ABSTRACT

The Effects of Heavy Resistance Exercise in Combination with Orally Administered Branched-Chain Amino Acids or Leucine on Insulin Signaling and Akt/mTOR Pathway Activity in Active Males

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Purpose: To determine if activity of the insulin signaling pathway is increased during lower body resistance exercise due to supplemental BCAA or leucine ingestion. Methods: 30 recreationally trained males (22.5yrs; 81.1kg) were randomly assigned to 1 of 3 groups: Leucine (60mg/kg/bw), BCAA (120mg/kg/bw), or non-caloric placebo. Participants performed 4 sets of leg press and leg extension at 80% 1RM to failure (at least 8 reps). Supplements were ingested at 3 time points: 30 minutes prior to RE, and immediately pre- and post-RE. Venous blood was sampled at baseline (Pre); immediate pre- and post-exercise, 30 minutes post-exercise; 2hours post-exercise, and 6 hours post-exercise for serum glucose, insulin, GH, and IGF-1. Muscle biopsies were obtained at baseline, and 30 minutes post, and 2 and 6 hours post-exercise for IRS-1, Akt, mTOR, 4E-BP1, and P70-S6K. Skeletal muscle variables were transformed to delta values and analyzed using a 3 (group) x 4 (time points) repeated measures MANOVA. Univariate ANOVAs (Bonferroni adjusted) were utilized as follow-up tests to the MANOVA. Post-hoc tests of the interaction effects demonstrated in the ANOVA were analyzed using

independent samples T-tests. Results: Neither BCAA or leucine significantly increased any of the 4 serum variables. A group x time interaction for IRS-1 phosphorylation demonstrated that the leucine group was significantly elevated at 2hr post and 6hr post when compared to the BCAA group ($p < .05$). A group x time interaction for 4E-BP1 phosphorylation demonstrated that the leucine and BCAA groups were both elevated at the 2hr post in comparison with the placebo ($p < .05$). BCAA was significantly greater than leucine at 6 hours ($p < .05$) as well. No interactions were observed for Akt, mTOR, or P70-S6K. Summary: The results indicate that BCAA and leucine supplementation with RE increased the phosphorylation status of 4E-BP1 at 2 hours post-exercise and the BCAA group increased the phosphorylation of 4E-BP1 greater than leucine ($p < .05$) at 6 hours post-exercise.

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by

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LIST OF ABBREVIATIONS

1-RM – one repetition maximum

4E-BP1 - eukaryotic initiation factor 4E binding protein 1

AKT – protein kinase B

BCAA – branched-chain amino acids

eIF – eukaryotic initiation factor

g – gram

GDP – guanine diphosphate

GH – growth hormone

GTP – guanine triphosphate

IGF-1 – insulin-like growth factor-1

IRS-1 – insulin receptor substrate-1

kg – kilogram

kJ – kilojoule

km - kilometer

m – meter

mg – milligram

mL – milliliter

mM – millimolar

mRNA – messenger ribonucleic acid

mTOR – mammalian target of rapamycin

P70-S6K - P70 Ribosomal Protein S6 Kinase

PI-3 kinase – phosphotidylinositol 3-kinase

RDA – recommended dietary allowance

RP S6 – ribosomal protein S6

RPE – rating of perceived exertion

Ser – serine

Thr – threonine

tRNA – transfer ribonucleic acid

U – units or international units

VO_2 max – maximal oxygen uptake

µg - microgram

µL – microliter

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CHAPTER ONE

Introduction

Leucine, along with isoleucine and valine, are collectively called the branched-chain amino acids (BCAAs). BCAAs constitute approximately one-third of all muscle protein (Mero, 1999). Specifically, leucine comprises approximately 5-10% of the total protein content (Mero, 1999). It has been demonstrated that leucine's oxidation rate is significantly higher when compared to isoleucine or valine (Mero, 1999). Furthermore, out of the three BCAAs, leucine has been the most studied secondary to the belief that it plays the most significant role in stimulating protein synthesis (J. C. Anthony, Anthony, & Layman, 1999; J. C. Anthony, Yoshizawa et al., 2000; Crozier, Kimball, Emmert, Anthony, & Jefferson, 2005; Kimball & Jefferson, 2006). The observed increase in protein synthesis is attributed to an increased efficiency in translation initiation (Bolster, Kimball, & Jefferson, 2003). Different animal models have shown that increased translation initiation facilitates protein synthesis (J. C. Anthony et al., 1999; J. C. Anthony, Yoshizawa et al., 2000; Bolster, Kimball et al., 2003; Crozier et al., 2005).

Translation initiation in humans is regulated by eukaryotic initiation factors (Bolster, Kimball et al., 2003; Kimball, Farrell, & Jefferson, 2002). Two of the steps involved with translation initiation in humans are subject to regulation. One is the binding of initiator methionyl-$tRNA_i$ (met-$tRNA_i$) to the 40S ribosomal subunit and the second being the binding of the mRNA to the 40S ribosomal subunit (Bolster, Kimball et al., 2003; Kimball et al., 2002). At this point in time it doesn't appear that leucine or other amino acids play a major role in regulating the binding of met-$tRNA_i$ to the 40S

ribosomal subunit (J. C. Anthony, Anthony, Kimball, Vary, & Jefferson, 2000; Vary, Jefferson, & Kimball, 1999). The binding of the mRNA to the 40S ribosomal subunit is regulated in part by three different initiation factors that are grouped together and communally called eIF4F (Bolster, Kimball et al., 2003; Kimball et al., 2002). Of the possible mechanisms that regulate the binding of the mRNA to the 40S ribosomal subunit, two may be mediated by leucine and/or insulin (Blomstrand, Eliasson, Karlsson, & Kohnke, 2006; Bolster, Kimball et al., 2003; Kimball & Jefferson, 2006).

The first mechanism that appears to regulate the mRNA binding step to the 40S ribosomal subunit is the sequestration of eIF4E by a binding protein called 4E-BP1 (Blomstrand et al., 2006; Bolster, Kimball et al., 2003; Kimball & Jefferson, 2006). 4E-BP1 becomes activated when it is hypophosphorylated. Hypophosphorylated 4E-BP1 will bind to eIF4E and effectively make it inactive (Blomstrand et al., 2006; Bolster, Kimball et al., 2003; Kimball & Jefferson, 2006). If eIF4E becomes inactive it cannot bind to eIF4G and ultimately this will not allow the mRNA to bind to the 40S ribosomal subunit (Bolster, Kimball et al., 2003; Kimball & Jefferson, 2006). This will inhibit translation initiation. Conversely, when 4E-BP1 is hyperphosphorylated, it will not bind to eIF4E and this will allow translation initiation to ensue (Blomstrand et al., 2006; Bolster, Kimball et al., 2003; Kimball & Jefferson, 2006).

Another mechanism that has the ability to play a regulatory role in the binding of the mRNA to the 40S ribosomal subunit is through the activation ribosomal protein S6 (rpS6) (Blomstrand et al., 2006; Bolster, Kimball et al., 2003; Kimball & Jefferson, 2006). When rpS6 becomes phosphorylated via $p70^{S6k}$, it facilitates the translation of a certain group of mRNAs called TOP mRNAs (Bolster, Kimball et al., 2003). Several

mRNAs including translation elongation factors 1A and 2, eIF4G, and ribosomal proteins, all fall under the TOP mRNA umbrella (Bolster, Kimball et al., 2003). These particular mRNAs have a distinctive series of pyrimidine residues near the 5'-cap that give them there unique characteristics (Bolster, Kimball et al., 2003). However, the exact mechanism by which the phosphorylation of rpS6 actually facilitates the translation of these particular TOP mRNAs remains unknown (Bolster, Kimball et al., 2003).

It appears at this time that the proteins in the Akt-mTOR signal transduction pathway helps regulate translation initiation. Some of the proteins that are involved in this signal transduction pathway include: PI-3 kinase, phosphoinositide-dependent protein kinase (PDK1), Akt (which is also referred to as protein kinase B [PKB]), mTOR, 4E-BP1 and $p70^{S6k}$ (Bolster, Kimball et al., 2003; Kimball et al., 2002). Several variables such as growth factors, and leucine, and resistance exercise appear to influence this pathway. Insulin and insulin-like growth factor I (IGF-1) are anabolic hormones known to regulate mTOR through its activation of upstream enzymes, such as IRS-1 and Akt (Kimball et al., 2002). The protein kinase mTOR has been shown to activate/phosphorylate its downstream targets 4E-BP1 and ribosomal protein S6. It has also been reported that resistance training, which can lead to an increase in protein synthesis, is most likely mediated through the PI-3 kinase-mTOR pathway (Bolster, Kimball et al., 2003).

Hyperphosphorylated forms of 4E-BP1 and $p70^{S6k}$ have been shown to facilitate protein synthesis while hypophosphorylated forms of these proteins have shown to have a negative effect on protein synthesis (J. C. Anthony, Yoshizawa et al., 2000; Bolster, Kimball et al., 2003; Crozier et al., 2005; Gautsch et al., 1998; Vary et al., 1999). The

BCAAs and more specifically, leucine, seem to help regulate protein synthesis through its ability to enhance translation initiation (J. C. Anthony, Anthony et al., 2000; J. C. Anthony et al., 1999; J. C. Anthony, Lang et al., 2002; J. C. Anthony, Yoshizawa et al., 2000; T. G. Anthony, Anthony, Yoshizawa, Kimball, & Jefferson, 2001; Bolster, Vary, Kimball, & Jefferson, 2004; Crozier et al., 2005). Specifically, leucine has been shown to stimulate protein synthesis through the hyperphosphorylation of 4E-BP1 as well as increasing the phosphorylation of $p70^{S6k}$ (J. C. Anthony, Anthony et al., 2000; J. C. Anthony, Yoshizawa et al., 2000; T. G. Anthony et al., 2001; Shah, Anthony, Kimball, & Jefferson, 2000). Additionally, removing leucine while all other amino acids were maintained at supraphysiological levels led to a decrease in protein synthesis by 40% in rats (Vary et al., 1999). In another study, of the three BCAAs, leucine was the most effective in stimulating the phosphorylation of the 4E-BP1 and $p70^{S6k}$ in rats that were food deprived (J. C. Anthony, Yoshizawa et al., 2000).

Only a limited amount of human studies have examined the effects of resistance exercise and BCAAs/leucine supplementation on protein synthesis (Blomstrand & Saltin, 2001; Koopman et al., 2005). Even less research has been performed studying the various proteins in the Akt-mTOR pathway in response to oral BCAAs/leucine administration and resistance exercise (Karlsson et al., 2004). One study using human participants and a lower body resistance exercise session that ingested BCAAs led to a 3.5 fold increase in $p70^{S6K}$ phosphorylation and significantly increased ribosomal protein S6 during the recovery period as well (Karlsson et al., 2004).

There is evidence to suggest that both oral and intravenous administration of essential amino acids, and leucine alone, can facilitate the release of insulin (J. C.

Anthony, Lang et al., 2002; Floyd, Fajans, Conn, Knopf, & Rull, 1966a, 1966b). However, other studies demonstrate that leucine alone does not increase serum insulin (J. C. Anthony, Anthony et al., 2000; J. C. Anthony et al., 1999; J. C. Anthony, Yoshizawa et al., 2000; Greiwe, Kwon, McDaniel, & Semenkovich, 2001). It has been shown that insulin administration alone stimulated protein synthesis in the gastrocnemius muscle of perfused rat hindlimb by approximately two times the baseline values (Kimball, Jurasinski, Lawrence, & Jefferson, 1997). The observed stimulation of protein synthesis was related to a 12-fold increase in the amount of eIF4G bound to eIF4E (Kimball et al., 1997). Other research has shown that insulin suppression decreased protein synthesis regardless of the nutritional condition and age of the rats (Prod'homme et al., 2005). Equally important, reduced insulin levels were associated with the dephosphorylation of 4E-BP1, increased binding of the 4E-BP1-eIF4E inactive complex and hypophosphorylation of eIF4E, p70S6k and PKB (Prod'homme et al., 2005). Ultimately, the literature suggests that leucine stimulates translation initiation at least in part through the protein kinase mTOR pathway, and that both leucine and insulin signaling most likely come together in this pathway to induce a maximal response (J. C. Anthony, Anthony, Kimball, & Jefferson, 2001; J. C. Anthony, Lang et al., 2002; Rennie, Bohe, Smith, Wackerhage, & Greenhaff, 2006).

Purposes of the Study

The overall purpose of this investigation is to determine if activity of the insulin signaling pathway is increased during a single bout of lower body resistance exercise due to supplemental BCAA or leucine ingestion and a concomitant increase in serum insulin

levels. Specifically, however, the primary purposes of the proposed study are to investigate the effects of:

1) BCAA or leucine in conjunction with an acute bout of lower body resistance training on IRS-1, Akt, mTOR, p70S6k, and 4EBP-1.

2) BCAA or leucine in conjunction with an acute bout of lower body resistance training on serum glucose, insulin, IGF-1, and GH.

Hypotheses

H_1: BCAA supplementation, combined with lower body resistance exercise, will not differ from placebo in regard to the levels of serum glucose.

H_2: Leucine supplementation, combined with lower body resistance exercise, will not differ from placebo in regard to the levels of serum glucose.

H_3: BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to serum levels of glucose.

H_4: BCAA supplementation, combined with lower body resistance exercise, will not increase the serum levels of insulin, as compared to the placebo.

H_5: Leucine supplementation, combined with lower body resistance exercise, will not increase the serum levels of insulin, as compared to the placebo.

H_6: BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to the serum levels of insulin.

H_7: BCAA supplementation, combined with lower body resistance exercise, will not increase phosphorylated IRS-1 when compared to the placebo.

H_8: Leucine supplementation, combined with lower body resistance exercise, will not increase phosphorylated IRS-1 when compared to the placebo.

H_9: BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to phosphorylated IRS-1.

H_{10}: BCAA supplementation, combined with lower body resistance exercise, will not increase phosphorylated Akt when compared to the placebo.

H_{11}: Leucine supplementation, combined with lower body resistance exercise, will not increase phosphorylated Akt when compared to the placebo.

H_{12}: BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to phosphorylated Akt.

H_{13}: BCAA supplementation, combined with lower body resistance exercise, will not differ from placebo in regard to the levels of serum IGF-1.

H_{14}: Leucine supplementation, combined with lower body resistance exercise, will not differ from placebo in regard to the levels of serum IGF-1

H_{15}: BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to serum levels of IGF-1.

H_{16}: BCAA supplementation, combined with lower body resistance exercise, will not differ from placebo in regard to the levels of serum GH.

H_{17}: Leucine supplementation, combined with lower body resistance exercise, will not differ from placebo in regard to the levels of serum GH.

H_{18}: BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to serum levels of GH.

H_{19}: BCAA supplementation, combined with lower body resistance exercise, will not increase phosphorylated mTOR when compared to the placebo.

H_{20}: Leucine supplementation, combined with lower body resistance exercise, will not increase phosphorylated mTOR when compared to the placebo.

H_{21}: BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to phosphorylated mTOR.

H_{22}: BCAA supplementation, combined with lower body resistance exercise, will not increase phosphorylated p70S6k when compared to the placebo.

H_{23}: Leucine supplementation, combined with lower body resistance exercise, will not increase phosphorylated p70S6k when compared to the placebo.

H_{24}: BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to phosphorylated p70S6K.

H_{25}: BCAA supplementation, combined with lower body resistance exercise, will not increase phosphorylated 4EBP-1 when compared to the placebo.

H_{26}: Leucine supplementation, combined with lower body resistance exercise, will not increase phosphorylated 4EBP-1 when compared to the placebo.

H_{27}: BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to phosphorylated 4E-BP1.

Delimitations

This study will be completed using the following guidelines:

1.) Approximately 30, recreationally active, but non-resistance-trained males (18-35 y) will participate in this study.

2.) Participants will be recruited from the student population at Baylor University by flyers posted throughout campus and through local gyms and health clubs.

3.) Muscle biopsies from the lateral thigh will be collected prior to exercise, 30 minutes post-exercise, 2 hours post-exercise, and 6 hours post-exercise.

4.) Fasting venous blood will be collected prior to exercise, 30 minutes after ingesting the supplement or placebo, immediately post-exercise, 30-minutes post-exercise, 2 hours post-exercise, and 6 hour post-exercise.

5.) Participants will complete 4 sets of 8-10 reps of both leg press and leg extension at 80% 1RM.

6.) Participants will be randomly assigned to one of three supplement groups: BCAA, leucine or placebo.

7.) All participants in the study will not participate in any other forms of vigorous exercise during the duration of the study and will not modify their nutritional intake in any manner.

8.) All testing will be in the Exercise and Biochemical Nutrition Lab and Exercise and Sport Nutrition Lab at Baylor University in the Marrs-McLean Gym according to all policies and procedures within each respective laboratory.

Limitations

1.) The sample size will be limited to those who come forward to participate in the study, which could limit the scope of conclusions that can be inferred to a larger population.

2.) The motivation and willingness of each participant to maximally exert themselves during the familiarization session to establish 1 RM and during the acute resistance exercise session.

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

4.) 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 fast for eight hours prior to reporting for testing.

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

3.) Participants will be physically active, but not resistance trained.

4.) All participants will follow all instructions throughout the familiarization session and the acute exercise bout and maximally exert themselves while completing the familiarization session and the acute exercise bout.

5.) All assay reagents and equipment used in the sample analysis are accurate and reliable in quantification of the criterion variables.

6.) All methods are previously established and are accurate and reliable methods for determination of the criterion variables.

Definitions of Key Terms

1. Akt – This protein is also known as protein kinase B (PKB) and is a downstream target of IRS-1. Akt is a key protein in cell signaling and specifically has the ability to phosphorylate serine 2448 on mTOR.

2. Branched-chain amino acids (BCAA) - A specific group of essential amino acids that have branched side chains and includes: leucine, isoleucine, and valine.

3. Eukaryotic initiation factors (eIF) – A special set of proteins that help regulate translation initiation.

4. Eukaryotic initiation factor 4A (eIF4A) – One of the three eukaryotic initiation factors that comprise the eukaryotic initiation factor 4F complex (eIF4F). Eukaryotic initiation factor 4A, which is a mRNA helicase, is vital for the process of translation initiation to occur.

5. Eukaryotic initiation factor 4E (eIF4E) – A key protein in the Akt-mTOR signal transduction pathway. When eIF4E is activated, it can bind to eIF4G and help

facilitate translation initiation. Eukaryotic initiation factor 4E binding protein (4E-BP1) regulates the activity of this protein.

6. Eukaryotic initiation factor 4E binding protein 1 (4E-BP1) – This protein is a key factor in the Akt-mTOR signal transduction pathway. When hypophosphorylated, it binds eIF4E and prevents it from combing with eIF4G. This ultimately inhibits translation initiation. However, when it becomes hyperphosphorylated, most likely via mTOR, it does not bind eIF4E and allows translation initiation to ensue.

7. Eukaryotic initiation factor 4F complex (eIF4F) – A combination of three eukaryotic initiation factors that include eIF4A, eIF4G, and eIF4E. The complete eIF4F complex helps regulate the binding of mRNA to the 40S ribosomal subunit.

8. Eukaryotic initiation factor 4G (eIF4G) – One of the three eukaryotic initiation factors that comprise the eukaryotic initiation factor 4F complex (eIF4F). Eukaryotic initiation factor 4G serves as a docking protein for eIF4A and eIF4E. When the complete eIF4F complex comes together it promotes the binding of mRNA to the 40S ribosomal subunit and ultimately translation initiation.

9. Growth hormone (GH) – A hormone that is released from the anterior pituitary and has the ability to enhance the release of IGF-1 from the liver. Furthermore, it may have the ability to influence the Akt-mTOR cell signaling pathway through the activation of IRS-1.

10. Insulin – An anabolic hormone that is released from the pancreas and when it binds to its receptor, it instigates various metabolic processes and can activate the proteins in the Akt-mTOR cell signaling pathway.

11. Insulin-like growth factor-1 (IGF-1) – A growth factor that has the ability to bind to its receptor, and to a lesser affinity, the insulin receptor, and activate the proteins in the Akt-mTOR cell signaling pathway.

12. Insulin receptor substrate-1 (IRS-1) – A cytoplasmic protein that is stimulated by insulin, insulin-like growth factor-1, and possibly growth hormone. IRS-1 has the ability to bind to and activate (phosphorylate) Akt.

13. Initiator methionyl transfer RNA (met-tRNAi) – The met-tRNAi carries the amino acid methionine and binds to the 40S ribosomal subunit. This binding of the met-tRNAi to the 40S ribosomal subunit is vital in facilitating the translation of mRNA into protein and is one of the steps of translation initiation that can be regulated in humans.

14. Leucine – An essential amino acid that is one of the three branched chain amino acids. Leucine is thought to help regulate protein synthesis.

15. Mammalian Target of Rapamycin (mTOR) – Is a protein kinase that is involved with cell signaling and plays a role in regulating translation initiation. It is thought to be an activator of the downstream targets 4E-BP1 and P70-S6K.

16. Messenger RNA (mRNA) – The DNA of a gene is transcribed into mRNA. The mRNA is carried to the ribosome and serves as a template for the synthesis of polypeptides (proteins).

17. Phosphorylation – The process of adding a phosphate to a compound or protein through an enzyme. Through the procedure of adding a phosphate, a protein/enzyme can be activated or deactivated.

18. Protein balance – Also known as protein turnover and net protein balance, is the sum of protein synthesis and protein breakdown.

19. Protein degradation - Also known as protein breakdown and proteolysis. This is the process of breaking down proteins into their individual amino acids. During states of muscle atrophy, protein breakdown exceeds muscle protein synthesis.

20. Protein synthesis – The process of translating mRNA in protein as directed by the genetic code. During states of muscle hypertrophy, protein synthesis exceeds muscle protein breakdown.

21. P70 Ribosomal Protein S6 Kinase (also denoted as S6K1, P70-S6K, and $p70^{S6K}$) – This 70 kilodalton protein kinase is a key enzyme in the Akt-mTOR signal transduction pathway. It is thought to be activated by mTOR and appears to activate ribosomal protein S6.

22. Ribosomal protein S6 (rpS6) – A downstream target of P70-S6K and when it is activated seems to facilitate the translation of certain mRNAs including eIF4G, and translation elongation factors 1A and 2.

23. Translation initiation – The first of three steps involved in the process of translating mRNA into proteins. The process of translation also includes elongation and termination.

24. 40S ribosomal subunit – An integral subunit of the 80S initiation complex. The 40S ribosomal subunit is a part of the ribosome where mRNA is translated into proteins

CHAPTER TWO

Literature Review

Introduction

Currently, leucine is one of the most popular sports supplements marketed to both athletes and those attempting those improve lean body accretion. Leucine, along with isoleucine and valine, are collectively called the branched-chain amino acids (BCAAs). BCAAs constitute approximately one-third of all muscle protein (Mero, 1999). Specifically, leucine comprises approximately 5-10% of the total protein content (Mero, 1999). Out of the three BCAAs, leucine has been the most studied secondary to the belief that it plays the most significant role in stimulating protein synthesis (Kimball & Jefferson, 2006). Furthermore, it appears that its oxidation rate is significantly higher when compared to isoleucine or valine (Mero, 1999). In fact, leucine's oxidation rate rises during whole body exercise and contributes approximately 3-4% of the energy required for exercise and roughly 1% of the energy needed at rest (Hood & Terjung, 1990). It is has been shown that the serum and plasma values of leucine can be significantly decreased by approximately 11-33%, 5-8%, and 30% following aerobic, anaerobic, and strength exercises respectively (Mero, 1999). Additionally, there have also been observed decreases in the skeletal muscle content of leucine following taxing aerobic exercise (Blomstrand & Newsholme, 1992; Mero, 1999). However, when leucine levels in the plasma approximate normal physiological values (approximately 116-343 μmol/L) (Greiwe et al., 2001; Mero, 1999), it has been shown to activate key mediators of protein synthesis in humans (Greiwe et al., 2001). As we have discovered

through research, amino acids can stimulate protein synthesis in skeletal muscle secondary to an increase in translation initiation (Vary et al., 1999). Furthermore, it has been reported that the branched chain amino acids (leucine, isoleucine and valine) alone can facilitate protein synthesis as well as a complete mixture of amino acids (Vary et al., 1999). Additionally, when given orally to rats in the fasted state the branched-chain amino acids are especially effective in stimulating translation initiation (Kimball & Jefferson, 2001). Leucine alone appears to play the most significant role in facilitating translation initiation in skeletal muscle. More specifically, leucine appears to stimulate protein synthesis in skeletal muscle by increasing both the activity and synthesis of specific proteins involved in translation of mRNA into actual protein (J. C. Anthony et al., 2001). Ultimately, the literature suggests that leucine stimulates translation initiation at least in part through the protein kinase mTOR pathway, and that both leucine and insulin signaling come together in this pathway to induce a maximal response (J. C. Anthony et al., 2001; Rennie et al., 2006).

Therefore, even though studies involving BCAAs will be discussed in this review of literature, the primary focus will involve the role of leucine alone on exercise performance / training adaptations, the regulation of protein synthesis, translation initiation, its impact on the Akt-mTOR signal transduction pathway, and insulin secretion.

Applied Studies Involving BCAA and Leucine Administration in Humans

It has been hypothesized that BCAAs and to a greater extent, leucine, may provide an ergogenic benefit for various types of exercise. Additionally, there is some evidence to suggest that BCAAs may aid in the preferential loss of visceral adipose tissue

in individuals that are attempting to lose weight through dieting. Only a handful of studies in humans to this date have examined the effects of supplementing leucine on muscular endurance, power, and strength. It will be the focus of this section to examine some of the research that studied the effects of BCAA and leucine supplementation on exercise. However, the emphasis will be placed on studies involving leucine administration.

Aerobic Based Exercise

As mentioned above, leucine oxidation is increased during exercise and is indicative of increased net protein breakdown (Tipton & Wolfe, 1998). To illustrate that point, Lamont, McCullough, and Kalhan (1999) demonstrated that both endurance trained and sedentary males experienced an increase in leucine oxidation during one hour of exercise at 50% VO_2 max. Interestingly, based on body weight, the endurance trained athletes had a significantly greater leucine rate of appearance during both exercise and recovery and an increased leucine oxidation at all times as compared with the participants in the sedentary cohort (Lamont et al., 1999). However, based on fat-free tissue mass, all of these between-group differences for leucine kinetics were eliminated (Lamont et al., 1999). Thus, the investigators concluded that when leucine kinetics are expressed per unit of fat-free tissue mass, there were no significant difference during rest, exercise, or recovery when comparing endurance-trained and sedentary humans (Lamont et al., 1999). Interestingly, endurance trained males, however, have been shown to oxidize leucine significantly more than endurance trained females during a submaximal exercise test (Phillips, Atkinson, Tarnopolsky, & MacDougall, 1993). The participants of this study weren't administered any protein or leucine supplement. They only consumed a

diet that was isoenergetic with their habitual intake for 10 days prior to the study. The diet was standardized, however, at 0.86 grams of protein per kg of body weight per day (Phillips et al., 1993).

A study by Blomstrand and Newsholme (1992) examined the effects of administering BCAAs to individuals running in a 30 km cross-country race and a full marathon. The researchers wanted to look at the effect BCAA ingestion and a placebo had on plasma and muscle concentrations of aromatic amino acids and BCAAs. The participants that ran in the 30 km cross-country race were given BCAAs at an absolute dose of 7.5 grams (isoleucine-15%, valine-50%, and leucine-35%) or placebo. Conversely, individuals who ran in the marathon were given BCAAs at an absolute dose of 12 grams (isoleucine-25%, valine-40%, and leucine-35%) or placebo. When BCAAs were ingested in both the 30 km run and the marathon group, the plasma and muscle concentration of these amino acids increased (Blomstrand & Newsholme, 1992). However, in the placebo group the concentration of BCAAs decreased in the plasma and did not change in the muscle (Blomstrand & Newsholme, 1992). Furthermore, in the placebo group, both types of exercise caused a 20-40% increase in the muscle concentration of the tyrosine and phenylalanine (Blomstrand & Newsholme, 1992). These two aromatic amino acids can be used as markers of net protein breakdown because they are not taken up or metabolized by skeletal muscle (Blomstrand & Newsholme, 1992). The authors further point out that when BCAAs were given in the two exercise conditions, these increases of tyrosine and phenylalanine were not observed (Blomstrand & Newsholme, 1992). In conclusion, this study suggests that BCAAs given

during long duration aerobic exercise might help mitigate the rate of protein breakdown (Blomstrand & Newsholme, 1992).

In another study conducted by Blomstrand, Andersson, Hassmen, Ekblom, and Newsholme (1995) five male endurance-trained participants performed exhaustive exercise on a cycle ergometer at 75% of their VO_2 max after reduction of their muscle glycogen stores (Blomstrand et al., 1995). The subjects were either administered 7 grams of BCAAs in a 6% carbohydrate solution, just the carbohydrate solution alone, or a flavored water placebo (Blomstrand et al., 1995). The investigators reported that there were no significant differences in performance when the subjects were given BCAAs plus carbohydrate or carbohydrate alone during exercise. Additionally, BCAAs plus carbohydrate and carbohydrate alone did not significantly affect the exercise-induced increase in the plasma and muscle concentration of aromatic amino acids seen in the placebo group (Blomstrand et al., 1995). The authors suggest that neither BCAA nor carbohydrate decreased the net protein breakdown during exercise (Blomstrand et al., 1995).

BCAA ingestion may improve how hard an individual feels he/she is working during exercise as measured by the rating of perceived exertions (RPE). One study examined the effect of performing exhaustive exercise on a cycle ergometer approximately 70% of their maximal oxygen uptake for 60 minutes and then followed by another 20 minutes of maximal exercise (Blomstrand, Hassmen, Ek, Ekblom, & Newsholme, 1997). The participants were male endurance-trained cyclists who were experimentally placed in a glycogen depleted state before the testing began. The participants were either given an oral solution of BCAAs or a placebo. The study

concluded that consuming an oral solution of BCAAs had a 7% decrease in RPE and a 15% decrease in ratings of mental fatigue as compared to the placebo (Blomstrand et al., 1997). Additionally, the ratio of free tryptophan/BCAAs (which higher ratios may play a role in fatigue) significantly increased during and after the exercise test in the placebo group, but remained unchanged in the BCAA group (Blomstrand et al., 1997). It was also reported that even though subjective ratings of RPE and mental fatigue may have improved, there was no difference in the exercise performance between the two trials determined by the amount of work done in the last 20 minutes of maximal exercise (Blomstrand et al., 1997).

Varnier et al. (1994) examined the effects of BCAA or saline infusion [300 mg/kg bodyweight (leucine - 15.7 g/L, isoleucine – 12.9 g/L, valine 12.0 g/L) infusion rate 260 mg/kg/hour] 70 minutes before an incremental endurance exercise (increases of 35 W every 4 minutes) to exhaustion while the participants were in a glycogen depleted state. The authors concluded that BCAA infusion while in a glycogen depleted state, secondary to a 20 hour fast, had no significant effect on total work performed or maximal oxygen uptake (Varnier et al., 1994).

In a study by Tang (2006), researchers set out to determine the effects of branched-chain amino acid (BCAA) supplementation or placebo on urinary urea nitrogen, hydroxyproline (HP), and 3-methylhistidine (3MH) concentrations after performing 25 minutes of a breast stroke exercise (65-70% maximum heart rate reserve) followed by a 600 m crawl stroke competition (Tang, 2006). Nineteen male college swimmers were either given a placebo or 12 g of BCAAs/day; in capsules (leucine 54%, isoleucine 19%, valine 27%) for 15 days while performing moderate intensity (60-70%

HRRmax) swimming and rowing for approximately 1.5 hours/day (Tang, 2006). The exercise program prescribed to the swimmers did not significantly affect urinary urea nitrogen, HP, and 3MH concentrations (Tang, 2006). Twenty hours after the last testing session, blood and urine samples were collected again. Significant increases in the concentrations of urinary urea nitrogen, HP, and 3MH, which are markers of proteolysis, were found in the placebo group, but not in the BCAA group (Tang, 2006). The author concluded that the observed muscle proteolysis observed in the placebo group, was prevented by BCAA supplementation (Tang, 2006).

Anaerobic/Resistance Training

One study by Crowe, Weatherson, and Bowden (2006) examined the effects of an oral leucine supplement on both power and endurance performance measures of outrigger canoeists. Thirteen competitive outrigger canoeists (three male and ten female) were given either capsulated leucine (45 mg/kg/day) or a placebo for six weeks while they performed their normal training regimen. The participants were tested both prior to and following the six week training session. The testing consisted of anthropometric assessment, a 10 second upper body power test and a row to exhaustion at 70% of their max aerobic power (Crowe et al., 2006). During the max row test, the rate of perceived exertion (RPE), heart rate, and plasma BCAA and tryptophan values were also measured. The investigators reported several findings. Leucine supplementation led to significantly elevated levels of leucine in the blood (Crowe et al., 2006). Upper body work and power increased in both the placebo and leucine groups as compared to baseline (Crowe et al., 2006). However, individuals in the leucine supplementation group had significantly greater power as compared to the placebo group (Crowe et al., 2006). Additionally, the

leucine group had significantly longer rowing to exhaustion times and a lower RPE than did the placebo group (Crowe et al., 2006). Leucine did not have a significant effect on heart rate, anthropometric measurements, or the plasma tryptophan to BCAA ratio (Crowe et al., 2006).

Pitkanen et al. (2003) examined the effects of leucine supplementation in competitive male power athletes during a strength exercise session (SES) and a maximal anaerobic running exercise (MARE) session until exhaustion. The participants performed either the strength training or anaerobic running twice, with seven days between testing sessions. The study participants consumed either a drink containing leucine (100 mg/kg of body weight before and during SES or 200 mg/kg of body weight before MARE) or a placebo (Pitkanen et al., 2003). Blood analyses revealed that in both the SES and the MARE groups, plasma leucine was significantly higher in the leucine supplemented group than in the placebo group both before and after the respective exercise sessions (Pitkanen et al., 2003). However, neither the performance variable (i.e., counter movement jump) measured after the SES nor the running performance in the MARE group was significantly different from that seen in the placebo group (Pitkanen et al., 2003). The researchers concluded that consuming leucine before or before and during exercise sessions does facilitate increases in blood leucine concentration (Pitkanen et al., 2003). However, the supplementation does not appear to affect acute physical performance at the doses given in this study (Pitkanen et al., 2003).

Weight Loss

One study examined the effects of BCAA supplementation on weight loss, body composition, and performance variables while in a hypocaloric state. Mourier et al.

(1997) investigated 25 competitive wrestlers who restricted their caloric intake to 28 kcal/kg/body weight/day for a total of 19 days. Different diets were utilized to determine the effects of caloric restriction on performance variables and body composition compared to a control diet. Anthropometric measurements of weight, percent body fat, and adipose tissue (AT) distribution determined via magnetic resonance imaging (MRI) were measured before and after the dietary intervention. Interestingly, the participants in the hypocaloric high-BCAA experienced a significantly greater loss of both body weight (-4 kg), body composition (-17.3%), and abdominal visceral adipose tissue (-34.4%) as compared to the other groups (Mourier et al., 1997). The hypocaloric high-BCAA group consumed approximately 20% protein, 60% carbohydrate, and 20% fat, with a protein supplement enriched with BCAAs [0.9 g/kg of body weight/day (51.9 g BCAA/100 g protein: 76% leucine, 19.9% isoleucine, and 5% valine)] (Mourier et al., 1997). The control diet consisted of 40 kcal/kg per day (approximately 12% protein, 55% carbohydrate, and 33% fat) (Mourier et al., 1997). As reported, there were no changes in aerobic (VO2max), anaerobic capacities (Wingate test), and in muscular strength between the different diet groups (Mourier et al., 1997). The researchers concluded that the combination of a moderately hypocaloric diet, in addition to BCAA supplementation, promoted significant improvements in body composition and preferential losses of visceral adipose tissue (Mourier et al., 1997). Equally important, it allowed the athletes to preserve high level of athletic performance (Mourier et al., 1997).

Overview of Protein Synthesis

Even though the effects of leucine supplementation does not seem overly promising in regards to acute exercise performance, it may play a role in helping

maintain or increase lean body mass by stimulating protein synthesis in muscle. Net protein balance, also called protein turnover, is the combination of net protein synthesis plus net protein degradation (Layman, 2002). During periods of skeletal muscle hypertrophy, in which muscle enlargement occurs, the overall net balance of synthesis and breakdown is positive (Layman, 2002). Conversely, when net protein balance is negative, the rate of degradation is greater than the rate of synthesis. It is largely agreed upon that when an individual is in a fasted state, the body is in a net negative protein balance, with protein breakdown predominating (Phillips, Tipton, Aarsland, Wolf, & Wolfe, 1997; Wolfe, 2006). However, when a meal containing whole protein or essential amino acids is consumed and plasma levels of amino acids increase, the protein balance changes from a negative to a positive state (Borsheim, Tipton, Wolf, & Wolfe, 2002; Wolfe, 2006). The degree to which protein synthesis is increased following a rise in plasma amino acids is contingent upon several factors such as: the age of the individual, the amount of amino acids consumed, and the hormonal profile (Wolfe, 2006). Equally important, the change in protein synthesis is also predicated upon whether or not the amino acids were consumed as a bolus or a constant intake (Wolfe, 2006).

In general, the protein synthesis response to exercise is contingent upon the mode, intensity, and duration of the exercise. Early studies, using fasted rats, have shown that longer duration aerobic exercise, both swimming and running, leads to a decrease in protein synthesis and an increase in degradation (Dohm, Kasperek, Tapscott, & Beecher, 1980). Additionally, these findings also demonstrated that the extent of the decrease in protein synthesis was directly proportional to the intensity and duration of the exercise bout (Dohm et al., 1980). Similarly, Gautch et al (1998) have also reported similar

decreases in protein synthesis following longer duration exercise (i.e. 2 hours) in fasted rats. As mentioned above, Tipton and Wolfe (1998) have reported that leucine oxidation is increased during exercise and is indicative of increased net protein breakdown. Furthermore, leucine oxidation has been shown to be markedly increased during exercise (45% of VO_2 max to exhaustion) when the participants were fasted for 36 hours as compared to 14 hours (Knapik et al., 1991).

It is fairly common to measure protein synthesis and degradation both before and following an exercise bout. Typically, after endurance exercise, whole body protein degradation is normally reduced from resting levels (Tipton & Wolfe, 1998). Conversely, whole body protein breakdown and leucine oxidation are increased following eccentric exercise (Tipton & Wolfe, 1998). However, in both endurance and eccentric exercise, whole body protein synthesis most likely increases, but may remain unchanged (Tipton & Wolfe, 1998).

Protein synthesis is also frequently measured during resistance exercise as well. In human trials, where fasted participants perform resistance training, protein synthesis has been shown to decrease while protein degradation increases (Tipton & Wolfe, 1998). However, the fractional synthetic rate (FSR) of protein in skeletal muscle can be increased up to 48 hours shortly after the cessation of exercise (Phillips et al., 1997). Still, the rate of muscle protein breakdown also remains elevated following resistance exercise as well (Borsheim et al., 2002; Rennie & Tipton, 2000; Wolfe, 2006). Again, without adequate amino acids, the net protein balance improves after weight training, but it still remains negative (Wolf, 2006). Therefore, essential amino acids are needed to stimulate protein synthesis and mitigate protein breakdown following an exercise bout

(Tipton, Ferrando, Phillips, Doyle, & Wolfe, 1999; Tipton & Wolfe, 1998). In fact, one study has shown that as little as 6 grams of essential amino acids, without carbohydrates, effectively stimulated protein synthesis following resistance exercise (Borsheim et al., 2002). Interestingly, it seems that when amino acids are taken in conjunction with resistance training, it promotes a synergistic effect in relation to protein synthesis (Wolfe, 2006). In other words, it leads to a greater rate of protein synthesis than taking into account the effects of resistance training alone plus the effects of amino acid ingestion alone (Wolfe, 2006). Furthermore, additional animal studies, which will later be discussed in detail, demonstrate that leucine alone can stimulate protein synthesis via its ability to enhance translation imitation (J. C. Anthony, Anthony et al., 2000; J. C. Anthony et al., 1999; J. C. Anthony, Yoshizawa et al., 2000; T. G. Anthony et al., 2001).

The philosophy by some in regards to protein intake is, if a little is good than more has got to better. However, this does not necessarily seem to be the case. It has been reported that athletes who are attempting to gain muscle mass, in some cases consume four to five times the amount of protein than the RDA suggests (Layman, 2002). However, it appears that the upper limit for skeletal muscle hypertrophy is approximately 0.5 kg per week (Layman, 2002). If this true, then only about 15 grams of protein can be incorporated into skeletal muscle for any given day, considering approximately 20% of the change in weight due to hypertrophy comes from actual protein (Layman, 2002). For active individuals, it appears that a protein intake of 1.4-2.0 g/kg of body weight per day to be sufficient in most cases to support protein synthesis (Lemon, 1991; Mero, 1999). Thus, larger amounts of protein consumption do not seem to be more efficacious in regards to stimulating protein synthesis or to facilitate skeletal muscle accretion.

Mechanisms that Control Translation Initiation

Translation initiation in mammals is regulated by specialized proteins called eukaryotic initiation factors (Bolster, Kimball et al., 2003). In comprehensive reviews Bolster, Kimball, and Jefferson (2003), as well as Kimball, Farrell, and Jefferson (2002) report that there are two specific steps of translation initiation that have the ability to be regulated in vivo. The first step of translation initiation that is subject to regulation is the binding of the initiator methionyl- tRNA complex to the 40S ribosomal subunit (Bolster, Kimball et al., 2003; Kimball et al., 2002; Layman, 2002). This aforementioned complex is comprised of eukaryotic initiation factor 2 (eIF2), guanosine triphosphate (GTP), and methionyl- tRNA (met-tRNA) (Bolster, Kimball et al., 2003; Kimball et al., 2002). Eventually the GTP which is associated with the eIF2 is hydrolyzed to GDP and ultimately this complex (GDP-eIF2) is released form the 40S ribosomal subunit (Bolster, Kimball et al., 2003; Kimball et al., 2002). If this process is to repeat itself, the GDP that was associated with eIF2 needs to be replaced with GTP in order for another cycle of initiation to occur (Bolster, Kimball et al., 2003; Kimball et al., 2002). This exchange process is regulated by eukaryotic initiation factor 2B (eIF2B) (Bolster, Kimball et al., 2003; Kimball et al., 2002). Eukaryotic initiation factor 2B is also subject to regulation by the direct phosphorylation of the ε–subunit on the actual eIF2B protein or by the process of phosphorylating of the α–subunit of its substrate, eIF2 (Bolster, Kimball et al., 2003). When eIF2α becomes phosphorylated, it can inhibit the exchange of GDP for GTP an ultimately translation initiation (Bolster, Kimball et al., 2003; Kimball et al., 2002). Interestingly, depending on which residue is actually phosphorylated on eIF2Bε can determine whether it actually facilitates or inhibits this exchange of GDP for GTP

(Bolster, Kimball et al., 2003). Overall, when eIF2B is inhibited it ultimately does not allow the exchange of GDP for GTP. This in turn, would decrease the translation of the majority of mRNAs (Bolster, Kimball et al., 2003). Even though the entire aforementioned process of binding the initiator methionyl- tRNA complex to the 40S ribosomal subunit is one of the two steps that are subject to regulation, neither increased amounts of leucine nor the other amino acids seem to play a major role in regulating this step (J. C. Anthony, Anthony et al., 2000; Vary et al., 1999).

The second step of translation initiation that is subject to regulation is the binding of the mRNA to the 40S ribosomal subunit (Bolster, Kimball et al., 2003; Kimball et al., 2002). This step appears to be much more affected by leucine administration. Three initiation factors regulate the binding of the mRNA to the 40S ribosomal subunit (Bolster, Kimball et al., 2003; Kimball et al., 2002). These three initiation factors are communally called eIF4F and consists of eIF4A (a RNA helicase), eIF4G (a scaffolding protein), and eIF4E (which binds to the m^7GTP cap on the mRNA) (Bolster, Kimball et al., 2003; Kimball et al., 2002). Of the possible mechanisms that regulate the binding of the mRNA to the 40S ribosomal subunit, two may be mediated by leucine and/or insulin (Blomstrand et al., 2006; Bolster, Kimball et al., 2003; Kimball & Jefferson, 2006). The first mechanism that appears to regulate the mRNA binding step to the 40S ribosomal subunit is the sequestration of eIF4E by a binding protein called 4E-BP1 (Blomstrand et al., 2006; Bolster, Kimball et al., 2003; Kimball & Jefferson, 2006). 4E-BP1 becomes activated when it is hypophosphorylated. Hypophosphorylated 4E-BP1 will bind to eIF4E and effectively make it inactive (Blomstrand et al., 2006; Bolster, Kimball et al., 2003; Kimball & Jefferson, 2006). If eIF4E becomes inactive it cannot bind to eIF4G

and ultimately this will not allow the mRNA to bind to the 40S ribosomal subunit (Blomstrand et al., 2006; Bolster, Kimball et al., 2003; Kimball & Jefferson, 2006). This will inhibit translation initiation. Conversely, when 4E-BP1 is hyperphosphorylated, it will not bind to eIF4E and this will allow translation initiation to ensue (Blomstrand et al., 2006; Bolster, Kimball et al., 2003; Kimball & Jefferson, 2006).

Another mechanism that has the ability to play a regulatory role in the binding of the mRNA to the 40S ribosomal subunit is the ribosomal protein S6 (rpS6) (Blomstrand et al., 2006; Bolster, Kimball et al., 2003; Kimball & Jefferson, 2006). When rpS6 becomes phosphorylated it facilitates the translation of a certain group of mRNAs called TOP mRNAs (Bolster, Kimball et al., 2003). Several mRNAs including translation elongation factors 1A and 2, eIF4G, and ribosomal proteins, all fall under the TOP mRNA umbrella (Bolster, Kimball et al., 2003). These particular mRNAs have a distinctive series of pyrimidine residues near the 5'-cap that give them there unique characteristics (Bolster, Kimball et al., 2003). However, the exact mechanism by which the phosphorylation of rpS6 actually facilitates the translation of these particular TOP mRNAs remains unknown (Bolster, Kimball et al., 2003).

Signal Transduction Pathways that Govern Translation Initiation

Researchers have discovered that activation or over expression of the proteins in the PI3-K/mTOR signaling pathway can increase cell size (Bolster, Kimball et al., 2003). Some of the proteins that are involved in this signal transduction pathway include: PI-3 kinase, phosphoinositide-dependent protein kinase (PDK1), Akt (which is also referred to as protein kinase B [PKB]), mTOR, and S6K1 ($p70^{S6K}$) (Bolster, Kimball et al., 2003; Kimball et al., 2002). Insulin and insulin-like growth factor I (IGF-1) are anabolic

hormones known to regulate mTOR through its activation of upstream enzymes, such as IRS-1 (Kimball et al., 2002). It has been reported that resistance training, which can lead to an increase in protein synthesis, is most likely mediated through the PI-3 kinase-mTOR pathway (Bolster, Kimball et al., 2003). In order to achieve a net increase in protein synthesis, translation must occur. It has been reported that in order for mRNA to undergo translation into protein, it must begin by mRNA binding to ribosomes to form polysomes (Bolster, Kimball et al., 2003). When translation initiation is stimulated, as in the case of BCAA ingestion, the proportion of ribosomal subunits in the polysomes increases (Bolster, Kimball et al., 2003). Different animal models have shown that increased translation initiation facilitates protein synthesis (J. C. Anthony et al., 1999; J. C. Anthony, Yoshizawa et al., 2000; Bolster, Kimball et al., 2003; Crozier et al., 2005). Furthermore, it appears that hypertrophy of skeletal muscle may be associated specifically with the activation or phosphorylation of PKB (Bolster, Kimball et al., 2003). The phosphorylation of PKB not only continues to facilitate the PI3-K/mTOR pathway, but also inhibits glycogen synthase kinase-3 (GSK-3), both of which help to promote translation initiation and subsequent hypertrophy (Bolster, Kimball et al., 2003). However, BCAA and leucine consumption does not appear to phosphorylate GSK-3 or Akt (Blomstrand et al., 2006; Liu, Jahn, Wei, Long, & Barrett, 2002; Liu et al., 2004). Finally, Akt is also known to phosphorylate Ser2448 on mTOR and this phosphorylation is also implicated in skeletal muscle hypertrophy (Bolster, Kimball et al., 2003; Kimball et al., 2002). Specifically, mTOR has been demonstrated to hyper-phosphorylate 4E-BP1 and $p70^{S6K}$. However, it appears that only the most highly phosphorylated forms of $p70^{S6K}$ lead to actual $p70^{S6K}$ activity in vivo (J. C. Anthony, Anthony et al., 2000). Once

4E-BP1 is inactivated (hyper-phosphorylated), S6K1 seems to play a role in increasing the protein synthesis following resistance training (Bodine et al., 2001; Bolster, Kimball et al., 2003; Kimball & Jefferson, 2006).

The Effects of Leucine on the AKT-mTOR Pathway and Protein Synthesis in Animal and Human Models

Protein synthesis is not only regulated by the transcription of DNA into messenger and ribosomal RNA, but also by the body's ability to translate the mRNA into actual peptides or proteins (Layman, 2002). At this time, it is thought that the increase in protein synthesis observed during hypertrophy, at least in part, is due to mechanisms involved in the regulation of the translation of mRNA into actual protein (Bolster, Kimball et al., 2003). Similarly, translation initiation is thought to be the rate limiting step in protein synthesis (Gallie, 2004). Furthermore, the mechanisms that govern translation initiation appear to play a crucial role during hypertrophy to resistance training (Bolster, Kimball et al., 2003). The BCAAs and more specifically, leucine, seem to help regulate protein synthesis through its ability to enhance translation initiation (J. C. Anthony, Anthony et al., 2000; J. C. Anthony et al., 1999; J. C. Anthony, Yoshizawa et al., 2000; T. G. Anthony et al., 2001; Crozier et al., 2005).

It should be noted that the majority of the published studies examining the effect of leucine administration on various enzymes in the Akt-mTOR pathway have used animal models. Currently, there are only a few select studies that have utilized human participants in regards to this subject matter. Therefore, this field of study warrants future investigations examining both acute designs elucidating the effects that leucine has on the Akt-mTOR pathway, translation initiation, and protein synthesis in humans.

Additionally and equally important, training studies examining the actual effect of leucine on lean tissue accretion needs to be addressed as well. The purpose of this section is to provide an overview of the studies that have investigated leucine's role in both translation initiation and protein synthesis.

Animal Models

In the 1970s, it was first discovered that leucine seemed to stimulate protein synthesis (Layman, 2002). The first study to suggest this finding was conducted by Buse and Reid in 1975. These investigators examined what effect the individual BCAAs had on protein synthesis when they were introduced into isolated rat hemidiaphragms (Buse & Reid, 1975). Initially, a combination of all three BCAAs (0.3 mM each) were added to media containing glucose and it subsequently stimulated protein synthesis (Buse & Reid, 1975). The investigators then decided to test valine, isoleucine, and leucine individually (Buse & Reid, 1975). Interestingly, when the BCAAs were tested individually, the researchers concluded for this particular study, that valine was ineffective, isoleucine was inhibitory, but 0.5 mM leucine increased protein synthesis (measured by the specific activity of muscle proteins during incubation with [14C]lysine or [14C]acetate) in hemidiaphragms from either fed or fasted rats incubated with or without insulin (Buse & Reid, 1975). The authors summarized their novel findings by stating that leucine alone, may play an essential role in the protein-sparing effect of amino acids (Buse & Reid, 1975).

As discussed in the sections above, translation initiation plays a pivotal role in regulating protein synthesis. Furthermore, the role that the Akt-mTOR signal transduction pathway has on influencing translation initiation has also been earlier

elucidated (Bolster, Kimball et al., 2003). Now, the question becomes, what role do the BCAAs and specifically leucine have on the Akt-mTOR pathway and protein synthesis? Before the studies involving leucine in relation to the Akt-mTOR pathway are mentioned, a couple of studies that examined variables of the Akt-mTOR pathway using whole protein and BCAAs will first be discussed. These studies helped pave the way for investigating leucine's role in regulating the Akt-mTOR pathway.

A study by Gautsch et al.(1998) examined the association of the mRNA cap binding protein, eIF4E, with a translation initiation inhibitor, 4E-BP1, and their effect on protein synthesis during recovery from exercise in rats. Fasting male rats performed treadmill running for 2 hours at 26 meters/minute. Immediately after exercise, the rats consumed saline, a carbohydrate-only meal, or a nutritionally complete meal (54.5% carbohydrate, 14% protein, and 31.5% fat) (Gautsch et al., 1998). The meals that were given to the rats were isocaloric in nature, approximately 44 kJ, and represented approximately 15% of their daily energy intake. Both the rats that exercised and the non-exercised controls were evaluated 1 hour following the cessation of exercise (Gautsch et al., 1998). The investigators reported that muscle protein synthesis was reduced by 26% after exercise. Equally important, there was a fourfold increase in the amount of eIF4E associated to 4E-BP1, a translational repressor, and a 71% decrease in the association of eIF4E with eIF4G (Gautsch et al., 1998). However, when the animals were fed the complete meal, but not the carbohydrate meal following exercise, muscle protein synthesis increased to that equal to the non-exercised controls (Gautsch et al., 1998). This result occurred even though both the carbohydrate only and complete meal groups had similar plasma concentrations of insulin (Gautsch et al., 1998). The authors also

reported that the association between eIF4E and 4E-BP1 was inversely related to the association between eIF4E and eIF4G and positively correlated to skeletal muscle protein synthesis (Gautsch et al., 1998). The investigators concluded that the increase of muscle protein synthesis following exercise is associated with the availability of eIF4E, not bound to 4E-BP1 (Gautsch et al., 1998). Unbound eIF4E helps facilitate translation initiation via the binding of the mRNA to 40S ribosomal subunit.

The next year, Vary, Jefferson, and Kimball (1999) published the results of two studies in one manuscript. The first study examined the effects of supraphysiological [(10X) i.e., 10 times the physiological amount] amino acid concentrations on eukaryotic initiation factors eIF2B and eIF4E were compared with normal physiological concentrations (1X) of amino acids (Vary et al., 1999). The amino acids were infused into the rats' hind limbs. The authors reported that amino acid supplementation (10X) significantly stimulated protein synthesis two fold (Vary et al., 1999). Nevertheless, there were no reported changes in eIF2B activity, in the quantity of eIF4E associated with 4E-BP1, or in the phosphorylation of eIF4E and 4E-BP1 (Vary et al., 1999). However, the amount of eIF4E bound to eIF4G increased by 800% and the phosphorylation of eIF4E increased by 20% (Vary et al., 1999). The second study reported in this paper, took it one step further. The investigators examined the effect of removing just leucine from the supraphysiological concentration (10X) of plasma amino acids (Vary et al., 1999). By simply removing the leucine, the rate of protein synthesis decreased by 40% (Vary et al., 1999). This observed decrease in protein synthesis was related to a 40% decrease in eIF2B activity and a concomitant 80% decrease in the amount of eIF4E bound to eIF4G (Vary et al., 1999). Furthermore, the decrease in eIF4G bound to eIF4E

was attributed to the observed increase in 4E-BP1 bound to eIF4E and a reduced phosphorylation state of 4E-BP1 (Vary et al., 1999). However, the phosphorylation of eIF4E was not affected by the removal of leucine (Vary et al., 1999). The investigators concluded that the formation of the active eIF4E and eIF4G complex regulates protein synthesis in the skeletal muscle of rats when the amino acid concentration is greater than the normal physiological range (Vary et al., 1999). Furthermore, they state that the removal of leucine from the supraphysiological concentration (10X) of amino acids decreases protein synthesis in skeletal muscle via changes in eIF2B and possibly eIF4E (Vary et al., 1999).

In a study by Anthony, Anthony, and Layman (1999) demonstrated for the first time that orally administered leucine following aerobic exercise in rats stimulated recovery of skeletal muscle protein synthesis. The research team randomly assigned male rats into one of five different treatment groups which included: sedentary, food-deprived (SF); exercised, food-deprived (EF); exercised, fed a carbohydrate meal (EC); exercised, fed a leucine meal (EL); and exercised, fed a combination of carbohydrate and leucine (ECL) (J. C. Anthony et al., 1999). Immediately after exercise, all meals were administered by oral gavage. Both the EC and ECL meals were isocaloric and provided approximately 15% of their daily caloric intake. EL and ECL meals each provided approximately 270 mg leucine (J. C. Anthony et al., 1999). This is approximately the amount of leucine that these rats would consume in 24 hours (J. C. Anthony et al., 1999). Furthermore, the ECL was isonitrogenous with the EL meal (J. C. Anthony et al., 1999). The rats ran on a treadmill for 2 hours at 36 meters/minute and were killed 1 hour postexercise. Muscle samples were taken to determine the fractional rate of skeletal

muscle protein synthesis (J. C. Anthony et al., 1999). The study yielded the following results. The exercise session did not alter plasma glucose or insulin, but did decrease protein synthesis by 18% (J. C. Anthony et al., 1999). By refeeding a mixture of carbohydrate and leucine, plasma insulin relative to the EF and SF groups significantly increased (J. C. Anthony et al., 1999). Additionally, it also led to a complete recovery of muscle protein synthesis, which was not different from values observed in SF group (J. C. Anthony et al., 1999). By administering leucine alone following exercise, protein synthesis was restored to that in the SF group without increasing plasma insulin values (J. C. Anthony et al., 1999). The authors summarized their findings by stating that oral leucine consumption alone can stimulate muscle protein synthesis after exercise, independent of increased plasma insulin (J. C. Anthony et al., 1999).

One study by Anthony, Anthony, Kimball, Vary, and Jefferson (2000) set out to determine if oral leucine administration not only would stimulate protein synthesis, but would also accomplish this in part, via the activity of the Akt-mTOR pathway enzymes. In this particular study, rats were freely fed (F) or food deprived for 18 hours (J. C. Anthony, Anthony et al., 2000). The food-deprived rats were subsequently given saline (S), carbohydrate (CHO), leucine (L) or a mixture of carbohydrate plus leucine (CL). As described in the previous study (J. C. Anthony et al., 1999), CHO and CL meals were isocaloric and provided 15% of daily energy requirements. Both L and CL meals each delivered 270 mg leucine (J. C. Anthony, Anthony et al., 2000). The authors reported the following findings. In the S group, muscle protein synthesis was only 65% of that observed in the F group one hour following the consumption of their respective meals (J. C. Anthony, Anthony et al., 2000). Related to the observed decrease in protein synthesis,

the phosphorylation of eukaryotic initiation factor (eIF) 4E-binding protein 1 (4E-BP1), was decreased in the S group (J. C. Anthony, Anthony et al., 2000). As a result of the decreased phosphorylation of 4E-BP1, a significantly greater association of 4E-BP1 bound to eIF4E as compared to F was observed (J. C. Anthony, Anthony et al., 2000). Concomitantly, there was also a significant reduction in the formation of the active eIF4G and eIF4E complex in the S group when compared with rats in the F group. Following the consumption of leucine in both the L or CL groups, but not in the CHO group, protein synthesis in muscle was restored to that observed in the F group (J. C. Anthony, Anthony et al., 2000). Oral leucine administration (L or CL) also significantly increased the phosphorylation of 4E-BP1 three times higher than the S group (J. C. Anthony, Anthony et al., 2000). Ultimately, this repressed the formation of 4E-BP1.eIF4E and the active eIF4G.eIF4E complex was not different from the values observed in the F group (J. C. Anthony, Anthony et al., 2000). There were no significant differences, however, between the L and CL groups for the aforementioned dependent variables (J. C. Anthony, Anthony et al., 2000). Phosphorylated eIF4E was significantly greater in S and CHO as compared to the other experimental groups. Importantly, the authors further point out that when the rats were food deprived, it led to a greater electrophoretic mobility on the immunoblot analysis of phosphorylated $p70^{S6K}$ (J. C. Anthony, Anthony et al., 2000). According to the investigators, this finding suggests that $p70^{S6k}$ activation was decreased as compared to the freely fed control group (J. C. Anthony, Anthony et al., 2000). The CHO group yielded a small shift to a more slowly migrating form (J. C. Anthony, Anthony et al., 2000). Interestingly, both the L and CL increased the phosphorylation of $p70^{S6K}$ evidenced by the fact that the bands had the slowest electrophoretic mobility (J. C.

Anthony, Anthony et al., 2000). Interestingly, there were no significant differences in the eIF2α and eIF2β phosphorylation states between any groups (J. C. Anthony, Anthony et al., 2000). In the CHO and CL groups, serum insulin was increased by 2.6 and 3.7 times, respectively. However, insulin levels were not significantly different when comparing the L to the S group (J. C. Anthony, Anthony et al., 2000). The authors conclude by purporting that leucine appears to stimulate protein synthesis in skeletal muscle by increasing eIF4F formation and $p70^{S6K}$ independently of increases in insulin (J. C. Anthony, Anthony et al., 2000).

Another study from 2000 also set out to determine if orally administered leucine, isoleucine, or valine had the ability to stimulate skeletal muscle protein synthesis of food-deprived rats (J. C. Anthony, Yoshizawa et al., 2000). The second objective of the study was to determine if leucine increased protein synthesis, in part, through mTOR signaling (J. C. Anthony, Yoshizawa et al., 2000). This particular research design had two separate purposes. With one purpose, rats were food-deprived for 18 hours and then were orally administered saline or 270 mg valine, isoleucine or leucine (J. C. Anthony, Yoshizawa et al., 2000). With the second purpose, food-deprived rats were first injected intravenously with rapamycin (0.75 mg/kg), which is a specific inhibitor of mTOR, before leucine administration (J. C. Anthony, Yoshizawa et al., 2000). The investigators reported the following results (J. C. Anthony, Yoshizawa et al., 2000). The individual administration of valine, isoleucine and leucine to food-deprived rats did not change circulating insulin concentrations compared with food-deprived controls(J. C. Anthony, Yoshizawa et al., 2000). Out of the three BCAAs, only leucine significantly enhanced protein synthesis in skeletal muscle above that observed in the saline-treated control group (J. C. Anthony,

Yoshizawa et al., 2000). Equally important, leucine was the most successful among the BCAA at increasing phosphorylation of 4E-BP1 and $p70^{S6K}$ (J. C. Anthony, Yoshizawa et al., 2000). Furthermore, the hyperphosphorylation of 4E-BP1, due to leucine administration, facilitated the increased availability of eIF4E to form the active eIF4G and eIF4E complex (J. C. Anthony, Yoshizawa et al., 2000). The researchers reported that isoleucine also enhanced phosphorylation of 4E-BP1 and $p70^{S6K}$ as well, but to smaller degree than leucine (J. C. Anthony, Yoshizawa et al., 2000). In regards to the second arm of the study the investigators reported the following results (J. C. Anthony, Yoshizawa et al., 2000). The introduction of rapamycin prevented protein synthesis in both the leucine group and food-deprived rats (J. C. Anthony, Yoshizawa et al., 2000). Rapamycin also blocked the stimulatory effects of leucine on both the phosphorylation of $p70^{S6K}$ and eIF4F formation (i.e., the binding of eIF4F to eIF4G) (J. C. Anthony, Yoshizawa et al., 2000). The authors concluded that leucine, as compared to isoleucine and valine is unique due to ability to stimulate skeletal muscle protein synthesis in food-deprived rats (J. C. Anthony, Yoshizawa et al., 2000). Lastly, according to the investigators the results of this study were also novel because it was shown for the first time that leucine can stimulate translation initiation in vivo through mTOR (J. C. Anthony, Yoshizawa et al., 2000).

A study by Shah, Anthony, Kimball, and Jefferson (2000) investigated if orally administered leucine could reverse the translational inhibition brought on by glucocorticoids. The investigators injected 100 µg/100 g body weight of dexamethasone, which is a synthetic glucocorticoid, or an equal volume of a saline placebo (Shah et al., 2000). Following three hours of dexamethasone administration, one-half of the saline

control group and one-half of the dexamethasone-treated group were given a bolus of 2.5 ml/100 g body weight leucine (54.0 g/l) in distilled water (Shah et al., 2000). The scientists provided the following results. Dexamethasone administration acutely reduced skeletal muscle protein synthetic rates to 80% of the control values (Shah et al., 2000). Furthermore, an increased amount of eIF4E was bound to the translational suppressor, 4E-BP1 in the dexamethasone group, due to the hypophosphorylation of 4E-BP1 (Shah et al., 2000). Similarly, $p70^{S6K}$, specifically at T389, was also dephosphorylated as a result of dexamethasone (Shah et al., 2000). The researchers then discovered that by orally administering leucine, both eIF4E bound to 4E-BP1, and $p70^{S6K}$ were successfully brought back to the values observed in the control group (Shah et al., 2000).

In 2001, a study was conducted to investigate the role of leucine in the regulation of hepatic protein synthesis and ribosomal protein mRNA translation in rats (T. G. Anthony et al., 2001). Similar to other study designs, rats were either given orally administered saline (control) or 270 mg leucine, isoleucine or valine (T. G. Anthony et al., 2001). The investigators reported that the administration of any of the three BCAAs facilitated an increased phosphorylation of 4E-BP1 when compared with the saline control group (T. G. Anthony et al., 2001). Nonetheless, leucine administration proved to be most successful in stimulating the phosphorylation of 4E-BP1 and $p70^{S6K}$. It is very noteworthy to mention that even though leucine significantly enhanced the phosphorylation of 4E-BP1 and $p70^{S6K}$ to a greater degree than isoleucine or valine, there were no significant differences in the total protein synthesis rates between treatment groups (T. G. Anthony et al., 2001). This last finding is noteworthy because in another similar study (J. C. Anthony, Yoshizawa et al., 2000), performed by some of the same

researchers involved in this project, leucine did indeed significantly increase the rates of protein synthesis above that of isoleucine and valine (T. G. Anthony et al., 2001).

As, mentioned above, certain studies have shown that leucine appears to have the ability to stimulate protein synthesis independently of insulin (J. C. Anthony, Anthony et al., 2000; J. C. Anthony et al., 1999). However, the question becomes to what extent does insulin enhance protein synthesis? Anthony, Lang, et al. (2002) set out to answer this question using a rodent model. The investigators intravenously administered a primed, constant infusion of somatostatin (60 μg + 3 μ/kg per hour) or a placebo starting one hour before the administration of oral leucine (1.35 g L-leucine/kg) or saline (control) (J. C. Anthony, Lang et al., 2002). Somatostatin was utilized in this study because it has the ability to block the release of insulin from the pancreas. The rats were ultimately euthanized 15, 30, 45, 60, or 120 minutes following leucine administration (J. C. Anthony, Lang et al., 2002). It was found that when compared to the control group, serum insulin levels were increased between 15 and 45 minutes following leucine administration, but soon returned to basal values by 60 minutes (J. C. Anthony, Lang et al., 2002). When somatostatin was administered, it maintained the insulin concentrations observed at basal levels for the duration of the study (J. C. Anthony, Lang et al., 2002). The authors reported that protein synthesis was enhanced between 30 and 60 minutes, but when somatostatin was administered, protein synthesis was effectively blocked (J. C. Anthony, Lang et al., 2002). Fifteen minutes following the leucine consumption, the assembly of eIF4E bound to eIF4G as well as the hyperphosphorylation 4E-BP1, p70^{S6K}, and ribosomal protein S6 were all increased (J. C. Anthony, Lang et al., 2002). This effect lasted for at least 60 minutes. Interestingly, somatostatin reduced the leucine-

induced changes in 4E-BP1 and p70^{S6K} phosphorylation (J. C. Anthony, Lang et al., 2002). Moreover, it totally blocked the change in ribosomal protein S6 phosphorylation. Conversely, it had no effect on the amount of eIF4G bound to eIF4E (J. C. Anthony, Lang et al., 2002). The authors summarized their findings by stating that leucine does enhance protein synthesis as well as the phosphorylation of 4E-BP1 and p70^{S6K} , but these increases are facilitated by the temporary increase in serum insulin (J. C. Anthony, Lang et al., 2002). To the contrary, this assembly of eIF4G bound to eIF4E happens independently of increases in insulin, and this assembly appears insufficient to actually increase the rates of protein synthesis in rat skeletal muscle following leucine administration (J. C. Anthony, Lang et al., 2002).

Bolster, Vary, Kimball, and Jefferson (2004) set out to investigate the mechanisms by which leucine influences protein synthesis. Specifically, the purpose of this study was to elucidate the degree to which leucine and mTOR signaling regulates protein synthesis. Postabsorptive rats were perfused with food-deprived (1X) or superphysiologic (10X) concentrations of leucine with all other amino acids at 1X concentration (bolster 2004). The following observations were reported. First, protein synthesis in both the gastrocnemius and soleus perfused with 10X concentration of leucine was significantly higher compared with the group that received the 1X concentration of leucine (Bolster et al., 2004). Interestingly, the stimulatory effects of leucine on protein synthesis were unchanged by a specific inhibitor of PI3-kinase (LY 294002). As discussed earlier in this review, PI3-kinase is upstream of mTOR in the Akt-mTOR cell signaling pathway (Bolster et al., 2004). This reported result leads scientists to believe that leucine influences protein synthesis downstream of PI3-kinase.

Also, signaling through mTOR, which in this study was indirectly monitored by the phosphorylation status of 4E-BP1 or $p70^{S6K}$, was not any more enhanced by 10X concentration as compared with 1X concentration of leucine (Bolster et al., 2004). On the other hand, the binding of eIF4E to eIF4G and eIF4G (Ser-1108) phosphorylation were significantly increased by 10X leucine (Bolster et al., 2004). In conclusion, the study suggests that leucine appears to stimulate the binding of eIF4E to eIF4G and ultimately increases protein synthesis in skeletal muscle (Bolster et al., 2004). According to the investigator's summary, leucine promotes these aforementioned findings possibly by enhancing the phosphorylation of eIF4G through a signaling pathway independent of mTOR (Bolster et al., 2004).

In a study by Crozier, Kimball, Emmert, Anthony, and Jefferson (2005), oral leucine administration was investigated to determine the smallest effective dose needed to activate some of the key variables of the Akt-mTOR pathway. It had been previously established that an oral dose of 270 mg (1.35 g/kg of body weight) of leucine was effective in stimulating protein synthesis in food-deprived rats (J. C. Anthony, Yoshizawa et al., 2000). Knowing this, the researchers set out to determine whether smaller amounts of leucine could also stimulate protein synthesis. Thus, rats were given oral leucine to rats in varying amounts (0.068 to 1.35 g/kg body wt). The authors reported that leucine given at 0.135 g/kg still significantly increased protein synthesis greater than control group (Crozier et al., 2005). The observed increase in protein synthesis was related to increases in markers of mRNA translation initiation such as the increased phosphorylation of 4E-BP1, the association of eIF4G with eIF4E, and the phosphorylation of the $p70^{S6K}$ [Thr 389] (Crozier et al., 2005). Interestingly, following

leucine consumption, these aforementioned changes reached their peak levels when insulin levels were elevated. The authors concluded that oral leucine administration affects both mRNA translation and skeletal muscle protein synthesis via the modulation of previously mentioned markers of translation initiation (Crozier et al., 2005). These investigators concluded their findings by stating that relatively small doses of leucine were found to stimulate skeletal muscle protein synthesis in rats (Crozier et al., 2005). Furthermore, they state that future investigations examining how orally administered leucine plays a role in the regulation of skeletal muscle protein synthesis in humans will be feasible (Crozier et al., 2005).

Human Models

In a study by Greiwe, Kwon, McDaniel, and Semenkovich (2001) investigators studied the effects of insulin and leucine infusion on translation initiation, by specifically looking at both Akt, (phosphorylated at serine 473) and $p70^{S6K}$ (Greiwe et al., 2001). The research team infused 18 healthy men (n=10) and women (n=8) with leucine alone (n = 6), insulin alone (n = 6), or both leucine and insulin (n = 6) for 2 hours. (Greiwe et al., 2001). The amount of leucine that was infused was equal to 1 g/hour for two hours. Insulin was infused in an exponentially decreasing fashion until a rate of 40 $mU \cdot m^{-2} \cdot min^{-1}$ was obtained (Greiwe et al., 2001). Plasma insulin rose to nearly 400 pmol/l during the insulin-alone and leucine + insulin infusions. However, insulin remained unchanged as a result of the infusion with leucine alone (Greiwe et al., 2001). Additionally, the phosphorylation of $p70^{S6K}$ increased by 400% in response to leucine alone, 800% in response to insulin alone, and 1800% after the leucine plus insulin infusion (Greiwe et al., 2001). Another key finding in this study was the fact that insulin alone and leucine +

insulin infusions increased Akt phosphorylation (Greiwe et al., 2001). However, when leucine alone was infused it had no effect on Akt phosphorylation (Greiwe et al., 2001). The authors contended that the results of their study demonstrated that physiological concentrations of leucine and insulin activate $p70^{S6K}$, which seems to be an integral mediator of protein synthesis in human skeletal muscle (Greiwe et al., 2001). They summarized their results by inferring that leucine has the ability to stimulate protein synthesis via a nutrient signaling mechanism that is independent of insulin (Greiwe et al., 2001).

Blomstrand and Saltin (2001) administered either BCAAs or a placebo to seven male participants during 1 hour of ergometer cycle exercise (at approximately 75% of VO_2 max) and a 2-hour recovery period. Fifteen minutes before exercise, immediately before exercise, at 15, 30, 45, and 60 minutes of exercise, and at 15, 30, 60, and 90 minutes of recovery, the subjects ingested 150 ml of either a solution containing BCAAs (45% leucine, 30% valine, and 25% isoleucine) or a flavored water placebo (Blomstrand & Saltin, 2001). Each subjects in the BCAA group received a total of 100 mg BCAA/kg of body weight. The ingestion of BCAAs did not positively change the rate of exchange of the aromatic amino acids, tyrosine and phenylalanine, in the legs during exercise or augment their concentration in muscle (Blomstrand & Saltin, 2001). On the contrary, during the recovery period, there was a quicker decline in the muscle concentration of aromatic amino acids in the BCAA group as opposed to the placebo group [46% as opposed to 25% in the placebo group] (Blomstrand & Saltin, 2001). Furthermore, there was also a 32% decrease in the release of these amino acids from the legs during the 2-hour recovery in the BCAA group (Blomstrand & Saltin, 2001). The

scientists concluded that BCAA seem to have a protein-sparing effect during the recovery period following exercise, possibly through an increase in protein synthesis or possibly do to a decrease in protein breakdown (Blomstrand & Saltin, 2001).

A study conducted by Koopman et al. (2005) wanted to determine if leucine supplementation would further increase protein synthesis above that of protein plus carbohydrates, or carbohydrates alone, following lower body resistance training in humans. Therefore, eight male participants were randomized into three trials in which they consumed drinks (3ml/kg), every half hour for 5.5 hours, containing carbohydrate (CHO), carbohydrate and protein (CHO+PRO), or carbohydrate, protein, and free leucine (CHO+PRO+Leu) after completing 45 minutes of lower extremity resistance exercise (Koopman et al., 2005). The resistance exercise protocol consisted of eight sets of eight repetitions on the horizontal leg press machine and eight sets of eight repetitions on the leg extension machine both at 80% of their 1 repetition maximum (Koopman et al., 2005). The total amount of carbohydrate ingested in the CHO trial was equal to 1.65 g/kg of body weight. Individuals in the (CHO+PRO) trial also ingested 1.65 g/kg of carbohydrates plus 1.1 g/kg of protein hydrosylates (Koopman et al., 2005). The CHO+PRO+Leu trial ingested an additional .55 g/kg of leucine in conjunction with same relative amount carbohydrate and protein content in the (CHO+PRO) group (Koopman et al., 2005). The investigators reported that the insulin response was significantly higher in the CHO+PRO+Leu compared with the CHO and CHO+PRO trials (Koopman et al., 2005). By simply adding leucine in the CHO+PRO+Leu trial, the rate of protein oxidation was significantly lower than that observed in the CHO+PRO trial (Koopman et al., 2005). In addition, the whole body net protein balance was significantly greater in

the CHO+PRO+Leu trial, as compared to both the CHO+PRO and CHO trials (Koopman et al., 2005). Lastly, the mixed muscle fractional synthesis rate (FSR), calculated over a 6 hour recovery period following exercise, was significantly greater in the CHO+PRO+Leu as compared to the CHO trial and greater, however non-significantly, than that of the CHO+PRO trial (Koopman et al., 2005).

Karlsson et al. (2004) examined the effects of lower body resistance training by itself or in conjunction with the ingestion of BCAAs on $p70^{S6K}$ phosphorylation. The seven male participants performed 4 sets of 10 reps on a leg press at 80% of their 1-RM on two separate occasions (Karlsson et al., 2004). The participants ingested either BCAAs or a placebo solution during and following exercise. The supplementation protocol consisted of consuming a 150 ml of either BCAA or placebo before the warm-up, immediately before resistance exercise, 15 minutes after starting the resistance exercise, and at 15, 30, 60, and 90 minutes after the resistance exercise session (Karlsson et al., 2004). The BCAA trial consisted of consuming a total of 100 mg/kg and was comprised of 45% lecuine, 30% valine, and 25% isoleucine (Karlsson et al., 2004). The consumption of BCAAs significantly increased the plasma concentrations of all three BCAAs (Karlsson et al., 2004). The lower body resistance training alone led to a significant increase in $p70^{S6K}$ at Ser^{424} and/or Thr^{421} up to 2 hours following exercise (Karlsson et al., 2004). Additionally, the consumption of BCAAs increased $p70^{S6K}$ phosphorylation 3.5 times during the recovery period (Karlsson et al., 2004). Furthermore, phosphorylation at Thr^{389} was not increased immediately after exercise, but was significantly increased during recovery in individuals consuming BCAAs (Karlsson

et al., 2004). Lastly, the phosphorylation of ribosomal protein S6 was also increased during recovery in the BCAA group as opposed to the placebo (Karlsson et al., 2004).

Even though the next study that will be mentioned does not involve a nutritional intervention, but it is noteworthy because it involves the response of several enzymes in the Akt-mTOR pathway in response to concentric and eccentric resistance exercise alone. Eliasson et al. (2006) wanted to compare and contrast both eccentric and concentric contractions in regards to the effect it has on variables of the Akt-mTOR pathway and protein synthesis (Eliasson et al., 2006). Ten male participants performed 4 sets of 6 maximal eccentric contractions on one leg followed by 4 sets of 6 maximal concentric contractions on the contralateral leg (Eliasson et al., 2006). Additionally, Six extra subjects underwent the same exercise protocol, but with maximal concentric and submaximal eccentric exercise of identical force to that of the maximal concentric contractions (Eliasson et al., 2006). Muscle biopsies were obtained before, immediately following, and 1 and 2 hours following exercise in both lower extremities (Eliasson et al., 2006). The following were the reported results. The average peak force generated during the maximal eccentric exercise was 31% greater as opposed to the maximal concentric exercise (Eliasson et al., 2006). A two-to-eight fold increase in $p70^{S6K}$ and rp S6 phosphorylation resulted from the maximal eccentric contractions and lasted for 2 hours into the recover period following exercise (Eliasson et al., 2006). However, the phosphorylation states of Akt and mTOR remained unchanged as a result of maximal eccentric contractions (Eliasson et al., 2006). Furthermore, neither maximal concentric or submaximal eccentric contractions facilitated any significant changes in the phosphorylation states of Akt, mTOR, $p70^{S6K}$ and rp S6 up to 2 h following the cessation

of the exercise (Eliasson et al., 2006). The authors concluded by stating that a single bout of maximal eccentric contractions increases $p70^{S6K}$ in skeletal muscle through an Akt-independent pathway (Eliasson et al., 2006). Secondly, it appears that maximal eccentric as opposed to maximal concentric contractions appear to be more effective in enhancing protein synthesis without the benefit of nutritional intake (Eliasson et al., 2006).

Koopman, Zorenc, Gransier, Cameron-Smith, and van Loon (2006) examined the phosphorylation of P70-S6K and 4E-BP1 following resistance training in fasted humans. The eight male participants in this study performed eight sets of ten repetitions on both leg press and knee extension machines at 75% of their 1-RM. Muscle biopsies were obtained prior to and immediately following exercise and 30 and 120 minutes after the conclusion of exercise. The investigators reported the following observations. 4E-BP1 phosphorylation was significantly reduced immediately following resistance exercise (Koopman, et al., 2006). Conversely, P70-S6K significantly increased from baseline at 30 minutes into the recovery period after exercise (Koopman et al., 2006). However, even though P70-S6K increased, rp S6 did not significantly increase following exercise. Interestingly, the investigators discovered that P70-S6K phosphorylation was more pronounced in type II muscle fibers as opposed to type I fibers (Koopman et al., 2006). The authors summarized their findings by stating that resistance training seems to initially decrease 4E-BP1 phosphorylation while enhancing the phosphorylation of P70-S6K (Koopman et al., 2006). Additionally, these observations occurred without significantly increasing the phosphorylation of rp S6 phosphorylation (Koopman et al., 2006).

Another study by Coffey et al. (2006) set out to examine the response of proteins in the Akt-mTOR pathway in response to either resistance or aerobic exercise in seven strength-trained and six endurance-trained males (Coffey et al., 2006). This particular study utilized a cross over design which allowed each participant to complete 1 hour of cycling at 70% VO2peak and eight sets of five maximal repetitions of isokinetic leg extensions on a Kin-Com isokinetic dynamometer. Muscle biopsies were taken immediately before and after exercise bout, as well as, 3 hours after exercise (Coffey et al., 2006). All participants were fasted for 10-12 hours before exercise. The researchers concluded the following results. Akt phosphorylation was unchanged in both strength and endurance trained athletes following the resistance training session (Coffey et al., 2006). However, Akt phosphorylation significantly increased in the endurance trained participants, but not in the resistance trained individuals following the cycling session (Coffey et al., 2006). Similarly, P70-S6K phosphorylation significantly increased in endurance trained individuals, but not strength trained individuals following the resistance exercise session (Coffey et al., 2006). However, P70-S6K was relatively unchanged in both groups following the cycling session (Coffey et al., 2006). The authors concluded that resistance trained participants had an attenuated response to the specific enhancement of the Akt-mTOR pathway as compared to aerobic trained participants (Coffey et al., 2006).

A study by Dreyer et al. (2006) examined the effects of an acute bout of resistance training on various proteins in the Akt-mTOR pathway. Seven males and four females performed 10 sets of 10 repetitions on a knee extension machine at 70% of their 1-RM. Muscle biopsies were taken at baseline, immediately before and after exercise, as well as,

one and two hours post-exercise. The researchers reported the following results. Protein synthesis significantly decreased during exercise and was associated with a significant increase in AMP-activated protein kinase (AMPK) immediately following resistance exercise (Dreyer et al., 2006). AMPK remained elevated at one hour into the recovery period (Dreyer et al., 2006). The phosphorylation of 4E-BP1 significantly decreased immediately after resistance exercise and then only increased slightly (not significant) into the recovery period (Dreyer et al., 2006). Protein synthesis significantly increased by one hour post-exercise and remained elevated at two hours following exercise. The increase in protein synthesis in the recovery period was associated with an observed significant increase in P70-S6K at 2 hours post-exercise and a significant increase in Akt and mTOR at one hour post exercise (Dreyer et al., 2006). The authors concluded their findings by suggesting that the decrease in protein synthesis that is observed during resistance exercise is likely do to the increased activity of AMPK and the decreased activity of 4E-BP1 (Dreyer et al., 2006).

In a recent study, Fujita et al. (2007) examined muscle protein synthesis and several proteins in the Akt-mTOR pathway in non-exercising human males. A baseline muscle biopsy was taken and then the participants either consumed a leucine enriched essential amino acid-carbohydrate mixture (EAC) or in the case of the control group, no nutrients were consumed at all (Fujita et al., 2007). One hour later a second biopsy was obtained for a second time. The following results were reported (Fujita et al., 2007). The EAC supplement significantly increased protein synthesis and significantly increased the phosphorylation of Akt, mTOR, 4E-BP1, and $p70^{S6K}$ (Fujita et al., 2007). All of the aforementioned variables remained unchanged in the control group. Additionally, eEF2

phosphorylation was significantly reduced through the consumption of EAC supplement (Fujita et al., 2007).

The Role of Insulin on the Akt-mTOR Pathway and Translation Initiation

There is evidence to suggest that both oral and intravenous administration of essential amino acids, and leucine alone, can facilitate the release of insulin (J. C. Anthony, Lang et al., 2002; Floyd et al., 1966a, 1966b). However, other studies demonstrate that leucine alone does not increase serum insulin (J. C. Anthony, Anthony et al., 2000; J. C. Anthony et al., 1999; J. C. Anthony, Yoshizawa et al., 2000; Greiwe et al., 2001). As mentioned above in detail, several of the studies where leucine was given orally to rats, a common dose of 1.35 grams per kilogram of body weight was utilized (J. C. Anthony, Anthony et al., 2000; J. C. Anthony et al., 1999; J. C. Anthony, Lang et al., 2002; J. C. Anthony, Yoshizawa et al., 2000; T. G. Anthony et al., 2001; Crozier et al., 2005). One of the aforementioned studies that used this particular supplementation protocol reported a significant rise in insulin (J. C. Anthony, Lang et al., 2002). However, the levels given to rats are significantly more than most human studies. Therefore, in the case of leucine supplementation and the subsequent insulin response, caution must be used when trying to extrapolate the results of animal studies and applying them to humans.

It has been shown that insulin administration alone stimulated protein synthesis in the gastrocnemius muscle of perfused rat hindlimb by approximately two times the baseline values (Kimball et al., 1997). The observed stimulation of protein synthesis was related to a 12-fold increase in the amount of eIF4G bound to eIF4E (Kimball et al., 1997). Other research has shown that insulin suppression decreased protein synthesis

regardless of the nutritional condition and age of the rats (Prod'homme et al., 2005). Equally important, reduced insulin levels were associated with the dephosphorylation of 4E-BP1, increased binding of the 4E-BP1-eIF4E inactive complex and hypophosphorylation of eIF4E, p70S6k and PKB (Prod'homme et al., 2005). The authors concluded that insulin is necessary for the regulation of skeletal muscle protein synthesis regardless of age (Prod'homme et al., 2005). However, in a recent article by Chow et al. (2006), it was reported that even though there is equivocal data on the subject, insulin most likely attains muscle protein anabolism through the inhibition of muscle degradation, not through the increase in protein synthesis.

Both the effects of BCAAs and leucine alone on insulin secretion, as well as, insulin's influence on the Akt-mTOR pathway have been discussed in the results of several of the aforementioned studies (J. C. Anthony, Anthony et al., 2000; J. C. Anthony et al., 1999; J. C. Anthony, Yoshizawa et al., 2000; Gautsch et al., 1998; Greiwe et al., 2001). Depending on the source, the average normal fasting level of plasma insulin in non obese/non diabetic adults is approximately 13-15.8 to µU/ml (Goodner, Conway, & Werrbach, 1969; Mack, Skurnick, Sterling-Jean, Pedra-Nobre, & Bigg, 2004). Importantly, the study mentioned earlier by Koopman et al (2005), illustrates leucine's effect on insulin secretion, in humans, when combined with protein and carbohydrates. When leucine (.55 g/kg) was combined with protein hydrosylates (1.1 g/kg) plus carbohydrate (1.65 g/kg - 50% glucose and 50% maltodextrin) (CHO+PRO+Leu) it led to significantly higher insulin levels (~75µU/ml) as compared to the CHO+PRO (~65µU/ml) and CHO (~35µU/ml) trials (Koopman et al., 2005). It is also important to note that insulin levels increased in all groups during the first 15–30 min following the

initial beverage ingestion and the concentrations finally plateaued 3–4 hours into postexercise recovery (Koopman et al., 2005). Equally important, Karlsson et al. (2004) reported in their study, that in the two hour recovery period following exercise, the insulin concentration in the BCAA group was significantly increased as compared to the placebo group. In the recovery period, the highest level of insulin in the BCAA and placebo group was approximately 17 μU/ml and 13 μU/ml respectively (Karlsson et al., 2004). However, as the investigators point out, this increase is probably not physiologically relevant because the values still fell within normal variability (Karlsson et al., 2004).

Existing research leads us to believe that when protein synthesis is stimulated by essential amino acids, such as leucine, it is associated with an increase in the activation of mTOR, 4E-BP1, and $p70^{S6K}$ without requiring an increase of plasma insulin above 10μU/ml (Rennie et al., 2006). However, when plasma insulin is below 5μU/ml, essential amino acids seem to increase skeletal muscle protein synthesis through other mechanisms than mTOR (Rennie et al., 2006). Lastly, there is evidence to suggest that if insulin is increased to levels that are observed in a postprandial state, it seems to enhance signaling via the Akt-mTOR pathway, but does not seem to lead to further increases in protein synthesis (Rennie et al., 2006).

As reported by Rennie, Bohe, Smith, Wackerhage, and Greenhaff (2006) in their extensive review article, the relationship between amino acid and insulin availability and the ultimate stimulation of protein synthesis still remains unclear. They further conclude that it is still possible that amino acids may exert their effect through signaling pathways other than mTOR, possibly through eIF2B or some other unrecognized pathway (Rennie

et al., 2006). However, more research in this area needs to be performed to elucidate these theories. Ultimately, the literature suggests that leucine stimulates translation initiation at least in part through the protein kinase mTOR pathway, and that both leucine and insulin signaling most likely come together in this pathway to induce a maximal response (J. C. Anthony et al., 2001; J. C. Anthony, Lang et al., 2002; Rennie et al., 2006).

Summary

As discussed above, the BCAAs comprise approximately of 5-10% of the total protein content. While performing exercise, the oxidation rate of the three BCAAs increases. Some research has shown that BCAAs and leucine alone may improve the rate of protein breakdown, subjective rating of perceived exertions, and exercise performance in both aerobic and anaerobic exercise. However, the research findings from these studies are equivocal. It appears that out of the BCAAs, leucine is the most studied due to its role in protein synthesis. Research suggests that translation initiation, which is regulated by eukaryotic initiation factors, is the rate limiting step in protein synthesis. Furthermore, the Akt/mTOR cell signaling pathway appears to influence translation initiation. Hormones such as insulin, IGF-1, GH, as well as the branched-chain amino acid, leucine, appear to phosphorylate/activate various proteins in the Akt/mTOR pathway and ultimately enhance protein synthesis. Specifically, several studies utilizing animal models have demonstrated that leucine appears to enhance protein synthesis through the phosphorylation of 4E-BP1, $p70^{S6K}$, and rpS6. Insulin has also been shown to activate upstream proteins of the Akt/mTOR pathway including PI-3 kinase and Akt. Human studies have also demonstrated that various proteins in the Akt/mTOR pathway

may be phosphorylated through insulin and other growth factors, as well as leucine alone. Currently, it is not clear if resistance training alone facilitates increases in the Akt/mTOR. At this time it is thought that leucine and insulin signaling most likely come together in the Akt/mTOR pathway to induce a maximal response.

CHAPTER THREE

Methods

Participants

Thirty apparently healthy and physically active males between the ages 19-32 participated in the study. Physically active, for purposes of our study, was defined as not consistently participating in a lower body resistance training program in the last year. Furthermore, the participants could not have performed lower body resistance exercise more than twice in the last thirty days and no more than once in the two weeks prior to the experimental testing session. However, the participants could be active in recreational sports, or moderate physical activity. 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 and protein supplements) one month prior to the study were allowed to participate.

Familiarization and Baseline Strength Testing Session

During the familiarization session, participants completed a medical history questionnaire and personal information form (Appendix A and B) to help determine that they met the eligibility criteria. The participants were familiarized to the study protocol through both verbal and written explanation outlining the study design. Individuals that participated in the study read and signed university-approved documents granted by the Institutional Review Board for Human Subjects of Baylor University (Appendix C).

Participants were then subjected to an initial strength test using the trial-and-error method which assessed their lower extremity one repetition maximum (1-RM) on the isotonic hip/leg sled (Nebula Fitness, Inc., Versailles, OH) and isotonic knee extension (Body Masters, Inc., Rayne, LA) exercises. Once the 1-RM leg press and 1-RM knee extension was determined, the participants performed the actual resistance exercise protocol that they would participate in for the actual study. This was to ensure they could safely and effectively perform the resistance exercise regimen. The resistance training protocol, which is discussed below in greater detail, consisted of four sets of both leg press and leg extension at 80% 1RM to failure, with 150 seconds of rest between each set and exercise. At the conclusion of the entry/familiarization and baseline strength testing session, each participant was scheduled for an appointment time approximately one week later to commence the study.

Resistance Exercise Session Protocol

Approximately 1 week after the subject's familiarization session, the subjects performed an acute bout of lower body resistance exercise. Upon reporting to the lab, both a fasted baseline/pre-exercise muscle biopsy and blood sample were obtained. After this, each participant performed a warm up on a stationary bicycle and then performed two sets of leg press at 50% of 1RM. Following this, each participant completed four sets of both leg press and leg extension at 80% 1RM to failure (approximately 8-12 repetitions). Rest periods of 150 seconds between both sets and exercises and were given. This constituted approximately a 1:5 work-to-rest ratio (Baechle, 2000). Total exercise time took approximately 30 minutes. Participants refrained from lower-body resistance exercise for 72 hours prior to baseline testing, and were fasted for

approximately 8-10 hours upon starting the testing session. Furthermore they continued fasting (excluding the administered supplement) for the duration of the study session.

Supplementation

After baseline testing each subject was matched according to muscle strength and body mass and then randomized into one of three supplement groups (a placebo group, a BCAA group, and a leucine group) using a random number generator. Specifically, the placebo supplement was composed of 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 [approximately 12 grams (6 grams of leucine, 3 grams of isoleucine, 3 grams of valine). The leucine supplement was given at a dosage of 60 mg/kg of bodyweight (approximately 6 grams). The leucine supplement contained the same amount of leucine as the BCAA supplement and only differed from the BCAA supplement by the absence of isoleucine and valine. Thus, by our study design we were effectively able to assess the effect of leucine alone on our criterion variables.

Each supplement were 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 Biopsy Sampling

Before administering the first supplement or initiating the resistance exercise session, the participants underwent an initial (baseline) muscle biopsy. After shaving their right leg near the midpoint of the lateral thigh, rubbing alcohol was applied to sterilize the injection site. Initially, the participants were given a 1.5 ml injection of a local anesthetic comprised of 1% xylocaine while in a supine position. Approximately 10 minutes following a local anesthetic injection, betadine antiseptic was applied to the leg to sterilize the incision site. Using a 5 mm Bergstorm needle, percutaneous muscle biopsies (~ 40 mg) were 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 last three biopsies, the biopsy needle was introduced in approximately the same location as the initial biopsy. After removing the muscle sample, it was placed in a cryogenic storage tube and immediately stored at -80°C for later analysis. Muscle samples were also obtained at 30 minutes post-exercise, and at two and six hours following the exercise bout. Following each muscle biopsy, the incision was cleaned and closed via a sterile butterfly bandage. Equally important, an additional sterile bandage was placed over the wound to ensure that it was covered and ultimately protected. At the conclusion of the testing session, every participant was given extra butterfly and regular bandages. Furthermore, verbal instructions on proper wound care, as well as, a printed wound care instruction sheet (will be in appendix) were given to each participant at the conclusion of the testing session. The study participants were specifically instructed to leave their initial bandage and butterfly on for at least 24 hours and not to participate in strenuous lower body exercise for 48 hours following the testing

session. The participants were instructed to call the principle investigators if they had any questions or concerns regarding their biopsy incision.

Venous Blood Sampling

Before administering the first supplement or initiating the resistance exercise session, the participants underwent an initial (baseline) blood draw. Venous blood samples were obtained from the antecubital vein into 10 ml collection tubes using a standard Vacutainer™ apparatus. Blood was obtained at baseline, 30 minutes after ingesting the supplement/immediately prior to exercise, immediately after exercise, 30 minutes after exercise, and 2, and 6 hours following exercise. A standard bandage was placed over the site of the needle insertion following each blood draw. Blood samples were allowed to stand at room temperature for 10 min and then centrifuged at 2,400 rpm for 15 minutes. The serum was removed and frozen at -80°C for later analysis.

Serum Analyses

Serum glucose, insulin, free/bioactive insulin-like growth factor -1 (IGF-1), and free/bioactive growth hormone (GH) were all assessed in order to evaluate their effects on various proteins in the Akt-mTOR pathway. Insulin, IGF-1, and GH are all growth factors and may possibly influence the phosphorylation states of various enzymes in the Akt-mTOR pathway. Insulin, IGF-1, and GH were analyzed using enzyme-linked immunoabsorbent assays (ELISA). Glucose was assesed using a clinical chemistry analyzer. All serum analytes that were measured via ELSIA methods employed a Jitterbug orbital microplate shaker (Boekel Scientific, Philadelphia, PA) for the incubation periods and a MultiWash Advantage automated microplate washer (Tri

Continent Scientific, Grass Valley, CA) during the plate washing steps. All enzyme-linked immunoabsorbent assays were quantified using the Wallac Victor^{2} ™1420 multilabel microplate reader (Turku, Finland). The plate reader measured the optical density of every standard, control, and unknown sample. All standards, controls, and unknowns were assayed in duplicate and were read at 450 nm wavelength against a known standard curve. After the absorbances were measured, Mikrowin 2000 (Mikrotek Laborsysteme, Germany) data-reduction software was utilized to quantify the concentration of the specific analyte.

Glucose

Glucose was analyzed using the Dade Behring Dimensional Rxl Ananlyzer (Newark, DE). This clinical chemistry analyzer was calibrated daily using liquid assay multiqual (BIO-RAD, Hercules, CA) The protocol for this analyzer is as follows. Approximately, 70 µl of serum from a BD Vacutainer® serum seperator collection tube (SST™) was first added to a sample cup to prepare for the subsequent anaylses. This particular anaylzer (Dade Behring) uses a sonicating probe to mix the sample. To analyze glucose, via this particular analyzer, an adaptation of the hexokinase-glucose-phosphate dehydrogenase method utilzed by Kunst et al. (Kunst, A, Draeger, B, Ziegenhorn, J, UV-methods with hexokinase and glucose-6-phosphate dehydrogenase, Methods of Enzymatic Analysis, Vol VI, Bergmeyer, HU, Ed, Verlag Chemie, Deerfield, FL 1983 pp 163-172) was employed. Once daily, two levels of a quality control of known concentrations were assesed. According to the manufacture, the coeficcient of variation for within-run and between-day is 3% and 8% respectively.

Insulin

Serum samples were analyzed for insulin using an ELISA [Alpha Diagnostic International (Cat. # 0030; San Antonio, TX)]. The sensitivity for this particular ELISA assay was reported to be 1.5 µIU/ml. To begin the assay, 25 µl of standards and the unknown samples were pipetted in duplicate into designated wells on a microplate which contained an immobilized monoclonal anti-insulin antibody. The plate was subsequently covered and allowed to incubate in the microplate shaker for 30 minutes. Following the incubation period, the microplate was washed five times with approximately 300 µl of wash buffer (phosphate buffered saline and Tween-20). After the plate was washed, 200 µl of horseradish peroxidase substrate mix (HRP, H_2O_2 and TMB) was added to each well. The microplate incubated a second time for 15 minutes on the microplate shaker. Next, 50 µl of a stop solution (0.2 M sulfuric acid) was supplied to each individual well. The subsequent absorbances, which are directly proportional to the concentration of analyte in the sample, were measured at a wavelength of 450 nm. A set of six insulin standards which ranged from 0 to 200 µIU/ml were utilized to construct standard curve by plotting the net absorbance values of the standards against their respective protein concentrations. By applying a valid fitting cubic spline curve, the insulin concentrations in the unknowns were appropriately calculated. Intra-assay coefficient of variation was determined for every insulin ELISA kit that was analyzed. The percent coefficients of variation for the five kits were 5.2, 9.5, 8.4, 6.1, and 7.3 respectively. Therefore, the overall intra-assay percent coefficient of variation for the combined kits was 7.3%.

Free Insulin-Like Growth Factor-1(IGF-1)

Serum samples were analyzed for free/bioactive IGF-1 using an ELISA [Active® Bioactive ELISA by Diagnostic Systems Laboratories Inc. (DSL-10-9400; Webster, TX)]. This particular free IGF-1 ELISA is considered highly sensitive and was reported to be 0.06 ng/ml, and does not cross-react with albumins or GH binding proteins. This particular IGF-1 assay is an enzymatically amplified "two-step" sandwich-type immunoassay. The microplate contained wells that were previously coated with an immobilized anti-IGF-1 antibody. To begin the assay, 50 µl of standards, both a high and low control, and the unknown samples were pipetted in duplicate into designated wells on a microplate. Next, 20 µl of the free IGF-1 sample buffer (a protein-based (BSA) buffer with a non-mercury preservative) was also added to each well. The plate was subsequently covered and allowed to incubate in the microplate shaker for one hour. Following the incubation period, the microplate was washed five times with approximately 300 µl of wash buffer (buffered saline with a non-ionic detergent). After the plate was washed, 100 µl of the antibody-enzyme conjugate solution (free IGF-1 antibody conjugated to horseradish peroxidase in a protein based (BSA) buffer with a non-mercury preservative) was pipetted into each well. The microplate was incubated a second time for 30 minutes on the microplate shaker and was subsequently washed five times. Then, 100 µl of the tetramethylbenzidine (TMB) chromagen solution was added to each well and was followed by a ten minute incubation period. Finally, 100 µl of the 0.2M sulfuric acid stopping solution was added to every well. The subsequent absorbances, which are directly proportional to the concentration of analyte in the sample, were measured at a wavelength of 450 nm. A set of six free IGF-1 standards

which ranged from 0.05 to 9.0 ng/ml were utilized to construct standard curve by plotting the net absorbance values of the standards against their respective protein concentrations. By applying a valid fitting cubic spline curve, the free IGF-1 concentrations in the unknowns were appropriately calculated. Intra-assay coefficient of variation was determined for every IGF-1 ELISA kit that was analyzed. The percent coefficients of variation for the five kits were 5.1, 5.5, 5.5, 3.5 and 5.2 respectively. Therefore, the overall intra-assay percent coefficient of variation for the combined kits was 4.9%.

Growth Hormone (GH)

Serum samples were analyzed for free/bioactive GH using an ELISA [Active® Bioactive ELISA by Diagnostic Systems Laboratories Inc. (DSL-10-11100; Webster, TX)]. The sensitivity for this particular ELISA assay was reported to be 0.06 ng/ml, and does not cross-react with albumins or GH binding proteins. This particular bioactive GH assay is reported to be an enzymatically amplified "two-step" sandwich-type immunofunctional assay that employs a distinctive anti-hGH monoclonal antibody and biotinylated recombinant hGH binding protein that will bind to hGH receptor binding-site 2 and binding-site 1, respectively, on biologically active hGH molecules. To begin the assay, 50 µl of standards, both a high and low control, and the unknown samples were pipetted in duplicate into designated wells on a microplate. Next, 50 µl of assay buffer (a protein based (BSA) buffer with a non-mercury preservative) was also added to each well. The plate was subsequently covered and allowed to incubate in the microplate shaker for three hours. Following the incubation period, the microplate was washed five times with approximately 300 µl of wash buffer (buffered saline with a non-ionic detergent). After the plate was washed, 100 µl of the bioactive GH-biotin conjugate

solution was pipetted into each well. The microplate was then covered and incubated a second time overnight at 2-8 °C. The next morning, the microplate was washed five times. Then, 100 µl of the streptavidin-enzyme conjugate solution (streptavidin conjugated to the enzyme horseradish peroxidase in a protein-based buffer with a non-mercury preservative) was added to each well and was followed by a 30 minute incubation period. Finally, 100 µl of the 0.2M sulfuric acid stopping solution was added to every well. The microplate was again washed five times with wash solution. Next, 100 µl of the TMB chromagen solution was added to each well and was followed by a ten minute incubation period. Finally, 100 µl of the 0.2M sulfuric acid stopping solution was added to every well. The subsequent absorbances, which are directly proportional to the concentration of bioactive GH in the sample, were measured at a wavelength of 450nm. A set of six free IGF-1 standards which ranged from 0 to 25 ng/ml were utilized to construct standard curve by plotting the net absorbance values of the standards against their respective protein concentrations. By applying a valid fitting cubic spline curve, the bioactive GH concentrations in the unknowns were appropriately calculated. Intra-assay coefficient of variation was determined for every GH ELISA kit that was analyzed. The percent coefficients of variation for the five kits were 4.9, 2.2, 1.2, 2.3, and 4.1 respectively. Therefore, the overall intra-assay percent coefficient of variation for the combined kits was 2.9%.

Skeletal Muscle Cellular Extraction Protocol

Approximately one-half (20 mg) of each muscle sample was removed, weighed, and subsequently placed in an autoclaved microcentrifuge tube. The skeletal muscle samples were homogenized using a commercial cell extraction buffer (Cat. FNN0011,

Biosource International, Camarillo, CA) and a tissue homogenizer and a hand held Kontes tissue homogenizer. The cell extraction buffer was supplemented with 1mM phenylmethanesulphonylfluoride (PMSF) and a protease inhibitor cocktail (Cat. P2714, Sigma Chemical Company, St. Louis, MO). Ultimately, a 0.3 M PMSF stock solution in dimethyl sulfoxide (DMSO) was produced. Next, 17 µL of this stock solution was added to 5mL of cell extraction buffer to create a final concentration of 1 mM PMSF. Based on the manufacture, the protease inhibitor cocktail that was utilized was comprised of a combination of water- soluble protease inhibitors with broad specificity for the inhibition of serine, cysteine, and metallo-proteases. Per the manufacture's recommendations, 500 µL of protease inhibitor cocktail was added to 5 mL of cell extraction buffer. As a result of the unstable nature of PMSF, this solution was both created and added to the cell extraction buffer immediately before the process of muscle homogenization. The cell extraction buffer was added to each frozen muscle sample at a ratio of 20.8 mL per gram of muscle tissue. To create the final muscle homogenate, the cell extraction buffer was introduced to the muscle sample and the tissue was homogenized via a hand held Kontes pestle in an autoclaved microcentrifuge tube.

Skeletal Muscle Analyses

The skeletal muscle protein levels of phosphorylated IRS-1, AKT, mTOR, p70-S6K, and 4EBP-1 were assessed using phosphoELISA kits (Biosource International, Camarillo, CA) that are specifically designed to quantify the phosphorylation/activation states of various proteins involved in Akt-mTOR signal transduction pathway. However, it should be noted that the ELISA used to analyze the phosphorylated state of mTOR were created in our lab using commercially-available polyclonal raised against

phosphorylated mTOR (Biosource International, Camarillo, CA). These five dependent variables were analyzed using a phosphoELISA that is specific for to the phosphorylation state of each dependent variable. The phosphorylated amount of IRS-1, Akt, mTOR, $p70^{S6}$ kinase, and 4EBP-1 were quantified in an assay that is specific to proteins only phosphorylated at a specific amino acid residue(s). The phosphoELISA kits employed antibodies that can identify a specific epitope that is only present on a protein when it is phosphorylated at a particular amino acid position. The relative activity of IRS-1, Akt, mTOR, $p70^{S6}$ kinase, and 4EBP-was then assessed by dividing absolute activity by muscle wet-weight. The specific protocols for quantifying each dependent variable via phosphoELISA will discussed in detail in their respective sections below.

PhosphoELISA Analysis of IRS-1

Muscle homogenate samples were analyzed for phosphorylated IRS-1 [pS312] using a phosphoELISA kit [Biosource International, Camarillo, CA (Biosource-KH00521]. This sensitivity of this particular IRS-1 phosphoELISA was reported to be <1 Units/ml. This particular phosphoELISA kit is a solid phase sandwich ELISA. A monoclonal antibody specific for IRS-1, regardless of the phosphorylation state, has previously been coated on the wells of the microplate. Before adding the standards and unknowns, appropriate steps needed to be taken. Specifically, the frozen muscle homogenate samples were thawed and subsequently diluted 1:10 in standard diluent buffer based upon the manufacture's recommendations. Additionally, the standards were also reconstituted in standard diluent buffer (Phosphate buffer with animal protein combined with sodium azide and a red dye) before they could be loaded into the microtiter plate. To begin the procedure, 100 µL of standards and the diluted samples

were pipetted into the appropriate microplate wells. The plate was covered, allowed to incubate on plate shaker for two hours, and then underwent a wash cycle. The wash cycle consisted of four 30 second soak cycles using approximately 300 μL of wash buffer (phosphate buffer plus EDTA) during each soak cycle. Following the wash cycle, 100 μL of an anti-IRS-1 [pS312] detection antibody was applied to every well except the chromogen blank. Again, the plate was covered, allowed to incubate for one hour at room temperature and then washed in the same fashion as before. An anti-rabbit IgG-HRP working solution was created by adding 120 μL of the supplied anti-rabbit IgG-HRP (100x concentrate) to 12 mL of HRP diluent. This anti-rabbit working solution was added in the amount of 100 μL to each well except the chromogen blank. Similar to before the plate was covered, incubated for 30 minutes at room temperature, and then washed as previously described. Next, 100 μL of the stabilized TMB chromogen was pipetted into every well. The solution in the wells turned various intensities of blue due to the activity of the HRP. The intensity of the colored product is directly proportional to the concentration of the IRS-1 [pS312] present in the muscle homogenate. The plate was covered, placed in the dark, and incubated for 30 minutes at room temperature. Following the incubation, 100 μL of stop solution (1 N HCL) was added to every well. The solution in the wells turned from a blue to a yellow color. The subsequent absorbances, which are directly proportional to the concentration of phosphorylated IRS-1 [pS312] in the sample, were measured on a Wallac 1420 Victor 2 plate reader at a wavelength of 450 nm. A set of eight IRS-1 [pS312] standards which ranged from 0 to 100 Units/ml were utilized to construct standard curve by plotting the net absorbance values of the standards against their respective protein concentrations. As suggested by

the manufacturer, a valid fitting four parameter curve, using Mikrowin data-reduction software, was utilized to best fit the data points. The concentrations of phosphorylated IRS-1 [pS312] in the unknowns were appropriately calculated. The lower limit, turning slope, turning point, upper limit, and fitting error values for the three respective four-parameter curve fit statistics were (0.14641, 1.0976, 2661.3, 69.602, 0.012379), (0.086729, 1.0853, 3413, 79.47, 0.001538), and (0.17041, 1.3784, 273.72, 7.425, 0.017509) for the muscle IRS-1 [pS312] standard curve calculations. Intra-Assay coefficient of variation was determined for every IRS-1 ELISA kit that was analyzed. The percent coefficients of variation for the three kits were 10.9, 12.9, and 12.0 respectively. Therefore, the overall intra-assay percent coefficient of variation for the combined kits was 11.9%.

PhosphoELISA Analysis of AKT

Muscle homogenate samples were analyzed for phosphorylated AKT [pS473] using a phosphoELISA kit [Biosource International, Camarillo, CA, (Biosource- KH00111)]. This sensitivity of this particular AKT phosphoELISA was reported to be <0.8 Units/ml. This particular phosphoELISA kit is a solid phase sandwich ELISA. A monoclonal antibody specific for AKT, regardless of the phosphorylation state, has previously been coated on the wells of the microplate. Before adding the standards and unknowns, appropriate steps needed to be taken. Specifically, the frozen muscle homogenate samples were thawed and subsequently diluted 1:10 in standard diluent buffer based upon the manufacture's recommendations. Additionally, the standards were also reconstituted in standard diluent buffer (phosphate buffer with animal protein combined with sodium azide) before they could be loaded into the microtiter plate. To begin the procedure, 100

µL of standards and the diluted samples were pipetted into the appropriate microplate wells. The plate was covered, allowed to incubate on plate shaker for two hours, and then underwent a wash cycle. The wash cycle consisted of four 30 second soak cycles using approximately 300 µL of wash buffer (phosphate buffer plus EDTA) during each soak cycle. Following the wash cycle, 100 µL of an anti-AKT [pS473] detection antibody was applied to every well except the chromogen blank. Again, the plate was covered, allowed to incubate for one hour at room temperature and then washed in the same fashion as before. An anti-rabbit IgG-HRP working solution was created by adding 120 µL of the supplied anti-rabbit IgG-HRP (100x concentrate) to 12 mL of HRP diluent. This anti-rabbit working solution was added in the amount of 100 µL to each well except the chromogen blank. Similar to before the plate was covered, incubated for 30 minutes at room temperature, and then washed as previously described. Next, 100 µL of the stabilized TMB chromogen was pipetted into every well. The solution in the wells turned various intensities of blue due to the activity of the HRP. The intensity of the colored product is directly proportional to the concentration of the phosphorylated AKT [pS473] present in the muscle homogenate. The plate was covered, placed in the dark, and incubated for 30 minutes at room temperature. Following the incubation, 100 µL of stop solution (1 N HCL) was added to every well. The solution in the wells turned from a blue to a yellow color. The subsequent absorbances, which are directly proportional to the concentration of phosphorylated AKT [pS473] in the sample, were measured on a Wallac 1420 Victor 2 plate reader at a wavelength of 450 nm. A set of eight AKT [pS473] standards which ranged from 0 to 100 Units/ml were utilized to construct standard curve by plotting the net absorbance values of the standards against their

respective protein concentrations. As suggested by the manufacturer, a valid fitting four parameter curve, using Mikrowin data-reduction software, was utilized to best fit the data points. The concentrations of phosphorylated AKT [pS473] in the unknowns were appropriately calculated. The lower limit, turning slope, turning point, upper limit, and fitting error values for the three respective four-parameter curve fit statistics were (0.078724, 1.0691, 116.98, 2.9986, 0.012379), (0.14274, 1.4657, 44.099, 2.6064, 0.0026899), and (0.088387, 1.1725, 64.179, 2.2893, 0.0040962) for the muscle AKT [pS473] standard curve calculations. Intra-Assay coefficient of variation was determined for every Akt ELISA kit that was analyzed. The percent coefficients of variation for the three kits were 4.6, 3.9, and 7.9 respectively. Therefore, the overall intra-assay percent coefficient of variation for the combined kits was 5.5%.

PhosphoELISA Analysis of 4E-BP1

Muscle homogenate samples were analyzed for phosphorylated 4E-BP1 [pT46] using a phosphoELISA kit [Biosource International, Camarillo, CA, (Biosource-KH00691)]. This sensitivity of this particular AKT phosphoELISA was reported to be <0.8 Units/ml. This particular phosphoELISA kit is a solid phase sandwich ELISA. A monoclonal antibody specific for 4E-BP1, regardless of the phosphorylation state, has previously been coated on the wells of the microplate. Before adding the standards and unknowns, appropriate steps needed to be taken. Specifically, the frozen muscle homogenate samples were thawed and subsequently diluted 1:10 in standard diluent buffer based upon the manufacture's recommendations. Additionally, the standards were also reconstituted in standard diluent buffer (Phosphate buffer with animal protein combined with sodium azide and a red dye) before they could be loaded into the

microtiter plate. To begin the procedure, 100 μL of standards and the diluted samples were pipetted into the appropriate microplate wells. The plate was covered, allowed to incubate on plate shaker for two hours, and then underwent a wash cycle. The wash cycle consisted of four 30 second soak cycles using approximately 300 μL of wash buffer (phosphate buffer plus EDTA) during each soak cycle. Following the wash cycle, 100 μL of an anti-4E-BP1 [pT46] detection antibody was applied to every well except the chromogen blank. Again, the plate was covered, allowed to incubate for one hour at room temperature and then washed in the same fashion as before. An anti-rabbit IgG-HRP working solution was created by adding 120 μL of the supplied anti-rabbit IgG-HRP (100x concentrate) to 12 mL of HRP diluent. This anti-rabbit working solution was added in the amount of 100 μL to each well except the chromogen blank. Similar to before the plate was covered, incubated for 30 minutes at room temperature, and then washed as previously described. Next, 100 μL of the stabilized TMB chromogen was pipetted into every well. The solution in the wells turned various intensities of blue due to the activity of the HRP. The intensity of the colored product is directly proportional to the concentration of the phosphorylated 4E-BP1 [pT46] present in the muscle homogenate. The plate was covered, placed in the dark, and incubated for 30 minutes at room temperature. Following the incubation, 100 μL of stop solution (1 N HCL) was added to every well. The solution in the wells turned from a blue to a yellow color. The subsequent absorbances, which are directly proportional to the concentration of phosphorylated 4E-BP1 [pT46] in the sample, were measured on a Wallac 1420 Victor 2 plate reader at a wavelength of 450 nm. A set of eight 4E-BP1 [pT46] standards which ranged from 0 to 100 Units/ml were utilized to construct standard curve by plotting the

net absorbance values of the standards against their respective protein concentrations. As suggested by the manufacturer, a valid fitting four parameter curve, using Mikrowin data-reduction software, was utilized to best fit the data points. The concentrations of phosphorylated 4E-BP1 [pT46] in the unknowns were appropriately calculated. The lower limit, turning slope, turning point, upper limit, and fitting error values for the three respective four-parameter curve fit statistics were (0.21453, 1.7127, 36.654, 4.0689, 0.057065), (0.14269, 1.319, 49.038, 4.3205, 0.032502), and (0.19475, 1.2176, 256.65, 22.867, 0.0014661) for the muscle 4E-BP1 [pT46] standard curve calculations. Intra-Assay coefficient of variation was determined for every 4E-BP1 ELISA kit that was analyzed. The percent coefficients of variation for the three kits were 3.5, 4.8, and 5.2 respectively. Therefore, the overall intra-assay percent coefficient of variation for the combined kits was 4.5%.

PhosphoELISA Analysis of P70-S6K

Muscle homogenate samples were analyzed for phosphorylated p70-S6K [pT389] using a phosphoELISA kit [Biosource International, Camarillo, CA, (Biosource-KH00581]. This sensitivity of this particular AKT phosphoELISA was reported to be <0.71 Units/ml. This particular phosphoELISA kit is a solid phase sandwich ELISA. A monoclonal antibody specific for p70-S6K, regardless of the phosphorylation state, has previously been coated on the wells of the microplate. Before adding the standards and unknowns, appropriate steps needed to be taken. Specifically, the frozen muscle homogenate samples were thawed and pretreated. Specifically, in regards to the sample pretreatment, each sample was incubated on ice with an equal volume of sample treatment buffer (phosphate buffer) for 20 minutes. Following this pretreatment step, this

newly formed mixture was diluted 5-fold in standard diluent buffer (phosphate buffer with animal protein combined with sodium azide and a red dye). Additionally, the standards were also reconstituted in standard diluent buffer before they could be loaded into the microtiter plate. To begin the procedure, 50 µL of the standards and the pretreated diluted samples were pipetted into the appropriate microplate wells. Immediately after, 50 µL of an anti-p70-S6K [pT389] detection antibody was applied to every well except the chromogen blank. The plate was covered, allowed to incubate on plate shaker for three hours, and then underwent a wash cycle. The wash cycle consisted of four 30 second soak cycles using approximately 300 µL of wash buffer (phosphate buffer plus EDTA) during each soak cycle. Again, the plate was covered, allowed to incubate for one hour at room temperature and then washed in the same fashion as before. An anti-rabbit IgG-HRP working solution was created by adding 120 µL of the supplied anti-rabbit IgG-HRP (100x concentrate) to 12 mL of HRP diluent. This anti-rabbit working solution was added in the amount of 100 µL to each well except the chromogen blank. Similar to before the plate was covered, incubated for 30 minutes at room temperature, and then washed as previously described. Next, 100 µL of the stabilized TMB chromogen was pipetted into every well. The solution in the wells turned various intensities of blue due to the activity of the HRP. The intensity of the colored product is directly proportional to the concentration of the phosphorylated p70-S6K [pT389] present in the muscle homogenate. The plate was covered, placed in the dark, and incubated for 30 minutes at room temperature. Following the incubation, 100 µL of stop solution (1 N HCL) was added to every well. The solution in the wells turned from a blue to a yellow color. The subsequent absorbances, which are directly proportional to the concentration

of phosphorylated p70-S6K [pT389] in the sample, were measured on a Wallac 1420 Victor 2 plate reader at a wavelength of 450 nm. A set of eight p70-S6K [pT389] standards which ranged from 0 to 100 Units/ml were utilized to construct standard curve by plotting the net absorbance values of the standards against their respective protein concentrations. As suggested by the manufacturer, a valid fitting four parameter curve, using Mikrowin data-reduction software, was utilized to best fit the data points. The concentrations of phosphorylated p70-S6K [pT389] in the unknowns were appropriately calculated. The lower limit, turning slope, turning point, upper limit, and fitting error values for the three respective four-parameter curve fit statistics were (0.35754, 1.76, 80.894, 3.3707, 0.00076985), (0.4408, 1.7146, 57.897, 3.2928, 0.000089128), and (0.32731, 2.3501, 33.107, 2.0918, 0.0017999) for the muscle p70-S6K [pT389] standard curve calculations. Intra-Assay coefficient of variation was determined for every P70-S6K ELISA kit that was analyzed. The percent coefficients of variation for the three kits were 9.2, 10.4, and 9.9 respectively. Therefore, the overall intra-assay percent coefficient of variation for the combined kits was 9.8%.

PhosphoELISA Analysis of mTOR

The muscle protein expression of phosphorylated mTOR was assessed through the use of an ELISA. Initially, a polyclonal phosphospecific antibody for mTOR [pS^{2448}] was purchased from Biosource International, Camarillo, CA. Initially, 4 μg/mL of primary antibody diluted in coating buffer (1.24 g sodium carbonate in 100 mL of deionized water, pH 9.6) was added to the entire plate. The plates were then covered and allowed to incubate overnight at room temperature. The next day, the wells were washed 4 times with approximately 300 μL of wash buffer (wash solution contained 1 liter of

10X phosphate buffered saline with 50 µL of Tween-20). Next, 200 µL of a blocking buffer (500 mL solution of blocking buffer contained 50 ml 10X phosphate buffered saline, 5 g BSA, and deionized water) was added to every well. The blocking buffer was added to prevent the non-specific binding of non-relevant proteins to the bottom of each well. Again, the plate was covered, allowed to incubate for one hour at room temperature and the washed 4 times. After, the wash step, 50 µL of the samples were added to each well and were allowed to incubate at room temperature for one hour. This was followed by another wash cycle. Next, 120 µL of a secondary antibody (IgG conjugated to HRP) diluted to 10 µg/ml was added to 12 mL of dilution buffer (500 mL of dilution buffer contained 50 mL of 10X phosphate buffered saline, 250 µL of Tween-20, and .5 g BSA). Then, 100 µL of the IgG-HRP conjugate was added to each well. The plate was then incubated for one hour followed by another wash cycle was performed. After plate washing was finished, 100 µL stabilized TMB chromogen was added, which resulted in a blue color formation due to the reactivity of the HRP. The plates were then covered and placed in the dark for the last 30 minute incubation. The final step required the addition of 100 µL of a stop solution (1 N HCL) to every well. This resulted in a yellow color transformation. The subsequent absorbances, which are directly proportional to the concentration of phosphorylated mTOR [pS^{2448}] in the sample, were measured at a wavelength of 450nm. There were no standards used in this phosphoELISA and thus, no standard curve was created. Therefore, the absorbances relative to muscle weight were assessed and compared as delta changes. Samples for this particular assay were not run in duplicate. Therefore, coefficient of variation could not be assessed.

Dietary Records

The participants' diets were not standardized and participants were not asked to change their dietary habits during the course of the study. However, participants were required to keep dietary records for 48 hours prior to the resistance exercise testing session. These dietary records were evaluated with ESHA's Food Processor dietary assessment software program (Salem, OR) to determine the average daily macronutrient consumption of fat, carbohydrate, and protein in the diet prior to exercise.

Statistical Analyses

A one-way univariate analysis of variance (ANOVA) was utilized to compare the means of every baseline criterion variable in order to determine if significant differences were present between the three groups at the onset of the study. If a significant difference between the groups were discovered at baseline in any of the dependent variables, a Bonferroni pairwise comparison test was utilized to determine which groups were significantly different. Two sets of repeated –measures MANOVAs were analyzed (one for muscle and one for blood variables) based on dependent variables that were likely to be related to one another. In addition, the use of MANOVA analysis also prevents the chance of committing a Type I error that would result with the use of repeated univariate procedures. Therefore, statistical analyses were performed by utilizing [treatment groups (3) x time point (4)] and [treatment groups (3) x time point (6)] multivariate analysis of variance (MANOVA) mixed methods with repeated measures on the second factor to analyze the variables obtained from muscle and serum samples respectively. To evaluate the MANOVA analysis, the Wilks's Lambda statistic was utilized due to the fact it is one of the most powerful multivariate test statistics. A

test of the assumption of the sphericity was performed specifically using Mauchly's test of sphericity when examining the within-subjects effect of time. If the assumption of sphericity is not met for any of the dependent variables, the Huynh-Feldt correction factor was adopted throughout the analyses where appropriate. The Huynh-Feldt correction factor was utilized for the within-subjects factor in order to adjust the degrees of freedom to increase the critical f-value and thus to prevent a type I error. Following the initial MANOVA, separate ANOVAs for each criterion variable were utilized as follow-up tests. Significant differences in mean values for any main effects discovered in the individual ANOVAs were determined using a Bonferroni follow-up test to control for alpha inflation. Non-significant trends ($p \leq .10$) were reported as partial Eta squared to illustrate the effect size. Castevens et al. (Caststevens, Cohen, Newman, & Durmaine, 2006) state that the partial Eta squared statistic is indicative of the proportion of variance associated with a given variable. It was reported by O'Connor et al. (O'Conner et al., 2007), that a partial Eta squared of 0.51 is relatively strong, 0.24 is moderate and is 0.17 relatively weak. Significant interactions that were discovered in the ANOVA were determined using independent samples T-tests. All statistical procedures were performed using SPSS 15.0 software (Chicago, IL) and a probability level of ≤ 0.05 was adopted throughout to determine significance.

CHAPTER FOUR

Results

Participants

Thirty male participants volunteered for this study, and the demographic information is presented in Table 1. At baseline, there was no significant difference between groups in regards to age ($p = 0.068$), body mass ($p = 0.625$), or height ($p = 0.605$). All participants completed the required exercise protocol, blood draws, and skeletal muscle biopsies during the course of the study. Two participants had fasting and post-supplementation serum insulin values that were deemed as outliers (greater than 2.5 standard deviations above the mean). Therefore, the insulin values for those two participants were removed and subsequently replaced by the group mean as previously described (Kreider et al., 2003).

Table 1

Study Participant Demographics

Age	22.5 ± 3.4
Weight in kg	83.1 ± 10.4
Height in cm	178.4 ± 6.9

Note: Data are presented as means ± standard deviations

Initial Assessment for Baseline Differences of all Criterion Variables

At the onset of the data analysis, univariate ANOVAs were utilized to assess the differences of every criterion variable between the groups at baseline. Bonferroni multiple comparison tests were utilized to determine where the significance group differences occurred. There were no significant differences between any groups in regards to the demographic, dietary recall, resistance exercise, or blood baseline variables. Therefore, all analyses involving blood variables were analyzed via MANOVA using raw values. However, out of the five skeletal muscle variables, three were significantly different at baseline. Specifically, the significant differences at baseline were for IRS-1 ($F (2, 27) = 4.534$, $p = .020$, effect size = .251), Akt ($F (2, 27) = 5.269$, $p = .012$, effect size = .281), and mTOR ($F (2, 27) = 12.118$, $p = .001$, effect size = .473). While baseline discrepancies existed, no significant differences were found between the other two muscle variables, 4E-BP1 ($p = .086$) and p70-S6K ($p = .343$). Conversely, all analyses involving skeletal muscle variables were analyzed via MANOVA using delta values in order to correct for baseline differences.

Dietary Recall Analyses

Participants were instructed not change their current dietary intake before initiating this study. However, all participants were asked to record all food and beverage intake for the two days hours prior to the exercise testing session. Univariate ANOVAs were utilized to analyze all of the dietary variables. Table 2 illustrates that there were no significant differences in 48-hour dietary recalls were observed between groups for total daily caloric intake or the daily macronutrient intake of protein, carbohydrate, and fat.

Table 2

48 hour Dietary Recall

Variable	BCAA Group	Leucine Group	Placebo Group	p-value
Carbohydrate (g/day)	334 ± 116	314 ± 124	256 ± 75	p = 0.259
Fat (g/day)	116 ± 49	136 ± 52	95 ± 38	p = 0.158
Protein (g/day)	129 ± 57	152 ± 77	108 ± 39	p = 0.281
Total calories (kcal/day)	2883 ± 955	3121 ± 990	2304 ± 674	p = 0.124

Note. Data are presented as means ± standard deviations

Resistance Exercise Sessions

For the resistance exercise session, participants were required to perform four sets of both leg press and leg extension at 80% 1RM. Univariate ANOVAs were utilized to analyze all of the resistance exercise variables. Table 3 illustrates the results of the resistance exercise session for all participants in which there were no significant differences between the groups for leg press 1-RM, knee extension 1-RM, and total work load. Furthermore, there was no significant difference between groups with respect to relative intensity (percentage of 1-RM) used for repetitions during the leg press and knee extension.

Table 3

Resistance Exercise Variables

Variable	BCAA Group	Leucine Group	Placebo Group	p-value
1-RM Leg Press (kg)	918 ± 134	972 ± 183	924 ± 238	p = 0.786
1-RM Knee Extension (kg)	210 ± 15	222 ± 43	224 ± 60	p = 0.883
%1-RM Leg Press	80.0 ± 0	79.7 ± .89	79.1 ± 2.7	p = 0.501
%1-RM Knee Extension	80.0 ± 0	76 ± 6.6	73.9 ± 10	p = 0.158
Total Lifting Volume (kg)	18733 ± 4610	19714 ± 3540	18321 ± 6260	p = 0.811

Note. Data are presented as means ± standard deviations

Blood Variables

A two-way [treatment groups (3) x time point (6)] MANOVA mixed methods with repeated measures on the second factor was conducted to evaluate the effects of three different supplements (BCAAs, leucine alone, or placebo) on various blood markers that may impact the Akt-mTOR pathway. The criterion variables that were analyzed in this multivariate analysis were: blood glucose, insulin, IGF-1, and GH.

The within-subjects factor was time with six levels (baseline = PRE, immediately before exercise = IMMPRE, immediately post exercise = POST, 30 minutes post-exercise = 30MINPOST, and 2 hours post-exercise = 2HRPOST, 6 hours post-exercise = 6HRPOST). The between-subjects factor was group with three levels (BCAA, leucine, and placebo). All blood variables were analyzed using the raw data, not delta changes.

The time main effect, group main effect, and group x time interaction effect were analyzed using the multivariate criterion of Wilks' Lambda.

There was no significant multivariate main effect for group (Wilks' Lambda = .782, F (8, 48) = .787, p = .617, effect size = .116). However, the multivariate main effect for time was significant (Wilks' Lambda = .075, F (20, 8) = 4.901, p = .013, effect size = .925). Furthermore, there was no significant multivariate interaction for group x time (Wilks' Lambda = .065, F (40, 16) = 1.174, p = .376, effect size = .746). As a result of the aforementioned multivariate results, univariate ANOVAs were run with subsequent Bonferroni post-hoc tests to determine the main effects for time and group for glucose, insulin, IGF-1, and GH. The assumption of sphericity was not met for any of the blood variables [insulin (p = .001), GH (p < .001), IGF-1 (p < .001), and glucose (p < .001)].

Serum Glucose

No significant main effect for group was observed in regards to glucose (F (2, 27) = 2.011, p = .153, effect size = .130). There was a significant main effect for time (*F* (3.9, 104.2) = 4.746, p = .002, effect size = .149). Bonferroni pair-wise comparisons revealed that there were significant differences between the following time points: The 6HOURPOST time point was significantly lower than baseline (PRE) (p = .047), POST (p = .005), and 2HOURPOST (p = .006). Non-significant trends were observed between time points IMMPRE and POST (p = .082, effect size = .149), and time points POST and 30MINPOST (p = .099, effect size .149). See Table 4 for the main effect of time. Additionally, no significant interaction for the main effects of group and time for glucose levels was observed (*F* (7.7, 104.2) = .658, p = .722, effect size = .046).

Table 4

Glucose Values (mg/dL)

Group Assignment	PRE	IMM. PRE	POST	30 MIN POST	2 HOUR POST	6 HOUR POST
BCAA Group	91 ± 10	89 ± 4	93 ± 9	87 ± 10	90 ± 4	84 ± 5
Leucine Group	95 ± 8	91 ± 6	95 ± 8	90 ± 6	93 ± 5	90 ± 6
Placebo Group	94 ± 7	91 ± 8	98 ± 13	95 ± 11	92 ± 6	89 ± 6
Marginal Mean	93 ± 8 *	90 ± 6	95 ± 10 ¥	90 ± 10	92 ± 5 §	88 ± 6 *¥§

Note. Data are presented as means ± standard deviations. Values reported in the table are in mg/dl
A significant main effect for time was observed ($p = .02$). Values with identical symbols in the marginal means row represent that those time points are significantly different ($p \leq .05$) from one another.

Therefore, in regards to hypotheses one, which states that BCAA supplementation, combined with lower body resistance exercise, will not differ from placebo in regard to the levels of serum glucose, the hypothesis was accepted. Similarly, hypothesis two, which states that leucine supplementation, combined with lower body resistance exercise, will not differ from placebo in regard to the levels of serum glucose was accepted. Lastly, in regards to glucose hypothesis three, which states that BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to serum levels of glucose was accepted.

Serum Insulin

No significant main effect for group was observed in regards to insulin (*F* (2, 27) = .319, p = .730, effect size = .023). There was a significant main effect for time (*F* (3.9, 104.7) = 10.868, p = <.001, effect size = .287). Bonferroni post-hoc analysis revealed that there were significant differences between the following time points. The 2HRPOST insulin values were significantly less than baseline (PRE) insulin (p = .014) and IMMPRE (p = .010), POST (p <.001), and 30MINPOST (p = .001). Additionally, 6HOURPOST was significantly less than IMMPRE (p = .019) and POST (P = .006). Furthermore, non-significant trends were observed between time points IMMPRE and POST (p = .092, effect size = .287), and time points 30MINPOST and 6HOURPOST (p = .074, effect size = .287). See Table 5 for the main effect for time. Additionally, no significant group x time interaction was observed (*F* (7.8, 104.7) = 1.676, p = .115, effect size = .110).

Therefore, in regards to hypotheses four through six, the following were concluded. Hypothesis four which stated, BCAA supplementation, combined with lower body resistance exercise, will not increase the serum levels of insulin, as compared to the placebo was accepted. Furthermore, hypothesis five, which declared leucine supplementation, combined with lower body resistance exercise, will not increase the serum levels of insulin, as compared to the placebo was accepted. Finally, the sixth hypothesis which stated that BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to the serum levels of insulin was accepted.

Table 5

Insulin Values (mU/mL)

Group Assignment	PRE	IMM. PRE	POST	30 MIN POST	2 HOUR POST	6 HOUR POST
BCAA Group	19 ± 8	23 ± 10	25 ± 13	25 ± 14	19 ± 9	16 ± 6
Leucine Group	22 ± 19	19 ± 14	22 ± 14	18 ± 13	14 ± 14	16 ± 15
Placebo Group	22 ± 10	22 ± 11	27 ± 9	24 ± 9	18 ± 9	21 ± 13
Marginal Mean	21 ± 13 *	21 ± 11 §¥	25 ± 12 ↑^	22 ± 12 ד	17 ± 11 §*↑ד	18 ± 12 ¥^

Note. Data are presented as means ± standard deviations. Values in the table are in reported in μIU/ml. A significant main effect for time was observed (p = .001). Values with identical symbols in the marginal means row represent that those time points are significantly different (p ≤ .05) from one another.

Serum Bioactive Growth Hormone (GH)

No significant main effect for group was observed in regards to bioactive GH (*F* (2, 27) = .189, p = .829, effect size = .014). There was a significant main effect for time (*F* (3.6, 97.6) = 7.516, p = .001, effect size = .218). Bonferroni post-hoc analysis revealed that there were significant differences between the following time points. POST growth hormone was significantly greater than PRE (p = .004), IMMPRE (p = .003), and 2HOURPOST (p = .004). See Table 6 for the main effect of time. Additionally, no significant group x time interaction was observed (*F* (7.2, 97.6) = 1.476, p = .183, effect size = .099).

Table 6

Bioactive Growth Hormone (ng/mL)

Group Assignment	PRE	IMM. PRE	POST	30 MIN POST	2 HOUR POST	6 HOUR POST
BCAA Group	.41 ± .81	.63 ± .97	1.93 ± 2.24	1.49 ± 2.55	.23 ± .32	2.57 ± 4.00
Leucine Group	.03 ± .03	.48 ± 1.30	3.32 ± 4.21	1.24 ± 1.75	.06 ± .05	.31 ± .42
Placebo Group	.07 ± .09	.84 ± 1.33	2.23 ± 1.92	2.21 ± 3.78	.28 ± .76	.36 ± .56
Marginal Mean	.17 ± .48 *	.65 ± 1.18 ↑	2.49 ± 2.93 *↑¥	1.65 ± 2.75	.19 ± .47 ¥	1.08 ± 2.51

Note. Data are presented as means ± standard deviations. Values in the table are in reported in ng/ml. A significant main effect for time was observed (p < .001). Values with identical symbols in the marginal means row represent that those time points are significantly different (p ≤ .05) from one another.

Therefore, in regards to hypotheses 16 through 18, the following were concluded. Hypothesis 16 which stated, BCAA supplementation, combined with lower body resistance exercise, will not increase the serum levels of growth hormone, as compared to the placebo was accepted. Furthermore, hypothesis 17, which stated leucine supplementation, combined with lower body resistance exercise, will not increase the serum levels of growth hormone, as compared to the placebo was accepted. Finally, the18th hypothesis which stated BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to the serum levels of growth hormone was accepted.

Serum Free Insulin-like Growth Factor-1(IGF-1)

No significant main effect for group was observed in regards to free IGF-1 (F (2, 27) = .811, p = .455, effect size = .057). There was a significant main effect for time (F (2.3, 63.1) = 9.339, p = <.001, effect size = .257). Bonferroni post-hoc analysis revealed that there were significant differences between the following time points. Baseline (PRE) growth hormone was significantly greater than 6HOURPOST (p = .025). In addition, post-hoc tests results revealed that POST was significantly greater than IMMPRE (p = .007). Furthermore, 30MINPOST was also significantly greater than IMMPRE (p = .005). Furthermore, 6HOURPOST was significantly lower than POST (p = .001), 30MINPOST (p < .001), and 2HOURPOST (p < .001). There was a non-significant trend observed between PRE and 30MINPOST (p = .052, effect size = .257). See Table 7 for the main effect for time. Lastly, no significant group x time interaction was observed (F (4.7, 63.1) = .836, p = .522, effect size = .058).

Therefore, in regards to hypotheses 13 through 15, the following were concluded. Hypothesis 13 which stated, BCAA supplementation, combined with lower body resistance exercise, will not increase the serum levels of IGF-1, as compared to the placebo was accepted. Furthermore, hypothesis 14, which stated leucine supplementation, combined with lower body resistance exercise, will not increase the serum levels of IGF-1, as compared to the placebo was accepted. Finally, hypothesis 15 which stated BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to the serum levels of IGF-1 was accepted.

Table 7

Free IGF-1 (ng/mL)

Group Assignment	PRE	IMM. PRE	POST	30 MIN POST	2 HOUR POST	6 HOUR POST
BCAA Group	1.26 ± .83	1.19 ± .72	1.22 ± .77	1.43 ± .91	1.14 ± .74	.95 ± .64
Leucine Group	1.09 ± .49	1.07 ± .38	1.14 ± .47	1.17 ± .41	1.05 ± .41	.78 ± .33
Placebo Group	1.35 ± .43	1.21 ± .43	1.60 ± .54	1.46 ± .57	1.40 ± .46	1.10 ± .53
Marginal Mean	1.23 ± .60 *	1.16 ± .52 ↑¥	1.32 ± .62 ↑^	1.36 ± .65 ¥ §	1.19 ± .56 ¡	.94 ± .51 *^§¡

Note. Data are presented as means ± standard deviations. Values in the table are in reported in ng/ml. A significant main effect for time was observed ($p < .001$). Values with identical symbols in the marginal means row represent that those time points are significantly different ($p \leq .05$) from one another.

Muscle Variables

A two-way [treatment groups (3) x time point (4)] MANOVA mixed methods with repeated measures on the second factor was conducted to evaluate the effects of three different supplements (BCAAs, leucine alone, or placebo) on various muscle proteins that may impact the Akt-mTOR signal transduction pathway. The criterion variables that were analyzed in this multivariate analysis were: phosphorylated IRS-1, phosphorylated Akt, phosphorylated mTOR, phosphorylated 4E-BP1, and phosphorylated P70-S6K.

The within-subjects factor was time with four levels (baseline, 30 minutes post exercise, and 2 and 6 hours post-exercise), and due to significant baseline differences for IRS-1, Akt, and mTOR delta values were used for the analysis of all skeletal muscle variables. However, Table 8 reports the raw data obtained for each muscle dependent variable before delta changes were analyzed. The between-subjects factor was group with three levels (BCAA, leucine, and placebo). The main effects for group and time, and the group x time interaction were analyzed using the multivariate criterion of Wilks' Lambda.

There was a significant multivariate main effect for group (Wilks' Lambda = .209, F (10, 46) = 5.463, $p < .001$, effect size = .543). Equally important, the multivariate main effect for time was significant (Wilks' Lambda = .242, F (15, 13) = 2.719, $p = .039$, effect size = .758). Furthermore, there was also a significant multivariate interaction for group x time (Wilks' Lambda = .092, F (30, 26) = 1.993, $p = .039$, effect size = .697). As a result of the aforementioned multivariate results, univariate ANOVAs were run with subsequent Bonferroni post-hoc tests to determine the main effects for time and group for IRS-1, Akt, mTOR, 4E-BP1, and P70-S6K. Significant group x time interactions for IRS-1, Akt, mTOR, 4E-BP1, and P70-S6K that were discovered in the univariate ANOVA were determined using independent samples T-tests. The assumption of sphericity was not met for the muscle variables [IRS-1 ($p = .004$), Akt ($p < .001$), and mTOR-1 ($p < .001$)]. However, the sphericity assumption was met for 4E-BP1 ($p = .080$) and P70-S6K ($p = .162$). Therefore, the Huynh-Feldt correction was adopted throughout the following analyses where appropriate.

Table 8. *Raw Muscle data*

Variable	PRE		30 MIN POST		2 HOUR POST		6 HOUR POST	
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
IRS-1 *(u/mL/g)*								
BCAA	9059.1	3121.9	9940.7	4373.6	9605.1	4240.8	8108.3	4708.4
Leucine	8926.6	4700.3	12310.8	7596.4	20070.1	9330.4	22702.6	11728.1
Placebo	14734.0	6394.6	19956.9	10108.5	23438.2	12238.3	21499.4	11242.2
AKT *(u/mL/g)*								
BCAA	5903.0	2139.9	6219.8	2218.4	6023.5	2577.2	6507.1	3267.3
Leucine	3885.6	921.2	3835.6	1197.8	4151.3	2044.7	3540.8	740.4
Placebo	3928.4	1461.6	6201.8	3632.5	9713.5	7969.5	7106.4	4024.9
mTOR *(AU)*								
BCAA	88.4	12.1	111.9	27.5	99.9	13.9	111.1	19.2
Leucine	118.1	22.0	128.4	29.3	174.2	152.7	135.4	28.4
Placebo	125.4	17.9	145.7	86.6	140.5	29.2	140.2	41.5
4E-BP1 *(u/mL/g)*								
BCAA	4897.1	1692.8	5033.1	1560.9	5122.6	1997.6	5535.9	1851.0
Leucine	4939.4	1762.2	5110.8	1505.8	4666.1	1602.9	4340.9	1906.3
Placebo	6509.8	1853.1	5548.3	3078.1	4434.1	1862.9	4841.3	3230.0
$P70^{S6K}$ *(u/mL/g)*								
BCAA	35898.3	18093.8	39902.4	30398.5	28542.2	8100.7	24736.3	15784.2
Leucine	24660.2	19251.8	21974.2	19803.7	11974.2	4367.3	9582.2	3839.0
Placebo	28530.8	13378.7	22751.5	8395.3	27573.6	27292.4	16761.0	4580.3

Note. Data are reported as means ± standard deviations. All muscle variables are in U/mL per gram of skeletal muscle except for mTOR which is in absorbance units (AU) per gram of skeletal muscle.

Phosphorylated IRS-1

A significant main effect for group was observed in regards to IRS-1 (F (2, 27) = 7.941, p = .002, effect size = .370). Bonferroni post-hoc analysis revealed that the BCAA group was significantly less than the leucine group (p = .002). Additionally, the BCAA group was also significantly less than the placebo group (p = .028). See Figure 1.

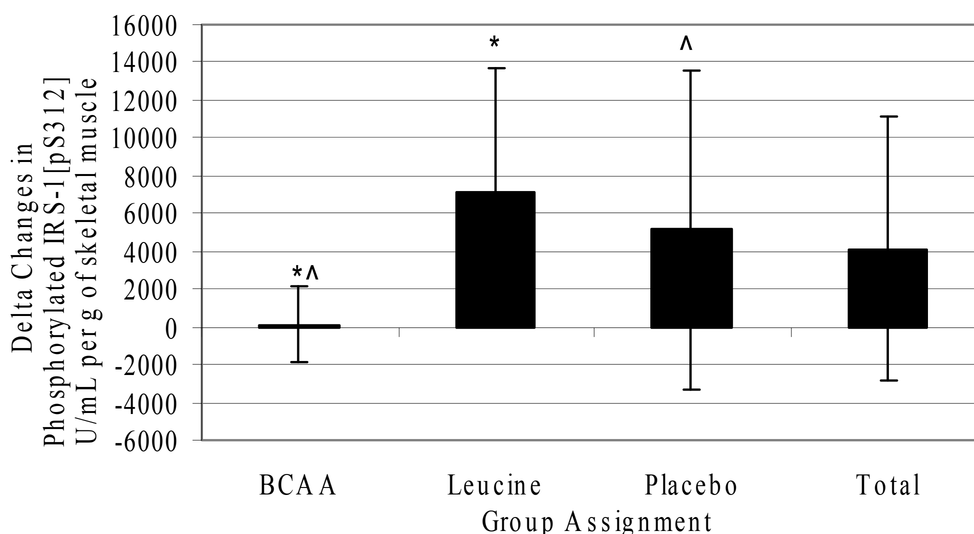

Figure 1. Group Differences in phosphorylated IRS-1 (U/mL/g). A significant main effect for group was observed (p = .002). Groups that have the same symbol (*, ^) are significantly different (p ≤ .05) from one other. The "Total" bar represents the mean of the three groups (BCAA, Leucine, and Placebo).

A significant main effect for time was observed in regards to IRS-1 (F (2.8, 74.2) = 5.741, p = .002, effect size = .175). Bonferroni post-hoc analysis revealed that in regards to delta changes, there were significant differences between the following time points. IRS-1 at 2HOURPOST (p = .007), and 6HOURPOST (p = .003) was significantly higher than PRE values. There was a non-significant trend observed between 30MINPOST and PRE (p = .052, effect size = .175). See Figure 2.

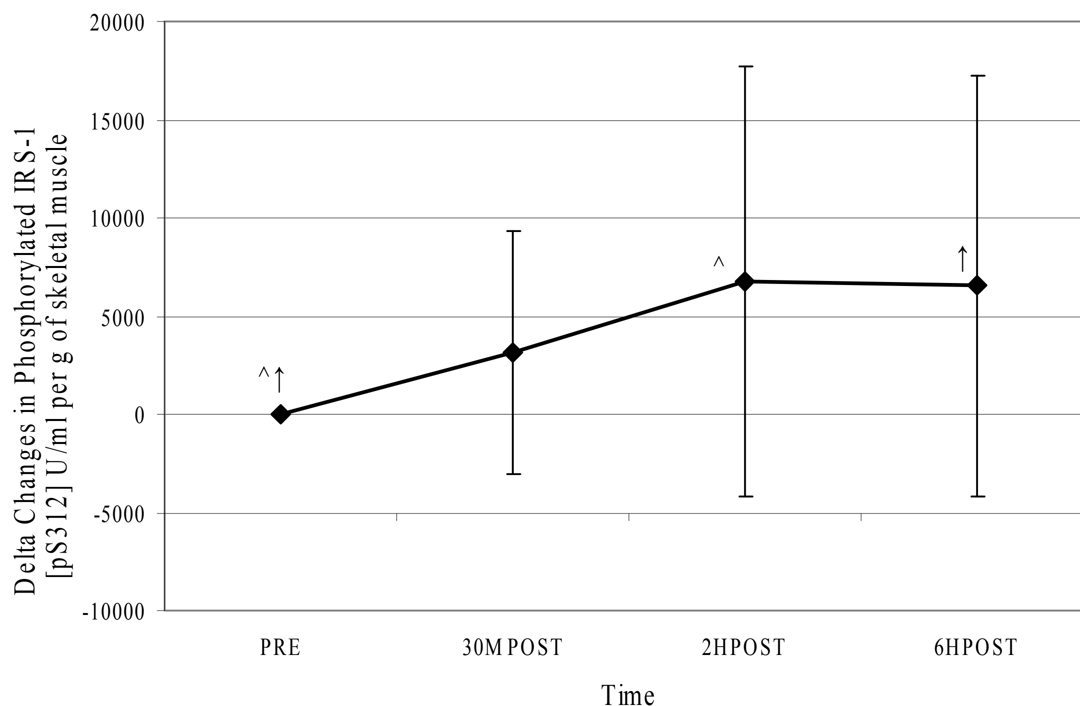

Figure 2. Delta values for phosphorylated IRS-1 (U/mL/g). This graph illustrates the overall mean for the main effect of time. A significant main effect for time was observed (p =.002). When two time points have the same symbol (↑, ^) it denotes that those time points are significantly different (p<.05) from one another.

A significant group x time interaction effect for IRS-1 was observed (*F* (5.5, 74.2) = 2.362, p = .043, effect size = .149). Independent samples t-tests were utilized to determine were the significant interactions occurred. There were significant interactions between the following points. When comparing delta values, the leucine group was significantly greater than the BCAA group at 2HOURPOST (*t* (9.9) = -3.5, p = .006) and at 6HOURPOST, (*t* (10.5) = -3.9, p = .003). Furthermore, the placebo group was significantly greater than the BCAA group at 6HOURPOST (*t* (10.9) = -2.3, p = .046). See Figure 3.

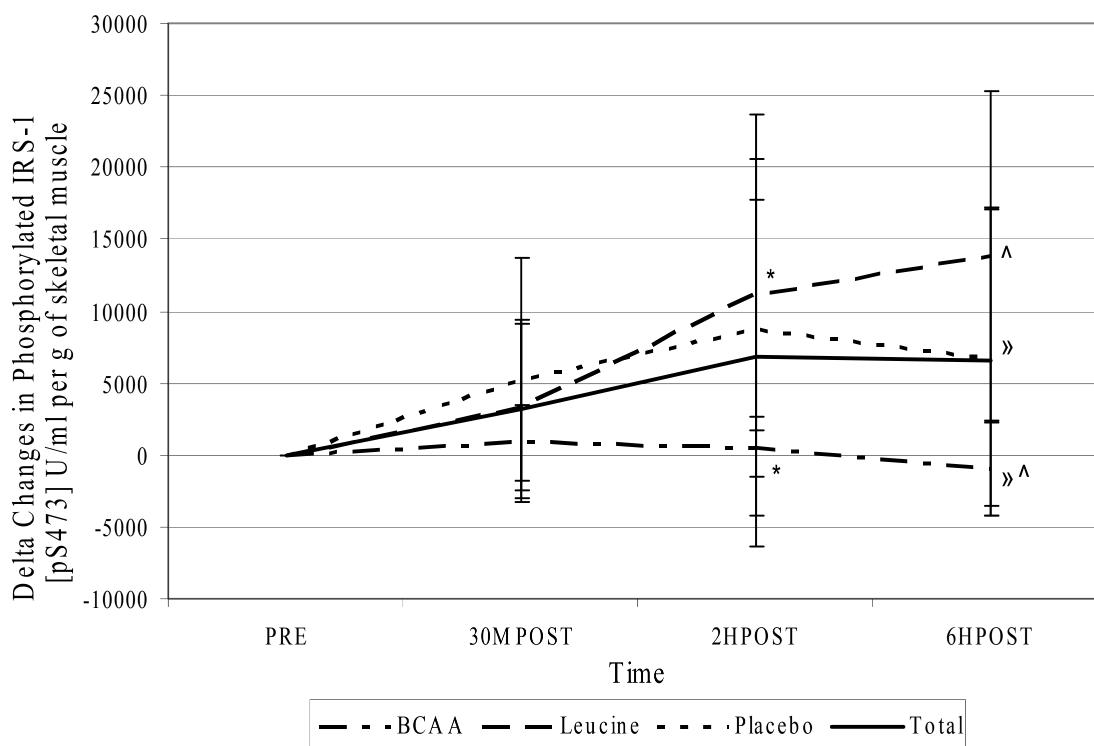

Figure 3. Group x Time interactions in delta values of phosphorylated IRS-1 (U/mL/g). A significant interaction effect was observed (p = .043). When two time points have the same symbol (*, », ^) it denotes that those time points are significantly different ($p \leq .05$) from one another.

Therefore, hypothesis seven which states BCAA supplementation, combined with lower body resistance exercise, will not increase phosphorylated IRS-1 when compared to the placebo was accepted. Even though leucine increased phosphorylated IRS-1 to a greater extent than the placebo, it was not significant. Therefore, hypothesis eight that states leucine supplementation, combined with lower body resistance exercise, will not increase phosphorylated IRS-1 when compared to the placebo was accepted. However, hypothesis nine which states BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to phosphorylated IRS-1 was rejected.

Phosphorylated Akt

A significant main effect for group was observed in regards to Akt (F (2, 27) = 11.745, p < .001, effect size = .465). Bonferroni post-hoc analysis revealed that the BCAA group was significantly less than the placebo group (p = .002). Additionally, the leucine was significantly less than the placebo group (p < .001). See Figure 4.

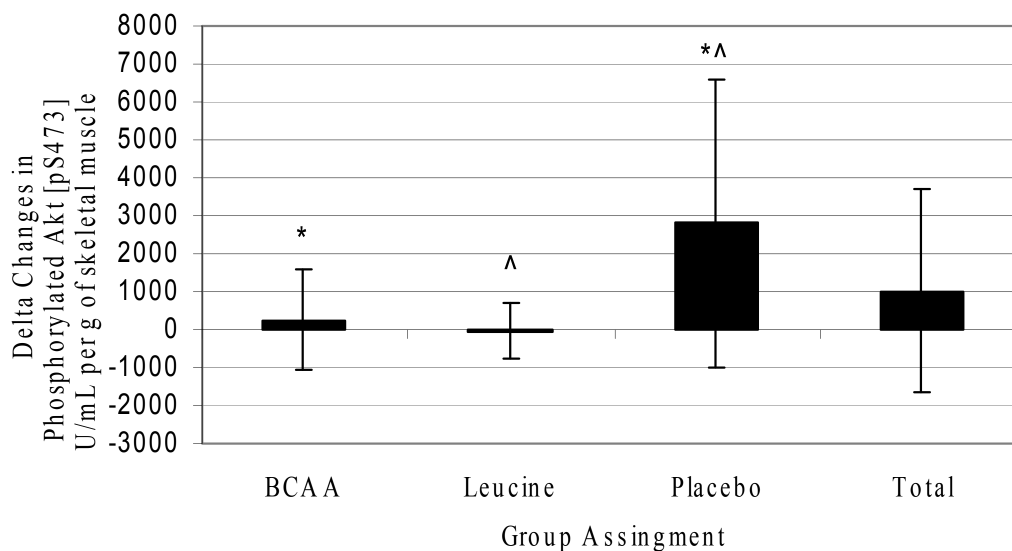

Figure 4. Group differences in phosphorylated Akt (U/mL/g). A significant main effect for group was observed (p < .001). Groups that have the same symbol (*,↑)are significantly different (p ≤ .05) from one another. The "Total" bar represents the mean of the three groups (BCAA, Leucine, and Placebo).

No significant main effect for time was observed in regards to phosphorylated Akt [pS473] (F (1.7, 46.6) = 2.358, p = .119, effect size = .080). See Figure 5. Furthermore, no significant group x time interaction was observed in regards to phosphorylated Akt [pS473] (F (3.5, 46.6) = 2.015, p = .125, effect size = .130). See Figure 6.

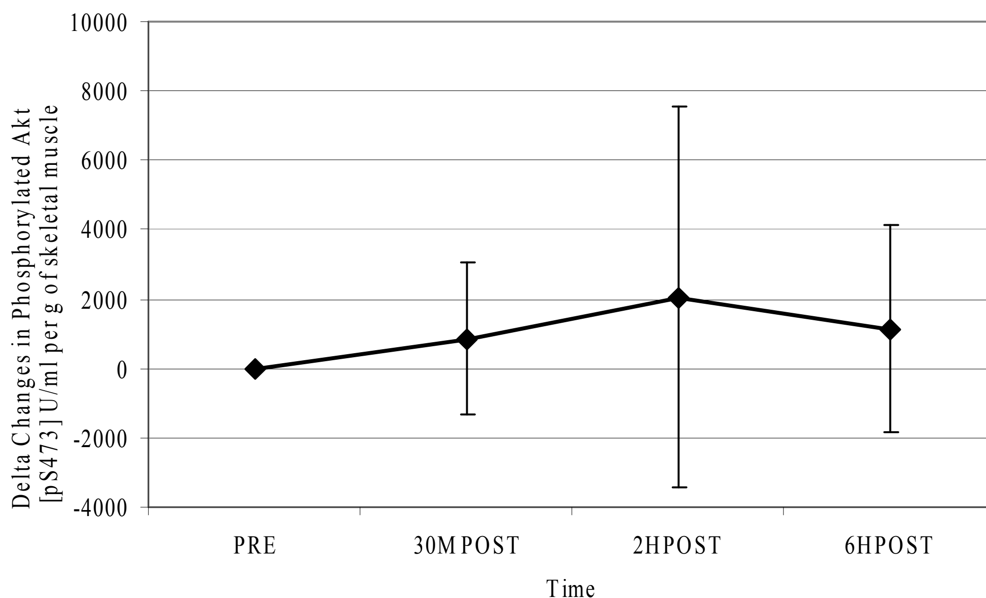

Figure 5. Delta values for phosphorylated Akt (U/mL/g). This graph illustrates the overall mean for the main effect of time. No significant main effect for time was observed (p = .119).

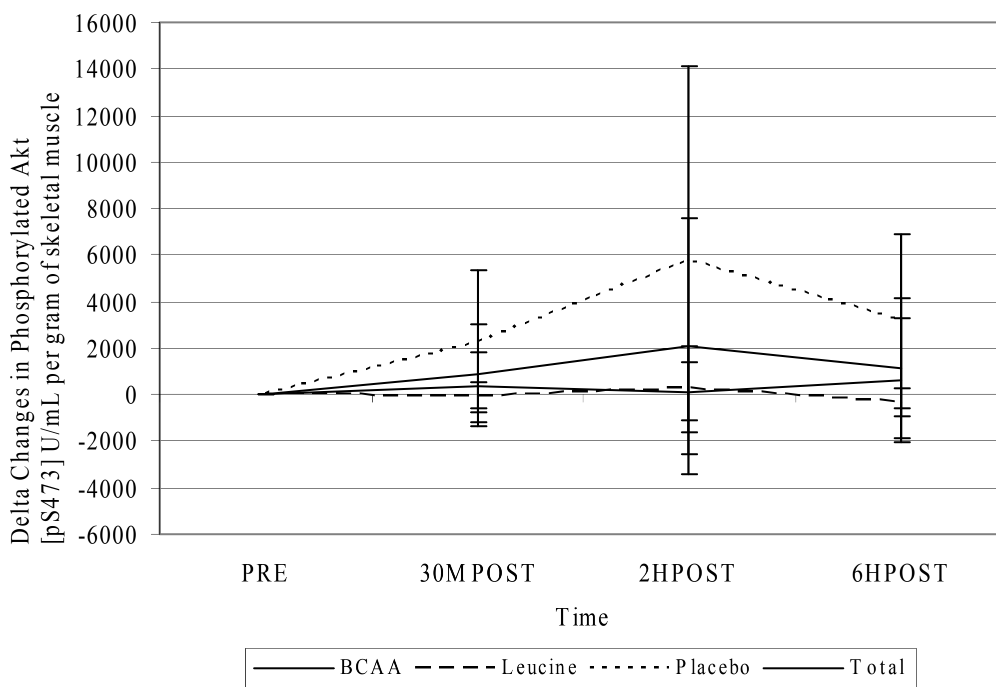

Figure 6. Group x Time interactions in delta values of phosphorylated Akt (U/mL/g). . No significant interaction effect was observed (p = .125).

In regards to the hypotheses for Akt, the following were concluded. Hypothesis 10 which states BCAA supplementation, combined with lower body resistance exercise, will not increase phosphorylated Akt when compared to the placebo was accepted. Also hypothesis 11 that states leucine supplementation, combined with lower body resistance exercise, will not increase phosphorylated Akt when compared to the placebo was accepted. Lastly, hypothesis 12 which states BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to phosphorylated Akt was accepted.

Phosphorylated mTOR

No significant main effect for group was observed in regards to phosphorylated mTOR (F (2, 27) = .192, p = .827, effect size = .014). See Figure 7.

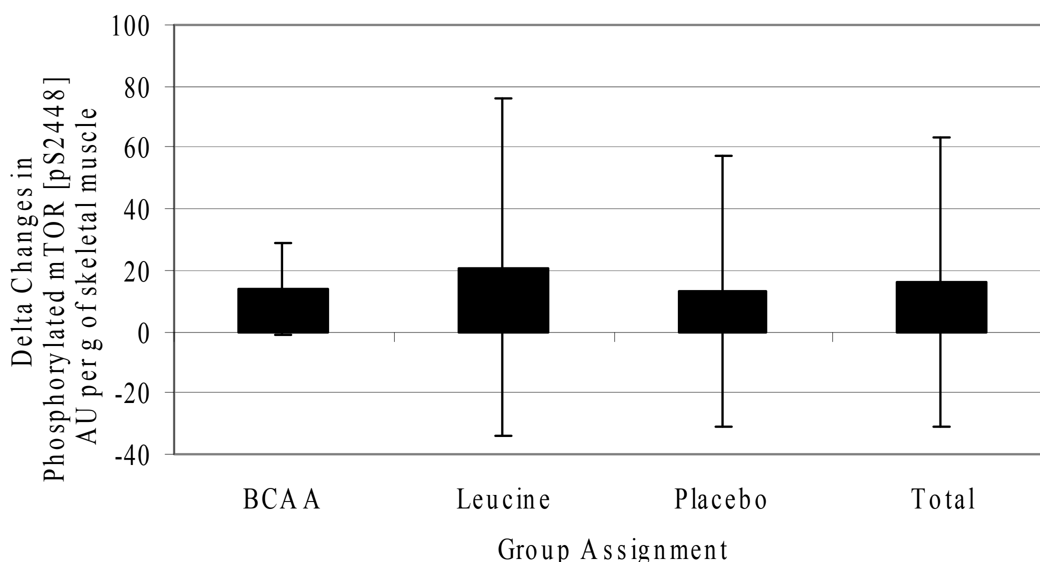

Figure 7. Group differences in phosphorylated mTOR (AU). No significant main effect for group was observed (p = .827). The “Total” bar represents the mean of the three groups (BCAA, Leucine, and Placebo).

No significant main effect for time was observed in regards to phosphorylated mTOR (*F* (2.1, 56.7) = 1.188, p = .314, effect size = .042). See Figure 8. No significant group x time interaction was observed in regards to phosphorylated mTOR (*F* (4.2, 56.7) = .595, p = .676, effect size = .042). See Figure 9.

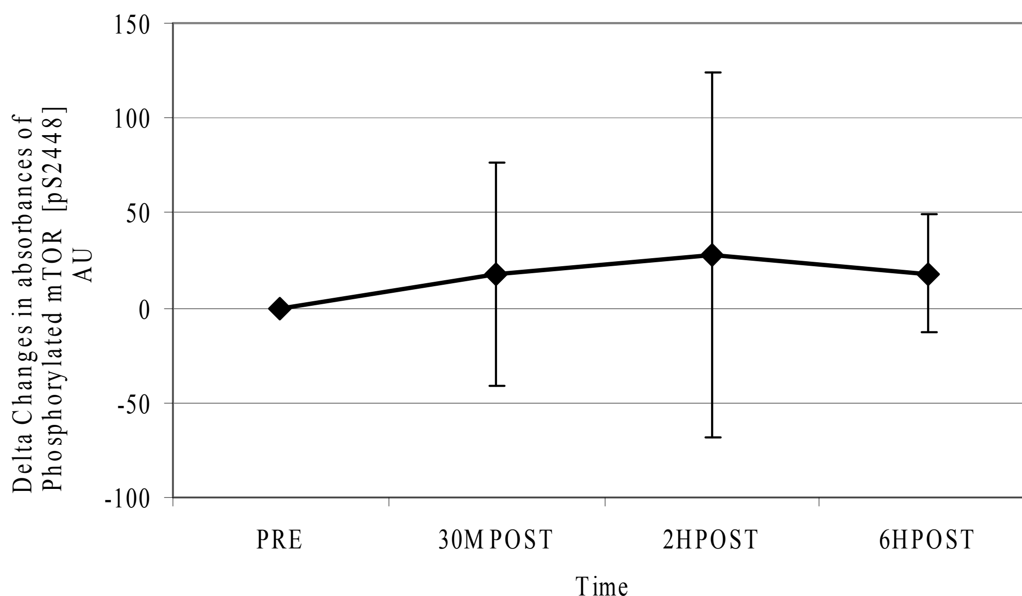

Figure 8. Delta values for phosphorylated mTOR (AU). This graph illustrates the overall mean for the main effect of time. No significant main effect for time was observed (p = .314).

In regards to the hypotheses relating to mTOR, the following were concluded. Hypothesis 19 which states BCAA supplementation, combined with lower body resistance exercise, will not increase phosphorylated mTOR when compared to the placebo was accepted. Hypothesis 20 that states leucine supplementation, combined with lower body resistance exercise, will not increase phosphorylated mTOR when compared to the placebo was accepted. Lastly, hypothesis 21 which states BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to phosphorylated mTOR was accepted.

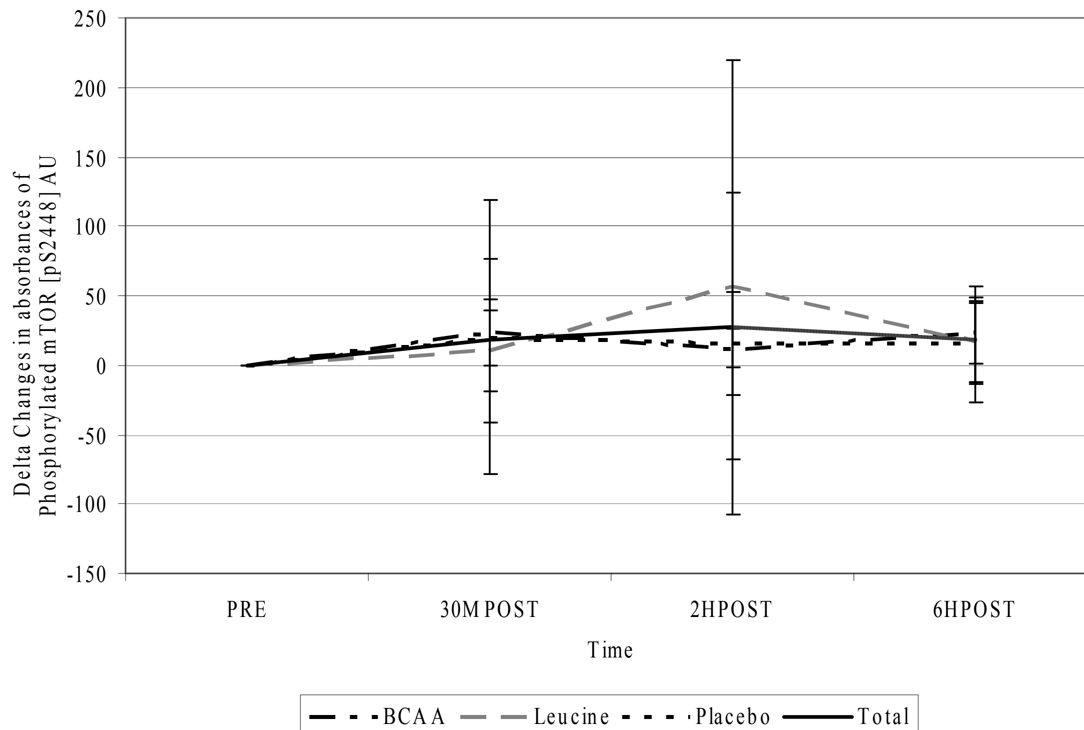

Figure 9. Group x Time interactions in delta values of phosphorylated mTOR (AU). No significant interaction effect was observed (p = .676)

Phosphorylated 4E-BP1

A significant main effect for group was observed in regards to 4E-BP1 (*F* (2, 27) = 7.667, p = .002, effect size = .362). Bonferroni post-hoc analysis revealed that the BCAA group was significantly greater than the placebo group (p = .002). Similarly, leucine was also significantly greater than the placebo group (p = .037). See Figure 10. However, no significant main effect for time was observed in regards to 4E-BP1 (*F* (2.9, 76.8) = 1.983, p = .127, effect size = .068). See Figure 11.

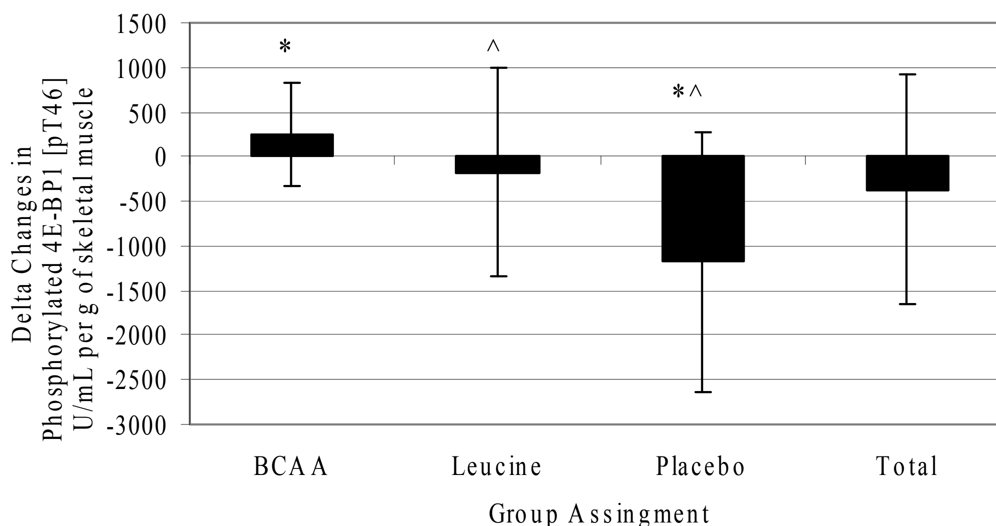

Figure 10. Group differences in phosphorylated 4E-BP1 (U/mL/g). A significant main effect for group was observed (p = .002). Groups that have the same symbol (*,^) are significantly different (p ≤ .05) from one another. The "Total" bar represents the mean of the three groups (BCAA, Leucine, and Placebo).

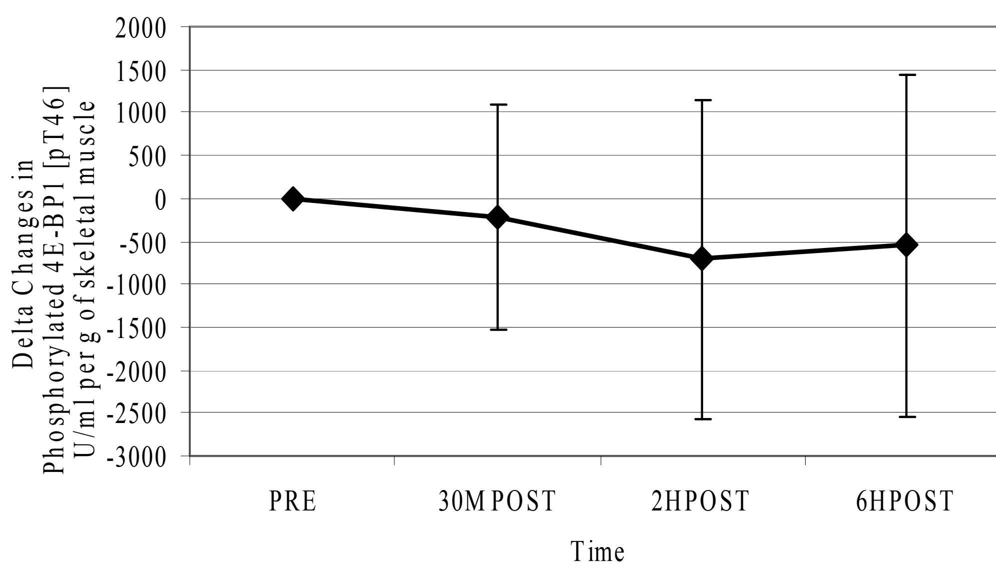

Figure 11. Delta values for phosphorylated 4E-BP1 (U/mL/g). This graph illustrates the overall mean for the main effect of time. No significant main effect for time was observed (p = .127).

A significant group x time interaction effect for 4E-BP1 was observed (*F* (5.7, 76.8) = 2.323, p = .044, effect size = .147). Independent samples t-tests were utilized to determine were the significant interactions occurred. There were significant interactions between the following points. At 6HOURPOST, the BCAA group was significantly

greater than the leucine group (t (11.4) = 2.3, p = .041). Furthermore, the BCAA group was significantly greater than the placebo group at 2HOURPOST (t (18) = 3.954, p = .001) and 6HOURPOST, (t (9.9) = 2.703, p = .022). Lastly, leucine was significantly greater than the placebo group at 2HOURPOST (t (18) = 2.249, p = .037). See Figure 12.

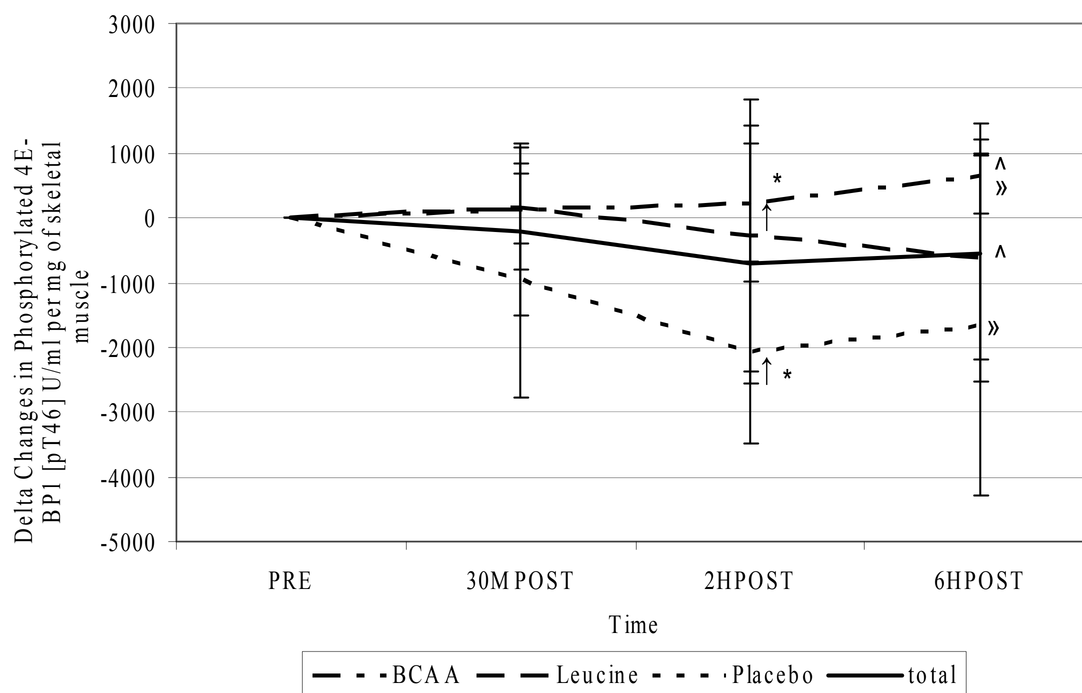

Figure 12. Group x Time interactions in delta values of phosphorylated 4E-BP1 (U/mL/g). A significant interaction effect was observed (p = .044). When two time points have the same symbol (*, », ^, ↑) it denotes that those time points are significantly different ($p \leq .05$) from one another.

With respect to the 4E-BP1 hypotheses the following results were concluded. Hypothesis 25 which states BCAA supplementation, combined with lower body resistance exercise, will not increase phosphorylated 4E-BP1 when compared to the placebo was rejected. Similarly, hypothesis 26 that states leucine supplementation, combined with lower body resistance exercise, will not increase phosphorylated 4E-BP1 when compared to the placebo was also rejected. Lastly, hypothesis 27 which states

BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to phosphorylated 4E-BP1 was rejected.

Phosphorylated P70^{S6K}

No significant main effect for group was observed in regards to P70-S6K (*F* (2, 27) = .197, p = .823, effect size = .014). See Figure 13.

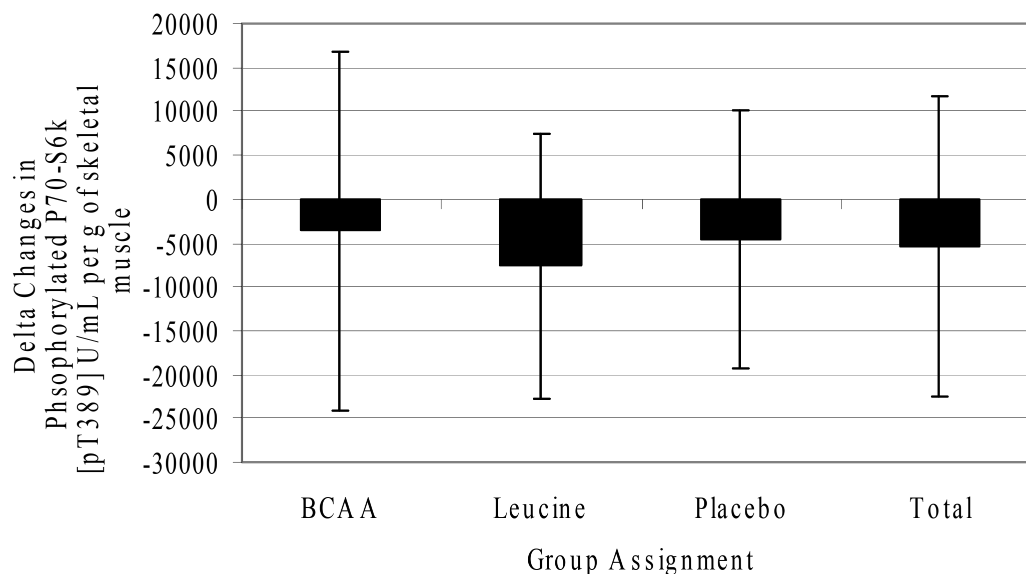

Figure 13. Group differences in phosphorylated P70-S6K (U/mL/g). No significant main effect for group was observed (p = .823). The "Total" bar represents the mean of the three groups (BCAA, Leucine, and Placebo).

A significant main effect for time was observed in regards to P70-S6K (*F* (3, 81) = 3.721, p = .015, effect size = .121). Bonferroni post-hoc analysis revealed that P70-S6K at 6HOURPOST was significantly lower than at PRE (p = .005). A non-significant trend was also observed between 30MINPOST and 6HOURPOST (p = .093, effect size = .121) was also observed. See Figure 14. Additionally, no significant group x time interaction was observed in regards to phosphorylated P70-S6K (*F* (6, 81) = .581, p = .745, effect size = .041). See Figure 15.

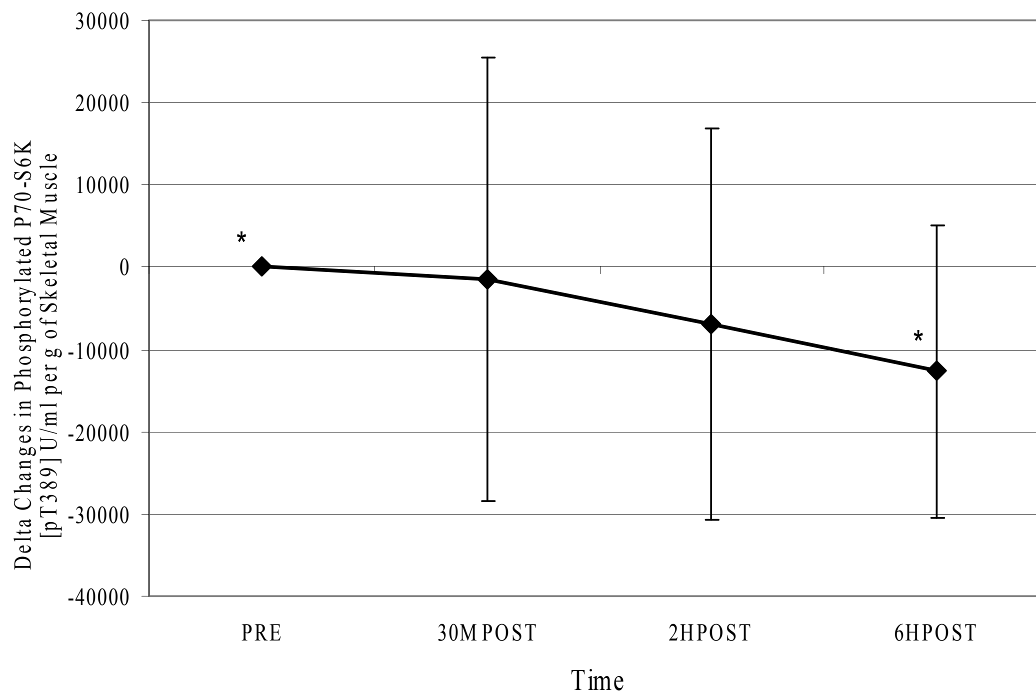

Figure 14. Delta values for phosphorylated P70-S6K (U/mL/g). This graph illustrates the overall mean for the main effect of time. A significant main effect for time was observed (p = .015). When two time points have the same symbol (*) it denotes that those time points are significantly different ($p \leq .05$) from one another.

In regards to the hypotheses relating to P70-S6K, the following results were concluded. Hypothesis 22 which states BCAA supplementation, combined with lower body resistance exercise, will not increase phosphorylated P70-S6K when compared to the placebo was accepted. Hypothesis 23 that states leucine supplementation, combined with lower body resistance exercise, will not increase phosphorylated P70-S6K when compared to the placebo was accepted as well. Lastly, hypothesis 24 which states BCAA and leucine supplementation, combined with lower body resistance exercise, will not differ in regard to phosphorylated P70-S6K was also accepted.

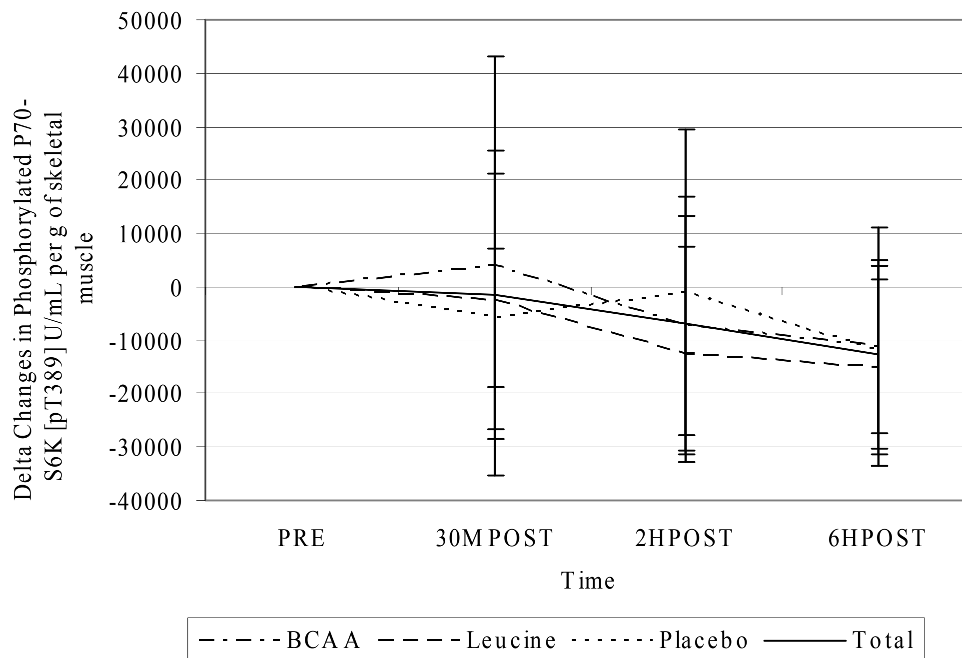

Figure 15. Group x Time interactions in delta values of phosphorylated P70-S6K (U/mL/g). No significant interaction effect was observed (p = .745).

CHAPTER FIVE

Discussion

The Akt-mTOR signal transduction pathway has been partially elucidated through various in vitro and animal studies (J. C. Anthony et al., 1999; J. C. Anthony, Yoshizawa et al., 2000; Crozier et al., 2005; Gautsch et al., 1998; Vary et al., 1999). To a much lesser extent, the pathway, in humans, has been partially elucidated following resistance exercise and/or amino acid supplementation (Coffey et al., 2006; Eliasson et al., 2006; Fujita et al., 2007; Karlsson et al., 2004; Koopman et al., 2006). The Akt-mTOR pathway, at this time, appears to regulate various protein kinases that mediate translation initiation. Resistance training and leucine supplementation seems to mediate their effects, in regards to protein synthesis, through this aforementioned pathway (Bolster, Kimball et al., 2003; Layman, 2002). However, much more research needs to be performed in humans to further elucidate the Akt-mTOR pathway in humans. The purpose of this study was to examine the effects of oral BCAA and leucine supplementation in conjunction with an acute bout of lower extremity resistance exercise, in humans, on several proteins of the Akt-mTOR pathway including: IRS-1, Akt, mTOR, 4E-BP1, and P70-S6K. Furthermore, serum glucose, insulin, IGF-1, and GH were also assessed, as these hormones seem to play a role in influencing the proteins of this particular pathway (Kimball et al., 2002). This study is unique because it combined either an oral leucine or BCAA supplement along with resistance exercise and examined both upstream and downstream targets in the Akt-mTOR cell signaling pathway. Additionally, several hormones that may influence the activity of various enzymes in this

pathway in humans were also quantified. The results of this study will be discussed in conjunction with the original hypotheses of the study.

Dietary Recall and Acute Resistance Exercise Session

As previously described, the participants of this study were initially screened to ensure that they were not currently taking any nutritional supplements, other than possibly multivitamins, at as part of the entrance criteria to participate. The participants were instructed not to alter their diet prior to initiating the study. However, 48 hour dietary recall assessments were utilized to examine macronutrient intake along with the total daily calories consumed in the participants' diets. Upon analysis, it was demonstrated that there were no significant differences between the individuals in the three supplement groups (i.e., BCAA, leucine, and placebo) in regards to the quantity of carbohydrate, protein, and fat consumed per day (Table 2). Equally important, there were no significant differences between the groups for the amount of total calories consumed daily.

The study incorporated an acute bout of lower extremity resistance exercise including four sets of both leg press and knee extension at 80% of their 1-RM. Upon analysis there was no significant differences in 1-RM leg press, 1-RM knee extension, the percent of 1-RM leg press and knee extension utilized during sets, and in total work between the three supplemental groups (Table 3). These aforementioned results, along with the fact that the participants were randomized into their respective experimental groups, based upon the 1-RM and body weight, helped to ensure that this study controlled for the effects that differences in dietary intake and exercise could have had on the dependent variables analyzed in this study.

Acute Serum Responses to Supplementation and Resistance Exercise

Glucose

Serum glucose was measured in all individuals participating in this study. Although, serum glucose does not directly impact the Akt-mTOR pathway, insulin, which is released in response to increases in serum glucose, has a direct impact on the Akt-mTOR pathway. Therefore, glucose was measured in this study to help account for possible changes that could be observed in serum insulin. Some research examining untrained young males have shown that glucose may not significantly increase following an acute bout of resistance exercise when given only a non-caloric placebo (Bird, Tarpenning, & Marino, 2006).

In this study there was no significant main effect for group in regards to glucose, meaning that neither BCAA nor leucine supplementation increased serum glucose to a significantly greater extent than the non-caloric placebo (Table 4). However, there was a significant main effect for time, demonstrating that glucose initially rose, although not significantly, following resistance exercise and then dropped significantly by six hours after the cessation of exercise. The observed transient increase in blood glucose following resistance exercise is not necessarily surprising. Due to the fact that the participants were in a carbohydrate fasted state, blood glucose may have slightly increased do to liver glycogenolysis/gluconeogenesis. The observation that blood glucose was significantly lower at six hours following exercise as compared to before exercise can be explained by the fact that they were still in a fasted state.

Insulin

Insulin is a powerful anabolic hormone that has various metabolic functions, as well as, the ability to instigate cell signaling via the Akt-mTOR pathway (Bolster, Kimball et al., 2003; Layman, 2002). Once insulin binds to its receptor it facilitates the activation of IRS-1 and its downstream targets in the aforementioned pathway (Layman, 2002). Some research has shown that insulin levels may not be greatly affected by resistance training alone (Bird et al., 2006; Crewther, Keogh, Cronin, & Cook, 2006). However, resistance training plus essential amino acids and more specifically BCAAs ingestion, have shown to significantly increase post-exercise insulin responses as compared to a placebo (Bird et al., 2006; Karlsson et al., 2004). Although transient increases in insulin, in some cases, might not be statistically significant, it does not necessarily mean that it is not physiologically relevant. It is thought that both leucine and insulin signaling most likely interact in the Akt-mTOR pathway to induce a maximal response (J. C. Anthony et al., 2001; J. C. Anthony, Lang et al., 2002; Rennie et al., 2006). In a study design similar to the present study, it was shown that in response to an acute bout of lower extremity resistance exercise in humans, insulin levels slightly increased (not significantly) even without caloric intake (Karlsson et al., 2004). The observed increase in insulin is quite possibly due to increased blood glucose due to liver glycogenolysis/gluconeogenesis that occurs with resistance exercise.

In this current study, there was no significant main effect for group in regards to insulin, meaning that neither BCAA nor leucine supplementation increased serum insulin to a greater extent than the non-caloric placebo (Table 5). As with glucose, there was a significant main effect for time. In regards to time, the results demonstrated that insulin

rose, although non-significantly, following exercise and then dropped significantly by six hours after the cessation of exercise, as compared to its peak levels observed immediately post exercise. These observed results nearly mirrored the glucose response. The fact that neither BCAA nor leucine did not significantly increase insulin values is not necessarily surprising. It has been shown that when leucine, carbohydrate alone, or leucine plus carbohydrate is orally consumed, the leucine alone group did not significantly increase insulin values more than the fasted controls (J. C. Anthony et al., 1999) (J. C. Anthony, Anthony et al., 2000). However, both the carbohydrate alone and leucine plus carbohydrate groups significantly increased serum insulin above that observed in the fasted controls (J. C. Anthony et al., 1999) (J. C. Anthony, Anthony et al., 2000). Additionally, it has been shown that leucine plus carbohydrate can produce a larger increase in serum insulin than just carbohydrate by itself (J. C. Anthony et al., 1999) (J. C. Anthony, Anthony et al., 2000). Therefore, in order to enhance the phosphorylation and subsequent activity of the Akt/mTOR pathway, combining carbohydrate with BCAAs/leucine is probably the most beneficial choice.

Growth Hormone (GH)

Several variables such as age, sex, training status and nutrition can influence the acute hormonal response following resistance training (Crewther et al., 2006). As discussed earlier, this study made attempts to control for these variables. By doing this, the influence of the leucine alone or BCAA ingestion on acute hormonal responses of GH and IGF-1, for example, can be more accurately elucidated. GH is a hormone that is released in a pulsatile fashion from the anterior pituitary gland in response to resistance exercise. (Godfrey, Madgwick, & Whyte, 2003; Nindl et al., 2006) and exerts much of its

anabolic effects via the release of IGF-1 from the liver. Once released, GH can increase the expression of IGF1-1 in skeletal muscle. Growth hormone may play a role in influencing the Akt-mTOR pathway directly by influencing the activity of IRS-1 and indirectly through the stimulation of IGF-1 (Sadowski, Wheeler, Wang, & Sadowski, 2001).

The results for this current study demonstrated that there was no significant main effect for group in regards to serum GH levels, meaning that neither BCAA nor leucine supplementation increased serum GH to a greater extent than the non-caloric placebo (Table 6). There was a significant main effect for time. In regards to time, the result demonstrated that baseline serum GH levels significantly increased immediately post exercise and then remained elevated for approximately two hours before decreasing significantly.

Insulin-Like Growth Factor-1 (IGF-1)

As previously mentioned, it has been established that resistance exercise can lead to an increase in serum IGF-1. However, the increase in IGF-1 is largely attributed to the action of GH and subsequently IGF-1 is secreted from the liver in a delayed response (Crewther et al., 2006; Godfrey et al., 2003). IGF-1 can bind to the insulin receptor and instigate the signaling cascade of the various protein kinases in the Akt-mTOR signal transduction pathway (Kimball et al., 2002). Therefore, if IGF-1 or insulin stimulates the activity of the Akt-mTOR pathway then it can possibly increase the efficiency of translation initiation in skeletal muscle.

The current study resulted in no significant main effect for group in regards to free IGF-1 (Table 7). There was, however, a significant main effect for time. IGF-1

significantly increased immediately following exercise and peaked at 30 minutes into the recovery session as compared to immediately pre-exercise. These results suggest that both GH and IGF-1 significantly increased due to the resistance exercise, but did not seem to be significantly influenced by either BCAA or leucine ingestion at the doses utilized in this study.

Cell Signaling Responses in Skeletal Muscle to Supplementation and Resistance Exercise

Insulin Receptor Substrate1 (IRS-1)

The insulin receptor substrate-1 (IRS-1) is an upstream protein in the Akt-mTOR signal transduction pathway. Once this protein is activated via phosphorylation from the insulin receptor by tyrosine kinase, it seems to bind to and activate the regulatory subunit of phosphatidylinositol 3-kinase (PI3-K). The activation of PI3-K, another integral protein in the Akt-mTOR pathway, is a known activator of Akt. Akt, also known as protein kinase B (PKB) has further downstream actions that will be discussed in more detail in the next section. Insulin, IGF-1, and most likely GH can mediate their effects on the Akt-mTOR pathway initially through IRS-1 activation.

The results of this study demonstrated that there was a significant main effect for group (Figure 1). Interestingly, both the leucine supplementation group as well as the placebo group had significantly higher levels of phosphorylated IRS-1 than those in the BCAA group. However, the leucine and placebo group did not significantly differ from one another. As mentioned earlier, if IRS-1 is activated, it in turn appears to ultimately activate Akt. However, Eliasson et al. (2006) observed that both lower extremity concentric and eccentric muscle (both sub-maximal and maximal) contractions, without

nutritional intake, did not lead to increases in either phosphorylated Akt or mTOR. This indirectly suggests that resistance training alone may not activate IRS-1 in humans. Similarly, it has shown by Brozinick and Birnbaum (1998) that in rodents, insulin but not muscle contraction, stimulated Akt. Furthermore, IRS-1 activation does not seem to be significantly affected by BCAA or leucine consumption. Leucine seems to exert its effects on the downstream targets such as mTOR (J. C. Anthony, Yoshizawa et al., 2000) (Vary, Anthony, Jefferson, Kimball, & Lynch, 2007). IRS-1 activation is often accomplished via insulin and IGF-1 binding to the insulin receptor (Kimball et al., 2002). In this current study, there was a significant main effect for time for IRS-1 (Figure 2). IRS-1 phosphorylation was significantly increased at both 2 and 6 hours following exercise. Furthermore, there was a non-significant trend between baseline and 30 minutes following exercise. In other words, IRS-1 appeared to increase over time when examining the main effect of time. Similarly, this current study demonstrated that IGF-1 significantly increased following resistance training. In the same way, insulin, although non-significantly, also increased immediately following exercise. These two hormones are probably the most likely the cause for the observed increase in IRS-1 phosphorylation. Interestingly, the leucine group had significantly higher levels of phosphorylated IRS at both 2 and 6 hours following resistance exercise as compared to the BCAA group (Figure 3). Furthermore, IRS-1 phosphorylation was significantly higher in the placebo group at 6 hours after exercise than the BCAA group. Although it was not significant, the placebo group had higher levels of IGF-1 at every time point and higher levels of insulin at 6 hours post-exercise when compared to the BCAA group. This might partially explain why the placebo group had higher levels of IRS-1 activation

than the BCAA group. It remains unclear why leucine alone had a greater effect on IRS-1 phosphorylation than did the BCAA group, in which both groups received the relative amount of leucine (60 mg/kg of body weight).

Akt

The current study also examined the effects of BCAA and leucine supplementation along with resistance exercise on Akt. As mentioned above, Akt is downstream of IRS-1 in the Akt-mTOR signal transduction pathway, and it appears that Akt has the ability to phosphorylate mTOR (Scott, Brunn, Kohn, Roth, & Lawrence, 1998; Sekulic et al., 2000). Moreover, an in vitro study specifically demonstrated that Akt can phosphorylate mTOR on Ser 2448 (Sekulic et al., 2000). Isolated rat muscle that was subjected to high frequency stimulation, which mimics the effects of resistance training did increase the phosphorylation of Akt at Ser 473 (Atherton et al., 2005). Also, rats that were subjected to an acute bout of resistance training have shown peak increases in phosphorylated Akt at Ser 473 approximately 10 minutes after the conclusion of exercise (Bolster, Kubica et al., 2003). As mentioned above, it does not appear that leucine or BCAAs seem to directly influence the proteins upstream of mTOR. Equally important, resistance training alone does not seem to influence the activity of Akt in humans (Coffey et al., 2006; Eliasson et al., 2006). However, one study did demonstrate an increase in Akt activation one hour following a resistance exercise bout, even though no significant increases were observed immediately after exercise or two hours following exercise (Dreyer et al., 2006). However, more research involving humans is needed to support these findings.

IGF-1 has been shown in cell culture studies to activate Akt and promote myotube hypertrophy (Rommel et al., 2001; Vyas, Spangenburg, Abraha, Childs, & Booth, 2002). The results of the current study demonstrated that there was a significant group main effect for Akt. Specifically, both the BCAA and leucine supplemented groups had significantly less phosphorylated Akt as compared to the placebo (Figure 4). Again, even though it was not statistically significant, at every time point where blood was sampled, IGF-1 levels were higher in the placebo group as compared to both the BCAA and leucine groups. This may have influenced the activity of the upstream proteins such as IRS-1 and PI-3 kinase, which in turn could increase the phosphorylation of Akt. If there was more IGF-1 binding to the insulin receptor in the placebo group, it may have lead to the observed results seen in Akt phosphorylation. There were no significant differences between the leucine and BCAA groups. Furthermore, there was no significant main effect for time or any significant interaction effects that were observed in this study (Figures 5-6).

Mammalian Target of Rapamycin (mTOR)

Mammalian target of rapamycin (mTOR) is another major protein kinase in the Akt-mTOR signal transduction pathway. In animal models it has been shown that leucine supplementation given to rats in fasted state can signal the downstream proteins 4E-BP1 and P70-S6K through a mTOR dependent fashion (J. C. Anthony et al., 2001; J. C. Anthony, Yoshizawa et al., 2000). In isolated rat muscle (non-fasted), high frequency stimulation, which mimics the effects of resistance training has also been shown to increase the phosphorylation of mTOR at Ser 2448 (Atherton et al., 2005). However, it has also been shown that rats subjected to an acute resistance training bout, had increased

activation of downstream proteins such as 4E-BP1 and P70-S6K without a concomitant increase in mTOR phosphorylation at Ser 2448 (Bolster, Kubica et al., 2003). This has led some to believe that 4E-BP1 and P70-S6K may be also activated in humans without a concomitant increase in phosphorylation of mTOR. Only a small number of studies to date have examined mTOR activation in humans in regards to resistance exercise or nutrition (Eliasson et al., 2006; Fujita et al., 2007). One study utilizing non-exercising humans demonstrated that leucine combined with carbohydrates can significantly increase mTOR signaling to a greater extent than the placebo (Fujita et al., 2007). However, the actual impact of leucine alone could have been made if the investigators would have utilized another experimental group without the confounding effects of carbohydrates. Eliasson et al. (2006) investigated different resistance exercise intensities and contractions without nutritional intervention and reported that mTOR may not be directly influenced by resistance exercise alone.

The results of this current study yielded that there were no significant main effect for group or time in regards to mTOR phosphorylation (Figures 7-8). Equally important, there were no significant group x time interactions as well (Figure 9). Simply stated, the data suggests that when combined with an acute bout of resistance exercise, both BCAAs and leucine ingestion (120 mg/kg and 60 mg/kg respectively), did not seem to impact the phosphorylation of mTOR to a greater extent than the placebo. Even though mTOR increased in humans in the study by Eliasson et al. (2006) mentioned above, it is not an equivalent comparison, because the participants in that study were also administered carbohydrates which confounds the results. As briefly discussed above, it may be

possible, however, to stimulate either 4E-BP1 or P70-S6K through a mTOR independent mechanism.

4E-BP1

Another downstream target of mTOR is 4E-BP1. The effects of 4E-BP1 on translation initiation have been studied in animal models (J. C. Anthony, Lang et al., 2002; J. C. Anthony, Reiter et al., 2002; J. C. Anthony, Yoshizawa et al., 2000; Vary et al., 2007; Vary et al., 1999). However, less has been done in humans with regards to resistance training (Dreyer et al., 2006; Fujita et al., 2007; Koopman et al., 2006). It has been shown that 4E-BP1 has the ability, when hyperphosphorylated, to prevent the binding of eIF4E and ultimately promote translation initiation. Conversely, when 4E-BP1 is hypophosphorylated, translation initiation can be suppressed. The current study measured 4E-BP1 phosphorylation in response to an acute bout of resistance exercise combined with BCAA or leucine administration. Dreyer et al. (2006) reported that subjects who participated in an acute bout of resistance exercise had decreased levels of phosphorylated 4E-BP1 at Thr 37/46 immediately following exercise. They attributed this decrease to a concomitant increase in AMP-activated protein kinase (AMPK). However, the levels of phosphorylated 4E-BP1 slightly increased (non-significantly) at the 2 hour post-exercise time point. Furthermore, the investigators speculate that the decrease in protein synthesis observed during resistance exercise can be attributed to these two aforementioned findings (Dreyer et al., 2006). However, no amino acid supplement was utilized in this aforementioned study. Another study mentioned earlier by Fujita et al. (2007), also measured 4E-BP1 in non-exercising humans. The authors reported that 1 hour following the consumption of a leucine enriched essential amino

acid-carbohydrate mixture, 4E-BP1 and other variables were significantly increased as compared to the non-caloric placebo. Even though, their study is one of the few that has measured 4E-BP1 in humans that have consumed leucine, the present study can not be directly compared to the current study because their subjects also consumed carbohydrates which can confound the comparison.

In relation to the current findings, the following results in 4E-BP1 were observed. There was a significant main effect for group (Figure 10). Specifically, the placebo group had significantly lower levels of phosphorylated 4E-BP1 as compared to both the BCAA and leucine group. Furthermore, there were no significant differences between the BCAA and leucine group with respect to the group main effect, even though the BCAA group had slightly higher levels of phosphorylated 4E-BP1 than the leucine group. This intuitively makes sense because the same amount of leucine is in both the BCAA and leucine groups. This data suggest that BCAA and leucine alone may have helped attenuate the decrease in phosphorylated 4E-BP1 seen in the placebo group due to resistance exercise. The current findings align themselves with Dreyer's (2006) results suggesting that resistance exercise may lead to a transient decrease in phosphorylated 4E-BP1. No main effect for time was observed for 4E-BP1 (Figure 11). However, over time, although not significant, 4E-BP1 phosphorylation mildly decreased until 2 hours post exercise and then slightly increased at 6 hours following exercise. Interestingly, there was, however, a significant group x time interaction with respect to phosphorylated 4E-BP1 (Figure 12). The results demonstrated that at 2 hours post, the phosphorylation levels of 4E-BP1 in both the BCAA and leucine group were significantly greater than the placebo group. By 6 hours post exercise the BCAA group was significantly greater than

the leucine and placebo group. Hence, these results suggest that in the initial stages of recovery from resistance exercise both leucine and BCAAs may have attenuated the decrease in the phosphorylated 4E-BP1 to a greater extent than placebo. Interestingly, by 6 hours following resistance exercise, individuals in the BCAA group had significantly higher levels of phosphorylated 4E-BP1 than the placebo or leucine groups. This data suggests that the other two BCAAs, isoleucine and valine may also help prevent the decline in phosphorylated 4E-BP1. Anthony, Yoshizawa, et al. (2000) reported that when fasted rats were orally administered leucine, isoleucine, or valine, leucine led to the greatest levels of phosphorylated 4E-BP1. However, they also reported that to a lesser extent, that isoleucine also phosphorylated 4E-BP1 (Anthony, Yoshizawa, et al., 2000). Therefore, it may be the effect of isoleucine in combination with leucine that led to the results that were observed in this study at the 6 hour post-exercise time point.

P70-S6K

Along with 4E-BP1, P70-S6K is another downstream target of mTOR. P70-S6K has the ability to activate/ phosphorylate ribosomal protein S6 (Coffey et al., 2006; Karlsson et al., 2004). When this occurs, it has been shown to contribute to an increase in translation initiation (Bolster, Kimball et al., 2003). Several animal models have examined the effects of leucine administration on P70-S6K (J. C. Anthony, Reiter et al., 2002; J. C. Anthony, Yoshizawa et al., 2000; Yoshizawa, Hirayama, Sekizawa, Nagasawa, & Sugahara, 2002). The preponderance of animal research demonstrates that leucine appears to increase the phosphorylation of P70-S6K (J. C. Anthony, Reiter et al., 2002; J. C. Anthony, Yoshizawa et al., 2000; Yoshizawa et al., 2002). However, only a few studies, in humans, have examined the effects of resistance exercise combined with

and without nutritional intervention on P70-S6K (Coffey et al., 2006; Eliasson et al., 2006; Karlsson et al., 2004; Koopman et al., 2006). Karlsson et al. (2004) reported significant increases in P70-S6K phosphorylation at Thr 389 during the recovery period, following an acute bout of lower extremity resistance exercise, in those who consumed the BCAA supplement (100 mg/kg of body weight = 45% leucine, 30% valine, and 25% isoleucine). However, resistance exercise alone did not alter P70-S6K phosphorylation at Thr 389. In a study by Coffey et al. (2006) investigators reported that resistance trained males, after an overnight fast, that performed eight sets of five repetitions on a Kin Com dynamometer showed no increases in P70-S6k phosphorylation. However, when aerobic trained males performed the same protocol, a significant increase in P70-S6k phosphorylation was observed (Coffey et al., 2006). The exact same pattern that was observed in P70-S6K phosphorylation was mirrored in the phosphorylation of S6 protein as well (Coffey et al., 2006). The participants in this present study were recreationally trained, meaning they were physically active, but they did not participate in a regular resistance exercise regimen.

The results of the present study demonstrate that there was no significant main effect for group in regards to P70-S6K (Figure 13). In other words, the administration of BCAA or leucine in this present study did not significantly change the phosphorylated states of P70-S6K to a greater extent than the placebo. There was, however, a significant main effect for time (Figure 14). A significant decrease in phosphorylated P70-S6K was observed at 6 hours into the recovery period as compared to baseline. Also a significant trend was observed between the 30 minute post and 6 hour post exercise. These results suggest that there was a decline in P70-S6K phosphorylation in the recovery period

following the resistance training. Lastly, there were no significant group x time interactions observed for P70-S6K (Figure 15).

This study yielded results that contradict those found in a similarly designed study by Karlsson et al.(2004). Our results demonstrated that P70-S6K [pT389] actually decreased in all groups (BCAA, leucine, and placebo), although non-significantly, in the 2 hour recovery period following exercise, where the Karlsson (2004) study demonstrated increases in P70-S6K [pT389] at 2 hours into the recovery period for those who consumed BCAAs. Furthermore, P70-S6K [pT389] decreased significantly by 6 hours post exercise, where as the Karlsson (2004) study stopped measuring at the 2 hours following exercise. Other human studies have measured P70-S6K phosphorylated at other sites such as Ser 424/Thr 421 (Koopman et al., 2006) and without nutritional intervention (Coffey et al., 2006; Koopman et al., 2006). However, it is difficult to compare the results of this current study to the studies by Koopman et al.(2006) and Coffey et al (2006)due to the fact that they did not measure P70-S6K based upon both BCAA or leucine ingestion combined with resistance exercise. Thus, more research needs to be conducted to clarify the response of P70-S6K following resistance training combined with BCAAs or leucine alone.

Conclusions

Therefore, in the current investigation both upstream and downstream proteins in the Akt-mTOR signal transduction pathway in response to concomitant amino acid consumption and resistance exercise were examined. The results of our study, in regards to P70-S6K, are somewhat equivocal to the research that has been reported in the past in a similar study design by Karlsson et al (2004). The fact that 4E-BP1 phosphorylation

was elevated in both the BCAA and leucine groups gives credence to the possible rationale in supplementing with these amino acids around resistance training. However, more acute research examining humans is needed in this area to further elucidate these pathways that regulate protein synthesis. Furthermore, longer duration training studies are also needed to study the chronic hypertrophic effects that have been observed only at an acute level to this point.

APPENDICES

APPENDIX A

Personal Demographic Form

Baylor University

Exercise and Sport Nutrition Laboratory

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

BAYLOR UNIVERSITY
ESNL
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

Informed Consent

BAYLOR UNIVERSITY

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

Principal Investigator: Darryn S. Willoughby, PhD, FACSM, FISSN, CSCS
Associate Professor, Department of HHPR, Baylor University

Co-investigators: Bill Campbell, PhD (c), CSCS
Exercise and Biochemical Nutrition Lab, Baylor University

Paul La Bounty, PhD (c), MPT, CSCS,
Exercise and Sport Nutrition Lab, Baylor University

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

IRB Proposal

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.

09/10/05

Signature of Principal Investigator 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

Muscle Biopsy Wound Care

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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