DISSERTATION

# POST EXERCISE PROTEIN FEEDING DOES NOT ALTER MOLECULAR MARKERS OF TRANSLATION INITIATION OR MEASURES OF SKELETAL MUSCLE FUNCTION

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY NICOLE R. STOB ENTITLED POST EXERCISE PROTEIN FEEDING DOES NOT ALTER MOLECULAR MARKERS OF TRANSLATION INITIATION OR MEASURES OF SKELETAL MUSCLE FUNCTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

POST EXERCISE PROTEIN FEEDING DOES NOT ALTER MOLECULAR MARKERS OF TRANSLATION INITIATION OR MEASURES OF SKELETAL MUSCLE FUNCTION

Resistance exercise stimulates skeletal muscle protein synthesis (SMPS) *and* breakdown; the pathway that predominates post-exercise appears to be influenced by nutrient intake before, during, and following exercise. Accordingly, insulin and amino acids have been shown to stimulate SMPS, appearing to activate proteins involved in translation initiation termed eukaryotic initiation factors (eIFs) (Jefferson & Kimball, 2003; Kimball, et al., 2002). An increase in translation initiation appears to be characterized by increased phosphorylation of eIF4E binding protein-1 (4EBP1) and decreased phosphorylation of eIF2$\alpha$ (Jefferson & Kimball, 2003). This study was undertaken to determine the effect of ingesting carbohydrate (CHO) or carbohydrate+protein (CP) on the phosphorylation of skeletal muscle 4EBP1 and eIF2$\alpha$ following eccentric exercise. CP ingestion was hypothesized to significantly increase the phosphorylation of 4EBP1 and decrease the phosphorylation of eIF2$\alpha$ in comparison to CHO. Thirty-six untrained adult

males performed eccentric leg extensions at 130% of their concentric one-repetition maximum followed by consumption of water (CON), CHO, or CP. There was no significant time effect (4EBP1 p=.15, eIF2$\alpha$ p=.49) or treatment effect (4EBP1 p=.43, eIF2$\alpha$ p=.46) for phosphorylation of 4EBP1 or eIF2$\alpha$, nor was there a significant time by treatment interaction (4EBP1 p=.44, eIF2$\alpha$ p=.69). Therefore, the ingestion of CHO or CP following intense eccentric exercise does not appear to alter the phosphorylation of 4EBP1 or eIF2$\alpha$.

Eccentric exercise typically induces delayed onset muscle soreness (DOMS) within 24 hours. Several methods of preventing and/or treating DOMS have been proposed; no single method has proven effective, however. In this area, nutrition has not been extensively studied. This study investigated the affect of post-exercise nutrition (CON, CHO, or CP) on muscle soreness, maximal leg extension, and vertical jump following eccentric exercise. It was hypothesized that the CP group would improve the time-course of recovery from eccentric exercise when compared with the CHO or CON groups. Despite significant time effects for skeletal muscle soreness, strength, and vertical jump (soreness and strength p<.001, vertical jump p=.01), no differences were observed between the treatment groups for these variables. Therefore, post-exercise

nutrition may not be effective in alleviating DOMS or promoting recovery of muscular performance.

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# CHAPTER 1

# INTRODUCTION

In the body, protein serves several different purposes. Many body tissues, including skeletal muscle and organs, are comprised largely of protein; proteins also make up enzymes, many hormones, receptors, transporters, and cell structure (cytoskeleton and extracellular matrix). In addition, amino acids can provide energy to the body under certain circumstances. Inasmuch as there is no "energy" storage site for protein in the body (unlike triglyceride and glycogen), proteins are constantly in a state of flux. One to two percent of total body protein is turned over each day in the human body (Murray, Granner, Mayes, & Rodwell, 2000) and dietary amino acids not readily used in biosynthetic pathways are quickly oxidized. Accordingly, developing an understanding of the regulation of whole body protein balance has implications both for athletes and in clinical populations with disorders of energy and/or protein metabolism.

## Skeletal Muscle Protein Balance

Skeletal muscle protein balance is the sum of synthesis and degradation and is affected by several stimuli including fasting, feeding, exercise, aging, and hormonal influences. Under normal conditions, the body is able to keep this balance tightly regulated (Liu & Barrett, 2002). At the molecular level, an

increase in skeletal muscle protein synthesis is indicated by the activity of several eukaryotic initiation factors (eIFs) in the muscle. It is known that both insulin and amino acids stimulate the phosphorylation of eIF4E binding protein-1 (4EBP1) by mammalian target of rapamycin (mTOR). This results in the dissociation of 4EBP1 from eIF4E and the union of eIF4E and eIF4G, resulting in the formation of eIF4F (Figure 1).

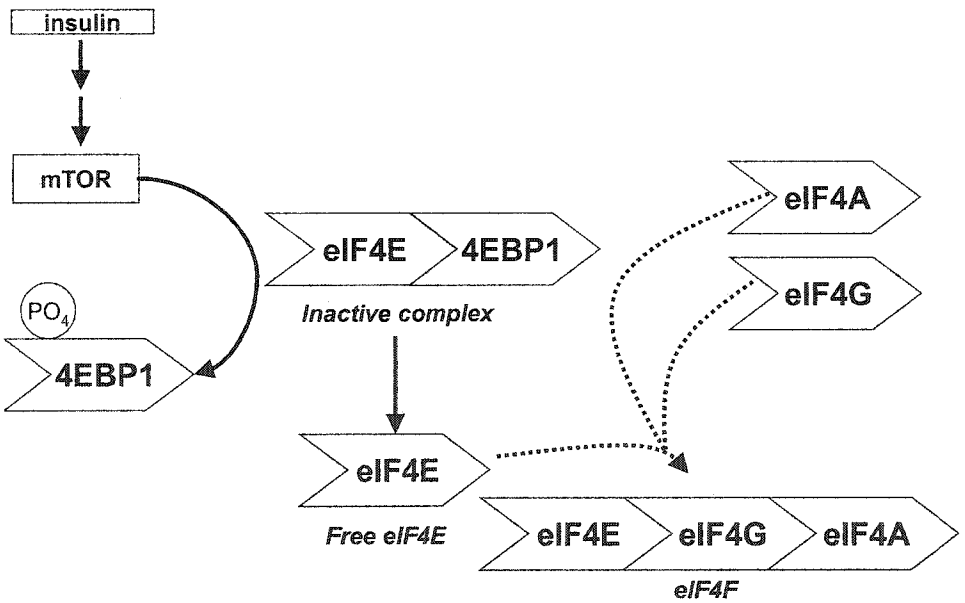



Figure 1: Formation of eIF4F. mTOR=mammalian target of rapamycin; $PO_4$=phosphorylate; eIF=eukaryotic initiation factor; 4EBP1=eukaryotic initiation factor 4E binding protein 1.

The end result is increased formation of eIF4F, which is ***necessary*** for increased skeletal muscle protein synthesis. An additional point of control for translation initiation involves eIF2 (Figure 2).

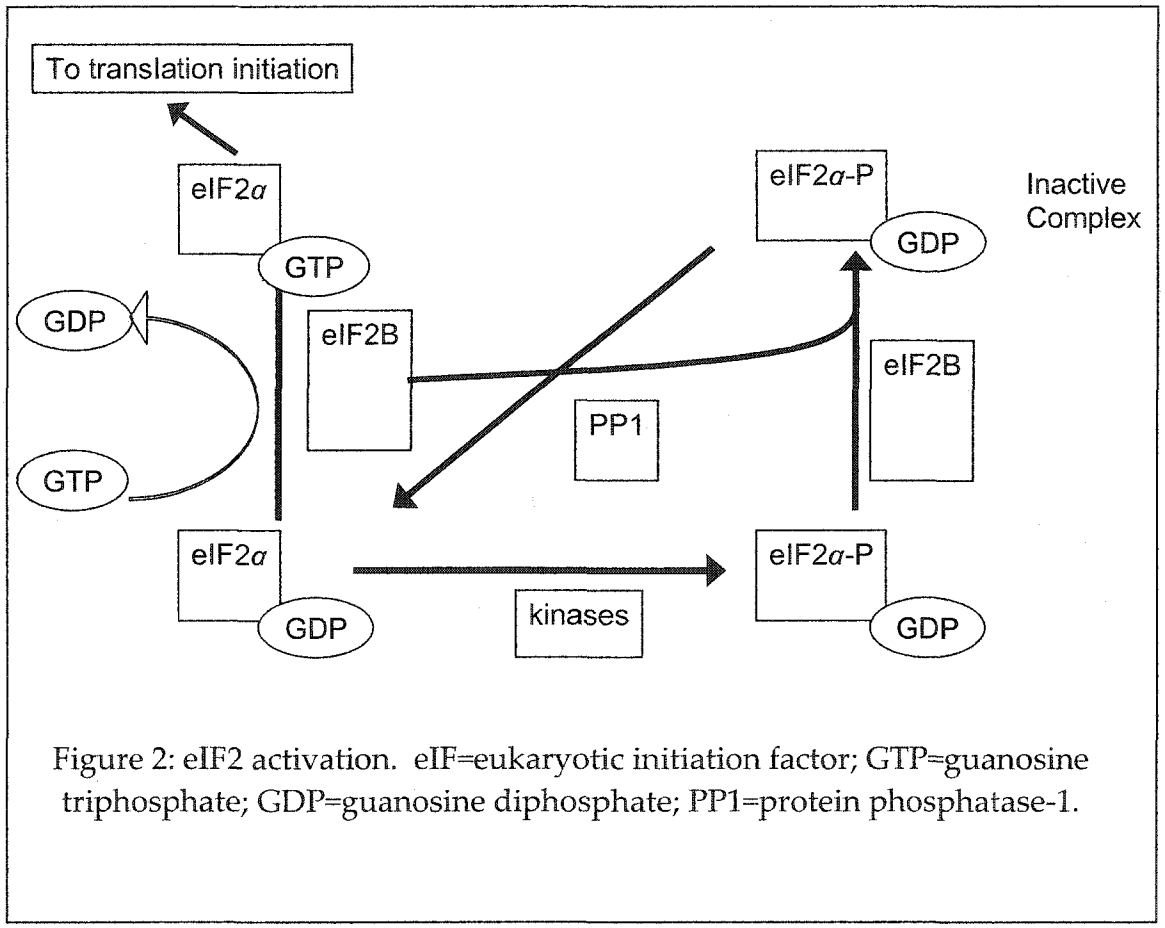



Figure 2: eIF2 activation. eIF=eukaryotic initiation factor; GTP=guanosine triphosphate; GDP=guanosine diphosphate; PP1=protein phosphatase-1.

Phosphorylation of the alpha ($\alpha$) subunit of eIF2 causes it to inhibit its former substrate, eIF2B. As a result, the ternary complex of methionyl-tRNA, eIF2, and GTP cannot enter translation initiation and protein synthesis is depressed. Thus, phosphorylated 4EBP1 can be taken as a molecular marker that

translation initiation has been activated, while phosphorylated eIF2$\alpha$ reflects inhibited translation initiation. Translation initiation, however, involves multiple proteins aside from these; therefore, a comprehensive look at translation initiation should involve more than 4EBP1 and eIF2$\alpha$.

Resistance exercise stimulates both skeletal muscle protein synthesis and breakdown, and the pathway that predominates after a bout of resistance exercise appear to be influenced by nutritional practices (Biolo, Fleming, Maggi, Wolfe, 1995a). Much remains to be discovered, however, about the specifics of post-exercise nutrition to optimize skeletal muscle protein synthesis. In particular, details on timing, energy, and macronutrient content of post-exercise meals all remain to be answered.

In addition to changes in skeletal muscle protein metabolism, resistance exercise can also induce skeletal muscle soreness. After a prolonged time off or with the presentation of a new exercise, nearly all athletes experience some skeletal muscle soreness. Eccentric muscle action, the lengthening of the muscle when resistive forces are applied, typically results in delayed onset muscle soreness (DOMS). DOMS can present as minor muscle stiffness to pain that restricts movement and may develop in parallel with strength loss (Cheung, Hume, & Maxwell, 2003). DOMS is proposed to be a result of several different factors including lactic acid build up in the muscle, muscle damage, connective

tissue damage, inflammation, muscle spasm, and enzyme efflux (Cheung, et al., 2003).

Several methods of preventing or relieving DOMS have been investigated; surprisingly, the influence of post-exercise nutrition on DOMS and strength recovery after eccentric exercise has not been extensively studied. In the current study, the influence of the intake of a protein and carbohydrate beverage immediately following and 30 minutes post-exercise on DOMS and strength recovery is investigated.

Specific Aims and Hypotheses

*Specific Aim #1:*

Determine the effect of post-exercise protein and carbohydrate feeding, in comparison with a carbohydrate feeding and a water control, on the phosphorylation state of selected members of the eukaryotic initiation factor (eIF) family.

*Hypothesis #1:*

Feeding protein and carbohydrate following a single bout of exhaustive eccentric exercise will result in a greater phosphorylation of 4EBP1 and a decreased phosphorylation of eIF2$\alpha$. This implies that there will be a greater interaction of eIF4E with eIF4G (active complex) relative to the eIF4E-4EBP1

(inactive complex) when compared to feeding only carbohydrate or a water control. In addition, the entry of the ternary complex into translation initiation will not be inhibited by phosphorylated eIF2$\alpha$. This is taken to mean that protein feeding will stimulate the formation of a more active protein translation complex at the ribosome, an essential molecular component to protein synthesis as well as promote the synthesis of the 43S subunit necessary for the progression of translation initiation.

*Specific aim #2:*

Determine the affect of a protein and carbohydrate feeding, in comparison with a carbohydrate feeding and a water control, on subjective assessments of muscle soreness 24, 48, 72, and 96 hours after a single bout of exhaustive eccentric exercise and single-leg one-repetition maximum leg extension performance and vertical jump performance 48 and 96 hours after a single bout of exhaustive eccentric exercise.

*Hypothesis #2:*

Feeding protein and carbohydrate after a single bout of exhaustive eccentric exercise will decrease subjective muscle soreness, increase single-leg one-repetition maximum leg extension performance, and increase vertical jump

performance earlier than with the consumption of a carbohydrate beverage or a water control.

# CHAPTER II

## LITERATURE REVIEW

When dietary protein is consumed, the peptide bonds are hydrolyzed, yielding its constituent amino acids which can meet several fates in the body (Murray, et al., 2000; Figure 3).

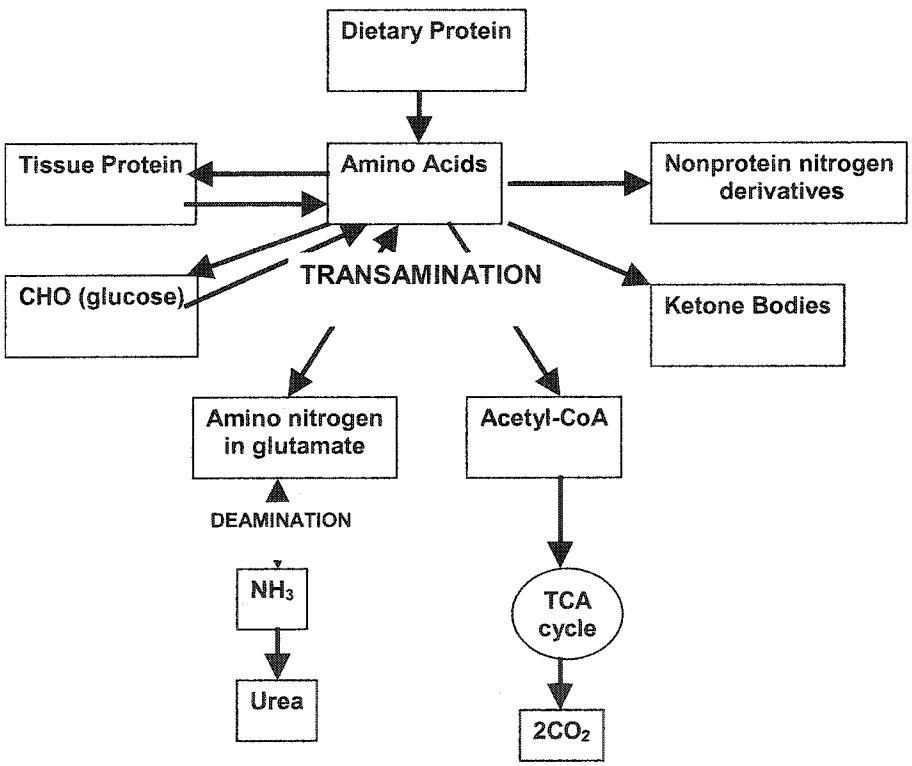



Figure 3: Protein Metabolism. CHO=carbohydrate; TCA=tricarboxylic acid; $CO_2$=carbon dioxide; CoA=conenzyme A.

Adapted from Murray, Granner, Mayes, & Rodwell. (2000). Harper's Biochemistry.

Amino acids cannot be stored in the body, unlike glucose and free fatty acids, the breakdown products of carbohydrate and fat. That is, glucose and free fatty acids can be stored in the body as glycogen and triglycerides, respectively, in muscle, liver, and adipose tissue; these stored nutrients can be utilized as energy sources in times of need. While all tissues have small pools of free amino acids, protein is unique among the three macronutrients in that there is no storage site for dietary protein that can be thought of as at least temporarily metabolically inert (as would be the case for stored trigyceride or glycogen, which solely serve the purpose of a storage form of fuel). Instead, ingested protein is primarily utilized to form new cellular proteins, enzymes, and other protein compounds (Brosnan, 2003). Amino acids may be converted to acetyl-CoA (or other intermediates that can enter the tricarboxylic acid cycle in anaplerotic reactions) in tissues that need to produce energy. When there is a need for glucose in the body, gluconeogenic tissues (i.e., liver and kidney) can convert the carbon skeletons of amino acids to glucose by way of gluconeogenesis. Ketone bodies can also be produced from amino acids in order to fuel the brain during prolonged fasting/starvation. Excess amino acids may be deaminated and the nitrogen-containing portions excreted in the urine as urea; amino acids may also contribute to formation of other nitrogen-containing substances such as purines (nucleotides), hexosamine products, et cetera. Finally, tissue formation may be

the fate of amino acids; in this case, the formation of skeletal muscle tissue is of interest. Interestingly, selected amino acids (leucine in particular) can act not only as substrates for tissue formation but as signals promoting skeletal muscle protein synthesis as well (Liu, Jahn, Wei, Long, & Barrett, 2002).

In order for amino acids to be used as substrates, they must travel by way of arteries to skeletal muscle and be taken up by the tissue (Figure 4).

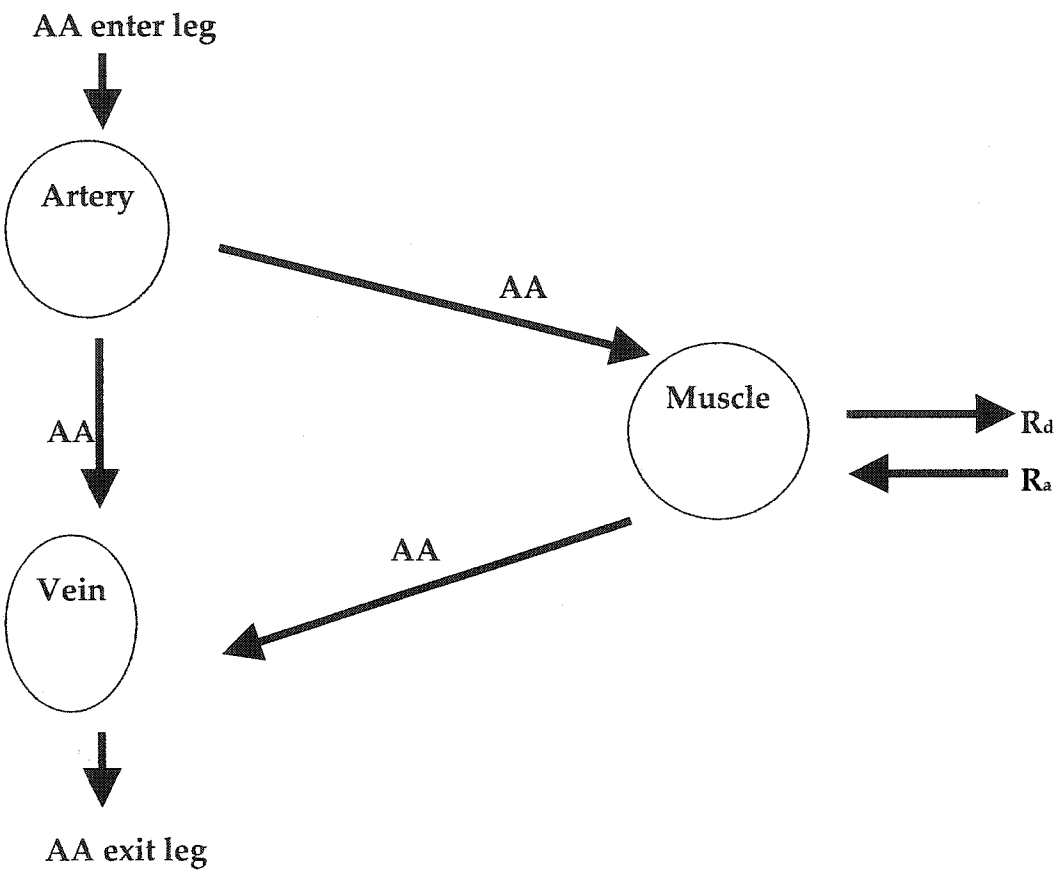



Figure 4: Amino Acid Kinetics. AA=amino acids; $R_d$=rate of disappearance; $R_a$=rate of appearance.

Adapted from Biolo, et al. (1995). Transmembrane transport and intracellular kinetics of amino acids in human skeletal muscle. American Journal of Physiology. 268:E75-E84.

When amino acids are used for protein synthesis in skeletal muscle, the rate of disappearance ($R_d$) of amino acids from the blood increases in comparison to a state in which protein synthesis is not stimulated. During a situation in which

protein degradation is taking place, the rate of appearance ($R_a$) of amino acids from muscle into the blood will increase.

In addition to their obvious role as substrates for protein synthesis, recent research suggests that amino acids, most notably leucine, stimulate translation initiation, the pivotal first stage of protein synthesis, via a rapamycin sensitive pathway (Jefferson & Kimball, 2001; Nygren & Nair, 2003; Wolfe, 2001). Rapamycin is a pharmacological probe which inhibits the activity of the mammalian target of rapamycin (mTOR), a serine/theronine kinase involved in intracellular phosphorylation cascades in response to a variety of hormonal stimuli. The sensitivity of amino acid-induced translation initiation to rapamycin provides evidence that amino acids stimulate translation initiation via mTOR. Substrates (or targets) for mTOR include p70S6kinase and 4EBP1, both of which increase the rate of translation initiation by altering the state of several eIFs involved in protein synthesis (Lawrence, 2001). Thus, several of the molecular mediators of the amino acid induced increase in translation have been identified, although few direct measurements of these have been made in human skeletal muscle (Liu, et al., 2002).

Timing of Amino Acid Intake

It is well known that post-exercise glycogen resynthesis is most efficient if carbohydrate is replenished within two hours after the exercise bout (Ivy, Katz, Cutler, Sherman, & Coyle, 1988). Recent work suggests that this phenomenon may apply to protein as well (Wolfe, 2002). That is, skeletal muscle protein synthesis may be maximized following exercise if amino acids are provided to the muscle within a specific window of time. Esmarck, Andersen, Olsen, Mizuno, and Kjaer (2001) investigated the degree of muscular hypertrophy after 13 weeks of resistance training in elderly men. Subjects ingested an amino acid supplement immediately after or two hours following each exercise session. The group ingesting the supplement immediately after exercise had a significant increase in cross sectional area and mean fiber area while the two hour group had no significant increases in these measures.

Following one hour of moderate intensity recumbent bicycling, subjects ingested an oral supplement (10g protein, 8g carbohydrate, 3g fat) immediately or three hours later (Levenhagen, Gresham, Carlson, Maron, Borel, & Flakoll, 2001). Essential and nonessential amino acids were taken up by the muscle when the supplement was ingested immediately after exercise, while they were released when the supplement was ingested three hours after exercise. Although both treatments stimulated protein synthesis in the leg and whole body, the

group ingesting the supplement immediately after exercise had a three-fold increase, while the three hour group only increased by 14%. These studies indicate that post-exercise protein supplementation may be beneficial for muscle protein recovery, but ingestion earlier after exercise, rather than later, appears to be more beneficial. While these studies are compelling, further work is needed in this area, particularly with reference to the regulation of translation initiation.

Therefore, as with glycogen resynthesis, the same amount and/or composition of amino acids ingested at different times during recovery from exercise does not appear to yield the same magnitude of protein synthesis. The specific time course of the proposed recovery window for protein remains to be elucidated. Moreover, the specific pathway(s) that may be sensitive to timing (i.e., stimulation of synthesis, inhibition of catabolism, skeletal muscle amino acid uptake) are also not known at this time (Kimball & Jefferson, 1988; Kimball & Jefferson, 1994).

Because there is not a clear mechanistic explanation for the affect of protein intake on skeletal muscle protein synthesis, it is important to investigate the pathways that may be utilized and how they may interact. It is proposed that amino acids and insulin influence skeletal muscle protein balance by different pathways which eventually converge and result in the phosphorylation

of mTOR, which leads to an activation of factors involved in skeletal muscle protein synthesis.

Liu, et al. (2002) infused a mixed amino acid solution into healthy males for a period of six hours. Prior to and after the infusion, the phosphorylation of protein kinase B (PKB/Akt), 4EBP1, and p70S6kinase were studied, along with forearm protein synthesis and degradation (via phenylalanine infusion). Protein synthesis was significantly increased after the infusion while protein degradation was unchanged. In parallel with the increase in skeletal muscle protein synthesis, the phosphorylation of both 4EBP1 and p70S6 kinase were significantly increased following the infusion; this provides evidence that measuring certain components of the pathways involved in protein synthesis (e.g., eIFs) may be sufficient to indicate the current state of protein metabolism in the muscle. Interestingly, the phosphorylation of PKB/Akt was not affected by amino acid infusion. Given that insulin administration does result in PBK/AKT phosphorylation, this observation suggests that amino acids may act through distinct proximal signaling steps, prior to the convergence with the insulin signaling cascade at mTOR. A more detailed look at both the insulin signaling and translation initiation pathways follow.

Regulation of Translation Initiation

Insulin is a hormone that is an important regulator of protein balance in skeletal muscle (Kimball & Jefferson, 1988; Mendez, Welsh, Kleijn, Myers, White, Proud, & Rhoads, 2001). Insulin serves to both inhibit protein breakdown and stimulate translation initiation (Nygren & Nair, 2003; Wolfe, 2001). Insulin stimulates translation initiation by a activating a serine-threonine kinase pathway, which ultimately phosphorylates mTOR. As discussed previously, the pathway by which insulin and amino acids activate mTOR are likely distinct from one another, although much remains to be elucidated. In addition, insulin has been shown to decrease the phosphorylation of eIF2$\alpha$, thereby permitting entry of the methionyl-tRNA into the translation initiation pathway. Insulin decreases skeletal muscle protein breakdown by inhibiting the release of amino acids from the muscle tissue into the blood (Gelfand & Barrett, 1987). Overall, the fed state (characterized by high amino acid and insulin levels) promotes amino acid flux from the intestine to other tissues while in a starved state, amino acids are shuttled from muscle to the kidney and liver for the primary purpose of serving as gluconeogenic precursors (Figure 5).

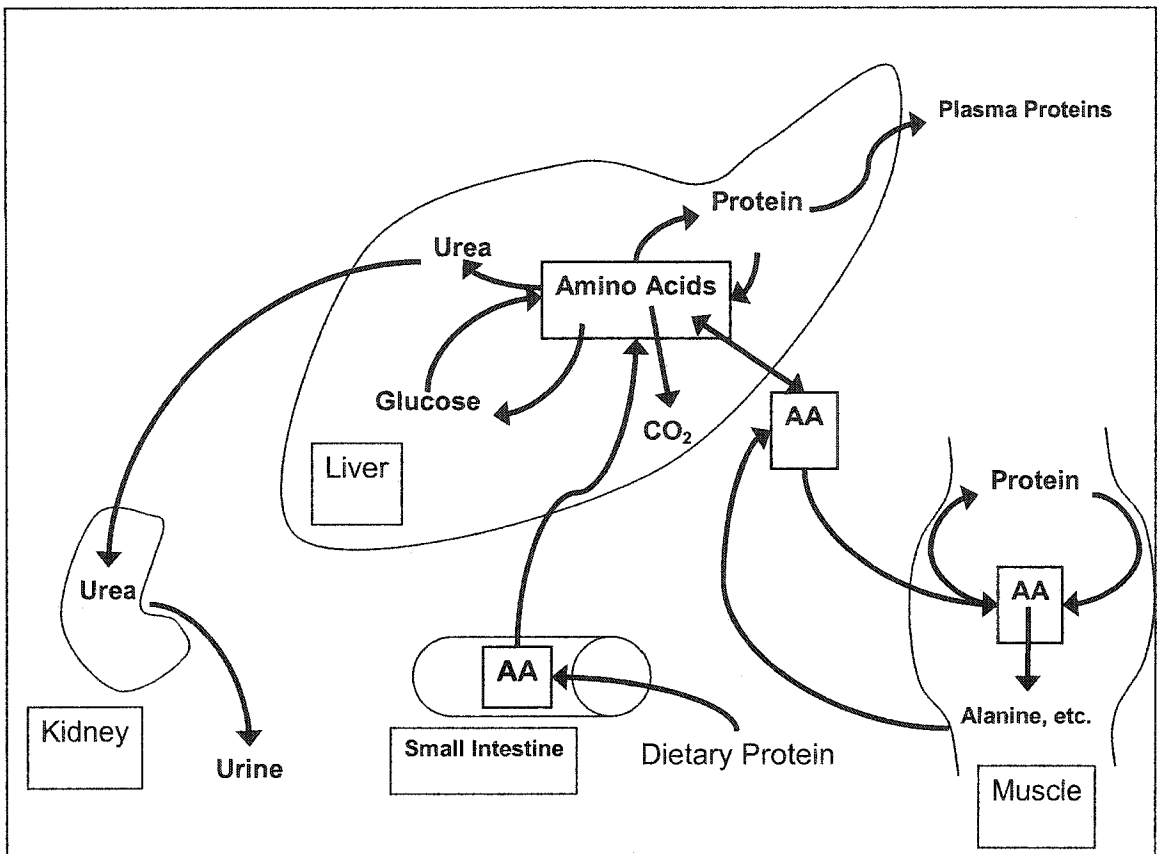



Figure 5: Interorgan Amino Acid Transport and Metabolism. AA=amino acids; $CO_2$=carbon dioxide.

Adapted from Murray, Granner, Mayes, & Rodwell. (2000). Harper's Biochemistry.

Insulin Signaling Pathway

The insulin signaling pathway (Figure 6) begins with insulin binding to the extra cellular $\alpha$-subunit of the insulin receptor, after which several actions of the insulin binding can result: glucose uptake into cells, inhibition of lipolysis, regulation of growth and differentiation of cells, gene activation and transcription, and synthesis of lipid, glycogen, or protein (Taha & Klip, 1999).

The pathway that leads to the synthesis of protein, namely translation initiation, is the focus of the following discussion.

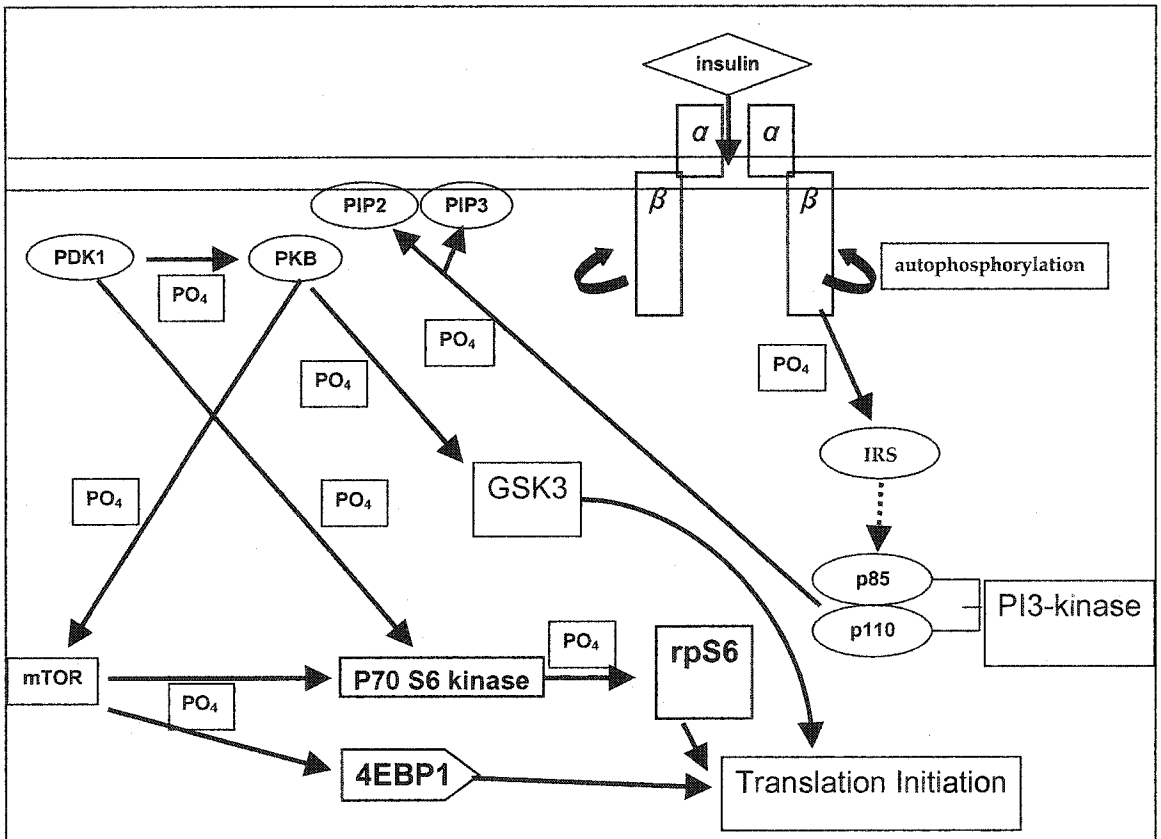



Figure 6: Insulin Signaling Pathway. See text for detailed description. α=alpha subunit of insulin receptor; β= beta subunit of insulin receptor; $PO_4$=phosphorylation; PIP=phosphatidylinositol phosphate; PDK=phosphoinositide dependent kinase; PKB=protein kinase B; GSK3=glycogen synthase kinase-3; mTOR=mammalian target of rapamycin; rpS6=ribosomal protein S6; 4EBP1=eukaryotic initiation factor 4E binding protein 1.

The binding of insulin to the insulin receptor results in autophosphorylation of tyrosine residues on the β-subunit of the receptor as well

as phosphorylation of insulin receptor substrate (IRS) proteins (Taha & Klip, 1999). IRS-1 then either binds to SH-2 domains of small proteins or docks on the p85 subunit of phosphatidylinositol-3 kinase (PI3-K) (Taha & Klip, 1999). The docking of IRS-1 activates PI3-K which allows for the phosphorylation of two phosphoinositides, creating phosphatidylinositol 3, 4, 5-triphosphate (PIP3) and phosphatidylinositol 3, 4-diphosphate (PIP2), which then activates downstream pathways, including translation initiation (Alessi & Downes, 1998).

These second messengers recruit phosphoinositide-dependent kinase (PDK1) and protein kinase B (PKB/Akt) to the membrane. PIP3 changes the conformation of PKB/Akt so that PDK1 can readily access the phosphorylation sites on PKB/Akt. Although the exact mechanism is unclear, PDK1 is also activated by PIP2 and PIP3 by interaction at the pleckstrin homology (PH) domain on PDK1. This situation then allows for PKB/Akt to be phosphorylated by PDK1 (Shah, Anthony, Kimball, & Jefferson, 2000). PI3-K can also activate two forms of protein kinase C (PKC$\zeta$ and PKC$\lambda$) which leads to translocation of GLUT4 to the membrane, facilitating glucose uptake into the cell, rather than leading to protein synthesis, thereby marking another divergence in the insulin signaling pathway (Taha & Klip, 1999).

With respect to protein synthesis, it is observed *in vitro* that PKB/Akt phosphorylates the mammalian target of rapamycin (mTOR) on serine(Ser)$^{2448}$,

activating it (Jefferson & Kimball, 2003; Shah, et al., 2000). mTOR then phosphorylates two separate intermediates, p70 S6 kinase/S6K1 (hereafter referred to as p70 S6 kinase) on threonine$^{389}$ (*in vitro*) and 4EBP1, creating yet another divergence in the pathway (Kimball, Farrell, & Jefferson, 2002). PDK1 also phosphorylates p70 S6 kinase, on threonine$^{229}$ (Alessi & Downes, 1998). Phosphorylated p70 S6 kinase then phosphorylates ribosomal protein (rp) S6 on several sites (Kimball, et al., 2002). Activation of rpS6 likely leads to increased translation of a class of mRNAs that have a terminal oligopyrimidine tract (TOP) sequence, which is approximately seven to 15 pyrimidine residues next to the 5′ cap structure (Jefferson & Kimball, 2003), although conflicting evidence has been provided regarding this function of rpS6 (Jefferson & Kimball, 2003). These mRNAs encode for proteins that are involved in mRNA translation, such as ribosomal proteins, eIF4G, PABP, and eukaryotic elongation factor-2. mTOR phosphorylation of 4EBP1 promotes its dissociation from another entity, eIF4E, which creates an environment favoring translation initiation.

PKB also phosphorylates glycogen synthase kinase 3 (GSK3), inactivating it. Active GSK3 phosphorylates eIF2Bε on serine$^{540}$, inactivating it and suppressing translation initiation. Therefore, PKB-mediated phosphorylation of GSK3 relieves its (GSK-mediated) inhibition of eIF2B. As a result, eIF2B is then able to catalyze the guanine nucleotide exchange on eIF2 and ternary complex

(met-tRNA-eIF2-GTP) formation can proceed. Thus, PKB simultaneously activates mTOR (which, in turn, activates p70S6 kinase and 4EBP1), while relieving GSK3 initiated inhibition of eIF2. The end result is an increased capacity for translation initiation.

Translation Initiation

Protein synthesis is made up of three phases, including translation initiation, elongation, and termination. Of the three phases, translation initiation is the pivotal step in protein synthesis; the degree to which translation initiation occurs determines the rate of overall protein synthesis (Raught & Gingras, 1999; Shah, et al., 2000).

Within translation initiation, there are three major steps involving several eukaryotic initiation factors (eIFs), including eIF1A, eIF2, eIF2B, eIF3, eIF4A, eIF4B, eIF4E, and eIF4G (Table 1, Figures 1 & 7).

Table 1: Selected eukaryotic initiation factors (eIFs) involved in translation initiation.

| eIF | Role |
|---|---|
| 1A | Stimulation of Met-tRNA and binding to 40S |
| 2 | Met-tRNA binding to 40S |
| 2B | GTP-GDP exchange on eIF2 |
| 3 | Ribosome dissociation, mRNA binding |
| 4A | RNA helicase, mRNA binding |
| 4E | Cap recognition, mRNA binding |
| 4G | Attach 4A & 4E, mRNA binding |

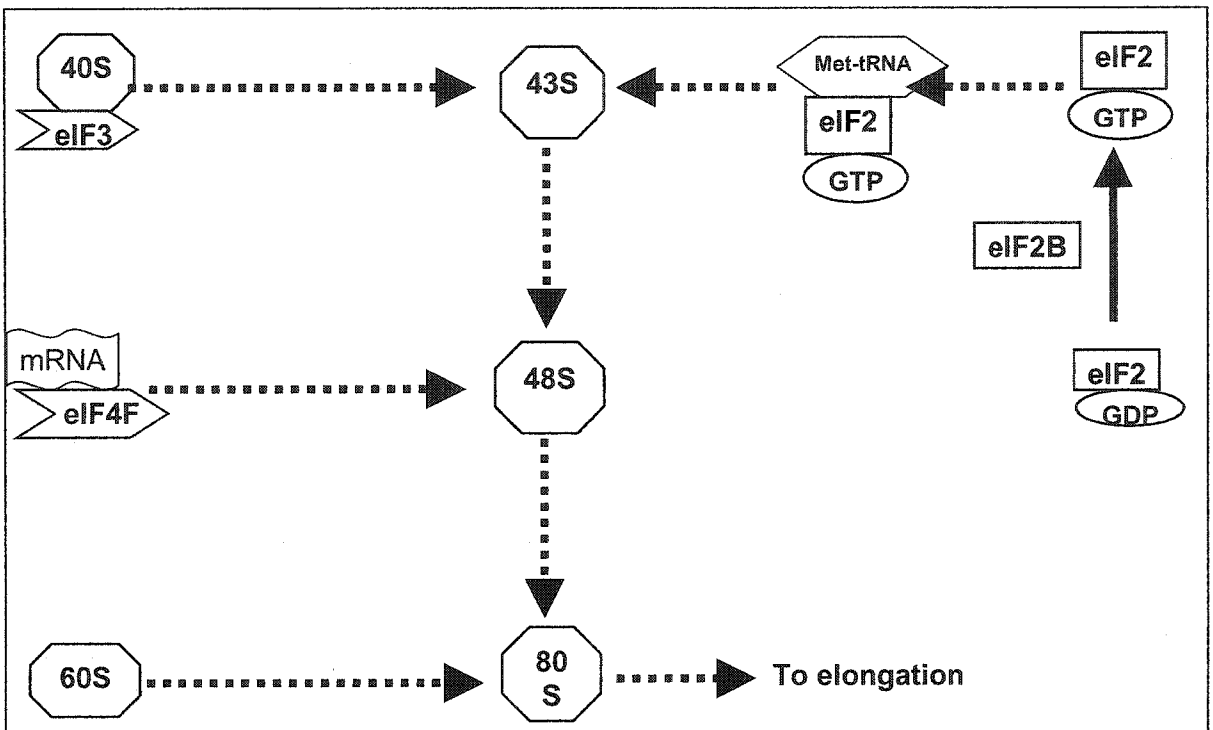



Figure 7: Translation Initiation. See text for detailed description. eIF=eukaryotic initiation factor; GTP=guanosine triphosphate; GDP=guanosine diphosphate; Met-tRNA=methionyl tRNA complex; mRNA=messenger RNA; S=subunit.

Adapted from: Shah, O. J., Anthony, J. C., Kimball, S. R., & Jefferson, L. S. (2000). 4E-BP1 and S6K1: translational integration sites for nutritional and hormonal information in muscle. American Journal of Physiology: Endocrinology and Metabolism, 279(4), E715-729.

To begin the process of translation initiation, the initiator methionyl tRNA (met-tRNA) must bind to the 40S ribosomal subunit. This is only accomplished in conjunction with eIF2, which transports met-tRNA to the subunit. eIF2 is only able to transport met-tRNA while associated with a GTP ligand; association with a GDP ligand renders eIF2 inactive. The exchange of GTP for GDP is catalyzed

by the guanine-nucleotide exchange factor eIF2B, thus making eIF2B an important regulator of translation initiation. However, when the alpha-subunit of eIF2 is phosphorylated by an eIF2$\alpha$ kinase, eIF2$\beta$ binds the $\delta$ and $\varepsilon$ subunits of eIF2B, inactivating it (Kimball, Heinzinger, Horetsky, & Jefferson, 1998). This renders eIF2B unable to catalyze GTP/GDP exchange; thus eIF2 is an inhibitor as well as a substrate of eIF2B (Figure 2). eIF2 is present in much larger quantities than eIF2B; therefore a small increase in the phosphorylation of eIF2$\alpha$ will result in a large increase in inactivated eIF2B (Asano, et al., 2001).

It has been shown in chinese hamster ovary (CHO) cells that components of the insulin signaling pathway can affect eIF2B (Welsh, Miller, Loughlin, Price, & Proud, 1998). That is, GSK3, an entity downstream of PI3-kinase, phosphorylates eIF2B$\varepsilon$ on serine$^{540}$, inactivating it. In opposition, insulin inactivates GSK3 by way of PI3-kinase, relieving the inhibition on eIF2B. Therefore, eIF2B is then able to catalyze the guanine nucleotide exchange on eIF2 and ternary complex (met-tRNA-eIF2-GTP) formation can proceed.

Once met-tRNA is associated with the 40S ribosomal subunit, mRNA must be recruited to bind to the newly formed 43S initiation complex. The recruitment of mRNA involves three eIFs: eIF4E, eIF4G, and eIF4A. These cumulatively make up eIF4F (Figure 1), which is the entity that binds to the met-tRNA complex; this step of ribosomal binding to the mRNA can be rate-limiting

in translation initiation (Haghighat, Mader, Pause, & Sonenberg, 1995; Raught & Gingras, 1999). Both the available quantity of eIF4E and the phosphorylation state of 4EBP1 determine whether eIF4F assembly occurs (Raught & Gingras, 1999). eIF4E is present in much smaller quantities (.01-.20 molecules/ribosome) than other eIFs (.50-3.0 molecules /ribosome). When translation is superfluous, eIF4E is bound by 4EBP1, and is unable to contribute to eIF4F formation. In response to translational stimuli (e.g., insulin or amino acids), 4EBP1 is phosphorylated by mTOR and dissociates from eIF4E, allowing it to interact with eIF4G.

Each component of eIF4F serves a distinct purpose and should be discussed. eIF4E is a 24-kDa polypeptide that was originally isolated as an $m^7$ cap binding complex referred to as cap binding protein I (Sonenberg, Ruprecht, Hecht, & Shatkin, 1979). All eukaryotic cellular mRNA are capped at the 5′ end by $m^7$GpppN cap structure (where N is any nucleotide), which serves as a recognition point for eIF4E interaction (Haghighat & Sonenberg, 1997; Scheper & Proud, 2002) (Figure 8).

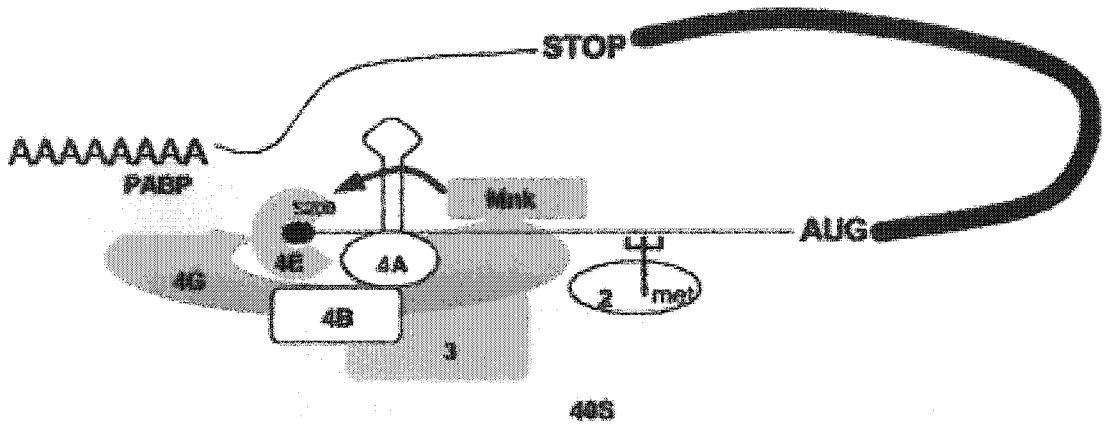



Figure 8: Assembly of eIF4F and the 5′-cap structure. PABP=poly(A)-binding protein; AUG=start codon; met=methionyl; S=subunit; Mnk= MAP kinase signal integrating kinase.

Source: Scheper & Proud. (2002). Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation? European Journal of Biochemistry, 269:5350-5359.

It is known that only capped mRNA are bound by eIF4E; uncapped viral RNAs are not (Sonenberg, et al., 1979). After the interaction of eIF4E with the cap, eIF4E recruits the translational machinery to the 5′ end of the mRNA (Raught & Gingras, 1999). A component of the machinery, eIF4G, then binds to eIF4E in a dorsal site overlapping the site previously occupied by 4EBP1. eIF4G is associated with eIF4A, the poly(A)-binding protein (PABP), eIF3, and MAP kinase signal integrating kinase (Mnk). The union of eIF4E with eIF4G and eIF4A results in eIF4F formation. The PABP binds to the N-terminus of eIF4G

and interacts with the mRNA on its poly(A)-tail, creating a circular structure out of the mRNA. eIF3 brings in the 40S ribosomal subunit and the met-tRNA complex with eIF2 and forms the 43S initiation complex when combined with eIF4F and mRNA. Mnk functions to phosphorylate eIF4E on serine[209]. The effect of the phosphorylation of eIF4E is a matter of debate. It has been reported that the phosphorylation both enhances and decreases the affinity of eIF4E for the mRNA cap structure, with more recent work suggesting the latter (Minich, Balasta, Goss, & Rhoads, 1994; Scheper, van Kollenburg, Hu, Luo, Goss, & Proud, 2002; Zuberek, et al., 2003). Mixed results have been obtained when studying eIF4E phosphorylation in relation to changes in other entities such as eIF4G and 4EBP1.

The bound mRNA is then unwound by another component of eIF4F, eIF4A, an ATP-dependent RNA helicase (Rozen, Edery, Meerovitch, Dever, Merrick, & Sonenberg, 1990). The unwinding allows binding between the 43S initiation complex and the mRNA, thus forming the 48S complex (Kleijn, Scheper, Voorma, Thomas, 1998). This process is stabilized by eIF3 preventing the interaction of the 40S and 60S subunits (Trachsel & Staehelin, 1979).

Further steps involve the addition of a 60S ribosomal subunit to the 48S subunit to form the 80S initiation complex and cleavage of several components formerly bound to the 48S complex, including the eIF2-GDP complex. This is

then reformed into the eIF2-GTP which is catalyzed by eIF2B (Figure 2). During the formation of the 80S initiation complex, eIF5 plays a role, and the 80S complex enters into the next step of protein synthesis, elongation (Kleijn, et al., 1998).

Regulation of eIF4F Formation

As stated previously, the phosphorylation state of 4EBP1 is a major determinant of the degree of formation of eIF4F (Figure 1). mTOR phosphorylation of 4EBP1 on threonine (Thr)$^{37}$ and Thr$^{46}$ allows further phosphorylation of 4EBP1 on Ser$^{65}$ and Thr$^{70}$ by an unidentified kinase, leading to an environment favoring the progression of translation initiation (Kimball, et al., 2002). It has been established that phosphorylation on Thr$^{37}$ and Thr$^{46}$ occurs first, followed by phosphorylation of Thr$^{70}$ and finally on Ser$^{65}$ (Gingras, et al., 2001). When 4EBP1 is hyperphosphorylated (4EBP1$\gamma$), it dissociates from eIF4E, allowing for the binding of eIF4G to eIF4E. The opposite, hypophosphorylated form of 4EBP1 (4EBP1$\alpha$), remains bound to eIF4E, inhibiting formation of eIF4F. 4EBP1 will not dissociate from eIF4E if phosphorylated on only Thr$^{70}$ or both Thr$^{70}$ and Ser$^{65}$; all four sites must be phosphorylated for dissociation to occur.

The phosphorylation state of 4EBP1 is affected by several variables including rapamycin, amino acids, and insulin. Rapamycin serves to inhibit the

phosphorylation of 4EBP1 through the inhibition of mTOR; in so doing, translation initiation is also inhibited. Conversely, amino acids and insulin both cause an increase in 4EBP1 phosphorylation, likely through mTOR, to increase rates of translation initiation.

The order in which the steps involved in the preparation for translation initiation as well as in translation initiation itself occurs has been debated; that is, whether eIF4F formation precedes the binding of eIF4E to the cap structure of the mRNA or if eIF4F is formed prior to the binding of eIF4E to the mRNA (Scheper, Voorma, & Thomas, 1992; Gingras, Raught, & Sonenberg, 1999). Despite differences of opinion in some areas, it is known that resistance exercise, amino acids, and insulin all influence skeletal muscle protein kinetics and should affect the state of eIFs in doing so (Kimball, et al., 2002).

Regulation of eIF2$\alpha$ Phosphorylation

A second mechanism by which translation initiation is regulated involves eIF2. It is known that the phosphorylation of the alpha subunit of eIF2 on serine$^{51}$ (serine$^{52}$ in humans) by one of four possible kinases causes a reduction in the rate of translation initiation by way of inhibition of eIF2B (Figure 2; Sood, Porter, Olsen, Cavener, & Wek, 2000; Sudhakar, Ramachandran, Ghosh, Hasnain, Kaufman, & Ramaiah, 2000). The affects of a variety of conditions have been

investigated in relation to eIF2$\alpha$ phosphorylation, although no measurements have been made in human skeletal muscle.

Male Sprague-Dawley rats took part in a resistance training protocol (detailed below) to determine effects on eIF2B activity and eIF2$\alpha$ phosphorylation (Bolster, Kubica, Crozier, Williamson, Farrell, Kimball, & Jefferson, 2003). At several points following the resistance exercise, no changes in eIF2$\alpha$ phosphorylation or eIF2B activity were observed; a significantly increased phosphorylation of 4EBP1 was observed, indicating that resistance exercise may affect translation initiation by way of the mTOR pathway rather than by way of eIF2.

The effects of starvation, insulin treatment, and diabetic conditions on eIF2 and eIF2B have also been investigated. Jeffrey, Kelly, Duncan, Hershey, & Pain (1990) investigated the effect of starvation and diabetic conditions in rats. After a 24 hour and a 48 hour fast, formation of the met-tRNA-eIF2-GTP ternary complex was decreased by 38% and 48%, respectively in comparison to a fed group ($p<.05$). Induction of diabetes also demonstrated a decrease in ternary complex formation, although this decrease was not significant due to marked variation between the rats. In order to explain these results, the ability of eIF2 to dissociate from GDP (indicating guanine nucleotide exchange activity) was also examined. As expected, the 48 hour fast decreased the dissociation of eIF2 and

GDP, which was restored with refeeding; no level of significance was noted. In addition, diabetic mice had decreased rates of dissociation in comparison with nondiabetic rats or diabetic rats chronically infused with insulin. Finally, the addition of eIF2B to starved rats markedly increased eIF2 and GDP dissociation while no change was seen in control rats.

The affect of insulin on eIF2B has been observed in Chinese hamster ovary (CHO) cells expressing the insulin receptor (Welsh, Stokes, Wang, Sakaue, Ogawa, Kasuga, Proud, 1997). Upon treatment with insulin, the activity of eIF2B doubled while GSK3 activity was decreased by 40%. When rapamycin, an mTOR inhibitor, was added in addition to the insulin, no changes were seen in the activity levels of eIF2B or GSK3 in comparison with insulin treatment only. This indicates that insulin's affect on eIF2B is independent of the mTOR pathway. The addition of wortmannin, a PI3-kinase inhibitor, decreased eIF2B and increased GSK3 activity to control levels, indicating that insulin's affect on eIF2B is PI3-kinase dependent. Interestingly, the level of eIF2$\alpha$ phosphorylation was not changed with insulin treatment, indicating that the activity of eIF2B in this study was modified by a mechanism other than that of eIF2$\alpha$ phosphorylation.

A later study in 1998 again observed the affect of insulin treatment on CHO cells (Welsh, Miller, Loughlin, Price, & Proud). As before, insulin

treatment increased the activity of eIF2B. Further study demonstrated that GSK3 phosphorylates eIF2B on its epsilon subunit, inactivating it. Administration of insulin causes the dephosphorylation of eIF2B and reverses the effect of GSK3. This indicates that insulin's affect on eIF2B (and protein synthesis) is dependent on PI3-kinase and is modulated through its inactivating influence on GSK3.

In 1999, Campbell, Wang, and Proud performed a study on CHO cells to determine if amino acids and/or glucose needed to be present for insulin to exert its effects on indicators of increased translation initiation. It was found that both glucose and amino acids were necessary for insulin to activate eIF2B; insulin doubled the activity of eIF2B when glucose and amino acids were present ($p<.005$ versus no insulin). In contrast, only amino acids were necessary for insulin to increase the phosphorylation of p70S6 kinase and 4EBP1. These results indicate that general mRNA translation may require the presence of both glucose and amino acids while the translation of specific mRNAs (mediated by p70S6 kinase) requires the presence of only amino acids.

Ehrlich ascites tumor cells were either fed or deprived of glutamine in a 1987 study by Scorsone, Panniers, Rowlands, and Henshaw. Leucine incorporation (an indicator of protein synthesis) and the percent of phosphorylated eIF2$\alpha$ present in the cells were measured. As expected, leucine incorporation was markedly higher in the fed cells at 20, 40, and 60 minutes after

the addition of leucine. In addition, the percent of phoshphorylated eIF2$\alpha$ was 22% in the fed cells and 37% in the amino acid deprived cells, indicating an inhibition of translation initiation in the latter, as would be expected with amino acid deprivation.

An early study by Cox, Redpath, and Proud (1988) investigated the effect of a 48-hour fast in rats on the phosphorylation of eIF2$\alpha$ and the activity of eIF2B. Despite a decrease in the rate of protein synthesis in the gastrocnemius, no changes in the activity of eIF2B or the phosphorylation of eIF2$\alpha$ were observed.

Rats were fasted for a period of 18 hours followed by refeeding of a complete diet to determine affects on translation initiation (Yoshizawa, Kimball, Jefferson, 1997). No significant changes were observed in the phosphorylation state of eIF2$\alpha$ or the activity of eIF2B, although an increase in the phosphorylation of 4EBP1 was observed. These studies support the idea that nutritionally-driven changes in skeletal muscle protein synthesis occurs by way of the mTOR pathway rather than by way of eIF2.

The affect of two amino acids, leucine and histidine, on eIF2B activity and eIF2$\alpha$ phosphorylation was investigated in L6 myoblasts (Kimball, Horetsky, & Jefferson, 1998). The cells were split into three groups: those maintained in a serum-free Dulbecco's modified Eagle's medium (DMEM), and those in either a histidine or leucine-free DMEM. Insulin was added to the medium along with

replacement of histidine or leucine to some of the cells which were initially deprived of them. The phosphorylation of eIF2$\alpha$ was significantly decreased when cells lacking leucine were provided with leucine ($p<.05$); the phosphorylation of eIF2$\alpha$ in those cells given leucine did not differ from the control that contained leucine for the duration of the study. Although not significant, there was a large decrease in the phosphorylation of eIF2$\alpha$ when histidine was provided to histidine-deprived cells; the phosphorylation level did not differ from the cells provided histidine for the duration of the study. The activity of eIF2B was significantly increased in the cells when amino acids were replaced; the increased eIF2B activity levels did not differ from the levels in cells provided the amino acids for the duration of the study. These results were accompanied by the observation that protein synthesis was significantly increased when amino acids were provided to previously deprived cells and synthesis levels did not differ significantly from control levels. This study provides evidence that, in culture, amino acids can stimulate protein synthesis by way of eIF2.

The affects of other factors on eIF2 have been investigated. Administration of dexamethasone, a glucocorticoid, resulted in a 59% decrease in rat skeletal muscle protein synthesis (Shah, Kimball, & Jefferson, 2000). No change was seen in the activity of eIF2B or the phosphorylation of eIF2$\alpha$; changes

in the phosphorylation of 4EBP1 were observed, however. In vitro, nitric oxide has been shown to decrease protein synthesis; concurrently an increase in the phosphorylation of eIF2$\alpha$ was observed (Kim, Son, Hong , Green, Chen, Tzeng, Hierholzer, & Billiar 1998). These studies imply that eIF2 may be a way in which many factors in the body affect protein synthesis.

Resistance Exercise and Skeletal Muscle Protein Kinetics

Resistance exercise is a profound physiological perturbation which stimulates significant increases in protein turnover by activating both protein anabolism and protein catabolism. The increased protein synthetic rate within minutes to hours post-exercise may be due to increased translation of mRNA already existing in the muscle; an increased rate seen hours to days post-exercise may be due to an elevation in the amount of mRNA available for translation initiation (Kimball, et al., 2002). The increased rate of skeletal muscle protein breakdown is proposed to be related to the increased rate of protein synthesis (Wolfe, 2001). That is, as skeletal muscle protein synthesis is occurring, the free amino acid pool likely provides substrates to synthesize protein since, after resistance exercise, no exogenous source of amino acids is available if feeding does not take place. To compensate for the depletion of the free amino acid pool,

skeletal muscle protein breakdown may take place in order to maintain the intracellular concentration of amino acids.

It has been well established that resistance exercise stimulates both skeletal muscle protein synthesis and breakdown. Biolo, Maggi, Williams, Tipton, & Wolfe (1995b) demonstrated this phenomenon in five untrained yet healthy males. Subjects underwent a resting protocol or a resistance exercise protocol consisting of five sets of 10 repetitions (5x10) of leg press at a 12-repetition maximum (12 RM) weight and 4x8 duo squat, leg curl, and leg extension at 10RM. The exercises were completed in an average of 40 minutes. Both before and after exercise, subjects were infused with phenylalanine and underwent a vastus lateralis biopsy. The standard method to measure skeletal muscle protein synthesis is by determining phenylalanine balance in the tissue of interest. This is accomplished by infusing labeled phenylalanine into the body and subsequently removing muscle tissue by muscle biopsy for determination of phenylalanine enrichment in the muscle. It is appropriate to use phenylalanine for this purpose as it is not synthesized nor is it catabolized in the muscle and will therefore reflect overall protein synthesis in the muscle (Borsheim, Tipton, Wolf, & Wolfe, 2002). Specifically, if phenylalanine enters the muscle ($R_a$), it must exit either via a vein or be incorporated into the muscle in the process of protein synthesis. Likewise, if unlabeled phenylalanine exits the muscle ($R_a$), it is

an indication of skeletal muscle protein breakdown. Immediately after exercise, skeletal muscle protein synthesis had increased (108%, $P<0.05$), as did proteolysis (51%, $p<.05$). The increase in synthesis is proposed to be due in part to an increased inward transport of amino acids into the muscle. As a result, muscle protein balance significantly increased overall ($p<.05$).

Sprague-Dawley rats have exhibited increased skeletal muscle protein synthesis following resistance training as well. Fluckey, Vary, Jefferson, and Farrell (1996), Farrell, Hernandez, Fedele, Vary, Kimball, and Jefferson (2000), Hernandez, Fedele, and Farrell (2000), and Kostyak, Kimball, Jefferson, and Farrell (2001) have measured skeletal muscle protein synthesis by phenylalanine infusion in sedentary and exercised rats. The exercise consisted of four sessions of 50 touches of a low bar followed by a high bar with an increasingly weighted vest in order to mimic a squat movement; one day of rest was given between each session.

Twelve rats were divided into an exercise group or a sedentary group and underwent the aforementioned exercise protocol (Fluckey, et. al., 1996). The exercise group had a significantly greater level of skeletal muscle protein synthesis in the gastrocnemius and the soleus in comparison with the sedentary group 16 hours postexercise ($p<.05$).

Hernandez, et al. (2000) studied the skeletal muscle protein synthesis of rats 1, 3, 6, 12, or 24 hours post-exercise by phenylalanine infusion followed by gastrocnemius and soleus extraction. All groups were fasted five hours prior to infusion. It was found that skeletal muscle protein synthesis was significantly greater in the exercised group than the sedentary group 12 and 24 hours after exercise in the gastrocnemius; this occurred at the 24 hour mark only in the soleus ($p<.05$). Therefore the stimulation of skeletal muscle protein synthesis as a result of resistance exercise may occur a period of hours after exercise rather than immediately following.

Farrell, et al. (2000) measured skeletal muscle protein synthesis by phenylalanine infusion and gastrocnemius extraction 16 hours after the cessation of exercise. Skeletal muscle protein synthesis in the gastrocnemius was 25% higher in the exercised group in comparison with the sedentary group ($p<.05$). Utilizing the same protocol, Kostyak, et al. (2001) observed significantly increased skeletal muscle protein synthesis in rats postexercise ($p<.05$). Clearly, resistance training does serve to increase skeletal muscle protein synthesis in rats.

Pitkänen, et al. (2003) investigated the effect of lower extremity resistance exercise on skeletal muscle protein synthesis and breakdown in humans. Subjects were randomly allocated to an exercise group or a sedentary (control)

group. All subjects were fasted for a period of 10 hours after which phenylalanine infusion was begun in order to determine skeletal muscle protein synthesis. Fifty-five minutes after the infusion began, subjects in the exercise group took part in 50 minutes of lower extremity resistance exercise while the control group remained sedentary. Following the exercise period, all subjects had several blood draws and one muscle biopsy during the period of 135 to 165 minutes after the start of the infusion (period one) and again between 270 and 300 minutes after the start of the infusion (period two).

Protein synthesis, breakdown, and net protein balance in the vastus lateralis were determined for period one and period two. During period one, no significant differences were found between the control and exercise groups for protein synthesis, breakdown, or net balance. In period two, however, the exercise group had significantly greater rates of protein synthesis and breakdown in comparison with the control group ($p<.05$). There was, however, no significant difference for net protein balance between the groups for period two. These results support the notion that resistance exercise exerts its effects on protein synthesis and breakdown later, rather than earlier, in recovery. Because resistance exercise stimulates both protein synthesis and breakdown, there may be no net change in protein balance in comparison with sedentary individuals in the absence of exogenous amino acids.

Six regular resistance trainers performed single-arm resistance exercise consisting of four sets of preacher curls, concentration curls, and single arm biceps at 80% of 1RM (MacDougall, Gibala, Tarnopolsky, MacDonald, Interisano, & Yarasheski, 1995). This was followed by leucine infusion for determination of protein synthetic rate in the biceps brachii. At four and 24 hours after exercise, the exercised arm had a significantly greater rate of protein synthesis than the sedentary arm ($p<.05$); no significant difference was observed at 36 hours. It is clear that resistance exercise stimulates protein synthesis; the peak may be at 24 hours with a return to basal levels by 36 hours after exercise.

From these studies it is observed that resistance exercise causes an increase in skeletal muscle protein synthesis; this increase appears to occur later rather than earlier after exercise. Despite the establishment of the role that resistance exercise has upon skeletal muscle protein synthesis, there remains little data on how the timing of amino acid intake may interact with resistance exercise to alter skeletal muscle protein kinetics.

Amino Acids and Skeletal Muscle Protein Kinetics

Amino acids serve as substrates for skeletal muscle protein synthesis; there is conflicting evidence on the affect amino acids have as stimulators of protein synthesis. An early study by Preedy and Garlick (1986) investigated the

effect of intravenous infusion of an amino acid mixture on skeletal muscle protein synthesis in rats fasted for 12 hours. After one hour of the infusion, phenylalanine was injected 10 minutes prior to death to determine skeletal muscle protein synthesis rates. There was no significant increase in skeletal muscle protein synthesis; it is possible that amino acids require another factor (i.e., insulin) to stimulate skeletal muscle protein synthesis.

The affect of amino acid feeding on skeletal muscle protein synthesis and breakdown was measured in 41 male Wistar rats (Balage, et al., 2001). Rats underwent a 17 hour fast followed by a one hour feeding of a 25% amino acid /protein meal with diazoxide, a phenylalanine injection, and a gastrocnemius excision two hours post feeding. The diazoxide caused an acute insulin deficiency in the rats, there by eliminating any effect of insulin. In comparison with a postabsorptive group (17 hour fast with no further treatments), no significant increase in skeletal muscle protein synthesis was observed; skeletal muscle protein degradation was significantly greater in the fed group in comparison with postabsorptive rats ($p<.05$). These results do not support amino acids having an anabolic affect on skeletal muscle when administered in the absence of insulin.

Male Sprague-Dawley rats were split into four treatment groups and skeletal muscle protein synthesis was measured via phenylalanine infusion in a

study by Anthony, et al. (2002a). Two of the treatments groups included 1) somatostatin-saline (control) and 2) somatostatin-leucine. The somatostatin was used to inhibit insulin release after leucine administration in the treatments. Rats were euthanized at 15, 30, 45, 60, or 120 minutes after the two-hour infusion. There was no difference in skeletal muscle protein synthesis between the control group and the somatostatin-leucine group 30 minutes after leucine infusion. This again supports the notion that another factor, perhaps insulin, may be required for the stimulation of skeletal muscle protein synthesis after fasting or that protein synthesis may stimulated at an earlier or later time after the administration of amino acids.

A study involving the reduction of plasma amino acids and the effect on skeletal muscle protein synthesis was performed in swine (Kobayashi, et al., 2003). Briefly, Yorkshire swine were given a phenylalanine and leucine infusion which was continued throughout the experiment. Four hours into the infusion, hemodialysis was begun to reduce plasma amino acid concentrations by ~40%; hemodialysis was continued for a period of four hours. Additionally, in one group of swine, a mixed amino acid solution was infused during the final two hours of hemodialysis. Phenylalanine utilization for skeletal muscle protein synthesis was significantly decreased in both groups when hemodialysis was started; this decrease remained significant throughout hemodialysis in the group

receiving no amino acids. In the group receiving amino acids, there was a significant increase phenylalanine utilization during the final two hours of hemodialysis (during the mixed amino acid infusion) in comparison with the first two hours. Therefore, reducing plasma amino acid concentrations markedly has a detrimental affect on skeletal muscle protein synthesis but can be restored with the administration of mixed amino acids.

Anthony, Yoshizawa, Anthony, Vary, Jefferson, and Kimball (2000) fasted rats for a period of 18 hours before administering either saline, valine, isoleucine, or leucine in order to determine which of the amino acids most effectively stimulates skeletal muscle protein synthesis. Phenylalanine was injected 50 minutes after the amino acid dose for determination of protein synthesis. Leucine was the only amino acid that had a significantly greater rate of protein synthesis than the saline treated rats ($p<.05$), indicating that its presence in a mixed amino acid solution may be necessary to stimulate skeletal muscle protein synthesis.

Following a 12 hour fast, young (34$\pm$4 years) and old (67$\pm$2 years) subjects ingested 15 grams of essential amino acids to determine any age-related differences in the protein synthetic response to oral amino acid administration (Paddon-Jones, et. al., 2004). The fractional synthesis rate in the vastus lateralis, measured by phenylalanine infusion, was significantly increased in both groups

by .04%/hour ($p<.05$ versus no amino acid ingestion). Despite this similarity, the young subjects achieved a higher peak femoral artery phenylalanine concentration, while the old subjects maintained their phenylalanine concentrations significantly higher than baseline 90 minutes longer than the young subjects. Therefore, the protein synthetic response to amino acid ingestion seems to occur both in young and old subjects, although the time course response may differ.

It appears that the infusion of amino acids after a prolonged fast has mixed affects on skeletal muscle protein synthesis. Specifically, an increase in synthesis may not be seen immediately after an infusion but may be seen a period of hours later. The presence of another factor with amino acids, such as insulin, may be necessary to stimulate skeletal muscle protein synthesis.

Insulin and Skeletal Muscle Protein Kinetics

Insulin is a hormone that has been shown to both increase skeletal muscle protein synthesis and have no effect on it. In a resting state, infusion of insulin into the femoral artery of humans resulted in significantly increased protein synthesis ($p<.05$) in the vastus lateralis as measured by phenylalanine incorporation into the muscle (Biolo, Williams, Fleming, & Wolfe, 1999). Insulin had no affect on protein degradation in the muscle.

Balage, et al. (2001) investigated the effect of insulin on skeletal muscle protein synthesis and breakdown in male Wistar rats. The rats underwent a 17 hour fast followed by a one hour feeding, a phenylalanine injection, and a gastrocnemius excision two hours post feeding. The feeding protocol was a 0% protein diet + a vehicle in order to stimulate only insulin production. In comparison with a postabsorptive group (17 hour fast with no further treatments), the insulin group did not significantly differ in skeletal muscle protein synthesis or degradation.

A comparison of skeletal muscle protein synthesis between nondiabetic and diabetic sedentary rats gives an indication of the influence insulin has on protein synthesis at rest (Fedele, et al., 2000; Kostyak, et al., 2001). The diabetic rats had significantly lower levels of insulin than the nondiabetic rats in the study ($p<.05$). Rates of skeletal muscle protein synthesis in the gastrocnemius were significantly lower in the diabetic rats ($p<.05$), supporting the idea that insulin plays a role in protein synthesis in skeletal muscle.

A starvation/refeeding study was performed on female mice to determine the role of insulin in the stimulation of skeletal muscle protein synthesis (Svanberg, Zachrisson, Ohlsson, Iresjö, & Lundholm, 1996). All mice were fasted for 18 hours and were refed for a period of three hours. Prior to refeeding, some mice were given insulin-neutralizing antibodies (M 8309) in order to compare the

affect of refeeding with and without insulin. The mice given anti-insulin had a significantly lower rate of protein synthesis ($p<.05$) than those who were refed only. Exogenous insulin was also provided to fasted mice (without refeeding) but no change was seen on protein synthetic rates. As a result, insulin likely plays a role in skeletal muscle protein synthesis, although it may not be a direct one. Additionally, the level at which insulin begins to have an affect on skeletal muscle protein synthesis remains to be investigated.

Resistance Exercise, Amino Acids, and Skeletal Muscle Protein Synthesis

The combination of resistance exercise and amino acids has proven to be successful in stimulating protein synthesis in skeletal muscle. Tipton, Ferrando, Phillips, Doyle, and Wolfe (1999) investigated three males and three females after lower-body resistance training (5x10 incline leg press; 4x8 duo-squat, leg curls, leg extension at 75% 1RM). Subjects consumed one liter (100 ml every 18-20 minutes) of a 1) mixed amino acid drink, 2) essential amino acid drink, or 3) a placebo drink beginning approximately one hour after exercise. During consumption of the drinks, net muscle protein balance was negative in the placebo group and positive in the mixed amino acid ($p<.05$ vs. placebo) and essential amino acid ($p<.01$ vs. placebo) groups. These results indicate that this combination of resistance exercise and amino acids does stimulate skeletal

muscle protein synthesis; further, essential amino acids may be more potent stimuli than nonessential amino acids.

Another study by Tipton, Borsheim, Wolf, Sanford, and Wolfe (2003) investigated chronic skeletal muscle protein balance (24 hours) with and without exercise and amino acid ingestion. Subjects took part in two 24 hour trials: a rest protocol and an identical protocol with the addition of amino acid ingestion and resistance exercise. The 24 hour fractional synthetic rate (as determined by phenylalanine enrichment in muscle) was significantly greater in the exercise group in comparison with the rest group ($p=.003$). In addition, protein balance was calculated for a three hour period which included a resistance exercise session and consumption of 15 grams of essential amino acids immediately before and one hour after exercise. The difference in phenylalanine exchange between the exercised and rested group was the same at the 24 hour measurement and the three hour measurement. This indicates that acute measurements of skeletal muscle protein synthesis may be representative of longer (i.e. 24 hour) period. Although the individual effects of amino acids and resistance exercise cannot be gleaned from this study, it is apparent that combination of amino acids and resistance exercise is a potent stimulator of skeletal muscle protein synthesis.

Intravenous amino acid infusion at rest and after resistance exercise was done to determine the affect of amino acids and resistance exercise on skeletal muscle protein synthesis (Biolo, Tipton, Klein, & Wolfe, 1997). Six untrained men were infused with ~0.15 grams amino acids/kilogram body weight/hour for a period of three hours while resting or after resistance exercise. The resistance exercise protocol consisted of 5x10 incline leg press at 12RM and 4x8 Nautilus duo-squats, leg curls, and leg extensions at 10RM. Vastus lateralis protein synthesis was determined by phenylalanine enrichment in the muscle. The infusion of amino acids at rest increased protein synthesis by 141±45%; after exercise, however, protein synthesis increased by 291±42% with the addition of amino acids, which was significantly greater than the increase at rest ($p<.05$). These results indicate that amino acids alone can stimulate skeletal muscle protein synthesis. In addition, performing resistance exercise prior to ingestion of amino acids will further increase skeletal muscle protein synthesis by a significant margin.

Resistance Exercise, Insulin, and Skeletal Muscle Protein Synthesis

It is clear that resistance exercise and insulin, at times, stimulate skeletal muscle protein synthesis independently; the focus is now turned to the combination of these two stimuli on protein synthesis in the muscle. Five

healthy males were studied both in a postabsorptive and a postexercise state for determination of skeletal muscle protein synthesis (Biolo, et al., 1999). Subjects performed intense lower body resistance training consisting of 5x10 incline leg press at 12RM, 4x8 Nautilus duo squats, leg curls, and leg extensions at 10RM. The insulin and exercise group had significantly greater fractional synthesis rates than the insulin only group at rest and the group with no insulin and no exercise ($p<.05$). Therefore adding the influence of resistance exercise to that of insulin causes a greater increase in protein synthesis than the influence of insulin alone.

Diabetic and nondiabetic rats performed resistance exercise with low/high bar touches as described previously for determination of the influence of resistance exercise and insulin on skeletal muscle protein synthesis (Farrell, Fedele, Vary, Kimball, Lang, & Jefferson, 1999). Skeletal muscle protein synthesis rates were determined 16 hours after exercise cessation by phenylalanine enrichment in the gastrocnemius. Both the diabetic and nondiabetic rats significantly increased protein synthesis rates in the gastrocnemius after exercise ($p<.05$) and there was no significant difference between the two groups. This occurred despite insulin levels in the diabetic rats being less than half that of the nondiabetic rats. These results indicate that moderately reduced levels of insulin do not affect skeletal muscle protein synthesis rates after intense resistance exercise.

Kostyak, et al. (2001) also observed the influence of insulin and resistance exercise on skeletal muscle protein synthesis in diabetic and nondiabetic rats. Utilizing the same exercise protocol as Farrell, et al. (1999), protein synthesis in the gastrocnemius was significantly increased 16 hours after exercise in only the nondiabetic rats. This indicates that higher levels of insulin may be necessary for an increase in skeletal muscle protein synthesis after resistance exercise and provides conflicting evidence regarding the influence of insulin and resistance exercise on skeletal muscle protein synthesis.

Amino Acids, Insulin, and Skeletal Muscle Protein Synthesis

The combination of amino acids and insulin has been shown to consistently stimulate skeletal muscle protein synthesis. Preedy and Garlick (1986) fed a group of 12 hour fasted rats an amino acid and glucose combination *ad libitum*. After one hour of feeding and an injection of phenylalanine, it was determined that feeding rats amino acids and glucose in combination significantly stimulated skeletal muscle protein synthesis in comparison with the control rats and the rats who received amino acids only. This is likely due to the insulin response to the feeding, as the insulin levels of the *ad lib* fed group were significantly greater than both the control group, which was not fed ($p<.01$), and the amino acid only group ($p<.01$).

Male Sprague-Dawley rats were split into four treatment groups and skeletal muscle protein synthesis was measured via phenylalanine infusion in a study by Anthony, et al. (2002a). Rats were treated with 1) somatostatin-saline (control), 2) somatostatin-leucine (AA), 3) vehicle-saline (insulin), or 4) vehicle-leucine (AA+insulin) and killed at various timepoints (15, 30, 45, 60, or 120 minutes after infusion). The somatostatin was used to inhibit insulin release after leucine administration in the treatments. When comparing the groups at 30 minutes after infusion, no difference in skeletal muscle protein synthesis was found between the control, AA, or insulin groups. The AA+insulin group did have a significantly higher ($p<.05$) rate of skeletal muscle protein synthesis than any of the other groups, supporting the idea that amino acids and insulin work together to stimulate skeletal muscle protein synthesis.

Skeletal muscle protein synthesis and breakdown was measured in 41 male Wistar rats who underwent a 17 hour fast followed by a one hour feeding, a phenylalanine injection, and a gastrocnemius excision two hours post feeding (Balage, et al., 2001). The rats were split into four groups: 1) 25% protein diet + vehicle (insulin+AA), 2) 25% protein diet + diazoxide (AA), 3) 0% protein diet + vehicle (insulin), or 4) 0% protein diet + diazoxide (control). The diazoxide caused an acute insulin deficiency in AA and control groups, there by eliminating the effect of insulin in these treatments. In comparison with a

postabsorptive group (17 hour fast with no further treatments), only the insulin+AA group significantly increased skeletal muscle protein synthesis ($p<.05$). Skeletal muscle protein degradation was only significantly decreased in the insulin+AA group in comparison with postabsorptive rats ($p<.05$). From these results it can be stated that the combination of insulin and amino acids serves to promote protein anabolism both by stimulating synthesis and decreasing degradation.

Male Sprague-Dawley rats were subjects in a 1997 study by Kimball, Jurasinski, Lawrence, and Jefferson investigating the affect of insulin presence with an amino acid infusion on skeletal muscle protein synthesis, as measured by phenylalanine infusion. Rats underwent a 90 minute hindlimb perfusion of erythrocytes, albumin, glucose, phenylalanine, and amino acids with or without insulin. At the end of the perfusion, the gastrocnemius was excised and homogenized. The group in which insulin was included had a 1.6-fold higher rate of skeletal muscle protein synthesis than the group without insulin, supporting the premise that insulin plays a part in stimulating skeletal muscle protein synthesis.

Yoshizawa, Kimball, Vary, and Jefferson (1998) investigated skeletal muscle protein synthesis in rats starved for 18 hours. After the fast, rats were given either a 0% protein diet or a 20% protein diet for one hour (diets were

isocaloric). The insulin response was not statistically different between the groups. Skeletal muscle protein synthesis, as measured by phenylalanine incorporation into the gastrocnemius, was significantly greater ($p<.05$) in the group fed the 20% protein diet in comparison with the 0% protein diet. This again indicates that insulin and amino acids may work together to stimulate skeletal muscle protein synthesis.

A study involving humans has also shown amino acids and insulin to be effective stimulators of skeletal muscle protein synthesis (Tipton, et al., 1999). Subjects ingested a 500 ml solution containing 13.4 grams of essential amino acids and 35 grams of sucrose. The composition of this drink allowed for the influence of both amino acids and insulin to be observed. A biopsy of the vastus lateralis was performed before and two hours after ingestion of the drink; free intracellular concentrations of amino acids in the muscle were then determined. Nitrogen balance increased from -495$\pm$128 nmol/ml before the drink to 416$\pm$140 nmol/ml after ingestion and arterial amino acid levels increased markedly after ingesting the drink. The ingestion of amino acids and carbohydrate, promoting an insulin response, has been shown repeatedly as an appropriate way in which to increase skeletal muscle protein synthesis.

Resistance Exercise, Insulin, Amino Acids, and Skeletal Muscle Protein Synthesis

With the knowledge that resistance exercise, amino acids, and insulin all affect skeletal muscle protein kinetics, it is important to look at these influences and how they may act in combination to affect protein kinetics. Leucine infusion was used to determine skeletal muscle protein synthesis rates in twelve healthy males who were regular resistance trainers (Chesley, MacDougall, Tarnopolsky, Atkinson, & Smith, 1992). The resistance exercise protocol consisted of 4-6x12 bicep curls at 80% of 1RM performed with one arm while the contralateral arm remained sedentary. A primed continuous infusion of leucine was begun .68 hours or 20.4 hours post-exercise for a period of six hours. In addition, 50% of individual mean energy intake was given two hours before and throughout the leucine infusion to ensure a steady rate of appearance of endogenous calories and protein. Biopsies of the bicep were taken at two hours after the start of infusion and at the end of the infusion.

Skeletal muscle protein synthesis rates were significantly elevated by 50% in the exercised bicep in comparison with the control bicep by in the four hour post-exercise group ($p<.05$) and by 109% in the 24 hour post-exercise group ($p<.05$). This was due to a significant increase ($p<.05$) in RNA activity, defined as the amount of protein synthesized per hour per microgram of RNA, in the muscle. Insulin concentrations, however, were not reported and were likely

affected by feeding; therefore the individual effects of exercise and feeding are unknown in this study.

Six subjects performed 10x8 of leg press and 8x8 leg extension at 80% of 1RM on two occasions followed by ingestion of a drink either one hour or three hours after exercise in a study investigating skeletal muscle protein synthesis (Rasmussen, Tipton, Miller, Wolf, & Wolfe, 2000). The drink consisted of 35 grams of sucrose and six grams of essential amino acids and was designed to increase intramuscular free concentrations of essential amino acids in the same ratio that they are required for skeletal muscle protein synthesis. Sixty minutes prior to exercise, phenylalanine infusion was begun; muscle biopsies were performed on the vastus lateralis in order to determine phenylalanine enrichment in the muscle. Skeletal muscle protein synthesis was significantly increased one to two hours after exercise when the drink was ingested one hour post exercise, while the significant increase was seen three to four hours after exercise when the drink was ingested three hours post exercise ($p<.05$). This was accompanied by spikes in insulin levels at similar time points, there by demonstrating an association between the three variables and skeletal muscle protein synthesis.

An essential amino acid-carbohydrate beverage was consumed by six healthy humans before or after lower body resistance exercise to determine its

effects on skeletal muscle protein synthesis (Tipton, Rasmussen, Miller, Wolf, Owens-Stovall, Petrini, & Wolfe, 2001). After consumption of the beverage before or after exercise, insulin levels were significantly increased. Rate of appearance ($R_a$), disappearance ($R_d$), and net balance of phenylalanine were determined through several blood and muscle samples taken at rest, during exercise, and after exercise. The $R_a$ of phenylalanine did not differ between the before and after groups at any of the specified time points. The $R_d$ of phenylalanine, however, was significantly greater in the before group both during exercise and one hour after exercise ($p<.05$). This was also the case for net phenylalanine balance. Therefore, it appears that ingestion of amino acids and carbohydrate has a stronger effect on skeletal muscle protein synthesis when ingested prior to exercise, although net phenylalanine balance did change from negative during exercise to positive one hour after exercise in the group consuming the beverage after exercise.

The effects of an amino acid (AA), carbohydrate (CHO), or an amino acid and carbohydrate (MIX) beverage were observed in 10 humans following lower body resistance exercise (Miller, Tipton, Chinkes, Wolf, & Wolfe, 2003). The beverages were ingested one and two hours after exercise, which was both preceded and followed by muscle biopsies and blood draws. The area under the curve for insulin was significantly greater for the CHO and the MIX group in

comparison with the AA group ($p<.001$). Net uptake of phenylalanine was significantly greater during the first two hours after ingestion of the first drink in both the AA and MIX group in comparison with the CHO group ($p<.05$); no significant differences were found during the third hour. When combining the three hours, the MIX group had a significantly greater net phenylalanine uptake in comparison with the CHO and AA groups. Under these condition, it appears that the sum of the effects of CHO and AA equal that of the effect of MIX on protein synthesis.

Amino Acids, Insulin, and the Markers of Skeletal Muscle Translation Initiation

With the knowledge that amino acids, insulin, and resistance exercise influence protein kinetics in skeletal muscle, it is of interest to investigate markers of skeletal muscle translation initiation (phosphorylation state of 4EBP1 and the association of eIF4E with 4EBP1 and eIF4G) and their response to these influences. Adult female mice (control, type 1 diabetes model, type 2 diabetes model) were fasted for 18 hours and refed standard laboratory chow for three hours (Svanberg, Jefferson, Lundholm, & Kimball, 1997). Hindlimb muscles were extracted and the phosphorylation state of 4EBP1 was determined by immunoprecipitation with a 4EBP1 antibody followed by immunoblot analysis. In all three groups of mice, the highly phosphorylated form of 4EBP1 ($\gamma$) was

significantly greater ($p<.001$) in a refed versus a starved state. These results indicate that 4EBP1 phosphorylation is occurring at a greater rate in a fed state than in a fasted state.

In the same study, several other indicators of translation initiation were observed by Western blot analysis. The association of 4EBP1 with eIF4E was found to be significantly greater ($p<.01$) in the starved group in comparison with the fed group in all three types of mice. This goes along with the previous results, as highly phosphorylated forms of 4EBP1 dissociate from eIF4E, which was observed in the fed mice. With the dissociation of 4EBP1 from eIF4E, it is expected that the association of eIF4G with eIF4E would increase. This phenomenon was observed, as the fed groups had significantly greater association of eIF4G with eIF4E in comparison with the starved groups ($p<.01$). The phosphorylation state of eIF4E was not significantly altered with fasting or refeeding. Taken together, these results indicate that skeletal muscle translation initiation occurs at a greater rate in a fed state. Because the results were the same for the three types of mice, it can be said that high levels of insulin may not be required for stimulation of skeletal muscle translation initiation, but likely still plays a role.

Balage, et al. (2001) investigated the effect of amino acids, insulin, and the combination of amino acids and insulin on several indicators of skeletal muscle

protein synthesis in Wistar rats. All rats were fasted for a period of 17 hours followed by a refeeding for one hour consisting of 1) 25% amino acids (AA+insulin), 2) 25% amino acids plus diazoxide (AA), 3) 0% amino acids (insulin), 4) 0% amino acids plus diazoxide (control), or 5) no refeeding (postabsorptive). The diazoxide served to inhibit insulin release and only the AA+insulin and the insulin group had significantly higher insulin levels than the postabsorptive group; the AA+insulin group also had a significantly greater insulin level than the insulin group ($p<.05$). The phosphorylation state of 4EBP1 was determined by immunoprecipitation and immunoblot analysis. The only group to have a significantly higher amount of the $\gamma$ form of 4EBP1 than the postabsorptive group was the AA+insulin group, indicating a greater activation of translation initiation.

Given the pattern of 4EBP1 phosphorylation, it would be expected that groups with high levels of phosphorylation would have an increased amount of eIF4E association with 4EBP1. Accordingly, the amount of 4EBP1 associated with eIF4E in the AA+insulin group was significantly lower than postabsorptive group, while the control group had a significantly higher association than the postabsorptive group. The associations of eIF4E with eIF4G would be expected to be opposite of the 4EBP1 eIF4E associations. As expected, the AA+insulin group had the highest association of eIF4E and eIF4G and was significantly

greater than the postabsorptive group ($p<.05$). All other groups had levels that were significantly lower than the postabsorptive group ($p<.05$). The phosphorylation of eIF4E was also observed: the AA+insulin and control groups had significantly lower levels of eIF4E phosphorylation than the postabsorptive group, with the AA+insulin being significantly lower than the control group. These results indicate that both amino acids and insulin are necessary for alterations in the eIF4s involved in protein synthesis.

A study by Anthony, et al. (2002b) investigated whether amino acids can regulate translation initiation without insulin. Control and diabetic rats (alloxan-induced) were fasted for 18 hours followed by an oral gavage of saline or leucine. Insulin was then administered to the diabetic rats in an amount of 0 pmol/kg + 0 pmol/min/kg, 160 pmol/kg + 4.0 pmol/min/kg, or 800 pmol/kg + 20 pmol/min/kg for one hour; control rats received 0.155 mol/l NaCl, 0.2% BSA. The 4.0 pmol/kg and 20 pmol/kg were chosen to restore insulin to levels of normal rats who were given saline or leucine, respectively.

The phosphorylation of 4EBP1 was assessed by percentage of 4EBP1 in the $\gamma$ form to the total amount of 4EBP1. 4EBP1$\gamma$ represents the form which is the most highly phosphorylated and does not bind eIF4E. Control (nondiabetic) rats and the diabetic rats given 4.0 pmol/kg and 20 pmol/kg had significantly greater levels of 4EBP1$\gamma$ when administered leucine in comparison with saline.

Therefore amino acids (leucine) and insulin may work together to increase the phosphorylation of 4EBP1. As expected, the amount of 4EBP1 associated with eIF4E significantly decreased when comparing saline to leucine in the same three groups; the diabetic rats receiving no insulin did not demonstrate a significant change. As 4EBP1 dissociates from eIF4E, eIF4G takes its place, which is evidenced by the significant increase in 4EBP1-eIF4G association in the control, 4.0 pmol/kg, and 20 pmol/kg groups receiving leucine in comparison with rats receiving saline. Therefore it appears that some level of insulin is necessary to stimulate eIFs in the presence of amino acids.

In a similar study, the phosphorylation state of PHAS-I (4EBP1) was of interest in five *ad libitum* fed rats and 20 fasted rats (Long, Saffer, Wei, & Barrett, 2000). The fasted rats were administered one of four treatments for a period of three hours: 1) saline (control), 2) insulin+dextrose (insulin), 3) 10% Travesol (mixed amino acid solution) (AA), or 4) insulin and 10% Travesol (ins+AA). After infusion, the gastrocnemius was excised and immunoblotted for analysis of PHAS-I phosphorylation. Results were expressed as the amount of PHAS-I$\alpha$ in comparison to total PHAS-I. PHAS-I$\alpha$ is the form that most readily associates with eIF4E; it is the hypophosphorylated form. The AA group had a significant decrease in PHAS-I$\alpha$ in comparison with the saline group. The insulin group, however, showed no significant difference in PHAS-I phosphorylation in

comparison with the saline group. There was a significant decrease in PHAS-I$\alpha$ in the AA+insulin group, although this was not greater than the AA only group. Therefore, in this study, insulin seems to have no individual effect on PHAS-I phosphorylation, nor does it exert an added effect on PHAS-I when combined with amino acids; conflicting results have been provided, however (Kimball, 1997).

As previously described, Anthony, et al. (2000) fasted rats for a period of 18 hours before administering either saline, valine, isoleucine, or leucine. Leucine had a significantly greater rate of protein synthesis than the saline treated rats ($p<.05$). Additionally, in comparison with the saline controls and the other amino acids, leucine had a significantly greater proportion of 4EBP1$\gamma$, a significantly lower amount of 4EBP1 associated with eIF4E, and had a significantly greater amount of eIF4G associated with eIF4E in comparison with the saline and valine groups. There was no significant difference in the phosphorylation of eIF4E between any of the groups. These results indicate that, of the three branched-chain amino acids, leucine is the most likely to stimulate eIFs.

Because of these results, a second study was done to determine the effect of leucine in combination with rapamycin on these eIFs in order to determine if leucine acts via mTOR. Rats were fasted for 16 hours followed by administration

of rapamycin or a vehicle. After two hours, half of the rats in each group were given leucine while the other half received saline. As expected, rapamycin abolished the effects of leucine in several ways. The leucine group had a significantly greater amount of 4EBP1γ in comparison with the control group; the addition of rapamycin caused the amount of 4EBP1γ to decrease below control levels. The association of 4EBP1 with eIF4E was significantly lower in the leucine group versus the control group, which was negated in the presence of rapamycin. Leucine significantly increased the amount of eIF4G associated with eIF4E over the control group while rapamycin eliminated this effect as well. The phosphorylation of eIF4E was significantly lower in the leucine group in comparison with all of the other groups. These results suggest that leucine works to stimulate skeletal muscle protein synthesis through a rapamycin sensitive pathway (i.e., via mTOR).

As described previously, Kimball, et al. (1997) stimulated protein synthesis in gastrocnemius of rats during a 90 minute hindlimb perfusion with erythrocytes, albumin, glucose, phenylalanine, and amino acids with or without insulin (control). Along with a higher rate of protein synthesis, the insulin group had a 3.3-fold greater amount of PHAS-I (4EBP1)γ (as a percent of total PHAS-I), the hyperphosphorylated form, than the control group. In addition, the insulin group had 60% less PHAS-I associated with eIF4E than the control group. To

strengthen these findings, it was also reported that the insulin group had a significantly greater amount of eIF4E associated with eIF4G. Insulin also caused a significant decrease in the amount of eIF4E present in the phosphorylated form in relation to total eIF4E content ($p<.001$). Based on these findings, it appears that both insulin and amino acids are necessary to cause changes in the eIFs of interest.

Several markers of protein synthesis were investigated in male Sprague-Dawley rats by Anthony, et al. (2002a). Male Sprague-Dawley rats were infused with one of the following treatments: 1) Somatostatin-Saline (Control), 2) Somatostatin-Leucine (AA), 3) Vehicle-Saline (Insulin), or 4) Vehicle-Leucine (AA+insulin). Somatostatin was used to inhibit insulin release after leucine administration. Rats were then killed at 15, 30, 45, 60, or 120 minutes after infusion. All reported results were obtained 30 minutes after infusion. Both groups receiving leucine significantly increased 4EBP1 phosphorylation; the AA+insulin group had a significantly greater level of 4EBP1 phosphorylation than the AA only group. The association of eIF4E, 4G, and 4EBP1 was significantly changed in the groups receiving leucine (4EBP1 association with eIF4E decreased and eIF4G association with eIF4E increased); there was no significant difference between these groups. There were no significant differences in the phosphorylation of eIF4E between the groups. This study

demonstrated that amino acids alone have an effect on markers of protein synthesis; in addition, insulin may have a synergistic effect on the phosphorylation of 4EBP1.

At the cellular level there is also evidence of increased translation initiation as a result of the influence of amino acids and/or insulin (Patti, Brambilla, Luzi, Landaker, & Kahn, 1998). When FAO hepatocytes were treated with amino acids and/or insulin, PHAS-I$\gamma$ was the dominant form of PHAS-I present. When treated with rapamycin, the insulin and amino acid groups expressed predominantly PHAS-I$\alpha$. The insulin and amino acid group also expressed more PHAS-I$\alpha$, although not as strongly as the other groups. Therefore insulin and amino acids alone and in combination serve to cause PHAS-I phosphorylation via an mTOR mediated pathway.

As previously described, Farrell, et al. (2000) employed rats in a resistance exercise protocol along with phenylalanine infusion to determine rates of skeletal muscle protein synthesis. In addition, several eukaryotic initiation factors were investigated. Despite observing a greater level of protein synthesis in the exercised versus the sedentary group, there was no difference between the groups for the phosphorylation of 4EBP1, the association of eIF4E with 4EBP1, the association of eIF4E with eIF4G, nor the phosphorylation of eIF4E. Due to these results, it may be postulated that another eIF may be involved in the

stimulation of protein synthesis by way of resistance exercise that was not observed in this study, although a lack of data is available regarding this pathway.

In order to determine which metabolic intermediates amino acids and insulin affect en route to 4EBP1 or eIF4E, humans were infused with a mixed amino acid solution, a physiological dose of insulin, or a pharmacological insulin dose (Liu, Wu, Nicklas, Jahn, Price, & Barrett, 2004). Upon analysis of skeletal muscle extracted before and after the infusion, it was found that amino acids enhanced the phosphorylation of p70S6 kinase but did not affect the phosphorylation state of GSK3 or glycogen synthase activity. GSK3 phosphorylation was also not affected by physiological hyperinsulinemic levels, but glycogen synthase activity was increased. The pharmacological dose of insulin increased the phosphorylation of GSK3 and p70S6 kinase as well as the activity of glycogen synthase. These observations indicated that amino acids likely affect translation initiation by an mTOR/4EBP1 pathway while insulin, depending on the dose, may act via the mTOR/4EBP1 pathway or by way of GSK3.

In addition to the mTOR pathway, the eIF2 pathway is of interest when observing increases in the rate of translation initiation in skeletal muscle. Vary, Jefferson, & Kimball (1999) observed the effect of a supraphysiological amino

acid infusion on 4EBP1 and eIF2B in rat hindlimbs. In comparison to a physiological level of amino acids, skeletal muscle protein synthesis was doubled and the association of eIF4E-eIF4G increased by eight-fold. Surprisingly, no change in the amount of phosphorylated 4EBP1 was observed. Additionally, there was no change in the activity of eIF2B. These results indicate that the amino acid induced elevation in protein synthesis acts by way of eIF4E and not through eIF2.

An additional study reported by Vary, et. al. (1999) involved maintaining supraphysiological levels of several amino acids in rat hindlimb while removing leucine, a well-known stimulator of protein synthesis. Upon the removal of leucine, a 40% decrease in protein synthesis was observed ($p<.001$). Along with this, a 40% decrease in eIF2B activity ($p<.05$), and an 80% decrease in the amount of the eIF4E-eIF4G complex ($p<.01$) were observed. As would then be expected, a significant decrease in 4EBP1 phosphorylation ($p<.05$) as well as a significant increase in the eIF4E-4EBP1 complex ($p<.01$) was observed. Taken together, the results of these studies indicate that an increase in protein synthesis (modulated by amino acids) occurs by way of increasedeIF4E-eIF4G complex formation. In contrast, a decrease in protein synthesis (due to a lack of amino acids) occurs by effecting both eIF4E complexes and the activity of eIF2B.

Delayed Onset Muscle Soreness

Nearly all individuals participating in unaccustomed exercise or taking part in exercise after a prolonged period of inactivity experience DOMS. Concentric, isokinetic, and eccentric exercise can result in DOMS, although eccentric exercise seems to be the most significant contributor. Additionally, the intensity of the exercise appears to be more of a determinant of the severity of DOMS than the duration of exercise (Cheung, et al., 2003). DOMS can present a broad range of symptoms, from muscle stiffness to severe pain, which develop over a characteristic timeline. Following eccentric exercise, DOMS has its onset within 24 hours of the exercise bout, typically peaks between 24 and 72 hours, and subsides within five to seven days after exercise (Cheung, et al., 2003). Alleviation of DOMS may be important for some athletic populations that train and compete regularly so as to avoid compromising performance.

*The progression of DOMS*

Smith, et al. (1994) observed the progression of DOMS in 26 untrained men after three sets of 12 repetitions of the eccentric phase of the chest press at 80% of 1RM. A significant time effect for DOMS was observed ($p<.001$) with onset occurring before 24 hours, peak soreness at approximately 48 hours and a return to baseline levels within 144 hours (6 days). In a study involving women,

26 subjects performed 70 single-arm eccentric contractions of the elbow flexors in order to induce DOMS (Cleak & Eston, 1992). Soreness was significantly increased 24 hours after exercise and peaked at approximately 72 hours after exercise. Subjects began to feel no soreness by 192 hours (8 days) after exercise. Twenty-four and 48 hours after a bout of eccentric leg curl exercise (10 sets of six repetitions at 100% of concentric 1RM), 19 subjects rated their perceived muscle soreness (Tokmakidis, Kokkinidis, Smilios, & Douda, 2003). Muscle soreness had significantly increased by 24 hours and remained as such at 48 hours after exercise ($p<.05$).

*DOMS and Muscular Strength*

Strength measurements in the days following eccentric exercise tend to decrease, often mirroring the increase in muscle soreness (Miles & Clarkson, 1994). This has been shown to occur with eccentric, isometric, and concentric strength. In the study by Smith, et al. (1994), concentric chest press strength was reduced following an eccentric exercise protocol. The greatest decrease in strength was seen at 48 hours after exercise, when it decreased by an average of nine percent ($p<.001$); this was also the time point at which soreness perception was the greatest. After this point, strength increased but still remained significantly lowered at 96 hours ($p=.003$) and 192 hours ($p=.037$) after exercise.

Eccentric leg curl exercise was performed by 19 subjects in order to induce DOMS and observe concentric strength recovery following the exercise (Tokmakidis, et al., 2003). Concentric leg curl strength was significantly decreased immediately after exercise as well as 24 and 48 hours after exercise, corresponding to the onset of muscle soreness in the subjects.

Cleak and Eston (1992) also measured strength following eccentric exercise of the elbow flexors. Isometric strength decreased at approximately 20 minutes after exercise ($p<.01$) and peaked by 24 hours after exercise, when strength was 46% of pre-exercise levels ($p<.01$). Strength remained markedly reduced at 72 hours after exercise, when soreness perception was at its peak. Complete recovery of strength was not achieved by day 11, although there was marked improvement by day seven.

Eccentric torque and muscle soreness were measured at 0, 2, 4, 6, 20, 24, 48, 72 hours after the performance of 300 eccentric quadriceps repetitions in a study by MacIntyre, Sorichter, Mair, Berg, and McKenzie (2001). DOMS increased after exercise, peaking at 48 hours; eccentric torque decreased until 24 hours after exercise and then began to recover. Therefore it is seen that strength and soreness may mirror each other in the early days after DOMS-inducing exercise, although the loss of soreness generally occurs more quickly than strength recovery.

*Causes and Treatments of DOMS*

Several causes of DOMS have been proposed (lactic acid build up in the muscle, muscle damage, connective tissue damage, inflammation, muscle spasm, and enzyme efflux), but it is currently believed that one single factor cannot be responsible for the development of DOMS (Cheung, et al., 2003); it is suggested that a combination of the proposed causes is what precipitates DOMS. Cheung, et al.(2003) have combined several theories to create a comprehensive mechanism of DOMS development. The first step involves structural proteins in the muscle being disturbed at the z-lines as a result of high tension produced during eccentric contractions. Along with this, the connective tissue at the myotendinous junction and the muscle fibers near to it undergo high amounts of strain. When the sarcolemma is damaged, calcium accumulates and inhibits cellular respiration, disturbing calcium equilibrium and decreasing ATP production. Proteolytic enzymes are activated as a result of the high calcium levels and the z-lines of the sarcomeres, troponin, and tropomyosin are broken down. Inflammation causes an increase in the number of neutrophils in the blood within hours. Markers of the connective tissue and muscle damage move into the interstitium and plasma and attract monocytes to the area. These monocytes become macrophages, histamine production increases, and an increase in neutrophils at the injury site is observed. The monocyte/macrophage

numbers peak at 48 hours and the macrophages make prostaglandin, sensitizing the nerve endings to mechanical, chemical, or thermal stimulation. Phagocytosis and cellular necrosis causes accumulation of histamine, potassium, and kinins which contributes, along with elevated pressure and an increase in temperature, to possible activation of nociceptors in the muscle tendon junction and the muscle fibers. This chain of events is proposed to characterize the symptoms associated with DOMS.

Treatment of DOMS has also been investigated and includes several possible modalities including massage (Hilbert, Sforzo, & Swensen, 2003), anti-inflammatory medications (Tomakidis, et al., 2003), stretching (Lund, Vestergaard-Poulsen, Kanstrup, & Sejrsen, 1998a; Johansson, Lindstrom, Sundelin, & Lindstrom, 1999), cryotherapy (Paddon-Jones & Quigley, 1997), ultrasound (Craig, Bradley, Walsh, Baxter, & Allen, 1999), hyperbaric oxygen therapy (Mekjavic, Exner, Tesch, & Eiken, 2000), and exercise (Dannecker, Koltyn, Riley, & Robinson, 2002).

Two hours after six sets of 10 repetitions of eccentric hamstring exercise, participants in a study by Hilbert, et al. (2003) received a 20-minute massage or a control treatment without massage. Muscle soreness intensity was significantly increased in both groups at six, 24, and 48 hours following exercise. At 48 hours, however, the intensity of soreness was significantly lower in the massaged group

in comparison with the control group. Although massage did not obliterate muscle soreness following eccentric hamstring exercise, it aided in decreasing the intensity of soreness at a time when muscle soreness is typically at its peak (48 hours post-exercise).

The affects of the anti-inflammatory medication ibuprofen on muscle soreness following eccentric leg curl exercise was investigated by Tomakidis, et al. (2003). Subjects performed six sets of 10 repetitions of the leg curl at 100% of their concentric 1RM. Following this, subjects ingested either ibuprofen (400mg every eight hours for a period of 48 hours) or a placebo pill at the same time points. Muscle soreness was significantly increased in both groups at 24 and 48 hours after exercise ($p<.05$). At 24 hours, the ibuprofen group had a significantly lower muscle soreness rating than the placebo group ($p<.05$). The ibuprofen group also had a lower soreness rating than the placebo group at 48 hours, but this did not reach statistical significance. Anti-inflammatory medications may not be effective in totally eliminating DOMS but may act to reduce the soreness 24 hours after exercise.

Stretching, both pre and post-exercise, has also been examined as a possible alleviator of DOMS (Lund, et al., 1998; Johansson, et al., 1999). Seven women performed eccentric quadriceps exercise to exhaustion with stretching performed both before and immediately after the exercise (Lund, et al., 1998).

Muscle soreness measurements were recorded daily for the following seven days. Muscle pain peaked 48 hours after exercise in both groups but no significant differences were observed for muscle pain between the groups at any time points.

Johansson, et al. (1999) investigated the affect of pre-exercise stretching on DOMS development following the performance of 10 sets of 10 repetitions of eccentric knee flexion exercise with both legs. Prior to exercise, the subjects stretched the hamstring group of one leg for a period of 20 seconds four separate times. Muscle soreness was evident at 24 hours and peaked at 48 hours after exercise in both groups, although there was no significant difference between the groups at any time point.

Following the performance of 64 eccentric elbow flexions with each arm, subjects immersed one arm in an ice-water bath for 20 minutes five separate times; each immersion was followed by a 60 minute rest period (Paddon-Jones & Quigley, 1997). Muscle soreness peaked at 48 hours following exercise in both arms and sustained until 120 hours (5 days) after exercise. There was no significant difference in muscle soreness between the arms at any time point. Therefore, cryotherapy does not appear to be effective in alleviating DOMS following eccentric exercise.

Two intensities of ultrasound were administered following eccentric elbow flexor exercise to determine if ultrasound has any affect on DOMS (Craig, et al., 1999). In addition to the two groups receiving ultrasound, a control group (no treatment) and a placebo group (sham insonation) also rated muscle soreness at 48 hours after the eccentric exercise bout. Supporting the notion that ultrasound does not have an affect on DOMS, no significant differences were found for muscle soreness between the groups.

In order to determine the affect of hyperbaric oxygen therapy on DOMS, subjects performed eccentric elbow flexor exercise followed by exposure to a hyperoxic (100% oxygen) or a normoxic (8% oxygen) environment (Mekjavic, et al., 2000). Exposure to the environments occurred ten minutes after exercise and each day for six successive days for a period of 60 minutes; muscle soreness was rated each day. Muscle soreness peaked in both groups at 48 hours ($p<.001$), but no significant difference in muscle soreness was observed between the groups at any time point. Therefore, hyperbaric oxygen therapy appears to not have a positive affect on DOMS relief following eccentric exercise.

Endurance exercise has been investigated as an aid to relieve DOMS as well (Dannecker, et al., 2002). Forty-eight hours after eccentric elbow flexor exercise (up to 15 sets of 10 repetitions), subjects performed 20 minutes of cycling at 80% of $VO_2$ max or watched a video during the corresponding time period.

Both groups reported significant increases in DOMS following the exercise ($p<.05$) with a near-significant ($p=.05$) decrease following the endurance exercise or video. Throughout the study, no significant differences were found between the groups.

Despite investigations into several factors that may aid in alleviating DOMS after eccentric exercise, the affect of post-exercise nutrition on DOMS and strength recovery has not been widely studied. In the present study, It is hypothesized that individuals consuming an amino acid and carbohydrate beverage immediately and 30 minutes after intense eccentric exercise will experience alleviation of DOMS earlier than those ingesting water or carbohydrate only.

# CHAPTER 3

# MATERIALS AND METHODS

Prior to beginning this study, approval was obtained from the Colorado State University Human Research Committee. Additionally, all subjects completed an informed consent form (Appendix A) and a health history questionnaire (Appendix B). All study procedures were thoroughly explained to each subject and all subjects were informed that their participation was completely voluntary and that they could withdraw from the study at any time.

## Subject Recruitment

Forty-six adult males, aged 20-34, were recruited for the present study; thirty six subjects met the criteria for the study and completed the entire protocol. The remaining ten subjects were removed from the study due to non-compliance. Subject recruitment was accomplished by use of recruitment fliers (Appendix C) posted on the campus of Colorado State University and at local businesses in Fort Collins, Colorado. All subjects were nonsmokers, free of metabolism-altering medications, nonvegetarian, and had not participated in resistance training within the last 12 months. Subjects were randomly allocated into one of three treatment groups: control (CON), carbohydrate (CHO), or

carbohydrate plus protein (CP). There were no significant differences ($p<.05$) between the groups for age, height, or mass (Table 2).

Table 2: Subject characteristics. No significant differences ($p>0.05$) were observed between the groups for age, height, or mass. Data expressed as mean±standard deviation and (range).

| Variable | CP group | CHO group | CON group |
|---|---|---|---|
| **Subject #** | 12 | 12 | 12 |
| **Age (years)** | **22.7±3.2** (20-29) | **22.0±1.1** (21-25) | **24.3±4.9** (20-34) |
| **Height (cm)** | **176.0±7.3** (163.8-185.4) | **177.8±7.1** (169.6-191.1) | **177.8±6.5** (168.5-190.5) |
| **Mass (kg)** | **86.6±20.2** (61.3-133.6) | **77.6±16.7** (62.1-116.8) | **77.2±12.4** (60.2-100.5) |

Preliminary Testing

All subjects reported for preliminary testing at least three days prior to starting the study protocol. Height was measured with a wall-mounted stadiometer to the nearest 0.1 centimeter, body weight was measured to the nearest 0.1 kilogram on a Health O Meter beam balance scale (Precision Weighing Balances, Bradford, MA), and body fat (percent and absolute fat mass)

was measured by dual energy x-ray absorptionmetry following the DPX-IQ Imaging Densitometer User's Manual (DEXA, DPX, Lunar Radiation, Madison, WI) using DPX-IQ X-Ray Bone Densitometer with Smart Scan™ software version 4.7e (Lunar Corp., Madison, WI). A vastus lateralis muscle biopsy was also taken.

Vertical jump was performed from a standing position alongside a wall with no approach steps; the best of five jumps was recorded. Concentric knee extension 1RM was performed after the vertical jump, in order to avoid fatigue, on a Cybex Magnum machine (CYBEX International, Inc. Medway, MA, USA). Subjects performed the first repetition at 25% of their body weight and increased weight by 2.3 or 3.4 kilograms (or more if the subject felt they could do so) there after in an effort to reach their 1RM weight in six or less repetitions. In performing the exercise, subjects were instructed to perform the concentric contraction on their own, while the test administrators lowered the weight in order for the subject to avoid eccentric fatigue. All results from the concentric 1RM and vertical jump were recorded on the SF 1RM record sheet (Appendix D) and the SF VJ Record sheet (Appendix E).

Muscle biopsies were taken from the vastus lateralis, on the lateral aspect of the thigh. Subjects were instructed to remain supine throughout the procedure. The subject was asked to contract the muscle while the incision site

was marked with a fingernail. The site to be biopsied was shaved (if necessary), and visually inspected. The area was cleaned with betadine swabs in triplicate by an assistant, while the principal investigator donned sterile surgical gloves. A fenestrated sterile field was placed over the biopsy site (in all cases that follow, an assistant opened the sterile package without touching the contents, so that only the principal investigator was in contact with instruments/materials used for the biopsy). The skin was numbed with a topical anesthetic (ethyl chloride), followed by administration of lidocaine subcutaneously in four injections using a sterile three cubic centimeter syringe and 25g needle. Ample time was allowed for numbing of the area to occur. An incision, generally six to eight mm, was made through the skin and fascia using a sterile #11 surgical blade. Sterile 4x4 gauze was placed over the incision while a Bergstrom biopsy needle was assembled and attached to a 60 cubic centimeter plastic syringe. The needle was inserted into the incision and through the fascia into the belly of the muscle. On verbal command, the assistant applied suction through the Bergstrom needle, extracting muscle tissue. This was repeated for a total of three times. The needle was removed from the incision and the muscle tissue quickly removed from the needle, frozen in liquid nitrogen, placed in a labeled cryovial, and temporarily placed in liquid nitrogen until placement into a -80°C freezer. Following the biopsy, pressure was maintained over the incision site for 15 minutes, followed

by five minutes of ice and pressure. The area around the incision was cleaned with alcohol and the incision pulled on either side and a steri-strip was applied to close the incision. A band-aid was applied over the steri-strip in a cross formation. The steri-strip and band-aid were covered with a several pieces of 2x2" gauze. A "pressure wrap" was created by taping the 2x2 gauze over the incision site and taping around the leg. Written instructions for care include leaving the pressure wrap on for eight to 12 hours and leaving the steri-strip on for a minimum of three days were orally explained to the subjects and provided in writing. A contact phone number was provided to all subjects in the event of complications.

Vertical jump and concentric knee extension 1RM were not done on days in which the muscle biopsy was performed in order to avoid any effects these tests might have on one another (e.g. muscle biopsy inhibiting maximum force output in the 1RM).

Study Day #1 Feeding

All subjects had a controlled dietary intake on study day #1; meals and snacks were provided by the study administrators in labeled bags and all uneaten food was returned the next day. Total caloric need was determined by the Harris Benedict equation determination of basal metabolic rate times 1.5 to

account for activity (Harris & Benedict, 1919). Total calories allowed was rounded to the nearest of the following caloric levels: 2500, 2700, 3000, 3200, and 3500. All subjects received identical meals while the snacks varied in size and composition to account for different total caloric intakes between the subjects (Appendix F). The macronutrient composition of each subject's controlled intake was identical: 60% of calories from carbohydrate, 25% from fat, and 15% from protein.

Five-day Study Period

All subjects reported to South College gym in a fasted state between the hours of 5:45am and 10:00am on day #1 of the study period (Figure 9).

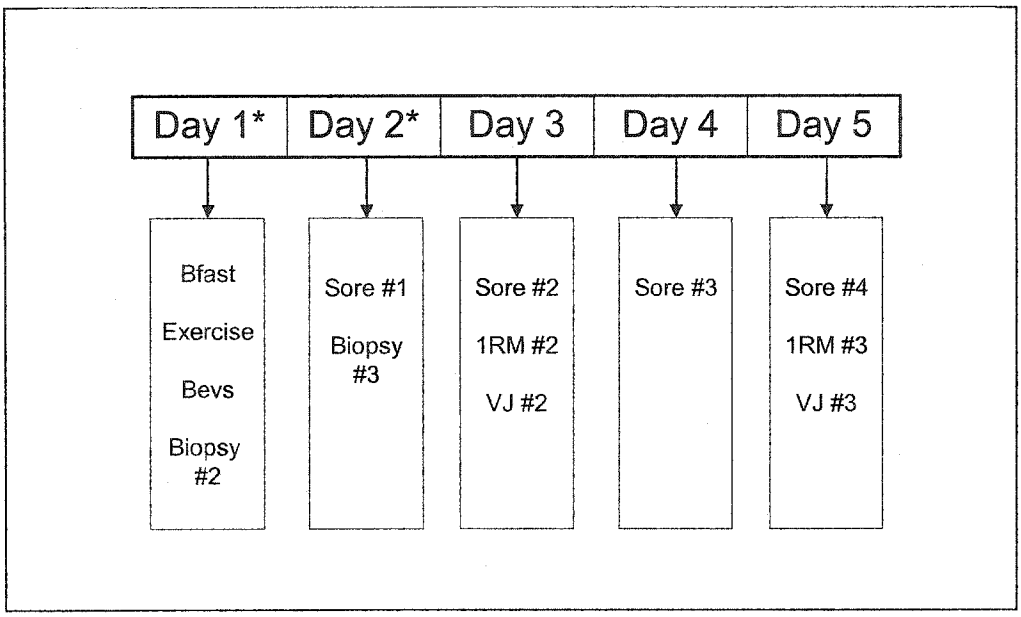



Figure 9: Five-day study protocol. Bfast=breakfast; Exercise= 10x10 eccentric exercise protocol; Bevs=consumption of treatment beverages; Sore= subjective assessment of skeletal muscle soreness; 1RM= one-repetition maximum testing; VJ=vertical jump testing. *Specific timing protocol used.

Upon arrival, subjects were given a standard breakfast consisting of 681 calories (Appendix F) and allowed 15 minutes to eat all food provided. A 30 minute digestion period followed. Prior to beginning exercise, subjects warmed up for 10 minutes on a cycle ergometer.

The resistance exercise protocol consisted of the eccentric phase of the leg extension exercise; 10 sets of 10 repetitions at 130% of the concentric leg extension 1RM was the goal. Subjects were allowed to warm up with a light resistance and then began the exercise protocol. In order to allow the subjects to perform only the eccentric phase of the exercise, test administrators would lift

the weight up and the subject would confirm that he was ready for the resistance to be transferred, at which time he would then slowly lower the bar (over a count to six) to the starting position. Each set was performed to fatigue or a maximum of 10 repetitions. A period of three minutes was allowed between sets and when the subject could complete no more repetitions, the exercise session was ended. A subject was allowed to achieve less than 10 repetitions in a set and still proceed to the next set if he felt that more repetitions could be completed. All sets and repetitions were recorded and the total number of repetitions calculated (SF 10x10 Exercise Record Sheet, Appendix G); the average number of repetitions performed by each group appears in Table 3.

Table 3: Average number of repetitions performed by each treatment group. Results reported as mean±standard deviation. No significant differences were observed between the treatment groups ($p>0.05$).

| Treatment | Repetitions |
|---|---|
| CP | 31.3±17.5 |
| CHO | 37.0±26.5 |
| CON | 29.5±14.3 |

Following exercise, subjects received a beverage divided into two equal doses. Immediately following exercise, subjects received the first beverage, which

consisted of water (CON, n=12), carbohydrate (CHO, n=12), or carbohydrate+protein (CP, n=12). The CP group received 1.0 grams of carbohydrate/kilogram body weight and 0.3 grams of protein/kilogram body weight, which was converted into a volume of the beverage to be given to the subjects. The CHO group received 1.0 g/kg of a glucose-fructose mix; the CON group received an isovolumetric amount of water. Subjects were then allowed minimal activity (e.g., walking); a second dose of the same beverage was consumed 30 minutes after exercise ended. Each beverage provided was half of the calculated amount required by the subject; average nutritional intakes, by group, are reported in Table 4.

Table 4: Average nutritional intake from post-exercise treatment. Data reported as mean±standard deviation.

| Treatment | Grams CHO | Grams protein | Total calories |
|---|---|---|---|
| CP | 86.6 ± 19.3 | 21.6 ± 4.8 | 432.9 ±96.5 |
| CHO | 79.7 ± 16.4 | 0.0 ± 0.0 | 318.6 ± 65.6 |
| CON | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |

Two hours after the cessation of exercise, subjects reported to Hartshorn Health Center for their second muscle biopsy. Subjects were sent home with a

cooler containing the food they were to eat for the rest of the day and instructed to eat the meals in order and eat as much as possible in the cooler; any uneaten food was to be returned the next day. Subjects were asked to avoid planned physical activity in order to not confound future assessments of muscle soreness.

Twenty-four hours after exercise had ended, subjects again reported to Hartshorn Health Center for their final muscle biopsy. In addition, subjects subjectively rated the soreness in their exercised quadriceps as a result of the exercise performed (Muscle Soreness Questionnaire, Appendix H). Ratings ranged from "No pain" to "Worst possible pain you have experienced" and subjects were asked to make a mark along a continuous line between the two. Ratings were quantified by measuring how many millimeters away the mark was from the left side of the scale.

On day three of the study period, subjects reported to the Human Performance Lab Clinical/Research Laboratory or Hartshorn Health Center for a second subjective rating of quadriceps muscle soreness. All subjects then reported to South College gym for another assessment of concentric knee extension 1RM and vertical jump.

On day four a subjective rating of quadriceps muscle soreness was completed. On the final day of the protocol, subjects reported to South College

gym where they completed a vertical jump, concentric knee extension 1RM, and a subjective rating of quadriceps muscle soreness.

Measurement of skeletal muscle translation initiation markers

*Homogenization of muscle tissue*

Muscle samples were removed from -80°C, weighed, and homogenized on ice in seven volumes of homogenization buffer with a mortar and pestle. Upon completion of homogenization, the slurry was transferred to a 1.5 microliter (µl) or 1.7µl eppendorf tube and centrifuged at 4°C for 10 minutes at 10,000 rpm. The supernatant (cell lysate) was then aspirated, placed in a labeled eppendorf tube, supernatant volume recorded, and returned to -80°C. Any remains of the homogenization were placed in biohazard.

*Immunoblotting*

The Bio Rad Electrophoresis cassette was set up by placing a short glass plate on a glass spacer plate and sliding them into the casting frame, making sure that plates were flush with bottom of the casting frame. The casting frame was locked and placed in a casting stand on top of a piece of filter paper and a gray rubber gasket. A running gel solution was placed between the glass plates with a small pipette. dd$H_2O$ was added with a small pipette to top off the plates.

Excess running gel solution was left in a flask at room temperature to be assured of polymerization, which took approximately 30 minutes.

After polymerization of the running gel, the water was poured off from the plates. Excess water was removed from the plates by placing filter paper in between the plates. Stacking gel solution was added in between the glass plates with small pipette up to the top of the small glass plate followed by insertion of a comb (0.75mm) between the plates for formation of lanes. The stacking gel solution was allowed to polymerize (~30 minutes) and was determined by placing excess solution at room temperature and observing for polymerization. Plates were removed from the casting frame after the stacking gel had polymerized and placed into the electrode assembly with the short plate facing inward. The electrode assembly with the plates was placed into a clamping frame and locked into place. The clamping frame was then placed in a mini tank which was filled with 1X running buffer and prerun for 10 minutes at 150 volts (V).

All samples (i.e., prepared muscle tissue) were loaded onto the gel at 65°C in a volume of 20µl, with the exception of the rainbow molecular weight marker (Amersham Biosciences, Piscataway, NJ), which was loaded in a volume of 10µl. The loaded gel was run for 10 minutes at 70V followed by 70-90 minutes at 100V.

Gels were removed from the mini tank, cut off of the spacer plate, and transferred to filter paper while immersed in transfer buffer. The transfer set-up was then assembled (gray holder, fiber pad, filter paper, gel, PVDF membrane, filter paper, fiber pad, clear holder) and transferred in transfer buffer at 100 V for one hour.

After one hour, the transfer set-up was disassembled and the membrane was removed using forceps. The right corner of the membrane was cut, indicating which side of the membrane the proteins were on. The membrane was placed in ~50 ml of tween-tris buffered saline (TBST) in a small container and placed on a shaker for five minutes. After shaking, the TBST was poured off and 50 ml of 5% blocking buffer was added to the same container. This was blocked on a shaker for one hour at room temperature.

*Immunological Procedures*

After blocking, the blocking buffer was poured off and 5% blocking buffer with the primary antibody (rabbit polyclonal p-eIF2$\alpha$ or p-4EBP1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added in a working dilution of 1:250. This was blocked for one hour at 4°C on a shaking platform.

After the primary antibody block, the antibody solution was poured off and the membrane washed three times (five minutes at 4°C on a shaker platform)

with ~50 ml of fresh TBST for each wash. After the final wash, a 5% blocking buffer with a secondary antibody (anti-rabbit IgG-HRP; Cell Signaling Technology, Inc., Beverly, MA) was added at a 1:5,000 dilution. The membrane was blocked in the secondary antibody and blocking buffer at 4°C for one hour on a shaking platform. After the secondary antibody block, the membrane was again washed three times with ~50 ml TBST. Blocking peptides for the corresponding proteins [p-eIF2$\alpha$ (Ser 52) or p-4EBP1 (Ser65/Thr70); Santa Cruz Biotechnology, Inc., Santa Cruz, CA] were run initially to ensure specificity (Figure 10).

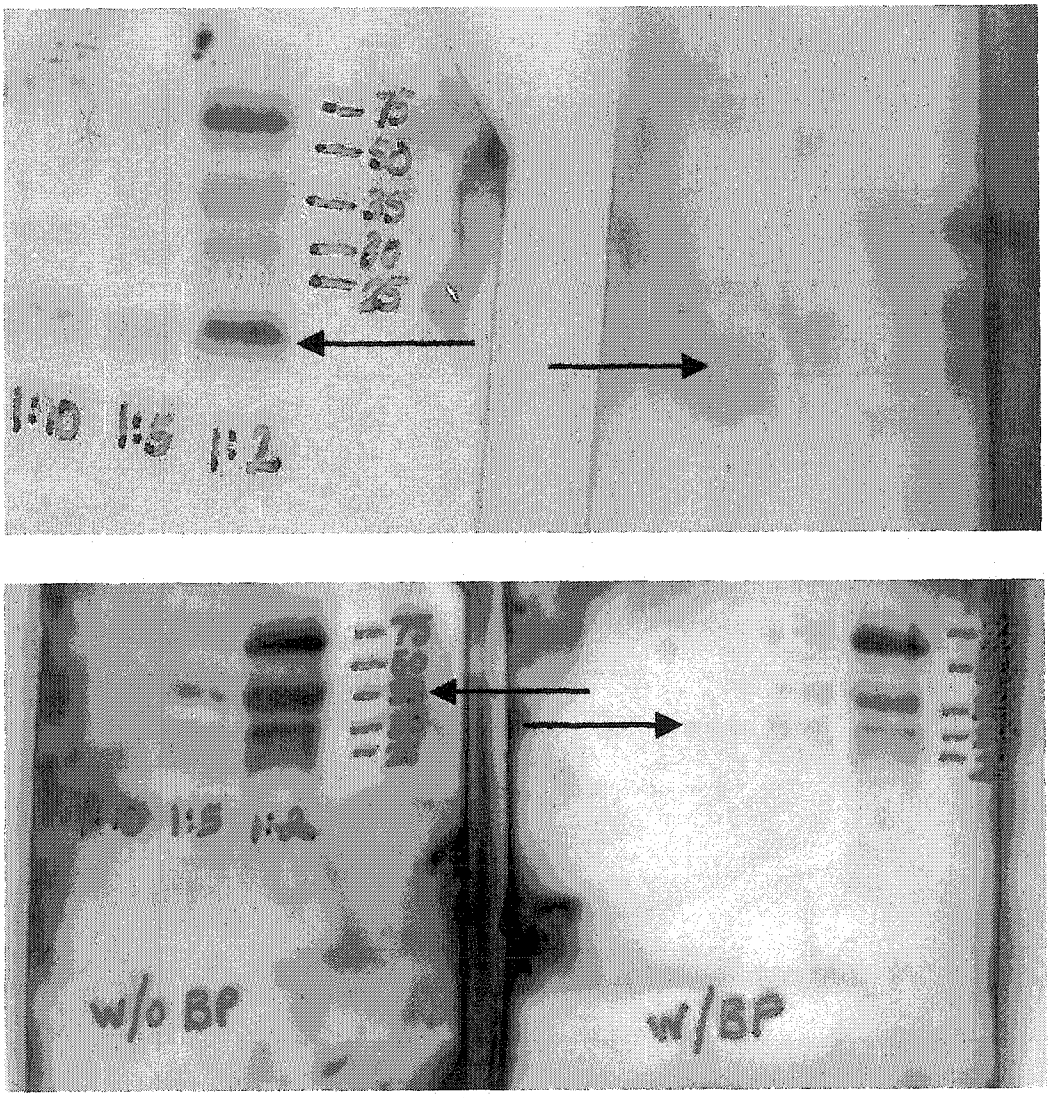



Figure 10: Blocking peptide example. Left side of each figure without blocking peptide, right side with blocking peptide. Arrows indicate location of bands corresponding to protein. A) *Top*: 4EBP1 blocking. B) *Bottom*: eIF2$\alpha$ blocking.

The PVDF membrane was removed with a forceps after the final wash, the edge wicked on a kimwipe, and placed protein side up on saran wrap on a table top. Two ml of ECL+Plus were placed on the membrane for five minutes.

*Membrane Imaging*

After five minutes of incubation with the ECL, each membrane was placed in the UVP EpiChem$^3$ Darkroom (UVP BioImaging Systems, Inc. Upland, CA). The membrane was centered and focused upon using LabWorks Image Acquisition and Analysis Software (UVP BioImaging Systems, Inc. Upland, CA). The length of exposure and times of image acquisition were set; the images were then captured and saved for analysis. One image from each series chosen and the protein bands analyzed for density using LabWorks.

Statistical Methods

A 3x3 repeated measures analysis of variance was computed to compare differences on 4EBP1 and eIF2$\alpha$ phosphorylation between treatment condition (CP, CHO, CON) and time (pre-exercise, 2 hours post-exercise, 24 hours post-exercise). A 3x3 repeated measures analysis of variance was computed to compare differences on one-repetition maximum and vertical jump between treatment condition (CP, CHO, CON) and time (pre-exercise, 48 hours post-

exercise, 96 hours post-exercise). A 3x4 repeated measures analysis of variance was computer to compare differences on post-exercise skeletal muscle soreness between treatment condition (CP, CHO, CON) and time (24, 48, 72, and 96 hours post-exercise). Where appropriate, Bonferroni's procedure was used for post-hoc testing. Significance was set at $p<.05$. Data are presented as mean $\pm$ standard deviation. The Statistical Package for the Social Sciences 12.0 (SPSS 12.0; SPSS, Chicago, Ill) was used for all statistical procedures.

# CHAPTER 4

## THE AFFECT OF POST-EXERCISE PROTEIN INTAKE ON PHOSPHORYLATION OF eIF2α and 4EBP1 IN HUMAN IN SKELETAL MUSCLE

To be submitted to American Journal of Physiology: Endocrinology and Metabolism

**Abstract**

Resistance exercise stimulates skeletal muscle protein synthesis *and* breakdown, and the pathway that predominates post-exercise appears to be influenced by nutrient intake before, during, and following exercise. Accordingly, insulin and amino acids have been shown to stimulate skeletal muscle protein synthesis, and appear to activate of a family of related proteins termed eukaryotic initiation factors (eIFs) involved in translation initiation (Jefferson & Kimball, 2003; Kimball, et al., 2002). Two steps that appear to characterize increased translation initiation include increased phosphorylation of eIF4E binding protein-1 (4EBP1) and decreased phosphorylation of eIF2α (Jefferson & Kimball, 2003). This study was undertaken to determine the effect of ingesting carbohydrate or carbohydrate+protein on the phosphorylation of skeletal muscle 4EBP1 and eIF2α following eccentric resistance exercise. It was hypothesized that the ingestion of carbohydrate+protein would significantly increase the phosphorylation of 4EBP1 and decrease the phosphorylation of

eIF2α in comparison to carbohydrate only. Thirty-six untrained adult males completed 10x10 eccentric leg extensions followed by consumption of water (CON), carbohydrate (CHO), or carbohydrate+protein (CP). Skeletal muscle biopsies were taken prior to and two and 24 hours following the exercise bout. There was no significant time effect (4EBP1 p=.15, eIF2α p=.49) or treatment effect (4EBP1 p=.43, eIF2α p=.46) for phosphorylation of 4EBP1 or eIF2α, nor was there a significant time by treatment interaction (4EBP1 p=.44, eIF2α p=.69). Therefore, the ingestion of CHO or CP following intense eccentric exercise does not appear to alter the phosphorylation of 4EBP1 or eIF2α at two or 24 hours post-exercise.

**Introduction**

Skeletal muscle protein balance is the sum of synthesis and degradation and is affected by numerous stimuli including fasting, feeding, exercise, aging, and hormones. Under normal conditions, the body is able to tightly regulate this balance (Liu & Barrett, 2002). At the molecular level, an increase in skeletal muscle protein synthesis is related to the activity of several eukaryotic initiation factors (eIFs) in the muscle. It is known that both insulin and amino acids stimulate the phosphorylation of eIF4E binding protein-1 (4EBP1) by mammalian target of rapamycin (mTOR) (Kimball, et al., 1998). This results in the

dissociation of 4EBP1 from eIF4E and the union of eIF4E and eIF4G, resulting in the formation of eIF4F, a necessary step in translation initiation (Kimball, et al., 1998). An additional point of control for translation initiation involves eIF2. Phosphorylation of the alpha ($\alpha$) subunit of eIF2 causes it to inhibit its former substrate, eIF2B. As a result, the methionyl tRNA complex cannot enter translation initiation and protein synthesis is depressed (Kimball, et al., 1998). Thus, increased phosphorylation of 4EBP1 and decreased phosphorylation of eIF2$\alpha$ can both be taken as signs that translation initiation may be enhanced

Resistance exercise stimulates both skeletal muscle protein synthesis and breakdown, and the pathway that predominates after a bout of resistance exercise appears to be influenced by what nutrients are consumed prior to, during, and following the exercise bout (Biolo, Fleming, Maggi, Wolfe, 1995a). Additionally, consuming protein at different time points following exercise may result in different time courses of increased protein synthesis. That is, following resistance exercise, humans have shown increases in skeletal muscle protein synthesis at varying points corresponding to the timing of the consumption of protein post-exercise, from one to 24 hours following the exercise bout (Chesley, et al., 1992; Rasmussen, et al., 2000). Post-exercise nutritional influences on skeletal muscle protein synthesis are not fully understood, however. Questions remain regarding the optimal timing, energy, and macronutrient content of post-

exercise nutrition to maximize protein synthesis. Moreover, while there is extensive data on skeletal muscle protein synthesis in humans (i.e., direct measurements of synthesis by phenylalanine infusion; Biolo, et al., 1995; Borsheim, et al., 2002; Chesley, et al., 1992; Rasmussen, et al., 2000; Tipton, et al., 1999), little work has focused on the regulation of translation initiation in humans. That is, measurements of proteins involved in translation initiation in humans are scarce, as only a few studies (Liu, et al., 2001; Liu, et al., 2002) have investigated this prior to the current study. The present study was undertaken to determine the effect of post-exercise protein feeding on the phosphorylation of 4EBP1 and eIF2$\alpha$. It was hypothesized that feeding protein and carbohydrate following a single bout of exhaustive eccentric exercise would result in a greater phosphorylation of 4EBP1 and a lesser phosphorylation of eIF2$\alpha$, which implies that there will be a greater interaction of eIF4E with eIF4G (active complex) relative to the eIF4E-4EBP1 (inactive complex) when compared to feeding only carbohydrate or a water control. In addition, this implies that the entry of the methionyl-tRNA complex will not be inhibited by eIF2$\alpha$. This is taken to mean that protein feeding immediately following resistance exercise will stimulate the formation of a more active protein translation complex at the ribosome, an essential molecular component to protein synthesis.

## Materials and Methods

Prior to beginning this study, approval was obtained from the Colorado State University Human Research Committee. Additionally, all subjects completed an informed consent form and a health history questionnaire. All study procedures were thoroughly explained to each subject and all subjects were informed that their participation in the study was completely voluntary and that they could withdraw from the study at any time.

### Subject Recruitment

Forty-six adult males, aged 20-34, were recruited for the present study; thirty six subjects met the criteria for the study and completed the entire protocol. The remaining ten subjects were removed from the study due to non-compliance. All subjects were nonsmokers, free of metabolism-altering medications, nonvegetarian, and had not participated in resistance training within the last 12 months. Subjects were randomly allocated into one of three groups: control (CON, n=12), carbohydrate (CHO, n=12), or carbohydrate plus protein (CP, n=12).

Preliminary Testing

All subjects reported for preliminary testing at least three days prior to starting the study protocol. Height was measured with a wall-mounted stadiometer to the nearest 0.1 centimeter, body weight was measured to the nearest 0.1 kilogram on a Health O Meter beam balance scale (Precision Weighing Balances, Bradford, MA), and body fat (percent and absolute fat mass) was measured by dual energy x-ray absorptionmetry following the DPX-IQ Imaging Densitometer User's Manual (DEXA, DPX, Lunar Radiation, Madison, WI) using DPX-IQ X-Ray Bone Densitometer with Smart Scan™ software version 4.7e (Lunar Corp., Madison, WI).

Percutaneous muscle biopsies were taken at three time points: at least 48 hours prior to the exercise bout and two and 24 hours following the exercise bout. A Bergstrom needle with suction was used for extraction; tissue was taken from the vastus lateralis, on the lateral aspect of the thigh.

Study Day #1 Feeding

All subjects had a controlled dietary intake on study day #1; meals and snacks were provided by the study administrators in labeled bags and all uneaten food was returned the next day. Total caloric intake was determined by the Harris Benedict equation estimation of basal metabolic rate times an activity

factor of 1.5 (Harris & Benedict, 1919). Total calories allowed was rounded to the nearest of the following caloric levels: 2500, 2700, 3000, 3200, and 3500. All subjects received identical meals while the snacks varied in size and composition to account for differences in total caloric intakes between the subjects. The macronutrient composition of each subject's controlled intake was identical: 60% of calories from carbohydrate, 25% from fat, and 15% from protein.

Five-day Study Period

All subjects reported to the testing site in a fasted state between the hours of 5:45am and 10:00am on day #1 of the study period. Upon arrival, subjects were given a standard breakfast consisting of 681 calories (Appendix F) and allowed 15 minutes to eat all food provided. A 30 minute digestion period followed. Prior to beginning exercise, subjects warmed up for 10 minutes on a cycle ergometer.

The resistance exercise protocol consisted of the eccentric phase of the leg extension exercise; 10 sets of 10 repetitions at 130% of the concentric leg extension 1RM was the goal. Subjects were allowed to warm up with a light resistance and then began the exercise protocol. In order to allow the subjects to perform only the eccentric phase of the exercise, test administrators would lift the weight up and the subject would confirm that he was ready for the resistance

to be transferred, at which time he would then slowly lower the bar (over a count to six) to the starting position. Each set was performed to fatigue or a maximum of 10 repetitions. A period of three minutes was allowed between sets and when the subject could complete no more repetitions, the exercise session was ended. A subject was allowed to achieve less than 10 repetitions in a set and still proceed to the next set if he felt that more repetitions could be completed. All sets and repetitions were recorded and the total number of repetitions calculated (SF 10x10 Exercise Record Sheet, Appendix G).

Following exercise, subjects received a beverage divided into two equal doses. Immediately following exercise, subjects received the first beverage, which consisted of water (CON, n=12), carbohydrate (CHO, n=12), or carbohydrate+protein (CP, n=12). The CP group received 1.0 grams of carbohydrate/kilogram body weight and 0.3 grams of protein/kilogram body weight, which was converted into a volume of the beverage to be given to the subjects. The CHO group received 1.0 g/kg of a glucose-fructose mix; the CON group received an isovolumetric amount of water. Subjects were then allowed minimal activity (e.g., walking); a second dose of the same beverage was consumed 30 minutes after exercise ended. Each dose provided was half of the calculated amount required by the subject. The mean amounts of energy,

carbohydrate, and protein provided to each group in the two beverage doses combined are reported in Table 4.

Two hours after the cessation of exercise, subjects reported for their second muscle biopsy. Subjects were sent home with a cooler containing the food they were to eat for the rest of the day and instructed to eat the meals in order and to eat as much as possible in the cooler; any uneaten food was to be returned the next day. Subjects were asked to avoid planned physical activity in order to not confound future assessments of muscle soreness. Twenty-four hours after exercise had ended, subjects reported for their final muscle biopsy.

Measurement of Skeletal Muscle Translation Initiation Markers

*Homogenization of muscle tissue*

Muscle samples were removed from -80°C, weighed, and homogenized on ice in seven volumes of homogenization buffer with a mortar and pestle. Upon completion of homogenization, the slurry was transferred to a 1.5 microliter (μl) or 1.7μl eppendorf tube and centrifuged at 4°C for 10 minutes at 10,000 rpm. The supernatant (cell lysate) was then aspirated, placed in a labeled eppendorf tube, supernatant volume recorded, and returned to -80°C. Any remains of the homogenization were placed in biohazard.

*Immunoblotting*

Eukaryotic initiation factors from the skeletal muscle homogenates were analyzed by western blot on a 12-15% Tris-HCl Ready Gel Precast Gel (Bio-Rad Laboratories, Inc., Hercules, CA). Membranes were incubated in primary antibody (rabbit polyclonal p-eIF2$\alpha$ (Ser 52) or p-4EBP1 (Ser65/Thr 70); Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and secondary antibody (anti-rabbit IgG-HRP; Cell Signaling Technology, Inc., Beverly, MA). Blocking peptides for the corresponding proteins [p-eIF2$\alpha$ (Ser 52) or p-4EBP1 (Ser65/Thr70); Santa Cruz Biotechnology, Inc., Santa Cruz, CA] were run initially to ensure specificity. Protein bands were quantified using LabWorks Image Acquisition and Analysis Software (UVP BioImaging Systems, Inc. Upland, CA).

Statistical Methods

A 3x3 repeated measures analysis of variance was computed to compare differences on 4EBP1 and eIF2$\alpha$ phosphorylation between treatment condition (CP, CHO, CON) and time (pre-exercise, 2 hours post-exercise, 24 hours post-exercise). Where appropriate, Bonferroni's procedure was used for post-hoc testing. Significance was set at $p<.05$. Data are presented as mean $\pm$ standard deviation. The Statistical Package for the Social Sciences 12.0 (SPSS 12.0; SPSS, Chicago, Ill) was used for all statistical procedures.

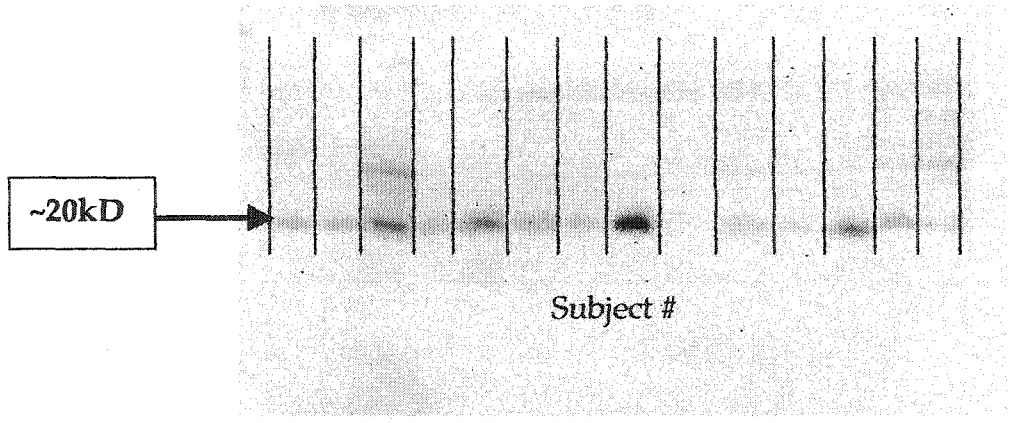
## Results

Subject Characteristics

Subject characteristics are shown in Table 2. There were no significant differences between the groups for age, height, or body mass ($p>.05$).

4EBP1 Phosphorylation

Due to undetectable levels of phosphorylated 4EBP1 in several subjects, treatment group numbers are reduced for 4EBP1 phosphorylation (CP n=6, CHO n=9, CON n=5). The time*treatment interaction effect was nonsignificant for 4EBP1 phosphorylation [$F$ (4,34)=0.29, $p$=0.44, $\eta_p^2$=.03]. The main effect for time was nonsignificant for 4EBP1 phosphorylation [$F$ (2,34)=1.26, $p$=0.15, $\eta_p^2$=.07]. The main effect for treatment was nonsignificant for 4EBP1 phosphorylation [$F$ (2,17)=0.14, $p$=0.43, $\eta_p^2$=.02]. Mean±standard deviation of each treatment and time combination are reported in Table 5 and shown in Figure 11.



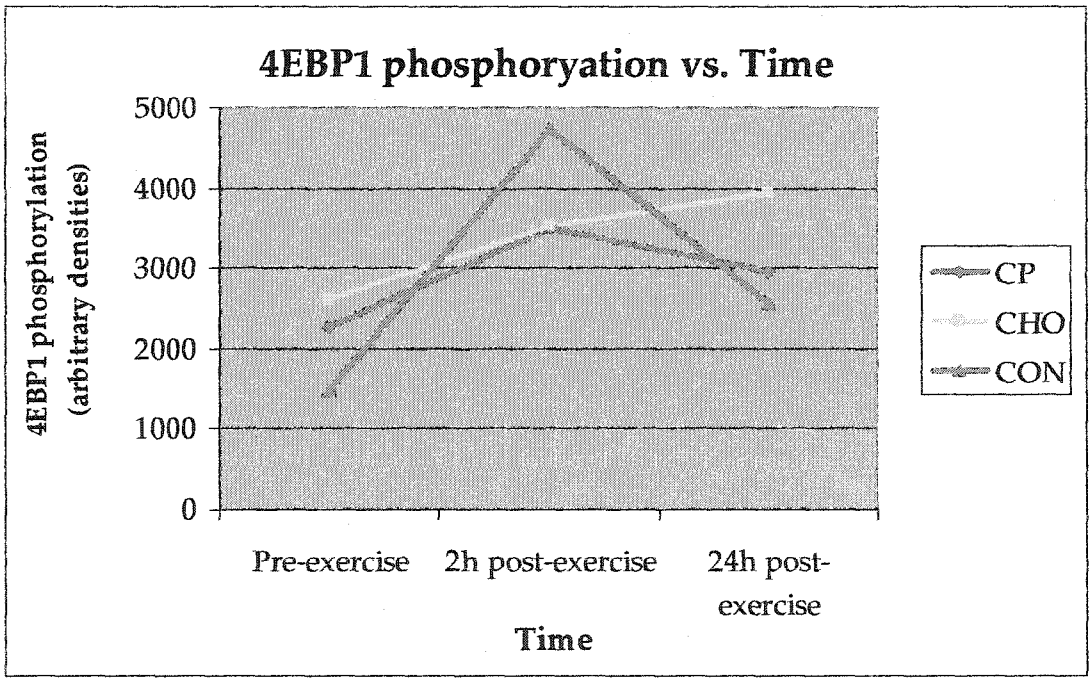



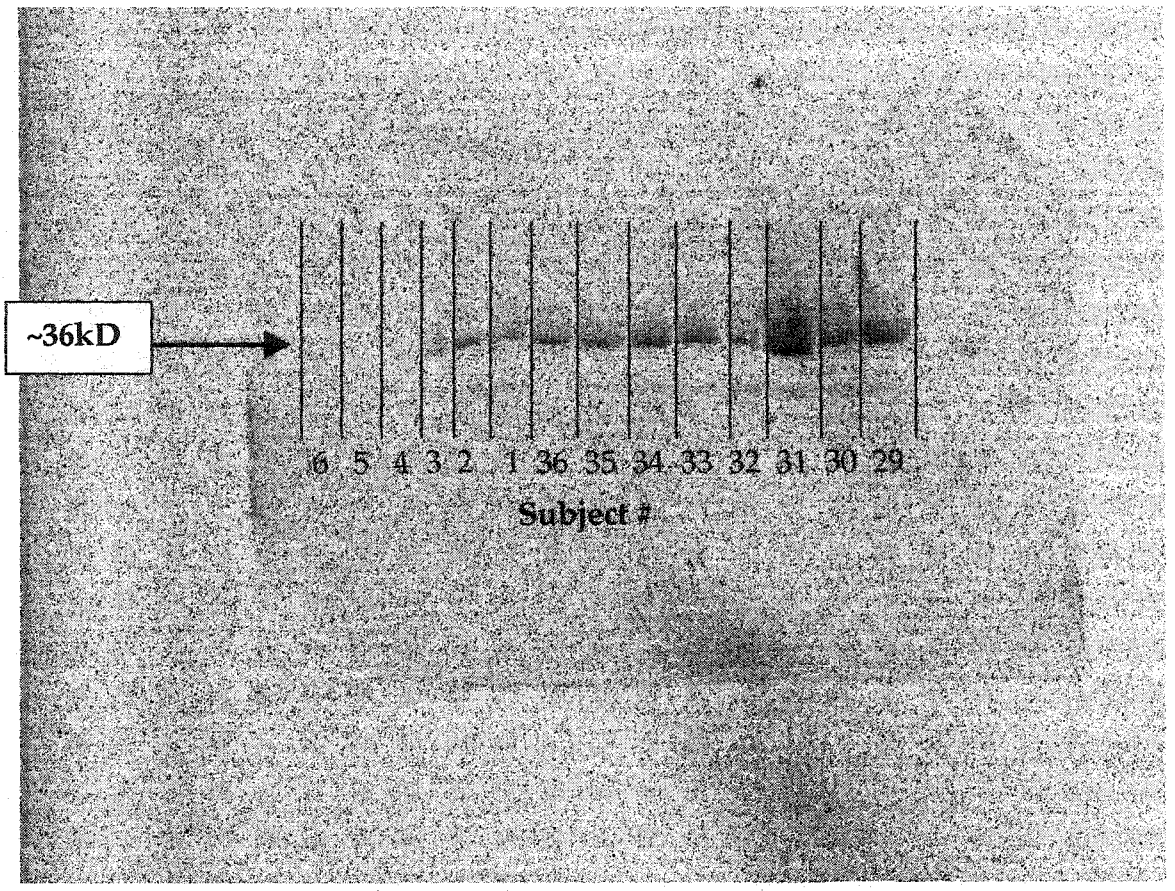

Figure 11: a) *Top:* Representative 4EBP1 western blot (subjects 15-28, pre-exercise). b) *Bottom:* 4EBP1 phosphorylation vs. Time.

Table 5: 4EBP1 phosphorylation. Pre=pre-exercise, 2h=2 hours post-exercise, 24h= 24 hours post-exercise. Data presented as mean±SD.

| | 4EBP1 phosphorylation (arbitrary densities) |
|---|---|
| CP pre | 2262.2 ± 2634.8 |
| CP 2h | 3487.8 ± 3477.2 |
| CP 24h | 2952.8 ± 3498.2 |
| CHO pre | 2603.0 ± 2467.0 |
| CHO 2h | 3531.9 ± 3193.2 |
| CHO 24h | 3948.0 ± 5397.4 |
| CON pre | 1471.6 ± 1767.8 |
| CON 2h | 4734.7 ± 3504.0 |
| CON 24h | 2551.2 ± 4052.3 |

eIF2$\alpha$ phosphorylation

The time*treatment interaction effect was nonsignificant for eIF2$\alpha$ phosphorylation [$F$ (4,60)=.57, $p$=.69, $\eta_p^2$=.04]. The main effect for time was nonsignificant for eIF2$\alpha$ phosphorylation [$F$ (2,60)=.95, $p$=.49, $\eta_p^2$=.02]. as was the main effect for treatment [$F$ (2,30)=.80, $p$=.46, $\eta_p^2$=.05]. Mean±standard deviation of each treatment and time combination are reported in Table 6 and shown in Figure 12.



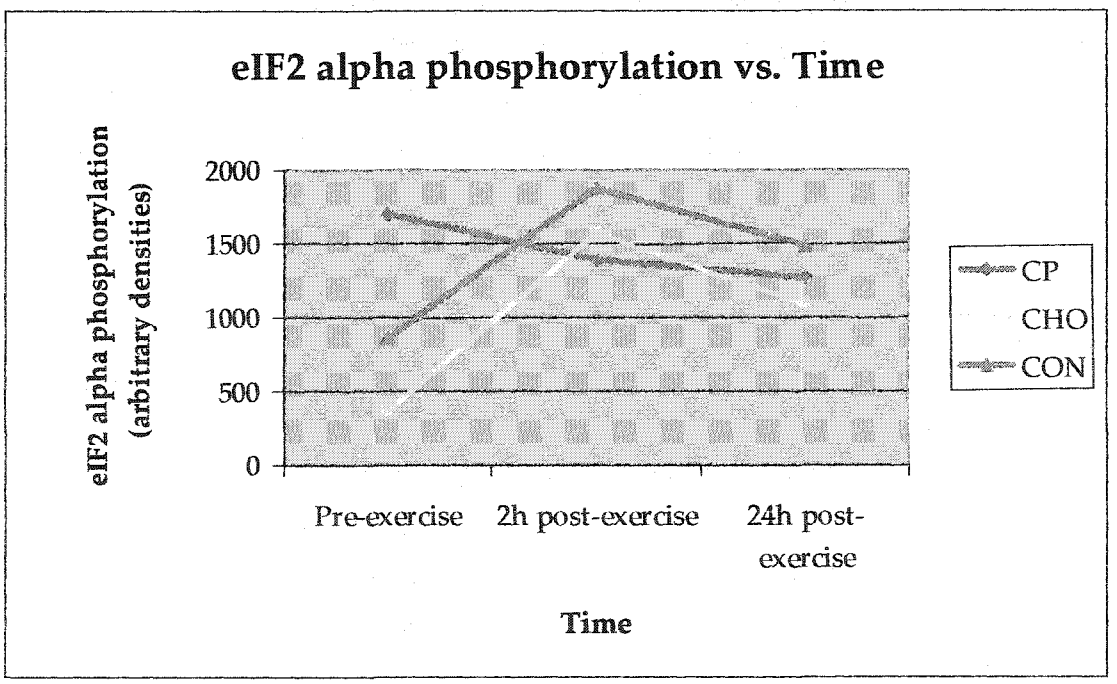




Figure 12: a) *Top:* Representative eIF2$\alpha$ western blot. (Subjects 29-36, pre-exercise; subjects 1-6, 2h post-exercise). b) *Bottom:* eIF2$\alpha$ phosphorylation vs. Time.

Table 6: eIF2α phosphorylation. Pre=pre-exercise, 2h=2 hours post-exercise, 24h= 24 hours post-exercise. Data presented as mean±SD.

| | eIF2α phosphorylation (arbitrary densities) |
|---|---|
| CP pre | 1699.9 ± 3156.0 |
| CP 2h | 1386.1 ± 1922.7 |
| CP 24h | 1272.5 ± 1036.4 |
| CHO pre | 359.1 ± 810.6 |
| CHO 2h | 1570.2 ± 1819.0 |
| CHO 24h | 1099.4 ± 852.9 |
| CON pre | 870.9 ± 1173.8 |
| CON 2h | 1875.0 ± 2514.4 |
| CON 24h | 1493.1 ± 1243.9 |

## Discussion

No significant time, treatment, or time*treatment interaction was observed for the phosphorylation of 4EBP1 or eIF2α in the present study. This occurred despite the presence of well-known stimulator(s) of skeletal muscle protein synthesis: resistance exercise, carbohydrate (insulin), and protein (amino acids) (Anthony, et al., 2002; Balage, et al., 2001; Biolo, et al., 1995; Farrell, et al., 2000; Hernandez, et al., 2000; Kimball, et al., 1997; Long, et al., 2000).

4EBP1 phosphorylation

The phosphorylation state of 4EBP1 has been shown to increase following stimulation of protein synthesis, in order to allow for the union of eIF4E with eIF4G and eIF4A, although the majority of this work has been done in rodents infused with amino acids (Anthony, et al., 2002; Kimball, et al., 1997; Long, et al., 2000). Each of these studies utilized four treatment groups: control, amino acids (AA), insulin, and AA+insulin. Anthony, et al., (2002) infused rats with somatostatin or a vehicle for a period of one hour followed by infusion of leucine or saline. Thirty minutes following the second infusion, the AA and AA+insulin groups significantly increased the phosphorylation of 4EBP1; the AA+insulin group had a significantly greater phosphorylation level than the AA group. Kimball, et al., (1997) infused rats with one of the four treatments for 95 minutes and found that the insulin group had a 3.3 fold increase in the highly phosphorylated form of 4EBP1, 4EBP1$\gamma$. Long, et al., (2000) utilized a three hour infusion period, observing 4EBP1 phosphorylation with the four treatments. The AA and AA+insulin groups had a decreased level of 4EBP1$\alpha$, the form of 4EBP1 that is least likely to bind eIF4E.

Balage, et al., (2001) also used rodents but incorporated a one hour oral feeding paradigm (following a 17 hour fast) to investigate the effect of amino acids on 4EBP1 phosphorylation two hours following the cessation of feeding.

As with the infusion studies, a significant increase in the phosphorylation of 4EBP1 was observed, indicating that one hour of oral administration of amino acids is sufficient to increase 4EBP1 phosphorylation as well. Because of the paucity of research in humans, it is unknown if 4EBP1 responds differently in humans compared to rodents. Liu, et al. (2002), however, did observe an increase in 4EBP1$\beta$ and $\gamma$ (the highly phosphorylated forms of 4EBP1) in response to a six hour amino acid infusion in humans.

Following the union of eIF4E, eIF4G, and eIF4A, the newly formed eIF4F is then able to bind the mRNA cap structure and enter translation initiation (Kimball, et al., 2002). In the current study, it was expected that the CP and CHO groups, due to the stimulus of insulin (and amino acids in the CP group), would demonstrate an increased phosphorylation of 4EBP1 as an indication of the upregulation of translation initiation; this was not the case, however, as a significant time effect was not observed. Additionally, it was hypothesized that the CP group would demonstrate a significantly greater level of 4EBP1 phosphorylation in comparison with the CHO and CON groups due to the ingestion of protein following exercise; however, a significant treatment effect was not observed. It is worth noting that the variability in the subject data was quite great (Table 5 and Table 6), which likely contributed to the finding of nonsignificance for both 4EBP1 and eIF2$\alpha$ phosphorylation.

The possibility that skeletal muscle protein synthesis was not stimulated seems unlikely, as the resistance exercise protocol performed by the subjects was comparable to other human studies (Biolo, et al., 1995) and rodent studies (Farrell, et al., 2000; Hernandez, et al., 2000) in which skeletal muscle protein synthesis was increased following the intense lower body resistance exercise. In particular, the CP group would have been expected to increase skeletal muscle protein synthesis based on the ingestion of protein following the exercise bout. On average, the CP group consumed 22 grams of protein following the exercise bout; a significant increase in skeletal muscle protein synthesis in humans has been observed with oral ingestion of as little as six grams of protein when measured at one and three hours following exercise (Rasmussen, et al., 2000).

It is well known that eccentric exercise causes significant muscle damage (Cheung, et al., 2003), which may have had an effect on post-exercise protein synthesis in the current study, although this is unknown. This type of exercise was chosen for the present study as another end point of interest was skeletal muscle soreness, which is another recognized effect of eccentric exercise (Clarkson, Byrnes, McCormick, Turcotte, & White, 1986).

It must, however, be taken into account that the phosphorylation of 4EBP1 is not the only molecular action involved in increasing the rate of translation initiation. Therefore it may be the case, as was expected, that translation

initiation was upregulated as a result of the aforementioned stimuli, but was not reflected in a change in 4EBP1 phosphorylation levels. As mentioned previously, the majority of studies investigating 4EBP1 phosphorylation have utilized rodents rather than human skeletal muscle. It is possible that humans do not rely to the same extent on the phosphorylation of 4EBP1 to increase levels of translation initiation, although one study has reported increased levels of 4EBP1 phosphorylation following six hours of amino acid infusion in healthy humans (Liu, et al., 2002). However, several rodent studies have reported increases in 4EBP1 phosphorylation as a result of amino acid infusions ranging from 90 minutes to three hours; two hours following a one hour oral feeding, an increase in the phoshphorylation of 4EBP1 was also observed in rats (Anthony, et. al., 2002; Balage, et al., 2001; Long, et al., 2000). In the current study, measures of 4EBP1 phosphorylation were taken at two and 24 hours post-exercise; it is possible that changes in this parameter occurred between these two time points, thereby eluding measurement. These time points were chosen, however, in order to capture both the immediate and delayed effects on activation of eIFs, as no work in human 4EBP1 or eIF2$\alpha$ had been published at this point. Further work should focus on additional post-exercise measurement times. Additionally, translating the effectiveness of an amino acid infusion in rodents to an oral feeding in humans is difficult. Similarly, a one hour feeding in rodents

(Balage, et al., 2001) is likely different than two five minute beverage consumptions, as done in the present study. Clearly more work in human skeletal muscle tissue is needed to help define the role that 4EBP1 phosphorylation does play in humans.

If it is the case that the phosphorylation of 4EBP1 does play the same role in rodents and humans, it may be possible that ample levels of eIF4F were already formed and able to bind the mRNA cap structure sufficiently. Additionally, an upregulation of translation initiation may have been indicated by other proteins in the pathways of interest. Specifically, the eIF4E "control point" of translation initiation is often regarded as control for translation *specific* mRNAs (Kimball, et al., 1998). For example, fibroblast cells that overexpress eIF4E promote the translation of growth-promoting proteins such as ornithine decarboxylase (Rousseau, Kaspar, Rosenwald, Gehrke, & Sonenberg, 1996). A protein that is regarded to control *global* protein synthesis may be a more appropriate target.

eIF2$\alpha$ phosphorylation

It has been postulated that steps involving eIF2 are those that control global protein synthesis at the level of translation initiation (Kimball, et al., 1998; Kimball, 2002). It would be expected that, when protein synthesis is stimulated,

the phosphorylation of eIF2$\alpha$ would decrease, thereby freeing eIF2B and allowing it to facilitate the guanine nucleotide exchange on eIF2 from GDP to GTP. The eIF2-GTP complex could then enter into translation initiation as part of the methionyl-tRNA complex. This phenomenon has been observed in L6 myoblasts provided with leucine for a period of two hours (Kimball, et al., 1998) and an increase in eIF2$\alpha$ phosphorylation has occurred in as little as ten minutes in Ehrlich ascites tumor cells which were deprived of amino acids or glucose for a period of 60 minutes (Scorsone, et al., 1987). In addition, a wealth of work has been performed in *Saccharomyces cerevisiae* documenting an increased eIF2$\alpha$ phosphorylation in response to amino acid deprivation (Hinnebusch, 1997). The characterization of a mammalian kinase for eIF2$\alpha$ has recently been accomplished in mouse liver and termed mammalian GCN2 (MGCN2) (Berlanga, Santoyo, & Haro, 1999). When human 293T cells were exposed to recombinant MGCN2, an increase in the phosphorylation of eIF2$\alpha$ was observed (Berlanga, et al., 1999). However, when comparing a fasted versus an orally fed state in rodents, no changes in the phosphorylation of eIF2$\alpha$ were observed, although a decreased association of eIF2 with GDP occurred in some cases (Cox, et al., 1988; Jeffrey, et al., 1990; Yoshizawa, et al., 1997). Cox, et al. (1988) and Jeffrey, et al. (1990) incorporated a 48 hour fast followed by refeeding or compared with a fed group. Cox, et al. (1988) observed a decreased skeletal

muscle protein synthesis in the fasted group but no change in eIF2$\alpha$ phosphorylation or the activity of eIF2B. Jeffrey, et al. (1990) observed a decreased association of eIF2 with GDP following the 48 hour fast. Yoshizawa, et al. (1997) observed no change in eIF2$\alpha$ phosphorylation or eIF2B activity following an 18 hour fast in rodents, but did observe an increased 4EBP1 phosphorylation. Therefore, modulation of eIF2 may be able to occur by a way distinct from that of eIF2$\alpha$ phosphorylation, possibly through desphosphorylation of the epsilon ($\varepsilon$) subunit of eIF2B, which has been shown to occur in culture in the presence of insulin (Welsh, et al., 1998). In the current study, an increased phosphorylation of eIF2$\alpha$ was not observed, as no significant effect for time, treatment, or time*treatment interaction was observed. No data has been published in humans prior to this study investigating this phenomenon.

Similarly, the enhanced protein synthetic effect of resistance exercise does not seem to act by way of eIF2, but rather by way of increased 4EBP1 phosphorylation (Bolster, et al., 2003). This study was performed in rodents and therefore is not necessarily applicable to human studies. With the additional protein stimulus in the CP group, an increase in eIF2$\alpha$ phosphorylation would be logical but is not supported by previous rodent studies.

It is important to note that no single member of the eIF family can be taken as a definitive indicator of the rate of translation initiation in skeletal

muscle. It is possible that another protein(s) in these pathways may be a better indicator of changes in the rate of translation initiation, although most sources site the formation of eIF4F and the binding of eIF2 to the met-tRNA complex as the major control points for translation initiation (Kimball, et al., 2002). Nonetheless, measurements of additional protein such as p70S6 kinase or GSK-3 may shed more light on the present observations; clearly, a direct measurement of skeletal muscle protein synthesis would have proven invaluable for interpretation of the results. Future studies which combine tracer-determined rates of protein synthesis with the measurement of markers of translation initiation in human skeletal muscle will provide valuable clarification for the importance of each step toward the biological endpoint (protein anabolism).

Additionally, the timing of protein intake in relation to an exercise bout may influence protein synthesis. As with glycogen resynthesis, the same amount and/or composition of amino acids ingested at different times during recovery from exercise may not yield the same magnitude of protein synthesis (Chesley, et al., 1992; Rasmussen, et al., 2000). The specific time course of the proposed recovery window for protein remains to be elucidated, although the current study's timing of beverage consumption immediately and 30 minutes following exercise is reasonable. Rasmussen, et al., (2000) provided a protein beverage one and three hours following a bout of resistance exercise, observing a significant

increase in skeletal muscle protein synthesis one hour following each ingestion (i.e. at two and four hours post-exercise). Additional studies have observed significant increases in human skeletal muscle protein synthesis following resistance exercise when oral protein ingestion occurred at varying time points including ~40 minutes, one hour, two hours, and 20 hours following exercise (Borsheim, et al., 2002; Chesley, et al., 1992; Tipton, et al., 1999). Although it is unknown if the current study protocol increased skeletal muscle protein synthesis, it is clear that 4EBP1 and eIF2$\alpha$ were unaffected at two and 24 hours post-exercise, regardless of the subjects' nutritional status.

Study Limitations

A direct measurement of skeletal muscle protein synthesis was not performed in the current study. Clearly, measurements of this nature would have been valuable for interpretation of the results of the eIF observations. Because these data were not obtained, it is not possible to discuss any relationship between eIF activation/inactivation and the physiological endpoint – protein synthesis.

In order to analyze the levels of phosphorylated 4EBP1 and eIF2$\alpha$ in the skeletal muscle tissue, western blots were performed. Due to the fact that the proteins of interest have not been analyzed using a phospho-specific antibodies

in human tissue previous to this study, no specific protocol is published for this particular assay. As a result, several difficulties were encountered in the process of establishing the proper protocol for these phospho-specific proteins in human tissue (e.g., proper antibody dilutions, incubation times, exposure times). It is clear that more work needs to be done in this area to help establish a specific protocol for these proteins (as well as others involved in translation initiation) in human tissue.

The current study was not able to be blinded due to the fact that each treatment, most notably the CP treatment, had a characteristic appearance and taste that may have been familiar to the subjects. Although it is likely that this did not affect the levels of eIFs present in the muscle, a subject who was aware of which treatment they were receiving may have felt that they "should" perform well on the strength and power tests and/or feel less soreness post-exercise. Additionally, owing to the same reasons and due to the fact that the study investigators prepared the treatments themselves, they were unable to be blinded to the treatments received by each subject.

Females were not included in the current study in order to avoid any impact the menstrual cycle may have had on the outcome measures. Therefore, any inferences made from the results can only be applied to a male population between the ages of 20 and 33.

## Conclusion

No significant changes were found in the phosphorylation of 4EBP1 or eIF2$\alpha$ following resistance exercise and post-exercise nutrition; therefore, the ingestion of a carbohydrate and protein beverage following a bout of intense eccentric exercise does not appear to significantly affect these markers of translation initiation. Because there is no clear mechanistic explanation regarding how post-exercise protein intake alters skeletal muscle protein synthesis, it is important to investigate the pathways that may be utilized and how they may interact. Additionally, more work in human tissue is necessary to determine responses specific to this species.

# CHAPTER 5

# THE AFFECT OF POST-EXERCISE PROTEIN INTAKE ON MUSCULAR SORENESS, CONCENTRIC STRENGTH, AND VERTICAL JUMP ABILITY

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**Abstract**

It is well established that eccentric exercise induces delayed onset muscle soreness (DOMS) within 24 hours after the exercise bout. DOMS can present as muscle stiffness, but can also result in debilitating pain, which may compromise further training or performance. Although the exact mechanism(s) of DOMS development remains to be determined, several methods of preventing and/or treating DOMS have been proposed. No single method, however, has proven to be a consistently effective treatment for DOMS. Surprisingly, nutrition as a preventive and treatment modality for DOMS has not been extensively studied. The current study investigated the affect of post-exercise nutrition (CON, CHO, or CP) on subjective assessments of muscle soreness, single-leg one-repetition maximum leg extension, and vertical jump at various time points following a single bout of exhaustive eccentric exercise. It was hypothesized that the CP group would improve the time-course of recovery from eccentric exercise when

compared with CHO or CON groups. Despite significant time effects for skeletal muscle soreness, strength, and vertical jump (soreness and strength $p<.001$, vertical jump $p=.01$), no differences were observed between the treatment groups for these variables at any time. This indicates that post-exercise nutrition may not be effective in alleviating DOMS or promoting recovery of muscular performance.

**Introduction**

After a prolonged period without training, or with the presentation of a new mode of exercise, nearly all individuals experience some skeletal muscle soreness. All types of muscular action (concentric, isometric, and eccentric) can result in soreness, although eccentric action is most likely to precipitate it (Clarkson, Byrnes, McCormick, Turcotte, & White, 1986). The soreness in question typically begins after some period of hours, and peaks at 24-48h after the bouts of exercise. This delayed-onset muscle soreness (DOMS) can present as minor muscle stiffness ranging to pain that restricts movement and is typically accompanied by strength loss (Cheung, et al., 2003). The soreness typically begins near the musculotendinous joint and spreads throughout the muscle over time (MacIntyre, Reid, & McKenzie, 1995).

The mechanism of DOMS has been suggested to be due to several different factors including lactic acid build up in the muscle, muscle damage, connective tissue damage, inflammation, muscle spasm, and enzyme efflux (Cheung, et al., 2003). The hypothesis that lactic acid build up in the muscle contributes to DOMS has largely been dismissed, as lactic acid levels typically return to basal levels within one hour following exercise while DOMS occurs much later following exercise. Typically, post-exercise lactate levels have not correlated with muscular pain; in downhill runners, elevations in lactic acid were not seen post-exercise despite the presence of soreness (Schwane, Watrous, Johnson, & Armstrong, 1983). Additionally, the muscle spasm theory remains under debate, as assessment techniques are questioned. Eccentric muscle action, the lengthening of the muscle when resistive forces are applied, involves approximately twice the force production of isometric action while only about 10% more cross bridges are formed (Faulkner, Brooks, & Opiteck, 1993); this may result in mechanical disruption of the muscle tissue or connective tissue. Disruption of this nature may cause delayed soreness in the muscle and/or a decreased maximal force production in the days following the eccentric exercise (Armstrong, 1984).

DOMS may present pain that is severe enough to hinder further training and/or performance. Interestingly, several methods of preventing or relieving

DOMS have been investigated, including massage (Hilbert, Sforzo, & Swensen, 2003), anti-inflammatory medications (Tomakidis, Kokkinidis, Smilios, & Douda, 2003), stretching (Lund, Vestergaard-Poulsen, Kanstrup, & Sejrsen, 1998; Johansson, Lindstrom, Sundelin, & Lindstrom, 1999), cryotherapy (Paddon-Jones & Quigley, 1997), ultrasound (Craig, Bradley, Walsh, Baxter, & Allen, 1999), hyperbaric oxygen therapy (Mekjavic, Exner, Tesch, & Eiken, 2000), and exercise (Dannecker, Koltyn, Riley, & Robinson, 2002). These investigations have provided mixed results, and as such, no single method has not proven to be a reliable preventor of or treatment for DOMS. It is likely that multiple factors act together to produce DOMS including the muscle damage, connective tissue damage, enzyme efflux, and the inflammation (Cheung, et al., 2003).

Despite investigations into factors that may aid in alleviating DOMS after eccentric exercise, the affect of post-exercise nutrition on DOMS and strength and power recovery has not been extensively studied. It is well established that oral amino acid feeding results in increased skeletal muscle protein synthesis, and this can occur in a dose dependent manner (Wolfe, 2002). For instance, 6 grams and 40 grams of essential amino acids were administered orally following resistance exercise and resulted in an increase in net muscle protein balance from negative to positive (Borsheim, Tipton, Wolf, & Wolfe, 2002; Tipton, Ferrando, Phillips, Doyle, & Wolfe, 1999). This increased rate of protein synthesis

following exercise may contribute to an alleviation of DOMS by repairing damaged tissue.

A recent study on post-exercise protein nutrition in military recruits reported a decreased level of muscle soreness when receiving protein in comparison with a placebo or carbohydrate (Flakoll, Judy, Flinn, Carr, & Flinn, 2004). This would bode well for populations such as the military in which DOMS may interfere with work performance. The current study was undertaken to determine the affect of a protein and carbohydrate beverage, in comparison with a carbohydrate only beverage and a water control, on subjective assessments of muscle soreness at 24, 48, 72, and 96 hours after a single bout of exhaustive eccentric exercise. In addition, the relation of post-exercise nutrition to the recovery of muscle performance, as assessed by concentric single-leg one-repetition maximum leg extension performance and vertical jump performance 48 and 96 hours after a single bout of exhaustive eccentric exercise, was studied. It was hypothesized that the consumption of the protein and carbohydrate beverage, after a single bout of exhaustive eccentric exercise, would decrease subjective muscle soreness, increase a concentric single-leg one-repetition maximum leg extension performance, and increase vertical jump performance earlier than with the consumption of a carbohydrate only beverage or a water control.

## Materials and Methods

Prior to beginning this study, approval was obtained from the Colorado State University Human Research Committee. Additionally, all subjects completed an informed consent form and a health history questionnaire. All study procedures were thoroughly explained to each subject and all subjects were informed that their participation in the study was completely voluntary and that they could withdraw from the study at any time. No significant differences ($p<.05$) were observed between the study groups for age, height, or mass (Table 2).

### Subject Recruitment

Forty-six adult males, aged 20-34, were recruited for the present study; thirty six subjects met the criteria for the study and completed the entire protocol. The remaining ten subjects were removed from the study due to non-compliance. All subjects were nonsmokers, free of metabolism-altering medications, nonvegetarian, and had not participated in resistance training within the last 12 months. Subjects were randomly allocated into one of three groups: control (CON, n=12), carbohydrate (CHO, n=12), or carbohydrate plus protein (CP, n=12).

Preliminary Testing

All subjects reported for preliminary testing at least three days prior to starting the study protocol. Height was measured with a wall-mounted stadiometer to the nearest 0.1 centimeter, body weight was measured to the nearest 0.1 kilogram on a Health O Meter beam balance scale (Precision Weighing Balances, Bradford, MA), and body fat (percent and absolute fat mass) was measured by dual energy x-ray absorptionmetry following the DPX-IQ Imaging Densitometer User's Manual (DEXA, DPX, Lunar Radiation, Madison, WI) using DPX-IQ X-Ray Bone Densitometer with Smart Scan™ software version 4.7e (Lunar Corp., Madison, WI).

Vertical jump was performed from a standing position alongside a wall with no approach steps; the best of five jumps was recorded. Concentric knee extension 1RM was performed after the vertical jump, in order to avoid fatigue, on a Cybex Magnum machine (CYBEX International, Inc. Medway, MA, USA). Subjects performed the first repetition at 25% of their body weight and increased weight by 2.3 or 3.4 kilograms (or more if the subject felt they could do so) thereafter in an effort to reach their 1RM weight in six or less repetitions. In performing the exercise, subjects were instructed to perform the concentric contraction on their own, while the test administrators lowered the weight in order for the subject to avoid eccentric fatigue.

Study Day #1 Feeding

All subjects had a controlled dietary intake on study day #1 in order to assure identical dietary macronutrient composition; meals and snacks were provided by the study administrators in labeled bags and all uneaten food was returned the next day. Total caloric intake was determined by the Harris Benedict equation determination of basal metabolic rate times 1.5 to account for activity (Harris & Benedict, 1919). Total calories allowed was rounded to the nearest of the following caloric levels: 2500, 2700, 3000, 3200, and 3500. All subjects received identical meals while the snacks varied in size and composition to account for differences in total caloric intakes between the subjects. The macronutrient composition of each subject's controlled intake was identical: 60% of calories from carbohydrate, 25% from fat, and 15% from protein. Each subject's actual energy intake was determined by accounting for any food waste from the prepared meals.

Five-day Study Period

All subjects reported to South College gym in a fasted state between the hours of 5:45am and 10:00am on day #1 of the study period. Upon arrival, subjects were given a standard breakfast consisting of 681 kilocalories (69.1% carbohydrate, 14.3% protein, 18.0% fat) and allowed 15 minutes to eat all food

provided. A 30 minute digestion period followed. Prior to beginning exercise, subjects warmed up for 10 minutes on a cycle ergometer.

The resistance exercise protocol consisted of the eccentric phase of the leg extension exercise; with a goal of completing 10 sets of 10 repetitions at 130% of the concentric leg extension 1RM. Subjects were allowed to warm up with a light resistance and then began the exercise protocol. In order to allow the subjects to perform only the eccentric phase of the exercise, test administrators would lift the weight up and the subject would confirm that he was ready for the resistance to be transferred, at which time he would then slowly lower the bar (over a count to six) to the starting position. Each set was performed to fatigue or a maximum of 10 repetitions. A period of three minutes was allowed between sets and when the subject could complete no more repetitions, the exercise session was ended. A subject was allowed to achieve less than 10 repetitions in a set and still proceed to the next set if he felt that more repetitions could be completed. All sets and repetitions were recorded and the total number of repetitions calculated.

Following exercise, subjects received a beverage divided into two equal doses. Immediately following exercise, subjects received the first beverage, which consisted of water (CON, n=12), carbohydrate (CHO, n=12), or carbohydrate+protein (CP, n=12). The CP group received 1.0 grams of carbohydrate/kilogram body weight and 0.3 grams of protein/kilogram body

weight, which was converted into a volume of the beverage to be given to the subjects. The CHO group received 1.0 g/kg of a glucose-fructose mix; the CON group received an isovolumetric amount of water. Subjects were then allowed minimal activity (e.g., walking); a second dose of the same beverage was consumed 30 minutes after exercise ended. Each beverage provided was half of the calculated amount required by the subject; average nutritional intakes, by group, are reported in Table 4.

Subjects were sent home with a cooler containing the food they were to eat for the rest of the day and instructed to eat the meals in order and eat as much as possible in the cooler; any uneaten food was to be returned the next day. Subjects were asked to avoid planned physical activity in order to not confound future assessments of muscle soreness.

Twenty-four, 48, 72, and 96 hours after exercise had ended, subjects reported to the laboratory and subjectively rated the soreness in their exercised quadriceps as a result of the exercise performed using a visual analog scale. Ratings ranged from "No pain" to "Worst possible pain you have experienced" and subjects were asked to make a mark along a continuous 101 mm line between the two descriptors. Soreness ratings were quantified by measuring how many millimeters away the mark was from the left side of the scale.

Forty-eight and 96 hours after exercise had ended, subjects reported to South College gym for assessments of concentric knee extension 1RM and vertical jump.

Statistical Methods

A 3x3 repeated measures analysis of variance was computed to compare differences on one-repetition maximum and vertical jump between treatment condition (CP, CHO, CON) and time (pre-exercise, 48 hours post-exercise, 96 hours post-exercise). A 3x4 repeated measures analysis of variance was computer to compare differences on post-exercise skeletal muscle soreness between treatment condition (CP, CHO, CON) and time (24, 48, 72, and 96 hours post-exercise). Where appropriate, Bonferroni's procedure was used for post-hoc testing. Significance was set at $p<.05$. Data are presented as mean $\pm$ standard deviation. The Statistical Package for the Social Sciences 12.0 (SPSS 12.0; SPSS, Chicago, Ill) was used for all statistical procedures.

**Results**

Subject Characteristics

A total of thirty-six males completed the entire protocol (CON=12, CHO=12, CP=12); 10 subjects removed themselves from the study prior to

completion. Subject characteristics are shown in Table 2. There were no significant differences between the groups for age, height, or body mass ($p>.05$).

Subjective Assessment of Muscular Soreness

The time*treatment interaction effect was nonsignificant for the subjective assessment of skeletal muscle soreness [$F$ (6,99)=.81, $p$=.56, $\eta_p^2$=.05, Figure 13]. The main effect for treatment was nonsignificant for subjective assessment of skeletal muscle soreness [$F$ (2,33)=.52, $p$=.60, $\eta_p^2$=.03]. The main effect for time was significant for the subject assessment of skeletal muscle soreness [$F$ (3,99)=43.32, $p$<.001, $\eta_p^2$=.57]. Significant differences were observed between pooled means at 24 and 48 hours post-exercise (39.1 mm vs. 49.8 mm), 24 and 96 hours post-exercise (39.1 mm vs. 13.7 mm), 48 and 72 hours post-exercise (49.8 mm vs. 26.6 mm), 48 and 96 hours post-exercise (49.8 mm vs. 13.7 mm), and 72 and 96 hours post-exercise (26.6 mm vs. 13.7mm, Figure 13). Mean±standard deviation of each treatment and time combination are reported in Table 7 and shown in Figure 13.

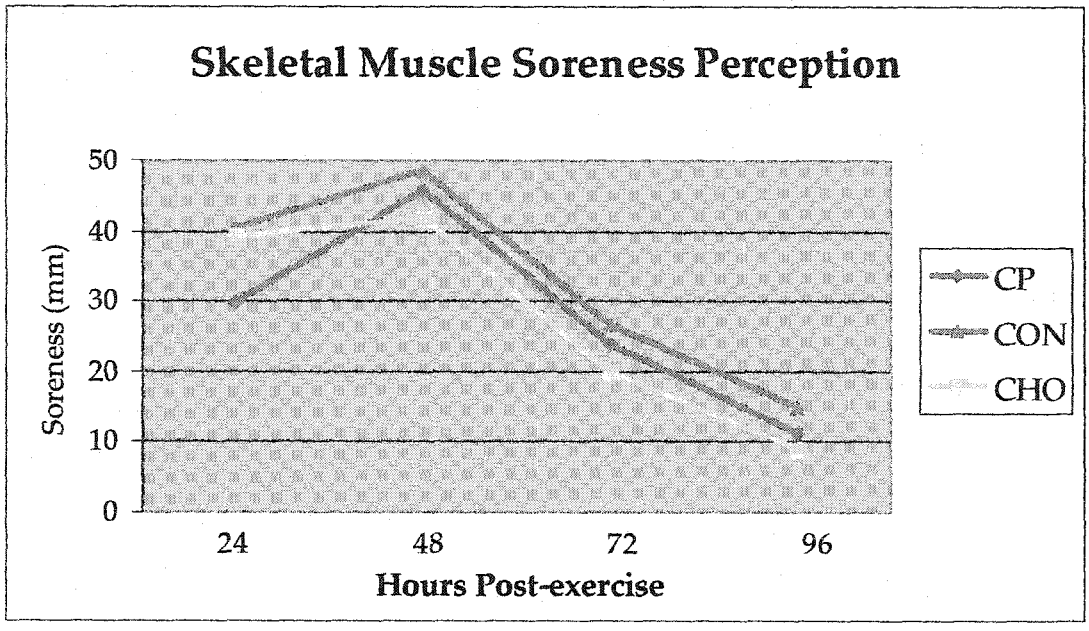


Figure 13: Post-exercise skeletal muscle soreness perception by group.

Table 7: Skeletal Muscle Soreness. 24h= 24 hours post-exercise, 48h=48 hours post-exercise, 72h=72 hours post-exercise, 96h=96 hours post-exercise. Data presented as mean±SD.

| | Skeletal Muscle Soreness (mm) |
|---|---|
| CP 24h | 29.8 ± 15.9 |
| CP 48h | 45.9 ± 19.7 |
| CP 72h | 23.8 ± 17.5 |
| CP 96h | 11.3 ± 12.9 |
| CHO 24h | 37.0 ± 21.2 |
| CHO 48h | 40.2 ± 24.0 |
| CHO 72h | 18.6 ± 19.1 |
| CHO 96h | 8.1 ± 11.6 |
| CON 24h | 43.3 ± 20.4 |
| CON 48h | 47.3 ± 25.5 |
| CON 72h | 24.2 ± 18.7 |
| CON 96h | 12.9 ± 14.3 |

Assessment of Skeletal Muscle Strength

The time*treatment interaction effect was nonsignificant for skeletal muscle strength [$F$ (4,66)=1.67, $p$=.17, $\eta_p^2$=.09, Figure 14]. The main effect for treatment was nonsignificant for subjective assessment of skeletal muscle soreness [$F$ (2,33)=.57, $p$=.57, $\eta_p^2$=.03]. The main effect for time was significant for skeletal muscle strength [$F$ (2,66)=20.08, $p$<.001, $\eta_p^2$=.38]. Significant differences were observed between pooled means at pre-exercise and 48 hours post-exercise

(53.3 kg vs. 42.5 kg) and 48 and 96 hours post-exercise (42.5 kg vs. 50.0 kg, Figure 14). Mean±standard deviation of each treatment and time combination are reported in Table 8 and shown in Figure 14.

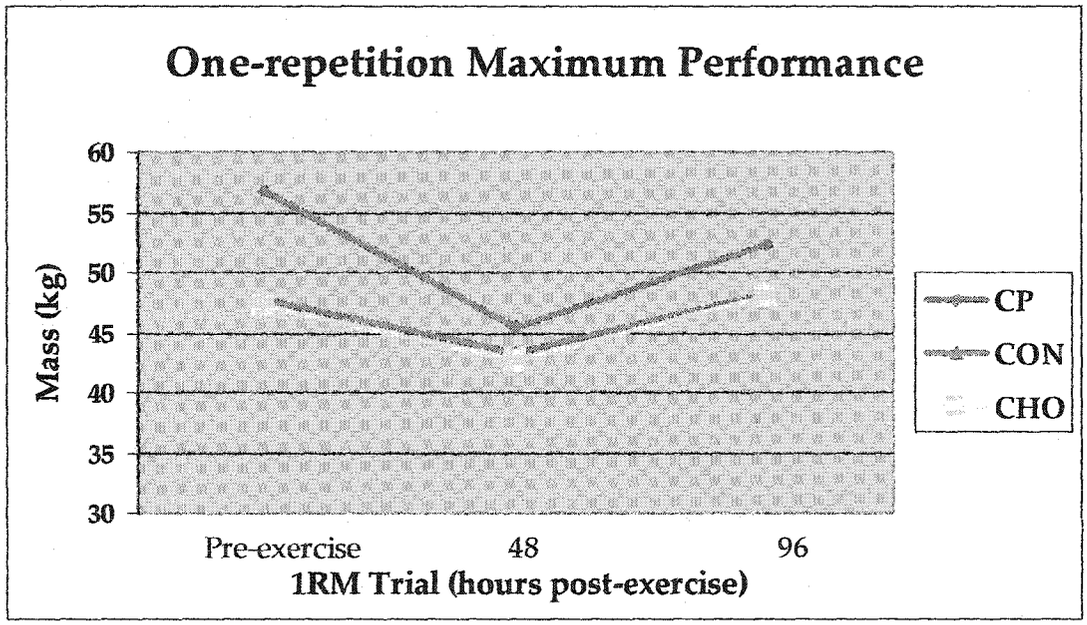


Figure 14: One-repetition maximum performance by group.

Table 8: 1RM performance. Pre=pre-exercise, 48h=48 hours post-exercise, 96h= 96 hours post-exercise. Data presented as mean±SD.

| | 1RM Performance (kg) |
|---|---|
| CP pre | 56.72 ± 11.15 |
| CP 48h | 45.45 ± 17.40 |
| CP 96h | 52.37 ± 16.08 |
| CHO pre | 49.53 ± 12.70 |
| CHO 48h | 44.98 ± 10.57 |
| CHO 96h | 50.57 ± 11.70 |
| CON pre | 48.30 ± 10.31 |
| CON 48h | 42.75 ± 9.31 |
| CON 96h | 48.49 ± 8.97 |

Assessment of Vertical Jump

The time*treatment interaction effect was nonsignificant for vertical jump [$F$ (4,66)=2.08, $p$=.09, $\eta_p^2$=.11, Figure 15]. The main effect for treatment was nonsignificant for vertical jump [$F$ (2,33)=.21, $p$=.81, $\eta_p^2$=.01]. The main effect for time was significant for vertical jump [$F$ (2,66)=4.61, $p$=.01, $\eta_p^2$=.12]. A significant difference was observed between the pooled means at pre-exercise and 48 hours post-exercise (51.5 cm vs. 47.9 cm, Figure 17). Mean±standard deviation of each treatment and time combination are reported in Table 9 and shown in Figure 15.

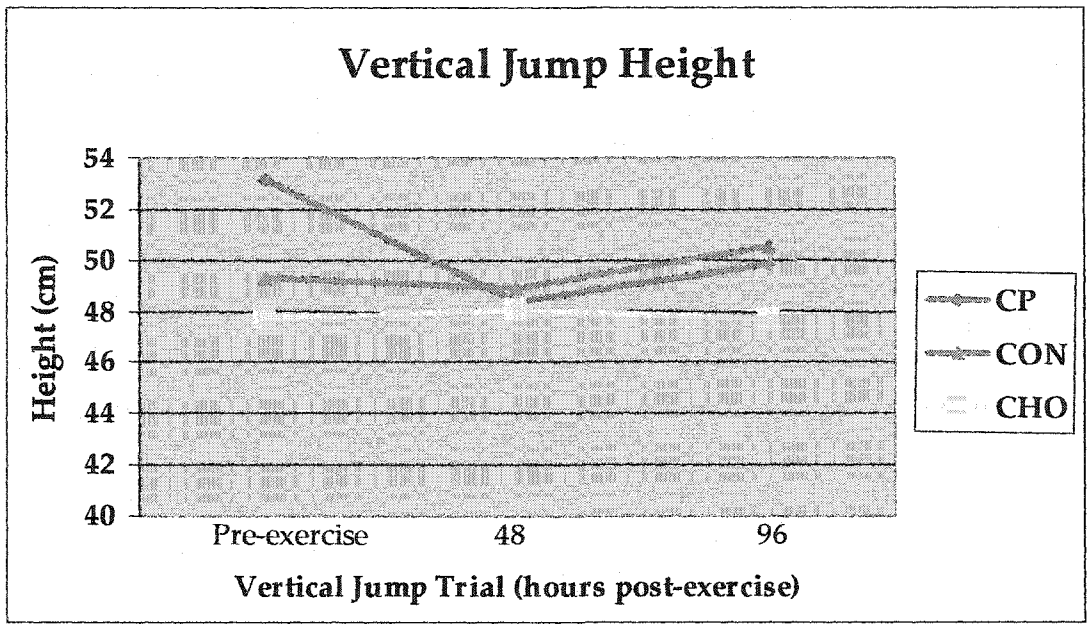


Figure 15: Vertical jump performance by group.

Table 9: Vertical jump performance. Pre=pre-exercise, 2h=2 hours post-exercise, 24h= 24 hours post-exercise. Data presented as mean±SD.

| | Vertical Jump Performance (cm) |
|---|---|
| CP pre | 53.13 ± 10.88 |
| CP 2h | 48.37 ± 12.80 |
| CP 24h | 49.85 ± 13.27 |
| CHO pre | 48.26 ± 10.15 |
| CHO 2h | 48.47 ± 6.54 |
| CHO 24h | 47.84 ± 8.27 |
| CON pre | 51.12 ± 7.64 |
| CON 2h | 48.68 ± 8.55 |
| CON 24h | 50.80 ± 6.27 |

## Discussion

In the present study, post-exercise feeding of protein and carbohydrate had no effect on DOMS in comparison with a water control or a carbohydrate only feeding. Additionally, neither strength nor power was recovered earlier with the protein and carbohydrate feeding in comparison with the water control or carbohydrate only feeding. It has been well established that after unaccustomed eccentric exercise, DOMS is likely to develop (cf. Armstrong, 1984). Several causes of DOMS have been proposed (lactic acid build up in the muscle, muscle damage, connective tissue damage, inflammation, muscle spasm, and enzyme efflux), but it is currently believed that no single factor can be responsible for the development of DOMS (Cheung, et al., 2003). Regardless of the etiology, several methods of treatment have been evaluated for the prevention and/or alleviation of DOMS, including massage (Hilbert, Sforzo, & Swensen, 2003), anti-inflammatory medications (Tomakidis, Kokkinidis, Smilios, & Douda, 2003), stretching (Lund, Vestergaard-Poulsen, Kanstrup, & Sejrsen, 1998; Johansson, Lindstrom, Sundelin, & Lindstrom, 1999), cryotherapy (Paddon-Jones & Quigley, 1997), ultrasound (Craig, Bradley, Walsh, Baxter, & Allen, 1999), hyperbaric oxygen therapy (Mekjavic, Exner, Tesch, & Eiken, 2000), and exercise (Dannecker, Koltyn, Riley, & Robinson, 2002). Despite the array of potential treatments for DOMS, none have provided unequivocal success in

remedying DOMS. Surprisingly, the affect of post-exercise nutrition on the full or partial prevention of DOMS has not been studied and is therefore the primary purpose of the current study.

In the current study subjects were provided with a control, carbohydrate, or carbohydrate and protein beverage immediately after and 30 minutes following intense eccentric exercise in order to determine the affect of post-exercise nutrition on DOMS, concentric quadriceps strength recovery, and recovery of vertical jump ability. It was hypothesized that individuals receiving both carbohydrate and protein would experience less pain associated with DOMS, an earlier recovery of strength, and an earlier recovery of vertical jump ability in comparison with those receiving a control or carbohydrate only beverage.

Subjective Assessment of Muscular Soreness

DOMS is typically sensed within 24 hours after exercise, with the peak of soreness occurring at 48 hours after exercise. Within five to seven days, the soreness has typically withdrawn or markedly alleviated (Cheung, et al., 2003). This was observed in the current study, as there was a significant main effect for time ($p<.001$) for self-reported/subjective soreness; additionally, the time effect accounted for 57% of the overall variability in soreness ratings. Specifically,

soreness was present by 24 hours after the eccentric exercise and peaked at 48 hours after the eccentric exercise in all groups (Figure 13); the average increase in soreness from 24 to 48 hours was significant ($p=.04$). By 72 hours after exercise, soreness had significantly decreased from 48 hours ($p<.001$) and continued to significantly decrease from 72 to 96 hours after exercise ($p<.001$). This pattern of DOMS development and alleviation follows that traditionally seen following eccentric exercise (Smith, et al., 1994; Cleak & Eston, 1992; Tokmakidis, et al., 2003).

Importantly, the current study provided no evidence that DOMS was alleviated as a result of post-exercise nutrition. There was no significant difference in soreness perception in any of the groups at any time points; therefore both the combination of carbohydrate and protein and carbohydrate alone have no greater affect on DOMS than water alone (control).

Because DOMS was measured only at 24 hour intervals, we cannot exclude the possibility that there was a significant difference in perceived muscle soreness between the groups at time points other than those studied (e.g., at 36 hours). In addition, a different feeding schedule may have provided significant results. That is, pre-exercise feeding of protein or feeding during the exercise bout may allow for different protein action within the muscle to affect recovery. Alternatively, feeding for a longer period of time following exercise would

provide a larger amount of protein which may allow for improved recovery. Further research should address these points.

Assessment of Skeletal Muscle Strength

It is typical to observe a decrease in strength following intense eccentric exercise that may not be recovered for a period of days (Cleak & Eston, 1992; Howell, Chleboun, & Conatser, 1993; Smith, et al., 1994; Tokmakidis, et al., 2003); a significant time effect for strength was observed in the present study ($p<.001$, Figure 14). This effect accounted for 38% of the overall variability in 1RM performance. The decrease in strength following exercise is proposed to result from fatigue and/or injury to the muscle. However, it is likely that fatigue is not a factor for more than a few hours after an exercise bout. Because the first assessment of strength occurred at 48 hours after the exercise bout, fatigue can be ruled out as a possible reason for the loss of strength in the present study (Faulkner, et al., 1993).

It has been suggested that with full recovery from fatigue the best method for quantifying the severity of injury in the muscle is by measuring the amount of force that can be produced at varying time points. On average, the subjects' concentric 1RM knee extension strength decreased by 13.34% (6.74 kg) 48 hours after exercise, which is similar to losses in concentric strength in the chest press (-

9.0%), and knee flexion (-11.7%) at 48 hours following eccentric exercise (Smith, et al., 1994; Tokmakidis, et al., 2003). By 96 hours, subjects regained an average of 5.72 kg of the loss, resulting in only a 2.01% decrement from pre-exercise values. In comparison with other data, the recovery of strength occurred quite rapidly in the present study (Howell, et al., 1993); other studies have reported not seeing a complete regain of strength until a period of weeks after exercise. It may have been beneficial to measure strength recovery for a longer period of time following the eccentric exercise bout in order to determine when full recovery was attained and how this related to other recovery measures taken in the study.

On average, strength was at its lowest point at 48 hours after exercise, thereby implying that muscular injury was greatest at this time point also. Forty-eight hours following exercise is the same time point at which muscle soreness was the greatest; therefore it is also possible that as a direct result of the soreness, subjects were unable to perform strength testing to the best of their ability. There were no differences in strength between any of the groups at any time points, however, demonstrating in this study that the post-exercise nutrition model tested herein does not affect strength recovery following exercise (Figure 14).

Assessment of Vertical Jump

Vertical jump is a measure of anaerobic/maximum muscular power (Baechle & Earle, 2000). As expected, as significant time effect was observed for vertical jump in the present study (p=.01, Figure 16); this accounted for 12% of the overall variability in vertical jump performance. On average, vertical jump performance significantly decreased between pre-exercise and 48 hours after exercise (-4.2%, p=.01), but did not significantly differ from baseline levels at 96 hours after exercise (-1.5%). As with the strength testing, it is likely that fatigue did not play a factor in the subjects' ability to generate power for the jumps as the first assessment was performed at 48 hours after the exercise bout. Therefore it is likely that muscular injury was responsible for the significant decrease in vertical jump height at 48 hours following the exercise bout.

Subjects in the current study were able to recover maximal muscular power by 96 hours after the eccentric exercise bout, which is comparable to other studies focusing on power recovery after eccentric exercise (Sargeant & Dolan, 1987).

Study Limitations

The current study was not able to be blinded due to the fact that each treatment, most notably the CP treatment, had a characteristic appearance and

taste that may have been familiar to the subjects. Although it is likely that this did not affect the levels of eIFs present in the muscle, a subject who was aware of which treatment they were receiving may have felt that they "should" perform well on the strength and power tests and/or feel less soreness post-exercise. Additionally, owing to the same reasons and due to the fact that the study investigators prepared the treatments themselves, they were unable to be blinded to the treatments received by each subject.

Females were not included in the current study in order to avoid any impact the menstrual cycle may have had on the outcome measures. Therefore, any inferences made from the results can only be applied to a male population between the ages of 20 and 33.

The resistance exercise protocol used was based largely on the subjects' willingness to continue with the exercise. As a result, a wide variation in the number of repetitions completed was amassed; this translates into different amounts of resistance exercise performed which may have had an impact on the magnitude of the stimulus that the resistance exercise provided for protein synthesis and/or breakdown. In addition, different levels of soreness may have resulted due to differences in the amount of exercise performed, which may have confounded the affect of the beverages on the alleviation of soreness.

Following the eccentric exercise bout, subjects were advised to avoid planned physical activity so as to avoid alleviating muscular soreness in this way. Subjects were not, however, advised to avoid aspirin or anti-inflammatories during this time. Had subjects used either of these following the eccentric exercise bout, soreness may have been alleviated earlier in comparison to those not using such substances. Although we cannot say if subjects did use these substances, it is quite clear from the subjective soreness ratings that soreness was present in the subjects.

**Conclusion**

The current study investigated the affect of post-exercise protein and carbohydrate intake on subjective assessments of muscle soreness, single-leg one-repetition maximum leg extension, and vertical jump at various time points following a single bout of exhaustive eccentric exercise. It was hypothesized that the consumption of a protein and carbohydrate beverage would improve the time-course of recovery from eccentric exercise when compared with the consumption of a carbohydrate only beverage or a water control. However, no differences were observed among the groups for subjective muscle soreness, one-repetition maximum performance, or vertical jump performance at any time points throughout the study. This indicates that post-exercise nutrition may not

be effective in alleviating DOMS or promoting recovery of muscular performance.

# CHAPTER 6

# FUTURE DIRECTIONS, STUDY LIMITATIONS, AND SUMMARY

## Future Directions

### *Markers of Translation Initiation*

Future studies investigating translation initiation in humans (both male and female) are needed, as this type of data is scarce. Prior to the present study, only one protein (4EBP1) involved exclusively in translation initiation has been measured in human tissue (Liu, et al., 2002). In this case, the measurement of 4EBP1 was performed by observing the three specific forms of 4EBP1 ($\alpha$, $\beta$, and $\gamma$) using an antibody for 4EBP1 rather than observing the phosphorylated form of 4EBP1 with a phospho-specific antibody. Due to the complexity and interplay of the pathways of interest (Figure 16), the investigation of additional proteins in these pathways (e.g. GSK-3, p70S6 kinase, etc.) and their response to resistance exercise and the influence of post-exercise nutrition on skeletal muscle protein synthesis are necessary. Measurements of several proteins within a single study is difficult, however, as human muscle biopsies generally afford only limited muscle tissue extraction.

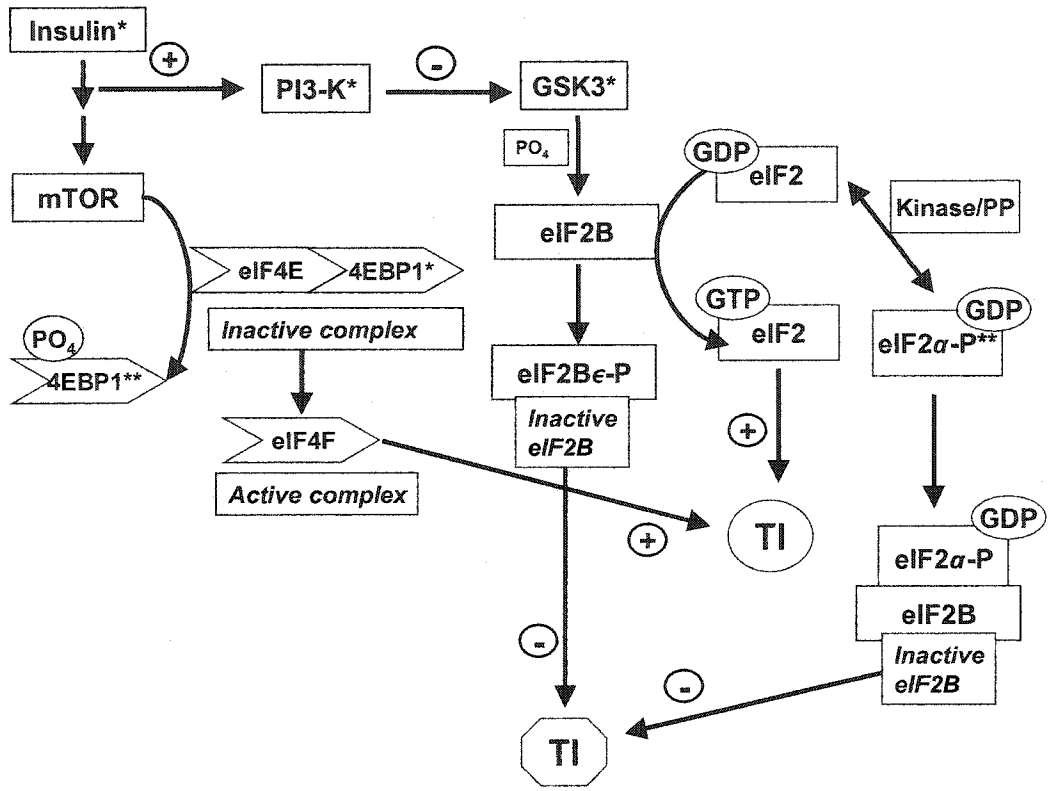


Figure 16: Proposed interaction between insulin signaling and translation initiation. See details in text. *Measured previously in humans. **Measured in human tissue in the present study.

The timing of nutrient intake should also be further elucidated. That is, utilizing protocols of pre-, during, and post-exercise macro-nutrient intake and their possible differential effects on proteins involved in translation initiation are necessary. Additionally, the timing of measurements of the proteins should be addressed. The only known human study on translation initiation proteins has reported increased levels of 4EBP1 phosphorylation following six hours of amino

acid infusion in healthy humans (Liu, et al., 2002). However, several rodent studies have reported increases in 4EBP1 phosphorylation as a result of amino acid infusions ranging from 90 minutes to three hours; two hours following a one hour oral feeding, an increase in the phoshphorylation of 4EBP1 was also observed in rats (Anthony, et. al., 2002; Balage, et al., 2001; Long, et al., 2000). However, when comparing a fasted versus an orally fed state in rodents, no changes in the phosphorylation of eIF2$\alpha$ were observed after 18-48 hours of fasting, although a decreased association of eIF2 with GDP occurred in some cases (Cox, et al., 1988; Jeffrey, et al., 1990; Yoshizawa, et al., 1997). The current study's measurements were performed at two and 24 hours following exercise; it is quite possible that changes in the phosphorylation states of the proteins occurred between these time points. Consequently, a studies in which several muscle biopsies are taken at various time points, as has been modeled in rodents (Anthony, et al., 2002), would help to define the time course of protein phosphorylation following anabolic stimuli. Finally, future studies which combine tracer-determined rates of protein synthesis with the measurement of markers of translation initiation in human skeletal muscle will provide valuable clarification for the importance of each step toward the biological endpoint (protein anabolism).

*Skeletal Muscle Function*

In order to provide more information on the time course of post-exercise skeletal muscle function, future studies should measure strength, power, and soreness recovery for longer periods of time following exercise (i.e., until strength and power are fully recovered and soreness is completely alleviated). In addition, a different feeding schedule may provide significant results. That is, pre-exercise feeding of protein or feeding during the exercise bout may allow for different protein action within the muscle to affect recovery. Alternatively, feeding for a longer period of time following exercise would provide a larger amount of protein which may allow for improved recovery.

Study Limitations

A direct measurement of skeletal muscle protein synthesis was not performed in the current study. Clearly, measurements of this nature would have been valuable for interpretation of the results of the eIF observations. Because these data were not obtained, it is not possible to discuss any relationship between eIF activation/inactivation and the physiological endpoint – protein synthesis.

In order to analyze the levels of phosphorylated 4EBP1 and eIF2$\alpha$ in the skeletal muscle tissue, western blots were performed. Due to the fact that the

proteins of interest have not been analyzed using a phospho-specific antibodies in human tissue previous to this study, no specific protocol is published for this particular assay. As a result, several difficulties were encountered in the process of establishing the proper protocol for these phospho-specific proteins in human tissue. Specifically, the proper amount of protein to load onto the gel should be determined; the present study loaded 50 micrograms of protein, which is a typical loading amount. The proper antibody dilutions (for human tissue) also need to be determined, as the dilutions given in the literature have been successful only in rodents. The length of time to incubate the membranes at various steps throughout the protocol is also not established (i.e. primary and secondary antibody incubations and blocking time). Although inevitably variable, a range for proper membrane exposure times also should be established. It is clear that more work needs to be done in this area to help establish specific protocols for these proteins (as well as others involved in translation initiation) in human tissue. It is important to note that no single member of the eIF family can be taken as a definitive indicator of the rate of translation initiation in skeletal muscle. It is possible that another protein(s) in these pathways may be a better indicator of changes in the rate of translation initiation.

The current study was not able to be blinded due to the fact that each treatment, most notably the CP treatment, had a characteristic appearance and taste that may have been familiar to the subjects. Although it is likely that this did not affect the levels of eIFs present in the muscle, a subject who was aware of which treatment they were receiving may have felt that they "should" perform well on the strength and power tests and/or feel less soreness post-exercise. Additionally, owing to the same reasons and due to the fact that the study investigators prepared the treatments themselves, they were unable to be blinded to the treatments received by each subject.

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resulted due to differences in the amount of exercise performed, which may have confounded the affect of the beverages on the alleviation of soreness.

Following the eccentric exercise bout, subjects were advised to avoid planned physical activity so as to avoid alleviating muscular soreness in this way. Subjects were not, however, advised to avoid aspirin or anti-inflammatories during this time. Had subjects used either of these following the eccentric exercise bout, soreness may have been alleviated earlier in comparison to those not using such substances. Although we cannot say if subjects did use these substances, it is quite clear from the subjective soreness ratings that soreness was present in the subjects.

Summary

No significant time, treatment, or time*treatment interaction was observed for the phosphorylation of 4EBP1 or eIF2$\alpha$ in the present study. This occurred despite the presence of well-known stimulator(s) of skeletal muscle protein synthesis: resistance exercise, carbohydrate (insulin), and protein (amino acids). It may be that skeletal muscle protein synthesis was not altered in the present study, although this seems unlikely, given that several anabolic stimuli were present.

The resistance exercise protocol in the current study induced DOMS as expected, and had a significant effect on strength and power. However, no differences were observed among the treatment groups for subjective muscle soreness, one-repetition maximum performance, or vertical jump performance. This indication that post-exercise nutrition may not be effective in alleviating DOMS or promoting recovery of muscular performance contributes to the wealth of information regarding ineffective treatments for DOMS.

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# APPENDIX A

# INFORMED CONSENT FORM

COLORADO STATE UNIVERSITY
INFORMED CONSENT TO PARTICIPATE IN A RESEARCH PROJECT

**TITLE OF PROJECT: Post-exercise protein feeding: role in recovery from eccentric exercise.**

**NAME OF PRINCIPAL INVESTIGATOR:** Matthew Hickey, Ph.D.

**NAME OF CO-INVESTIGATOR:** Gay Israel, Ed.D.
Chris Melby, Dr. P.H.

**CONTACT NAME AND PHONE NUMBER FOR QUESTIONS/PROBLEMS:** Matt Hickey, Ph.D. 491-5727

**SPONSOR OF THE PROJECT:** Slimfast Nutrition Institute

**PURPOSE OF THE RESEARCH:** The purpose of the present study is to determine how the consumption of protein following resistance exercise may help reduce muscle soreness.

**PROCEDURES/METHODS TO BE USED:** You will be asked to complete a health history questionnaire prior to participation, which will take about 15-20 minutes. If you agree to participate and the investigators determine you are eligible, you will be scheduled for several visits to the Human Performance Clinical/Research Laboratory (HPCRL), Hartshorn Health Center, and to South College Gym. You will not be able to participate in this study if you are currently using prescription or over-the-counter medications known to alter metabolism, diet/nutritional supplements containing amino acids, creatine, steroid derivatives, or tobacco. These will be determined from a health history questionnaire you will complete before starting the study. Should you meet any of these exclusion criteria, we will fully inform you as to the reason for the exclusion.

The preliminary tests include:
1). **Body composition (fat and lean tissue)** - This will be performed using a machine called a dual energy X-ray absorptiometer (DEXA). This unit uses 2 low energy X-rays to determine the amount of body fat you have. You should be advised that the amount of radiation exposure in this procedure is very low, about 1/10,000 of the normal radiation exposure you receive yearly from what is called "background" radiation from the environment. Put another way, the exposure from a DEXA scan is less than the normal exposure in a flight from Denver to Chicago, and about 1/250$^{th}$ the exposure from a normal stomach X-ray you might receive at a hospital. This test will be performed in room 124 in the HPCRL. You will be asked to lie quietly on a bed in shorts and a T-shirt for about 15 minutes while the scan is performed. **This procedure will be performed once at the beginning of the study.**

2). **Fasting Blood sample:** You will be asked to report to the HPCRL following a 10-12 hour fast for a blood sample. You will lie on a cot, and a blood sample will be taken from a vein in your forearm. We will be taking a 10 cc sample, which is equivalent to about 2 teaspoons. The blood will be analyzed for the presence of the enzyme creatine kinase, which is frequently released from muscle following weight lifting.. This procedure will take about 15 minutes. **This procedure will be performed 5 times**: once during the screening period at the beginning of the study, and at 2, 24, 48, and 72 hours after the weight lifting session. The blood samples obtained

at 24, 48, and 72 hours after the exercise bout will also follow an overnight fast. You will only be asked to refrain from eating for the 10 hour period prior to the blood samples at 24, 48, and 72h.



3). **One repetition-maximum test:** Your muscle strenGTh will be determined using a standard weight lifting machine located in Room 110 in South College Gym. Your maximum leg extension strenGTh will be determined as follows:. You will be asked to sit in an upright position in the weight machine. The investigators will place weights on the machine (a padded bar from the machine will be located just above your foot on the front of your leg). You will be asked to RAISE the weights only. This process will be repeated 4-5 times until the maximum amount you can lower is determined. **The one repetition maximum will be repeated twice, at 48 and 96 hours after the weight lifting session.**

4). **Vertical Jump test:** You will be asked to complete a vertical jump test either at Moby Gym or at South College in Room 110. The vertical jump test will involve you jumping and touching a marker mounted on a wall. You will be asked to complete 5 jumps, and we will record the best of those 5 jumps as your vertical jump. This test is expected to take 10-15 minutes to complete. **The vertical jump test will be repeated twice, at 48 and 96 hours after the weight lifting session. The testing will take place at the same time of day as (immediately following) the one-repetition maximum testing.**

5). **Muscle Biopsy:** You will be asked to report to Hartshorn Clinic on the CSU campus for a muscle biopsy. The biopsy will be obtained from the vastus lateralis, which is a large muscle in your thigh. The procedure involves numbing the skin using lidocaine, an anesthetic similar to novacaine, which you may have received at the dentist If you are allergic to novacaine or similar types of anesthetics, or if have had any reaction to anesthetics, you should notify Professor Hickey immediately and should not participate in this study. After numbing the skin, a small incision (less than the width of a pencil) is made in the skin over the muscle. The biopsy is obtained using a sterile needle. The muscle sample obtained is generally ~ ½ the size of an eraser on the end of a pencil. This procedure will take 30-45 minutes, including preparation time. It is not uncommon to experience some mild soreness in the muscle that lasts for about a day. You should NOT restrict your activity, although you should also not perform any unusual or extremely vigorous activity for a few days. You will be provided with written instructions regarding proper care of the incision, and a telephone contact should you have any questions. **This procedure will be performed 3 times: once during the preliminary phase, and at 2 and 24 hours after the weight-lifting session. Please note that all 3 biopsies will be obtained from the same leg (the exercised leg).**

6). **Diet Analysis:** You will be asked to complete a 4 day diet diary in which all food and drink you eat will be recorded. We will analyze your diet using a computer program and determine the percentage of fat, carbohydrate and protein as well as vitamin and mineral status. **This procedure will be performed once during the screening phase to determine your normal diet intake and determine your eligibility for the study. The diet analysis will be repeated for the 4 day period surrounding the weight-lifting session (the day prior and 3 days following the exercise bout, during which time we will provide a research menu which has selections of food items that are acceptable for your diet during the study).**

7). **Weight lifting Session:** Approximately 1 week after the preliminary tests are completed, you will be asked to report to Room 110 in South College Gym for a weight lifting session. You will be asked to complete leg extension exercise (in which you LOWER the weight only) to the point of fatigue. These will be conducted in sets of ten repetitions, with a 3 minute rests between sets.

We anticipate that you will complete 8-10 sets of ten repetitions. **This weight lifting session is designed to make your leg sore, and it will remain sore for 3-4 days after this exercise session.**

**8). Diet supplement:** You will be provided with a liquid diet supplement to be consumed following the weight lifting session. You will be asked to consume the supplement immediately after finishing the exercise session and 30 minutes after finishing the session. These are the only times during the study that you will consume the supplement.



**RETENTION OF BLOOD AND MUSCLE SAMPLES**

You should understand that we plan to keep any extra muscle and blood samples that are not used in the analysis for this study. In other words, if we have any "extra" blood or muscle we will keep them in a freezer in our lab. It is very possible that we will use all of the blood and muscle obtained in this study and will have none left, but in the event that we do, we would like your permission to keep the samples in the event that they can be used for further research. We will use these samples in the future solely for additional research on obesity and metabolism; specifically, all future research will simply be an extension of what we hope to accomplish with the current study. We may simply analyze your blood for the presence of other hormones or metabolites, or analyze your muscle for other enzymes, etc. We have NO plans to store DNA in this study. Your stored samples will be coded in such a way that your confidentiality will be maintained. Only the Principal Investigator (Professor Hickey) will have access to the coding system for your samples. There is a possibility that your samples may be shipped to other departments on the CSU campus, or to colleagues at other Universities for assistance with analysis. Under such circumstances, the same coding system will be used, so researchers in other labs will not be able to identify you. We do not anticipate ANY commercial product development from your tissue, the samples will be used solely for research purposes. You should be advised that we do NOT have plans to recontact you in the future regarding any additional analyses, but will seek full approval of the CSU Regulatory Compliance Office prior to initiating any further research on your samples.

By checking "Yes" below and signing on the accompanying line, you are agreeing to allow the investigators retain any muscle and blood samples obtained during this study. If you do not wish the investigators to retain any samples, please check the box marked "No" and also sign on the accompanying line.

The investigators may keep any muscle or blood samples obtained during the course of this study for future research on obesity and metabolism YES NO

_____

Signature Date

**RISKS INHERENT IN THE PROCEDURES:**

**1). DEXA:** The risks associated with the DEXA are very low. The radiation you will receive in this study is less than 1/3000$^{th}$ of the FDA limit for annual exposure. Put another way, you could

receive 3000 DEXA scans in a single year and still not meet the FDA limit for radiation exposure. The more radiation you receive over the course of your life, the more is the risk of having cancerous tumors or of inducing changes in genes. The radiation in this study is not expected to greatly increase these risks, but the exact increase in such risks is not known. Women who are pregnant or could be pregnant should receive no unnecessary radiation and should not participate in this study.

**2). Blood Samples:** The risks associated with blood drawing include hematoma/bruising, slight risk of infection, local soreness, and fainting. These are all very minor risks and if present, are generally resolved in less than a day.



**3). Muscle Biopsy:** The risks associated with the muscle biopsy include discomfort, localized soreness, bruising, infection, and minor scarring. In addition, it is possible that you may become lightheaded or faint. Some discomfort and localized soreness are likely, but generally last only 24-48h. Temporary scarring is also expected, the natural course of wound healing varies substantially from individual to individual, but the scar will only be 8-10 mm long, and is generally difficult to distinguish within 8-12 months after the biopsy. The risk of bruising is low, and infections are extremely rare.

**5). One repetition maximum testing:** There is some risk on a muscle injury during this testing, but it is very low. All weight lifting will be conducted on a weight machine, which limits the range of motion at your joints. In addition, because we are using a weight machine and not free-weights, it is not possible for you to injure yourself by "dropping" the weights.

**6). Vertical Jump Testing:** The vertical jump testing is a simple procedure that carries little risk of injury. You will be jumping from a flat surface. Because of the muscle soreness that is expected following the weight lifting session, your vertical jump tests conducted at 48 and 96 hours post-exercise are likely to be more painful for your exercised leg than your pre-exercise jumps.

## 7). Diet Supplement: There is no risk associated with the diet supplement used in this study. It is a commercially available liquid diet supplement that has been on the market for several years.

**BENEFITS:**
You will receive diet information, body composition data, and strenGTh data.

**COMPENSATION:**
You will be paid $150.00 upon completion of this study. Should you decide not to complete the study, you will be paid $25 for completion of the preliminary tests, $25 for completion of the weight lifting session, and $25 for completion of the post-training tests. In addition, you will be paid $25 for each biopsy ($75 TOTAL).

**CONFIDENTIALITY:**
Your data will be coded and kept in a locked file cabinet on the CSU campus. A copy of the coded data must be sent to the sponsor of this project. However, you will note be identified in relation to your data at any point.

**LIABILITY:**
We do not anticipate any medical complications related to this research. However, you should be aware that because Colorado State University is a publicly-funded, state institution, it may have only limited legal responsibility for injuries as a result of participation in this study under a Colorado law known as the Colorado Government Immunity Act (Colorado Revised Statutes, section 24-10-101, et seq.). In addition, under Colorado law, you must file any claims against the University within 180 days after the date of the injury. Please be aware **that for** this study **the University** has made special arrangements to provide initial medical coverage for any injuries that are directly related to your participation in this research project. The research project will provide for the coverage of reasonable expenses for emergency medical care related to the treatment of research-related injuries, if necessary. In the event that you are injured as a direct result of participation in this study, you should report research-related injuries to Professor Hickey (970-491-5727-O, 970-204-1304-H) and to Hartshorn Health Services on the Colorado State University Campus (970-491-7121) Questions concerning treatment of subject's rights may be directed to Celia S. Walker at (970) 491-1563.



**PARTICIPATION:**
Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participating at any time without penalty or loss of benefits to which you are otherwise entitled. Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing __5__ pages.

_____ _____
Participant Name (print) Date Participant Signature

_____ _____
Investigator or co-investigator Signature Date

# APPENDIX B

# HEALTH HISTORY QUESTIONNAIRE

**Human Performance Clinical/Research Laboratory**
**COLORADO STATE UNIVERSITY**
**CONFIDENTIAL HEALTH HISTORY QUESTIONNAIRE**

**STUDY**_____ **DATE**_____ **SUBJECT ID #**_

**Reviewed by (must be PI):**_____

**PLEASE PRINT**

**GENERAL MEDICAL HISTORY**

Do you have any current medical conditions? YES NO If Yes, please explain:

Have you had any major illnesses in the past? YES NO If Yes, please explain:

Have you ever been hospitalized or had surgery? YES NO If Yes, please explain:
(include date and type of surgery, if possible)

Are you currently taking any medications, including aspirin, hormone replacement therapy, or other over-the-counter medications?

YES NO If Yes, please explain:

| Medication | Reason | Times taken per Day | Taken for how long? |
|---|---|---|---|

Have you ever had an EKG? YES NO If Yes, please explain:

Have you been diagnosed with diabetes? YES NO If Yes, please explain:

Age at diagnosis_____

**FAMILY HISTORY**

| | Age (if alive) | Age of Death | Cause of Death |
|---|---|---|---|
| Father | _____ | _____ | _____ |
| Mother | _____ | _____ | _____ |
| Brothers/Sisters | _____ | _____ | _____ |
| | _____ | _____ | _____ |
| | _____ | _____ | _____ |
| | _____ | _____ | _____ |

Do you have a family history of any of the following: (Blood relatives only, please give age at diagnosis if possible)

| | YES | NO | Relation | Age at Diagnosis |
|---|---|---|---|---|
| a. High blood pressure | | | | |
| b. Heart Attack | | | | |
| c. Coronary bypass surgery | | | | |
| d. Stroke | | | | |
| e. Diabetes | | | | |
| f. Obesity | | | | |

**TOBACCO HISTORY** (check one)

None
Quit (when)_____
Cigarette
Cigar

**CURRENT TOBACCO USE**
(if applicable)

| | # per day |
|---|---|
| Cigarette | _____ |
| Cigar | _____ |

| | |
|---|---|
| Pipe | Pipe _____ |
| Chew Tobacco | Chew Tobacco _____ |
| Snuff | Snuff _____ |

**Total years of tobacco use**_____

## CARDIORESPIRATORY HISTORY

| | YES | NO |
|---|---|---|
| Are you presently diagnosed with heart disease? | | |
| Do you have any history of heart disease? | | |
| Do you have a heart murmur? | | |
| Occasional chest pain or pressure? | | |
| Chest pain or pressure on exertion? | | |
| Episodes of fainting? | | |
| Daily coughing? | | |
| High blood pressure? | | |
| Shortness of breath? | | |
| At rest? | | |
| lying down? | | |
| After 2 flights of stairs? | | |
| Do you have asthma? | | |
| Do you have a history of bleeding disorders? | | |
| Do you have a history of problems with blood clotting? | | |

***If you checked YES to any of the above, you will be asked to clarify your response by an investigator so we can be sure to safely determine your ability to participate.***

## MUSCULOSKELETAL HISTORY

| | YES | NO |
|---|---|---|
| Any current muscle injury or illness? | | |
| Any muscle injuries in the past? | | |
| Do you experience muscle pain at rest? | | |

Do you experience muscle pain on exertion?

Any current bone or joint (including spinal) injuries?

Any previous bone or joint (including spinal) injuries?

Do you ever experience painful joints?

Do you ever experience swollen joints?

Do you ever experience edema (fluid build up)?

Do you have pain in your legs when you walk?

***If you checked YES to any of the above, you will be asked to clarify your response by an investigator so we can be sure to safely determine your ability to participate.***

**NUTRITIONAL SURVEY**

How many times do you usually eat per day? _____

What time of day do you eat your largest meal? _____

How many times per week do you eat out? _____

**How many times per week do you normally eat the following:**

| | | | |
|---|---|---|---|
| Ground beef____ | Sausage_____ | Bacon_____ | Beef_____ |
| Pork_____ | Cheese_____ | Fish_____ | Poultry_____ |
| Shellfish | Fried Foods_____ | Breads_____ | Cereals_____ |
| Fruits_____ | Vegetables_____ | Eggs_____ | Desserts_____ |

Other_____ (describe)

**How many servings per week of the following do normally consume:**

| | | | |
|---|---|---|---|
| Whole milk_____ | 2% Milk | Skim milk_____ | Buttermilk_____ |
| Coffee_____ | Tea_____ | Soft-Drinks_____ | Beer_____ |
| Wine_____ | Liquor_____ | Water_____ | |

Have you ever dieted? YES NO

If YES, have you dieted within the past 12 months or are you currently on a diet?

YES NO

If YES, please describe the diet:

a). Name (if applicable):_____

b). Prescribed by a Physician/nutritionist? YES NO

c). Have you lost weight? YES NO

d). Duration of diet_____

What was your weight 12 months ago? _____

What is your current weight?_____

Have you dieted other than in the past 12 months? YES NO

If YES, please answer the following:

a). How many times have you dieted?

b). How old were you?

c). Weight loss (amount)?

***You may be asked to complete a more detailed diet survey if you are volunteering for a research study.***

**PHYSICAL ACTIVITY SURVEY**

Compared to a year ago, how much regular physical activity do you get? (Check one)

Much less

Somewhat less

about the same

somewhat more

much more

Have you been exercising regularly for the past three months? YES NO

If YES, what type of exercise do you regularly participate in? (check those that apply)

| | **Days per week** | **Minutes per session** | **Intensity** (1=easy, 10=very hard) |
|---|---|---|---|
| Walking | _____ | _____ | _____ |
| Running | _____ | _____ | _____ |
| Cycling | _____ | _____ | _____ |
| Swimming | _____ | _____ | _____ |
| Aerobics | _____ | _____ | _____ |
| Weight Training | _____ | _____ | _____ |
| Martial Arts | _____ | _____ | _____ |
| Other (describe) | _____ | _____ | _____ |

**EDUCATION**

Please check the highest degree obtained:

Grade School
Junior High
High School
College Degree
Master's Degree
Doctorate

# APPENDIX C

# SUBJECT RECRUITMENT FLIER

# RESEARCH SUBJECTS NEEDED

## HUMAN PERFORMANCE CLINICAL/RESEARCH LABORATORY

**Male volunteers ages 20-40 who are NOT regularly weight training are sought for a research study to investigate the effect of dietary protein on muscle soreness.**

**The study includes:**

- **strenGTh testing**
- **a single supervised weight training session**
- **supervised diet (including a liquid diet supplement)**
- **body composition measurements**
- **3 muscle biopsies**
- **5 blood samples**

**Volunteers will be compensated for the time committed to the study.**

**For more information, please contact:**
**Matt Hickey, Ph.D. at 491-5865,**
**Or Nicole Stob at 491-5865, nrstob@lamar.colostate.edu**

# APPENDIX D

## SF 1RM RECORD SHEET

**Subject** _____

**Date** _____

**1RM #**_____

# SF 1RM record sheet

1. **Leg (circle): Left Right**
2. **Subject's weight:** _____
3. **Beginning testing weight (25% of subject's weight):** _____

| Repetition | Weight |
|---|---|
| #1 (from above) | |
| #2 | |
| #3 | |
| #4 | |
| #5 | |
| #6 | |
| | |
| | |
| **Final 1RM** | _____ **pounds**<br>_____ **kilograms** |

***The subject must use the same leg throughout the study! Ask if they have had a biopsy and, if so, which leg it was done on and use that leg!**

***Remember that the leg extension machine is in pounds and the subject's weight may be recorded as kg, so you may have to convert the body mass to pounds.**

***Please try to keep the # of reps to 6. Write in more reps if necessary.**

# APPENDIX E

# SF VJ RECORD SHEET

**Subject** _____

**Date** _____

**VJ #**_____

# SF VJ record sheet

| | **Reach height** |
|---|---|
| | |
| **Jump** | **Height** |
| #1 | |
| #2 | |
| #3 | |
| #4 | |
| #5 | |
| **Final VJ** (best VJ-reach) | _____ **inches** _____ **centimeters** |

# APPENDIX F

# SAMPLE STUDY DIET

**SlimFast 3000 kcal diet**

**Subject** _____

**Date of diet** _____

| **Breakfast (eaten at South College):** | |
|---|---|
| Orange juice, Minute Maid | 210 g |
| Bagel, Lender's | 102 g |
| Banana | 110 g |
| 2% milk | 300 g |
| Cream cheese | 16 g |
| | |
| **Lunch:** | |
| Bread, 100% whole wheat | 68 g |
| Turkey breast, Healthy Choice | 83 g |
| Swiss cheese | 32 g |
| Mayo, regular | 7 g |
| Mustard | 4 g |
| Lettuce, iceberg | 8 g |
| Chips Ahoy! Cookies | 77 g |
| Orange juice, Minute Maid | 250 g |
| | |
| **Snack:** | |
| Chips Ahoy! Cookies | 19.25 g |
| Yogurt, Dannon low-fat | 227 g (1 container) |
| 2% milk | 300 g |
| | |
| **Dinner:** | |
| Macaroni & Cheese, Lean Cuisine | 283 g (1 package) |
| Bread, 100% whole wheat | 34 g |
| Green beans, frozen | 75 g |
| Margarine, stick, salted | 7 g |
| Orange juice, Minute Maid | 250 g |
| | |
| **Snack:** | |
| Yogurt, Dannon low-fat | 227 g (1 container) |
| Grapes, red | 65 g |

*Breakfast is eaten at South College prior to the exercise protocol.
*Subject must refrain from eating for 2 ½ hours after they consume the 2nd beverage.
*All food in the cooler must be eaten!
*Subject must return cooler with any uneaten food the following morning.

# APPENDIX G

## SF 10X10 EXERCISE RECORD SHEET

Subject _____

Date _____

# SF 10x10 Exercise Record Sheet

4. **Leg (circle): Left Right**

5. **Subject's 1RM: _____**

6. **Testing weight (130% of 1RM): _____**

| Set | Reps |
|---|---|
| #1 | |
| #2 | |
| #3 | |
| #4 | |
| #5 | |
| #6 | |
| #7 | |
| #8 | |
| #9 | |
| #10 | |

***The subject must use the same leg throughout the study! Ask what leg they have used for biopsy/1RM.**

***Each set is to fatigue or a max of 10 reps; the number of sets is to fatigue or a max of 10 sets.**

# APPENDIX H

# MUSCLE SORENESS QUESTIONNAIRE

**Subject #**_____

**Date:**_____

**Testing Day:**_____

## Muscle Soreness Questionnaire

Please rate the muscle soreness that you are experiencing today by marking a line on the following scale.