variables were transformed to delta values and analyzed via a 3 (group) x 4 (time points) repeated measures MANOVA. Univariate ANOVAs (Bonferroni adjusted) were conducted as follow-up tests to the MANOVA. Post-hoc tests of the interaction effects

demonstrated in the ANOVA were investigated via an independent samples T-test. Results: Neither BCAA or leucine increased the secretion of the 4 serum variables. A group x time interaction relative to ERK1/2 activation indicated that the BCAA group was significantly elevated at the 2hr post and 6hr post time points in comparison with the Leucine ($p < .05$) and Placebo groups ($p < .001$). A group x time interaction for IRS-1 activation indicated that the Leucine group was significantly elevated at 2hr post and 6hr post in comparison with the BCAA ($p < .05$) group. No group x time interactions were observed for MEK1 or SHP-2. Summary: BCAA supplementation increased the phosphorylation status of ERK1/2 in conjunction with RE at 2 and 6 hours post-exercise. Leucine supplementation did not have any effect on ERK1/2 activation.

The Effects of Branched-Chain Amino Acid and Leucine Ingestion on the ERK1/2 MAP Kinase Signal Transduction Pathway in Conjunction with an Acute Bout of Heavy Resistance Exercise

by

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A Dissertation

Approved by the Department of Health, Human Performance and Recreation

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LIST OF ABBREVIATIONS

BCAA – branched chain amino acids

ERK1/2 – extracellular regulated kinases one and two

IRS-1 – insulin receptor substrate one

MAPK – mitogen activated protein kinases

MEK1 – mitogen activated protein kinase kinase

mTOR – mammalian target of rapamycin

raf – mitogen activated protein kinase kinase kinase

SHP-2 – *src* homology protein two

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daughter Sage, who encourages me to be efficient with my time so that I can spend as much time with her and Jo as possible. I would also like to thank my parents, John and Karen Campbell for their support and guidance. It is my desire to make both of you proud and to make all of your selfless sacrifices worthwhile. To Gwen, I can't thank you enough for your overwhelming generosity and kindness to both Jo and I and we look forward to raising our daughters together. Lastly, I would like to thank my in-laws, Lyn and Doris Shiery for continuously supporting Jo and I in multiple ways.

DEDICATION

I would like to dedicate this dissertation to my grandfather, Robert Wolfe Campbell and my grandmother, Alberta Campbell. My grandpa has always and will always serve as a primary source of motivation for me. He is the hardest-working, family-centered individual that I know, and I am proud to be a Campbell because of everything that he is. When I was a boy, I could not spend enough time with my grandpa, and the worst thing about following a career is not being able to spend time with my grandpa and family. I will never forget all of the time you spent with me as a boy while we fished, hunted, or just played hard. More importantly, you have taught me how to be a grandpa to my own grandchildren someday and how important family is. I also want to thank my grandma for being a perfect model of what a wife and mother should be. I love both of you very much and cherish every moment that my family and I get to spend with each of you.

CHAPTER ONE

Introduction

Increasing lean body mass (skeletal muscle hypertrophy) possesses many benefits. Relative to disease, an increase in lean body mass may prevent some of the debilitating atrophic effects of AIDS, cancer, and sarcopenia. In addition, skeletal muscle hypertrophy can improve body composition, enhance sports performance, and increase quality of life. One of the primary ways to increase skeletal muscle mass is via mechanical stimulation, which is often accomplished with resistance exercise.

Skeletal muscle hypertrophy is regulated by at least three major molecular processes: 1) satellite cell activity; 2) gene transcription; and 3) protein translation. Recently, two potential cellular pathways have been implicated for cellular growth and development in response to muscle contraction, the PI3-K/Akt/mTOR pathway and the extracellular signal-related kinase mitogen activated protein kinase (ERK1/2 MAPK) pathway (Glass, 2003; Hornberger & Esser, 2004). Once activated, these pathways lead to phosphorylation of several downstream targets responsible for activation of transcriptional and translational factors that serve as the molecular basis for muscle adaptation. ERK1/2 phosphorylates various transcription factors and downstream targets such as p90 ribosomal S6 kinase (p90rsk), MAPK interacting kinase (Mnk1/2), and mitogen- and stress-activated protein kinase (MSK1/2), which are associated with gene transcription and protein translation (Pearson et al., 2001; Seger & Krebs, 1995; Widegren, Ryder, & Zierath, 2001).

The PI3-K/Akt/mTOR pathway exerts its impact on protein synthesis via its effects on translation initiation. The mammalian target of rapamycin (mTOR) is a key regulatory element in this pathway (Hay & Sonenberg, 2004). Specifically, phosphorylated/activated mTOR has been demonstrated to phosphorylate 4E-BP1 and $p70^{S6k}$ (Hay & Sonenberg, 2004; von Manteuffel et al., 1997; T. Xu et al., 2004). Once phosphorylated, 4E-BP1 and $p70^{S6k}$ play a role in increasing the protein synthesis following resistance training.

Several growth factors have been demonstrated to activate the PI3-K/Akt/mTOR and the ERK1/2 MAPK pathway leading to skeletal muscle hypertrophy. Insulin, and to a lesser extent insulin-like growth factor-1 (IGF-1), play a strong regulatory role in activating the PI3-K/Akt/mTOR pathway (Brozinick & Birnbaum, 1998; Fanzani, Colombo, Giuliani, Preti, & Marchesini, 2006). Likewise, insulin, IGF-1, and growth hormone have been shown to activate the ERK1/2 MAPK pathway (Foulstone, Huser, Crown, Holly, & Stewart, 2004; Jorgensen et al., 2006; Osman et al., 2000), but the primary stimulus for activating this pathway in humans seems to be through mechanical stress. Of the anabolic hormones that activate these pathways, insulin has shown some capacity to increase the activation of both pathways (Cheng, Dube, Gu, & Tremblay, 2002). It appears as if insulin is able to increase the activity of the ERK1/2 MAPK pathway via a cross-talk mechanism originating from the PI3-K/Akt/mTOR pathway by way of the activation of the protein tyrosine phosphatase Shp-2. Shp-2 is a widely expressed cytoplasmic tyrosine phosphatase. Shp-2 activation has been shown repeatedly to activate the ERK1/2 MAPK pathway in non-human cell cultures, although the exact mechanism is debated and not yet fully understood (Shi, Lu, & Feng, 1998; Shi, Yu, Park, Marshall, & Feng, 2000; Takada et al., 1998).

Similar to insulin, the branched chain amino acids (BCAAs) also activate the PI3-K/Akt/mTOR pathway at the mTOR level (Rennie, Bohe, Smith, Wackerhage, & Greenhaff, 2006). Quite possibly, however, the amino acid leucine alone may be the specific amino acid responsible for activating the PI3-K/Akt/mTOR pathway at the mTOR level (Anthony, Yoshizawa et al., 2000). More research needs to be conducted to determine if all three BCAAs or leucine alone is responsible for activating mTOR and its downstream targets. It also appears that both the BCAAs (or solely leucine) and insulin are required in order to induce a maximal response relative to protein synthesis via the PI3-K/Akt/mTOR pathway. While many studies have linked BCAA ingestion with increased PI3-K/Akt/mTOR activity, there has only been one clinical study investigating the effects of BCAA supplementation in conjunction with resistance exercise on the ERK1/2 MAPK pathway in humans (Karlsson et al., 2004). In addition, no clinical studies exist that have investigated the effects of leucine alone in conjunction with resistance exercise on the activation status of the ERK1/2 MAPK pathway. Therefore, the present study (which introduces a supplemental leucine intervention and observes its effects on the activation status of the ERK1/2 MAPK pathway in conjunction with resistance exercise) is novel in its design and methodology.

Purposes of the Study

The primary purpose of this investigation is to determine if leucine or BCAA ingestion increases the activation of the ERK1/2 MAPK pathway to an extent greater than that which resistance exercise would elicit alone. A secondary purpose of this study is to determine a possible mechanism responsible for a BCAA or leucine induced activation of the ERK1/2 MAPK pathway, if an increase in activation is observed. Given the fact that

multiple studies have shown that leucine is able to slightly increase serum insulin levels in humans, and the ability of insulin to activate the ERK1/2 MAPK pathway via crosstalk from the PI3-K/Akt/mTOR pathway, this aspect of explaining a possible BCAA or leucine induced activation of the ERK1/2 MAPK pathway will be explored. Specifically, however, the primary purposes of the proposed study are to investigate the effects of:

1). Leucine supplementation in conjunction with an acute bout of lower-body resistance exercise on the activity of the ERK1/2 MAPK pathway (MEK1 and ERK1/2). A BCAA group will also be included in this study, but the only purpose for the addition of this group is to investigate whether or not leucine alone is responsible for any increases in the ERK1/2 MAPK pathway, or if the other BCAAs also play a role.

2). Leucine supplementation in conjunction with an acute bout of lower-body resistance exercise on the activity of IRS-1 and the protein phosphatase SHP-2 (as a possible cross-talk mechanism between the PI3-K/Akt/mTOR and ERK1/2 MAPK pathways).

Hypotheses

H_1: BCAA supplementation will not increase the phosphorylated (activation) state of MEK1 when compared to placebo in conjunction with lower-body resistance exercise.

H_2: Leucine supplementation will not increase the phosphorylated (activation) state of MEK1 when compared to placebo in conjunction with lower-body resistance exercise.

H_3: BCAA and leucine supplementation will not differ in regard to their increasing the phosphorylated state of MEK1 in conjunction with lower-body resistance exercise.

H_4: BCAA supplementation will not increase the phosphorylated (activation) state of ERK1/2 when compared to placebo in conjunction with lower-body resistance exercise.

H_5: Leucine supplementation will not increase the phosphorylated (activation) state of ERK1/2 when compared to placebo in conjunction with lower-body resistance exercise.

H_6: BCAA and leucine supplementation will not differ in regard to their increasing the phosphorylated state of ERK1/2 in conjunction with lower-body resistance exercise.

H_7: BCAA supplementation will not increase the phosphorylated (activation) state of IRS-1 when compared to placebo in conjunction with lower-body resistance exercise.

H_8: Leucine supplementation will not increase the phosphorylated (activation) state of IRS-1 when compared to placebo in conjunction with lower-body resistance exercise.

H_9: BCAA and leucine supplementation will not differ in regard to their increasing the phosphorylated state of IRS-1 in conjunction with lower-body resistance exercise.

H_{10}: BCAA supplementation will not increase the phosphorylated (activation) state of SHP-2 when compared to placebo in conjunction with lower-body resistance exercise.

H_{11}: Leucine supplementation will not increase the phosphorylated (activation) state of SHP-2 when compared to placebo in conjunction with lower-body resistance exercise.

H_{12}: BCAA and leucine supplementation will not differ in regard to their increasing the phosphorylated state of SHP-2 in conjunction with lower-body resistance exercise.

Delimitations

This study was completed using the following guidelines:

1). Apparently healthy, recreationally trained males between the ages of 18-32 participated in this study.

2). Participants were recruited from the student population at Baylor University and in the Waco community by flyers posted throughout campus and through local gyms and health clubs.

3). Participants conducted the lower body resistance training session in the Exercise and Biochemical Nutrition Laboratory.

4). Muscle biopsies from the lateral thigh were collected prior to exercise, 1 hour after exercise, 2 hr post-exercise, and 6 hr post-exercise.

5). Venous blood samples were obtained pre supplementation, 30 minutes post supplement ingestion, immediately postexercise, 30 minutes postexercise, two hours postexercise, and six hours postexercise.

6). All participants were randomly assigned to ingest one of three supplements: 1) BCAA (isoleucine, leucine, and valine); 2) leucine; 3) placebo.

7). All participants performed lower body resistance exercise consisting of four sets of leg press on an isotonic hip/leg sled and four sets of leg extension on an isotonic leg extension machine.

8). All participants in the study were instructed to not participate in any other forms of vigorous exercise for four days prior to the resistance training session and were instructed to not modify their nutritional intake in any manner.

Limitations

1). This study compared the effects of leucine and BCAA ingestion and a lower body resistance training bout on the activation of the ERK1/2 MAP kinase pathway.

2). The motivation and willingness of each participant to maximally exert themselves during the lower-body resistance exercise bout.

3). The results obtained from this investigation can be generalized to young, resistance trained males.

4). The sensitivity of the technologies and protocols utilized to identify quantifiable changes in the criterion variables.

5). The daily schedules of each participant and the inherent circadian rhythms that exist for all humans as a result of slightly different testing times, stresses, etc.

Assumptions

1). Participants will be apparently healthy with no contraindications to any of the prescribed treatments involved with this protocol.

2). Participants were physically active, but not resistance trained.

3). Each participant maximally exerted themselves during the lower-body resistance exercise bout.

4). Each participant did not participate in intense lower body resistance exercise or intense aerobic activity for the three days prior to the investigational period.

5). Participants arrived to the testing session in a fasted state (10-12 hours).

6). All assay reagents and equipment that was used in the sample analysis was accurate and reliable in quantification of the criterion variables.

7). All methods, which were previously established, will be accurate and reliable methods for determination of the criterion variables.

8). Participants were truthful in their responses regarding past anabolic steroid and ergogenic aid use.

Definition of Terms

1). Akt – also known as protein kinase B, is a protein kinase. It is activated by a diverse array of growth factors and physiologic stimuli in a PI3-K-dependent manner

2). BCAA – Branched chain amino acids. Consists of three amino acids: leucine, isoleucine, and valine.

3). ERK1/2 – Extracellular signal regulated kinase (also known as MAPK). ERK1 and ERK2 are two closely related isoforms. ERK1 (also known as p44 MAP kinase), is a 44 kDa protein and ERK2, (also known as p42 MAP kinase), is a 42 kDa protein. Both ERK1 and ERK2 are activated through the phosphorylation of a threonine and a tyrosine residue within the activation loop by MEKs.

4). ERK1/2 MAPK Pathway - is a signal transduction pathway that couples intracellular responses to the binding of growth factors to cell surface receptors. It typically refers to three main protein kinases, Raf, MEK1, and ERK1/2.

5). 4E-BP1 - Eukaryotic initiation factor 4E binding protein 1 (also known as PHAS-I), is the predominant member of a family of eIF4E binding proteins whose binding affinity to eIF4E is regulated by its phosphorylation.

6). Gene Transcription - the process through which a DNA sequence is enzymatically copied by an RNA polymerase to produce a complementary RNA (the transfer of genetic information from DNA into RNA).

7). IRS-1 - Insulin receptor substrate-1 (IRS-1), a cytoplasmic adaptor protein which plays a key role in mediating metabolic and proliferative signaling arising from stimulation by insulin, IGF-1, IGF-2, and cytokines such as IL-4. It has a molecular weight of 165 kDa.

8). Leucine - One of three branched-chain amino acids (the others are valine and isoleucine) that enhance energy, increase endurance, and aid in muscle tissue recovery and repair.

9). MEK1 – also known as ERK kinase, MAPK kinase, and MKK1. It is a member of the MEK family of dual specificity protein kinases which phosphorylates ERK1 and ERK2 at the conserved threonine and tyrosine residues of the activation loop.

10). mTOR – mammalian target of rapamycin. It is a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription. mTOR integrates the input from multiple upstream pathways, including insulin, growth factors (such as IGF-1 and IGF-2), and mitogens.

11). PI3-K - Phosphoinositide 3-kinase. Are a family of related kinases/enzymes that are capable of phosphorylating the 3 position hydroxyl group of the inositol ring of phosphatidylinositol.

12). Protein Translation - the synthesis of protein from mRNA

13). Raf-1 – a 74 kDa cytoplasmic serine/threonine protein kinase that transduces signals from the cell surface receptors to other cytoplasmic members of the MAPK pathway (directly phosphorylating MEK) proteins and ultimately to the nucleus

14). p70s6k - p70 Ribosomal Protein S6 Kinase (also known as S6K1, RPS6KB1 or p70(S6K)-alpha), is a member of the ribosomal S6 kinase (RSK) family of serine/threonine kinases. It has a molecular weight of 70 kDa.

CHATPER TWO

Review of Literature

Physical and Chemical Properties of Leucine

Leucine ((S)-2-amino-4-methyl-pentanoic acid) is an essential amino acid with a non-polar side chain (Figure 1), possesses a molecular mass of 131.18 $g \cdot mol^{-1}$, and is classified as a ketogenic amino acid. Due to the branching of its two side chain methyl groups, it is one of three amino acids (along with isoleucine and valine) known as the branched-chain amino acids (BCAAs). The BCAAs make up about one-third of muscle protein (Mero, 1999). Of the three branched-chain amino acids, leucine has been the most thoroughly investigated because its oxidation rate is higher than that of isoleucine and valine (Mero, 1999). The current recommended dietary intake of leucine is 14 mg/kg bodyweight/day. While amino acids are most commonly thought of as fundamental building blocks to protein molecules, leucine participates in metabolism in diverse ways including as a: 1) substrate for protein synthesis, 2) fuel, 3) metabolic signal (Layman, 2002). Another area in which leucine differs from the other common amino acids is its disproportionate incorporation into proteins (accounting for approximately 9%) of muscle amino acids (RDA, 1989).

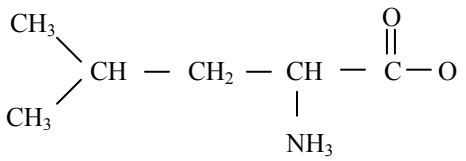

Figure 1. The chemical structure of leucine.

Relative to sports performance, many scientific studies have investigated the effects of the branched-chain amino acids, but very few have studied the effects of leucine alone on sports performance. As Mero (1999) indicated in his comprehensive review of leucine: "Caution must be paid when interpreting the limited number of studies in this area [leucine supplementation] since, in many cases, leucine has been supplemented as part of a mixture of branched-chain amino acids." For this reason, whenever possible, an attempt of this review will be to discuss only those studies in which leucine alone was supplemented. A few studies have investigated leucine as part of a mixture (usually BCAAs) in comparison with leucine alone, with the intent of demonstrating that leucine alone was responsible for any observed effects. These types of studies will also be discussed throughout this review.

Leucine Supplementation and Exercise Performance

Of the clinical trials that have investigated leucine supplementation on exercise performance, the results have been contradictory. Pitkanen et al. (2003) supplemented competitive male power athletes with leucine or placebo under two different exercise parameters, a strength exercise session (SES) or a maximal anaerobic running exercise session (MARE). The SES exercise protocol consisted of 90 minutes of jumps and heavy resistance exercise (squats, calf raises, and bench press) followed by a performance evaluation, which consisted of a counter movement jump, performed 5 minutes after the SES protocol. Thus, the main dependent variable of the SES protocol was the recorded flight time during the jump. The MARE exercise consisted of repeated 20-second sprints on a treadmill with a recovery of 100 seconds between the runs. The initial treadmill speed (4.08m x s^{-1}, 4^{o} slope) was increased by 0.38m x s^{-1} for each consecutive run until

exhaustion. In this investigation, each subject visited the test laboratory twice and consumed, in random order, either the leucine drink for SES (100mg x kg/bodyweight; one half of the drink before and the other half of the drink in the middle of the strength exercise session) or a leucine drink for MARE (200mg x kg/bodyweight before the anaerobic exercise session), or placebo drinks. For the SES, there were no significant differences for the performance variable of explosive strength (a counter movement jump), indicating that leucine supplementation at a dosage of 100mg x kg/bodyweight had no effect on this exercise performance variable. For MARE, there was no significant difference relative to running velocity between the leucine and placebo group, indicating that leucine supplementation at a dosage of 200mg x kg/bodyweight had no effect on an acute bout of anaerobic running performance. Other findings reported from this study showed that following the SES and MARE, the leucine supplementation resulted in increases in blood leucine values (11% in SES and 28% in MARE), with corresponding decreases in the other branched-chain amino acids – valine (42% in SES and 14% in MARE) and isoleucine (61% in SES and 25% in MARE). This increase in blood leucine content demonstrated in this investigation may have important considerations relative to the role that leucine possesses in cell-signaling and protein synthesis. The role of leucine in cell signaling and protein synthesis is described below (see *Leucine Ingestion and Protein Synthesis*).

In another study which supplemented athletes with leucine, Crowe, Weatherson, & Bowden (2006) gave outrigger canoeists 45 mg/kg per day of leucine for a 6 week period. In this investigation, both anaerobic and aerobic variables were measured before and after 6 weeks of chronic leucine supplementation. A row to exhaustion at 70-75% maximal aerobic power served as the criterion measure of endurance performance. In

addition, ratings of perceived exertion were recorded every 5 minutes both before and after the supplementation period. In relation to anaerobic assessments, relative peak power (watts/kg) and total work (J/kg) were determined via an "all out" 10-second arm crank test. Following the supplementation protocol, the leucine group significantly increased their rowing time to exhaustion (~77.6 to 88.3 minutes) as well as significantly decreasing RPE (14.5 to 12.9) as compared to the placebo. Upper-body power and work significantly increased in both groups after supplementation but power was significantly greater after leucine supplementation compared to placebo (6.7 vs. 6.0 watts/kg). These results are in contrast to the aforementioned Pitkanen et al. (2003) study, which showed no performance increases in explosive strength or anaerobic running following an acute ingestion of greater amounts of supplemental leucine. One possible explanation for the differences in performance outcomes of the two aforementioned studies is the time period for which the leucine supplementation was ingested. The Crowe et al. (2006) investigation instructed their study participants to chronically ingest leucine for a 6-week period, but the Pitkanen et al. (2003) study administered a single or double dose of leucine on the day of exercise testing. Mero et al. (1997) also administered leucine supplementation to athletes over a period of 10 weeks. In this investigation, male track and field power athletes were given leucine at a dosage of 50-mg/kg body weight per day during training. It is important to clarify that the main dependent variable of interest was not exercise performance, but rather to examine the effects of leucine supplementation on the amino acid and hormone profile during 10 weeks of indoor track and field training. However, a jumping test (as a reflection of speed strength of the leg extensor muscles) was evaluated using a counter movement jump at baseline, mid-point (5-weeks), and end of the leucine supplementation period (10-weeks). The authors reported that there were

no significant differences between the leucine and placebo groups in the counter movement jump on the 3 test occasions.

Taking these three aforementioned studies together, it appears that leucine supplementation does not significantly increase lower-body anaerobic exercise performance (either when leucine supplementation is given acutely on the day of performance evaluation (Pitkanen et al., 2003) or when given chronically over a period of 10 weeks (Mero et al., 1997), but has been shown to significantly improve aerobic and upper-body anaerobic exercise performance when ingested chronically over a period of 6 weeks (Crowe et al., 2006).

Another recent study utilizing leucine ingestion was conducted by Coburn et al. (2006). Unlike the previous studies discussed above, this investigation did not supplement with leucine alone, but rather added supplemental leucine to a whey protein supplement. This study divided the participants into three groups: a supplement group (20 grams of whey protein plus 6.2 grams of leucine), a carbohydrate placebo group (26.2 grams of maltodextrin), and a control group. The supplement and placebo groups performed unilateral training of the leg extensor muscles with the nondominant limb for 8 weeks (3-5 sets of 6 repetitions at 80% 1RM three times per week). The control group performed no resistance training during the 8-week intervention. Three dependent variables were assessed pretraining and post training: 1) the strength of each limb; 2) muscle cross-sectional area of the quadriceps femoris (via magnetic resonance imaging); and 3) body composition. The results indicated significant increases in strength for both limbs in the whey protein/leucine group but only the trained limb in the carbohydrate placebo group. The increase in strength for the trained limb of the whey protein/leucine group was greater than that for the trained limb of the carbohydrate placebo. There was

no significant increase in strength for either limb in the control group. There were no differences between the supplement groups for quadriceps cross-sectional area. Also, there were no significant changes in body composition for the three groups. The authors concluded that leucine and whey protein supplementation may provide an ergogenic effect, which enhances the acquisition of strength beyond that achieved with resistance training and a carbohydrate placebo.

Leucine Supplementation and Blood Concentrations of Leucine following Acute Exercise Bouts and Chronic Exercise Training

In addition to assessing performance variables, each of the aforementioned studies also evaluated serum/plasma leucine concentrations in conjunction with leucine supplementation and exercise testing/training. Leucine supplementation results in increases in serum leucine concentrations or prevents the decline in plasma leucine as a result of physical training. Mero et al. (1997) reported that leucine supplementation of 50 mg/kg bodyweight per day prevented a decrease in serum concentrations of leucine during 10 weeks of heavy training in male power athletes. In the 6 week study conducted Crowe et al. (2006) utilizing outrigger canoeists, leucine supplementation (45 mg/kg bodyweight) resulted in significant increases in plasma leucine following an aerobic row to exhaustion test. In the Pitkanen et al. (2003) study, leucine supplementation (administered acutely on the day of exercise testing at a dosage 200 mg/kg bodyweight) resulted in significant increases in blood leucine concentrations following both jumps and heavy resistance exercise as well as repeated 20 second sprints. Table 1 summarizes the studies investigating leucine supplementation and its effects on exercise performance and blood concentrations.

Table 1. *Leucine Supplementation and Exercise Performance*

Reference	Leu Dosage	Exercise DVs	Results	Blood Response
Pitkanen (2003)	200 mg/kg (single dose)	Counter movement jump (CMJ); Run until exhaustion (RUE)	No improvement in CMJ; no improvement in RUE	Sig. increase in blood leucine concentrations
Crowe (2006)	45 mg/kg (daily for 6 wks)	Row to exhaustion (RTE); Upper-body peak power (PP) and total work (TW)	Sig. increase in RTE and PP; no improvement in TW	Sig. increase in blood leucine concentrations
Mero (1997)	50 mg/kg (daily for 10 wks)	Counter movement jump (CMJ)	No improvement in CMJ	Prevented a decrease in serum concentrations of leucine during 10 wks of heavy training

While exercise performance following acute and chronic leucine supplementation is not consistently improved, each of these studies are in agreement relative to increases in serum levels of leucine following physical activity (or preventing a decrease in plasma leucine as a result of physical training). Additional leucine resulting from supplementation has three basic fates in skeletal muscle: oxidation, incorporation into protein, and accumulation in the intracellular free amino acid pool. These last two fates of leucine in skeletal muscle, incorporation into protein (i.e. protein synthesis) and an accumulation in the intracellular free amino acid pool (allowing leucine to exert its effects on certain cell signaling pathways) have been an intense area of scientific inquiry in recent years. Consistent with these areas of investigation is the hypothesis that leucine possesses a regulatory role in protein synthesis and promotes skeletal muscle hypertrophy. Following is an overview of the mechanisms associated with skeletal

muscle hypertrophy with an emphasis placed on protein synthesis and translation initiation. It is important to note, however, that even though there is a multitude of studies demonstrating that leucine enhances skeletal muscle protein synthesis, to date there are no studies that have shown that leucine supplementation alone leads to significant increases in lean body mass in conjunction with resistance training.

Skeletal Muscle Hypertrophy

Skeletal muscle hypertrophy is characterized by increases in protein mass per fiber (Nader, Hornberger, & Esser, 2002). This increased accumulation of protein results from a net increase in protein synthesis relative to breakdown (i.e. a positive net protein balance). Attaining such a positive net protein balance is regulated by at least three major molecular processes: 1) satellite cell activity; 2) gene transcription; and 3) protein translation. Of these processes, satellite cell activity is a prerequisite for the addition of new sarcomeres. Satellite cells are activated during the process of skeletal muscle hypertrophy and are thought to proliferate, differentiate, and then fuse with existing myofibers (Hawke, 2005; Schultz & McCormick, 1994). This integration of the satellite cells with the existing myofibers and subsequent increase in myonuclei content sets the stage for an overall potential increase in genetic transcription and protein translation of skeletal muscle specific genes.

Muscle specific gene transcription and protein translation involve numerous physiological processes. Among these, increases in hormonal concentrations of insulin, IGF-1, testosterone, and growth hormone have been associated with skeletal muscle hypertrophy (Kraemer & Ratamess, 2005). Signal transduction pathways that have been shown to exert transcriptional control include calcineurin, CaMK, MAPK, PKC, NF-κB,

and AMPK (Wackerhage & Woods, 2002). Activated signal transduction pathways are able to activate or increase the expression of transcription factors which regulate skeletal muscle genes or are able to increase the rate of translation (Gautsch et al., 1998). One mechanism of intense study relative to increasing the rate of protein translation is the Akt-mTOR pathway (Bodine et al., 2001;Gautsch et al., 1998; Glass, 2003; Kimball & Jefferson, 2004). In the past few years, however, increased attention has been placed on the mitogen activated protein kinases (MAPK) pathway relative to skeletal muscle hypertrophy (Karlsson et al., 2004; Widegren, Wretman, Lionikas, Hedin, & Henriksson, 2000; Williamson, Gallagher, Harber, Hollon, & Trappe, 2003; Yu, Blomstrand, Chibalin, Krook, & Zierath, 2001). Once activated, the MAPK pathway leads to phosphorylation of the downstream targets responsible for activation of transcriptional factors that serve as the basis for muscle adaptation (Creer et al., 2005). A more in-depth discussion of the MAPK pathway is found below in the section titled *Mitogen Activated Protein Kinases (MAPK)*.

Overview of Translation Initiation

The translation of mRNA to protein (i.e. protein translation) occurs in three distinct phases: initiation, elongation, and termination (Jefferson & Kimball, 2001). All three of these phases can undergo regulation, but initiation and elongation seem to be the most tightly controlled (Jefferson & Kimball, 2001). Of the several steps in initiation, two have been shown to be subject to regulation *in vivo* (Bolster, Kimball, & Jefferson, 2003):

1) the binding of initiator methionyl-*t*RNA$_i$ (met-*t*RNA$_i$) to the 40S ribosomal subunit.

2) the binding of mRNA to the 40S ribosomal subunit

Due to the fact that leucine appears to exert influence over both of these regulatory steps, this aspect of protein translation will be highlighted here. Following this, a discussion of how insulin secretion as well as leucine supplementation contributes to regulating translation initiation will be highlighted.

Formation of the eIF-4F Complex

The binding of mRNA to the 40S ribosomal subunit is mediated by a heterotrimeric complex of three initiation factors collectively referred to as eukaryotic initiation factor 4F (eIF-4F) (Bolster et al., 2003; Jefferson & Kimball, 2001; Raught & Gingras, 1999). The three proteins that constitute the eIF-4F complex are eIF-4A, eIF-4G, and eIF-4E. eIF-4A is an RNA helicase. The eIF-4G protein is a scaffold protein, which binds eIF-4E and eIF-4A thereby linking these proteins together and stabilizing the formation of the eIF-4F complex. eIF-4E is a protein that binds to the m^7GTP cap located at the 5'-end of the mRNA (Bolster et al., 2003; Jefferson & Kimball, 2001). Formation of the eIF-4F complex is regulated by the reversible binding of eIF-4E to the translational repressor 4E-binding protein 1 (4E-BP1) (Jefferson & Kimball, 2001). Binding of 4E-BP1 to eIF-4E prevents association of eIF-4E with eIF-4G and thus precludes formation of the active eIF-4F complex (Jefferson & Kimball, 2001). The interaction between eIF-4E and 4E-BP1 is regulated by phosphorylation of 4E-BP1, whereby hypophosphorylated forms of 4E-BP1 bind to eIF-4E but hyperphosphorylated forms do not (Figure 2) (Bolster et al., 2003).

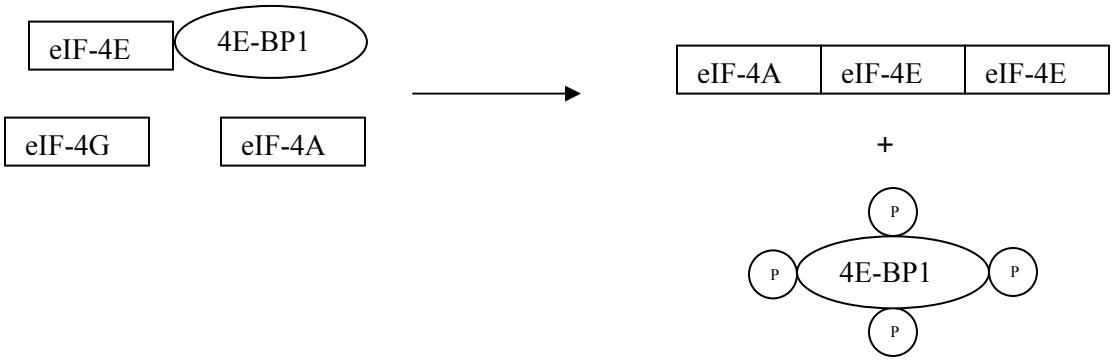

Figure 2. Well characterized mechanisms regulating translation initiation. Hyperphosphorylation of 4E-BP1 allows the association of the heterotrimeric complex eIF-4F to occur.

p70S6kinase Regulation

In addition to the formation of the eIF-4F complex, which regulates mRNA binding to the 40S ribosomal subunit, one other mechanism that is also affected by leucine availability is the phosphorylation of ribosomal protein S6 by the protein p70s6 kinase (p70S6k). For mRNA to be translated into protein, it must first associate with ribosomes to form translationally competent structures referred to as polysomes (Bolster et al., 2003). Ribosomes are the organelles on which mRNAs are translated and consist of two subunits (a large subunit and a small subunit). In eukaryotes, the large subunit is the 60S subunit and the small subunit is the 40S subunit. Each ribosomal subunit consists of rRNA (ribosomal RNA, encoded by rRNA genes) and ribosomal proteins and collectively constitute the machinery on which new proteins are assembled (i.e. translated) in the cell (Dufner & Thomas, 1999). Together, they play a fundamental role in all phases of translation, and the regulation of their activities contributes significantly to the regulation of translation. Ribosomal protein S6 (rpS6) is part of the small (40S) ribosomal subunit, and when phosphorylated it has been correlated with increased protein synthesis following growth factor stimulation (Dufner & Thomas, 1999). In addition, phosphorylated rpS6 is selectively associated with polysomes (Jefferson & Kimball,

2001; Duncan & McConkey, 1982a, 1982b). Currently, the mechanism explaining how rpS6 enhances translation is still a mystery, but it is interesting to note that rpS6 is positioned near the mRNA binding site on the 40S ribosomal subunit and is thus located in a position that may permit a role in mRNA selection (Bolster et al., 2003).

As stated above, p70S6K is an rpS6 kinase and therefore rpS6 phosphorylation and activation are regulated through p70S6K activity. The activity of p70S6K is controlled by its phosphorylation state with enhanced phosphorylation producing a general increase in kinase activity (Dufner & Thomas, 1999; Nader et al., 2002). When activated (phosphorylated), p70S6K up-regulates ribosomal biosynthesis and enhances the translational capacity of the cell (An et al., 2003) and has been implicated in load-induced skeletal muscle hypertrophy (Xu et al., 2004). Other studies have also implicated p70S6K in the control of cell growth via increased mRNA translation (Montagne et al., 1999; Radimerski et al., 2002).

mTOR Activity

Both of the aforementioned compounds (4E-BP1 and p70S6K) that regulate translation initiation possess the same upstream regulator, the mammalian target of rapamycin (mTOR) which is a serine/threonine protein kinase that regulates cell growth, protein synthesis, and transcription (Hay & Sonenberg, 2004). Activation of mTOR has been shown to increase protein translation by activating p70S6K and inhibiting the activity of 4E-BP1 (Glass, 2003). In a study investigating rpS6 and 4E-BP1 phosphorylation, von Manteuffel et al. (1997) reported that increased 4E-BP1 phosphorylation is controlled by a parallel signalling pathway that bifurcates immediately upstream of p70s6k, with the two pathways sharing a common rapamycin-sensitive

activator, mTOR. Figure 3 demonstrates the role of mTOR relative to p70S6K and 4E-BP1 regulation.

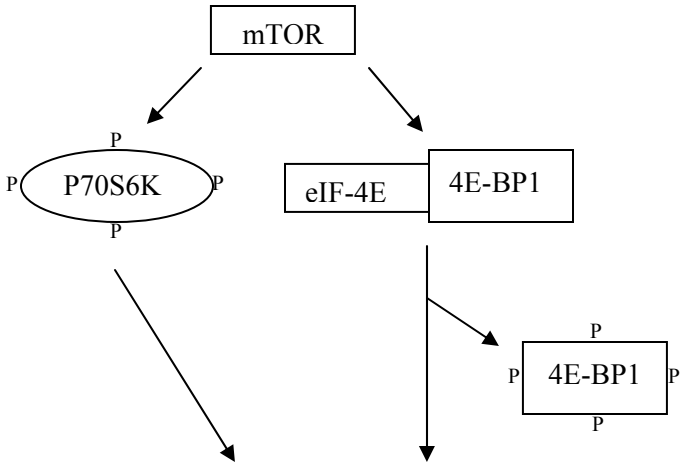

Figure 3. mTOR activation phosphorylates both P70S6K (activating it) and 4E-BP1 (resulting in the releasing of eIF-4E) leading to increases in the rate of translation initiation.

Insulin Signaling

Much of the information that is known about regulation of translational efficiency (i.e. translation initiation) in skeletal muscle has been obtained from the study of growth factors such as insulin (Nader et al., 2002). Insulin and other growth factors mediate a wide spectrum of biological responses including cell division, regulation of gene expression, glucose transport, glycogen synthesis, protein synthesis, and antilipolysis (Layman, 2002). Due to the specificity of this review, the focus will remain on the effects of insulin signaling on protein synthesis and the synergistic relationship with leucine that exists to amplify increases in the rates of translation initiation. Also, in the MAPK section below, a discussion of how insulin secretion affects the MAPK pathway will be summarized.

Insulin is one of the most anabolic hormones identified in the body. Insulin binding to its receptor results in receptor autophosphorylation on tyrosine residues and the tyrosine phosphorylation of insulin receptor substrates (e.g. IRS and Shc) by the insulin receptor tyrosine kinase. The insulin receptor (IR) belongs to a subclass of a large family of protein tyrosine kinases (Cheng et al., 2002; Schlessinger, 2000). The insulin receptor is a transmembrane protein comprising two extracellular α subunits that serve as insulin-binding sites and two transmembrane β subunits linked by disulphide bonds (Cheng et al., 2002). Upon binding to insulin, the intrinsic kinase activity of the receptor is increased, and the IR undergoes autophosphorylation on several tyrosine residues located on the cytoplasmic portion of the β subunits (Cheng et al., 2002; White & Kahn, 1994). This promotes phosphorylation of a family of substrates that includes insulin receptor substrate-1 (IRS-1), IRS-2, IRS-3, and IRS-4 (Cheng et al., 2002; Ogawa, Matozaki, & Kasuga, 1998; White, 1998). In human skeletal muscle, IRS-1 and IRS-2 appear to be the two most abundant proteins from the IRS family, with IRS-1 being the principal insulin receptor substrate (Kirwan & Jing, 2002). IRS-1 is responsible for some, if not all, of insulin's biological actions (White & Kahn, 1994).

Once the IR activates IRS-1, IRS-1 becomes heavily tyrosine phosphorylated and then subsequently activates phosphatidylinositol 3-kinase (PI3-kinase). Following PI3-kinase activation, protein kinase B (PKB or Akt) is activated following its recruitment to the plasma membrane of cells by a PI3-kinase dependent mechanism (Alessi & Downes, 1998). Akt provides one of the critical links between upstream signals generated upon activation of insulin receptors and their cellular consequences (Alessi & Downes, 1998). Similar to many of the other proteins in the insulin signaling cascade, the primary

mechanism of Akt activation is via protein phosphorylation (Kohn, Takeuchi, & Roth, 1996).

Following Akt activation/phosphorylation, it has been shown that mTOR is subsequently activated (Kimball, Farrell, & Jefferson, 2002). More specifically, it appears that mTOR is phosphorylated by the PI3-kinase regulated protein kinase Akt at two COOH-terminal sites (Thr2446 and Ser2448), with Ser2448 being the major phosphorylation site (Scott, Brunn, Kohn, Roth, & Lawrence, 1998; Sekulic et al., 2000). In summary, the proteins that constitute the insulin signaling pathway, both the upstream protein kinase (PI3-kinase) as well as the downstream kinase Akt, work in concert through mTOR thereby inducing the phosphorylation of 4E-BP1 and p70S6K and subsequently activate/amplify protein translation (Gingras, Kennedy, O'Leary, Sonenberg, & Hay, 1998). Figure 4 highlights how insulin signal transduction is signaled through mTOR, which is an upstream controller of both 4E-BP1 and p70S6K (Kimball, Horetsky, & Jefferson, 1998).

Insulin Signaling and Protein Synthesis

Many studies have demonstrated that insulin is associated with increases in the rates of protein synthesis, and some have investigated specific mechanisms responsible for such observations. Xu et al. (1998) isolated pancreatic beta cells from male Sprague-Dawley rats and incubated them with elevated levels of glucose in order to increase endogenous levels of insulin and determine if the levels of 4E-BP1 phosphorylation were observed. Xu et al. (1998) reported that glucose stimulates 4E-BP1 phosphorylation via insulin interacting with its own receptor on the beta cell which may serve as an important mechanism for autoregulation of protein synthesis by translation.

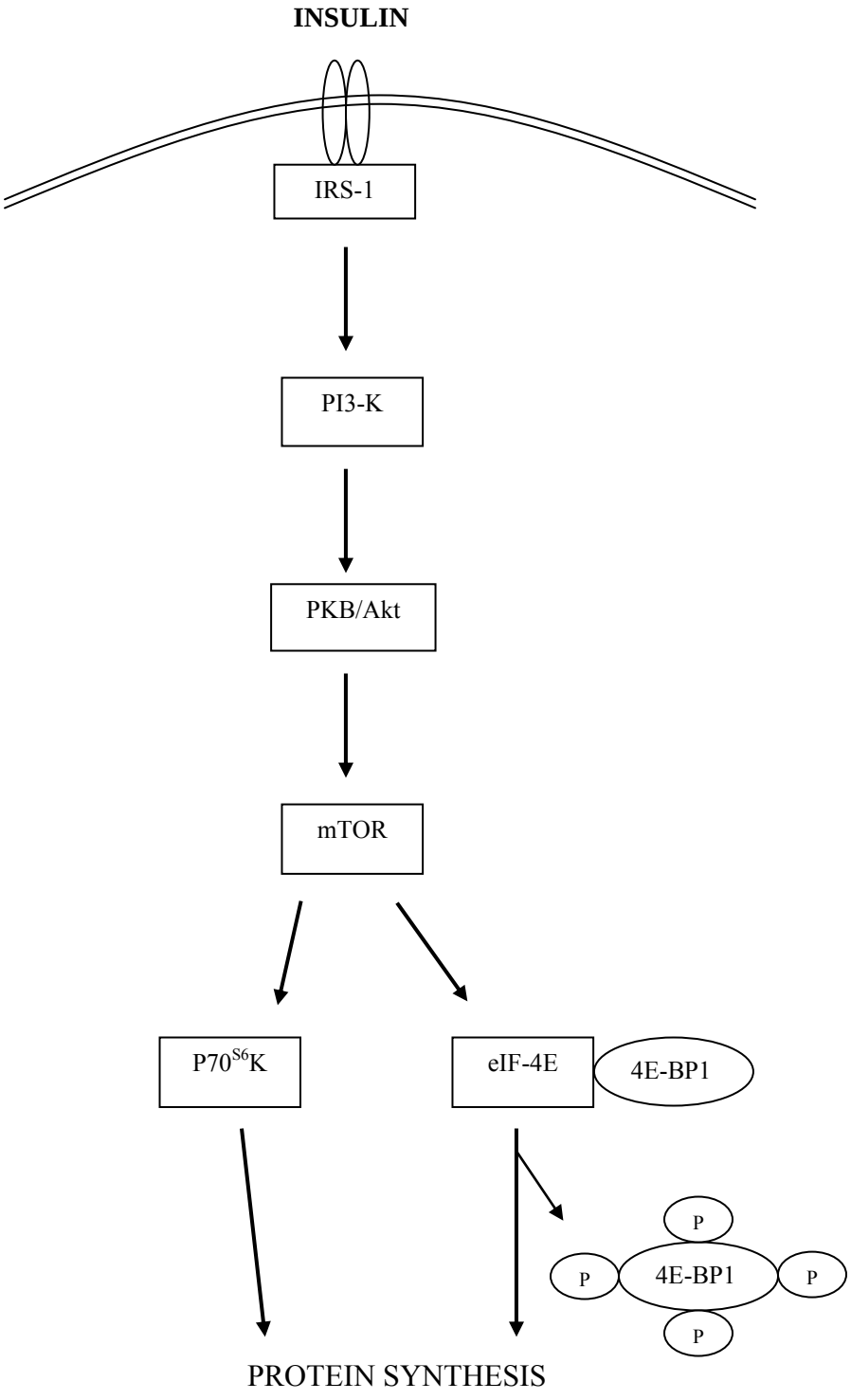

Figure 4. Insulin signal transduction pathway. IRS-1 = insulin receptor substrate; PI3-K = phosphatidylinositol-3 kinase; PKB/Akt = protein kinase B (also known as Akt); mTOR = mammalian target of rapamycin; $P70^{S6}K$ = ribosomal protein S6 kinase; eIF-4E = eukaryotic initiation factor-4E; 4E-BP1 = eIF-4E binding protein 1.

Similarly, Mendez, Myers, White, & Rhoads, (1996) utilized a myeloid progenitor cell line to determine insights relative to the mechanisms with which insulin stimulates protein synthesis. They concluded that the phosphorylation of both 4E-BP1 and p70S6K requires IRS-1-mediated stimulation of PI3-kinase (Mendez et al., 1996). Jefferson, Koehler, & Morgan (1972) reported that perfusion of psoas muscle from fasted rats with buffer containing insulin reduced the concentration of ribosomal subunits and increased phenylalanine incorporation (which are both markers of increased translational capacity).

These studies and others like them may lead to the conclusion that elevated insulin levels consistently lead to increases in protein synthesis. However, as Hillier, Long, Jahn, Wei, & Barrett (2000) has stated: "….there exists an interesting and unexplained dichotomy that arises from studies examining insulin's actions on muscle protein synthesis *in vitro* and *in vivo*. Abundant in vitro data clearly indicate that insulin strongly stimulates protein synthesis in a variety of cells and perfused organs and that insulin acts primarily to enhance mRNA translation. However, using a variety of methods investigators have almost without exception reported that insulin at physiological concentrations does not stimulate whole body or muscle protein synthesis *in vivo*."

In fact, several studies have infused insulin causing physiological hyperinsulinemia in order to elicit increases in protein synthesis in humans. Biolo, Declan Fleming, & Wolfe (1995) infused insulin (at a rate of 0.15 μU/min per 100 ml/leg) into the femoral artery to increase femoral venous insulin concentration (from 10 to 77 μU/ml) with minimal systemic perturbations. The fractional synthesis rate of muscle protein significantly increased following the insulin infusion compelling the authors to conclude that insulin promoted muscle anabolism, primarily by stimulating

protein synthesis (Biolo et al., 1995). Hillier, Fryburg, Jahn, & Barrett (1998) raised forearm insulin concentrations 1,000-fold above basal levels while maintaining euglycemia for a four hour period. The resultant extreme hyperinsulinemia strongly reversed postabsorptive muscle's phenylalanine balance from a net release to a net uptake. The authors concluded that this marked anabolic effect resulted from a dramatic stimulation of protein synthesis and a modest decline in protein degradation. To help explain the mechanism behind the physiological hyperinsulinemia's effect on increasing protein synthesis, Hillier et al. (2000) measured the phosphorylation of p70s6k and 4E-BP1 (two key proteins that regulate messenger ribonucleic acid translation and protein synthesis and discussed above) in healthy adults following an insulin infusion. The insulin infusion elevated baseline insulin levels from 6 to 53 μU/ml and significantly increased p70s6k, but did not affect 4E-BP1 phosphorylation in muscle.

While it appears that normal, physiological elevations in insulin alone fails to increase protein synthesis *in vivo*, when amino acids (particularly leucine) are administered along with elevations in insulin, elevations in protein synthesis (and activation of key insulin signaling molecules) are observed. Preceding a discussion on the synergistic relationship between leucine and insulin will be a brief review of how leucine ingestion alone impacts protein synthesis and its regulation of key enzymes associated with translation initiation.

Leucine Ingestion and Protein Synthesis

Isolated Rat Muscle Preparations

Emerging data suggests that leucine supplementation ingested shortly following physical activity may enhance the anabolic status of the post-exercise period. It appears

that the effects that leucine exerts on the anabolic status are attributed to increases in protein synthesis. In a comprehensive review, Layman (2002) stated that: "The first evidence that leucine could stimulate muscle protein synthesis appeared in the 1970's. Using isolated diaphragm muscle and perfused hindlimb preparations, researchers demonstrated that supplementing the plasma or media with a complete mixture of essential amino acids stimulated protein synthesis (Fulks, Li, & Goldberg, 1975; Li & Jefferson, 1978). Further evaluation (utilizing isolated rat muscle) of the impact of individual amino acids revealed that the stimulatory effect of the complete mixture could be reproduced by the single amino acid leucine (Buse & Reid, 1975; Hong & Layman, 1984; Tischler, Desautels, & Goldberg, 1982)."

Animal Models

The first study to demonstrate that orally administered leucine stimulates recovery of skeletal muscle protein synthesis after exercise was conducted by Anthony, Anthony, & Layman, 1999). Using multiple groups of exercised rats (food deprived, carbohydrate only, leucine only) and comparing them to a sedentary, food deprived group of rats serving as the control group, the investigators required that the non-control groups of rats run on a motorized treadmill for two hours at 36 meters/min. The two-hour exercise bout reduced skeletal muscle protein synthesis 18% as compared to the sedentary, food deprived group of rats. Immediately following the exercise bout, the carbohydrate and leucine meals were administered by oral gavage. The amount of leucine given to the rats was 270 mg. Feeding carbohydrate alone (which increased insulin levels about 3-fold) did not promote recovery of muscle protein synthesis. In contrast, the rats fed leucine

only following the acute exercise bout reversed the depression in protein synthesis one hour after the exercise bout (Anthony et al., 1999).

In an attempt to elucidate a potential mechanism for leucine's effect on restoring protein synthesis, Anthony, Anthony, Kimball, Vary, & Jefferson (2000) conducted a similar study. Male rats were freely fed (protein synthesis control group) or food deprived for 18 hours. Following the 18 hours of food deprivation (which resulted in a decrease in protein synthesis), rats were administered saline, carbohydrate only, or leucine only (1,350 mg/kg of bodyweight). One hour after the meal administration, the authors reported that provision of carbohydrate alone did not affect muscle protein synthesis. In contrast, administration of leucine alone stimulated complete recovery of muscle protein synthesis as compared to the freely fed rats. In addition to measuring rates of protein synthesis, the authors also investigated potential mechanisms responsible in the protein synthetic machinery by measuring the activity (phosphorylation status) of the translational repressor 4E-BP1 as well as p70S6K. It was reported that administration of leucine resulted in a three-fold greater 4E-BP1 phosphorylation (thereby allowing the formation of the ternary complex eIF-4F and subsequently allowing the binding of mRNA to the 40S ribosomal subunit) as compared to the carbohydrate only and saline groups. In relation to activation of p70S6K, it was reported that leucine also activated (phosphorylated) p70S6K (Anthony, Anthony et al., 2000).

Given the results of the studies conducted by Anthony, Anthony et al. (2000) and Anthony et al. (1999) in which leucine administration resulted in increases in protein synthesis via 4E-BP1 and p70S6K phosphorylation, Anthony, Yoshizawa et al. (2000) conducted another study to determine the role of mTOR (which is directly upstream of both of these proteins; Figure 4) and its response to leucine administration in rats. To

investigate the role of mTOR signaling in the stimulation of translation initiation and protein synthesis *in vivo*, food-deprived rats were injected with rapamycin, a specific inhibitor of mTOR, two hours prior to leucine administration (1,350 mg/kg of bodyweight). The authors reported that rapamycin inhibited protein synthesis in the leucine-treated rats. Additionally, rapamycin prevented the stimulatory effects of leucine on hyperphosphorylating both 4E-BP1 and p70S6K. These observations led the authors to conclude that leucine-dependent stimulation of translation initiation *in vivo* occurs via a rapamycin-sensitive pathway (via mTOR) (Anthony, Yoshizawa et al., 2000).

A second aim of this study was to determine whether leucine is unique among the BCAAs in its ability to stimulate protein synthesis in skeletal muscle of food-deprived rats (Anthony, Yoshizawa et al., 2000). In order to determine this, food-deprived rats were orally administered leucine, isoleucine, valine (at a dosage of 1,350 mg/kg of bodyweight) or saline. From this intervention, only leucine stimulated protein synthesis relative to the saline-treated controls. In contrast, neither valine nor isoleucine administration affected rates of protein synthesis. Leucine was also the most effective among the BCAA in its ability to hyperphosphorylate 4E-BP1, increasing its phosphorylation state five-fold greater when compared to controls. Isoleucine was only able to increase the phosphorylation state of 4E-BP1 three-fold, and valine did not increase 4E-BP1 phosphorylation. In relation to p70S6K, leucine was again the most effective BCAA in its ability to stimulate p70S6K phosphorylation. Isoleucine administration also promoted phosphorylation of p70S6K, but to a lesser extent than leucine. Valine did not alter p70S6K phosphorylation compared with control rats (Anthony, Yoshizawa et al., 2000). The authors concluded that leucine is unique among the BCAA in its ability to stimulate protein synthesis in muscle of food-deprived rats.

In each of these aforementioned studies using food-deprived rats, the amount of leucine supplementation was 1,350 mg/kg of bodyweight (which is considerably higher than the supplemental leucine given to humans when performance variables were assessed). For comparison, these studies supplemented humans with 200 mg/kg (Pitkanen et al., 2003), 45 mg/kg (Crowe et al., 2006), and 50 mg/kg (Mero et al., 1997) of bodyweight. To elucidate the minimal dose of leucine required to stimulate protein synthesis in skeletal muscle and to identify the biomarkers (4E-BP1 and p70S6K) of mRNA translation that mediate such a response, Crozier, Kimball, Emmert, Anthony, & Jefferson (2005) administered (by oral gavage) leucine at concentrations ranging from 68 mg/kg to 1,350 mg/kg of bodyweight to rats who were food-deprived for 18 hours. Specifically, there were five levels of leucine supplementation (68, 135, 338, 675, and 1,350 mg/kg of bodyweight) and a control group (which were administered NaCl). Protein synthesis (as measured in the gastrocnemius 30 minutes following supplementation) was increased by 31, 30, 37, and 43% as compared to control values at the 135, 338, 675, and 1,350 mg/kg of bodyweight, respectively. Protein synthetic rates were significantly elevated in all groups except the 68 and 338 mg/kg of bodyweight groups [probably due to the small sample size in the 338 mg/kg group (n = 6 vs. 10-12)]. 4E-BP1 phosphorylation was increased in the gastrocnemius of rats fed leucine compared to controls, with significant differences from controls observed at all doses of leucine aside from the lowest dose (68 mg/kg bodyweight). In addition, the dissociation of 4E-BP1 from eIF-4E was observed 30 minutes after supplementation and was significant in all doses of leucine, even the 38-mg/kg dose. The phosphorylation of p70S6K was significantly greater than control values at all doses investigated. The authors concluded that the ability of small doses of leucine supplementation that resulted in significant

elevations in protein synthesis suggests that future research on the regulation of skeletal muscle protein synthesis by orally administered leucine will be feasible in humans (Crozier et al., 2005). The following section discusses those investigations that have supplemented leucine in humans and that have measured changes in the rates of protein synthesis.

Human Models

There are few, if any, studies that have investigated the effects of leucine supplementation alone on protein synthesis in humans. There are however, multiple studies that have investigated the impact of BCAAs on protein synthesis as well as additional leucine being added to a total protein supplement with subsequent analysis of protein synthesis or relevant translation initiation enzyme markers. As Mero (1999) had indicated in his review, caution must be taken when discussing the impact of the results of these types of studies as the outcomes may not be completely explained by the leucine that is supplemented along with the other amino acids or proteins. Following is a discussion of a few of these types of studies with the exception of those studies that also administered a carbohydrate source – these studies will be discussed in the following section (see *Synergism between Leucine and Insulin relative to Protein Synthesis* below).

One of the first studies to look at the effects of leucine on the Akt/mTOR pathway in humans was conducted by Greiwe, Kwon, McDaniel, & Semenkovich (2001). This study tested the hypothesis that leucine and insulin stimulate translation initiation in human skeletal muscle by phosphorylating p70S6K. The morning after an overnight fast, subjects received a leucine infusion (into the antecubital vein) at a constant rate of 1 gram per hour for two hours (equating to approximately 28 mg/kg of bodyweight).

Phosphorylation of p70S6K increased 4-fold in response to leucine alone, indicating that physiological concentrations of leucine activate a key mediator of protein synthesis in human skeletal muscle (Greiwe et al., 2001).

Rieu et al. (2006) designed an investigation to assess the effects of dietary leucine supplementation on muscle protein synthesis and whole body protein kinetics in elderly individuals. Twenty elderly men (~70 years of age) were studied in the postabsorptive state after an overnight fast. The men were divided into two groups: a control diet and a leucine supplemented diet. Both diets provided 10.2 kcal, 0.4 g casein protein, 1.3 g carbohydrate (maltodextrin) and 0.36 g fat (vegetable oil) per kg of bodyweight. The leucine group was supplemented with 52 mg/kg of bodyweight and was also supplemented with isoleucine (11.6 mg/kg) and valine (6.8 mg/kg) to maintain their plasma levels at postprandial values. Also, the control diet was supplemented with alanine (71 mg/kg) in order to supply the same amount of nitrogen as the leucine diet. The respective diets were administered for 5 hours and were ingested as 15 small meals (aliquots of 50 ml) given every 20 minutes. The authors reported that leucine supplementation improved myofibrillar muscle protein fractional synthesis rate measured at the end of the feeding period (0.083 +/- 0.008 versus 0.053 +/- 0.009% per hour, in the leucine and control groups, respectively, $P < 0.05$). Further, the authors concluded that the observed effect was due only to increased leucine availability because only plasma free leucine concentration significantly differed between the control and leucine-supplemented groups (Rieu et al., 2006).

Similarly, Katsanos, Kobayashi, Sheffield-Moore, Aarsland, & Wolfe (2006) enriched an essential amino acid (EAA) mixture with leucine and measured the fractional synthetic rate in a cohort of young and elderly subjects. The EAA mixture contained

26% leucine and the leucine enriched EAA mixture contained 41% leucine. Fractional synthetic rate increased following amino acid ingestion in both the 26% and the 41% in the young groups. In contrast, in the elderly, fractional synthetic rate did not increase following ingestion of 26% leucine EAA mixture but did increase following ingestion of the 41% leucine EAA mixture. The authors concluded that increasing the proportion of leucine in a mixture of EAA can reverse an attenuated response of muscle protein synthesis in elderly but does not result in further stimulation of muscle protein synthesis in young subjects (Katsanos et al., 2006). Figure 5 summarizes the role that leucine exerts on the Akt/mTOR pathway leading to enhanced protein synthesis.

Synergism between Leucine and Insulin relative to Protein Synthesis

In his comprehensive review titled '*Role of leucine in protein metabolism during exercise and recovery*', Layman (2002) stated that: "It appears that leucine has a synergistic role with insulin as regulatory factors in the Akt/mTOR signal transduction pathway. Insulin serves to activate the signal pathway, while leucine is essential to enhance or amplify the signal for protein synthesis at the level of peptide initiation factors". As was shown above (Hillier et al., 2000), insulin is often not sufficient alone to stimulate muscle protein synthesis in postabsorptive conditions unless supraphysiological levels of insulin (> 100 μIU/mL) are provided (Layman, 2002). Also, leucine has been repeatedly shown to increase protein synthesis, even in the absence of elevated levels of insulin, but multiple studies have shown that there is an enhanced synthetic protein rate when insulin levels are increased in conjunction with leucine supplementation (Anthony et al., 1999; Anthony et al., 2002; Crozier et al., 2005; Greiwe et al., 2001). In another

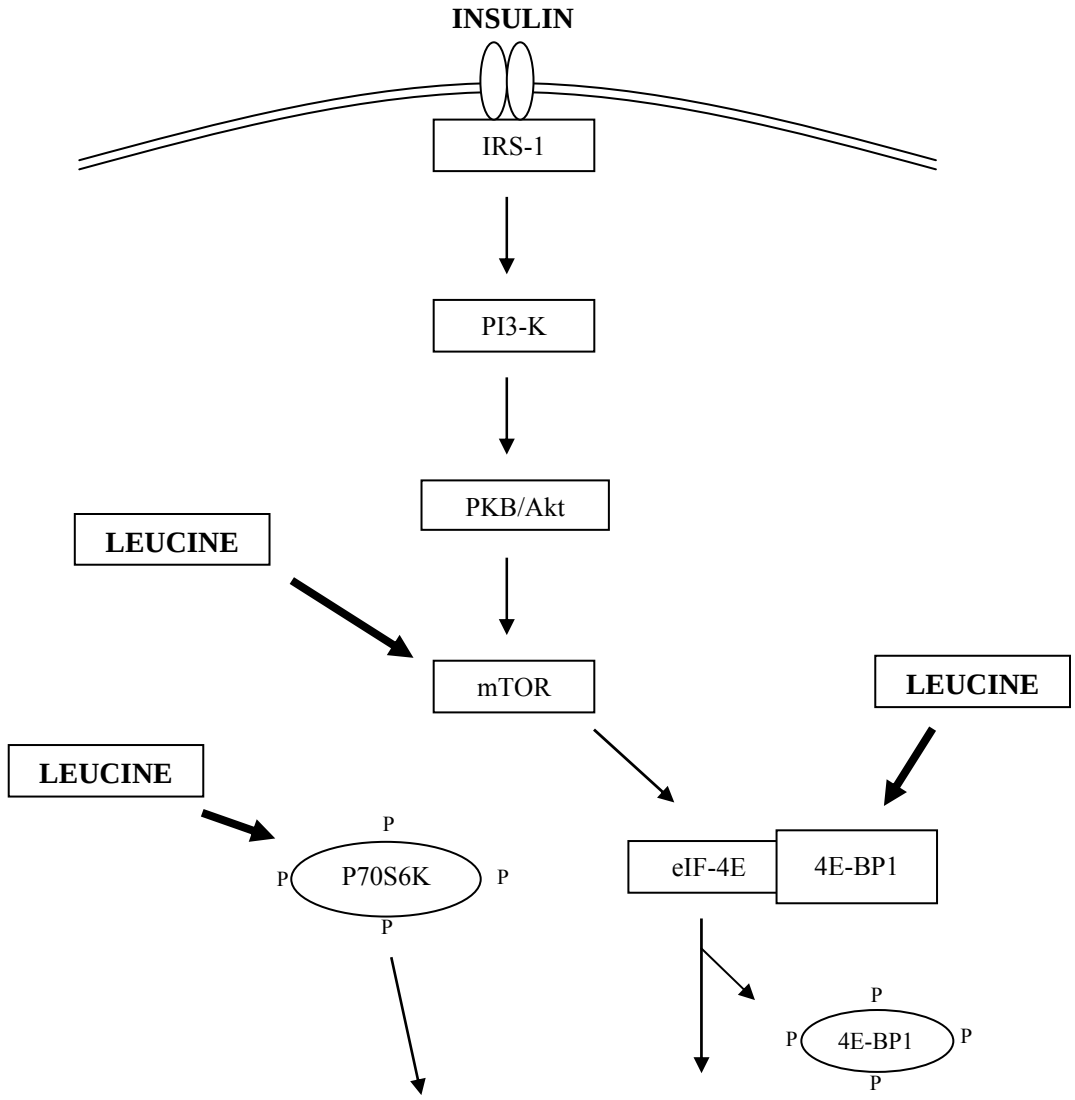

Figure 5. The effects of leucine on the Akt-mTOR pathway leading to increases in protein synthesis via translation initiation. IRS-1 = insulin receptor substrate; PI3-K = phosphatidylinositol-3 kinase; PKB/Akt = protein kinase B (also known as Akt); mTOR = mammalian target of rapamycin; $P70^{S6}K$ = ribosomal protein S6 kinase; eIF-4E = eukaryotic initiation factor-4E; 4E-BP1 = eIF-4E binding protein 1. P = phosphorylation.

review article, Kimball et al. (2002) stated that "the full response of translation initiation and protein synthesis to increased amino acid availability following exercise is not observed in absence of a minimal concentration of insulin". Following are specific investigations demonstrating the synergistic relationship that exists between insulin and

leucine relative to protein synthesis or enzymatic markers associated with protein synthesis in both animal and human models.

Animal Models

Anthony et al. (1999) compared four groups of exercised rats (food deprived, carbohydrate only, leucine only, and carbohydrate plus leucine) to a sedentary, food-deprived group of rats serving as the control group. Each group of rats performed a two-hour aerobic exercise regimen. Immediately following the exercise bout, the carbohydrate (2.63g), leucine (270 mg), and carbohydrate plus leucine (which was isocaloric with the carbohydrate meal and insonitrogenous with the leucine meal) meals were administered by oral gavage. One hour post-feeding, protein synthetic rates were measured. Feeding carbohydrate alone increased insulin levels about 3-fold. In contrast, feeding leucine alone did elevate insulin levels. Feeding a combination of carbohydrate and leucine resulted in a five-fold stimulation of plasma insulin. The two-hour endurance exercise bout induced a reduction of protein synthesis by 18% in the exercise, food deprived rats as compared to the non-exercised, sedentary fed rats. While the carbohydrate meal group did not promote recovery of muscle protein synthesis, both the leucine only group and the carbohydrate plus leucine groups reversed the depression in protein synthesis. In addition, the carbohydrate plus leucine feeding exerted the largest increases in protein synthesis.

Anthony et al. (2002) designed a similar study in order to assess the contribution of insulin to the protein synthetic response of leucine supplementation. Based on this group's previous work, they had demonstrated that leucine is responsible for phosphorylating both 4E-BP1 and p70S6K (Anthony, Anthony et al., 2000; Anthony,

Yoshizawa et al., 2000). In this study, the investigators stabilized insulin at fasting levels with the pancreatic hormone inhibitor somatostatin. Specifically, rats were food deprived for 18 hours (resulting in a reduction in protein synthesis) and were intravenously administered somatostatin one hour before administration of leucine (1,350 mg/kg bodyweight) or saline (control). Following administration of leucine, dependent variable (protein synthesis, 4E-BP1 and p70S6K phosphorylation states) assessments were made at 15, 30, 45, 60, and 120 minutes post-leucine administration. In the control group, protein synthesis was significantly increased 30 and 60 minutes after leucine supplementation. This increase in protein synthesis was inhibited by somatostatin (which inhibited elevations in insulin) in those rats that consumed the same amounts of leucine. Relative to 4E-BP1 and p70S6K phosphorylation, somatostatin partially attenuated the leucine-induced changes in 4E-BP1 and p70S6K phosphorylation. Overall, the results suggest that the leucine-induced enhancement of protein synthesis and the phosphorylation states of 4E-BP1 and S6K1 are facilitated by the transient increase in serum insulin (Anthony et al., 2002). A similar study in which insulin levels were controlled was conducted by Balage et al. (2001). Some differences in this study were the administration of BCAAs instead of leucine and the use of diazoxide injections (rather than somatostatin) to control insulin elevations in rat skeletal muscle. Regardless of these study design differences, the conclusions made by Balage et al. (2001) were comparable to Anthony et al. (2002) in that both insulin and amino acids are required to stimulate protein synthesis, inhibit protein degradation, and regulate the interactions between eIF4E and 4E-BP1 or eIF4G in response to feeding.

In the Crozier et al. (2005) study discussed above, the group of rats receiving the highest dosage of leucine supplementation (1,350 mg/kg bodyweight) resulted in the

largest elevations in insulin concentrations as compared to those groups receiving a lower dose of leucine. Subsequently, it was this group that attained the highest rates of protein synthesis, leading the authors to state that the maximal changes occurred when circulating insulin concentrations were elevated.

Human Models

There are also a few examples in human studies that demonstrate how adding insulin (or carbohydrate which subsequently increases insulin concentrations) to a leucine or protein supplement increases protein synthesis or key mediators of protein synthesis (p70S6K) in human skeletal muscle. Greiwe et al. (2001) tested the hypothesis that leucine and insulin stimulate translation initiation in human skeletal muscle by phosphorylating p70S6K. The morning after an overnight fast, the investigators infused healthy adults with leucine alone, insulin alone, or both leucine and insulin for 2 hours. The amount of leucine infused into the subjects was approximately 28 mg/kg of bodyweight. Phosphorylation of p70S6K increased 4-fold in response to leucine alone, 8-fold in response to insulin alone, and 18-fold after the leucine + insulin infusion. These results show that physiological concentrations of leucine and insulin activate a key mediator of protein synthesis in human skeletal muscle, and that there is a synergistic effect on the activation of this mediator when leucine and insulin are infused together (Greiwe et al., 2001).

Koopman et al. (2005) designed a study to determine post-exercise muscle protein synthesis following the combined ingestion of carbohydrate with or without protein and/or free leucine. Eight male subjects were randomly assigned to three trials in which they consumed drinks containing carbohydrate, carbohydrate and protein (whey protein),

or carbohydrate, protein, and free leucine following 45 minutes of resistance exercise. The resistance exercise consisted of 8 sets of 8 repetitions at 80% 1RM on a leg press machine and 8 sets of 8 repetitions at 80% 1RM on a leg extension machine. The three supplements were ingested during a six-hour post-exercise period and each subject received a beverage volume of 3ml /kg every thirty minutes to ensure a given dose of 0.3 g carbohydrate, 0.2 g/kg of protein, and 0.1 g/kg of free leucine corresponding with the group placement. Following this protocol, it was reported that plasma insulin response was significantly higher in the carbohydrate-protein-leucine group compared with the carbohydrate only and carbohydrate-protein trials. In relation to fractional synthetic rate measured over the six-hour period of post-exercise recovery, there was a significant elevation in the carbohydrate-protein-leucine trial as compared to the carbohydrate only trial with intermediate values observed in the carbohydrate-protein trial (but not significantly different than the carbohydrate only group). The authors concluded that coingestion of protein and leucine with carbohydrate stimulates muscle protein synthesis compared with the intake of carbohydrate only (Koopman et al., 2005). Further, this observation was made in an environment in which insulin levels were elevated to the greatest extent, thereby adding to the evidence that leucine has a synergistic role with insulin as regulatory factors in increasing protein synthesis.

Koopman et al. (2006) conducted another study in which two groups of supplements were compared: carbohydrate only and carbohydrate plus protein and free leucine. Instead of utilizing a resistance exercise bout as in their previous study, they introduced simulated activities of daily living for a 30-minute time period in both elderly and young men and measured rates of protein synthesis resulting from these activities and ingestion of the supplements. The results indicated that mixed-muscle protein synthesis

rates were significantly greater in the carbohydrate plus protein and free leucine than in the carbohydrate only experiment in both the young and the elderly subjects, with no significant differences between groups. This study adds to the growing amount of scientific evidence that insulin alone (induced by carbohydrate ingestion) is not sufficient to increase rates of protein synthesis, but rather is optimized when protein and free leucine are added to the nutritional bolus.

Nutritional Interventions and Gene Transcription Events in Conjunction with Exercise

As was stated above, attaining a positive net protein balance (which can ultimately lead to increases in skeletal muscle hypertrophy) is regulated by at least three major molecular processes: 1) satellite cell activity; 2) gene transcription; and 3) protein translation. Numerous scientific investigations discussed above have demonstrated consistently that the amino acid leucine is able to increase rates of protein translation via enhancing translation initiation. Additionally, it was well demonstrated above that leucine has a synergistic role with insulin as regulatory factors in the Akt/mTOR signal transduction pathway, which are associated with increases in protein translation. Relative to one of the other regulatory mechanisms responsible for skeletal muscle hypertrophy, gene transcription, much less scientific research has been conducted in response to exercise (particularly resistance exercise). Also, research investigating nutritional interventions and the subsequent effects that it may induce on regulation of gene transcription in response to exercise are nearly non-existent. Therefore, any scientific inquiry investigating nutritional interventions in this area would be novel. Following is a review of one of the cell-signaling pathways (mitogen activated protein kinase pathway) that is believed to exert transcriptional control in response to exercise.

Mitogen Activated Protein Kinases (MAPK)

Overview

Signal transduction pathways that have been shown to exert transcriptional control include calcineurin, CaMK, MAPK, PKC, NF-κB, and AMPK (Wackerhage & Woods, 2002). Signal transduction is used to describe the transfer of signals and stresses form the outside or inside of the cell usually by kinase or phosphatase cascades or other signalling processes to cytosolic or nuclear targets (Wackerhage & Woods, 2002). These targets either change cellular processes such as energy metabolism or regulate genes, which results in a changed muscle phenotype (Wackerhage & Woods, 2002). One of the primary ways in which these signal transduction pathways exert their effects on transcription is via nuclear localization, in which a nuclear localization signal is activated and translocated into the nucleus. Inside the nucleus, the signaling protein either acts as a transcription factor itself or it activates transcription factor that will bind regulatory DNA sequences. Ultimately, the activation of transcription factors leads to an increase in (muscle specific) gene expression, which subsequently may lead to an increase in translation. One such signal transduction/cell signalling pathway under intense study relative to exercise is the mitogen activated protein kinases (MAPK) pathway.

Evidence for the role of activated MAPK (specifically ERK1/2 – discussed below) affecting the phenotypic control of skeletal muscle has been demonstrated repeatedly (Higginson et al., 2002; Murgia et al., 2000; Shi et al., 2007). More specifically, some of these studies implicate the MAPK signal transduction pathway in promoting fiber type shifts in skeletal muscle (Higginson et al., 2002; Murgia et al., 2000). It cannot be overstated that there is very little research on the MAPK signal transduction pathway in human skeletal muscle, with most of the scientific literature

conducted in cell culture and intact animal models. Following an overview of the MAPK cell-signalling pathway, an attempt will be made to focus on those studies that utilized an exercise intervention and used human subjects.

The MAPK cascade is a ubiquitously expressed intracellular network of proteins that form a major signaling system by which cells transduce extracellular cues into intracellular responses (Aronson et al., 1997; Seger & Krebs, 1995). The MAPK pathways are specifically classified into three main pathways: the c-Jun NH2-terminal kinase (JNK) (also known as stress-activated protein kinases), the p38 family, and the extracellular signal-related kinase (ERK 1/2) pathways (Widegren et al., 2001). The JNK and p38 MAPK pathways are referred to as stress-activated kinases, and ERK1/2 are described as kinases involved in growth factor stimulation (Cohen, 1997; Widegren et al., 2000). The ERK pathway has been shown to be activated (phosphorylated) in response to mechanical stress, with studies reporting an increase in ERK 1/2 phosphorylation after running (Yu, Blomstrand, Chibalin, Krook et al., 2001) and cycling (Aronson et al., 1997; Krook et al., 2000; Osman et al., 2000; Widegren et al., 1998). More recently, an increase in ERK 1/2 phosphorylation has been shown in response to resistance exercise in humans (Creer et al., 2005; Karlsson et al., 2004; Williamson et al., 2003).

Raf-MEK-ERK Pathway (ERK1/2)

Each MAPK cascade contains at least three protein kinases in series that culminate in the activation of a multi-functional MAP kinase. MAP kinases are regulated by phosphorylation cascades (Pearson et al., 2001). Two upstream protein kinases activated in series lead to activation of a MAP kinase, and additional kinases may also be required upstream of this three-kinase module (Xu et al., 1997). Relative to the ERK

pathway (known as the Raf-MEK-ERK pathway and mitogen-activated protein (MAP) kinase cascade), the three protein kinases in series are MAPK kinase kinase (also known as Raf), MAPK kinase (also known as MEK), and MAPK (also known as ERK).

ERK1 and ERK2 are proteins of 43 and 41 kDa that share approximately 85% sequence homology (Pearson et al., 2001). The two residues (tyrosine and threonine) which are phosphorylated and consequently activate the kinases are separated by a glutamate residue in both ERK1 and ERK2 (Osman et al., 2000). ERK1 and ERK2 are activated by a pair of closely related MEKs, MEK1 and MEK2 (MAPK kinase). MEK1 and MEK2 also share approximately 85% sequence homology, but have unique inserts in their C-terminal domains (Pearson et al., 2001). MEK1 (43.5 kDa) contains 393 amino acid residues and MEK2 (44 kDa) contains 400 amino acid residues (Zheng & Guan, 1993a). The MEKS are phosphorylated/activated by a family of protein kinases known as the Raf family. The Raf family of kinases is composed of A-Raf, B-Raf, and Raf-1. Of the 3 Rafs, Raf-1 has been the most studied. Raf-1 (74 kDa) is a cytoplasmic serine/threonine protein kinase that transduces signals from the cell surface receptors to other cytoplasmic members of the MAPK pathway (directly phosphorylating MEK) proteins and ultimately to the nucleus (Pumiglia et al., 1995). In summary, activated Raf directly phosphorylates and activates MEK, which in turn directly phosphorylates and activates ERK.

Nuclear Substrates of ERK1/2 Relative to Skeletal Muscle Hypertrophy

There are several cellular substrates that have been identified for ERK1/2. ERK1/2 has been shown to target membrane proteins, cytoplasmic proteins, and nuclear proteins such as transcription factors. Unlike Raf-1 and MEK, when ERK1/2 is

stimulated it is relocalized from the cytoplasm to the nucleus. Since many of the nuclear substrates of MAPK are transcription factors, MAPK nuclear translocation is thought to represent a crucial step in the modulation of gene expression (Brunet et al., 1999).

Relative to ERK1/2 activation of transcription factors, some of the most studied are AP-1 and Elk1. A consequence of MAPK activation is the formation of AP-1 (Activator Protein-1), which binds to the promoter regions of immediate response genes governing cell proliferation and differentiation (Hess, Angel, & Schorpp-Kistner, 2004). The transcriptionally active complex AP-1 is formed by the dimerization of Jun and Fos proteins (Hess et al., 2004). Elk-1 (Ets-like protein-1) is a member of the Ets family of transcription activators and its activation depends on phosphorylation of Ser383 and Ser389 in the transcriptional activation domain by ERK1/2 (Li et al., 2003). In the nucleus, Elk-1 forms a ternary complex with the serum response factor (SRF) protein and the serum response element promoter region (Barrett et al., 2006). In animal models, phosphorylation of Elk-1 by the ERK1/2 pathway is necessary for c-fos gene activation during cardiac myocyte hypertrophy (Babu, Lalli, Sussman, Sadoshima, & Periasamy, 2000). At this point, there is not enough scientific data to directly relate the transcription factors AP-1 and Elk1 (which are activated by ERK1/2) to an increase in skeletal muscle hypertrophy. Since both of these transcription factors have been associated with cell proliferation, their role in skeletal muscle hypertrophy is most likely an indirect one due to the fact that human skeletal muscle fibers hypertrophy rather than proliferate. Because there is very little research relative to AP-1 and Ekk-1 in human skeletal muscle, more research needs to be conducted on these transcription factors and the associated cellular responses.

Protein Kinase Substrates of ERK1/2 Relative to Skeletal Muscle Hypertrophy

Another potential role for ERK1/2 relative to skeletal muscle hypertrophy is its activation of specific protein kinases. The direct downstream kinases that are activated by ERK1/2 are comprehensively called the MAPK activated protein kinase (MAPKAPK) family. This family of kinases activated by ERK1/2 can be categorized into three subclasses: the RSKs (Rsk1, Rsk2, Rsk3), the Mnks (Mnk1 and Mnk2), and the MSKs (Msk1 and Msk2).

The RSK (or p90rsk) family includes three members, RSK1-3, which shows 75-80% homology at the amino acid level (Pan, Devaux, & Ray, 2004). Of the RSKs, RSK1 (heretofore referred to as p90rsk) has been the most studied and is directly phosphorylated by ERK1/2 in the cytosol. p90rsk (RSK1) is an S6 protein kinase that may be associated with the stimulation of protein synthesis (Chen, Tung, Abate, & Blenis, 1993; Frodin & Gammeltoft, 1999; Gavin & Schorderet-Slatkine, 1997). p90rsk itself, once phosphorylated, is able to translocate to the nucleus and phosphorylate Fos proteins and Elk1 (Chen, Abate, & Blenis, 1993; Chen, Sarnecki, & Blenis, 1992; Chen, Tung et al., 1993). In response to exercise of various modes such as resistance training (Creer et al., 2005), endurance exercise (Yu, Blomstrand, Chibalin, Krook et al., 2001), and cycling exercise (Aronson et al., 1997), p90rsk activity has been shown to increase along with the upstream components of the ERK1/2 MAPK pathway in humans. An important substrate of p90rsk relative to translation is ribosomal protein 6 (which was responsible for giving the enzyme its name) (Seger & Krebs, 1995). However, the physiological role of p90rsk in this phosphorylation may be limited, as other studies show that most of the ribosomal S6 phosphorylation in vivo occurs by another protein kinase,

p70rsk, which is activated by a different pathway (the Akt-mTOR pathway) (Seger & Krebs, 1995).

The other two families of kinases, the Mnks and MSKs, when phosphorylated by ERK1/2, are associated with gene transcription and protein translation (Aronson et al., 1997; Creer et al., 2005; Pyronnet, 2000; Waskiewicz et al., 1999; Williamson et al., 2003). Mnk1 is a protein kinase that is involved in the regulation of protein synthesis through its phosphorylation of eukaryotic translation initiation factor 4E (eIF4E) at Ser209 (Ueda, Watanabe-Fukunaga, Fukuyama, Nagata, & Fukunaga, 2004). eIF4E is a key participant in regulation of translation initiation, serving as the mRNA 5' cap-binding protein. eIF4E and its phosphorylation are generally enhanced by agents that activate translation (Wang et al., 1998). Also, eIF4E phosphorylation may favor its entry into initiation complexes (Wang et al., 1998; Waskiewicz, Flynn, Proud, & Cooper, 1997) by strengthening this initiation factor's affinity for 7-methylguanosine cap structures, directing ribosomes to the 5' ends of mRNAs and enhancing translation efficiency and potentially upregulating protein synthesis. However, other investigations (Flynn & Proud, 1996) have shown that increased phosphorylation of eIF4E does not lead directly to the release of 4E-BP1, a condition in which entry into initiation complexes would not be favored (Ueda et al., 2004). In addition, Knauf, Tschopp, & Gram (2001) have also demonstrated that eIF4E phosphorylation is not crucial to the formation of the initiation complex.

Similar to Mnks, MSKs (mitogen- and stress-activated protein kinase) are kinases activated downstream of the ERK1/2 pathway. There are 2 isoforms of MSK in mammalian cells, termed MSK1 and MSK2. The MSKs are localized to the nucleus and are required for the phosphorylation of cAMP response element-binding protein (CREB

(cAMP response element-binding protein) and histone H3 (Darragh et al., 2005). CREB is a transcription factor that has been shown to play a key role in learning and memory (Lonze & Ginty, 2002; West, Griffith, & Greenberg, 2002). Another transcription factor substrate that is activated by MSK phosphorylation is AP-1. MSKs also phosphorylate histone H3. Histone phosphorylation is associated with the immediate early genes c-fos and c-myc, and plays a part in chromatin remodeling and transcription of these genes (Dunn, Espino, Drobic, He, & Davie, 2005). An overview of the ERK1/2 MAPK and its downstream nuclear and protein kinase substrates is shown in figure 6.

Activation of the MAPK Pathway via Growth Factor Stimulation

The MAPKs are central transducers of extracellular signals from hormones, growth factors, cytokines, and environmental stresses (Canagarajah, Khokhlatchev, Cobb, & Goldsmith, 1997). The three main hormones/growth factors that have been shown to activate the ERK1/2 MAPK pathway are growth hormone, insulin-like growth factor-1 (IGF-1), and insulin. Literature to support the effects of growth hormone activating the ERK1/2 signalling cascade have been demonstrated repeatedly in adipocytes (Anderson, 1992; Campbell, Pang, Miyasaka, Saltiel, & Carter-Su, 1992; Hodge et al., 1998; Winston & Bertics, 1992). In a recent study that utilized in vivo human skeletal muscle, the investigators gave an intravenous bolus of growth hormone and monitored the subsequent growth hormone signaling pathways (Jorgensen et al., 2006). As a result of the growth hormone bolus, activation of MAPK was observed in several lysates but without growth hormone dependency, compelling the investigators to conclude that the direct GH effects in muscle need further characterization (Jorgensen et al., 2006). Clinical studies demonstrating the role of IGF-1 activating ERK1/2 MAPK pathways

have been conducted in human skeletal muscle cell cultures (Foulstone et al., 2004), zebrafish embryonic cells (Pozios, Ding, Degger, Upton, & Duan, 2001), and trout muscle cells (Castillo, Ammendrup-Johnsen, Codina, Navarro, & Gutierrez, 2006). Insulin has also been shown to activate the ERK1/2 MAPK pathway. This effect has been demonstrated in rat skeletal muscle (Goodyear, Chang, Sherwood, Dufresne, & Moller, 1996; Napoli et al., 1998; Wojtaszewski, Lynge, Jakobsen, Goodyear, & Richter, 1999), rat mucosal (Marandi et al., 2001) and hepatomal cells (Xu et al., 2006), and rat adipocyte cell cultures (Martinez-deMena & Obregon, 2005). There has also been at least one clinical investigation conducted in humans in which elevations in insulin resulted in increases in ERK1/2 MAPK activation (Osman et al., 2000). In this study, seven healthy males underwent a 30-minute insulin infusion ($40mU \cdot m^{-2} \cdot min^{-1}$). In addition, skeletal muscle biopsies were taken pre and post insulin infusion. After the insulin infusion, ERK1/2 phosphorylation increased 141% and MEK1 phosphorylation increased 161%.

Cross-talk between Akt/mTOR and MAPK by Insulin

There are two hypotheses that explain how insulin signaling (and subsequent Akt/mTOR signaling) increases ERK1/2 MAPK activation directly downstream of the insulin receptor (IR). The two hypotheses differ in insulin's downstream substrate activation. Upon binding to insulin, the intrinsic kinase activity of the receptor is increased, and the IR becomes activated via autophosphorylation on several tyrosine residues located on the cytoplasmic portion of the β subunits (Cheng et al., 2002; White & Kahn, 1994). Subsequently, these phosphotyrosine residues, in their surrounding

sequence context, recruit signaling molecules containing SH2 (src homology 2) domains (Cheng et al., 2002).

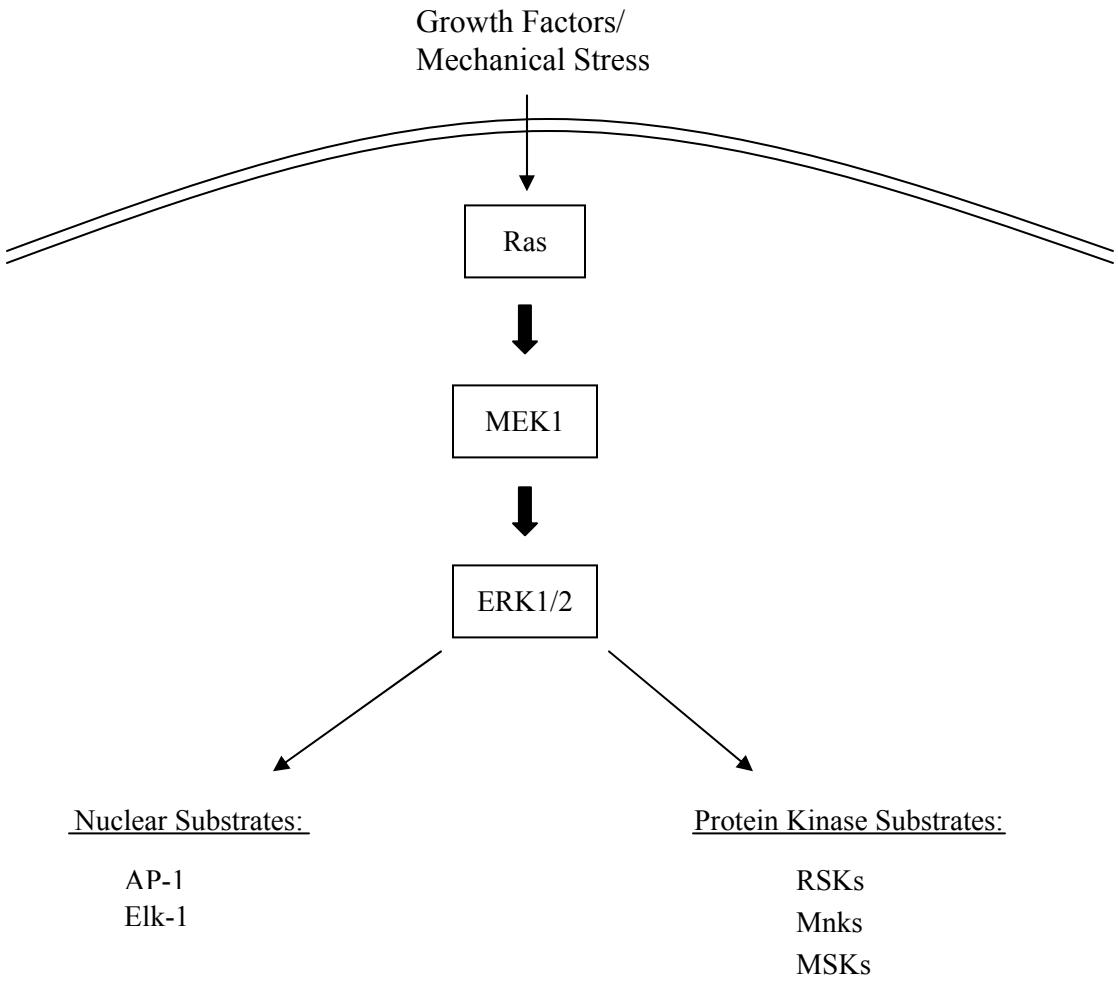

Figure 6. Overview of MAPK cell-signaling.

Shc (src homology 2/α collagen related) contains a SH2 domain and is a member of the adaptor protein family that apparently participates in multiple signal transduction pathways (Bourne, 1995). Once phosphorylated, Shc proteins may couple to the Ras signaling pathway through the formation of a Shc-Grb2-Sos complex (Cheng et al., 2002; Egan et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1992). The other downstream substrate that the IR may recruit resulting in subsequent MAPK activation is the protein

tyrosine phosphatase Shp-2. Shp-2 is a widely expressed cytoplasmic tyrosine phosphatase with two SH2 domains (Shi et al., 1998). Shp-2 activation has been shown repeatedly to activate the ERK1/2 MAPK pathway in non-human cell cultures, although the exact mechanism is debated and not yet fully understood (Noguchi, Matozaki, Horita, Fujioka, & Kasuga, 1994; Shi et al., 1998; Shi et al., 2000; Takada et al., 1998). More research needs to be conducted relative to Shp-2 and its role in mediating crosstalk between the PI3-K/Akt/mTOR and MAPK pathways.

Activation of the MAPK Pathway via Mechanical Stress

In addition to growth factor stimulation, the other well documented means of activating the ERK1/2 MAPK pathway is in response to mechanical stress (i.e. contraction). While ERK1/2 MAPK activity has been demonstrated in response to running exercise (Yu, Blomstrand, Chibalin, Krook et al., 2001; Yu, Blomstrand, Chibalin, Wallberg-Henriksson et al., 2001) and cycling exercise (Aronson et al., 1997; Krook et al., 2000; Osman et al., 2000; Richter et al., 2004; Widegren et al., 1998; Widegren et al., 2000; Yu et al., 2003), the following will discuss only those studies that have investigated the ERK1/2 MAPK response following resistance exercise in human subjects. Also, some of these studies investigated other aspects of cell signaling in addition to ERK1/2 MAPK, but the following discussion will only focus on those signal transduction events within the ERK1/2 MAPK pathway.

Williamson et al., (2003) examined the activation (phosphorylation) of ERK1/2 MAPK at rest and following exercise in both young (18-30 yrs) and elderly (70-80 yrs) subjects. The resistance exercise session consisted of knee extensions for 3 sets of 10 repetitions at 70% 1RM with 3-minute rest periods between sets. Skeletal muscle biopsy

samples were taken from the vastus lateralis muscle prior to and immediately after the resistance exercise session. Under resting conditions, the elderly men displayed significantly higher phosphorylation status of ERK1/2. The authors reported that the resistance exercise bout caused a 73% significant increase in ERK1/2 phosphorylation in the young men, but significantly decreased by 49% in the elderly group. This dichotomy was most likely explained by the apparent differences observed at rest (Williamson et al., 2003).

Thompson and coworkers (Thompson, Maynard, Morales, & Scordilis, 2003) examined ERK activation in response to 50 maximal voluntary eccentric contractions (MVC) of the elbow flexors performed with the dominant arm using a modified preacher curl exercise apparatus. Specifically, the subjects were instructed to resist maximally against the downward motion of a lever, allowing for an MVC throughout the entire range of motion. Each subject performed two sets of 25 MVCs at a constant rate of one contraction every 15 seconds with 2 minutes of rest between sets. The pre-exercise biopsy was taken from the dominant arm 2-3 weeks prior to the exercise bout and the post-exercise biopsy was taken from the non-dominant arm 48 hours post-exercise. This type of resistance exercise resulted in a significant increase (200%) in ERK activation. This data demonstrates that in humans, ERK activation is significantly elevated 2 days after high-force eccentric contractions of the elbow flexors (Thompson et al., 2003).

Creer et al., (2005) investigated the influence of muscle glycogen availability on ERK1/2 signaling after resistance exercise. To investigate this, experienced cyclists performed 30 repetitions of knee extension exercise at 70% of 1RM after a low or high carbohydrate diet (which resulted in low (~174) or high (~591) pre-exercise muscle glycogen content). Skeletal muscle biopsies were taken from the vastus lateralis before,

~20 seconds and 10 minutes following the resistance exercise bout. Immediately (~20 seconds) after the exercise bout, there were no changes in the phosphorylation status of ERK1/2 in either the low or high carbohydrate fed groups. After 10 minutes of recovery, there were significant elevations (1.2 fold) in both the low or high carbohydrate fed groups. These results demonstrate that resistance exercise increases the activation of the ERK1/2 signal transduction pathway at 10 minutes post-resistance exercise, but such activation appears to be unaffected by muscle glycogen content (Creer et al., 2005).

ERK1/2 MAPK Response to BCAA Supplementation and Resistance Exercise

The only study to date that has investigated the effects of nitrogen-containing nutrients on the ERK1/2 signal transduction pathway in conjunction with resistance exercise (RE) has been conducted by Karlsson et al., (2004). The purpose of their study was to investigate the effect of resistance exercise alone or in combination with oral intake of BCAAs on the phosphorylation state of ERK1/2. Seven healthy men performed leg press for 4 sets of 10 repetitions at a workload corresponding to 80% 1RM. There was also a 5-minute rest period between each set. Relative to the BCAA supplementation, the subjects ingested a 150ml solution of BCAA (45% leucine, 30% valine, 25% isoleucine) or flavored water at 7 time points (10 minutes and immediately before RE, during RE, and 15, 30, 60, and 90 minutes following RE). Skeletal muscle biopsies were taken at 4 time points (10 prior to RE, immediately after RE, and 1 and 2 hours following RE). Due to the effects of insulin on ERK1/2 activation, it is important to note that while the change in plasma insulin concentration during exercise was not significantly different between the placebo and BCAA groups, there was a significantly higher insulin response in the BCAA group during the 2-hour recovery period as

compared to the placebo group. The authors stated that this result was unlikely to be physiologically relevant, because this parameter was within normal variability. Relative to ERK1/2 activation, the resistance exercise bout led to a significant increase in ERK1/2 phosphorylation immediately after RE. At 1 and 2 hours post-RE, ERK1/2 phosphorylation was similar to pre-exercise levels. In relation to BCAA supplementation, the phosphorylation status of ERK1/2 was unaltered by BCAA ingestion.

Summary and Future Direction

The aforementioned review highlights the fact that leucine is a popular sports supplement due to its purported effects on cell-signaling and ability to increase rates of protein synthesis, and not solely for its acute effects on exercise performance. Multiple studies (mainly in rodent models) have demonstrated the ability of leucine to activate key regulatory elements in the Akt/mTOR signal transduction pathway, and that this regulation is amplified in the presence of minimal concentrations of insulin. Another signal transduction pathway, the ERK1/2 MAPK pathway, has been well defined in its activation following various modes of exercise including running, cycling, and resistance exercise. Currently, no studies exist examining the effects that supplemental leucine alone may have on the ERK1/2 signal transduction pathway in conjunction with resistance exercise. In fact, only two studies have been conducted (Creer et al., 2005; Karlsson et al., 2004) investigating the effects of nutritional interventions on this pathway in conjunction with resistance exercise in humans. Therefore, any clinical investigation that manipulates nutritional parameters in conjunction with resistance exercise in the

ERK1/2 MAPK pathway, specifically the well-documented cell-signaling amino acid leucine, would be novel and welcomed.

CHAPTER THREE

Methods

Participants

Thirty healthy and physically active males between the ages 19-32 participated in the study. For the purposes of the present study, physically active was defined as engaging in physical activity at least one time per week and not lower body-resistance trained (not consistently performing a lower-body resistance training program in the past year,not performing more than two lower-body resistance exercise sessions in the past 30 days, and no more than once in the two weeks prior to the investigation). Only participants considered as either low or moderate risk for cardiovascular disease and with no contraindications to exercise as outlined by the American College of Sports Medicine (ACSM) and/or who had not consumed any nutritional supplements (excluding multi-vitamins and protein supplements) one month prior to the study were allowed to participate. The participants were randomly assigned (via a random number generator) to one of three groups consisting of 10 subjects in the leucine group, 10 subjects in the branched-chain amino acid (BCAA) group, and ten subjects in the placebo group.

Entry/Familiarization and Baseline Strength Testing Session

During the familiarization session, participants completed personal and medical history questionnaires (appendices 1 and 2) and underwent a general physical examination to determine whether they met eligibility criteria. Participants meeting entry criteria were familiarized to the study protocol by way of a verbal and written explanation outlining the study design. Eligible participants who agreed to participate in the study

read and signed an informed consent statement (appendix 3) granted by the Institutional Review Board for Human Subjects of Baylor University (appendix 4). Participants were then subjected to initial strength tests using the trial-and-error method to assess their leg one repetition maximum (1RM) on the isotonic hip/leg sled (Nebula Fitness, Inc., Versailles, OH) and isotonic leg extension (Body Masters, Inc., Rayne, LA) exercises. A rest-period of approximately 150 seconds was utilized between each 1RM attempt. After the leg press 1RM was established, the leg extension 1RM was obtained in the same manner without any warm-up sets. Once the 1RM leg press and 1RM leg extension were determined, the participants were asked to perform four sets of their determined 80% 1RM on the leg press and four sets of their determined 80% 1RM on the leg extension with approximately 150 seconds of rest periods between sets and exercises to familiarize them with the protocol. At the conclusion of the entry/familiarization and baseline strength testing session, each participant was given an appointment time approximately one week later to begin the study. In addition, each participant was instructed to refrain from lower-body resistance exercise for 72 hours and to fast for 8-10 hours prior to baseline testing.

Resistance Exercise Session Protocol

Approximately one week after the subject's familiarization session, the participants engaged in an acute bout of lower body resistance exercise. Upon reporting to the laboratory, participants donated a fasted baseline/pre-exercise muscle biopsy and blood sample. After this, each participant warmed up on a stationary bicycle for five minutes and then performed two sets of leg press at 50% of 1RM. Following this, each participant performed the resistance exercise intervention with consisted of four sets of

both leg press and leg extension at 80% 1RM to failure (approximately 8-12 repetitions). If a participant was unable to accomplish at least eight repetitions on any given set, the investigators subsequently lowered the resistance enabling the participant to achieve the desired number of repetitions. This technique was performed in order to ensure that the total workload remained consistent between each of the three experimental groups. Between each set and exercise, each participant utilized a rest period of approximately 150 seconds. These rest periods constituted an approximate 1:5 work-to-rest ratio (Baechle & Earle, 2000). Total exercise time was approximately 30 minutes.

Supplementation

After baseline testing, each subject was randomized and matched according to muscle strength and body mass into one of three supplement groups (leucine, BCAA, or placebo). Specifically, the placebo supplement contained deionized water flavored with non-caloric Crystal Light®. The BCAA supplement (50% leucine, 25% isoleucine, and 25% valine) was given at a dosage of 120 mg/kg of bodyweight. The leucine supplement was given at a dosage of 60 mg/kg of bodyweight. The leucine supplement contained the same amount of leucine as the BCAA supplement and only differed from the BCAA supplement in the absence of isoleucine and valine. The overall purpose of having both a BCAA group and a leucine group was to attribute any observed changes that occur in both of these groups to the amino acid leucine alone. Each supplement was orally ingested at three time points, approximately 30 minutes apart, in equal doses dissolved in 150 ml of the same non-caloric flavored water as the placebo (Crystal Light®). After the initial blood draw and muscle biopsy, and 30 minutes prior to the resistance exercise

bout, the first dose was ingested. The second and third doses were ingested immediately pre-exercise and immediately post-exercise, respectively.

Muscle Biopsies

Immediately prior to the resistance exercise bout, but following the 8-12 hour fast, participants underwent an initial/baseline muscle biopsy. In addition to a baseline sample, subsequent muscle biopsies were extracted 30 minutes, two hours, and six hours after the exercise bout. Using a 5mm Bergstrom needle, percutaneous Biopsies (~40mg) were extracted under local anesthesia of 1% Xylocaine from the middle portion of the muscle at the midway point between the patella and the greater trochanter of the femur at a depth between 1 and 2 cm. For the remaining three biopsies, the same incision site was used and attempts were made to extract tissue from approximately the same location as the initial biopsy. After removal, the muscle biopsy samples were placed in a cryogenic storage tube and immediately frozen and subsequently stored at -80^{o}C for later analysis. Written instructions for post-biopsy care were given to the participants (appendix 5). The participants were instructed to leave the bandage over the incision for 24 hours. The participant was further advised to refrain from vigorous physical activity for 48 hours following the last biopsy procedure. If needed, the subjects were instructed to take a non-prescription analgesic medication such as Ibuprofen to relieve pain. However, medications such as aspirin, Nuprin, Bufferin, or Advil were discouraged as these medications may have lead to ecchymosis at the biopsy site.

Venous Blood Sampling

Immediately prior to the resistance exercise bout, but following the 8-12 hour fast, participants underwent an initial/baseline blood draw. Blood samples were obtained using standard phlebotomy procedures using standard sterile venipuncture of an antecubital vein by laboratory technicians trained in phlebotomy. The phlebotomists and lab technicians wore personal protective clothing (latex gloves) when handling blood samples. Participants were seated in a phlebotomy chair and their arms were cleaned with a sterile alcohol wipe and sterile gauze. A standard rubber tourniquet was then placed on the brachium. The antecubital vein was then palpitated and a 23 gauge sterile needle attached to a plastic vacutainer holder was inserted into the vein using standard procedures. Once the samples were obtained, the vacutainer holder and needle were removed. The needle was discarded as hazardous waste in a plastics sharps container. The site of the blood draw was then cleaned with sterile gauze and a sterile Band-Aid was placed on the site. Blood was obtained at baseline, 30 minutes after ingesting the first supplement dosage, and immediately, 30 minutes, two and six hours after the resistance exercise bout. Blood samples were allowed to stand at room temperature for 10 minutes and then centrifuged at 2,400 rpm for 15 minutes. The serum was removed and frozen at -80^{o}C for later analysis. Figure 7 highlights the overall study design.

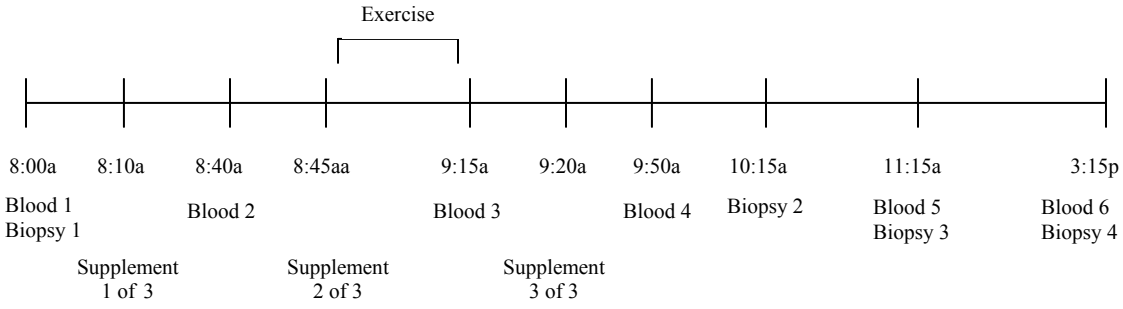

Figure 7. Overview of the study design.

Serum Analyses

Four blood markers were assessed: blood glucose, insulin, free/bioactive growth hormone (GH), and free/bioactive insulin-like growth factor -1 (IGF-1). Blood glucose was measured in order to help explain any changes over time that may have been observed relative to serum insulin concentrations. Since it has been demonstrated that insulin, GH, and IGF-1 can activate the ERK1/2 pathway, these three growth factors were analyzed. Each of these analytes was measured via enzyme linked immunosorbent assay (ELISA). To determine the optical density of all standards, controls, and unknowns, a Wallac 1420 Multilabel Counter (Turku, Finland) was used. All standards, controls, and unknowns were assayed and read at a 450nm wavelength against a known standard curve. Data analysis was performed using MicroWin microplate data-reduction software (Mikrotek Laborsysteme, Germany). All assays utilized a Jitterbug orbital microplate shaker (Boekel Scientific, Philadelphia, PA) and a MultiWash Advantage automated microplate washer (Tri Continent Scientific, Grass Valley, CA) for the incubation and washing steps, respectively.

Blood Glucose

Blood glucose was analyzed on a Dade Behring Dimension RxL® (Newark, DE). This automated clinical chemistry analyzer was calibrated using liquid assayed multiqual (Bio-Rad, Hercules, CA). In addition, on a daily basis two levels of quality control with known concentrations were performed. The procedure for the Dade Behring Dimension assay is as follows: Serum (~70µL) from a BD Vacutainer® serum separator tube (SST^{TM}) was added to a sample cup by the investigator. The dimension uses a sonicating probe to mix the sample, and the subsequent glucose analysis is based off of an

adaptation of the hexokinase-glucose-phosphate dehydrogenase method, presented as a general clinical laboratory method by Kunst, et al (Kunst, Draeger, & Ziegenhorn, 1983). According to the manufacturer, the coefficient of variation for within-run and between–day performance is 3 and 8%, respectively.

Serum Insulin

Serum insulin was analyzed by an ELISA kit [Alpha Diagnostic International (Cat. # 0030; San Antonio, TX)]. According to the manufacturer, the sensitivity for this assay was 1.5 μIU/ml. Initially, 25 μl of standards and serum samples were pipetted in duplicate into a previously coated microplate with monoclonal anti-insulin. After a 30-minute incubation on the microplate shaker, the microplate was washed five times with 300 μl wash buffer (phosphate buffered saline and tween-20). Following the wash step, 200 μl of horseradish peroxidase (HRP) substrate mix (TMB + H_2O_2) was added to each well and then the microplate incubated for an additional 15 minutes on the microplate shaker. Following this 15-minute incubation, 50 μl of an acidic stopping solution (0.2 M sulfuric acid) was added to each well. The degree of HRP-induced enzymatic turnover of the substrate was determined by wavelength absorbance measurement at 450 nm that is directly proportional to the concentration of insulin present in each well. A set of six insulin standards ranging from 0 to 200 μIU/ml was used to plot a standard curve of absorbances versus insulin concentration from which the insulin concentrations in the unknowns were calculated using a valid fitting cubic spline curve. Intra-assay coefficient of variation was determined for all five kits that were analyzed. The coefficient of variation for each individual kit were as follows: 5.2%, 9.5%, 8.4%, 6.1%, and 7.3%. For all insulin kits combined, the overall coefficient of variation was 7.3%.

Serum Growth Hormone

Serum free/bioactive GH was analyzed using an ELISA kit [Active® Bioactive ELISA by Diagnostic Systems Laboratories Inc. (DSL-10-11100; Webster, TX)]. According to the manufacturer, the sensitivity for this assay was 0.06 ng/ml, and does not cross-react with albumins or GH binding proteins. This assay was an enzymatically amplified "two-step" sandwich-type immunofunctional assay involving a unique anti-hGH monoclonal antibody and biotinylated recombinant hGH binding protein that bind, respectively, to hGH receptor binding-site 2 and binding-site 1 present on biologically active hGH molecules. In the first step of this assay, 50 µl of standards, controls (high and low), and serum samples were pipetted in duplicate into the wells along with 50 µl of assay buffer (a protein based BSA buffer with a non-mercury preservative). Next, this mixture was allowed to react with the anti-hGH monoclonal antibody immobilized in the microtitration wells. After a three-hour incubation on the microplate shaker, the plate was washed five times with wash buffer (buffered saline with non-ionic detergent). Following the wash step, 100 µl of bioactive GH-biotin conjugate was added to each well and the incubated overnight at 2-8^{o}C. After the overnight incubation, the wells were washed five times and then 100 µl of enzyme conjugate of streptavidin labeled with HRP was added to each well. Another 30-minute incubation on the microplate shaker followed and then the wells were washed one last time. Next, 100 µl of tetramethylbenzidine (TMB) chromogen solution was added and one last 10 minute incubation on the orbital microplate shaker ensued. Lastly, 100 µl of an acidic stopping solution (0.2 M sulfuric acid) was added to each well and the degree of HRP-induced enzymatic turnover of the substrate was determined by a wavelength absorbance measurement at 450 nm that is directly proportional to the concentration of bioactive GH present in each well. A set of

seven bioactive GH standards ranging from 0 to 25 ng/ml was used to plot a standard curve of absorbances versus bioactive GH concentration from which the bioactive GH concentrations in the unknowns were calculated using a valid fitting cubic spline curve. Intra-assay coefficient of variation was determined for all five kits that were analyzed. The coefficient of variation for each individual kit was as follows: 4.9%, 2.2%, 1.2%, 2.3%, and 4.1%. For all GH kits combined, the overall coefficient of variation was 2.9%.

Serum IGF-1

Serum free/bioactive IGF-1 was analyzed using an ELISA kit [Active® free IGF-1 ELISA by Diagnostic Systems Laboratories Inc. (DSL-10-9400; Webster, TX)]. According to the manufacturer, the sensitivity for this assay was 0.015 ng/ml, and does not cross-react with insulin growth factor binding proteins. This assay was an enzymatically amplified "two-step" sandwich-type immunoassay. In the first step of this assay, 50 µl of standards, controls (high and low), and serum samples were pipetted in duplicate along with 50-µl sample buffer (a protein based BSA buffer with a non-mercury preservative) into the wells (which were coated with anti-free IGF-1). After a one-hour incubation on the microplate shaker, the wells were washed five times with wash buffer (buffered saline with non-ionic detergent). Following the incubation and washing, the wells were treated with another anti-Free IGF-1 detection antibody labeled with the enzyme horseradish peroxidase. After a second 30-minute incubation and wash step (five times), the wells were incubated with 100 µl of TMB solution for 10 minutes. Lastly, 100 µl of an acidic stopping solution (0.2 M sulfuric acid) was added to each well and the degree of enzymatic turnover of the substrate was determined by a wavelength absorbance measurement at 450nm. The absorbance measured was directly proportional

to the concentration of free IGF-1 present. A set of seven free IGF-1 standards ranging from 0 to 10 ng/ml was used to plot a standard curve of absorbances versus free IGF-1 concentration from which the free IGF-1 in the unknown serum samples were calculated using a valid fitting cubic spline curve. Intra-assay coefficient of variation was determined for all five kits that were analyzed. The coefficient of variation for each individual kit was as follows: 5.1%, 5.5%, 5.5%, 3.5%, and 5.2%. For all free IGF-1 kits combined, the overall coefficient of variation was 4.9%.

Skeletal Muscle Analyses

Four skeletal muscle proteins and their activation state were assessed: MEK1, ERK1/2, IRS-1, and SHP-2. MEK1 and ERK1/2 were measured to determine if leucine supplementation amplified their activation status above that which would be expected from resistance exercise alone. In the advent that MEK1 or ERK1/2 were elevated, IRS-1 and SHP-2 were assessed to determine the extent to which, if at all, the insulin-signaling pathway contributed to their increased activation (a measure of potential cross-talk activity). The following describes the manner in which the skeletal muscle samples were prepared to analyze these muscle proteins.

Skeletal Muscle Preparation Using a Cell Extraction Protocol

Approximately one-half (22.5mg) of each muscle sample was collected, weighed, and placed in a separate microcentrifuge tube. Muscle samples were homogenized using a commercial cell extraction buffer (Cat. FNN0011, Biosource International, Camarillo, CA) and a tissue homogenizer. The cell extraction buffer was supplemented with 1mM phenylmethanesulphonylfluoride (PMSF) and a protease inhibitor cocktail (Cat. P2714, Sigma, St. Louis, MO). A 0.3 M stock solution in dimethyl sulfoxide (DMSO) was made

and 17μL of this stock was added to 5mL of cell extraction buffer to attain a final concentration of 1mM PMSF. The protease inhibitor cocktail was a mixture of water-soluble protease inhibitors with broad specificity for the inhibition of serine, cysteine, and metallo-proteases. After reconstitution, 500 μL of protease inhibitor was added to every 5 mL of cell extraction buffer. Due to the unstable nature of PMSF, this solution was both created and added to the cell extraction buffer just prior to use. Following the completion of the cell extraction buffer formulation, the buffer was added to each frozen muscle sample at a ratio of approximately 20.8 mL per gram of muscle tissue (500 μL of cell extraction buffer to each frozen muscle sample). Following the addition of the cell extraction buffer formulation, the muscle tissue was homogenized by hand with a Kontes pestle tissue homogenizer in a microcentrifuge tube. For the purposes of clarification, the skeletal muscle homogenized in supplemented cell extraction buffer will be referred to as "muscle homogenate".

MEK1, ERK1/2, and IRS-1 Analysis Overview

The phosphorylated levels of MEK1 (Cat. KHO0321), ERK1/2 (Cat. KHO0091), and IRS-1 (Cat. KHO0521) were assessed using phosphoELISA kits (Biosource International, Camarillo, CA) that are designed to quantify the activation states of these signal transduction proteins. From a general perspective, each of these kits utilizes the same procedures in that the sandwich ELISA begins with a polyclonal antibody precoated onto a 96-well mictrotiter plate. Samples are added and total protein is captured, similar to an immunoprecipitation reaction. The prediluted detection antibody is added next, which is phospho-site specific to either MEK1, ERK 1/2, or IRS-1. The sandwich is detected using anti-rabbit HRP-conjugated antibody followed with a colorimetric

substrate. To determine the optical density of all standards, controls, and unknowns, a Wallac 1420 Multilabel Counter (Turku, Finland) was used. All standards, controls, and unknowns were assayed and read at a 450 nm wavelength against a known standard curve. Data analysis was performed using MicroWin microplate data-reduction software (Mikrotek Laborsysteme, Germany). The relative activity of MEK1, ERK 1/2, and IRS-1 was then assessed by dividing absolute activity by muscle wet-weight. All assays utilized a Jitterbug orbital microplate shaker (Boekel Scientific, Philadelphia, PA) and a MultiWash Advantage automated microplate washer (Tri Continent Scientific, Grass Valley, CA) for the incubation and washing steps, respectively. Each specific phosphoELISA is discussed next.

MEK1

The MEK1 [pSpS218/222] kit is a solid phase sandwich ELISA. A monoclonal antibody specific for MEK1 (regardless of phosphorylation state) had been coated onto the wells of the microtiter strips provided. The MEK1 phosphoELISA detected and quantified the level of MEK1 protein when dually phosphorylated at the specific serine residues 218 and 222. Muscle homogenate pretreatment included the addition of sample treatment buffer that was included in the kit at a 1:1 ratio. Specifically, 10 μL sample treatment buffer was added to 10 μL of muscle homogenate. This 20 μL solution was then further diluted by adding 80 μL of standard diluent buffer that was included in the kit resulting in a 100 μL solution. Relative to muscle homogenate, these dilutions resulted in a final sample pretreatment dilution of 1:10. Following these dilutions, the MEK1 samples were ready for analysis.

Initially, 50 µL of standards and pre-treated diluted samples (muscle homogenate) were added to the wells. In addition, 50 µL of anti-MEK1 detection antibody was pipetted into each well and then the wells were incubated on the microplate shaker for two hours. Following this, the plate was washed four times (including a 30 second soak cycle with each wash) with wash buffer (phosphate buffer with EDTA). Next, 100 µL anti-rabbit IgG-HRP working solution was added to each well. Subsequently, a 30-minute incubation on the microplate shaker followed by washing the plate four times (including a 30 second soak cycle with each wash) with wash buffer was performed. The final steps included adding 100 µL of stabilized TMB chromogen to each well and allowing them to incubate on the orbital shaker for 30 minutes. Immediately following this, 100 µL of an acidic stopping solution (1 N hydrochloric acid) was added to each well and the degree of HRP-induced enzymatic turnover of the substrate was determined by a wavelength absorbance measurement at 450 nm. The measured optical density was directly proportional to the concentration of MEK1 present in the original specimen. The MEK1 [pSpS218/222] standard (prepared using purified, full length, recombinant, phosphorylated MEK1 protein) that was included in the kit was reconstituted to a concentration of 100 Units/ml. Serial dilutions of this high standard were made six times not including a zero concentration standard (consisting of diluent buffer) resulting in eight total standards (ranging from 0 to 100 Units/ml). These standards were used to plot a standard curve of absorbances utilizing a four-parameter algorithm. A total of three kits were utilized to run the appropriate analyses in duplicate on all 30 subjects. The fitting state for all three kits was valid. Curve-fit statistics for the four-parameter algorithm coefficients were as follows: Kit 1 - lower limit = 0.129; turning slope = 1.05; turning point = 116.77; upper limit = 6.87; and fitting error = 0.0011. Kit 2 - lower limit = 0.232;

turning slope = 1.522; turning point = 37.166; upper limit = 3.725; and fitting error = 0.006. Kit 3 - lower limit = 0.203; turning slope = 1.601; turning point = 35.556; upper limit = 3.752; and fitting error = 0.035. Intra-assay coefficient of variation was determined for all three kits that were analyzed. The coefficient of variation for each individual kit was as follows: 7.5%, 6.5%, and 9.7%. For all pMEK1 kits combined, the overall coefficient of variation was 7.9%.

ERK1/2

The ERK1/2 [pTpY185/187] kit is a solid phase sandwich ELISA. A monoclonal antibody specific for ERK1/2 (regardless of phosphorylation state) had been coated onto the wells of the microtiter strips provided. The ERK1/2 phosphoELISA detected and quantified the level of ERK1/2 protein when dually phosphorylated at the specific residues of threonine 185 and tyrosine 187. Muscle homogenate pretreatment included the addition of a denaturing sample treatment buffer (Cat. ST001, Biosource International, Camarillo, CA) that was included with the kit. The reason for this pretreatment is due to the fact that this Biosource kit efficiently detects ERK1/2 proteins in denatured cell extracts. In addition to adding the denaturing agent, standard diluent buffer was also added to the muscle homogenate. Specifically, 25 µL of the denaturing sample treatment buffer was added to 25 µL of muscle homogenate and incubated on ice for 20 minutes. This mixture was then diluted five-fold with 200 µL of standard diluent buffer. Relative to muscle homogenate, these dilutions resulted in a final sample pretreatment dilution of 1:10. Following these dilutions, the ERK1/2 samples were ready for analysis.

Initially, 100 µL of standards and pre-treated diluted samples (muscle homogenate) were added to the wells and incubated on the microplate shaker for two hours. Following this, the plate was washed four times (including a 30 second soak cycle with each wash) with wash buffer (phosphate buffer with EDTA). Next, 100 µL of anti-ERK1/2 (detection antibody) was added to each well and then incubated for one hour on the microplate shaker, followed by a washing of the wells (four times including a 30 second soak cycle with each wash) with wash buffer. Next, 100 µL of anti-rabbit IgG-HRP was added to each well and then incubated for 30 minutes on the microplate shaker, followed by another washing of the wells (four times including a 30 second soak cycle with each wash) with wash buffer. The final steps included adding 100 µL of stabilized chromogen to each well and allowing them to incubate on the orbital shaker for 30 minutes. Immediately following this, 100 µL of an acidic stopping solution (1 N hydrochloric acid) was added to each well and the degree of enzymatic turnover of the substrate was determined by a wavelength absorbance measurement at 450nm. The measured optical density was directly proportional to the concentration of ERK1/2 present in the original specimen. The ERK1/2 [pTpY185/187] standard (prepared using purified full length human recombinant active ERK1/2) that was included in the kit was reconstituted to a concentration of 100 Units/ml. Serial dilutions of this high standard were made six times not including a zero concentration standard (consisting of diluent buffer) resulting in eight total standards (ranging from 0 to 100 Units/ml). These standards were used to plot a standard curve of absorbances utilizing a four-parameter algorithm. A total of three kits were utilized to run the appropriate analyses in duplicate on all 30 subjects. The fitting state for all three kits was valid. Curve-fit statistics for the four-parameter algorithm coefficients were as follows: Kit 1 - lower limit = 0.170;

turning slope = 1.559; turning point = 31.799; upper limit = 3.768; and fitting error = 0.020. Kit 2 - lower limit = 0.066; turning slope = 0.908; turning point = 11500; upper limit = 328.42; and fitting error = 0.063. Kit 3 - lower limit = 0.059; turning slope = 1.064; turning point = 93.447; upper limit = 3.968; and fitting error = 0.003. Intra-assay coefficient of variation was determined for all three kits that were analyzed. The coefficient of variation for each individual kit was as follows: 2.8%, 2.3%, and 2.8%. For all pERK1/2 kits combined, the overall coefficient of variation was 2.6%.

IRS-1

The IRS-1 [pS312] kit is a solid phase sandwich ELISA. A monoclonal antibody specific for IRS-1 (regardless of phosphorylation state) had been coated onto the wells of the microtiter strips provided. The IRS-1 phosphoELISA detected and quantified the level of IRS-1 protein when phosphorylated at the specific serine residue 312. Muscle homogenate pretreatment included the addition of standard diluent buffer that was included in the kit to a dilution of a 1:10 ratio. Specifically, 90 µL of standard diluent buffer was added to 10 µL of muscle homogenate. Relative to muscle homogenate, this dilution resulted in a final sample pretreatment dilution of 1:10. Following this dilution, the IRS-1 samples were ready for analysis.

100 µL of standards and pre-treated diluted samples (muscle homogenate) were added to the well and then incubated on the microplate shaker for two hours. Following this, the plate was washed four times (including a 30 second soak cycle with each wash) with wash buffer. Next, 100 µL anti-IRS-1 [pS312] (detection antibody) was added to each well and then incubated for one hour on the microplate shaker, followed by a washing of the wells (four times including a 30 second soak cycle with each wash) with

wash buffer. Following this, 100 μL of anti-rabbit IgG-HRP working solution was added to each well. Subsequently, a 30-minute incubation on the microplate shaker followed by washing the plate four times (including a 30 second soak cycle with each wash) with wash buffer (phosphate buffer with EDTA) was performed. The final steps included adding 100 μL of stabilized TMB chromogen to each well and allowing them to incubate on the orbital shaker for 30 minutes. Immediately following this, 100 μL of an acidic stopping solution (1 N hydrochloric acid) was added to each well and the degree of HRP-induced enzymatic turnover of the substrate was determined by a wavelength absorbance measurement at 450nm. The measured optical density was directly proportional to the concentration of phosphorylated IRS-1 present in the original specimen. The IRS-1 [pS312] standard (prepared from phosphorylated recombinant IRS-1 C-terminal region) that was included in the kit was reconstituted to a concentration of 100 Units/ml. Serial dilutions of this high standard were made six times not including a zero concentration standard (consisting of diluent buffer) resulting in eight total standards (ranging from 0 to 100 Units/ml). These standards were used to plot a standard curve of absorbances utilizing a four-parameter algorithm. A total of three kits were utilized to run the appropriate analyses in duplicate on all 30 subjects. The fitting state for all three kits was valid. Curve-fit statistics for the four-parameter algorithm coefficients were as follows: Kit 1 - lower limit = 0.146; turning slope = 1.10; turning point = 2661.3; upper limit = 69.602; and fitting error = 0.012. Kit 2 - lower limit = 0.087; turning slope = 1.085; turning point = 3413; upper limit = 79.47; and fitting error = 0.002. Kit 3 - lower limit = 0.170; turning slope = 1.378; turning point = 273.72; upper limit = 7.425; and fitting error = 0.018. Intra-assay coefficient of variation was determined for all three kits that were analyzed. The coefficient of variation for each individual kit was as follows: 10.9%,

12.9%, and 12.0%. For all pIRS-1 kits combined, the overall coefficient of variation was 11.9%.

SHP-2

The activity of SHP-2 [pY542] was assessed through the use of an ELISA. Briefly, a primary antibody specific to phosphorylated SHP-2 [pY542] (cat. 44-554G, Biosource, Camarillo, CA) protein was diluted in coating buffer (1.24 g sodium carbonate in 100 ml of deionized water, pH 9.6) at a concentration of 4 µg/ml and added to all wells of the microplate. Following overnight incubation at room temperature, the wells were washed in 300 µL of washing buffer (1L of 10X phosphate buffered saline with 50 µL of tween-20) prior to the addition of a blocking buffer (500 mL of blocking buffer contains 50 mL 10X phosphate buffered saline, 5g BSA, and deionized water) to prevent the non-specific binding of non-relevant proteins to the bottom of each well. Next, 50 µL of undiluted muscle homogenate were then added to the wells, followed by an incubation period of one hour. After the incubation, the wells were washed four times (including a 25 second soak cycle with each wash) with wash buffer. Next, a secondary antibody (IgG conjugated to HRP) was diluted to 10 µg/ml in dilution buffer (500 mL of dilution buffer contains 50 mL of 10X phosphate buffered saline, 250 µL of tween-20, and .5g BSA), added to each well, and then was incubated for one hour at room temperature. Following this incubation, the wells were washed four times (including a 25 second soak cycle with each wash) with wash buffer. The final steps included adding 100 µL of stabilized TMB chromogen to each well and allowing them to incubate on the orbital shaker for 30 minutes. Immediately following this, 100 µL of an acidic stopping solution (1 N hydrochloric acid) was added to each well and the degree of HRP-induced

enzymatic turnover of the substrate was determined by a wavelength absorbance measurement at 450 nm. Differences between group and testing session absorbances relative to muscle weight were observed to determine protein content differences for phosphorylated SHP-2. Samples for SHP-2 were analyzed as single samples (not in duplicate), therefore, coefficient of variation analyses were not conducted.

Statistics

A one-way univariate analysis of variance was calculated on all dependent variables to determine if significant differences existed at baseline between the three groups. If a significant difference existed at baseline in any of the dependent variables, a test of bivariate comparisons was conducted utilizing the Bonferroni test to determine which groups were significantly different at baseline. Two sets of MANOVAs were analyzed for this experiment based on dependent variables that were likely to be related to one another. In addition, the use of a MANOVA analysis also prevents the increasing of Type I errors that would result with the use of repeated univariate procedures. Relative to the primary dependent variables, a 3 (group) x 6 (time points) (serum variables) or a 3 (group) x 4 (time points) (skeletal muscle variables) repeated measures multivariate analysis of variance (MANOVA) mixed methods with the repeated measures on the second factor was utilized. The MANOVA was used to control for alpha inflation of the subsequent univariate analysis of variance (ANOVA). ANOVA on each dependent variable were conducted as follow-up tests to the MANOVA. To control for alpha inflation of the ANOVA, the Bonferroni test was utilized. Post-hoc tests of the interaction effects demonstrated in the ANOVA were investigated via an independent samples T-test. In addition to reporting probability values, an index of effect size was

reported to reflect the magnitude of the observed effect. The index of effect size utilized was partial Eta squared (η^2), which estimates the proportion of variance in the dependent variable that can be explained by the independent variable. Partial Eta squared effects sizes were determined to be: weak = 0.17, medium = 0.24, strong = 0.51, very strong = > 0.70 as previously described by O'Connor (O'Connor et al., 2007). All statistical procedures were performed using SPSS 15.0 software (Chicago, IL) and a probability level of <0.05 was adopted throughout. In addition, for all statistical analyses not meeting the sphericity assumption for the within-subjects analyses, a Huynh-Feldt correction factor was applied to the degrees of freedom in order to adjust (increase) the critical F-value to a level that would prevent the likelihood of committing a type I error.

CHAPTER FOUR

Results

For body mass, age, and height, descriptive statistics were calculated using the mean (*M*) and standard deviation (*SD*). Summaries of these values are listed in table 2. A one-way univariate analysis of variance was calculated on all dependent variables to determine if significant differences existed at baseline between the three groups. If a significant difference existed at baseline in any of the dependent variables, a test of bivariate comparisons was conducted utilizing the Bonferroni test to determine which groups were significantly different at baseline. Significant differences existed at baseline for only two variables, IRS-1 ($F(2,27) = 4.53$, $p = .02$, effect size (η^2) = .25) and SHP-2 ($F(2,27) = 7.74$, $p = .002$, effect size (η^2) = .36). Regarding IRS-1, the placebo group ($M = 14.734$, $SD = 6.395$) was significantly higher than the leucine group ($M = 9.059$, $SD = 3.122$, $p = .047$) and the BCAA group ($M = 8.927$, $SD = 4.700$, $p = .041$). Relative to SHP-2, the leucine group ($M = 87.0$, $SD = 21.7$ absorbance units) was significantly lower than the BCAA group ($M = 122.1$, $SD = 30.3$, $p = .007$) and the placebo group ($M = 123.2$, $SD = 16.0$, $p = .005$). To adjust for these differences at baseline, all skeletal muscle variables were analyzed as delta values (testing session – baseline).

Total Lifting Volume and Macronutrient Intake

A univariate ANOVA was used to determine if there were differences between the supplement groups relative to resistance exercise variables and nutritional intake. No statistically significant differences were observed between the groups in total lifting volume or in the relative percentage of 1RM that was lifted for both the leg press and leg

Table 2. Subject Demographics

Physical characteristics	BCAA		Leu		Pla		
	M	*SD*	*M*	*SD*	*M*	*SD*	*p*-value
Height (cm)	178.0	7.4	180.0	5.7	176.8	7.7	> .05
Mass (kg)	85.4	12.1	80.8	8.2	83.0	11.2	> .05
Age (yrs)	24.4	4.4	22.0	2.4	21.0	2.4	> .05

Table 3. *Comparison of Resistance Exercise Variables between the Supplement Groups*

Resistance Exercise Variable	BCAA	Leucine	Placebo	p-value
Total lifting volume (kg)	18,733 ± 4,610	19,714 ± 3,540	18,320 ± 6,260	0.811
% 1RM Leg Press	80.0 ± 0.0	79.7 ± 0.89	79.1 ± 2.7	0.501
% 1RM Leg Extension	80.0 ± 0.0	76.0 ± 6.6	73.9 ± 10.0	0.158

Table 4. *Comparison of Nutritional Intake Variables between the Supplement Groups*

Nutritional Variable	BCAA	Leucine	Placebo	p-value
Total calories (kcal/day)	2,883 ± 955	3,121 ± 990	2,304 ± 674	0.124
Protein (g/day)	129 ± 57	152 ± 77	108 ± 39	0.281
Carbohydrate (g/day)	334 ± 116	314 ± 124	256 ± 75	0.259
Fat (g/day)	116 ± 49	136 ± 52	95 ± 38	0.158

extension (Table 3). Also, there were no statistically significant differences in macronutrient intake (total calories, carbohydrate, protein, and fat intake) between the groups (Table 4).

Serum Analyses

Data were inspected to identify any outliers that exceeded ± 2.5 standard deviations from the group mean for all serum variables. A total of two serum variables was determined to be outlier and were replaced with the group mean as previously described (Kreider et al., 2003). Four serum variables (glucose, insulin, GH, and IGF-1) were analyzed by multivariate analysis of variance (MANOVA). There were no multivariate main effects for group nor was there a multivariate group x time interaction, but there was a multivariate main effect for time. The multivariate main effect for time (Wilks' Lamda = .075, $F(20,8) = 4.91$, $p = .013$, effect size (η^2) = .925) was accompanied by significant univariate ANOVA for all serum variables. The main effect for time for each of these serum variables is shown in Figures 8 through 11. All time points will be referred to as follows: Pre = baseline measurement; 30min = 30 minutes following baseline measurement (preexercise); Post = immediate postexercise; 30min post = 30 minutes postexercise; 2hr post = 2 hours postexercise; 6hr post = 6 hours postexercise. For a complete summary of all serum data, refer to table 5.

Blood Glucose

Following the univariate ANOVA indicating a main effect for time ($F(3.9,104.2) = 4.7$, $p = .002$, effect size (η^2) = .149), pairwise comparisons were made utilizing a Bonferroni adjustment. These pairwise results revealed the following data for blood glucose (measured in mg/dl): 6hr post ($M = 87.7$, $SD = 6.0$) was significantly lower than

Pre (*M* = 93.3, *SD* = 8.2, *p* = .047), Post (*M* = 95.3, *SD* = 10.1, *p* = .005), and 2hr post (*M* = 91.6, *SD* = 5.2, *p* = .006).

Table 5. *Data for all serum variables.*

Variable	T1 M(SD)	T2 M(SD)	T3 M(SD)	T4 M(SD)	T5 M(SD)	T6 M(SD)	*p*-value
Glucose							
BCAA	91(10)	89(5)	93(9)	82(10)	90(4)	84(5)	group (g) .153
Leucine	96(8)	91(6)	95(8)	90(6)	93(5)	90(6)	time (t) .002
Placebo	94(7)	91(8)	98(12)	95(10)	92(6)	89(6)	g x t .722
Insulin							
BCAA	19(8)	23(10)	25(13)	25(14)	19(9)	16(6)	group (g) .153
Leucine	22(19)	19(14)	22(14)	18(13)	14(14)	16(15)	time (t) < .001
Placebo	22(10)	22(11)	28(9)	24(9)	18(9)	21(13)	g x t .730
GH							
BCAA	.41(.81)	.64(.97)	1.9(2.2)	1.5(2.6)	.23(.3)	2.57(4)	group (g) .829
Leucine	.03(.03)	.48(1.3)	3.3(4.2)	1.2(1.6)	.06(.1)	.30(.4)	time (t) < .001
Placebo	.07(.09)	.84(1.3)	2.2(1.9)	2.2(3.8)	.28(.8)	.36(.6)	g x t .183
IGF-1							
BCAA	1.3(0.8)	1.2(0.7)	1.2(0.8)	1.4(0.9)	1.1(.7)	0.9(.6)	group (g) .455
Leucine	1.1(0.5)	1.1(0.4)	1.1(0.5)	1.2(0.4)	1.0(.4)	0.8(.3)	time (t) < .001
Placebo	1.3(0.4)	1.2(0.4)	1.6(0.5)	1.5(0.6)	1.4(.5)	1.1(.5)	g x t .522

Note. T1 = baseline; T2 = 30 min after baseline; T3 = immediate post; T4 = 30 min post; T5 = 2hr post; T6 = 6hr post. Units of measurement for the serum markers: glucose = mg/dL; insulin uIU/mL; GH = ng/dL; IGF-1 = ng/dL. *p* values: group = main effect for group; time = main effect for time; g x t = group x time interaction.

Serum Insulin

Following the univariate ANOVA indicating a main effect for time ($F(3.9,104.7) = 10.9$, $p < .001$, effet size (η^2) = .287), pairwise comparisons were made utilizing a Bonferroni adjustment. These pairwise results revealed the following data for serum insulin (measured in μIU/ml): 2hr post (*M* = 16.9, *SD* = 10.9) was significantly lower than Pre (*M* = 21.27, *SD* = 13.1, p = .014), 30min (*M* = 21.4, *SD* = 11.5, *p* = .010), Post

(M = 25.1, SD = 12.0, p = < .001), and 2hr post (M = 22.3, SD = 12.2, p = .001). 6hr post (M = 17.7, SD = 11.7) was significantly lower than 30min (M = 21.4, SD = 11.5, p = .019) and Post (M = 25.1, SD = 12.0, p = .006).

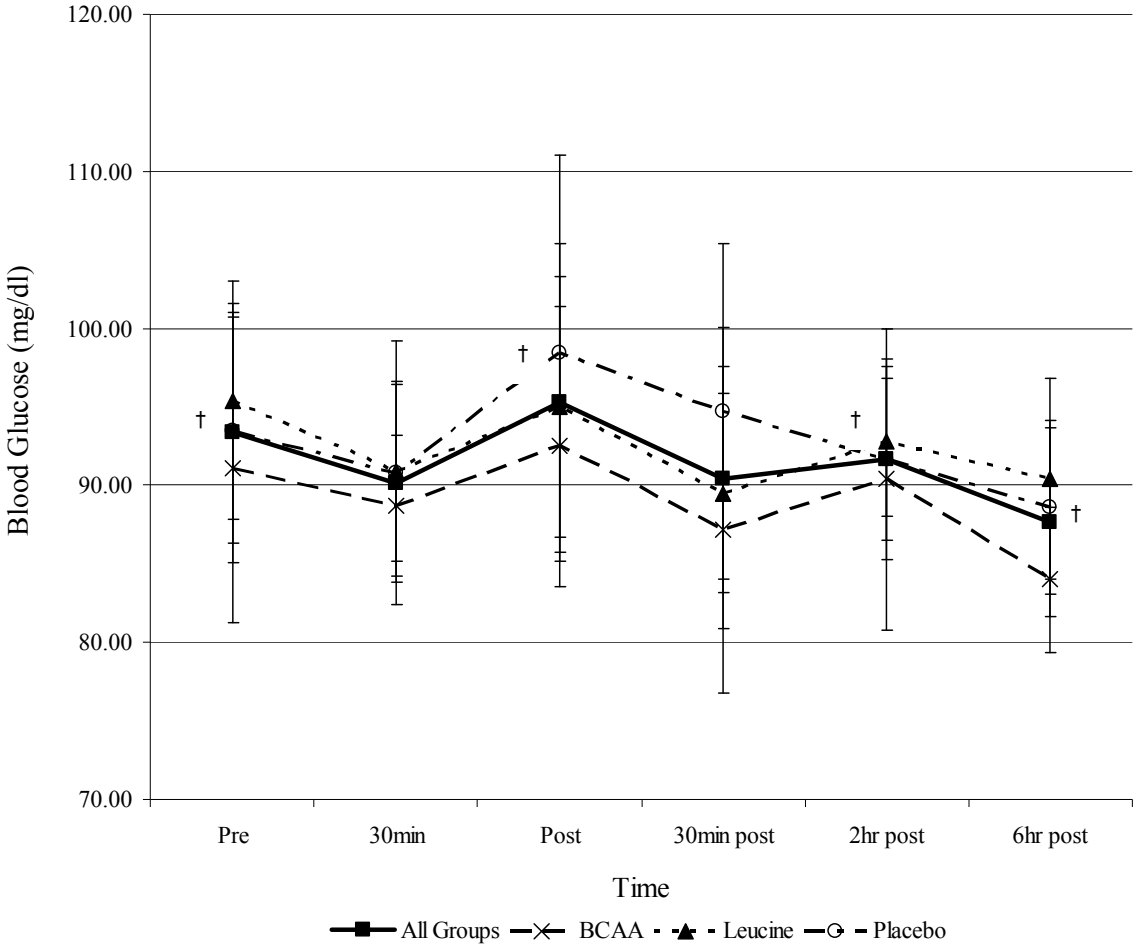

Figure 8. Changes over time (+*SD*) for all groups relative to blood glucose. † = Significant differences between time points irrespective of supplementation group representing the main effect for time. 6hr post was significantly lower than pre, post, and 2hr post (p < .05).

Serum GH

Following the univariate ANOVA indicating a main effect for time (F(3.6,97.6) = 7.5, p < .001, effect size (η^2) = .218), pairwise comparisons were made utilizing a Bonferroni adjustment. These pairwise results revealed the following data for serum GH (measured

in ng/ml): Post (M = 2.5, SD = 2.9) was significantly higher than Pre (M = 0.17, SD = 0.49, p = .004), 30min (M = 0.65, SD = 1.18, p = .003), and 2hr post (M = 0.19, SD = 0.47, p = .004).

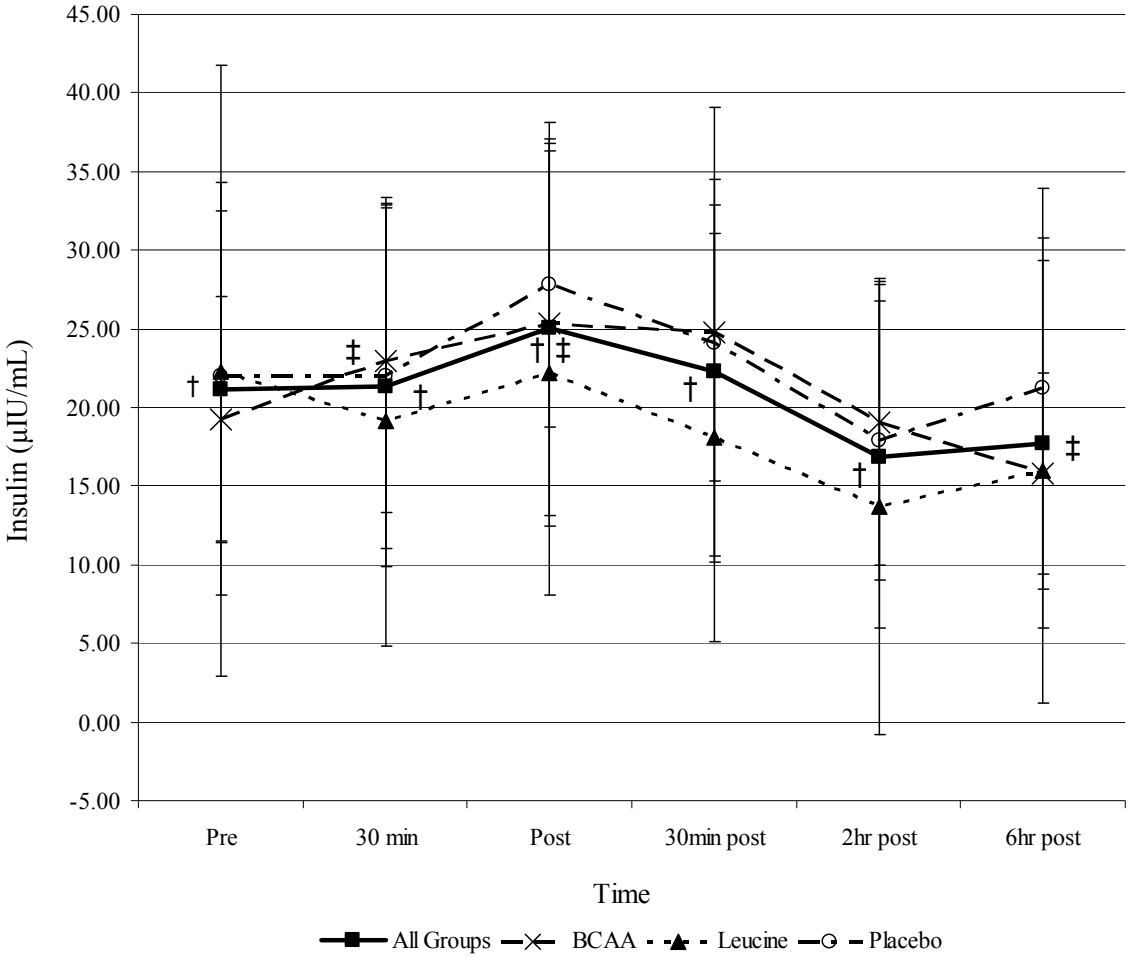

Figure 9. Changes over time (+*SD*) for all groups relative to serum insulin. † = Significant differences between time points irrespective of supplementation group representing the main effect for time. 2hr post was significantly lower than pre, 30min, post, and 30min post ($p < .05$). ‡ = Significant differences between time points irrespective of supplementation group representing the main effect for time. 6hr post was significantly lower than 30 min and post ($p < .05$).

Serum IGF-1

Following the univariate ANOVA indicating a main effect for time (F(2.3,63.1) = 9.3, $p < .001$, effect size (η^2) = .257), pairwise comparisons were made utilizing a Bonferroni adjustment. These pairwise results revealed the following data for serum

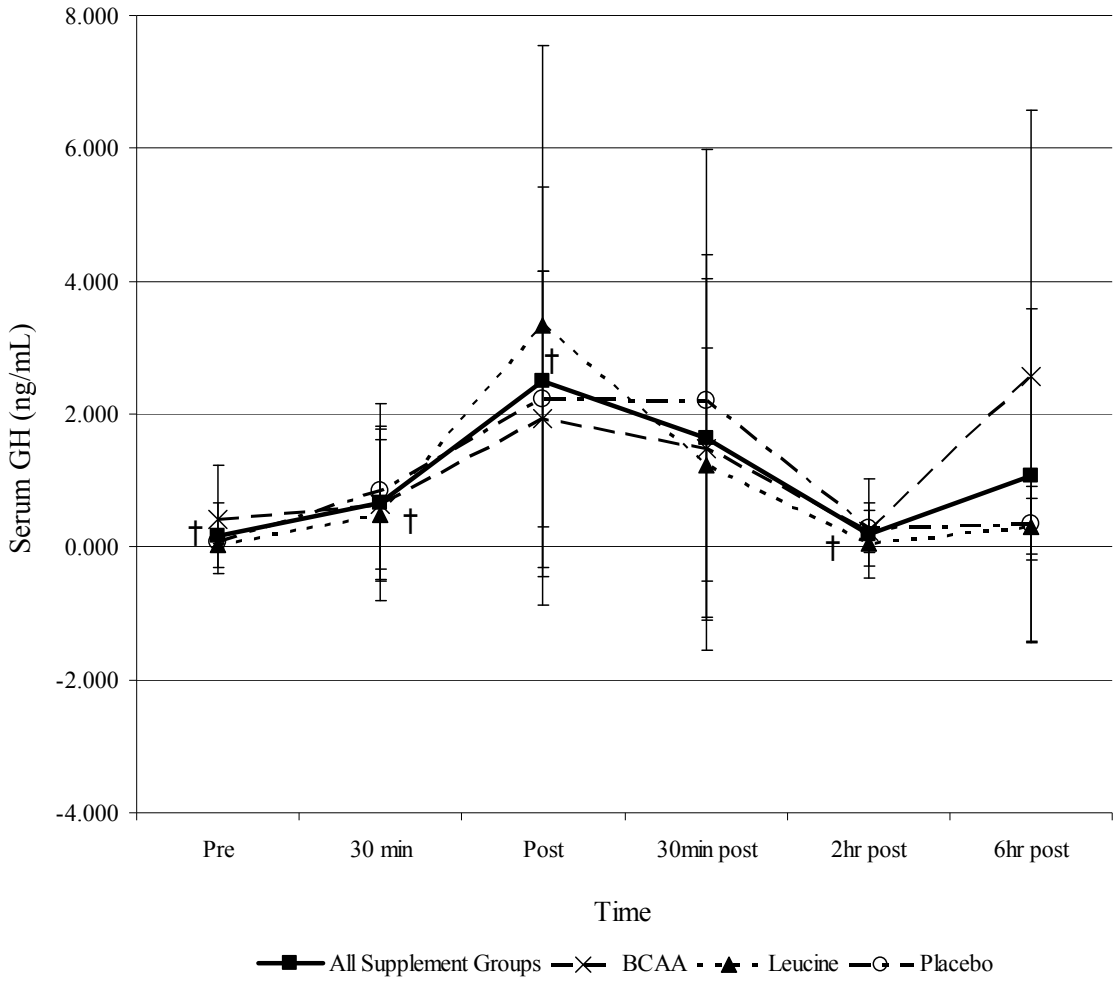

Figure 10. Changes over time (+*SD*) for all groups relative to serum GH. † = Significant differences between time points irrespective of supplementation group representing the main effect for time. Post was significantly greater than pre, 30 min, and 2hr post ($p < .005$).

IGF-1 (measured in ng/ml): 6hr post ($M = .94$, $SD = 0.51$) was significantly lower than Pre ($M = 1.23$, $SD = 0.60$, $p = .025$), Post ($M = 1.32$, $SD = 0.62$, $p = .001$), 30min post ($M = 1.36$, $SD = 0.65$, $p = < 001$) and 2hr post ($M = 1.20$, $SD = 0.47$, $p = 0.56$). In addition, 30min ($M = 1.16$, $SD = 0.52$,) was significantly lower than Post ($M = 1.32$, $SD = 0.62$, $p = .007$) and 30min post ($M = 1.36$, $SD = 0.65$, $p = .005$).

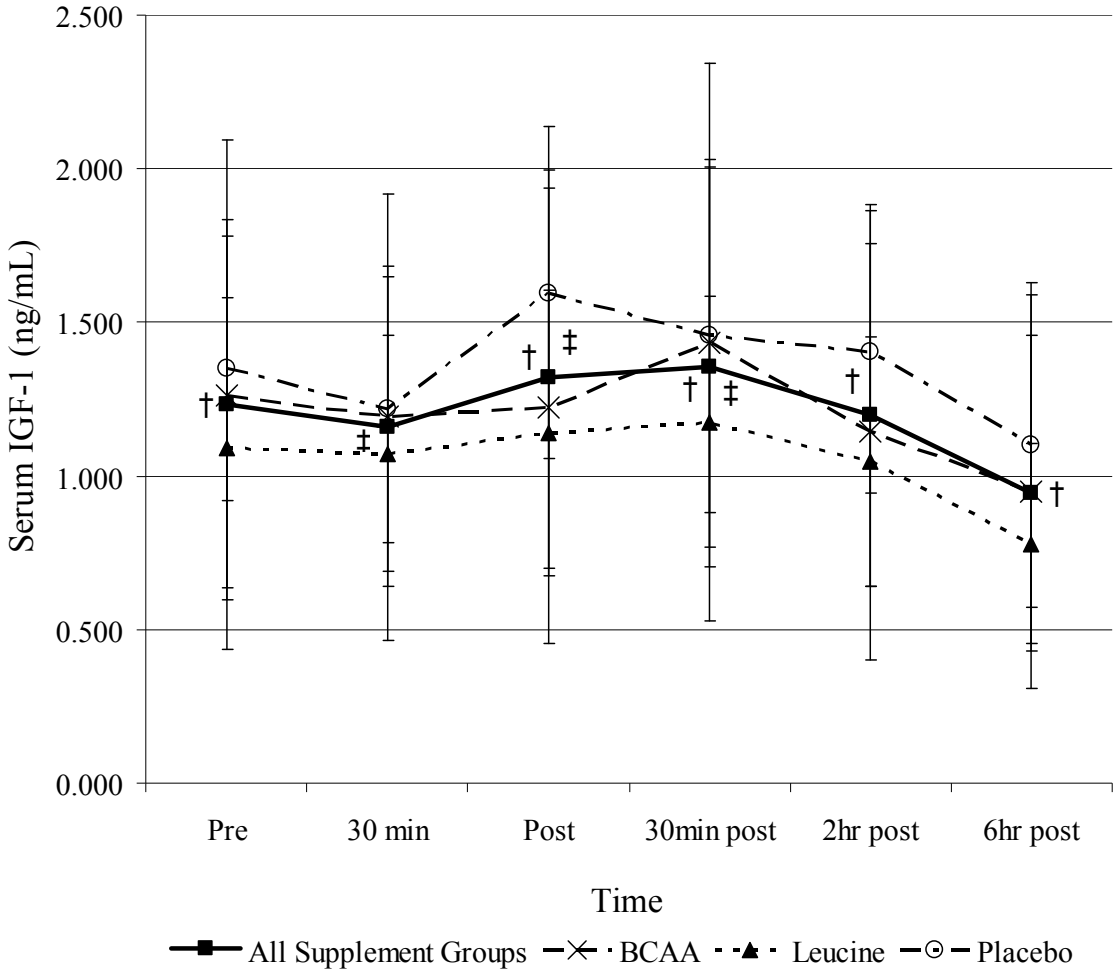

Figure 11. Changes over time (±*SD*) for all groups relative to serum IGF-1. † = Significant differences between time points irrespective of supplementation group representing the main effect for time. 6hr post was significantly lower than pre, post, 30min post, and 2hr post ($p < .05$). ‡ = Significant differences between time points irrespective of supplementation group representing the main effect for time. 30min was significantly lower than post and 30min post ($p < .05$).

Skeletal Muscle Protein Analyses

For each skeletal muscle protein variable, all reported units of measurement are in units of the variable of interest per mL of muscle homogenate per mg of skeletal muscle (U/mL/mg). Due to the differences at baseline for IRS-1 and SHP-2, all skeletal muscle variables were analyzed as delta values (testing session – baseline). For a complete summary of all raw skeletal muscle data, refer to Table 6. Four skeletal muscle protein

variables (MEK1, ERK1/2, IRS-1, and SHP-2) were analyzed by multivariate analysis of variance (MANOVA). The MANOVA analysis indicated a significant main effect for time (Wilks' Lamda = .300, F(12,16) = 3.11, p = .018, effect size (η^2) = .700), a significant main effect for group (Wilks' Lamda = .291, F(8,48) = 5.1, p < .001, effect size (η^2) = .460), and a significant group x time interaction (Wilks' Lamda = .132, F(24,32) = 2.3, p = .013, effect size (η^2) = .637). Due to the numeric transformation of the data as delta values, all time points for the skeletal muscle variables will be referred to as follows: Pre = baseline measurement minus baseline measurement; 30min post = 30 minutes following the resistance exercise minus baseline measurement; 2hr post = two hours following the resistance exercise minus baseline measurement; 6hr post = six hours following the resistance exercise minus baseline measurement.

MEK1

The MANOVA analysis that indicated a significant main effect for time (Wilks' Lamda = .300, *F*(12,16) = 3.11, *p* = .018, effect size (η^2) = .700) was accompanied by a significant univariate ANOVA main effect for time for phosphorylated MEK1 (*F*(2.1,55.3) = 8.6, *p* = .001, effect size (η^2) = .242). Follow-up univariate ANOVA revealed that there were no main effects for group or a group x time interaction relative to phosphorylated MEK1. Following the univariate ANOVA indicating a main effect for time in phosphorylated MEK1, pairwise comparisons were made utilizing a Bonferroni adjustment. These pairwise results revealed that there was a significant increase over time in phosphorylated MEK1 at 2hr post (*M* = 11.668, *SD* = 17.832 U/ml/mg, *p* = .005). Additionally, this increase in MEK1 phosphorylation at the 2hr post was significantly greater than the change in MEK1 phosphorylation over time observed at the 6hr post

Table 6. *Raw Data for all skeletal muscle variables*.

Variable	Pre		30min post		2hr post		6hr post	
	M	SD	M	SD	M	SD	M	SD
MEK-1								
BCAA	6.35	(3.48)	11.09	(6.96)	13.82	(7.56)	9.08	(3.03)
Leucine	11.46	(6.39)	15.94	(7.12)	32.38	(23.8)	17.35	(3.03)
Placebo	8.71	(7.28)	12.92	(9.44)	15.32	(11.5)	9.75	(8.38)
ERK1/2								
BCAA	8.36	(4.79)	18.16	(14.7)	16.26	(3.83)	17.65	(5.61)
Leucine	6.34	(3.21)	6.21	(2.17)	7.97	(5.81)	3.21	(1.01)
Placebo	5.76	(3.81)	6.84	(3.07)	4.22	(2.18)	3.82	(2.87)
IRS-1								
BCAA	9.06	(3.12)	9.94	(4.37)	9.61	(4.24)	4.24	(4.71)
Leucine	8.93	(4.70)	12.31	(7.60)	20.07	(9.33)	9.33	(11.7)
Placebo	14.73	(6.39)	19.96	(10.1)	23.44	(12.2)	12.24	(11.2)
SHP-2								
BCAA	86.96	(21.73)	102.75	(27.94)	89.48	(24.99	101.89	(29.13)
Leucine	122.12	(30.31)	130.27	(28.99)	176.01	(153.7)	134.94	(30.28)
Placebo	123.21	(16.01)	145.98	(73.46)	153.41	(43.67)	132.83	(40.57)

Note. Units of measurement for MEK1, ERK1/2, and IRS-1 are Units of variable/ml muscle homogenate/mg of skeletal muscle (U/ml/mg). Units for SHP-2 are Absorbance units.

value (M = 3.222, SD = 8.385 U/ml/mg, p = .013). Pairwise comparisons (Bonferroni adjusted) also revealed a statistical trend for phosphorylated MEK1 at 30min post (M = 4.47, SD = 8.86, p = .076, effect size (η_p^2) = .24). Figure 12 demonstrates the main effect for time relative to phosphorylated MEK1. Hypothesis number one (H_1) stated that: "BCAA supplementation will not increase the phosphorylated (activation) state of MEK1 when compared to placebo in conjunction with lower-body resistance exercise." The

statistical results of the present study revealed that there was no group main effect or an interaction effect that had the BCAA group increase the activation state of MEK1 to a greater extent as compared to the placebo group. Due to these findings, H_1 was accepted. Hypothesis number two (H_2) stated that: "Leucine supplementation will not increase the phosphorylated (activation) state of MEK1 when compared to placebo in conjunction with lower-body resistance exercise." Since there was no group main effect or an interaction effect that observed the Leucine supplementation group increasing the activation status of MEK1 as compared to the placebo group, H_2 was accepted. The last hypothesis (H_3) regarding MEK1 phosphorylation stated that: "BCAA and leucine supplementation will not differ in regard to their increasing the phosphorylated state of MEK1 in conjunction with lower-body resistance exercise." The results of the present study indicated that neither BCAA nor Leucine increased the phosphorylation state of MEK1. Due to these findings, H_3 was accepted.

ERK1/2

The MANOVA analysis that indicated a significant main effect for group (Wilks' Lamda = .291, $F(8,48) = 5.1$, $p < .001$, effect size (η^2) = .460), and a significant group x time interaction (Wilks' Lamda = .132, $F(24,32) = 2.3$, $p = .013$, effect size (η^2) = .637) was accompanied by a significant univariate ANOVA main effect for group ($F(2,27) = 12.2$, $p = < .001$, effect size (η^2) = .474) and a significant univariate ANOVA group x time interaction ($F(4.3,57.5) = 2.9$, $p = .024$, effect size (η^2) = .181) for phosphorylated ERK1/2. Follow-up univariate ANOVA revealed that there were no main effects for time relative to phosphorylated ERK1/2. Following the univariate ANOVA indicating a main effect for group, pairwise comparisons were made utilizing a Bonferroni adjustment.

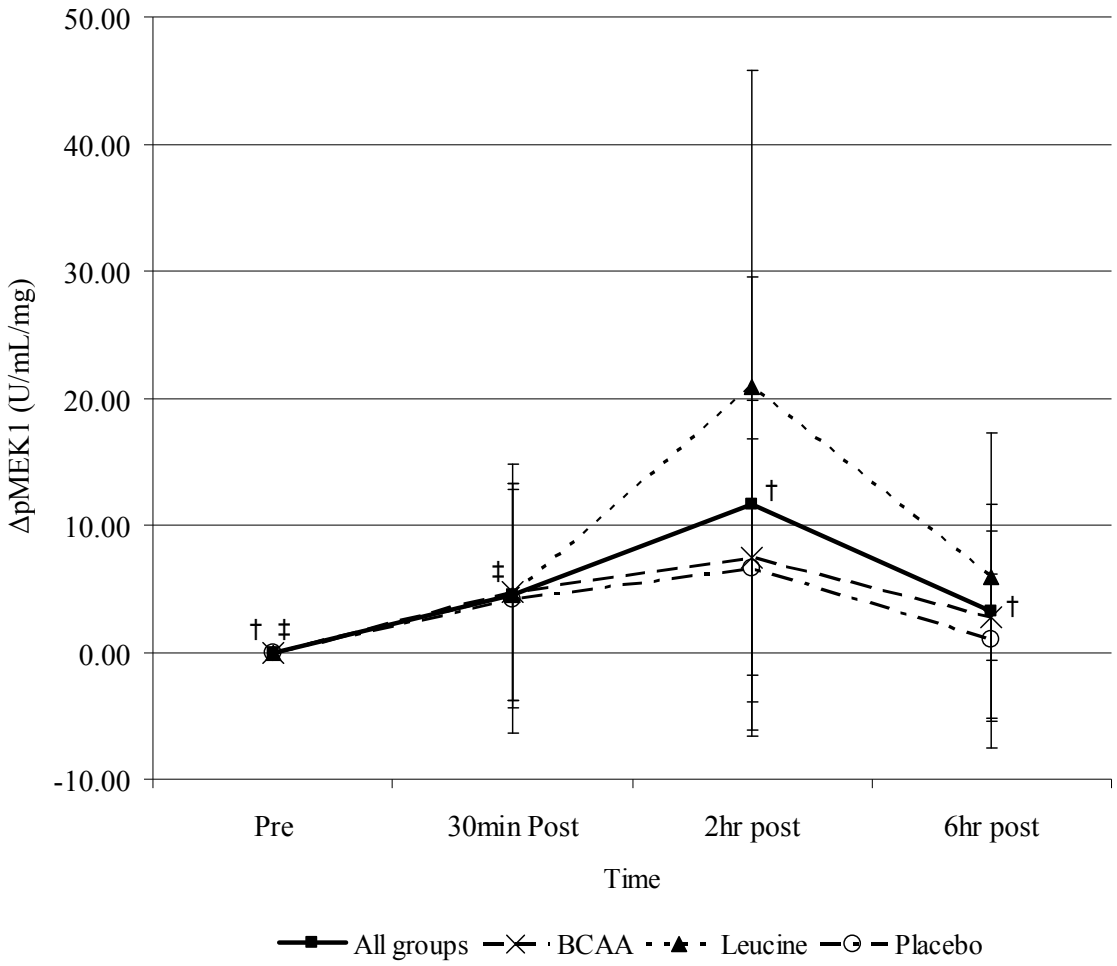

Figure 12. Changes over time (+*SD*) for all groups relative to phosphorylated MEK1. † = Significant differences between time points irrespective of supplementation group representing the main effect for time. MEK1 phosphorylation significantly increased at the 2hr post (p = .005). 2hr post was significantly greater than the changes in phosphorylated MEK1 observed at the 6hr post (p = .013). ‡ = Statistical trend indicating that phosphorylated MEK1 increased at 30min post (p = .076).

These pairwise results revealed that the BCAA group (M = 6.748, SE = 1.2 U/ml/mg) was significantly elevated as compared to the Leucine (M = -0.406, SE = 1.2 U/ml/mg, p = .001) and Placebo groups (M = -0.603, SE = 1.2 U/ml/mg, p = .001) (Figure 13). Following the univariate ANOVA indicating a group x time interaction, an independent samples t-test was utilized to determine where the significant interactions occurred. When comparing delta values, a significant difference occurred between the BCAA and Leucine group at 2hrs post ($t(18)$ = 2.2, p = .045) and 6hrs post ($t(18)$ = 5.5, p < .001).

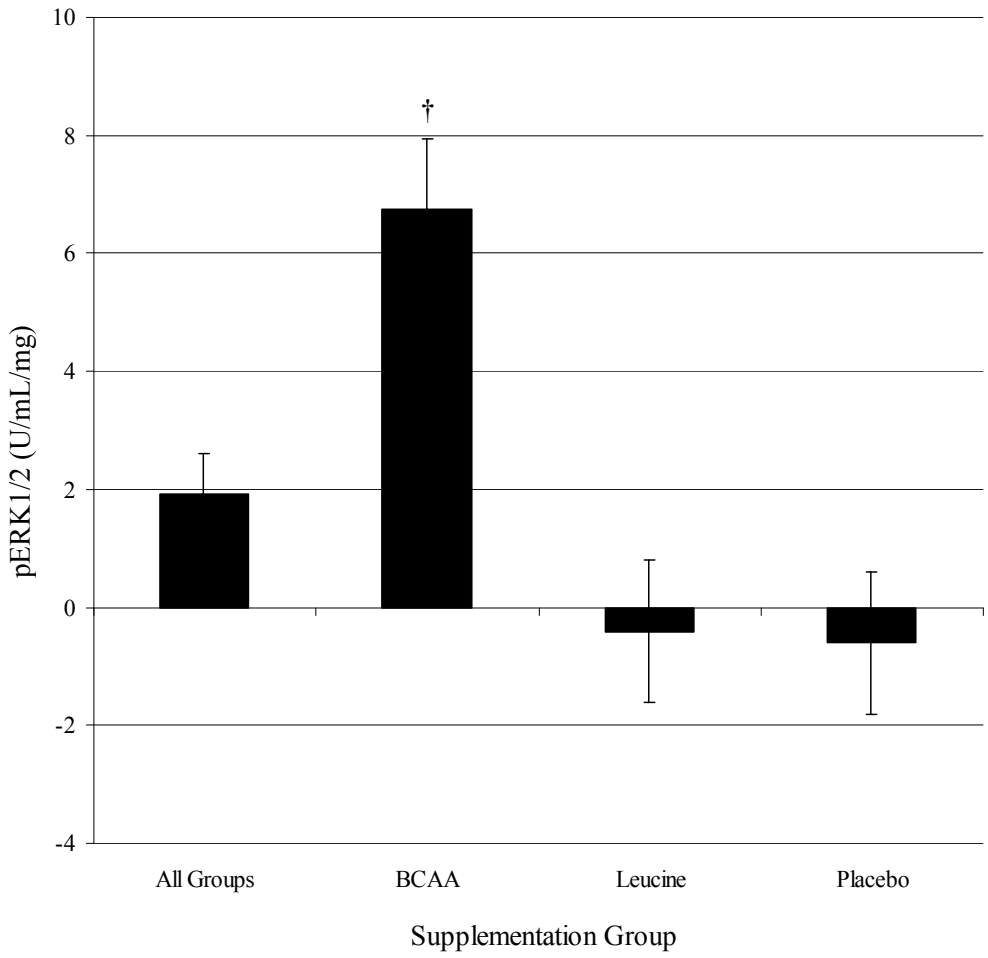

Figure 13. Significant group differences (+*SD*) irrespective of time representing the main effect for group. † = BCAA was significantly greater than leucine and placebo ($p = .001$).

Specifically, the BCAA group ($M = 9.80$, $SD = 15.88$ U/ml/mg) experienced a statistical trend ($t(18) = 1.911$, $p = .072$) at 30min post resistance exercise in comparison with the Leucine group ($M = -0.131$, $SD = 4.213$ U/ml/mg). Also, the BCAA group (2hrs post: $M = 7.903$, $SD = 6.613$; 6hrs post: $M = 9.287$, $SD = 6.543$ U/ml/mg) experienced significant increases in phosphorylated ERK1/2 as compared to the leucine group at 2hrs post ($M = 1.633$, $SD = 6.412$ U/ml/mg) and 6hrs post ($M = -3.126$, $SD = 2.948$ U/ml/mg). A significant difference occurred between the BCAA and Placebo groups at 2hrs post ($t(18) = 3.9$, $p = .001$) and 6hrs post ($t(18) = 4.5$, $p < .001$). On

average, those participants in the BCAA group (2hrs post: $M = 7.903$, $SD = 6.613$; 6hrs post: $M = 9.287$, $SD = 6.543$) experienced significantly greater levels of phosphorylated ERK1/2 as compared to the placebo group at 2hrs post ($M = -1.54$, $SD = 3.851$) and 6hrs post ($M = -1.943$, $SD = 4.47$) resistance exercise. These interaction effects are illustrated in figure 14. No significant differences were observed between the Leucine and Placebo groups.

Hypothesis number four (H_4) stated that: "BCAA supplementation will not increase the phosphorylated (activation) state of ERK1/2 when compared to placebo in conjunction with lower-body resistance exercise." The statistical results revealed that there was a main effect for group with the BCAA group demonstrating a significant increase in ERK1/2 phosphorylation irrespective of time as compared to the Placebo group ($p = .001$). The statistical analyses of delta values also revealed a group x time interaction, with the BCAA group experiencing a significant increase in ERK1/2 phosphorylation at the 2hrs post and 6hrs post time points as compared to the placebo group at 2hrs post and 6hrs post. Due to these findings, H_4 was not accepted. Hypothesis number five (H_5) stated that: "Leucine supplementation will not increase the phosphorylated (activation) state of ERK1/2 when compared to placebo in conjunction with lower-body resistance exercise." No significant differences were observed between the Leucine and Placebo groups. Therefore, H_5 was accepted stating that Leucine supplementation will not increase the phosphorylated (activation) state of ERK1/2 when compared to placebo in conjunction with lower-body resistance exercise). The last hypothesis (H_6) regarding ERK1/2 phosphorylation stated that: "BCAA and leucine supplementation will not differ in regard to their phosphorylated state of ERK1/2 in conjunction with lower-body resistance exercise." The statistical results revealed that

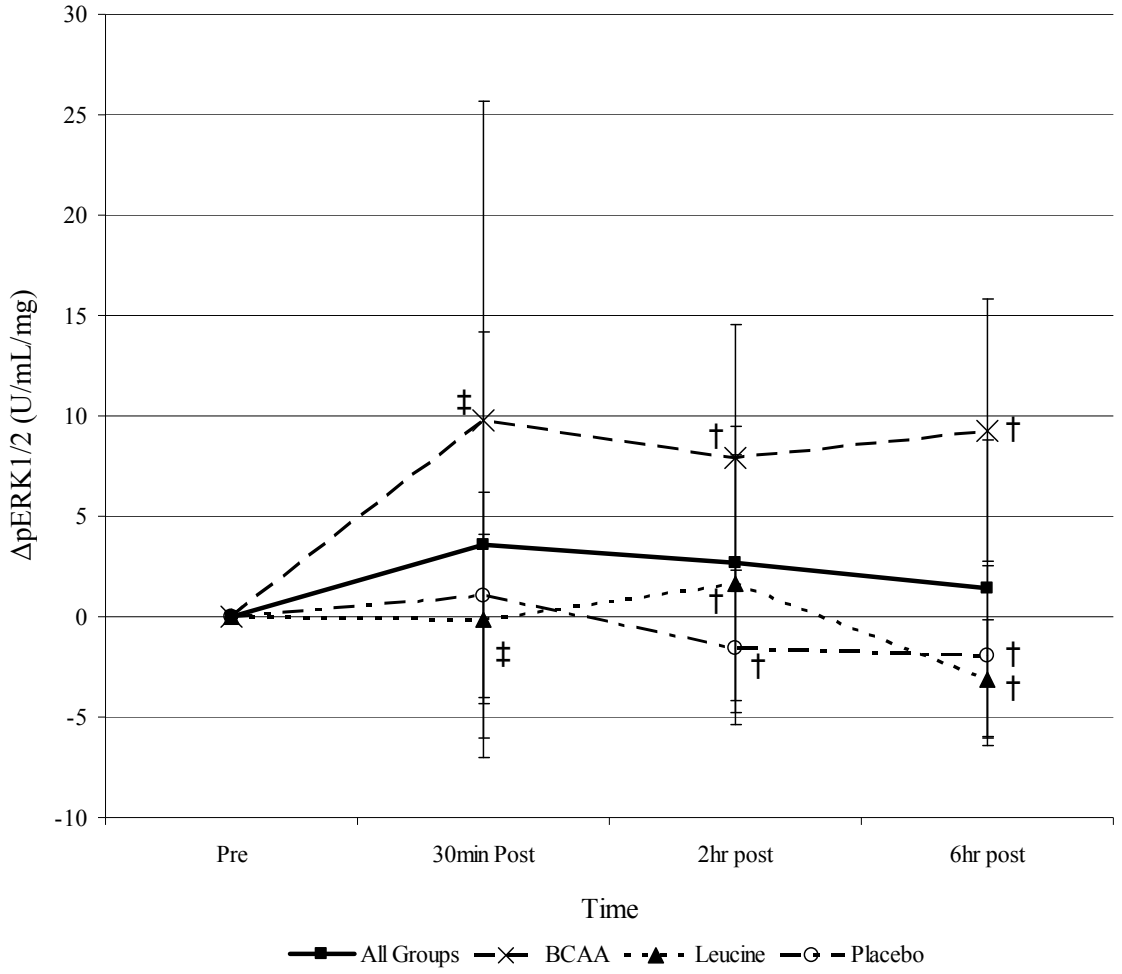

Figure 14. Group x time interaction. † = BCAA obtained significantly greater phosphorylated levels of ERK1/2 as compared to Leucine and Placebo at 2hr post and 6hr post ($p < .05$). ‡ = Statistical trend indicating that BCAA had greater phosphorylated ERK1/2 levels at 30min post as compared to the Leucine group ($p = .072$).

there was a main effect for group with the BCAA group demonstrating a significant ($p = .001$) increase in ERK1/2 phosphorylation irrespective of time as compared to the Leucine group. The statistical analyses also revealed a group x time interaction, with the BCAA group experiencing a significant increase in ERK1/2 phosphorylation at the 2hrs post and 6hrs post time points as compared to the leucine group at these same time points. Due to these findings, H_6 was not accepted.

IRS-1

The MANOVA analysis that indicated a significant main effect for group (Wilks' Lamda = .291, $F(8,48) = 5.1$, $p < .001$, effect size (η^2) = .460) was accompanied by a significant univariate ANOVA main effect for group for phosphorylated IRS-1 ($F(2,27) = 7.9$, $p = .002$, effect size (η^2) = .370). Following the univariate ANOVA indicating a main effect for group, pairwise comparisons were made utilizing a Bonferroni adjustment. These pairwise comparisons revealed that the BCAA group ($M = .119$, $SE = 1.28$) had significantly less phosphorylated IRS-1 as compared to the Leucine ($M = 7.076$, $SE = 1.28$, $p = .002$) and Placebo ($M = 5.173$, $SE = 1.28$, $p = .028$) groups across time. Figure 15 demonstrates the main effect for group in relation to phosphorylated IRS-1. The MANOVA analysis that indicated a significant main effect for time (Wilks' Lamda = .300, $F(12,16) = 3.11$, $p = .018$, effect size (η^2) = .700) was accompanied by a significant univariate ANOVA main effect for time for phosphorylated IRS-1 ($F(2.8,74.2) = 5.7$, $p = .002$, effect size (η^2) = .175). Following the univariate ANOVA indicating a main effect for time, pairwise comparisons were made utilizing a Bonferroni adjustment. These pairwise comparisons revealed the following data for phosphorylated IRS-1 (measured in Units/mL/mg). Specifically, there was a strong statistical trend in IRS-1 phosphorylation at the 30min post ($M = 3.163$, $SD = 6.173$, $p = .052$) time point. Also, the 2hr post ($M = 6.798$, $SD = 10.946$, $p = .007$) and 6hr post ($M = 6.530$, $SD = 10.716$, $p = .003$) time points demonstrated significant increases over time in relation to IRS-1 phosphorylation. Figure 16 demonstrates the main effect for time in regards to IRS-1. Also, the MANOVA analysis that indicated a significant group x time interaction (Wilks' Lamda = .132, $F(24,32) = 2.3$, $p = .013$, effect size (η^2) = .637) was accompanied by a significant univariate ANOVA group x time interaction ($F(5.5,74.2) = 2.4$, $p = .043$, effect size

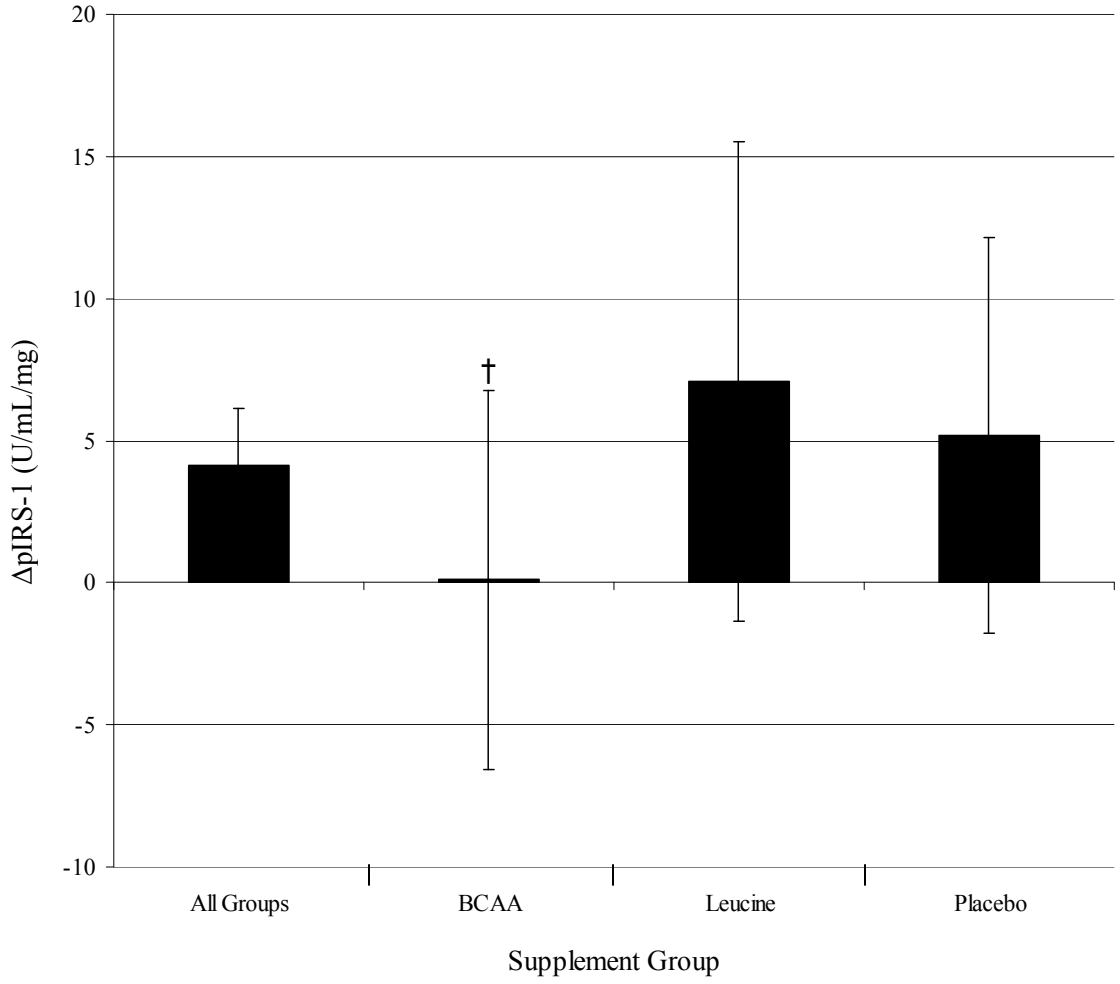

Figure 15. Significant group differences (+*SD*) irrespective of time representing the main effect for group. † = BCAA was significantly less than leucine and placebo relative to phosphorylated IRS-1 ($p < .05$).

$(\eta^2) = .149$) for phosphorylated IRS-1. Following the univariate ANOVA indicating a group x time interaction, an independent samples t-test was utilized to determine where the significant interactions occurred. When comparing delta values, a significant difference occurred between the BCAA and Leucine group at 2hrs post ($t(9.9) = -3.49$, $p = .006$) and 6hrs post ($t(10.5) = -3.88$, $p = .003$), with the leucine group ($M = 11.144$, $SD = 9.376$ Units/ml/mg; $M = 13.776$, $SD = 11.509$ Units/ml/mg) demonstrating significant increases in IRS-1 phosphorylation as compared to the BCAA group ($M = .546$, $SD = 2.055$ Units/ml/mg; $M = -0.951$, $SD = 3.323$ Units/ml/mg) at 2hr post and 6hr post, respectively. In addition, a significant interaction occurred between the BCAA and

Placebo group at 6hrs post ($t(10.9) = -2.3$, $p = .046$), with the placebo group ($M = 6.765$, $SD = 10.294$ Units/ml/mg) demonstrating a significant elevation as compared to the BCAA group ($M = -0.951$, $SD = 3.323$ Units/ml/mg). No significant differences were observed between the Leucine and Placebo groups relative to the phosphorylated states of IRS-1. These interaction effects are illustrated in Figure 16.

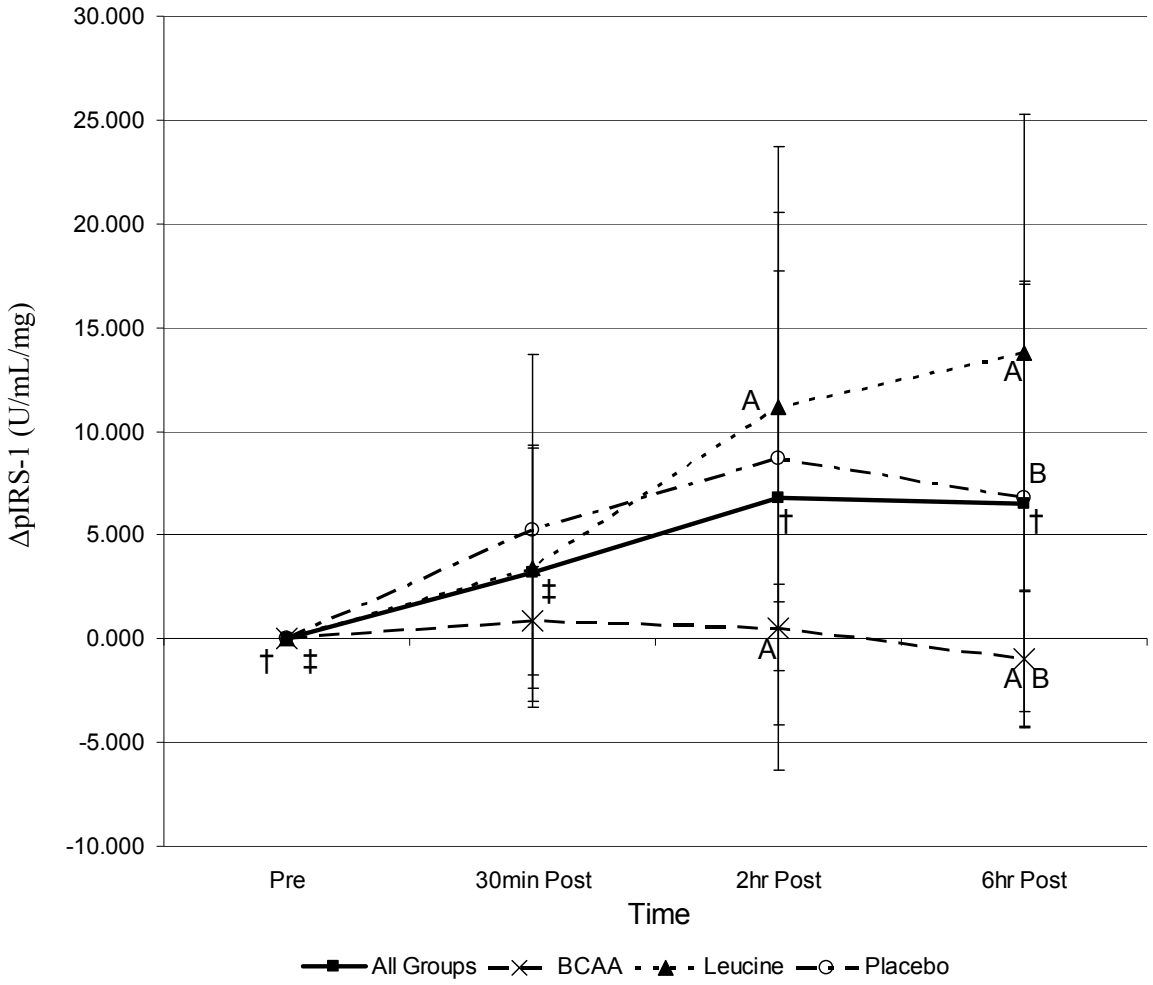

Figure 16. Main effects for time and group x time interactions relative to pIRS-1. † = Significant differences between time points irrespective of supplementation group representing the main effect for time. 2hr post and 6hr post were significantly greater than Pre ($p < .05$). ‡ = Statistical trend indicating that 30min post is greater than Pre ($p = .052$). Group x time interaction: A = Leucine had a significantly greater level of pIRS-1 as compared to BCAA at 2hr post and 6hr post ($p < .05$); B = Placebo had a significantly greater level of pIRS-1 as compared to BCAA at 6hr post ($p = .046$).

Hypothesis number seven (H_7) stated that: "BCAA supplementation will not increase the phosphorylated (activation) state of IRS-1 when compared to placebo in conjunction with lower-body resistance exercise." While a main effect for group was observed in relation to IRS-1 activation, the results revealed that BCAA group demonstrated significantly less phosphorylated IRS-1 as compared to the leucine and placebo groups. In addition, a group x time interaction revealed that at 6hrs post, the placebo group had significantly greater levels of phosphorylated IRS-1 as compared to the BCAA group. Due to these findings, H_7 was accepted.

Hypothesis number eight (H_8) stated that: "Leucine supplementation will not increase the phosphorylated (activation) state of IRS-1 when compared to placebo in conjunction with lower-body resistance exercise." No significant differences were observed between the Leucine and Placebo groups relative to the phosphorylated states of IRS-1. Due to these findings, H_8 was accepted.

Hypothesis number nine (H_9) stated that: "BCAA and leucine supplementation will not differ in regard to their increasing the phosphorylated state of IRS-1 in conjunction with lower-body resistance exercise. The results of the present indicated that there were significant group x time interactions between the BCAA and leucine groups at the 2hr post-exercise and 6hr post-exercise time points in relation to changes in IRS-1 phosphorylation over time. Specifically, the leucine group experienced significant increases in IRS-1 phosphorylation at these time points as compared to the BCAA group. In light of these findings, H_9 was not accepted.

SHP-2

The MANOVA analysis that indicated a significant main effect for time (Wilks' Lamda = .300, $F(12,16) = 3.11$, $p = .018$, effect size (η^2) = .700), a significant main effect for group (Wilkes lamda = .291, $F(8,48) = 5.1$, $p < .001$, effect size (η^2) = .460), and a significant group x time interaction (Wilks' Lamda = .132, $F(24,32) = 2.3$, $p = .013$, effect size (η^2) = .637) were not accompanied by a significant univariate ANOVA main effect for time, main effect for group, or a group x time interaction. Therefore, no further statistical investigations were made in relation to SHP-2. Figure 17 demonstrates the changes over time for each supplemental group relative to SHP-2 phosphorylation.

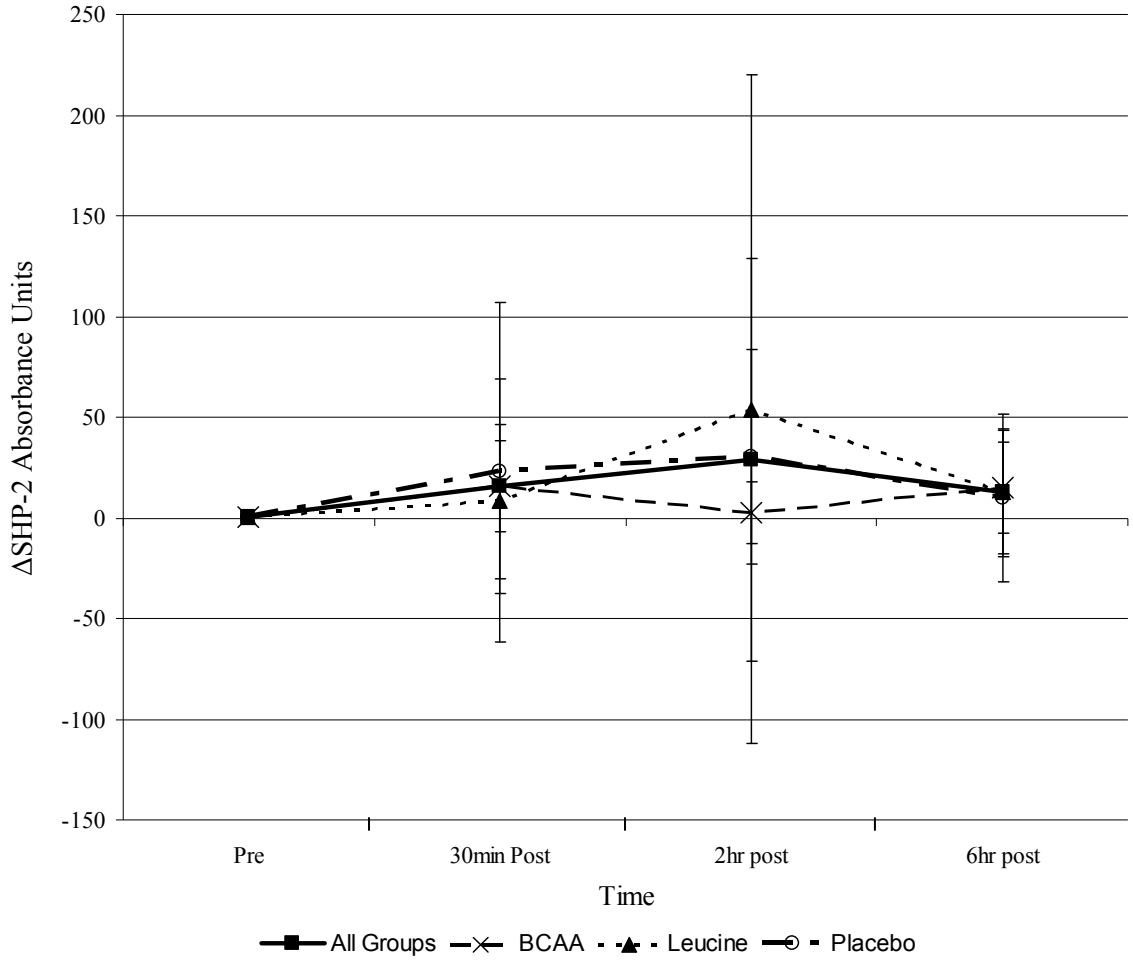

Figure 17. Changes in absorbance units over time for each supplemental group relative to SHP-2 phosphorylation.

Hypothesis number 10 (H_{10}) stated that: "BCAA supplementation will not increase the phosphorylated (activation) state of SHP-2 when compared to placebo in conjunction with lower-body resistance exercise." The results indicated that BCAA did not increase the activity of phosphorylated SHP-2 as compared to the placebo group. Therefore, H_{10} was accepted. Hypothesis number 11 (H_{11}) stated that: "Leucine supplementation will not increase the phosphorylated (activation) state of SHP-2 when compared to placebo in conjunction with lower-body resistance exercise." The results indicated that leucine ingestion did not increase the activity of phosphorylated SHP-2 as compared to the placebo group. In light of these findings, H_{11} was accepted. The last hypothesis to be tested was hypothesis number 12 (H_{12}), which stated: "BCAA and leucine supplementation will not differ in regard to their increasing the phosphorylated state of SHP-2 in conjunction with lower-body resistance exercise." The results indicated that BCAA and leucine supplementation did not increase the activity of phosphorylated SHP-2. Therefore, H_{12} was accepted.

CHAPTER FIVE

Discussion

Introduction

The primary purpose of this investigation was to determine if leucine ingestion increases the activation of the ERK1/2 MAPK pathway (including the phosphorylation state of the MAPK proteins MEK1 and ERK1/2) to an extent greater than that which resistance exercise would elicit alone. If such an increase in ERK1/2 MAPK pathway activity increased as a result of leucine ingestion, then a secondary purpose of this study was to determine a possible mechanism responsible for a the leucine induced activation of the ERK1/2 MAPK pathway. Specifically, the PI3-K/Akt/mTOR pathway is capable of increasing the activity of the ERK1/2 MAPK pathway, and one of the ways in which it accomplishes this is through the activation of IRS-1 and the subsequent activation of the protein phosphatase SHP-2. Therefore, as a secondary purpose of the investigation, the activation status of both IRS-1 and SHP-2 were studied.

The clinical investigations that have examined the effects of leucine supplementation in humans and its effects on exercise performance have consistently shown that leucine does not improve performance (Mero, 1999; Pitkanen et al., 2003). In addition, to date there are few no studies that have shown that leucine supplementation alone leads to significant increases in lean body mass in conjunction with resistance training. Despite the lack of scientific evidence linking leucine ingestion to improved performance, the studies demonstrating that leucine possesses a regulatory role in protein synthesis and promotes skeletal muscle hypertrophy have continued to make leucine a

popular sports supplement (Buse & Reid, 1975; Layman, 2002; Tischler et al., 1982). Multiple studies (mainly in rodent models) have demonstrated the ability of leucine to activate key regulatory elements in the Akt/mTOR signal transduction pathway (which is correlated to an increase in protein synthesis) (Anthony, Anthony et al., 2000; Anthony et al., 1999; Anthony, Yoshizawa et al., 2000; Crozier et al., 2005)).

Similar to the Akt/mTOR pathway, the ERK1/2 MAPK pathway has been well-defined in its activation following various modes of exercise including running (Yu 2001a, Yu 2001b) cycling (Aronson et al., 1997; Krook et al., 2000; Osman et al., 2000; Widegren et al., 1998), and resistance exercise (Creer et al., 2005; Thompson et al., 2003; Williamson et al., 2003). Currently, no studies exist examining the effects that supplemental leucine alone may have on the ERK1/2 signal transduction pathway in conjunction with resistance exercise. In fact, only two studies have been conducted (Creer et al., 2005; Karlsson et al., 2004) investigating the effects of nutritional interventions on this pathway in conjunction with resistance exercise in humans. The present study is the only investigation that we are aware of which has examined the effects of leucine supplementation in conjunction with resistance exercise on the effects of the ERK1/2 MAPK pathway. In this sense, the present study appears to be a novel investigation.

In early investigations, it was believed that all three of the BCAAs were necessary to activate the PI3-K/Akt/mTOR pathway, leading to increased rates of protein synthesis. In later investigations, it was determined that leucine alone was responsible for the increased activation of the PI3-K/Akt/mTOR pathway (Anthony et al., 2002; Greiwe et al., 2001). To date, only one study has been conducted on the effects of the BCAAs on the activation status of the ERK1/2 MAPK pathway in conjunction with resistance

exercise (Karlsson et al., 2004). For these two reasons (past studies investigating leucine alone or leucine as part of a BCAA mixture on cell-signaling pathways and the fact that currently only one study exists investigating the effects of BCAAs on the ERK1/2 MAPK pathway), a BCAA group was included in the present investigation.

ERK1/2 MAPK Pathway

MEK1

Since there was no group main effect or a group x time interaction relative to MEK1 activation, the changes observed in the phosphorylation/activation status of MEK1 were likely due to the mechanical stimulation induced by the lower-body resistance exercise session. This is the first investigation to study the effects of MEK1 phosphorylation in response to a resistance exercise protocol in humans. There are three other studies which have investigated the MEK1 activation response to exercise, but in each of these studies the mode of exercise utilized was cycling (Aronson et al., 1997; Osman et al., 2000; Widegren et al., 2000).

Aronson et al. (1997) designed an investigation to determine how a single bout of cycling exercise activates the MAP kinase pathway (MEK1 and ERK1/2). The exercise protocol consisted of 60 minutes of cycle ergometer exercise at a load corresponding to 70% of the subjects' VO_{2max}. MEK1 activation was sampled via skeletal muscle biopsies before and immediately (approximately 3-4 minutes) following the cycling exercise protocol. Following the 60 minute cycling exercise bout, MEK1 activation was significantly increased in comparison with baseline values.

In a similar study design, Osman et al. (2000) designed an investigation to determine how a single bout of cycling exercise and physiological hyperinsulinemia

activate the MAP kinase pathway (MEK1 and ERK1/2). The cycling exercise protocol employed by Osman et al., (2000) was 30 minutes in duration at a workload that was equivalent to 90% of the subjects' anaerobic threshold (~60% VO_{2max}). MEK1 activation was sampled via skeletal muscle biopsies before and immediately post-exercise. The 30-minute cycling exercise had no effect on the activation status of MEK1.

Widegren et al. (2000) instructed study participants to perform one-leg cycle ergometer exercise at workloads calculated to elicit ~40% of one-leg VO_{2max} for the first 30 minutes and ~75% of one-leg VO_{2max} for the following 30 minutes. Skeletal muscle biopsies were obtained at 30 and 60 minutes from the non-exercised leg and at 0, 30, and 60 minutes of cycle ergometry from the exercised leg (corresponding to an approximate immediate post-exercise muscle biopsy) and analyzed for MEK1/2 activity. Relative to the basal value, MEK1/2 phosphorylation increased 2.5-fold at the lower intensity and 4.8-fold at the high intensity. Also, Widegren et al. (2000) reported that there was a significant difference between the basal conditions, 40% of VO_{2max}, and 75% of VO_{2max} relative to MEK1/2 activation. There was no increase in MEK1/2 phosphorylation in the non-exercised leg regardless of intensity. The authors of this study concluded that the major finding in this study was that the exercise-induced activation of MEK1/2 occurs in an intensity-dependent manner.

The findings of the present study are in agreement with the findings of Aronson et al. (1997) and Widegren et al. (2000) in relation to a mechanical stress via an exercise bout resulting in an increase in MEK1 (or MEK1/2) activation/phosphorylation. The main differences between these studies are the mode of exercise (cycle ergometer vs. resistance exercise) and the time points for assessing MEK1-MEK1/2 activity (immediate post-exercise (Aronson et al., 1997; Widegren et al., 2000) or in the several hours

following an exercise bout (current study). The Osman et al. (2000) study reported findings that are inconsistent with the findings of the current study as well as those reported by Aronson et al. (1997) and Widegren et al. (2000). Perhaps the 30 minute cycle ergometer protocol at ~60% VO_{2max} utilized in the Osman investigation was not of sufficient duration to induce an increase in MEK1 phosphorylation. However, Widegren et al. (2000) reported significant increases in MEK1 phosphorylation following 30 minutes of cycle ergometry at an intensity of only 40% of VO_{2max}. Due to the small number of investigations studying the effects of exercise on the MAPK signal transduction protein kinase MEK1 and the inconsistent results that have been reported, more research need to be conducted. Table 7 summarizes each study that has been conducted and the time points for which MEK1 or MEK1/2 have been analyzed following an acute bout of either cycling exercise or resistance exercise.

Table 7. *MEK1 response to mechanical stress*

		Time Point of MEK1 Sampling			
Reference	Mode	Immediate	30 min	2 hour	6 hour
Aronson (1997)	Cycling	↑**	X	X	X
Osman (2000)	Cycling	NC	X	X	X
Widegren (2000)	Cycling	↑**	X	X	X
Current study	Resistance Exercise	X	NC*	↑**	NC

Note. The Widegren study assessed MEK1/2 and not solely MEK1. X = did not sample at that time point. NC = no changed in MEK1 phosphorylation status. NC* = statistical trend toward increasing MEK1 phosphorylation ($p < .10$). ↑** = significant increase in MEK1 (MEK1/2) phosphorylation ($p < .05$).

ERK1/2

The BCAA group significantly increased ERK1/2 phosphorylation without a subsequent increase in MEK1 activation. This is an interesting finding given the fact that phosphorylation and activation of MEK are required for activation of ERK1/2 (Zheng & Guan, 1993b). There are two possible explanations for this observation. The first explanation is the possibility that the BCAA's collectively activate ERK1/2 directly without activating the upstream regulators of the ERK1/2 MAPK pathway (Raf and MEK). The other explanation was suggested by Campbell, Wenderoth, Hauschka, & Krebs (1995) in which they found that, under certain conditions, the ERK1/2 activities may not follow their upstream activator MEK due to changes in phosphatase activity. Each of these possible mechanisms needs to be further investigated.

Karlsson et al. (2004) conducted the only other study in humans that investigated the effects of resistance exercise in combination with oral intake of BCAAs on the phosphorylation state of ERK1/2. Seven healthy men performed leg press for 4 sets of 10 repetitions at a workload corresponding to 80% 1RM. There was also a 5-minute rest period between each set. Relative to the BCAA supplementation, the subjects ingested a 150 mL solution of BCAA (45% leucine, 30% valine, 25% isoleucine) at a dosage of 100 mg/kg of bodyweight or flavored water at seven time points (10 minutes and immediately before resistance exercise, during resistance exercise, and 15, 30, 60, and 90 minutes following resistance exercise). Skeletal muscle biopsies were taken at four time points (10 minutes prior to resistance exercise, immediately after resistance exercise, and one and two hours following resistance exercise). Relative to ERK1/2 activation, the resistance exercise bout led to a significant increase in ERK1/2 phosphorylation immediately after resistance exercise. At one and two hours post- resistance exercise,

ERK1/2 phosphorylation was similar to pre-exercise levels. In relation to BCAA supplementation, the phosphorylation status of ERK1/2 was unaltered by BCAA ingestion.

The results of the present study are not in agreement with the Karlsson et al., (2004) study. The main finding of the Karlsson et al. (2004) investigation was that BCAA supplementation did not increase the activation status of ERK1/2 in comparison with a placebo group. In contrast, the present study observed a significant increase in ERK1/2 phosphorylation at two and six hours post-exercise in those subjects ingesting BCAAs in comparison with the placebo group. The BCAA supplementation differed slightly in the two investigations. The present study administered 120 mg/kg of bodyweight consisting of 50% leucine, 25% isoleucine, and 25% valine. The present study used this ratio of BCAAs as this is a ratio similar to that found in animal protein (Shimomura, Murakami, Nakai, Nagasaki, & Harris, 2004).

Karlsson et al. (2004) administered 100 mg/kg of bodyweight consisting of a slightly different BCAA composition (45% leucine, 25% isoleucine, and 30% valine). It is possible that the slight increase in the relative amount of BCAA supplementation in the present study surpassed a threshold that would activate the ERK1/2 activation status. However, a theoretical threshold for BCAA supplementation inducing ERK1/2 activation is only a possible explanation in an attempt to explain the differences between the current study and the Karlsson et al. (2004) study, and to date has not been investigated. Table 8 highlights some of the conflicting results found between the present study and the Karlsson (2004) study.

The fact that BCAAs increased ERK1/2 activation and leucine alone did not is an interesting finding. Unlike the effects of BCAAs and leucine alone on the PI3-

K/Akt/mTOR pathway, in which leucine alone increases the activity of this pathway, the present study showed no such increases in ERK1/2 MAPK pathway activity as a result of leucine supplementation. The only other related studies investigating the effects of leucine on ERK1/2 MAPK activity were conducted in cell cultures (rat hepatocyte cells) (Kimura & Ogihara, 2005; Perez de Obanos, Lopez Zabalza, Prieto, Herraiz, & Iraburu, 2006).

Table 8. *BCAA and ERK1/2 Activation*

		Time Point of ERK1/2 Sampling				
Reference	BCAA Dosage	Immediate	30min	1hr	2hr	6hr
Karlsson (2004)	100 mg/kg BW	NC	X	NC	NC	X
Present Study (2007)	120 mg/kg BW	X	NC*	X	↑**	↑**

Note. BW = body weight. NC = No change in ERK1/2 activation. X = no sample taken at that time point. ↑ = increase in ERK1/2 activation. * = trend ($p < .10$). ** = significant increase ($p < .05$).

The first of these studies investigated the effects of the BCAAs independently on the activation status of ERK1/2 in cultured rat hepatocytes (Kimura & Ogihara, 2005). The results of this study revealed that after leucine was added to the cell cultures, there was a significant increase in the phosphorylation level of ERK2 (but not ERK1) within 20 minutes. It was also determined that isoleucine and valine on their own did not stimulate ERK1/2 activity. The authors of this study explained that there is little evidence that leucine stimulates the ERK MAPK pathway directly, but rather stimulates the secretion of TGF-α which subsequently increases ERK2 activation (Kimura & Ogihara, 2005). The other investigation (Perez de Obanos et al., 2006) also treated hepatic cell cultures with leucine and measured (among other variables) the ERK2 activation response. The

authors reported that ERK2 phosphorylation was induced within five minutes of leucine treatment (Perez de Obanos et al., 2006). Caution must be made when interpreting these findings to the present study, however, as these findings were reported from cultured rat hepatocytes and not in humans *in vivo*.

As was stated before, the present study appears to be the first study to investigate the effects of leucine alone in conjunction with resistance exercise on the activation status of the ERK1/2 pathway in humans. The findings of the present study indicate the possibility that isoleucine, valine, or a combination of isoleucine and valine may be responsible for the observed effects of increasing the phosphorylation status of ERK1/2 in conjunction with resistance exercise. This proposed mechanism of ERK1/2 activation by isoleucine and valine are not in agreement with the findings that Kimura & Ogihara (2005) and Perez de Obanos et al. (2006) observed in cultured rat hepatocytes, in which only leucine augmented the activation of ERK2. More research needs to be conducted, preferably in human models, before any conclusions can be drawn relative to leucine and BCAA ingestion and their effects on ERK1/2 MAPK activation in conjunction with resistance exercise.

Growth Factor Stimulation of ERK1/2 Activation

Since ERK1/2 activation was found to increase in those subjects ingesting BCAAs, an important consideration was to investigate those hormones/growth factors that have been shown to increase ERK1/2 activity. The three main hormones/growth factors that have been shown to activate the ERK1/2 MAPK pathway are GH (Anderson, 1992; Campbell et al., 1992; Hodge et al., 1998; Winston & Bertics, 1992), IGF-1 (Castillo et al., 2006; Foulstone et al., 2004; Pozios et al., 2001), and insulin (Goodyear et

al., 1996; Napoli et al., 1998; Osman et al., 2000; Wojtaszewski et al., 1999). BCAA ingestion did not increase the levels of these hormones as compared to the leucine and placebo groups. Therefore, the possibility that ERK1/2 activation was due to a concomitant increase in circulating growth factors resulting from BCAA ingestion can likely be eliminated.

PI3-K/Akt/mTOR and ERK1/2 MAPK Crosstalk

IRS-1

Because there was an increase in ERK1/2 MAPK pathway activation at the level of ERK1/2, the present study was designed in that it investigated certain variables that may help explain a possible mechanism for the ERK1/2 responses observed. The cross talk between the PI3-K/Akt/mTOR and MAPK pathways occur at the level of the insulin receptor. Currently, two methods of cross-talk have been elucidated (Cheng et al., 2002). The first of these methods involves the recruitment of signaling molecules containing SH2 (src homology 2) domains by the insulin receptor. Once phosphorylated, Shc proteins may couple to the Ras signaling pathway through the formation of a Shc-Grb2-Sos complex (Cheng et al., 2002; Egan et al., 1993; N. Li et al., 1993) and subsequently activate the ERK1/2 MAPK pathway. The other method of cross talk between the PI3-K/Akt/mTOR and MAPK pathways occurs at the level of IRS-1 activation and the subsequent activation of the protein tyrosine phosphatase SHP-2. Once activated, SHP-2 binds with Grb2 and subsequently is able to activate the ERK1/2 MAPK pathway through Ras activation (Cheng et al., 2002; Z. Q. Shi et al., 1998; Z. Q. Shi et al., 2000). The latter of these two methods of PI3-K/Akt/mTOR-ERK1/2 MAPK crosstalk was investigated in the present study.

Despite the finding that insulin levels were not significantly elevated in response to BCAA ingestion, IRS-1 activity was significantly elevated. However, it is likely that the observed IRS-1 activation was a result of the mechanical stress imposed by the resistance exercise or by some other unexplained mechanism and not via increased insulin secretion.

SHP-2

What each of these findings relative to IRS-1 and SHP-2 activity indicate is that even though BCAA ingestion increased the activity of ERK1/2, it is likely not a result of cross talk between the PI3-K/Akt/mTOR and ERK1/2 MAPK pathways via IRS-1 and SHP-2. This is evident for three reasons: 1) serum insulin was not elevated in the BCAA group which would result in an increase in IRS-1 activity; 2) the group which demonstrated changes in ERK1/2 activity, the BCAA group, did not increase the activity of IRS-1; 3) SHP-2 was not elevated by BCAA ingestion and therefore was likely not involved in any cross-talk between the PI3-K/Akt/mTOR and ERK1/2 MAPK pathways. The other mechanism identified relative to cross-talk between the PI3-K/Akt/mTOR and ERK1/2 MAPK pathways exists at the insulin receptor level (Cheng et al., 2002). Since this pathway was not investigated in the current study, there is the possibility that this mechanism could have played a role in the observed ERK1/2 activation.

Summary and Future Direction

In summary, the results of the present study indicate that BCAA supplementation increases the phosphorylation status of ERK1/2 in conjunction with resistance exercise at 2 and 6 hours post-exercise. Leucine supplementation did not have any effect on ERK1/2 activation at any of the sampled time points. It appears that isoleucine, valine, or a

combination of these two BCAAs likely played a role in increasing the activity of ERK1/2 in conjunction with lower-body resistance exercise. It was also evident that cross-talk between the PI3-K/Akt/mTOR and ERK1/2 MAPK pathways at the level of IRS-1 and SHP-2 did not play a role in the increased levels of ERK1/2 that were observed. The findings in this study were novel in that this appears to be the first investigation to study the effects of leucine alone on the ERK1/2 MAPK pathway. This study also observed findings relative to BCAA ingestion and ERK1/2 activation that were not in agreement with a previous study (Karlsson et al., 2004) that utilized a similar study design. In light of these findings, more research needs to be conducted relative to nutritional interventions (specifically nitrogen-containing compounds) and their effects on the signal transduction pathways that regulate transcriptional control in human skeletal muscle.

APPENDICES

APPENDIX A

Personal Information Form – Baylor University

Personal Information

Name:

Address:

City: _____ State: _____ Zip Code _____ SS# _____

Home Phone: (___) _____ Work Phone: (___)_____

Beeper: (___) _____ Cellular(___) _____

Fax: (___) _____ email address: _____

Birth date: _____ / _____ / _____ Age: _____ Height: _____ Weight:

Exercise & Supplement History/Activity Questionnaire

1. Describe your typical occupational activities.

2. Describe your typical recreational activities

3. Describe any exercise training that you routinely participate.

4. How many days per week do you exercise/participate in these activities?

5. How many hours per week do you train?

6. How long (years/months) have you been consistently training?

7. When was the last time you ingested an anti-inflammatory product?

8. What was the reason you were taking an anti-inflammatory product?

APPENDIX B

Medical History Inventory

Directions. The purpose of this questionnaire is to enable the staff of the Exercise and Sport Sciences Laboratory to evaluate your health and fitness status. Please answer the following questions to the best of your knowledge. All information given is CONFIDENTIAL as described in the Informed Consent Statement.

Name:_____ Age _____Date of Birth_____

Name and Address of Your Physician:_____

MEDICAL HISTORY

Do you have or have you ever had any of the following conditions? (Please write the date when you had the condition in blank).

____ Heart murmur, clicks, or other cardiac findings?
____ Frequent extra, skipped, or rapid heartbeats?
____ Chest Pain of Angina (with or without exertion)?
____ High cholesterol?
____ Diagnosed high blood pressure?
____ Heart attack or any cardiac surgery?
____ Leg cramps (during exercise)?
____ Chronic swollen ankles?
____ Varicose veins?
____ Frequent dizziness/fainting?
____ Muscle or joint problems?
____ High blood sugar/diabetes?
____ Thyroid Disease?
____ Low testosterone/hypogonadism?
____ Gluacoma?

____ Asthma/breathing difficulty?
____ Bronchitis/Chest Cold?
____ Cancer, Melanoma/Skin Lesions?
____ Stroke or Blood Clots?
____ Emphysema/lung disease?
____ Epilepsy/seizures?
____ Rheumatic fever?
____ Scarlet fever?
____ Ulcers?
____ Pneumonia?
____ Anemias?
____ Liver or kidney disease?
____ Autoimmune disease?
____ Nerve disease?
____ Psychological Disorders?

Do you have or have you been diagnosed with any other medical condition not listed?
_____

Please provide any additional comments/explanations of your current or past medical history.
_____

Please list any recent surgery (i.e., type, dates etc.).
_____

List all prescribed/non-prescription medications and nutritional supplements you have taken in the last 3 months.

What was the date of your last complete medical exam?

Do you know of any medical problem that might make it dangerous or unwise for you to participate in this study?
(Including strength and maximal exercise tests) ____ If yes, please explain:
_____

Recommendation for Participation
____ No exclusion criteria presented. Participant is *cleared* to participate in the study.
____ Exclusion criteria is/are present. Participant is *not cleared* to participate in the study.

Signed: _____ Date: _____

APPENDIX C

Department of Health, Human Performance, & Recreation
Informed Consent Form

Title of Investigation: Effects of Branched-Chain Amino Acids or Leucine with Resistance Exercise on the Akt/mTOR and MAP Kinase Signal Transduction Pathways in Male Skeletal Muscle

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Richard Kreider, PhD, FACSM, FISSN
Department Chair, Department of HHPR, Baylor University

Melyn Galbreath, MS, RN
Exercise and Sport Nutrition Lab, Baylor University

Ronald Wilson, MD
Medical Supervisor for EBNL & ESNL

Sponsors: Exercise and Biochemical Nutrition Laboratory (Baylor University)

Exercise and Sport Nutrition Laboratory (Baylor University)

Rationale:

It is well known that resistance training can lead to skeletal muscle hypertrophy. Recently, scientists have been investigating the mechanisms behind such adaptations. In general, there are two major factors that promote protein synthesis: 1) DNA transcription of muscle specific genes (gene expression) and 2) the translation of these mRNA transcripts into functional proteins. The mechanisms by which skeletal muscle-specific genes are expressed involve the mitogen activated protein kinase (MAP kinase) signal transduction pathway and its activation of the muscle specific transcription factors known as the myogenic regulatory factors (MRFs). The MAP kinase pathway most influenced by resistance training is the ERK 1,2 pathway [1]. This pathway consists of Raf, Ras, MEK 1,2, and ERK 1,2. MEK 1,2 and ERK 1,2 will be evaluated in this study due to their role in growth factor stimulation and the adaptation to stress in response to skeletal muscle contraction involved with resistance exercise. The primary signal transduction pathway regulating translation is the Akt/mTOR pathway [2]. More specifically, some of the down stream targets of mTOR include $p70^{S6K}$, 4EBP-1, eIF4E and rpS6. The activation of these proteins will be evaluated in this study due to their role

in translation initiation. Interestingly, specific nutritional interventions in conjunction with resistance training lead to greater rates of protein synthesis. Branched-chain amino acids (leucine, isoleucine, and valine) have been shown to increase translation of skeletal muscle proteins via its activation of the Akt/mTOR pathway [3]. Of the three branched-chain amino acids, it appears that leucine plays the greatest role in promoting translation initiation. Specifically, leucine has been shown to activate mTOR and its down stream targets in direct fashion. The anabolic hormones insulin and insulin-like growth factor-1 (IGF-1), upon binding to their receptor, also have the ability to activate both the MAP kinase and Akt/mTOR signal transduction pathway. Another anabolic hormone, growth hormone (GH), has also been shown to cause activation of the MAP kinase pathway [4]. Several amino acids including the branched-chain amino acids have the ability to instigate an insulin response [5]. Therefore, the branched-chain amino acids can subsequently activate the MAP kinase (i.e. MEK 1,2; ERK 1,2) and Akt/mTOR pathways indirectly via insulin. There are two primary research questions: 1) In conjunction with resistance training, to what extent does leucine alone as compared to the branched-chain amino acids activate the downstream targets of the Akt/mTOR in humans? 2) In conjunction with resistance training, to what extent does insulin (in response to branched-chain amino acids or leucine ingestion) activate the MAP kinase (i.e. MEK 1,2; ERK 1,2) pathway. To date, there are few if any studies investigating the effects of branched-chain amino acid or leucine ingestion on these anabolic processes in humans.

Description of the Study:

I will be one of approximately 30 apparently healthy and physically active males between the ages of 18-30 years who will participate in this study. During an initial familiarization session, I will be informed of the requirements of the study and sign an informed consent statement in compliance with the Human Subjects Guidelines of Baylor University and the American College of Sports Medicine. A trained individual will examine me to determine if I am qualified to participate in this study. If I am cleared to participate in the study, I will be familiarized to the testing procedures. This session will take approximately 30 minutes to complete. Once I complete the familiarization session, I will be scheduled for maximal baseline testing and at that time instructed to refrain from exercise for 72 hours prior to baseline testing.

Prior to reporting to the EBNL for baseline assessments, I will complete a medical history questionnaire and undergo a general physical examination to determine whether I meet eligibility criteria. If I am eligible to participate in the study I will be familiarized to the study protocol by way of a verbal and written explanation outlining the study design. I will then be subjected to an initial leg strength test to assess my maximum leg strength level on the isotonic hip/leg sled (Nebula Fitness, Inc., Versailles, OH) and the leg extension machine (Body Masters, Inc., Rayne, LA) to be used in the study. Once my leg strength has been determined, I will then be asked to perform and practice 2 sets of leg presses. At this time I will be scheduled to return to the lab to perform the resistance exercises and will be instructed at this time to record my dietary intake for 48 hours prior to the testing session.

Once reporting to the lab for the resistance exercise session, I will turn in my 48-hour dietary record. I will then be weighed and have my blood pressure and heart rate measured. I will then be instructed to lie down on an observation table. I will have the biopsy site on my upper leg cleaned and shaven, followed by an injection of local

anesthetic (2% Xylocaine w/epinephrine). Following this, I have the first (pre-exercise) of 6 blood draws performed. During each blood sampling period, I will donate 2-3 teaspoons (10-15 milliliters) of venous blood by way of inserting a needle into my arm using sterile techniques by an experienced technician using standard phlebotomy procedures. This procedure may cause a small amount of pain when the needle is inserted into the vein as well as some bleeding and bruising. However, proper pressure will be applied upon removal to reduce bruising. I understand that I may also experience some dizziness, nausea, and/or faint if I am unaccustomed to having blood drawn. I understand that personnel who will be inserting the needle and taking my blood is experienced in phlebotomy (procedures to take blood samples) and are qualified to do so under guidelines established by the Texas Department of Health and Human Services. The process of inserting the needle and blood draws at each sampling point will take about 15-minutes. During the time I am having my blood drawn, the Xylocaine will take affect and a small region of my leg, about 2 inches in diameter, will become numb. I will then have the first (pre-exercise) of 4 biopsies. I will then be randomized to ingest one of three supplements: 1) Placebo group (non essential amino acids, glutamine, citrulline malate, Vitamin B6 and sucralose); 2) BCAA group (leucine, isoleucine, valine, glutamine, citrulline malate, Vitamin B6 and sucralose); or 3) Leucine group (leucine, non essential amino acids, glutamine, citrulline malate, Vitamin B6 and sucralose). Following this, I will rest for 30 minutes and then have a second blood draw followed by another ingestion of my assigned supplement. Next, I will warm up on a stationary bike for 5 minutes and then perform two warm-up sets of isotonic leg press for 8-10 reps at 50% of my 1 repetition maximum (1 RM). Upon completing my warm-up, I will then perform 4 sets of leg press for approximately 8-10 reps at 80% of my (1 RM). I understand I will rest for 150 seconds between each set of the leg press. I will rest 150 seconds following my final set of leg press. I will then perform 4 sets of knee extension for approximately 8-10 reps at 80% of my (1 RM) again resting 150 seconds between each set. Immediately following my exercise I will donate 2-3 teaspoons (10-15 milliliters) of venous blood (3rd blood draw) and then ingest my last assigned supplement. Thirty minutes later I will donate my 4th blood sample. At one hour after exercise I will have my 2nd second biopsy. Similarly, at two hours post exercise, I will have my blood taken for the 5th time and then have a 3rd biopsy taken. Finally at 6 hours after my exercise bout, I will have my blood taken for the 6th and final time and then have the 4th and final biopsy taken.

I understand that over the course of the study that I will have a total of six (6) blood draws and four (4) muscle biopsies performed in order to determine how my muscle adapts to training and the supplementation protocol. I understand that Darryn Willoughby, PhD (Director of the Exercise Biochemical and Nutrition Lab [EBNL] at Baylor University) will be responsible for the muscle biopsy procedure. The muscle biopsy procedure basically involves sterilizing and anesthetizing the biopsy site on the outside middle of my thigh. Then, a small incision (about 1 centimeter or the width of a finger) is made in my skin and fascia in order to expose the underlying muscle. Approximately 50 to 75 milligrams of muscle tissue is then extracted from the thigh muscle using a sterile muscle biopsy needle according to standard clinical procedures. Once the sample is obtained, the area of incision is cleaned and dressed. I will then be given a list of post-biopsy instructions on how to clean and care for the incision in order to promote healing. I understand that I will have to return to the lab within 24 to 72 hours so that the biopsy sites can be checked for infection and improper healing.

I understand that the lower extremity resistance training protocol will be supervised. I understand that if clinically significant side effects are reported from the exercise session, I will be referred to discuss the problem with Melyn Galbreath, RN who is the research nurse for the ESNL at Baylor University. Upon her discretion, I may be referred to discuss the matter with Dr. Ronald Wilson to determine whether any medical treatment is needed and/or whether I can continue in the study. I understand that if I fail to report my progress and health status to the research assistant I may be removed from the study.

I agree to do my best to: 1) follow the instructions outline by the investigators and 2) show up to all scheduled testing times. I agree not to take any nutritional supplements or performance enhancing aids during this study (i.e., vitamins/minerals, creatine, HMB, androstenedione, DHEA, etc). In addition, I agree not to take any non-medically prescribed medications and to report any medication that is prescribed for me to take during this study. I understand that if I take any other nutritional supplements or medications during the course of the study that may affect vitamin/mineral status, body composition, or blood hormone levels that I may be removed from the study.

Exclusionary Criteria

I understand that in order to participate in the study, a trained individual will examine me to determine whether I qualify to participate. I understand that I will not be allowed to participate in this study if: 1.) I have any known metabolic disorder including heart disease, diabetes, thyroid disease, or hypogonadism; 3.) I have a history of pulmonary disease, hypertension, liver or kidney disease, musculoskeletal disorders, neuromuscular or neurological diseases, autoimmune disease, cancer, peptic ulcers, or anemia; 4.) I am taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic (e.g., thyroid, insulin, etc), psychotropic, neuromuscular/neurological, or androgenic medications; 5.) I have a known bleeding disorder.

I have reported all nutritional supplements, medically prescribed drugs, and non-medically prescribed drugs that I am presently taking. I have completed medical history questionnaires and am not aware of any additional medical problems that would prevent me from participating in this study. I agree to report all changes in medical status, nutritional and/or pharmacological agents (drugs) that I take during the course of the investigation to Darryn Willoughby, PhD (254-710-3504). I understand that if I experienced any unexpected problems or adverse events from participating in this study I may be referred to discuss the problem with Melyn Galbreath, RN who is the research nurse for the ESNL at Baylor University. Upon her discretion, I may be referred to discuss the matter with Dr. Ronald Wilson to determine whether any medical treatment is needed and/or whether I can continue in the study.

Risks and Benefits

I understand that I will have about 2 teaspoons (10 milliliters) of blood drawn from a vein in my forearm using a sterile needle and blood tubes by an experienced phlebotomist six times during this study. This procedure may cause a small amount of pain when the needle is inserted into my vein as well as some bleeding and bruising. I may also experience some dizziness, nausea, and/or faint if I am unaccustomed to having blood drawn. I understand that I will also have 4 biopsies performed during this study and that

I will have a small incision on one leg. The biopsy procedure may cause a small amount of bleeding and/or pain as the incision is made and the sample is extracted from the muscle. However, once the anesthesia takes affect, there is usually only mild pressure and a small amount of bleeding as the needle is inserted and extracted. During the biopsy procedure, I may experience a slight localized cramping followed by brief and minor aching but these symptoms usually go away when the needle is withdrawn. Usually, subjects feel little or no sensation at all. I understand that although the muscle selected for biopsy (vastus lateralis) has no major blood vessels or nerves in the areas where the biopsy needle will be inserted; there is the rare occurrence of compressing or cutting small nerve branches, which can sometimes cause temporary tingling and numbness in the skin. These responses, when they have occurred, have been reported to dissipate in a few days or weeks. However, Dr. Willoughby has never had this occur with all of the muscle biopsies he has performed. After the needle is withdrawn, pressure is applied to the site of the incision to prevent any unwarranted bleeding (there is usually very little bleeding). With the incision being so small bleeding is slight; therefore, only a butterfly bandage is needed to close the incision, which will then be covered with a pressure bandage. I understand that I will be provided verbal and written instructions for post-biopsy care. I understand that I will be instructed to leave the butterfly bandage in place for 72 hours. However, I understand that I will be asked to report back to the lab 24 hours after the biopsy (unless unexpected bleeding or pain occurs) to have the old bandage removed, the incision inspected and a new bandage applied. I will be further advised to refrain from vigorous physical activity with my leg during the first 24 hours post-biopsy. After the biopsy, the muscle is likely to be moderately sore for about 24 hours. This soreness is similar to muscle soreness following unusually vigorous exercise or a muscle injury especially if muscle is compressed against a bone (e.g., "charley horse"). I understand that if I feel it necessary I may take a non-prescription analgesic medication such as Ibuprofen to relieve pain if needed. Complications accompanying this procedure are rare and no complications have been observed in subjects who have donated biopsies in the EBNL in previous studies. The primary risks, however, include bleeding, bruising, infection, and slight scarring of the skin. Some individuals may develop mild keloid scarring at the site of incision. Also, some individuals may have an allergic reaction to the anesthetic, such as a local rash, or difficulty breathing. I understand that I am required to inform the study investigators if I have had any prior allergic reactions to anesthesia (e.g. while in the hospital or during a dental visit).

I also understand that the exercise tests that will be performed may cause symptoms of fatigue, shortness of breath, and/or muscular fatigue/discomfort. The strength tests and resistance exercise protocol may also cause short-term muscle soreness and moderate fatigue for several days following the tests. I understand that I may also experience muscle strains/pulls during the exercise testing and/or training program. However, these risks will be similar to the risk of participating in my normal training program. I also understand that trained, non-physician exercise specialists certified in CPR will supervise exercise assessments. I understand that a telephone and an automated electronic defibrillator are in the laboratory in case of any emergencies and that there will be no less than two researchers working with me during each testing session. I understand that emergency procedures are posted in the lab in the unlikely event that any emergency may arise.

Alternative Treatments

This is not a medical treatment. Therefore, if medical treatment is needed, I must continue to obtain treatment for any medical problem I might have from my personal physician.

Costs and Payments

If I am a Baylor University student, I will not receive any academic credit for participating in this study. I understand that if I am an intercollegiate scholarship athlete I may not be eligible to receive payment to participate in this study. If eligible I will be paid $200 for completing the familiarization and all facets of the experimental testing sessions. I also understand that I will be given free blood assessments during the course of the study as described above and may receive information regarding results of these tests if I desire.

New Information

Any new information obtained during the course of this research that may affect my willingness to continue participation in this study will be provided to me. In addition, I will be informed of any unusual/abnormal clinical findings in which medical referral to my personal physician may be warranted. If I desire, I may request that this information be provided to my physician.

Confidentiality

I understand that any information obtained about me in this research, including questionnaires, medical history, laboratory findings, or physical examination will be kept confidential to the extent permitted by law. However, I understand in order to ensure that FDA regulations are being followed, it may be necessary for a representative of the FDA to review my records from this study which may include questionnaires, medical history, laboratory findings/reports, statistical data, and/or notes taken about my participation in this study. In addition, I understand that my records of this research may be subpoenaed by court order or may be inspected by federal regulatory authorities. I understand that data derived may be used in reports, presentations, and publications. However, I will not be individually identified unless my consent is granted in writing. Additionally, that confidentiality will be maintained by assigning code numbers to my files, limiting access to data to research assistants, locking cabinets that store data, and providing passwords to limit access to computer files to authorized personnel only. I understand that once blood samples are analyzed that they will be discarded.

Right to Withdrawal

I understand that I am not required to participate in this study and I am free to refuse to participate or to withdraw from the study at any time. Further, that my decision to withdraw from the study will not affect my care at this institution or cause a loss of benefits to which I might be otherwise entitled. If there is concern about my medical safety, I may be referred to seek medical attention.

Compensation for Illness or Injury

I understand that if I am injured as a direct result of taking part in this study, I should consult my personal physician to obtain treatment. I understand that the cost associated with the care and treatment of such injury will be the responsibility of me or my insurance carrier. In some cases, insurers may not reimburse claims submitted for a research-related injury resulting from medical procedures or treatments performed as part of a research study. I understand that Baylor University, the investigator's institutions, and the grant sponsor have not budgeted funds to compensate me for injury or illness that may result from my participation in this study and thus will not be accountable for illness or injury acquired during the course of this study. However, I may be referred to my personal physician if any clinically significant medical/psychological findings are observed during the course of this study.

I agree to indemnify and hold harmless Baylor University, its officers, directors, faculty, employees, and students for any and all claims for any injury, damage or loss I suffer as a result of my participation in this study regardless of the cause of my injury, damage or loss.

Statement on Conflict of Interest

I understand that researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

Voluntary Consent

I certify that I have read this consent form or it has been read to me and that I understand the contents and that any questions that I have pertaining to the research have been, or will be answered by Darryn Willoughby, PhD (Associate Professor, Department of Health, Human Performance & Recreation, 120 Marrs McLean Gymnasium, Baylor University, phone: 254-710-3504, Darryn_Willoughby@baylor.edu) or one of the research associates. My signature below means that I am at least 18 years of age and that I freely agree to participate in this investigation. I understand that I will be given a copy of this consent form for my records. If I have any questions regarding my rights as a research participant in this study, I may contact Baylor's University Committee for Protection of Human Subjects in Research. The chairman is Matt Stanford, Ph.D., Associate Professor of Psychology and Neuroscience, P.O. Box 97334, Waco, TX 76798-7334, phone number 254-710-2236.

Date _____ Participant's Signature _____

I certify that I have explained to the above individual the nature and purpose of the potential benefits and possible risks associated with participation in this study. I have answered any questions that have been raised and have witnessed the above signature. I have explained the above to the volunteer on the date stated on this consent form.

Date _____ Investigator's Signature _____

APPENDIX D

Application to the Baylor IRB
For Review of Research/Activity Proposal

Part 1: Signature Page

1. Name Darryn S. Willoughby, Ph.D.
2. Email Address (optional) Darryn_Willoughby@baylor.edu
3. Complete Mailing Address P.O. Box 97313
4. Position Associate Professor
5. Faculty Advisor (if researcher is Graduate Student) _____
6. Department/School HHPR
7. Telephone # x3504 FAX # x3527
8. Are you using participants in research (***Y*** or N) or in teaching exercises (Y or ***N***)?
9. Title of the research project/teaching exercise:

Effects of Varying Intensities of Single Leg Resistance Exercise on Signal Transduction and Skeletal Muscle Specific Gene Expression in Males

10. Please return this signed form along with all the other parts of the application and other documentation to the University Committee for Protection of Human Subjects in Research; Dr. Matt Stanford, Chairman, Department of Psychology and Neuroscience, Baylor University, P.O. Box 97334, Waco, Texas 76798-7334. If you have questions, or if you would like to see a copy of the OHRP Report on protection of human subjects in research, contact Dr. Stanford at extension 2961.

Signature of Principal Investigator

09/10/05
Date

_____
Signature of Faculty Advisor (required if researcher is a Graduate Student)

Departmental Review: _____
Department Chair or the Chair's Designate

Part 2: Introduction & Rationale

It is well known that resistance training can lead to skeletal muscle hypertrophy. Recently, scientists have been investigating the mechanisms behind such adaptations. In general, there are two major factors that promote protein synthesis: 1) DNA transcription of muscle specific genes (gene expression) and 2) the translation of these mRNA transcripts into functional proteins. The mechanisms by which skeletal muscle-specific genes are expressed involve the mitogen activated protein kinase (MAP kinase) signal transduction pathway and its activation of the muscle specific transcription factors known as the myogenic regulatory factors (MRFs). The MAP kinase pathway most influenced by resistance training is the ERK 1,2 pathway [1]. This pathway consists of Raf, Ras, MEK 1,2, and ERK 1,2. MEK 1,2 and ERK 1,2 will be evaluated in this study due to their role in growth factor stimulation and the adaptation to stress in response to skeletal muscle contraction involved with resistance exercise. The primary signal transduction pathway regulating translation is the Akt/mTOR pathway [2]. More specifically, some of the down stream targets of mTOR include $p70^{S6K}$, 4EBP-1, eIF4E and rpS6. The activation of these proteins will be evaluated in this study due to their role in translation initiation. Interestingly, specific nutritional interventions in conjunction with resistance training lead to greater rates of protein synthesis. Branched-chain amino acids (leucine, isoleucine, and valine) have been shown to increase translation of skeletal muscle proteins via its activation of the Akt/mTOR pathway [3]. Of the three branched-chain amino acids, it appears that leucine plays the greatest role in promoting translation initiation. Specifically, leucine has been shown to activate mTOR and its down stream targets in direct fashion. The anabolic hormones insulin and insulin-like growth factor-1 (IGF-1), upon binding to their receptor, also have the ability to activate both the MAP kinase and Akt/mTOR signal transduction pathway. Another anabolic hormone, growth hormone (GH), has also been shown to cause activation of the MAP kinase pathway [4]. Several amino acids including the branched-chain amino acids have the ability to instigate an insulin response [5]. Therefore, the branched-chain amino acids can subsequently activate the MAP kinase (i.e. MEK 1,2; ERK 1,2) and Akt/mTOR pathways indirectly via insulin. There are two primary research questions: 1) In conjunction with resistance training, to what extent does leucine alone as compared to the branched-chain amino acids activate the downstream targets of the Akt/mTOR in humans? 2) In conjunction with resistance training, to what extent does insulin (in response to branched-chain amino acids or leucine ingestion) activate the MAP kinase (i.e. MEK 1,2; ERK 1,2) pathway. To date, there are few if any studies investigating the effects of branched-chain amino acid or leucine ingestion on these anabolic processes in humans.

Part 3: Methodology

Participants

Approximately thirty apparently healthy and physically active males between the ages 18-30 will be allowed to volunteer to participate in the proposed study. Participants will undergo a mandatory medical exam by a Certified Exercise Physiologist (EPC) to determine whether they meet the entry criteria to participate in the study. Only participants considered as either low or moderate risk for

cardiovascular disease and with no contraindications to exercise as outlined by the American College of Sports Medicine (ACSM) and/or who have not consumed any nutritional supplements (excluding multi-vitamins) one month prior to the study will be allowed to participate. All eligible participants will be asked to provide oral and informed written consent based on university-approved documents and approval will be granted by the Institutional Review Board for Human Subjects of Baylor University. Additionally, all experimental procedures involved in the study will conform to the ethical consideration of the Helsinki Code. The purpose of the research, the protocol to be followed, and the experimental procedures to be used will be explained to each participant.

Study Site

All supervised testing and training will be conducted in the Exercise & Sport Nutrition Laboratory (ESNL) and Exercise and Biochemical Nutrition Laboratory (EBNL) in the Department of Health, Human Performance, and Recreation at Baylor University, in Waco, TX.

Independent and Dependent Variables

Table 1 and Figure 1 show the general research design protocol that will be administered in this study. The independent variables will be the ingestion of nutritional supplements comprising of BCAAs, leucine, or a placebo. Also, independent variables will include the number of blood samples and muscle biopsies obtained during the course of the study. Dependent variables evaluated from the muscle samples will include insulin receptor activation, IRS-1, MEK 1/2, ERK 1/2, AP-1, PI3-k, Akt, mTOR, $p70^{S6}$ kinase, rpS6, eIF 4E, and 4EBP-1. Dependent variables evaluated from the blood samples will include serum insulin, growth hormone, IGF-I, CBCs, and routine clinical chemistry markers to ensure safety.

Muscle Biopsies and Venous Blood Sampling

Immediately prior to each resistance exercise bout, but following a 8-12 hr fast, participants will undergo the initial/baseline muscle biopsy and blood draw. Percutaneous muscle biopsies (50-70 mg) will be obtained from the middle portion of the vastus lateralis muscle of the leg at the midpoint between the patella and the greater trochanter of the femur at a depth between 1 and 2 cm. For the remaining three biopsies, attempts will be made to extract tissue from approximately the same location as the initial biopsy by using the pre-biopsy scar, depth markings on the needle, and a successive incision that will be made approximately 0.5 cm to the former from medial to lateral. After removal, adipose tissue will be trimmed from the muscle specimens and muscle biopsy samples will placed in a cryogenic storage tube and flash frozen in liquid nitrogen and then stored at -80°C for later analysis. Muscle samples will be also be obtained one hour post exercise and at 2 and 6 hours after the exercise bout.

Upon entering the laboratory, a blood sample will be obtained. Thirty minutes after ingesting the supplement or placebo, a second blood draw will be taken. The final four

blood draws will be taken immediately post-exercise, and 30-minutes, two hours, and six hour post-exercise. Venous blood samples will be obtained from the antecubital vein into 10 ml collection tubes using a standard Vacutainer™ apparatus. Blood samples will be allowed to stand at room temperature for 10 min and then centrifuged at 2,400 rpm for 15 minutes. The serum will be removed and frozen at -20°C for later analysis. Whole blood will be analyzed the same day as donation to obtain complete blood counts for clinical safety.

Using enzyme-linked immunoabsorbent assays (ELISA), the serum levels of insulin, growth hormone, and IGF-1 will be determined. Also using ELISA, the skeletal muscle protein levels and activation status of insulin receptor, IRS-1, MEK 1/2, ERK 1/2, AP-1, PI3-k, Akt, mTOR, $p70^{S6}$ kinase, eIF-4E, and 4EBP-1 will be determined. rpS6 will be assayed via immunoblotting.

Supplementation

After baseline testing, each subject will be randomized and matched according to body mass into one of three supplement groups:

1) Placebo group (non essential amino acids, glutamine, citrulline malate, Vitamin B6 and sucralose)
2) BCAA group (leucine, isoleucine, valine, glutamine, citrulline malate, Vitamin B6 and sucralose)
3) Leucine group (leucine, non essential amino acids, glutamine, citrulline malate, Vitamin B6 and sucralose)

Supplementation will be ingested at three time points during the study. After the initial blood draw and muscle biopsy, the first oral dose will be ingested. The second and third oral doses will be ingested immediately pre-exercise and immediately post-exercise, respectively.

Entry/Familiarization and Baseline Strength Testing Session

Participants expressing interest in participating in this study will be interviewed on the phone to determine whether they appear to qualify to participate in this study. Participants believed to meet eligibility criteria will then be invited to attend an entry/familiarization and baseline strength testing session. At this time, participants will be instructed to refrain from lower-body resistance exercise for 72 hours prior to baseline testing. Once reporting to the lab, participants will complete a medical history questionnaire and undergo a general physical examination to determine whether they meet eligibility criteria. Participants meeting entry criteria will be familiarized to the study protocol by way of a verbal and written explanation outlining the study design. Eligible participants who agree to participate in the study will read and sign university-approved informed consent document. Participants will then be subjected to an initial strength test using our previously established trial-and-error method to assess their leg one repetition maximum (1-RM) on the isotonic hip/leg sled (Nebula Fitness, Inc., Versailles, OH) and isotonic leg extension (Body Masters, Inc., Rayne, LA) exercises. Once the 1-RM leg press has been determined, participants will be asked to perform and

practice 2-3 sets of the proposed resistance exercise session (see section for "Resistance Exercise Session") without blood or muscle sampling to familiarize them with the protocol and to also insure that they are able to complete the protocol before being formally admitted to the study. At the conclusion of the entry/familiarization and baseline strength testing session, each participant will be given an appointment time approximately one week later to begin the study.

Resistance Exercise Session Protocol

Participants will be subjected to an acute bout of lower body resistance exercise during the course of the study. Each participant will complete 4 sets of both leg press and leg extension at 80% 1RM. Rest periods will be 150 seconds between sets and 150 seconds between exercises.
Participants will have been fasted for approximately 8-10 hours upon starting the testing session and will continue fasting (excluding the administered supplement) for the duration of the study session (approximately 7 hours). The participants will report to the lab and will relax in a supine position on an observation table for 15 minutes. During this time they will have the baseline/pre-exercise muscle biopsy and blood sample obtained. The leg press will be conducted on an isotonic hip/leg sled (Nebula Fitness, Inc., Versailles, OH) and the leg extension will be conducted on an isotonic leg extension (Body Masters, Inc., Rayne, LA).

Dietary Records

The participants' diets will not be standardized and participants will be asked not to change their dietary habits during the course of the study. However, participants will be required to keep dietary records for 2 days prior to the resistance exercise session. These dietary records will be evaluated with the Food Processor dietary assessment software program to determine the average daily macronutrient consumption of fat, carbohydrate, and protein in the diet prior to exercise.

Statistical Analyses

Statistical analyses will be performed by utilizing a repeated-measures two-factor [treatment groups (3) x time point (4)] analysis of variance (ANOVA) will be used to analyze the variables determined from muscle samples. A repeated measures two-factor [treatment group (3) x time point (6)] ANOVA will be used to analyze the variables in the whole blood and plasma samples. Significant differences in mean values for main effects or interactions will be determined using a Newman-Keuls post hoc test. All statistical procedures will be performed using SPSS software and a probability level of <0.05 will be adopted throughout. However, to protect against Type I error, the conservative Hunyh-Feldt Epsilon correction factor will be used to evaluate observed within-group F-ratios.

References

1. Williamson D, Gallagher P, Harber M, Hollon C, Trappe S. Mitogen-activated protein kinase (MAPK) pathway activation: effects of age and acute exercise on human skeletal muscle. J Physiol. 2003, 547:977-87.

2. Bolster DR, Kimball SR, Jefferson LS. Translational control mechanisms modulate skeletal muscle gene expression during hypertrophy. Exerc Sport Sci Rev. 2003, 31(3):111-6.

3. Karlsson HK, Nilsson PA, Nilsson J, Chibalin AV, Zierath JR, Blomstrand E. Branched-chain amino acids increase p70S6k phosphorylation in human skeletal muscle after resistance exercise. Am J Physiol Endocrinol Metab. 2004 287(1):E1-7.

4. Liang L, Jiang J, Frank SJ. Insulin receptor substrate-1-mediated enhancement of growth hormone-induced mitogen-activated protein kinase activation. Endocrinology. 2000, 141(9):3328-36

5. Kimball SR, Farrell PA, Jefferson LS. Invited Review: Role of insulin in translational control of protein synthesis in skeletal muscle by amino acids or exercise. J Appl Physiol. 2002, 93(3):1168-80.

Research Team

Darryn S. Willoughby, PhD, FACSM, FISSN, CSCS. Dr. Willoughby is Associate Professor of Exercise and Muscle Physiology and Biochemistry in the Department of Health, Human Performance, and Recreation at Baylor University. Dr. Willoughby is an internationally recognized exercise biochemist and molecular physiologist. He has conducted a vast amount of research focusing on the biochemical and molecular regulatory mechanisms regarding exercise performance and nutrition. Dr. Willoughby is the principal investigator and will maintain complete oversight and perform all muscle biopsies.

Bill Campbell PhD(c), CSCS. Mr. Campbell is an exercise physiologist pursuing his Ph.D. in Exercise, Nutrition, and Preventative Health and serves as a research assistant in the EBNL. He will co-coordinate and lead the project and will be responsible for participant recruitment, day-to-day scheduling and testing, and data collection and analysis. Mr. Campbell will use part of this project as his dissertation work for completion of his PhD.

Paul LaBounty PhD(c), MPT, CSCS. Mr. LaBounty is a licensed physical therapist pursuing his Ph.D. in Exercise, Nutrition, and Preventative Health and serves as a research assistant in the ESNL. He will co-coordinate and lead the project and will be responsible for participant recruitment, day-to-day scheduling and testing, and data

collection and analysis. Mr. LaBounty will use part of this project as his dissertation work for completion of his PhD.

Richard B. Kreider, PhD, FACSM, FISSN. Dr. Kreider is Professor and Chair of the Department of Health, Human Performance, and Recreation at Baylor University. Dr. Kreider is an internationally recognized exercise scientist and has conducted a vast amount of research primarily focusing on the role of exercise and nutrition and health and performance. Dr. Kreider will serve as a consultant and assist in providing administrative oversight for the study.

Melyn Galbreath, MSN, RN. Ms. Galbreath is a nurse practitioner who serves as the laboratory nurse and is also pursuing her Ph.D. in Preventative Health. She provide the medical monitoring, approve subjects for entry into the study, review the reported side effects from the exercise and the biopsy, and be involved in data collection.

Ronald Wilson, MD. Dr. Wilson serves as medical supervisor for the ESNL, EBNL, and Center for Exercise, Nutrition & Preventive Health Research (CENPHR).

Procedures

Medical Monitoring. Interested participants will be invited to familiarization sessions. During this time, participants will sign consent forms and complete medical history information. Participants will then undergo a mandatory medical exam by a Certified Exercise Physiologist (EPC) to determine whether the participant meets entry criteria to participate in the study. This exam will include evaluating the medical and training history questionnaires and performing a general physical examination according to ACSM exercise testing guidelines. Based on this examination, participants will be assessed for their risk of cardiovascular disease and contraindications to exercise and then a recommendation will be made on whether the participant meets entry criteria and may therefore participate in the study. Trained, non-physician exercise specialists certified in CPR will supervise participants undergoing testing and assessments. A telephone is in the laboratory in case of any emergencies, and there will be no less than two researchers working with each participant during testing sessions. In the event of any unlikely emergency one researcher will check for vital signs and begin any necessary interventions while the other researcher contacts Baylor's campus police at extension 2222. Instructions for emergencies are posted above the phone in the event that any other research investigators are available for assistance. Participants will be informed to report any unexpected problems or adverse events they may encounter during the course of the study to Darryn S. Willoughby, Ph.D. or Melyn Galbreath, RN. If clinically significant side effects are reported, the participants will be referred to discuss the problem with Melyn Galbreath, RN who is a nurse practitioner and serves as the ESNL research nurse at Baylor University. If deemed necessary, Ms. Galbreath will refer the participant to Ronald Wilson, MD for medical follow-up. Dr. Wilson is one of the Sports Medicine physicians for Baylor University and is an adjunct Professor in the Department of HHPR. He has agreed to provide medical support and consultation for this study and to the ESNL. Dr. Wilson will evaluate the complaint and make a recommendation whether any medical treatment is needed and/or whether the participant can continue in the study. If

Dr. Wilson feels medical follow-up is necessary, the participant will be referred to obtain medical treatment from their personal physician. This is a similar referral/medical follow-up system that Baylor athletes are provided with the exception that participants in this study will not be provided medical care. New findings and/or medical referrals of unexpected problems and/or adverse events will be documented, placed in the participants research file, and reported to the Baylor IRB committee.

Estimated Energy Intake/Dietary Inventories. The participants' diets will not be standardized and participants will be asked not to change their dietary habits during the course of the study. However, participants will be required to keep dietary records for 48 hours prior to the resistance exercise session. The 48-hour dietary recalls will be evaluated using the Food Processor III Nutrition Software. The Food Processor dietary assessment software program will be used to determine the average daily macronutrient consumption of fat, carbohydrate, and protein in the diet prior to supplementation and exercise.

Strength Assessments. All strength/exercise tests will be supervised by certified lab assistants experienced in conducting strength/anaerobic exercise tests using standard procedures. Strength testing will involve the participants performing one repetition maximum (1 RM) on the isotonic hip/leg sled (Nebula Fitness, Inc., Versailles, OH) and the isotonic leg extension (Body Masters, Inc., Rayne, LA). Participants will warm-up (2 sets of 8 – 10 repetitions at approximately 50% of anticipated maximum) on the leg press. Participants will then perform successive 1 RM lifts starting at about 70% of anticipated 1RM and increasing by 10–20 lbs until the participant reaches their 1RM. Participants will be given 3 minutes of recovery between attempts and between exercises.

Blood Samples. Participants will donate approximately 2-3 teaspoons of fasting venous blood
(10-15 milliliters) during each of the 6 blood sampling periods into two serum separation vacutainer tubes (red tops) and approximately 2 teaspoons in a EDTA tube (lavender top). Blood samples will be obtained via veinapuncture from the antecubital vein using standard phlebotomy procedures by the research assistants trained in phlebotomy in compliance with guidelines established by the Texas Department of Health and Human Services. The nurse and lab technicians will wear personal protective clothing (gloves, lab coats, etc.) when handling blood samples. The blood collection tubes will be labeled and placed in a test tube rack. Laboratory technicians (who have received blood borne pathogen training and will be wearing personal protective clothing) will centrifuge the serum samples, transfer serum into labeled serum storage containers, and store at -20°C for later analysis. Participants will be laid down on an observation table. A tourniquet will be applied high on the brachium (upper arm) and will be tight enough to visibly indent the skin, but not cause the patient discomfort. The entry site will be thoroughly cleaned with an alcohol prep pad and allowed to dry. The participant will be instructed to lower their arm and make a fist several times in order to maximize venous engorgement. The appropriate vein will be selected for puncture. To puncture the vein, the 21-gauge needle will be held in the dominant hand. With the bevel up, enter the skin at about a 30-degree angle and in the direction of the vein. If the vein appears to "roll" (move around

freely under the skin), the veinapuncture will begin by applying counter tension against the skin just below the entry site using the thumb of the non-dominant hand. Upon filling of the vacutainer, the tourniquet will be released and the needle will be removed from the participants arm and pressure will be applied with sterile gauze pads. The vacutainers will be centrifuged and the serum will be processed and stored for later analysis. The whole blood will be analyzed daily to ascertain a complete blood count for clinical safety markers.

Muscle Biopsies. Percutaneous muscle biopsies (approximately 50-70 mg) will be obtained from the vastus lateralis of each participant's exercised thigh using our previously-established procedures by Dr. Darryn Willoughby, who has extensive experience in performing muscle biopsies as a part of his research. Samples will be extracted under local anesthesia of 2% Xylocaine with epinephrine from the middle portion of the muscle at the midway between the patella and the greater trochanter of the femur. For each biopsy, muscle tissue will be extracted from the same location by using the previous incision and depth markings on the needle. First, the participant will lie supine or assume a comfortable reclining position on a sterilized table. Once the extraction point is identified, the area will be shaved clean of leg hair, washed with an antiseptic soap and cleaned with rubbing alcohol. In addition, the biopsy site will further be cleansed by swabbing the area with Betadine (fluid antiseptic) and then draped. A small area of the skin approximately 2 cm in diameter will be anesthetized with a 1.0 mL subcutaneous injection of Xylocaine. Once anesthetized, a scalpel point will be used to produce the initial biopsy site by making an incision approximately 1 cm in length through the skin and underlying subcutaneous fat and fascia. Due to the localized effects of the anesthetic, the participant should feel no pain during this process. The biopsy needle will be advanced into the incision approximately 1 cm, and during this part of the procedure the subject may feel pressure to the thigh area. Once the muscle sample has been obtained, pressure will be immediately applied and the wound will and then bandaged. With the incision being so small bleeding is slight; therefore, only a butterfly bandage is needed to close the incision, which is then covered with a pressure bandage. The needle and scalpel blade will be discarded as hazardous waste in an appropriately-labeled plastic sharps container. The site of the biopsy will be cleaned with a sterile alcohol wipe and gauze. The alcohol wipe and gauze then will be discarded in a appropriately labeled biohazard waste receptacle. The tissue sample will be stored at –80°C for future analyses. Once the local anesthesia has taken effect (approximately 2-3 minutes) the biopsy procedure will take approximately 15-20 seconds. Written instructions for post-biopsy care will be given to the subjects. The participant will be instructed to leave the bandages on for 24 hours (unless unexpected bleeding or pain occurs) and asked to report to the lab within 24 hours to have the old bandages removed, the incision inspected and new bandages applied. The participant will be further advised to refrain from vigorous physical activity for 48 hours post-biopsy. These suggestions will minimize pain and possible bleeding of the area. If needed, the subject may take nonprescription analgesic medication such as Ibuprofen to relieve pain if needed. However, medications such as aspirin, Nuprin, Bufferin, or Advil will be discouraged as these medications may lead to ecchymosis at the biopsy site. Soreness of the area may occur for about 24 hours post-biopsy.

Equipment

***Digital Scale*.** Total body weight will be determined using a digital scale accurate to ±0.02 kg. The scale is calibrated by placing certified 25-kg weights and balancing the scale. Other than general instructions, special skills are not required to measure body weight.

Resistance Exercise Machines. Both 1-RM strength and the resistance exercise bouts will be performed on a isotonic leg/hip sled (Nebula Fitness, Inc., Versailles, OH) and a isotonic leg extension (Body Masters, Inc., Rayne, LA). Equipment and testing will be contained within the EBNL.

Muscle Biopsy Needle. The muscle biopsy technique will be performed with a 5-mm Bergstrom biopsy needle (shown in above photos).

Serum Hormone Analysis. Blood samples will also be used to assess serum hormone levels of growth hormone, IGF-1, and insulin. These assays will help evaluate the effects of the branched-chain amino acids/leucine supplementation and resistance exercises on the aforementioned hormones. These variables will be assessed photometrically using either enzyme-linked immunoabsorbent assays (ELISA), or enzyme immunoassys (EIA) with a Wallac Victor-1420 microplate reader. The assays will be performed at either 405 or 450 nm wavelength against a standard curve and known control values.

Serum branched-chain amino acid (BCAA) Analysis. Blood samples will also be used to assess BCAA levels in the blood utilizing high performance liquid chromatography (HPLC).

Participants

Recruitment

Approximately 30 apparently healthy, physically and recreationally active males between the ages of 18-30 years will be allowed to volunteer to participate in the proposed study. A recruitment flyer that will be posted on campus, at area fitness centers, and on the Internet. (http://www3.baylor.edu/HHPR/research/subjects/) and sent via campus mail is attached.

Selection Criteria

Participants will not be allowed to participate in the study if they:

1. Have not been physically active (minimum of 1 hours/week for 1 year);
2. Have any known metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism;
3. Have a history of pulmonary disease, hypertension, hepatorenal disease, musculoskeletal disorders, neuromuscular/neurological diseases, autoimmune disease, cancer, peptic ulcers, or anemia;

4. Are taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic (e.g., thyroid, insulin, etc), psychotropic, neuromuscular/neurological, or androgenic medications;
5. Have taken ergogenic levels of nutritional supplements that may affect muscle mass (e.g., creatine, HMB) or anabolic/catabolic hormone levels (e.g., androstenedione, DHEA, etc) within one month prior to the start of the study.
6. Have any absolute or relative contraindication for exercise testing or prescription as outlined by the American College of Sports Medicine;
7. Report any unusual adverse events associated with this study that in consultation with the supervising physician recommends removal from the study.

Compensation or Incentives

Participants completing all familiarization and testing sessions as well as turning in all required materials (i.e., dietary and training logs) in the study will be paid $200. Participants may receive information regarding results of these tests if they desire. If participants are Baylor students, they will not receive any academic credit for participating in this study.

Potential Risks

Subjects who meet eligibility criteria will be exposed to several muscle strength tests and a separate resistance exercise bout that may cause symptoms of fatigue, shortness of breath, and/or muscular fatigue/discomfort. The exercise tests may also cause short-term muscle soreness and moderate fatigue for several days following the tests. Subjects may also experience muscle strains/pulls during the exercise testing and/or training program. However, exercise sessions will be conducted by certified strength and conditioning specialists and monitored to ensure the subjects follow appropriate exercise guidelines. Subjects will donate about 2-3 teaspoons (10-15 milliliters) of venous blood six (6) times and will also donate four (4) muscle biopsies during the study using standard procedures. Both the blood sampling and biopsy procedure may cause a small amount of pain and some bleeding and bruising may occur. The subject may also experience some dizziness, nausea, and/or faint if are uncomfortable with needles and/or they are unaccustomed to having blood drawn. Subjects donating muscle biopsies may experience some anxiety before this procedure regarding a perception of pain or discomfort. The biopsy procedure may cause a small amount of bleeding and/or pain as the incision is made and the sample is extracted from the muscle. However, once the anesthesia takes affect, there is usually only mild pressure and a small amount of bleeding as the needle is inserted and extracted. During the biopsy procedure, subjects may experience a slight localized cramping followed by brief and minor aching but these symptoms usually go away when the needle is withdrawn. Frequently, subjects feel little or no sensation at all. Although the muscle selected for biopsy (vastus lateralis) has no major blood vessels or nerves in the areas where the biopsy needle will be inserted, there is the rare occurrence of compressing or cutting small nerve branches, which can sometimes cause temporary tingling and numbness in the skin. These responses, when they have occurred, have been reported to dissipate in a few days or weeks. However, it should be noted that Dr. Willoughby has

yet to see this occur with all of the biopsies he has performed. After the biopsy needle is withdrawn, pressure is applied to the site of the incision to prevent any unwarranted bleeding (there is usually very little bleeding). After the biopsy, the muscle is likely to be moderately sore for about 24 hours. This soreness is similar to muscle soreness following unusually vigorous exercise or a muscle injury especially if muscle is compressed against a bone (e.g., "charley horse"). Complications accompanying this procedure are rare and no complications have been observed in subjects who have donated biopsies in the EBNL in previous studies. The primary risks, however, include bleeding, hematoma (bruising), infection, and slight scarring of the skin. In addition, some individuals may have an allergic reaction to the anesthetic, such as a local rash, or difficulty breathing. To minimize these risks, the amount of anesthetic used will be approximately 2-4% of the maximal dose for a normal sized individual. Every precaution will be made to keep these risks to a minimum. Additionally, these potential risks can be prevented and/or treated with rest, ice, compression, elevation, and adhering to post-biopsy care instructions. Taking a mild nonprescription pain medication such as Ibuprofen, providing the subject can tolerate these medications, is also recommended for pain. In all these procedures, care is taken to employ precautions to avoid infection, including the "universal precautions" for the handling of blood and infectious materials. Muscle biopsies do not cause any permanent damage with the exception of a small scar, which should become undetectable over time, although some individuals may develop keloid scarring at the site of the incision. Researchers involved in collecting data represent trained, non-physician, certified exercise specialists (American Society of Exercise Physiologies Certified Exercise Physiologist, Certified Strength & Conditioning Specialists, Certified Athletic Trainers, and/or American College of Sports Medicine Health Fitness Instructor, Exercise Technologist, Exercise Specialists, or Program Director for Preventive and Rehabilitative Exercise Programs). All personnel involved in collecting data will be certified in CPR, which is also a condition to holding these professional certifications. Testing personnel will have a cell-phone available in case of any emergencies. In the event of any unlikely emergency one researcher will check for vital signs and begin any necessary interventions while the other researcher contacts 911. Researchers will follow school emergency procedures in case of an emergency.

Potential risks of this study are as follows: acute musculoskeletal injury resulting from 1-RM testing and acute injury resulting from the exercise sessions, and transient acute muscle pain and soreness from the exercise session and the muscle biopsies. Complications resulting from the muscle biopsy are rare. As with the blood draw, however, there is a risk of infection if the participant does not adequately cleanse the area for approximately 48 hours post biopsy. Participants will be instructed to cleanse the incision, without disturbing the butterfly bandage, with soap and water every 4-6 hours, pat the area dry and reapply a fresh adhesive pressure bandage. There is a potential risk of an allergic reaction to the Xylocaine. All participants will be asked if they have known allergies to local anesthetics (e.g. Lidocaine, Xylocaine, etc.) that they may have been previously given during dental or hospital visits. Participants with known allergies to anesthesia medications will not be allowed to participate in the study.

Injury due to 1- RM testing and exercise will be minimized by ensuring that all subjects adhere to correct lifting form while performing the exercise. In addition, only Dr.

Willoughby and co-investigators of this study will conduct the testing and exercise procedures. Participants will be made aware of the intensity and duration of the expected soreness due to the exercise sessions. Dr. Willoughby will conduct all muscle biopsies. While at the University of Southern Maine and Texas Christian University, he successfully completed this procedure approximately 500 times on both male and female participants ranging from 18-85 years of age without any complication. Conducting all testing and exercise sessions in the exercise physiology laboratory with only the essential personnel in attendance will protect privacy. Participants will donate 2-3 teaspoons (10-15 milliliters) of venous blood six (6) times and muscle biopsies four (4) times in a 24-hour time period during the study by experienced lab staff using standard procedures. These procedures may cause a small amount of pain when the needle is inserted into the vein as well as some bleeding and bruising. However, proper pressure will be applied upon removal to reduce bruising. The participant may also experience some dizziness, nausea, and/or faint if they are unaccustomed to having blood drawn. Even though the participants in this study will be physically active and healthy, and will be instructed to continue exercising, participants may also still experience short-term muscle soreness, moderate fatigue, and muscle strains/pulls during their routine resistance-training program.

Researchers involved in collecting data represent a licensed nurse practitioner, licensed physical therapist, and trained, non-physician, certified strength and conditioning specialists. All personnel involved in collecting data will be certified in CPR, which is also a condition to holding these professional certifications. A telephone and automated electronic defibrillator (AED) is located in the laboratory in case of any emergencies and there will be no less than two researchers working with each participant during testing. In the event of any unlikely emergency one researcher will check for vital signs and begin any necessary interventions while the other researcher contacts Baylor's campus police at extension 2222. Instructions for emergencies are posted above the phone in the event that any other research investigators are available for assistance.

Potential Benefits

Participants may also gain insight about their health and fitness status from the assessments to be performed during the resistance exercise bouts. The information obtained in this study will help expand the body of scientific knowledge involving the physiological responses of skeletal muscle to resistance training and concomitant BCAA/leucine supplementation. This information will be helpful to exercise physiologists, doctors, and researchers to increase their knowledge as well.

Assessment of Risk

The possibility of infection at the site of the blood draws and muscle biopsies performed in this study will be the greatest risk associated with the study. However, participants will be instructed on simple cleansing methods to prevent the possibility of infection. An additional risk associated with participating in this study will be performing the two bouts of resistance exercise. However, since the participants to be used in this study will all undergo medical screening, these risks would be no different than participating in their

own exercise programs. Therefore, the potential benefits of participating in this study outweigh the potential risks.

Compensation for Illness or Injury

Each participant will agree to indemnify and hold harmless Baylor University, its officers, directors, faculty, employees, and students for any and all claims for any injury, damage or loss suffered as a result of participation in this study regardless of the cause of injury, damage, or loss.

Confidentiality

Information obtained from this research (including questionnaires, medical history, laboratory findings, or physical examination) will be kept confidential to the extent permitted by law. However, according to FDA regulations, records will be open to FDA representatives to review if necessary. This may include questionnaires, medical history, laboratory findings/reports, statistical data, and/or notes taken throughout this study. Records of the research may also be subpoenaed by court order or may be inspected by federal regulatory authorities. Data derived from this study may be used in reports, presentations and publications. However, participants will not be individually identified unless they give their written consent.

Data Presentation & Publication

Data will be presented at an appropriate scientific conference (e.g., American College of Sports Medicine, International Society of Sports Nutrition, Experimental Biology, etc.) and published in a peer reviewed scientific journal (e.g., Medicine & Science in Sport and Exercise, Journal of Sport Science and Medicine, International Journal of Sport Nutrition and Exercise Metabolism, etc.).

Statement on Conflict of Interest

Researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

APPENDIX E

Wound Care for the Muscle Biopsy Procedure

Complications resulting from the muscle biopsy procedure are rare. Furthermore, after the procedure, you can reduce your risk of chance of infection by adhering to the following course of action for wound care:

For approximately 24 hours post biopsy

- leave the bandage(s) on for 24 hours (unless unexpected bleeding or pain occurs, which should be immediately reported to the lab)
- lightly clean around the bandage(s) if necessary
- report back to the lab within 24 hours to have the old bandage(s) removed, the incision inspected and new bandages applied
- refrain from vigorous physical activity during the first 48 hours post-biopsy

After the 24-hour follow-up (for approximately 72 hours post biopsy)

- leave the butterfly bandage in place
- adequately cleanse the area surrounding the bandage with soap and water every 4-6 hours, and pat the area dry
- reapply a fresh adhesive bandage

At approximately 72 hours post biopsy

- return to the lab
- allow the incision sight to be inspected and new bandages applied
- leave these bandages on for 24 hours (unless unexpected bleeding or pain occurs)
- return to normal hygiene practices unless complications arise

Possible Pain Side Effects

Soreness of the area may occur for about 24 hours post-biopsy. Following the procedures outlined above should significantly minimize pain and possible bleeding of the area. However, some subjects experience no significant pain post biopsy.

☺ If needed, the subject may take non-prescription analgesic medication such as Tylenol or Ibuprofen to relieve pain if needed.

☹ Medications such as aspirin, Advil, Bufferin, or Nuprin, are discouraged as they may lead to excess bruising at the biopsy site.

If any questions or complications arise please contact:

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