PROTEIN QUALITY AND DISTRIBUTION DETERMINES ANABOLIC SIGNALING, CELLULAR ENERGETICS, AND BODY COMPOSITION

BY

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DISSEIRATION

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ABSTRACT

Building and maintaining muscle is critical to the quality of life for adults and elderly. Physical activity and nutrition are important factors for long-term muscle health. In particular, dietary protein – including protein distribution and quality – are under-appreciated determinants of muscle health for adults. The most unequivocal evidence for the benefit of optimal dietary protein at individual meals is derived from studies of weight management. During the catabolic condition of weight loss, higher protein diets attenuate loss of lean tissue and partition weight loss to body fat when compared with commonly recommended high carbohydrate, low protein diets.

Muscle protein turnover is a continuous process in which proteins are degraded, and replaced by newly synthesized proteins. Muscle growth occurs when protein synthesis exceeds protein degradation. Regulation of protein synthesis is complex, with multiple signals influencing this process. The mammalian target of rapamycin (mTORC1) pathway has been identified as a particularly important regulator of protein synthesis, via stimulation of translation initiation. Key regulatory points of translation initiation effected by mTORC1 include assembly of the eukaryotic initiation factor 4F (eIF4F) complex and phosphorylation of the 70 kilodalton ribosomal protein S6 kinase (S6K1). Assembly of the eIF4F initiation complex involves phosphorylation of the inhibitory eIF4E binding protein-1 (4E-BP1), which releases the initiation factor eIF4E and allows it to bind with eIF4G. Binding of eIF4E with eIF4G promotes preparation of the mRNA for binding to the 43S pre-initiation complex.

Consumption of the amino acid leucine (Leu) is a key factor determining the anabolic response of muscle protein synthesis (MPS) and mTORC1 signaling to a meal. Research from this dissertation demonstrates that the peak activation of MPS following a complete meal is proportional to the Leu content of a meal and its ability to elevate plasma Leu.

Leu has also been implicated as an inhibitor of muscle protein degradation (MPD). In particular, there is evidence suggesting that in muscle wasting
conditions Leu supplementation attenuates expression of the ubiquitin-proteosome pathway, which is the primary mode of intracellular protein degradation. However, this is untested in healthy, physiological feeding models. Therefore, an experiment was performed to see if feeding isonitrogenous protein sources with different Leu contents to healthy adult rats would differentially impact ubiquitin-proteosome (protein degradation) outcomes; and if these outcomes are related to the meal responses of plasma Leu. Results showed that higher Leu diets were able to attenuate total proteasome content but had no effect on ubiquitin proteins.

This research shows that dietary Leu determines postprandial muscle anabolism. In a parallel line of research, the effects of dietary Leu on changes in muscle mass overtime were investigated. Animals consuming higher Leu diets had larger gastrocnemius muscle weights; furthermore, gastrocnemius muscle weights were correlated with postprandial changes in MPS (r=0.471, P<0.01) and plasma Leu (r=0.400, P=0.01). These results show that the effect of Leu on ubiquitin-proteosome pathways is minimal for healthy adult rats consuming adequate diets. Thus, long-term changes in muscle mass observed in adult rats are likely due to the differences in MPS, rather than MPD.

Factors determining the duration of Leu-stimulated MPS were further investigated. Despite continued elevations in plasma Leu and associated translation initiation factors (e.g., S6K1 and 4E-BP1), MPS returned to basal levels ~3 hours after a meal. However, administration of additional nutrients in the form of carbohydrate, Leu, or both ~2 hours after a meal was able to extend the elevation of MPS, in a time and dose dependent manner. This effect led to a novel discovery that decreases in translation elongation activity was associated with increases in activity of AMP kinase, a key cellular energy sensor.

This research shows that the Leu density of dietary protein determines anabolic signaling, thereby affecting cellular energetics and body composition.
DEDICATION

I would like to dedicate this work to my family, particularly my parents Floyd and Anita, as well as my brother Jake and his wife Stephanie for all of their guidance and support throughout this challenging endeavor.
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LIST OF ABBREVIATIONS

4E-BP1, eukaryotic initiation factor 4E binding protein 1
AMPK, AMP activated Protein Kinase
BCAA, branched chain amino acids
BCAT, branched-chain aminotransferase
BCKDH, branched-chain α-keto-acid dehydrogenase
BV, biological value
CaM, calmodulin
CHO, carbohydrate
DAAO, direct amino acid oxidation
EAA, essential amino acids
eEF2, eukaryotic elongation factor 2
eIF4E, eukaryotic initiation factor 4E
eIF4F, eukaryotic initiation factor 4F
eIF4G, eukaryotic initiation factor 4G
eIFs, eukaryotic initiation factors
FAO, Food and Agriculture Organization
GC-MS, gas chromatography mass spectroscopy
HPLC, high performance liquid chromatography
IAAO, indispensible amino acid oxidation
Leu, leucine
LKB1, liver kinase B1
MPS, muscle protein synthesis
mTORC1, mammalian target of rapamycin complex 1
NRC, National Research Council
PDCAAS, Protein Digestibility-Corrected Amino Acid Score
TSC1/TSC2, tuberous sclerosis complex 1 and 2
RDA, Recommended Dietary Allowance
Rheb, Ras homolog enriched in brain
S6K1, 70 kilodalton ribosomal protein S6 kinase
SPE, solid phase extraction
WHO, World Health Organization
This dissertation attempts to better define the role of dietary leucine (Leu) in regulation of skeletal muscle protein balance. While the threshold response of mammalian target of rapamycin (mTORC1) and muscle protein synthesis (MPS) to plasma Leu concentrations is established from studies using free Leu, the significance of this threshold activation in physiological meals with complex macronutrient and fiber mixtures is unknown. Accordingly, Chapter II will examine the importance of the Leu content of a meal on post-prandial plasma Leu concentrations, translation factors 70 kilodalton ribosomal protein S6 kinase (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), and MPS.

Much less is known about the regulation of muscle protein degradation (MPD). The primary mode of intracellular protein degradation is the ubiquitin-proteosome pathway. The process of ubiquitination involves covalent attachment of ubiquitin – a small, compact, highly conserved polypeptide – to proteins, effectively “tagging” them for selective destruction in proteolytic complexes called proteasomes. While some studies demonstrate a potential role of Leu in regulation of ubiquitin-proteosome signaling, virtually all research has been performed in vitro or muscle wasting models and is untested in healthy subjects under physiological conditions. Therefore, Chapter III will investigate if feeding isonitrogenous protein sources with different Leu contents to healthy adult rats will differentially impact key elements of the ubiquitin-proteosome pathway, and if changes in the ubiquitin-proteosome pathway relate to meal responses of plasma Leu.

While translation initiation and MPS responses shortly after a meal have been well characterized, much less is known about the duration of peak MPS following food intake. Therefore, Chapters IV and V will present a series of time-course experiments designed to determine the duration of muscle protein synthesis after a meal, the effects of dietary supplements to extend or enhance the duration of protein synthesis, as well as mechanisms ultimately regulating the post-meal duration of protein synthesis.
Chapter VI provides a summary of all findings plus thoughts and speculations about dietary implications and future research directions.
CHAPTER I: REVIEW OF LITERATURE

INTRODUCTION

This dissertation attempts to better define the role of dietary leucine (Leu) in regulation of skeletal muscle protein balance. The purpose of Chapter I is to briefly overview current knowledge for this area of research.

The primary role of skeletal muscle is control of movement, which ultimately underlies both physical and metabolic performance. However, the roles of skeletal muscle in metabolic performance and long-term health are rarely discussed (71). Increases in muscle metabolic activity may help attenuate obesity by raising energy expenditure – through voluntary movement and increased muscle protein turnover, an energy costly metabolic process (60).

Muscle is a primary tissue for insulin action, is an important site for glucose utilization, and provides substrate for hepatic gluconeogenesis (33). Insulin resistance is reported to originate within skeletal muscle due to inactivity and an abundance of cellular energy (28). This metabolic state results in the accumulation of intramuscular triglycerides and glucose, resulting in disturbances in glucose and insulin metabolism (53). Wolfe (70) demonstrated that fatty acid oxidation has minimal direct inhibitory effects on glucose metabolism. Conversely, glucose oxidation increases plasma insulin, resulting in decreased oxidation and transport of long chain fatty acids into the mitochondria (70). Exercise improves insulin sensitivity, in part by increased mitochondrial oxidation of glucose and fatty acids (30).

Building and maintaining muscle is critical to the quality of life for adults and elderly, who have a greater risk for muscle loss associated with aging, called sarcopenia (71). When an individual's muscle mass falls more than two standard deviations below the sex-specific mean obtained from normative data of premenopausal women and men under 50 years of age, the individual is determined to have sarcopenia (42). The prevalence of sarcopenia increases from 13–24% in persons 50-70 years of age to >50% in persons over 80 years of
Maintenance of muscle mass is a concern in the elderly as its decline is associated with a two to three times greater likelihood of functional impairment in activities of daily living such as shopping and performing household chores than in individuals who fall within a normal range of skeletal muscle tissue (26). The most severe consequence of sarcopenia occurs when an individual loses his or her capacity to remain functionally independent (21). Research suggests that sarcopenia may occur through alterations in protein balance (21, 65, 66).

Physical activity and nutrition are important factors for protein balance and long-term muscle health. In particular, dietary protein is an underappreciated determinant of muscle health for adults. Indeed, decreased quantity and quality of protein consumption in the elderly is associated with greater muscle loss and increased risk of sarcopenia (29). Conversely, older adults enhance muscle strength and size with greater consumption of dietary protein (13). These findings call into question the adequacy of current dietary protein recommendations for healthy adults (50).

**DIETARY PROTEIN GUIDELINES**

In the United States, protein requirements are based on the Recommended Dietary Allowance (RDA) (52) defined as the minimum amount of protein to maintain short-term nitrogen balance (0.8g/kg of body weight) using data from studies with young adults in ideal health and energy balance (52). These guidelines assume that the minimum dietary intake of indispensable or essential amino acids (EAA) to optimize nitrogen retention represents the protein requirement.

The requirement for dietary protein is determined by the proper amounts of each of the nine EAA. Hence, both the *quantity* and *quality* of proteins is important to determining protein requirements.

The Protein Digestibility-Corrected Amino Acid Score (PDCAAS) was introduced by Food and Agriculture Organization of the World Health Organization (FAO/WHO), and is the current international standard for protein quality assessment (59). The amino acid score is based on comparison of the
concentration of the first limiting essential amino acid in the test protein, with the concentration of that amino acid in a reference (scoring) pattern. The amino acid score obtained is then corrected for the digestibility of the test protein. The current consensus is that meeting the minimum requirements for lysine, threonine, methionine, and tryptophan, considered the most limiting amino acids in poor quality proteins, determines the amino acid score and will lead to a plateau of nitrogen retention (59). At the plateau of nitrogen balance, any further increase in plasma amino acids would stimulate increased oxidation and elimination of the “excess” amino acids, implying that protein quality above requirements does not matter.

These criteria are useful for determining the efficiency of protein utilization for growth or animal production but fail to reflect the diverse metabolic roles of amino acids and long-term health implications. Beyond the fundamental amino acid needs for synthesis of new proteins, amino acids participate in numerous metabolic roles including cell signaling. In many cases signaling pathways and their metabolic outcomes are sensitive to dietary intake. Examples of intracellular amino acid concentrations as determinants of cell signaling includes the roles of tryptophan as a precursor to the neurotransmitter serotonin, arginine stimulating epithelial production of nitrous oxide, and Leu triggering translation initiation of protein synthesis (34).

**GLOBAL REGULATION OF MUSCLE PROTEIN SYNTHESIS**

Regulation of protein synthesis is complex, with multiple signals influencing this process. First, messenger ribonucleic acid (mRNA) is moved from the nucleus to the sarcoplasm where it attaches to ribosomes and the accompanying ribosomal proteins. Once mRNA and ribosomes are assembled into an active complex, a new protein is constructed, a process known as translation. Translation is a three-step process involving initiation, elongation, and termination, and is regulated by eukaryotic initiation, elongation, and releasing factors, respectively. The initiation process requires multiple proteins including at least 12-eukaryotic-initiation factors (eIFs) that direct and facilitate
the process. During translation initiation, the mRNA containing the instructions for a new protein is assembled to a ribosome. The 40s ribosomal subunit is recruited to a selected mRNA and joined with the large 60s ribosome, forming a 80s ribosome competent to identify the translation start codon and begin elongation. Subsequently, transfer RNA (tRNA) brings individual amino acids to the ribosome, where they are linked one after another (translation elongation). Finally, translation is terminated when recognition of a stop codon causes disassociation of the ribosomal subunits from the mRNA (translation termination).

The mammalian target of rapamycin (mTORC1) is a protein complex identified as a particularly important regulator of translation. Key regulatory proteins for translation initiation effected by mTORC1 include assembly of the eukaryotic initiation factor 4F (eIF4F) complex and phosphorylation of the 70 kilodalton ribosomal protein S6 kinase (S6K1). Assembly of the eIF4F initiation complex involves phosphorylation of the inhibitory eIF4E binding protein-1 (4E-BP1), which releases the initiation factor eIF4E and allows it to bind with eIF4G. Binding of eIF4E with eIF4G promotes preparation of the mRNA for the binding to the 43S pre-initiation complex.

mTORC1 also increases translation elongation by activating the eukaryotic elongation factor 2 (eEF2) (11). eEF2 mediates ribosomal translocation, and is inhibited by phosphorylation at Thr56, catalyzed by eEF2 kinase (eEF2K) (54). eEF2K activity is dependent on its ability to bind to Ca2+ ions and calmodulin (CaM); while phosphorylation of eEF2K by protein kinases affects its ability to bind to CaM. mTORC1 inhibits eEF2K activity by phosphorylation of Ser366 (mediated by S6K1) (67), and Ser78, through a yet unidentified protein kinase (10).

Regulation of mTORC1 is not fully understood but is influenced by multiple regulatory proteins, including the tuberous sclerosis complex (TSC1 and TSC2) and Ras homolog enriched in brain (Rheb) (45). Rheb, a Ras-like GTPase, is an activator of mTORC1 in vivo. The action of Rheb is opposed by the TSC1/TSC2 complex, which acts as a Rheb GTPase, promoting the conversion of Rheb-GTP to Rheb-GDP, inhibiting Rheb’s stimulatory effects on mTORC1. TSC2 is
sensitive to growth factors and cellular energy but not to amino acids (4). In TSC2 knockout cells, amino acid deprivation still impairs mTORC1 signaling (61), suggesting that the primary site for amino acids is downstream from TSC2, possibly through Rheb (38).

Changes in cellular energy (AMP / ATP) are detected by the so called “cellular energy sensor” AMP activated Protein Kinase (AMPK). Three ways that AMP improves AMPK activity is by 1) making AMPK a better substrate for the upstream kinase, liver kinase B1 (LKB1); 2) allosteric activation of the phosphorylated enzyme; and 3) inhibition of dephosphorylation by protein phosphatases (22). Conversely, cellular ATP inhibits these processes (22). Phospho-AMPK acts to phosphorylate TSC1/2, resulting in inhibition of Rheb and mTORC1 signaling (69). AMPK is also known to stimulate eEF2K by phosphorylation of a Ser 398 residue, resulting in increased phosphorylation (inhibition) of eEF2 and translation elongation (9). Considering that protein turnover is a metabolically costly process, accounting for approximately one quarter of resting energy expenditure (68), it is logical that a cell would suppress protein synthesis activity during an energy deficit to preserve critical functions.

Hormones and growth factors are also important regulators of translation and mTORC1 signaling. A particularly important hormone is insulin, which activates Akt (also known as protein kinase B, PKB). Akt inhibits TSC1/2, allowing increased activation of Rheb and mTORC1 (25). During growth, anabolic hormones drive development of muscle and bone. However, as growth ends, the anabolic drive is lost and basal levels of hormones become largely ineffective in stimulating protein synthesis in structural tissues (32). Accordingly, in adult populations diet and physical activity become the limiting factors for maintaining muscle mass (32).

Consumption of dietary protein is known to stimulate translation and MPS. In particular, dietary Leu is known to be a critical determinant of MPS (3). Subsequent sections will analyze Leu metabolism, the mechanisms of Leu stimulated MPS, and dietary Leu experiments in clinical trials.
LEUCINE METABOLISM

Leu has numerous metabolic roles with particular importance in skeletal muscle. Like all amino acids, Leu is essential as a building block for synthesis of new proteins. Beyond this substrate role, Leu provides energy in the form of a keto-acid as a fuel for skeletal muscle (2, 58), modulates the insulin to mTORC1 signal cascade (39, 64), and serves as a donor of an amino group for production of alanine or glutamine (2, 23) (Fig. 1.1). In each of these pathways, the impact of Leu is proportional to availability and dependent on intracellular concentration.

The multiple roles of Leu illustrate the unique metabolism of branched-chain amino acid (BCAA) (8). Catabolism of most amino acids occurs in the liver facilitating disposal of the amino-nitrogen group in the urea cycle. However, the liver lacks the branched-chain aminotransferase (BCAT) enzyme required to initiate BCAA degradation. After a meal, amino acid degradation accelerates in the liver and enterocytes, blunting post-prandial swings in amino acid concentrations. Nearly 99% of glutamine, 75% of threonine and 50% of phenylalanine are lost to degradation or protein synthesis on the first pass through the visceral bed (18, 41). However, absence of the BCAT in liver results in over 70% of Leu and the other two BCAA, valine and isoleucine, reaching the systemic circulation. This is a striking metabolic difference for these amino acids which becomes even more remarkable with the realization that the three BCAA account for over 20% of total dietary protein.

Post-prandial changes in free Leu serve as an important nutrient signal to skeletal muscle. After a meal, plasma and intracellular BCAA concentrations rise in proportion to their meal content. The rise in intracellular Leu is the signal that triggers initiation of both muscle protein synthesis (MPS) and BCAA oxidation (60). Parallel activation of both the anabolic response for muscle protein synthesis and the degradation of the BCAA allows the post-prandial Leu concentration to initiate muscle protein synthesis, but assures that intracellular Leu levels return to baseline before the next meal (46).

Increased BCAA oxidation is triggered by transamination of Leu to ketoisocaproate (KIC) mediated by BCAT. Increased KIC levels in skeletal
muscle have been shown to increase oxidation of all three BCAA (Leu isoleucine, and valine) by allosteric activation of branched-chain α-keto-acid dehydrogenase (BCKDH), which is responsible for the non-reversible oxidation of all three BCAA into their respective keto acids (1, 20). While all three keto-acids of Leu, isoleucine, and valine can increase BCKDH activity, research shows that KIC is a much stronger stimulus (1).

**LEUCINE AND MUSCLE PROTEIN SYNTHESIS**

During stages of development, muscle and bone growth is driven by anabolic hormones including insulin, growth hormone, and steroid hormones (32, 43). Later in life, basal levels of hormones become mostly ineffective at stimulating muscle growth; rather, diet and exercise become limiting factors for maintaining optimal protein turnover for repair, remodeling and recovery (32, 43). In particular, dietary Leu becomes an important regulator of muscle protein health in adults (33).

Leu is a potent stimulator of mTORC1 kinase activity, resulting in improved translation (17, 45). Numerous animal studies demonstrate the importance of a rapid rise in plasma Leu to initiate muscle protein synthesis (17, 19). Crozier et al. (17) evaluated oral doses of free Leu ranging from 5% to 100% of the daily Leu requirement on regulation of translation initiation of muscle protein synthesis. They found that a single oral dose of Leu at 10% to 25% of the daily requirement produced maximum stimulation of muscle protein synthesis. They also found that the maximum stimulations of muscle protein synthesis related to maximum activation of the mTORC1 signal pathway at translation initiation factors p70S6K1 and eIF4E corresponding with 2- to 3-fold increases of plasma Leu above fasted values. Changes in plasma Leu act as a signal for activation of mTORC1 and represent an important criterion of protein quality for muscle health. Increases in plasma Leu above the threshold for mTORC1 activation produce no additional effects on initiation factors or maximum rates of muscle protein synthesis.
EVIDENCE FOR THE LEUCINE THRESHOLD IN CLINICAL TRIALS

As demonstrated, Leu is a potent stimulator of MPS. Indeed, research has shown evidence for a dietary “threshold” of Leu required for stimulation of MPS. Evidence for defining the adult meal threshold for Leu derives from studies of catabolic conditions induced by acute exercise (63), extended bed rest (48), or aging (56). Oral ingestion or intravenous administration of 15 g of EAA produces a hyperaminoacidemia sufficient to stimulate muscle protein synthesis and produces a whole body anabolic state (6). These studies provided a complete mixture of EAA containing at least 2.5 g of Leu. Subsequent studies have established that the response threshold for muscle protein synthesis requires at least 15 g of EAA (or approximately 30 g of protein), while a much larger protein meal (90 g of protein) does not produce additional stimulation (62). Doses of EAA less than 7.5 g (or approximately 15 g of protein and less than 1.0 g of Leu) fail to stimulate muscle protein synthesis (27, 49).

While Leu stimulates muscle protein synthesis in acute studies, the efficacy of prolonged administration is less clear. Leu supplementation in clinical trials with chronic conditions such as liver disease, sepsis, or frail elderly has not produced consistent long-term benefits (40, 51). Although chronic down regulation of muscle protein synthesis capacity is a likely complication with these studies. Still, sustained anabolic effects of Leu on muscle protein synthesis should produce changes in lean tissue mass.

The most unequivocal evidence for the benefit of optimal dietary protein and Leu at individual meals is derived from studies of weight management (33, 35, 36). Diets designed to provide at least 2.5 g of Leu per meal have been shown to be highly beneficial during weight loss because of their ability to modify body composition. During the catabolic condition of weight loss, higher protein diets attenuate loss of lean tissue and partition weight loss to body fat when compared with commonly recommended high carbohydrate low fat protein diets (35).
LEUCINE AND MUSCLE PROTEIN DEGRADATION

While the effects of Leu on protein synthesis are well defined, an equally pertinent factor for determining muscle health is regulation of muscle protein degradation (MPD). Muscle tissue mass represents the net balance between MPS and MPD. The continuous process of building and replacing muscle protein allows muscle to repair and adapt to environmental conditions. Net protein balance is positive during growth when MPS exceeds MPD, while net balance is negative during weight loss or aging, when MPD exceeds MPS (47). While there is evidence that MPD may be a major factor in protein turnover during aging (15), nearly all research on the effects of protein or amino acid supplementation on protein turnover has focused on MPS. Therefore, further research is needed to understand how nutrition impacts MPD.

In part, the lack of MPD research is due to the lack of accurate methods for measuring MPD. Previous studies have used whole body amino acid flux and turnover measurements, which may not accurately reflect amino acid metabolism in muscle (55). An alternate approach has been to examine key signals believed to be involved in the regulation of MPD. Specifically, research has focused on regulation of the ATP-dependent ubiquitin-proteosome-proteolytic pathway (37).

The ubiquitin-proteosome pathway was discovered in the late 1970s by Hershko, Ciechanover and colleagues (14, 24), who demonstrated its role in catalyzing the degradation of abnormal proteins. Now the ubiquitin-proteosome pathway is recognized to account for the majority of intracellular protein degradation (57). In particular, this system is responsible for degradation of short-lived regulatory proteins (24), and for the slow and progressive turnover of the long-lived proteins that comprise the majority of the cells enzymes and structure, respectively (44, 57). This system becomes more active during accelerated breakdown of myofibrilar proteins during diseased states (44).

The process of ubiquitination involves covalent attachment of ubiquitin – a small, compact, highly conserved polypeptide – to proteins, effectively “tagging” them for selective destruction in proteolytic complexes called proteasomes (12, 37). To elaborate, this process requires a concerted action of 3 enzymes known
as ubiquitin conjugating enzymes E1, E2, and E3. First, ubiquitin proteins are activated by ATP dependent formation of a thiol ester with the ubiquitin-activating enzyme E1, an abundant 110 kD protein. E1 then transfers the activated ubiquitin molecule to an ubiquitin E2 carrier protein, which are smaller 14-20 kD proteins with high specificities. Finally, ubiquitin is transferred from E2 to a ε-amino group of a lysine residue on the protein to be targeted for degradation. A family of E3 ubiquitin-conjugating enzymes mediates transfer of ubiquitin from E2 to the target protein. Subsequently, additional ubiquitin molecules are attached to the preceding ubiquitin molecule by E3, forming an ubiquitin polypeptide chain. Once 4 or more ubiquitin proteins have been attached to the target protein, the target protein is recognized for degradation by the proteolytic complexes called proteasomes. The 26S proteasome is considered the major proteolytic enzyme complex involved in intracellular protein degradation (37).

Since discovery of the ubiquitin-proteosome pathway, many studies have focused on its function and regulation – particularly, in states of muscle wasting. In this context, there is evidence suggesting that Leu has anti-proteolytic effects, including attenuation of the ubiquitin-proteosome pathway (72). For instance, Combaret et al. (16) demonstrated that Leu supplementation in elderly rats completely prevented the rise in ubiquitin-proteosome expression associated with age. Further, Baptista et al. (5) showed that Leu supplementation in rats with immobilized hind limbs, attenuated muscle loss, protein ubiquitination, and expression of ubiquitin proteins.

POSTPRANDIAL DURATION OF MUSCLE PROTEIN SYNTHESIS

Protein synthesis is an oscillating process, increasing after consumption of food, and decreasing in post-absorptive and fasting conditions. While complete meals providing ample protein and energy increase protein synthesis, it is unknown how long this response lasts, and what causes termination of the anabolic response. Bohé et al. (7) investigated this by intravenously infusing amino acids into adult humans for six hours. Plasma amino acid concentrations were raised 1.7 fold and then maintained throughout the allotted period of time.
MPS remained elevated 2.8 fold for 1.5 hours, but returned to basal levels for the remaining four hours. This suggests that mechanisms other than dietary amino acids determine the duration of protein synthesis. However, mechanisms underlying the discordance between elevated plasma amino acids and declined MPS are unknown.

SUMMARY

This dissertation seeks to further elucidate the role of dietary Leu in regulation of skeletal muscle protein balance. Current knowledge underscores that Leu is a critical regulator of MPS. The mechanism appears to be through stimulation of mTORC1 signaling and translation initiation. There is also evidence that Leu can attenuate MPD and ubiquitin-proteosome signaling. However, most of this research has been done with purified Leu formulas or in vitro, and is untested with whole foods using in vivo feeding protocols. This dissertation seeks to explore the role of dietary Leu in regulation of skeletal muscle protein balance using whole food feeding models.

There is ample research on the early signaling effects of Leu on MPS; however, research has shown discordance between elevated plasma Leu and MPS at the end of the absorptive period. The mechanisms underlying these results are also unknown and this dissertation will explore mechanisms regulating the duration of translational control of protein synthesis using a unique feeding model in adult rats.
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Fig. 1.1. Leucine and insulin regulation of muscle protein synthesis. IRS-1 (insulin receptor 1); PI3-K (Phosphoinositide 3-kinase); Akt (protein kinase B, or PKB); tuberous sclerosis complex 1 and 2 (TSC1/2); mTORC1 (mammalian target of rapamycin); eukaryotic initiation factor 4E binding protein 1 (4E-BP1); eukaryotic initiation factor 4E (eIF4E); Phosphorylated 70 kilodalton ribosomal protein S6 kinase (p70S6K1); ribosomal protein S6 (rpS6).
INTRODUCTION

Food proteins differ in many characteristics including amino acid content. Current measures of protein and amino acid requirements are based on minimum daily intake to maintain nitrogen balance and to prevent increases in plasma amino acids stimulating amino acid oxidation. These measurements focus on lysine, methionine, and threonine as the most limiting of the indispensable amino acids. The branched-chain amino acid (BCAA) leucine (Leu) is comparatively abundant in most proteins and unlikely to be limiting based on nitrogen balance or amino acid oxidation measurements.

Although Leu is unlikely to be limiting new metabolic roles are being defined for Leu intakes above minimum requirements. Among these roles, previous research suggests that a dietary threshold for Leu stimulation of muscle protein synthesis (MPS) may exist (14). Indeed, Leu is unique among amino acids as a modulator of the mTORC1 (mammalian target of rapamycin) signal cascade and MPS (8). Numerous in vivo studies demonstrate that oral or intravenous administration of free Leu stimulates mTORC1 activation of translation initiation factor eIF4E-BP1 (4E-BP1) and the 70 kilodalton ribosomal protein S6 kinase (S6K1). Titration studies of Leu regulation of MPS indicate that Leu stimulation of mTORC1 is dependent on increasing intracellular Leu concentrations and that stimulation requires approximately a doubling of plasma Leu above baseline (i.e. food deprived) values (5, 14).

While the threshold response of mTORC1 and MPS to plasma Leu concentrations is established from studies using free Leu, the significance of this threshold activation in physiological meals with complex macronutrient and fiber mixtures is unknown. Accordingly, the present experiments utilized a meal-feeding protocol with adult rats to examine the importance of the Leu content of a
meal on post-prandial plasma Leu concentrations, S6K1 and 4E-BP1 activation, and MPS stimulation. The test meals were designed to represent small breakfast meals (20% of daily energy) with a mixture of macronutrients (16% of energy from protein, 54% carbohydrates, 30% fats) consumed after a 12-h period of food deprivation. *Experiment 1* used four proteins (wheat gluten, soy isolate, egg white, and whey protein isolate) selected to provide a range of Leu concentrations from 6.8% to 10.9% (w/w). We hypothesized that the Leu content of these meals with limited protein quantity and energy would predict MPS outcomes. While *experiment 2* examined if supplementing the wheat protein meal (6.8% leu) with Leu to be equivalent to the whey protein meal (10.9% leu) would equalize peak plasma Leu concentration, signaling responses, and MPS.

**MATERIALS AND METHODS**

*Animals.* Male rats (250 ± 12 g, 9-11 weeks of age) were purchased from Harlan-Teklad (Indianapolis, IN) and maintained at 24°C with a 12-h light:dark cycle and free access to water. The animal facilities and protocol were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign.

*Meal training protocol.* Rats were trained to consume 3 meals/d consisting of a 4 g meal consumed between 07:00 and 07:20 h followed by free access to food from 13:00 to 14:00 and 18:00 to 19:00 (10). Rats consumed ~17 g/d of total diet equivalent to ad libitum intake. All diet treatments provided 16/54/30% of energy from protein, carbohydrates and fats, respectively.

*Experimental Treatments.* *Experiment 1* examined post-prandial changes in MPS, plasma leu, and translation factors in rats fed meals differing in source of protein: wheat (n=10), soy (n=10), egg (n=11), or whey protein (n=11) (Table 2.1). Wheat gluten diets were supplemented with lysine to meet National Research Council (NRC) requirements and all diets exceeded minimum indispensable amino acid requirements (Supplemental Table 2.5) and to equal the lysine content of the whey protein isolate (Table 2.2). A baseline food-
deprived control group was also adapted to meal-feeding using whey protein (n=10). Rats were fed the respective diets for 14 d. On the experiment day, rats were food-deprived for 12 h and then fed their normal 4 g breakfast meal. The food-deprived controls received no breakfast meal. The treatment groups received 0, 46, 54, 60, and 74 mg of Leu for the food-deprived controls, wheat, soy, egg, and whey groups, respectively. Rats were killed 90 min after consumption of the meal and blood and tissue samples collected. Tissues were then stored at -80°C for later analyses. MPS was measured at 0 and 90-min time-points as described below.

**Experiment 2** examined supplementing the wheat gluten meal with Leu to determine if matching Leu content of the wheat meal would yield similar anabolic responses as whey. In addition, time-course of changes in plasma Leu, MPS, and translation initiation factors were examined to evaluate digestion and absorption patterns for the wheat, whey and wheat + Leu meals. All rats were adapted to meal feeding using wheat gluten as the protein source. Wheat gluten was supplemented with lysine as in **Experiment 1**.

After 6 d of adaptation to meal feeding, rats were assigned to treatment groups based on body weight. Animals (n = 5-6 per group) were food deprived for 12 h and then groups randomly assigned to either food deprived controls or fed one of three 4 g meals with 16% protein coming from wheat gluten, wheat gluten supplemented with Leu (WGL) (Ajinomoto, Chicago, IL) (total = 10.9% Leu), or whey protein (Table 2.1). The whey protein and wheat gluten (WGG) conditions were both supplemented with glycine (Sigma-Aldrich, St. Louis, MO) to make test meals isonitrogenous and isoenergetic with the WGL condition. At 30, 90, and 135 min after consumption of the meal, rats were euthanized and blood and tissues harvested. Gastrocnemius muscles were dissected and snap frozen in liquid nitrogen. Tissues were then stored at -80°C until analyzed. MPS was measured at 0 and 90-min time-points.

**Administration of metabolic tracer and sample collection.** Protein synthesis was measured in gastrocnemius muscles using the flooding dose method (9). A 100% enriched L-[^2]H₅]-phenylalanine solution (150 mmol/L; Cambridge Isotopes,
Andover, MA) was administrated at 150 µmol/100 g body weight and injected via tail vein (1 mL/100 g body weight). After 10 min rats were killed by decapitation and hind limbs quickly removed and immersed in an ice-water mixture. Muscles were removed from cooled hind limbs, frozen in liquid N₂, and stored at -80°C.

**Determination of MPS.** Frozen muscle was powdered in liquid nitrogen and protein precipitated with cold (4°C) perchloric acid (30 g/L, 1 mL per 50 mg muscle tissue). The resulting supernatant and protein pellet were prepared for gas chromatography mass spectroscopy (GC-MS) analyzes as described previously (6, 12). Enrichment of L-[²H₅]-phenylalanine in the muscle hydrolysate was measured by GC-MS using a 6890N GC and a 5973N mass detector (Agilent Technologies Santa Clara, CA). Samples were run under electron impact ionization in splitless mode and phenylethylamine ions with mass-to-charge ratio (m/z) 106 (m + 2) and 109 (m + 5) were monitored for enrichment.

Muscle supernatants were used for determination of intracellular free phenylalanine enrichment. Free amino acids were purified by ion exchange resin solid phase extraction (SPE) using EZ:faastTM amino acid analysis sample testing kit (Phenomenex Inc. Torrance, CA, USA) and ²H₅-phenylalanine enrichment was determined using a propyl chloroformate derivative with GC-MS monitoring of ions at m/z 206 (m) and 211 (m+5) (13).

Rates of muscle protein synthesis (MPS) were determined from the rate of incorporation of L-[²H₅]-phenylalanine into total mixed muscle protein as described previously (4). The time from injection of the metabolic tracer until tissue cooling was recorded as the actual time for L-[²H₅]-phenylalanine incorporation. MPS, defined as the percentage of tissue protein renewed each day, were calculated according to the formula: MPS=(E₉ x 100)/(Eₐ x t) where t is the time interval between isotope injection and snap freezing of muscle expressed in days and E₉ and Eₐ are the enrichments of [²H₅]-phenylalanine in hydrolyzed tissue protein and in muscle free amino acids, respectively.

**Plasma measurements.** Plasma was obtained from trunk blood by centrifugation at 1800 x g for 10 min at 4°C. Plasma insulin concentrations were
analyzed using a commercial RIA kit for rat insulin (Linco Research, St. Charles, MO). Plasma glucose (Thermo Fisher Scientific, Middletown, VA) was determined by the glucose oxidase method. Plasma amino acid concentrations were analyzed by HPLC using a Waters 2475 Fluorescence detector (17).

**Western blot analysis.** Muscle supernatants were subjected to protein immunoblot analyses as described previously (1, 3) using rabbit polyclonal antibodies for 4E-BP1 (1:1,000 dilution) (Bethyl Labs, Montgomery, TX), S6K1 (1:1,000 dilution) (Bethyl Labs, Montgomery, TX), and Akt (1:1,000 dilution) (Cell Signaling, Boston, MA). Anti-rabbit IgG, HRP-linked secondary antibody was purchased from Cell Signaling (1:2,000 dilution) (Boston, MA).

**Statistical analysis.** All data were analyzed by SPSS 15.0 (Chicago, IL) software package for Windows. For **Experiment 1**, a one-way ANOVA was performed with the treatment groups as the independent variables. For **Experiment 2**, a one-way ANOVA was performed for MPS, with treatment groups as the independent variables. Other comparisons for **Experiment 2** utilized a 3 x 4 (ie. experimental groups: WGG, WGL, and whey x time: 0, 30, 90, and 135 min) repeated measures ANOVA to determine within and between group differences. When a significant overall effect was detected, differences among individual means were assessed using Fisher’s LSD post hoc test. Data sets were tested for normal distribution and variance homogeneity using Levene’s test. When variances were not homogeneous, means were compared using a Games-Howell test. Significance was set at P<0.05 for all statistical tests. All values are presented as means ± SEM.

**RESULTS**

**Experiment 1** compared isonitrogenous, isoenergetic meals containing wheat gluten, soy protein isolate, egg white protein, or whey protein isolate as the protein source on the potential to stimulate translation initiation and protein synthesis in skeletal muscle. After consumption of a 4 g meal providing 20% of daily energy and containing 16% of energy as protein, plasma Leu increased in rats fed egg or whey proteins but not in wheat or soy groups. Similar post-
prandial patterns occurred for each of the BCAA with whey producing the highest concentrations at 90 min after the meal (i.e. whey > egg > soy > wheat) (Table 2.3).

Other plasma amino acids varied among the groups largely in proportion to the amino acid content of the protein source (Table 2.3). Lysine was different between rats fed soy and whey; with rats fed whey having the greatest concentration of post-prandial lysine and rats fed soy having the lowest lysine levels. The wheat group reflects the diet supplementation. Plasma methionine concentrations were only increased in rats fed egg with the other groups not being different from food deprived controls (Table 2.3). Plasma threonine was increased by feeding egg or whey with highest concentrations observed in rats fed whey protein.

MPS increased in rats fed egg or whey with the highest value observed for the whey group (Fig. 2.1). The MPS response was consistent with phosphorylation of the mTORC1 signaling targets 4E-BP1 (Fig. 2.2A) and S6K1 (Fig. 2.2B). Phosphorylation of S6K1 increased after egg or whey meals but not wheat or soy with peak values obtained in the whey protein group. 4E-BP1 phosphorylation increased in all groups with whey greater than wheat or soy and egg greater than soy but not different from wheat or whey.

Plasma insulin concentrations were above baseline at 90 min after the meal in all groups except soy, which was not different from food-deprived controls (Table 2.3). Insulin was not significantly different between rats fed wheat, soy, egg, or whey. Similarly, plasma glucose was increased above baseline concentrations in all groups except for rats fed soy, which was not different from baseline, wheat, egg, or whey groups (Table 2.3).

Akt activation (i.e. phosphorylation at Ser473) was increased in rats fed soy compared with rats fed egg, whey, or the food-deprived controls (Fig. 2.3). Rats fed wheat had intermediate levels of Akt phosphorylation that was not different from baseline or soy groups but greater than egg or whey groups.

Experiment 2 compared meals containing wheat (WGG), wheat + Leu (WGL), or whey (10.9% Leu) on the potential to increase plasma Leu, signaling
responses, and MPS over a 135-min time-course. At all time-points, WGL and whey groups had greater increases in plasma Leu than WGG or food-deprived controls; but WGL and whey did not differ from each other (Table 2.4). Likewise, MPS increased in whey and WGL groups but not in WGG (Fig. 2.4).

Plasma amino acids were consistent with Experiment 1 except Ile and Val concentrations tended to be lower in the WGL group compared with WGG. Post-prandial insulin concentrations at 30 min were greater in whey protein and WGL fed rats, than WGG rats (Table 2.4). Likewise, Akt signaling tended to be greater 30-min post-meal in whey protein and WGL fed rats than WGG fed rats, but this did not reach statistical significance (p>0.05) (Fig. 2.5).

**DISCUSSION**

Leu is established as a signaling factor for translation initiation and MPS in skeletal muscle (1, 5, 11). However, the majority of this research has been done with large supplemental doses of free Leu, and the significance of this threshold activation in physiological meals with complex macronutrient and fiber mixtures is unknown. Accordingly, the present studies examined responses of plasma amino acids, translation initiation factors, and MPS in adult rats fed complete meals with protein sources selected for differences in Leu content. The primary finding of this research is that meals with different protein sources stimulate anabolic signaling and MPS in proportion to the Leu content of the meal and post-prandial changes in plasma Leu concentrations.

*Experiment 1* examined the MPS and anabolic signaling responses to isonitrogenous meals containing wheat, soy, egg, or whey proteins, which were selected to provide a range of Leu concentrations from 6.8% to 10.9% (w/w). The Leu content of the protein sources predicted plasma Leu concentrations and anabolic responses in skeletal muscle. Consistent with plasma Leu and MPS, post-prandial S6K1 phosphorylation increased in egg or whey treatment groups by 22% and 58%, respectively, whereas rats receiving wheat or soy had no increase in S6K1 phosphorylation. Phosphorylation of 4E-BP1 increased after the meal in all groups with maximum phosphorylation of 4E-BP1 in the whey
group. The present study demonstrates that after a small meal with limited protein content, MPS responds to post-prandial changes in plasma Leu concentrations that reflect the Leu content of the individual proteins. These findings support a concept for a minimum meal threshold for Leu to stimulate post-prandial MPS.

Further support for the importance of Leu content of protein for MPS is available from both animal (3, 14) and human (15, 18) studies. Norton et al. (14) conducted a dose response study examining wheat and whey proteins fed at 10%, 20% and 30% of dietary energy. At isonitrogenous intakes, whey protein produced higher levels of plasma Leu, mTORC1 signaling, and MPS than wheat. However, MPS was not different comparing meals with 30% wheat vs 20% whey consistent with the Leu difference between wheat (6.8% Leu) and whey (10.9% Leu). Similar support for Leu as a signal to initiate an anabolic meal response has been observed for young men consuming whey, soy and casein after exercise (18) and in older men consuming whey or casein hydrolysates (15).

In the present study with dietary protein limited to 16% of energy, the egg protein meal (8.8% Leu) increased plasma Leu to 146 µmol/L stimulating MPS, while soy protein (~8.0% Leu) did not increase plasma Leu or MPS. Anthony et al. (3) compared mTORC1 signaling and MPS in rats fed 20% soy vs whey after exercise. Interestingly, with dietary protein at 20% of energy, post-prandial plasma Leu in the soy group was 150 µmol/L representing approximately a doubling of Leu concentration above baseline and there was no difference in MPS between soy and whey. Further supporting a “threshold” of Leu intake required to increase plasma Leu and stimulate MPS.

While dietary Leu appears to be a critical factor to account for the post-meal MPS response, there is evidence that orally administered isoleucine (Ile) also activates mTORC1 signaling, although not as powerfully as Leu (1). In complete proteins, Ile is typically present in proportion to the Leu content, allowing the possibility that Ile may in part contribute to the mTORC1 signaling and MPS. However, in the current study, rats fed soy significantly increased post-prandial plasma Ile concentration with no corresponding increase in mTORC1
signaling or MPS. This is consistent with previous research that only Leu among the EAA was able to increase MPS when infused in rats (8). In the present study with complete meals, only the post-prandial changes in plasma Leu predicted changes in mTORC1 signaling and stimulation of MPS.

Beyond Leu content, food proteins differ in many characteristics including distribution of other indispensable amino acids, differences in gastric emptying or digestibility, and insulinogenic properties. Experiment 2 was designed to isolate the importance of Leu within a meal and to test the significance of gastric clearance and digestibility. Experiment 2 demonstrated that supplementing the wheat protein meal with Leu to equalize the Leu density of the meals (WGL vs whey) equalized the 90-min post-prandial MPS response (Fig. 4).

If gastric emptying and digestibility differed between wheat and whey protein, then the timing of peak plasma Leu concentrations should be different for the WGG and WGL vs Whey groups presumably with the WGG and WGL groups exhibiting delayed plasma amino acid appearances compared with the Whey treatment. However, for each of the treatment groups, peak plasma Leu concentration occurred 30 min after the meal, and there was a strong correlation with Leu content of the meal and peak plasma Leu concentration (r = 0.919; P < 0.05). Indeed, at all time-points, WGL and whey protein groups had greater rises in plasma Leu than WGG and fasted values and were not different from each other. Furthermore, the 30-min peak plasma Leu response for the WGL group achieved ~ 91% of the predicted value, based on the plasma leu response of the whey meal (which had equal dietary Leu to WGL). These finding are consistent with published digestibility values for wheat gluten (98%), whey protein isolate (97%), egg white protein (100%), and soy protein isolate (96%) (7) indicating that digestibility is not significantly different among these isolated proteins.

Insulin signaling was greatest 30-min post-meal in the whey and WGL groups relative to baseline and WGG groups. Akt signaling also tended to be greater 30 min post-meal in whey and WGL groups compared with the WGG group, but did not reach statistical significance (p>0.05). Prior research suggests that post-prandial increases in insulin play a permissive role in Leu-dependent
stimulation of protein synthesis and translation initiation (2). Therefore, the heightened insulin response in whey protein and WGL fed rats may contribute to greater rates of mTORC1 signaling and MPS.

In summary, Experiment 1 demonstrates that within a small meal, with protein quantity set at 16% of energy and limited by meal size, the Leu content of individual proteins determines translation initiation signaling in skeletal muscle and ultimately post-prandial MPS. To our knowledge this is the first study to show the significance of Leu as a factor of protein quality within meals containing complete macronutrient and fiber mixtures. While experiment 2 demonstrates that Leu supplementation of a meal containing a Leu-poor protein enhances translation initiation and MPS. These findings support the hypothesis that the Leu density of dietary proteins is important to adult protein utilization and highlights the need for long-term studies examining the impact of Leu density on muscle health and body composition.
REFERENCES


Fig. 2.1. Rates of muscle protein synthesis in gastrocnemius muscle of rats fed a complete meal containing wheat, soy, egg, or whey proteins. Data are means ± SEM; n = 9-10. Labeled means without a common letter differ, P<0.05.
Fig. 2.2. Phosphorylation states of 4E-BP1 (A) and p70S6K1 (B) in gastrocnemius muscle of rats fed a complete meal containing wheat, soy, egg, or whey proteins. Data are means ± SEM; n = 9-10. Labeled means without a common letter differ, P<0.05.
Fig. 2.3. Phosphorylation states of Akt in gastrocnemius muscle of rats fed a complete meal containing wheat, soy, egg, or whey proteins. Data are means ± SEM; n = 9-10. Labeled means without a common letter differ, P<0.05.
Fig. 2.4. Rates of muscle protein synthesis in gastrocnemius muscle of rats fed isonitrogenous meals containing wheat gluten supplemented with glycine (WGG), wheat supplemented with leucine (WGL), or whey protein supplemented with glycine. Data are means ± SEM; n = 5-6. Labeled means without a common letter differ, P<0.05.
Fig. 2.5. Phosphorylation states of Akt in gastrocnemius muscle of rats fed isonitrogenous meals containing wheat gluten supplemented with glycine (WGG), wheat supplemented with leucine (WGL), or whey protein supplemented with glycine. Data are means ± SEM; n = 5-6. Labeled means without a common letter differ within time-points, P<0.05. * Indicates different from fasted (P<0.05).
Table 2.1. *Composition of animal diets*

<table>
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<th>Component</th>
<th>Wheat Diet</th>
<th>Soy Diet</th>
<th>Egg Diet</th>
<th>Whey Diet</th>
</tr>
</thead>
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<tr>
<td></td>
<td>g/kg</td>
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<td></td>
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<td>0.0</td>
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</table>

<sup>1</sup>Vital Wheat Gluten purchased from Honeyville Grain, Honeyville, UT. 83.4% protein, 7.6% carbohydrate, 9% other.

<sup>2</sup>Soy Protein Isolate provided by Archer Daniels Midland Company, Decatur, IL. 91.6% protein, 1.4% carbohydrate, 7% other.

<sup>3</sup>Egg White Solids purchased from Harlan-Teklad, Madison, WI. 87.8% Protein, 4.5% carbohydrate, 7.7% other.

<sup>4</sup>Whey Protein Isolate provided by Perham, Perham, MN. 89.9% protein, 3.8% carbohydrate, 6.3% other.

<sup>5</sup>Vital Wheat Gluten supplemented with 6.3g L-lysine/100g protein to match Whey Protein Isolate.

<sup>6</sup>Mineral and Vitamin supplements (16) from Harlan-Teklad, Madison, WI.

<sup>7</sup>Egg White Solids supplemented with 16.0 mg biotin/kg diet.
Table 2.2. *Amino acid compositions of protein sources*

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Vital Wheat Gluten&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Soy Protein Isolate&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Egg White Solids&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Whey Protein Isolate&lt;sup&gt;4&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>g/100g Protein</td>
<td></td>
<td></td>
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<tr>
<td>Alanine</td>
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<td>6.8</td>
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</tr>
</tbody>
</table>

<sup>1</sup>Vital Wheat Gluten purchased from Honeyville Grain, Honeyville, UT. 83.4% protein, 7.6% carbohydrate, 9% other.

<sup>2</sup>Soy Protein Isolate provided by Archer Daniels Midland Company, Decatur, IL. 91.6% protein, 1.4% carbohydrate, 7% other.

<sup>3</sup>Egg White Solids purchased from Harlan-Teklad, Madison, WI. 87.8% Protein, 4.5% carbohydrate, 7.7% other.

<sup>4</sup>Whey Protein provided by Perham, Perham, MN. 89.9% protein, 3.8% carbohydrate, 6.3% other.

<sup>5</sup>Vital Wheat Gluten supplemented with 6.3g L-lysine/100g protein to match Whey Protein Isolate.
Table 2.3. Selected plasma essential amino acid\(^1\), insulin\(^2\), and glucose\(^3\) concentrations 90 min after feeding complete meals containing wheat, soy, egg, or whey protein\(^4\)

<table>
<thead>
<tr>
<th></th>
<th>Baseline(^5)</th>
<th>Wheat</th>
<th>Soy</th>
<th>Egg</th>
<th>Whey</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>84 ± 4.6(^c)</td>
<td>78 ± 4.3(^c)</td>
<td>84 ± 5.6(^c)</td>
<td>146 ± 8.4(^b)</td>
<td>192 ± 11.4(^a)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>56 ± 3.4(^d)</td>
<td>50 ± 2.9(^a)</td>
<td>74 ± 4.0(^c)</td>
<td>121 ± 6.3(^b)</td>
<td>144 ± 8.2(^a)</td>
</tr>
<tr>
<td>Valine</td>
<td>117 ± 8.2(^{cd})</td>
<td>95 ± 5.2(^d)</td>
<td>143 ± 8.1(^c)</td>
<td>295 ± 14.2(^b)</td>
<td>248 ± 13.7(^a)</td>
</tr>
<tr>
<td>(\sum) BCAA</td>
<td>257 ± 16.0(^{bc})</td>
<td>223 ± 12.1(^c)</td>
<td>301 ± 17.4(^b)</td>
<td>562 ± 28.5(^a)</td>
<td>584 ± 33.0(^a)</td>
</tr>
<tr>
<td>Lysine</td>
<td>510 ± 29.8(^{ab})</td>
<td>527 ± 23.8(^{ab})</td>
<td>419 ± 37.2(^b)</td>
<td>495 ± 35.6(^{ab})</td>
<td>549 ± 29.3(^a)</td>
</tr>
<tr>
<td>Methionine</td>
<td>51 ± 2.9(^{bc})</td>
<td>46 ± 2.8(^{bc})</td>
<td>38 ± 4.1(^c)</td>
<td>86 ± 6.8(^a)</td>
<td>52 ± 5.2(^b)</td>
</tr>
<tr>
<td>Threonine</td>
<td>252 ± 13.7(^c)</td>
<td>269 ± 30.4(^{bc})</td>
<td>349 ± 40.6(^{bc})</td>
<td>357 ± 28.3(^{ab})</td>
<td>538 ± 51.2(^a)</td>
</tr>
<tr>
<td>Insulin</td>
<td>140 ± 10.5(^b)</td>
<td>178 ± 9.0(^a)</td>
<td>161 ± 11.0(^{ab})</td>
<td>184 ± 12.0(^a)</td>
<td>170 ± 7.2(^a)</td>
</tr>
<tr>
<td>Glucose</td>
<td>7.8 ± 0.58(^b)</td>
<td>9.9 ± 0.50(^a)</td>
<td>8.5 ± 0.67(^{ab})</td>
<td>9.7 ± 0.77(^a)</td>
<td>9.4 ± 0.40(^a)</td>
</tr>
</tbody>
</table>

\(^1\)Plasma amino acids expressed as \(\mu\)mol/L  
\(^2\)Plasma insulin expressed as pmol/L  
\(^3\)Plasma glucose expressed as mmol/L  
\(^4\)Values expressed as means ± SEM, n=8-10. Labeled means without a common letter differ (P<0.05).  
\(^5\)12 h food-deprived controls.
Table 2.4. Post-prandial changes for plasma amino acids\(^1\) and plasma insulin\(^2,3\)

<table>
<thead>
<tr>
<th></th>
<th>Fasted(^4)</th>
<th>Whey</th>
<th>Wheat</th>
<th>Wheat+Leu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td>30</td>
<td>90</td>
<td>135</td>
<td>30</td>
</tr>
<tr>
<td>Leu</td>
<td>86±4</td>
<td>226±17^a</td>
<td>164±26^a</td>
<td>173±22^a</td>
</tr>
<tr>
<td>Ile</td>
<td>69±2</td>
<td>166±11^a</td>
<td>104±4^a</td>
<td>134±16^a</td>
</tr>
<tr>
<td>Val</td>
<td>117±5</td>
<td>234±17^a</td>
<td>161±5^a</td>
<td>186±19^a</td>
</tr>
<tr>
<td>Lys</td>
<td>608±24</td>
<td>1083±78</td>
<td>593±34</td>
<td>688±62</td>
</tr>
<tr>
<td>Met</td>
<td>49±2</td>
<td>102±6^a</td>
<td>62±2^a</td>
<td>80±5^a</td>
</tr>
<tr>
<td>Thr</td>
<td>309±9</td>
<td>594±73</td>
<td>567±18</td>
<td>554±38^a</td>
</tr>
<tr>
<td>Insulin</td>
<td>35±2</td>
<td>50±8^a</td>
<td>41±6^b</td>
<td>40±9</td>
</tr>
</tbody>
</table>

\(^1\) Plasma amino acids expressed as µmol/L  
\(^2\) Plasma insulin expressed as pmol/L  
\(^3\) Data are means ± SEM; N=5-6.  
\(^4\) 12 hour food-deprived controls.

Means without a common letter differ between treatments within time-points, (P<0.05).

* Indicates different from fasted (P<0.05)
Supplemental Table 2.5. *Comparison of test diet amino acid compositions with NRC requirements*\(^1\)\(^2\)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Wheat Diet</th>
<th>Soy Diet</th>
<th>Egg Diet</th>
<th>Whey Diet</th>
<th>NRC Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine + Tyrosine</td>
<td>11.5</td>
<td>17.0</td>
<td>16.8</td>
<td>10.7</td>
<td>1.9</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.1</td>
<td>4.2</td>
<td>3.9</td>
<td>3.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.1</td>
<td>8.1</td>
<td>9.0</td>
<td>10.5</td>
<td>3.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>11.5</td>
<td>13.6</td>
<td>14.9</td>
<td>18.5</td>
<td>1.8</td>
</tr>
<tr>
<td>Lysine(^3)</td>
<td>4.7(+10.6)</td>
<td>10.7</td>
<td>11.0</td>
<td>15.4</td>
<td>1.1</td>
</tr>
<tr>
<td>Methionine + Cysteine</td>
<td>6.5</td>
<td>4.4</td>
<td>13.9</td>
<td>7.6</td>
<td>2.3</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.4</td>
<td>6.5</td>
<td>7.6</td>
<td>10.9</td>
<td>1.8</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2.2</td>
<td>2.0</td>
<td>2.7</td>
<td>2.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Valine</td>
<td>7.6</td>
<td>8.0</td>
<td>11.5</td>
<td>10.2</td>
<td>2.3</td>
</tr>
</tbody>
</table>

\(^1\)Table 2-2 from Nutrient Requirements of Laboratory Animals Fourth Revised Edition (1).

\(^2\)Values calculated for 300 g rat at maintenance.

\(^3\)Vital Wheat Gluten supplemented with 10.6 g L-lysine/kg diet to match Whey Protein Isolate.

**SUPPLEMENTAL LITERATURE CITED**

INTRODUCTION

Chapter II demonstrated that consuming proteins with greater leucine (Leu) densities resulted in improved anabolic signaling and rates of muscle protein synthesis (MPS). However, while the effects of Leu on MPS are important, an equally pertinent factor for determining muscle health is regulation of muscle protein degradation (MPD). Muscle tissue mass represents the net balance between MPS and MPD. The continuous process of building and replacing muscle protein allows muscle to repair and adapt to environmental conditions. Net protein balance is positive during growth when MPS exceeds MPD, while net balance is negative during weight loss or aging, when MPD exceeds MPS (24). While there is evidence that MPD may be a major factor in protein turnover during aging (6), nearly all research on the effects of protein or amino acid supplementation on protein turnover has focused on MPS. Therefore, further research is needed to understand how nutrition impacts MPD.

In part, the lack of MPD research is due to the inability to accurately measure MPD. Previous studies have used whole body amino acid flux and turnover measurements, which may not accurately reflect amino acid metabolism in muscle (27). An alternate approach has been to examine key signals believed to be involved in the regulation of MPD. Specifically, research has focused on regulation of the ATP-dependent ubiquitin-proteosome-proteolytic pathway (18).

The ubiquitin-proteosome pathway was discovered in the late 1970s by Hershko, Ciechanover and colleagues (4, 16), who demonstrated its role in catalyzing the degradation of abnormal proteins. Now the ubiquitin-proteosome pathway is recognized to account for the majority of intracellular protein degradation (28). In particular, this system is responsible for degradation of short-lived regulatory proteins (16), and for the slow and progressive turnover of
the long-lived proteins that comprise the majority of the cell's enzymes and structures, respectively (20, 28). This system becomes more active during accelerated breakdown of myofibrilar proteins during diseased states (20).

The process of ubiquitination involves covalent attachment of ubiquitin – a small, compact, highly conserved polypeptide – to proteins, effectively “tagging” them for selective destruction in proteolytic complexes called proteasomes (3, 18). To elaborate, this process requires a concerted action of 3 enzymes known as ubiquitin conjugating enzymes E1, E2, and E3. First, ubiquitin proteins are activated by ATP dependent formation of a thiol ester with the ubiquitin-activating enzyme E1, an abundant 110 kD protein. E1 then transfers the activated ubiquitin molecule to an ubiquitin E2 carrier protein, which are smaller 14-20 kD proteins with high specificities. Finally, ubiquitin is transferred from E2 to a ε-amino group of a lysine residue on the protein to be targeted for degradation. A family of E3 ubiquitin-conjugating enzymes mediates transfer of ubiquitin from E2 to the target protein. Subsequently, additional ubiquitin molecules are attached to the preceding ubiquitin molecule by E3, forming an ubiquitin polypeptide chain. Once 4 or more ubiquitin proteins have been attached to the target protein, the target protein is recognized for degradation by the proteolytic complexes called proteasomes. The 26S proteasome is considered the major proteolytic enzyme complex involved in intracellular protein degradation (18).

Only a single E1 protein has been identified (15); while estimates for as many as 500 E2 targeting enzymes may exist for selection of individual proteins (14). A small number of E3s have been described (about 10) (18). In particular, two E3 ligases studied extensively in muscle are muscle RING-finger protein-1 (MURF1), which targets myofibrilar protein degradation (5); and Atrogin1, which targets degradation of the transcription factor MyoD (17).

E2s have specificity for the proteins, which they bind to; likewise, E3s also have specific binding sites for the E2 enzyme, the ubiquitin proteins they bind to, as well as the protein “targeted” for degradation. Accordingly, it is believed that E3s alone, or together with E2s, accounts for the specificity of the ubiquitin degradation process (18).
The 26S proteasome consists of a catalytic 20S and a regulatory 19S subunit, and is considered the major proteolytic enzyme complex involved in intracellular protein degradation (18). The catalytic 20S proteasome subunit is a large 600 kD particle, comprising up to 1% of cell proteins (18). These proteins have a barrel-shaped structure, consisting of four stacked rings, each comprised of subunits surrounding a central cavity (8). Inside are two rings composed of β subunits, which enclose a large central chamber containing the sites where the proteins are degraded; while the two outer rings are composed of α subunits (called PSMAs), which cover a small opening where protein substrates must enter (8). Seven different α and ten different β proteasome genes have been identified in mammals (22).

To enter the proteasome, proteins must first be unfolded (8). Protein unfolding is handled by the regulatory 19S proteasome subunits (700 kD) (18), which are located on either end of the 20S proteasome. The 19S subunit provides specificity to proteolysis by binding ubiquinated substrates and catalyzing the degradation of polypeptides into the 20S subunit (8). There are six different ATPases located on the 19S complex suggesting that this process is ATP dependent (13).

Once unfolded proteins enter the 20S subunit, they are “cut” multiple times into small peptides ranging from 3 to 25 residues in length, before the next protein is degraded (1). This progressive process of protein degradation is presumably designed to prevent partially digested proteins from accumulating and causing cell damage (18). Collectively, the specificity and energy requirements of the ubiquitin-proteasome pathway make it ideal for selective regulation of the degradative process.

Since discovery of the ubiquitin-proteasome pathway, many studies have focused on its function and regulation – particularly, in states of muscle wasting. In this context, there is evidence suggesting that the essential amino acid Leu has anti-proteolytic effects, including attenuation of the ubiquitin-proteasome pathway (30). For instance, Combaret et al. (7) demonstrated that Leu supplementation in elderly rats completely prevented the rise in ubiquitin-
proteosome expression associated with age. Further, Baptista et al. (2) showed that Leu supplementation in rats with immobilized hind limbs, attenuated muscle loss, protein ubiquitination, and expression of E3 ligases MURF1 and Atrogin1.

While these studies demonstrate a potential role of Leu in regulation of muscle protein degradation, virtually all research on Leu regulation of ubiquitin-proteosome has been performed in vitro, or in muscle wasting models (9, 10), and is untested in healthy subjects under physiological conditions. This study was designed to determine if feeding isonitrogenous protein sources with different Leu contents to healthy adult rats would differentially impact ubiquitin-proteosome (protein degradation) outcomes; and if these outcomes are related to the meal responses of plasma Leu. This study of protein degradation was a component of a larger research project designed to evaluate if long-term consumption of higher Leu diets would result in sustained increases in MPS and changes in muscle mass. Results for MPS and changes in muscle mass have been reported previously for this study (23) and will be briefly summarized here for clarity.

**MATERIALS AND METHODS**

**Animals.** Thirty-nine male Sprague-Dawley rats (Harlan-Teklad, Indianapolis, IN) with a mean body weight of 270 ± 10 g, 9-11 weeks of age were housed individually in stainless steel wire-bottomed cages and maintained at 24°C with 12 h reverse light cycle (light: 1900-0700 h) and free access to water. Rats were fed during the dark cycle time-periods and fasted during the light cycle periods. The animal protocol was approved by the University of Illinois Institutional Animal Care and Use Committee.

**Meal training protocol.** Rats were trained to consume three meals each d using a modified AIN-93G diet (54% of energy from carbohydrate, 16% protein, and 30% fat) (25). The meal pattern consisted of a 4 g breakfast meal consumed between 0700-0720 h, followed by free access to food between 1300-1400 h and 1800-1900 h for an adaptation period of 6 d. Rats consumed ~18 g / d of diet during the adaptation period. After adaptation, rats were randomized to one of
four treatment diets containing either wheat gluten, egg protein isolate, or whey protein isolate as the protein source (n = 13 per group) with protein at 16% of energy. Wheat gluten was supplemented with lysine in order to meet National Research Council guidelines for maintenance and to approximate the lysine content of the whey isolate. The breakfast meals contained 46, 60, and 74 mg of leu, respectively, for the wheat, egg, and whey groups. The composition of the treatment diets is shown in Table 3.1.

In order to optimize handling of energy and to normalize food intake between treatment groups, the treatment diets were fed at 80% of ad libitum intake (14 g/d). This was accomplished by feeding three daily meals of discrete amounts: a 4 g morning meal consumed between 0700-0720 h, followed by a 4 g afternoon meal 1300-1400 h and a 6 g evening meal from 1800-1900 h.

**Experimental Treatments.** The animals consumed the treatment diets for 11 weeks, at which point, rats were food deprived for 12 h and then either euthanized by decapitation (food deprived) (n = 5 per group) or fed their normal 4 g morning meal (n = 8 per group). Ninety min after consumption of the meal, rats were euthanized by decapitation, and then blood and tissues were harvested. Gastrocnemius tissues were weighed prior to being snap frozen in liquid nitrogen. Tissues were then stored at -80°C until further analysis.

**Administration of metabolic tracer and tissue collection.** MPS was measured in skeletal muscle using the flooding dose method (12). A 100% enriched L-[2H5]phenylalanine solution (150 mmol/L; Cambridge Isotopes, Andover, MA) was administered at 150 mmol/100 g body weight and injected via the tail vein (1 mL/100g body weight). After 10 min, animals were killed by decapitation, blood was collected in pre coated EDTA tubes, and hind limbs were quickly removed and immersed in an ice-water mixture. Gastrocnemius muscles were removed from cooled hind limbs, frozen in liquid N2, and stored at -80°C.

**Measurement of MPS.** Frozen muscle tissue was powdered in liquid nitrogen and protein was precipitated with cold (4°C) perchloric acid (30 g/L, 1 mL/50 mg muscle tissue). The resulting supernatant and protein pellet were prepared for analysis by GC-MS as described previously (11, 19). The
enrichment of L-[2H5]phenylalanine in the muscle hydrolysate was measured by GC-MS using a 6890N GC and a 5973N mass detector (Agilent Technologies, Santa Clara, CA). The samples were analyzed under electron impact ionization and in splitless mode, and phenylethylamine ions at mass:charge ratio 106 (m + 2) and 109 (m + 5) were monitored for enrichment analysis.

The muscle supernatant was used to determine intracellular free phenylalanine enrichment. Free amino acids were purified by ion exchange resin solid-phase extraction using EZ:faast amino acid analysis sample testing kit (Phenomenex, Torrance, CA) and ²H5-phenylalanine enrichment was determined using a propyl chloroformate derivative with GC-MS by monitoring the ions at mass:charge ratio 206 (m) and 211 (m + 5) (21).

MPS was determined using the rate of incorporation of L-[²H5]phenylalanine into total mixed muscle protein as described previously (19). The time from injection of the metabolic tracer until tissue cooling was recorded as the actual time for L-[²H5]phenylalanine incorporation. MPS, defined as the percentage of tissue protein renewed each day, were calculated according to the formula: MPS = (E_b x 100) / (E_a x t), where t is the time interval between injection and cooling of sampled tissue expressed in days and E_b and E_a are the enrichments of [²H5]Phe in hydrolyzed tissue protein and in muscle free amino acids, respectively.

*Plasma measurements.* Plasma was obtained from trunk blood by centrifugation at 1800 X g; 10 min at 4°C. Plasma insulin concentrations were analyzed using a commercial RIA kit for rat insulin (Millipore, Billerica, MA). Plasma amino acid concentrations were determined by HPLC using a Waters 2475 Fluorescence detector (26).

*Western blot analysis.* Muscle supernatants were subjected to protein immunoblot analysis as previously described (12). Rabbit polyclonal antibodies were used for total PSMA2 (1:3,000 dilution) (a portion of the outer ring of the 20S proteasome) (Cell Signaling, Boston, MA), total UBE2L3 (1:1,000 dilution) (an E2 ligase) (Cell Signaling, Boston, MA), Muscle RING-finger protein-1 (MURF1) (1:2,000 dilution) (an E3 ligase) (Santa Cruz Biotechnology, Santa
Cruz, CA), Atrogin 1 (an E3 ligase) (1:500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA), and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000 dilution) (Cell Signaling, Boston, MA). Anti-rabbit IgG, HRP-linked secondary antibody was purchased from Cell Signaling (1:2,000 dilution) (Boston, MA).

Statistical analysis. Data are expressed as mean ± SEM. All variables were evaluated for normality and homogeneity of variance by Levene’s test. A one-way ANOVA was performed with the dietary treatment as the independent variable. When a significant effect was detected, differences among means were determined using an LSD post hoc test or a Games-Howell post hoc test when variances were not equal. The level of significance was set at \( P < 0.05 \). All analyses were performed using SPSS Version 15.0 (Chicago, IL).

RESULTS

The primary purpose of this study was to determine if feeding adult rats protein sources with different Leu contents would differentially regulate ubiquitin-proteosome protein concentrations.

After 11 weeks of feeding, rats were fed a breakfast meal providing ~30% of daily energy and with 16% of energy as protein. Plasma Leu increased in animals fed egg and whey proteins but not the wheat group. Other plasma amino acids varied among the groups largely in proportion to the amino acid content of the protein source. No essential amino acid was significantly below baseline post-prandially. Plasma insulin concentrations were above baseline at 90-min post meal, but did not differ between treatment groups (Table 3.2).

Total PSMA2 was measured as an estimate of proteasome degradative capacity. Likewise, total UBE2L3 (an E2 ligase), MURF1 (an E3 ligase), and Atrogin 1 (an E3 ligase) proteins were measured as estimates of ubiquitin ligase degradative capacities.

PSMA2 significantly decreased in rats fed egg and whey compared to animals fed wheat (\( P < 0.05 \)) (Fig. 3.1a), suggesting decreased total proteasome degradative capacity in rats consuming higher Leu diets. Conversely, no
differences were detected in the ubiquitin ligases UBE2L3, MURF1, or atrogin1 (Fig. 3.1b-d), suggesting that ligase capacity was not affected by treatments.

A parallel purpose of this research was to determine if long-term consumption of higher Leu diets would result in sustained increases in MPS, and changes in muscle mass overtime (23). Briefly, post-prandial MPS was significantly greater in animals fed egg and whey than animals fed wheat, with no significant difference between the egg and whey groups (Fig. 3.2). Gastrocnemius muscle weights were also greater in animals fed egg and whey compared to animals fed wheat (Fig. 3.3).

**DISCUSSION**

The study examined if feeding isonitrogenous protein sources with different Leu contents to healthy adult rats would differentially impact ubiquitin-proteosome (protein degradation) outcomes; and if these outcomes are related to the meal responses of plasma Leu. The primary finding was that total PSMA2 (a portion of the outer ring of the 20S proteasome) significantly decreased in rats fed egg and whey compared to animals fed wheat (Fig. 3.1a), suggesting decreased total proteasome degradative capacity in rats consuming higher Leu diets. However, there was no effect of diet treatment on any of the regulatory ubiquitin ligases, including E2 (UBE2L3), and E3 ligases (MURF1, or atrogin1) (Fig. 3.1b-d). These results suggest that protein quality within accepted dietary ranges has minimal regulatory effects on MPD in healthy adult rats.

A parallel purpose of this research was to determine if long-term consumption of higher Leu diets would result in sustained increases in MPS, and changes in muscle mass overtime (23). Consistent with short-term feeding studies presented in **Chapter II**, after 11 weeks of meal feeding, animals fed egg and whey protein isolates had greater post-prandial increases in muscle protein synthesis (Fig. 3.2) and plasma Leu (Table 3.2). Furthermore, rats consuming whey or egg protein had larger gastrocnemius muscle weights than those consuming wheat protein (Fig. 3.3), and gastrocnemius weights correlated with post-prandial changes in MPS ($r = 0.471$, $P < 0.01$) and plasma Leu ($r = 0.400$, $P$...
These findings demonstrate that short-term rises in post-prandial Leu are crucial for increasing MPS and contribute to long-term optimization of muscle mass.

There is evidence suggesting that Leu has anti-proteolytic effects, including attenuation of the ubiquitin-proteosome pathway (30); however, most of this research is in vitro, or in muscle wasting models (9, 10), and is untested in healthy subjects or under physiological conditions. Therefore, the present experiment sought to test these theories using a meal-feeding protocol with healthy adult rats. This study found significant effects of diet on PSMA2 (Fig. 3.1a) but all measures of ubiquitin ligases were unchanged (Fig. 3.1b-d). These results suggest that the Leu effects on ubiquitin-proteosome pathways are more important in situations of muscle wasting, when this pathway becomes more active (2) (Fig. 3.4). While the decrease in PSMA2 by higher Leu diets may support greater muscle anabolism, collectively these results indicate that MPS is more responsive to this meal feeding protocol in healthy adults, than MPD. Accordingly, the changes in muscle mass observed overtime (Fig. 3.3) is likely due to the differences in MPS, rather than MPD (Fig. 3.2).

For the proteins used in this study only the wheat glutens lysine content was below the National Research Council (NRC) requirement for the adult rat (29). This specific requirement was corrected by supplementing the wheat gluten diet with lysine to the level of the concentration in whey protein (Table 3.3). All other EAA exceeded NRC guidelines. The unique regulatory role of Leu may in part explain the inconsistencies in comparison of optimal needs for Leu compared with current measures of minimum protein requirements for nitrogen balance or indispensable amino acid oxidation (IAAO) measurements. These standard measurements for protein requirement interpret postprandial increases in plasma amino acids as inefficient and excessive protein intake. While increased amino acid oxidation may reflect reduced efficiency of nitrogen retention of dietary protein and may provide an accurate representation of minimum requirements for an EAA such as lysine with no known metabolic signaling or regulatory roles, the extrapolation that protein intakes above the
minimum amount for short-term nitrogen balance are physiologically meaningless is not consistent with the regulatory role of Leu in MPS.

In summary, this experiment supports the contention that Leu intakes above current requirements are beneficial for maximizing anabolic signaling in muscle tissue, and supports long term preservation of muscle mass. Furthermore, in this healthy adult rat-feeding model, ubiquitin-proteosome is less responsive to meal feeding than MPS. This finding is contrary to studies using muscle-wasting models, where MPD and ubiquitin-proteasome activity are typically increased, but significantly attenuated when Leu is added into the diet. These results suggest that Leus effects on ubiquitin-proteosome pathways are minimal for healthy adult rats consuming adequate diets. Thus, long-term changes in muscle mass observed in adult rats are likely due to the differences in MPS, rather than MPD.
REFERENCES


Fig. 3.1. Ubiquitin proteasome results for rats fed complete meals containing wheat, egg, or whey proteins for 11 weeks. Differences reported for PSMA2 (outer ring of the 20S proteasome) (A), Atrogin1 (B), Muscle RING-finger protein-1 (MURF1) (C), and UBE2L3 (an E2 ligase) (D), with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. Data are means ± SEM; n = 13. Labeled means without a common letter differ, $P < 0.05$. 
Fig. 3.2. Post-prandial rates of muscle protein synthesis (MPS) for rats fed complete meals containing wheat, egg, or whey proteins for 11 weeks. Data are means ± SEM; n = 5-8. Labeled means without a common letter differ, $P < 0.05$. 

MPS (%/d)
Fig. 3.3. Gastrocnemius muscle weights (g) of rats fed complete meals containing wheat, egg, or whey proteins for 11 weeks. Data are means ± SEM; n = 13. Labeled means without a common letter differ, $P < 0.05$. 

![Gastrocnemius muscle weights](image-url)
Fig. 3.4. Theoretical role of leucine regulation of ubiquitin-proteosome pathway. Evidence in muscle wasting populations show leucine supplementation can attenuate ubiquitin-proteosome expression; but research from this chapter showed minimal effects. UB (ubiquitin); MURF-1 (muscle RING-finger protein-1).
<table>
<thead>
<tr>
<th>Component</th>
<th>Wheat Diet</th>
<th>Egg Diet</th>
<th>Whey Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vital Wheat Gluten(^1)</td>
<td>190.2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Egg White Solids(^2)</td>
<td>0.0</td>
<td>195.6</td>
<td>0.0</td>
</tr>
<tr>
<td>Whey Protein Isolate(^3)</td>
<td>0.0</td>
<td>0.0</td>
<td>188.8</td>
</tr>
<tr>
<td>L-Lysine(^4)</td>
<td>10.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Cornstarch</td>
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\(^1\) Vital Wheat Gluten purchased from Honeyville Grain, Honeyville, UT. 83.4% protein, 7.6% carbohydrate, 9% other.

\(^2\) Egg White Solids purchased from Harlan-Teklad, Madison, WI. 87.8% Protein, 4.5% carbohydrate, 7.7% other.

\(^3\) Whey Protein Isolate provided by Perham, Perham, MN. 89.9% protein, 3.8% carbohydrate, 6.3% other.

\(^4\) Vital Wheat Gluten supplemented with 6.3g L-lysine/100g protein to match Whey Protein Isolate.

\(^5\) Mineral and Vitamin supplements from Harlen-Teklad, Madison, WI.

\(^6\) Egg White Solids supplemented with 16.0 mg biotin/kg diet.
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<th>Baseline&lt;sup&gt;2&lt;/sup&gt;</th>
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<tr>
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<sup>1</sup>Values expressed as means ± SEM, n=5-8. Labeled means without a common letter differ (P < 0.05).
<sup>2</sup>12 h food-deprived control.
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<td>11.5</td>
<td>10.2</td>
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</table>

1. From Nutrient Requirements of Laboratory Animals Fourth Revised Edition (29).
2. Values calculated for 300 g rat at maintenance
3. Amino acid values expressed as g/kg diet
CHAPTER IV:
LEUCINE OR CARBOHYDRATE SUPPLEMENTATION REDUCES AMPK AND EEF2 PHOSPHORYLATION AND EXTENDS POSTPRANDIAL MUSCLE PROTEIN SYNTHESIS IN RATS

INTRODUCTION

Muscle protein synthesis (MPS) is rapidly stimulated after consumption of a protein-containing meal, and is associated with activation of translation initiation by hormone and nutrient signals (29). Key regulatory points of translation initiation include assembly of the eukaryotic initiation factor 4F (eIF4F) complex and phosphorylation of the 70 kilodalton ribosomal protein S6 kinase (S6K1). Stimulation of these initiation factors is potentiated by insulin and the essential amino acid (EAA) leucine (Leu) (4, 5). After a meal, plasma concentrations of Leu and insulin trigger the activation of eIF4F and S6K1. Assembly of the eIF4F initiation complex involves phosphorylation of the inhibitory eIF4E binding protein-1 (4E-BP1), which releases the initiation factor eIF4E and allows it to bind with eIF4G. Binding of eIF4E with eIF4G promotes preparation of the mRNA for binding to the 43S pre-initiation complex. The postprandial rise in insulin activates the PI3K – Akt – mTORC1 (phosphoinositide 3 kinase - protein kinase B - mammalian target of rapamycin complex 1) signaling cascade (4), while Leu acts downstream of Akt to stimulate mTORC1, through an as yet unidentified mechanism (12). Apart from the postprandial rise in plasma Leu, MPS does not appear to be limited by amino acids beyond that required to maintain basal levels (31), or by eIF2-mediated formation of the 43S pre-initiation complex (6, 24).

While the initial translation initiation response shortly after a meal (< 2 h) has been well characterized, much less is known about what determines the duration of peak MPS following food intake. Two recent studies (8, 28) using

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1 Chapter IV is adapted from: Wilson GJ, Layman DK, Moulton CJ, Norton LE, Anthony TG, Proud CG, Rupassara SI, and Garlick PJ. Leucine or carbohydrate supplementation reduces AMPK and eEF2 phosphorylation and extends postprandial muscle protein synthesis in rats. Am J Physiol Endocrinol Metab. In-press: 2011. AJP grants the rights to the author(s) for any research article published in the Am J Physiol Endocrinol Metab to be reprinted in the author(s) thesis.
precisely timed meals containing intact proteins confirmed that the early postprandial rise of MPS (< 2 h) corresponded to increases in plasma insulin and Leu, as well as assembly of eIF4F and phosphorylation of S6K1 and 4E-BP1, but found that MPS declined by 3 h, despite continued elevations of plasma Leu, mTORC1 signaling, and translation initiation. This discordance between rates of MPS and initiation factor activity suggests that events other than eIF4F complex assembly and S6K1 phosphorylation determine the postprandial MPS response. In both experiments, the postprandial rise and fall of plasma insulin paralleled changes in MPS activity, suggesting that a decline in plasma insulin may limit MPS activity ~3 h after a meal.

Consistent with the meal-feeding experiments, Bohé et al. (9) infused a complete amino acid mixture into adult volunteers for 6 h. The continuous infusion increased plasma amino acids 1.7-fold above basal levels and stimulated MPS for ~2 h, but MPS subsequently returned to baseline in spite of the continuous infusion of amino acids. Contrary to these findings, Wilson et al. (36) infused a large dose of Leu to neonatal pigs for 24 h, along with replacement amino acids to maintain basal levels, and found that MPS remained elevated for the entire infusion period. This discrepancy highlights the need for additional research to elucidate factors that determine the duration of the postprandial anabolic period.

Potential explanations for the discordance between initiation signals and the duration of MPS after a meal include: 1) factors or steps within mRNA translation become refractory (unresponsive) to external stimuli, 2) reduced availability of amino acids, or 3) reduced signaling from insulin or other important stimuli. The purpose of this study was to determine if the postprandial period of muscle anabolism can be extended by supplemental doses of Leu, carbohydrates, or both; or if MPS becomes refractory to anabolic stimuli and requires a period of time to reset before it can be stimulated again. A better understanding of the relationship between MPS and the mechanisms leading to active mRNA translation in muscle may have implications for maintenance of lean tissue during weight loss or aging, for treatment of patients suffering trauma
or prolonged bed rest, as well as for acceleration of muscle development in athletes.

MATERIALS AND METHODS

**Animals.** Thirty-four Male Sprague-Dawley rats (269 ± 7 g, 9-11 weeks of age; Harlan-Teklad, Madison, WI) were housed individually and maintained at 24°C with a 12 h reverse light cycle (light period: 1900-0700 h). Rats were fed during the dark period and had free access to water. The animal protocol and facilities were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign.

**Meal training protocol.** Baseline diets provided 20%, 50%, and 30%, of energy from protein, carbohydrates, and fats, respectively (Table 4.1). Rats were trained to consume 3 meals/d consisting of 4 g meals at 0700 h and 1300 h and a 6 g meal at 1800 h. These meals provided 80% of daily ad libitum intake, which insured that energy intake was equal and that all food was consumed rapidly (within 20 min of introduction) (Fig. 4.1). This food restriction protocol does not alter development of lean tissue but reduces accumulation of body fat (20).

**Experimental Treatments.** After 7 d of meal training, rats were food deprived for 12 h (1900 h - 0700 h), and then euthanized pre-meal at time 0 (food-deprived), or at 90 or 180 min after the 4 g test meal. Based on previous research (28), the 90 min time point represented peak MPS activity, while the 180 min time point reflected a period of time when mTORC1 signaling and initiation factor activity was elevated, but MPS returned to baseline values. At 135 min after the test meal, rats were administered a 5 ml oral gavage of carbohydrates (CHO), leucine (Leu), CHO + Leu (LC), or a Sham (water) control (Fig. 4.1).

The CHO supplement contained 2.63 g of carbohydrates (1.315 g glucose + 1.315 g sucrose) in distilled water, which was slightly greater than the carbohydrate content of the 4 g meal (~2.2 g). The Leu supplement contained 270 mg of Leu in distilled water, which was equivalent to the daily amount of Leu consumed by rats of this age and strain with free access to AIN-93 powdered diet.
(Harlan-Teklad, Madison, WI) (19), and nearly three times the Leu content of the 4 g meal (~100 mg). The LC supplement contained 2.36 g of carbohydrate (1.18 g glucose + 1.18 g sucrose) and 270 mg of Leu in distilled water, which was isocaloric with the CHO gavage and isonitrogenous with the Leu gavage. The CHO and LC supplements supplied ~15% of daily energy intake for this age and strain of rat (19). The amounts and timing of the supplements were based on previous research that produced maximal Leu- and insulin-induced stimulations of translation initiation and MPS 45 min after oral gavage (2, 3, 6, 12).

Administration of metabolic tracer and sample collection. MPS was measured in skeletal muscle using the flooding dose method (18). A 100% enriched L-[\(^2\)H\(_5\)]phenylalanine solution (150 mmol/L; Cambridge Isotopes, Andover, MA) was administered at 150 mmol/100 g body weight and injected via tail vein (1 mL/100g body weight). After 10 min, animals were euthanized by decapitation, blood was collected in pre-coated EDTA tubes, and hind limbs were quickly removed and immersed in an ice-water mixture. Gastrocnemius muscles were removed from cooled hind limbs, frozen in liquid N\(_2\), and stored at -80°C.

Determination of MPS. Frozen muscle tissue was powdered in liquid nitrogen and protein was precipitated with cold (4°C) perchloric acid (30 g/L, 1 mL/50 mg muscle tissue). The resulting supernatant and protein pellet were prepared for MPS analysis as described previously (16, 26). The enrichment of L-[\(^2\)H\(_5\)]phenylalanine in the muscle hydrolysate was measured by GC-MS using a 6890N GC and a 5973N mass detector (Agilent Technologies, Santa Clara, CA). Samples were analyzed under electron impact ionization in splitless mode and the mass:charge ratio of phenylethylamine ions at 106 (m + 2) and 109 (m + 5) were monitored for enrichment.

The muscle supernatant was used to determine intracellular free phenylalanine enrichment. Free amino acids were purified by ion exchange resin solid-phase extraction using EZ : faast amino acid analysis sample testing kit (Phenomenex, Torrance, CA) and \(^2\)H\(_5\)-phenylalanine enrichment was determined using a propyl chloroformate derivative with GC-MS by monitoring the ions at mass:charge ratio 206 (m) and 211 (m + 5) (27).
MPS was assessed from the rate of incorporation of L-[\(^{2}\text{H}5\)]phenylalanine into total mixed muscle protein as described previously (26). The time from injection of the tracer until tissue cooling was recorded as the actual time for L-[\(^{2}\text{H}5\)]phenylalanine incorporation. MPS, defined as the percentage of tissue protein renewed each day, were calculated according to the formula: MPS = (Eb x 100) / (Ea x t), where t is the time interval between injection and cooling of sampled tissue expressed in days and Eb and Ea are the enrichments of \([^{2}\text{H}5]\)Phe in hydrolyzed tissue protein and in muscle free amino acids, respectively.

*Plasma measurements.* Plasma was obtained from trunk blood by centrifugation at 1800 x g for 10 min at 4°C. Plasma insulin concentrations were analyzed using a commercial RIA kit for rat insulin (Millipore, Billerica, MA). Plasma amino acid concentrations were determined by HPLC using a Waters 2475 Fluorescence detector (Milford, MA) (30).

*Western blot analysis.* Muscle supernatants were subjected to protein immunoblot analysis as previously described (12). Rabbit polyclonal antibodies were used for total and phospho-S6K1 (Thr389) (1:1,000 dilution), total and phospho-4E-BP1 (Thr37/46) (1:1,000 dilution), total and phospho-eEF2 (Thr56) (1:25,000 dilution), total and phospho-AMPK\(\alpha\) (Thr172) (adenosine monophosphate-activated protein kinase) (1:2,000 dilution), total and phospho-ACC (Ser79) (acetyl-CoA carboxylase) (1:2,000 dilution), total and phospho-Akt (Ser473) (1:1,000 dilution), total and phospho-eIF2\(\alpha\) (Ser51) (eukaryotic initiation factor 2\(\alpha\)) (1:250 dilution), as well as total and phospho-eIF2B\(\epsilon\) (phospho-serine 535) (eukaryotic initiation factor 2B epsilon) (Invitrogen, Camarillo, CA) (1:1,000 dilution). All primary antibodies used were purchased from Cell Signaling (Boston, MA), unless stated otherwise. Anti-rabbit IgG, HRP-linked secondary antibody was also purchased from Cell Signaling (1:2,000 dilution) (Boston, MA).

*Nucleotide analysis.* Adenosine 5’-monophosphate (AMP) and adenosine 5’-triphosphate (ATP) were determined by HPLC analysis as described in a technical note CN-039 by Phenomenex (Torrance, CA). Briefly, frozen gastrocnemius muscle was ground under liquid nitrogen and 50 mg of sample
was combined with 1 ml of MeOH in water (25:75) and vortexed. This sample was centrifuged at 10,000 RPM at 4 °C for 5 min and the supernatant extracted. The supernatant was dried and then reconstituted in phosphate buffered saline (PBS). The sample was then passed through a Strata X-AW 33u Polymeric Weak Anion Solid Phase Extraction Sorbent (Phenomenex, Torrance, CA). Ten microliter aliquots of the final muscle extract were injected onto the HPLC using a Phenomenex Gemini 5u C18 110A Column 150 x 4.6 mm with temperature set at °25. A single mobile phase was used at a flow rate of 1 ml / min with 25% acetonitrile / 10mM KH2PO4 pH 7.0 (pH was adjusted with potassium hydroxide (KOH) / 5mM tetrabutylammonium chloride (TBAC). Nucleotide peaks were measured by UV detection at wavelength 260 nm. Peaks were identified by comparison with external standards (Sigma-Aldrich, St. Louis, MO).

Statistical analysis. All data were analyzed by SPSS 15.0 software package for Windows (Chicago, IL). A one-way ANOVA was performed with the postprandial times and oral gavage treatments as the independent variables. When a significant overall effect was detected, differences among individual means were assessed using LSD post hoc test. For plasma amino acids, a one-way ANOVA was performed for meal effects (0, 90, and 180 min) and supplement effects (Sham, CHO, Leu, and LC at 180 min). Correlations were determined by linear regression (Pearson correlation). The level of significance was set at $P < 0.05$ for all statistical tests. Values are presented as means ± SEM.

RESULTS

This experiment evaluated the potential for supplemental Leu or carbohydrates to extend the peak MPS response after a meal. Consistent with previous research (28), MPS increased at 90 min after a 4 g test meal but then returned to food-deprived values at 180 min (Sham). Supplementation with Leu, CHO, or LC 135 min after the test meal significantly increased MPS at 180 min above Sham rats and equal to meal-fed rats at 90 min (Fig. 4.2).
To understand the mechanisms explaining these findings, changes in plasma amino acid concentrations were first examined. As expected, plasma Leu and EAAs increased 90 min after the meal and remained elevated at 180 min (Table 4.2).

Administering the Leu supplements at 135 min elevated plasma Leu at 180 min; however, Leu and LC also depressed plasma isoleucine and valine below sham values (Fig. 4.3). Likewise, the CHO gavage lowered plasma branched-chain amino acids (BCAA; Leu, isoleucine, and valine) below sham levels. The supplements had no effect on levels of other measured plasma EAA, including methionine, threonine, and lysine, relative to Sham control (Fig. 4.3).

Phosphorylation of S6K1 (Thr389) was measured as a marker of mTORC1 signaling. Consistent with previous reports (8, 28), relative phosphorylation of S6K1 (Thr389) in muscle increased at 90 min ($P < 0.05$) and remain elevated at 180 min (sham control) compared with food deprived rats (time 0) ($P < 0.05$) (Fig. 4.4). Likewise, supplemental treatments maintained S6K1 activation at 180 min. From 0 to 90 min, there was a significant correlation between S6K1 phosphorylation and MPS ($r = 0.843$, $P < 0.05$). However, from 90 to 180 min after the meal, no statistical correlation was present ($r = 0.163$, $P > 0.05$). Similar results were seen for phospho-4E-BP1 (Thr37/46) (Fig. 4.4).

To evaluate other steps in translation initiation, phosphorylation of regulatory sites in eIF2α (Ser51) and eIF2Bε (Ser535) were examined as markers of eIF2 activation. No changes in phosphorylation of either of these proteins were found (data not shown).

Consistent with earlier publications (8, 28) plasma insulin rose at 90 min post-meal and returned to food-deprived (time 0) values at 180 min (Sham) (Fig. 4.5). The CHO and LC supplements maintained elevated insulin at 180 min, while the Leu supplement was not different from the Sham group at 180 min. Phosphorylation of Akt (Ser473) was measured as an indicator of insulin signaling but no differences in Akt activation were detected among the groups at 180 min (data not shown).
Since the decline in MPS at 180 min post-meal (Sham) was not related to changes in essential amino acids, plasma insulin, or phosphorylation of S6K1, eIF2α, or eIF2Bε, the phosphorylation of eEF2 (Thr56) was measured, for an indication of translation elongation activity. After the meal, eEF2 phosphorylation decreased from 0 to 90 min reflecting activation of elongation for MPS, but returned to food-deprived (time 0) values at 180 min post-meal (Sham control) consistent with inhibition of elongation (Fig. 4.6) and the decline in MPS. All three supplements (Leu, CHO, and LC) reduced eEF2 phosphorylation at 180 min relative to Sham control. Across all treatment groups, there was an inverse association between phospho-eEF2 and MPS ($r = -0.500$, $P < 0.05$).

Phosphorylation of AMPKα (Thr172) and its substrate ACC (Ser79) and the nucleotide ratio of AMP/ATP were measured as indicators of cellular energy status (Fig. 4.7). In meal-fed rats, phosphorylation of muscle AMPKα and ACC (21) declined at 90 min as compared to food deprived rats reflecting increased energy availability, but increased at 180 min reflecting decreased energy availability. All three of the supplemental treatments maintained reduced phospho-AMPKα and ACC to levels similar to the 90 min post-meal values. Similarly to changes in phospho-AMPKα and ACC, the ratio of AMP/ATP (allosteric regulator of AMPK (22) and a direct measure of cellular energy status) declined at 90 min post-meal but increased above food deprived values at 180 min. Each of the supplements served to maintain a reduced ratio of AMP/ATP at 180 min, consistent with the behaviors of AMPKα and ACC.

**DISCUSSION**

This study demonstrates that Leu or CHO supplements provided ~2 h after consumption of a complete meal can extend the postprandial anabolic period of MPS (Fig. 4.2). Further, the findings of this study suggest that the incongruity between MPS and mTORC1 signaling 180 min after a meal is associated with activation of AMPK (Fig. 4.7) and reduced translation elongation activity (Fig. 4.6). These data suggest that temporal changes in skeletal muscle energy state following meal feeding influence the period of muscle anabolism.
Further, the energy status of muscle can be enhanced by post-meal nutrition supplements to extend the period of muscle anabolism.

Translation initiation is a critical regulatory step for stimulating the rise in MPS after a meal, and is activated by nutrient signals derived from changes in plasma insulin and Leu and propagated through the PI3K – Akt – mTORC1 signaling cascade (4). Triggering of initiation and maximum MPS activity occurs within 30 to 60 min following administration of free Leu (6) and 60 to 90 min following ingestion of intact proteins (8, 28). Two recent studies (8, 28) using precisely timed meals containing intact proteins confirmed that the early postprandial rise of MPS (< 2 hr) corresponded to increases in plasma insulin and Leu, as well as phosphorylation of S6K1 and 4E-BP1 and assembly of the eIF4F initiation complex, but found that MPS declined after 3 h, despite continued elevations of plasma Leu, mTORC1 signaling, and translation initiation. The present study is consistent with these reports, finding a postprandial rise in plasma Leu and significant correlation between phospho-S6K1 and MPS \((r = .843, P < 0.05)\) from 0 to 90 min after the test meal (Fig. 4.4). However, from 90-180 min post-meal, the correlation between phospho-S6K1 and MPS disappears \((r = .163, P > 0.05)\) (Fig. 4.4). Likewise, similar results were found for phospho-4E-BP1 (Fig. 4.4). Furthermore, no changes in the phosphorylation states of eIF2α or eIF2Bε were found, suggesting that eIF2 is unlikely to be a regulatory factor for postprandial changes in MPS. Collectively, these findings are consistent with the hypothesis that translation initiation signaling via mTORC1 facilitates the initial rise in MPS after a meal; however, factors in addition to translation initiation are required to sustain peak MPS 180 min after a meal (8,28).

Potential explanations for the discordance between initiation signals and the postprandial duration of MPS include: 1) factors or steps within mRNA translation become refractory (unresponsive) to external stimuli, 2) reduced availability of amino acids, or 3) reduced signaling from insulin or other important signals. In the present study, all three nutrient supplements were able to extend the duration of peak MPS demonstrating that mRNA translation is not refractory
to anabolic stimuli, but rather limited by one or more regulatory factors. Additional measures were taken determine what factors limited the postprandial duration of MPS, and why either Leu, or CHO supplements overcame these limitation(s).

Results showed that plasma essential amino acids remained elevated at 180 min after the meal (sham control) relative to the 90 min time point and that Leu or CHO treatments were sufficient to extend MPS without providing additional amino acids (Table 4.2) establishing that availability of essential amino acids was not a primary limitation during the decline in MPS.

Likewise, insulin does not appear to be a critical factor in extending MPS (Fig. 4.5). As expected, the carbohydrate treatments (CHO and LC) increased plasma insulin concentration at 180 min compared to the sham control, however the Leu treatment – which prolonged the duration of MPS at 180 min – did not maintain elevated insulin concentration at 180 min. There were no differences between groups in phosphorylation of the insulin-signaling target Akt at 180 min (data not shown). However, research has previously shown that peak insulin and Akt responses occur 30 min after a complete meal (14), and < 20 min after administration of these supplements (4). Therefore, the time-point measurements used in this study most likely missed peak insulin and Akt responses. Thus, the influence of insulin signaling on the duration of MPS remains unresolved.

Another possible determinant of the post-meal reduction in MPS is a decrease in translation elongation. In this experiment, phosphorylation of eEF2 (Thr56) was used as an indicator for translation elongation activity. Results showed increased phosphorylation of eEF2 in the sham control, indicating inhibition of eEF2 at 180 min after the test meal (Fig. 4.6). Conversely, phosphorylation of eEF2 was reduced by each of the supplements at 180 min consistent with sustained elevation of MPS after the meal.

Proud and colleagues have shown inhibition of eEF2 and protein synthesis in response to mild energy deficits is associated with activation of AMPK, which stimulates the regulatory kinase eukaryotic elongation factor 2 kinase (eEF2K) to subsequently phosphorylate (inhibit) eEF2 (11, 23). The ability of AMPK to respond to changes in AMP/ATP – with AMP stimulating AMPK, and
ATP inhibiting it – allows this kinase to be an effective cellular energy sensor (22). Measurements of AMPKα phosphorylation, the ratio of AMP/ATP, and phosphorylation of ACC were taken to determine if energy status of muscle could be a limiting factor for MPS 180 min after a meal (Fig. 4.7). Consistent with the energy hypothesis, each of the biomarkers for cellular energy status reflected energy stress in the cell and produced a pattern of change nearly identical with that observed for eEF2 (Fig. 4.6). Further, there was a significant inverse correlation across treatments between MPS and AMPKα phosphorylation ($r = -0.581$, $P < 0.05$). Collectively, these results suggest that energy is a likely determinate of the duration of postprandial MPS, and that eEF2 is the regulatory step responding to energy status.

When considering the upstream regulators of translation initiation and elongation, there appears to be a paradox in these signals. Indeed, AMPK and mTORC1 are both regulators of translation initiation and elongation, but act in opposing ways – with AMPK inhibiting translation initiation (7) and elongation (10), and mTORC1 stimulating these processes (33). The findings that both mTORC1 (8,28) and AMPK (Fig. 4.7) are activated during the postprandial decline in MPS suggests that AMPK and energy become the dominant signals. Further, the finding that eEF2, a key regulator of translation elongation, is inhibited coinciding with down-regulation of MPS, indicates that the AMPK inhibitory effect on eEF2 overrides the stimulatory effects of mTORC1.

Support for this hierarchal form of regulation is provided from research by Proud and colleagues (10, 11, 23, 25, 32). In a variety of cell lines (10, 23, 25, 32) these authors have shown that under mild energy deficits, AMPK activation results in strong and rapid inhibition of eEF2 and translation elongation, with no corresponding changes in phosphorylation of proteins involved in the initiation stages of mRNA translation, including eIF2, p70S6K1, 4E-BP1, and assembly of the eIF4F initiation complex. Contrary to these findings, Dennis et al. (13) demonstrated inhibition of mTORC1 signaling using higher concentrations of the AMPK agonist 2-DOG (10 fold greater than Proud and colleagues) (2-deoxyglucose). Likewise, Williamson et al. (35) reported inhibition of mTORC1
signaling by AMPK using higher levels of AICAR (double the amount of Proud and colleagues) (5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside). Overall, the current study and the published reports by Proud and colleagues suggest that the physiological regulation of cellular energy status by AMPK exerts a dominant effect over the anabolic stimulation of MPS by mTORC1.

Considering that protein turnover is a metabolically costly process, accounting for approximately one quarter of resting energy expenditure (34), it is logical that a cell would suppress protein synthesis activity during an energy deficit to preserve critical functions. Furthermore, when considering that translation elongation accounts for the majority (> 99%) of the energy used for polypeptide assembly (10, 11, 29), it appears logical that the cell would regulate translation elongation to protect energy status.

The finding that the large Leu supplement was able to decrease AMPKα phosphorylation is consistent with the work from Du et al. (15), who showed that Leu treatment in C2C12 myoblast cells resulted in a 36% decrease in the AMP/ATP ratio, a 28% reduction in phospho AMPKα, and a 43% reduction in AMPK activity. Leu can provide energy (ATP) to muscle by conversion to ketoisocaproate (KIC) and its subsequent oxidation via the TCA cycle. Increased KIC levels in skeletal muscle have also been shown to increase oxidation of all three BCAA (Leu isoleucine, and valine) by allostERIC activation of branched-chain α-keto-acid dehydrogenase (1, 17). In support of the hypothesis that Leu increased BCAA oxidation, the Leu-containing supplements in the present experiment reduced isoleucine and valine concentrations by 50-150%, while other plasma EAAs remained unchanged relative to sham control (Fig. 4.3).

In summary, intake of a Leu or CHO supplements ~2 h after consumption of a complete meal can extend the duration of the postprandial rise in MPS. Further, the incongruity between MPS and mTORC1 signaling at 180 min after the meal does not reflect a refractory period or decreased sensitivity to anabolic stimuli, but rather, an increase in AMPK activity and a decrease in translation elongation activity. Improved understanding of factors that regulate the duration of MPS may have implications for maintenance of lean tissue during weight loss.
or aging, for treatment of patients suffering trauma or prolonged bed rest, as well as for acceleration of muscle development in athletes. As such, the roles of eEF2 and AMPK in regulation of postprandial MPS warrant further investigation.
REFERENCES


Fig. 4.1. Experimental design and timeline. Treatment groups were intubated at 135 min after the meal with water (sham), carbohydrates (CHO), leucine (Leu), or combination of CHO + Leu (LC).
Fig. 4.2. Postprandial changes for muscle protein synthesis (MPS). Treatment groups were intubated at 135 min after the meal with water (sham), carbohydrates (CHO), leucine (Leu), or combination of CHO + Leu (LC). Data are means ± SEM; n = 4-6. Means without a common letter differ, (P < 0.05).
Fig. 4.3. Percent difference of selected plasma amino acid concentrations from control (sham treatment) for each supplement. Data are means ± SEM; n = 6. Treatment groups were intubated at 135 min after the meal with water (sham), carbohydrates (CHO), leucine (Leu), or combination of CHO + Leu (LC), with measurements taken 180 min after the meal. Means without a common letter differ, ($P < 0.05$). * Indicates that amino acid differs from sham ($P < 0.05$).
Fig. 4.4. Postprandial changes in the relative phosphorylation state of ribosomal S6 protein kinase 1 (phospho-S6K1 (Thr389) / total-S6K1) and eIF4E binding protein-1 (phospho-4E-BP1 (Thr37/46) / total 4E-BP1). Treatment groups were intubated at 135 min after the meal with water (sham), carbohydrates (CHO), leucine (Leu), or combination of CHO + Leu (LC). Data are means ± SEM; n = 4-6. Means without a common letter differ, (P < 0.05).
Fig. 4.5. Postprandial changes in plasma insulin. Treatment groups were intubated at 135 min after the meal with water (sham), carbohydrates (CHO), leucine (Leu), or combination of CHO + Leu (LC). Data are means ± SEM; n = 4-6. Means without a common letter differ, ($P < 0.05$).
Fig. 4.6. Postprandial changes in the relative phosphorylation state of eukaryotic elongation factor 2 (phospho-eEF2 (Thr56) / total eEF2). Treatment groups were intubated at 135 min after the meal with water (sham), carbohydrates (CHO), leucine (Leu), or combination of CHO + Leu (LC). Data are means ± SEM; n = 4-6. Means without a common letter differ, (P < 0.05).
Fig. 4.7. Postprandial changes in the relative phosphorylation states of adenosine monophosphate-activated protein kinase alpha (phospho-AMPKα (Thr172) / total AMPKα), acetyl-CoA carboxylase (phospho-ACC (Ser79) / total ACC), and nucleotide ratio of AMP/ATP (adenosine 5′-monophosphate / adenosine 5′-triphosphate). Treatment groups were intubated at 135 min after the meal with water (sham), carbohydrates (CHO), leucine (Leu), or combination of CHO + Leu (LC). Data are expressed as percent change from fasted (time 0) values. Data are means ± SEM; n = 4-6. Means without a common letter differ, (P < 0.05).
Table 4.1. *Meal composition*

<table>
<thead>
<tr>
<th>Components</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey Protein Isolate¹</td>
<td>1228.0</td>
</tr>
<tr>
<td>Corn starch</td>
<td>290.0</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>134.1</td>
</tr>
<tr>
<td>Sucrose</td>
<td>101.5</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>140.9</td>
</tr>
<tr>
<td>Cellulose</td>
<td>53.7</td>
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<tr>
<td>Mineral Mix²</td>
<td>37.6</td>
</tr>
<tr>
<td>Vitamin Mix²</td>
<td>10.7</td>
</tr>
<tr>
<td>Choline biturate</td>
<td>2.7</td>
</tr>
<tr>
<td>TBHQ³</td>
<td>0.014</td>
</tr>
</tbody>
</table>

¹Whey protein provided by Perham, Perham, MN. (89.9% protein, 3.8% carbohydrate, 6.3% other).

²Purchased from Harlen-Teklad, Madison, WI.

³TBHQ, tertiary butylhydroquinone.
Table 4.2. Postprandial concentrations of selected essential amino acids in plasma following meal consumption

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0(^1)</th>
<th>90</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>200±18(^{b})</td>
<td>297±31(^{a})</td>
<td>312±19(^{a})</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>100±11(^{b})</td>
<td>150±16(^{a})</td>
<td>148±12(^{a})</td>
</tr>
<tr>
<td>Valine</td>
<td>171±19(^{a})</td>
<td>221±25(^{a})</td>
<td>243±13(^{a})</td>
</tr>
<tr>
<td>Lysine</td>
<td>569±33(^{b})</td>
<td>724±61(^{a})</td>
<td>595±38(^{ab})</td>
</tr>
<tr>
<td>Methionine</td>
<td>72±8(^{a})</td>
<td>88±6(^{a})</td>
<td>88±5(^{a})</td>
</tr>
<tr>
<td>Threonine</td>
<td>799±96(^{a})</td>
<td>982±71(^{a})</td>
<td>993±68(^{a})</td>
</tr>
</tbody>
</table>

Values expressed as means ± SEM; n = 4-6.

Means without a common letter differ, (\(P < 0.05\)).

\(^{1}\)12 h food deprived controls
CHAPTER V:
DOSE AND TIME DEPENDENT EFFECTS OF LEUCINE OR
CARBOHYDRATE SUPPLEMENTS TO REDUCE AMPK AND EEF2
PHOSPHORYLATION AND EXTEND POSTPRANDIAL MUSCLE PROTEIN
SYNTHESIS IN RATS

INTRODUCTION

Studies investigating nutritional regulation of muscle protein synthesis (MPS) have shown that consumption of the essential amino acid leucine (Leu) is a key factor determining the anabolic response of MPS to a meal (15). Most studies investigating Leu stimulated protein synthesis have focused on early postprandial responses (< 2 h); however, much less is known about what determines the duration of peak MPS following food intake. In Chapter IV, research investigating the postprandial anabolic period was discussed (29). Using an adult rat-feeding model, it was demonstrated that MPS increased rapidly after a breakfast meal but declined by 180 min in spite of continued elevations of plasma amino acids, plasma Leu, and translation initiation signals. The decline in MPS was inversely associated with the phosphorylation states of translation elongation factor 2 (eEF2), the “cellular energy sensor” adenosine monophosphate-activated protein kinase alpha (AMPKα), and the AMPKα substrate acetyl-CoA carboxylase (ACC) and with increases in the ratio of AMP / ATP. Supplementation with a large dose of Leu (270 mg, triple the amount of the breakfast meal), carbohydrates (2.63 g, slightly more than the breakfast meal), or a combination of both 135 min after the meal were all able to maintain peak rates of MPS through 180 min post-meal, and prevent rises in phosphorylation of eEF2, AMPKα, and ACC, as well as the ratio of AMP / ATP. These findings are consistent with the proposed mechanism that the cell regulates protein synthesis during mild energy deficits through activation of AMPKα which stimulates the regulatory kinase eukaryotic elongation factor 2 kinase (eEF2K) to subsequently phosphorylate (inhibit) eEF2 (9, 20).
Leu and carbohydrates are both energy substrates in skeletal muscle but would be expected to have different impacts on cellular energy and signaling. Yet, previous research (29) demonstrated that both substrates were effective at increasing energy and extending post-prandial MPS. Two limitations of the initial study were that maximum oral doses of the supplements were given and only one time point was measured. Therefore, the present experiments were designed to extend the initial findings by examining the impact of Leu and carbohydrate supplements on MPS, cellular energy status, and signaling elements using multiple doses and time-points.

MATERIALS AND METHODS

Animals. Ninety-two male Sprague-Dawley rats (256 ± 8 g, 9-11 weeks of age; Harlan-Teklad, Madison, WI) were housed individually and maintained at 24°C with a 12 h reverse light cycle (light period: 1900-0700 h). Rats were fed during the dark period and had free access to water. The animal protocol and facilities were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign.

Meal training protocol. Baseline diets provided 20%, 50%, and 30%, of energy from protein, carbohydrates, and fats, respectively. Rats were trained to consume 3 meals/d consisting of 4 g meals at 0700 h and 1300 h and a 6 g meal at 1800 h. These meals provided 80% of daily ad libitum intake, which insured that energy intake was equal and that all food was consumed rapidly (within 20 min of feeding). This food restriction protocol does not alter development of lean tissue but reduces accumulation of body fat (18).

Experiment 1 evaluated specific responses of MPS, AMPK, and eEF2 to four energy levels of oral supplements of carbohydrates and BCAA. After 7 d of meal training, rats were food deprived for 12 h (1900 h - 0700 h), and then euthanized pre-meal at time 0 (food-deprived), or at 90 or 180 min after the 4 g control meal. Based on previous research (25), the 90 min time point represented peak MPS activity, while the 180 min time point reflected a period of time when MPS declined. At 135 min post-meal, rats were gavaged with 270 mg Leu
(Leu270), 80 mg Leu, 40 mg isoleucine and 40 mg of valine (Leu80), 2.63 g carbohydrates (CHO2.6), 1 g carbohydrates (CHO1.0), or water (Sham control).

The CHO2.6 supplement contained 2.63 g of carbohydrates (1.315 g glucose + 1.315 g sucrose) in distilled water, which was similar to the carbohydrate content of the 4 g control meal (~2.2 g). The CHO1.0 supplement contained 1 g of carbohydrates (0.5 g glucose + 0.5 g sucrose) in distilled water. The Leu270 supplement contained 270 mg of Leu in distilled water, which is equivalent to the daily amount of Leu consumed by rats of this age and strain with free access to AIN-93 diet (Harlan-Teklad, Madison, WI) (17). The Leu80 supplement contained 80 mg of Leu, 40 mg of isoleucine and 40 mg of valine, which approximated the amount of branched-chain amino acids (BCAA) in the control meal.

The amounts and timing of the supplements was based on previous research that produced maximal Leu and insulin-induced stimulations of translation initiation and MPS 45 min after oral gavage in the fasted state (3, 4, 6, 10). The addition of isoleucine and valine to the Leu80 supplement served to balance the BCAA formula (Leu80) to determine how this compared to Leu270, which lacked isoleucine and valine.

Experiment 2 evaluated supplement effects on MPS, AMPK, and eEF2 responses at multiple time points after oral supplements to determine the duration of signaling responses. After 7 d of meal training, rats were food deprived for 12 h (1900 h - 0700 h), and fed the 4 g control meal. At 135 min after the meal, rats were administered a 5 ml oral gavage of CHO1.0, Leu80, or water (Sham), as previously described. Rats were sacrificed at 3 time points: 20, 45, and 120 min post-gavage (155, 180, and 255 min post-meal, respectively). These time-points were selected based on previous experiments, and designed to observe maximal anabolic signaling and MPS responses 20-45 min after oral gavage, and a decline in MPS and anabolic signaling 120 min after oral at oral bolus (3, 4, 6, 10).

Administration of metabolic tracer and sample collection. MPS was measured in skeletal muscle using the flooding dose method (16). A 100%
enriched L-[\textsuperscript{2}H\textsubscript{5}]phenylalanine solution (150 mmol/L; Cambridge Isotopes, Andover, MA) was administered at 150 mmol/100 g body weight and injected via tail vein (1 mL/100g body weight). After 10 min, animals were euthanized by decapitation, blood was collected in pre-coated EDTA tubes, and hind limbs were quickly removed and immersed in an ice-water mixture. Gastrocnemius muscles were removed from cooled hind limbs, frozen in liquid N\textsubscript{2}, and stored at -80°C.

*Determination of MPS.* Frozen muscle tissue was powdered in liquid nitrogen and protein was precipitated with cold (4°C) perchloric acid (30 g/L, 1 mL/50 mg muscle tissue). The resulting supernatant and protein pellet were prepared for MPS analysis as described previously (13, 22). The enrichment of L-[\textsuperscript{2}H\textsubscript{5}]phenylalanine in the muscle hydrolysate was measured by GC-MS using a 6890N GC and a 5973N mass detector (Agilent Technologies, Santa Clara, CA). Samples were analyzed under electron impact ionization in splitless mode and the mass:charge ratio of phenylethylamine ions at 106 (m + 2) and 109 (m + 5) were monitored for enrichment.

The muscle supernatant was used to determine intracellular free phenylalanine enrichment. Free amino acids were purified by ion exchange resin solid-phase extraction using EZ : faast amino acid analysis sample testing kit (Phenomenex, Torrance, CA) and \textsuperscript{2}H\textsubscript{5}-phenylalanine enrichment was determined using a propyl chloroformate derivative with GC-MS by monitoring the ions at mass:charge ratio 206 (m) and 211 (m + 5) (23).

MPS was assessed from the rate of incorporation of L-[\textsuperscript{2}H\textsubscript{5}]phenylalanine into total mixed muscle protein as described previously (22). The time from injection of the tracer until tissue cooling was recorded as the actual time for L-[\textsuperscript{2}H\textsubscript{5}] phenylalanine incorporation. MPS, defined as the percentage of tissue protein renewed each day, were calculated according to the formula: 

\[
MPS = \frac{E_b \times 100}{E_a \times t},
\]

where t is the time interval between injection and cooling of sampled tissue expressed in days and Eb and Ea are the enrichments of \textsuperscript{2}H\textsubscript{5}Phe in hydrolyzed tissue protein and in muscle free amino acids, respectively.
Plasma measurements. Plasma was obtained from trunk blood by centrifugation at 1800 x g for 10 min at 4°C. Plasma insulin concentrations were analyzed using a commercial RIA kit for rat insulin (Millipore, Billerica, MA). Plasma amino acid concentrations were determined by HPLC using a Waters 2475 Fluorescence detector (Milford, MA) (27).

Western blot analysis. Muscle supernatants were subjected to protein immunoblot analysis as previously described (10). Rabbit polyclonal antibodies were used for total and phospho-AMPKα (Thr172) (1:2,000 dilution) (adenosine monophosphate-activated protein kinase), total and phospho-ACC (1:2,000 dilution) (Ser79) (acetyl-CoA carboxylase), total and phospho-eEF2 (1:25,000 dilution) (Thr56), total and phospho-4E-BP1 (1:1,000 dilution) (Thr37/46), and total and phospho-Akt (1:1,000 dilution) (Ser473). All primary antibodies used were purchased from Cell Signaling (Boston, MA), unless stated otherwise. Anti-rabbit IgG, HRP-linked secondary antibody was also purchased from Cell Signaling (1:2,000 dilution) (Boston, MA).

Nucleotide analysis. Adenosine 5'-monophosphate (AMP) and adenosine 5'-triphosphate (ATP) were determined by HPLC analysis as described in a technical note CN-039 by Phenomenex (Torrance, CA). Briefly, frozen gastrocnemius muscle was ground under liquid nitrogen and 50 mg of sample was combined with 1 ml of MeOH in water (25:75) and vortexed. This sample was centrifuged at 10,000 RPM at 4 °C for 5 min and the supernatant extracted. The supernatant was dried and then reconstituted in phosphate buffered saline (PBS). The sample was then passed through a Strata X-AW 33u Polymeric Weak Anion Solid Phase Extraction Sorbent (Phenomenex, Torrance, CA). Ten microliter aliquots of the final muscle extract were injected onto the HPLC using a Phenomenex Gemini 5u C18 110A Column 150 x 4.6 mm with temperature set at 25°C. A single mobile phase was used at a flow rate of 1 ml / min with 25% acetonitrile / 10mM KH2PO4 pH 7.0 (pH was adjusted with potassium hydroxide (KOH) / 5mM tetrabutylammonium chloride (TBAC). Nucleotide peaks were measured by UV detection at wavelength 260 nm. Peaks were identified by comparison with external standards (Sigma-Aldrich, St. Louis, MO).
Statistical analysis. All data were analyzed by SPSS (version 19) statistical software package for Windows. Experiment 1 data was analyzed using a one-way ANOVA, with the postprandial times and dietary treatments as the independent variables. When a significant overall effect was detected, differences among individual means were assessed using LSD post hoc test. Experiment 2 data was analyzed using a two-way ANOVA to assess main effects for gavage treatments (Leu80, CHO1.0, and Sham) and time (20, 45, and 120 min post-gavage) as the independent variables. When a significant main effect was detected, differences among treatment groups were assessed with LSD post-hoc test. The level of significance was set at $P < 0.05$ for all analysis. Data are reported as mean ± S.E.

RESULTS

Experiment 1 evaluated the potential for supplemental Leu or carbohydrates to extend the peak MPS response after a meal, and if there was a dose response. Consistent with previous research (29), experiment 1 showed that MPS increased at 90 min after a 4 g control meal but returned to baseline by 180 min post-meal (Sham). Leu270 or CHO2.6 supplements given at 135 min after the meal maintained MPS at rates similar to peak values at 90 min post-meal (Fig. 5.1a). Supplementation with Leu80 or CHO1.0 maintained MPS above baseline (ie Sham group, $P < 0.05$) but lower than the 90 min post-meal peak value ($P < 0.05$) (Fig. 5.1a).

Phosphorylation of AMPKα (Thr172) and ACC (Ser79) (Table 5.1) and the nucleotide ratio of AMP / ATP (Fig. 5.2a) were measured as indicators of cellular energy status. At 90 min after the control meal, phosphorylation of muscle AMPKα ($P = 0.15$) and ACC ($P < 0.05$) tended to decline reflecting increased energy availability and then increased at 180 min consistent with declining energy availability ($P < 0.05$). The Leu270 supplement reduced phospho-AMPKα and ACC and reduced the AMP / ATP ratio relative to the 90 min time-point. The Leu80 supplement suppressed phospho-ACC ($P < 0.05$) but phospho-AMPKα and AMP / ATP were not different from the 180 min sham control. Both
carbohydrate supplements, CHO2.6 and CHO1.0, maintained reduced phospho-AMPKα relative to the 90 min time-point, with the greatest effect coming from the CHO2.6 supplement. Similar results were seen for both carbohydrate supplements for phospho-ACC and AMP / ATP.

Phosphorylation of eEF2 (Thr56) was measured as a biomarker of translation elongation (Table 5.1). After the meal, eEF2 phosphorylation tended to decrease from 0 to 90 min ($P = 0.15$) consistent with stimulation of MPS, but increased at 180 min post-meal (Sham control) ($P < 0.05$), suggesting inhibition of elongation. The Leu270 supplement reduced eEF2 phosphorylation at 180 min relative to Sham control ($P < 0.05$), while the Leu80 supplement elicited an intermediate response that was not statistically different from with Sham or 90 min peak values. Both CHO2.6 and CHO1.0 supplements reduced eEF2 phosphorylation at 180 min relative to the Sham control ($P < 0.05$).

Phosphorylation of 4E-BP1 (Thr37/46) was measured as a biomarker of translation initiation and a specific indicator of mammalian target of rapamycin (mTORC1) signaling (Table 5.1). Consistent with previous findings (29), relative phosphorylation of 4E-BP1 (Thr37/46) in muscle increased at 90 min ($P < 0.05$) and remained elevated at 180 min (sham control) compared with food deprived rats (time 0) ($P < 0.05$) with no additional activation after either the Leu or CHO treatments.

Plasma insulin rose at 90 min post-meal and returned to food-deprived (time 0) values at 180 min (Sham) (Table 5.1). The CHO2.6 and CHO1.0 supplements restored insulin to 90 min peak insulin values, while the Leu270 and Leu80 supplements had no effect on insulin relative to sham control. Phosphorylation of Akt (Ser473) was measured as an indicator of insulin signaling but no differences in Akt activation were detected among the groups (Table 5.1).

As expected, plasma Leu and EAAs increased 90 min after the meal and remained elevated at 180 min (Table 5.2). The Leu270 supplement elevated plasma Leu; however, the supplement also depressed plasma isoleucine and valine below sham values, with no effect on other amino acids relative to sham
The Leu80 supplement elevated plasma Leu, isoleucine, and valine above sham control, with no effect on other amino acids relative to sham. Lastly, both carbohydrate supplements had no effect on plasma amino acids relative to sham.

Experiment 2 evaluated effects of Leu80 and CHO1.0 supplements on MPS, energy status, and signaling outcomes at multiple time-points post-gavage. Both Leu80 and CHO1.0 treatments maintained MPS above sham values (ie. 180 min sham control) at 20 and 45 min post-gavage (two-way ANOVA $P < 0.05$ main effects for treatment and time, with no interaction $P = 0.16$) (Fig. 5.1b) but MPS declined to baseline values by 120 min post-gavage (Fig. 5.1b).

The CHO1.0 treatment tended to suppress phospho-AMPKα ($P = 0.13$) (Table 5.3) and AMP / ATP ($P = 0.09$) (Fig. 5.2b) relative to Sham control by 20 min post gavage, but significantly reduced phospho-AMPKα (two-way ANOVA $P < 0.05$ main effect for time, with a significant interaction), phospho-ACC (two-way ANOVA $P < 0.05$ main effect for treatment), and AMP / ATP (two-way ANOVA $P < 0.05$ main effect for treatment) by 45 min post-gavage. All of these values were similar to Sham controls at 120 min.

The Leu80 supplement suppressed phospho-AMPKα ($P < 0.05$), AMP / ATP ($P < 0.05$), and tended to decrease phospho-ACC ($P = 0.12$) relative to sham control at 20 min post-gavage ($P < 0.05$); however, phospho-AMPKα, and AMP / ATP returned to sham levels at 45 and 120 min post-gavage for the Leu80 treatment. Phospho-ACC remained depressed 45 min post-gavage for the Leu80 treatment relative to sham control ($P < 0.05$) and returned to sham values 120 min post-gavage consistent with a temporal sequence for energy signaling.

Phosphorylation of eEF2 (Thr56) was lower at 20 and 45 min post-gavage after the CHO1.0 treatment relative to sham, but rose to sham values 120 min post-gavage (two-way ANOVA $P < 0.05$ main effect for treatment) (Table 5.3). While the Leu80 treatment transiently decreased phospho-eEF2 at 20 min post-gavage relative to sham control but phosphorylation increased by 45 min.

There was a trend for the CHO1.0 supplement to increase phosphorylation of 4E-BP1 (Thr37/46) relative to the Leu80 treatment and sham.
control (two-way ANOVA $P < 0.05$ main effect for treatment) however, post hoc analysis did not detect significant differences among treatments, within time-points (Table 5.3).

Plasma insulin was increased by the CHO1.0 supplement 45 and 120 min post-gavage, relative to Sham control (two-way ANOVA $P < 0.05$ main effect for treatment) (Table 5.3). While the Leu80 supplement had no effect on plasma insulin relative to Sham control. No differences were found for phosphorylation of Akt (Ser473) (Table 5.3).

As anticipated, the Leu80 supplement increased plasma BCAA (Leu, isoleucine, and valine) above sham control at 20 and 45 min post-gavage, but returned to sham control values at 120 min post-gavage (two-way ANOVA $P < 0.05$ main effects for treatment and time, with a significant interaction). While the CHO1.0 supplement had no effect on plasma BCAA relative to sham control (Table 5.4). Plasma lysine and methionine peaked 20 min post-gavage (two-way ANOVA $P < 0.05$ main effect for time), but there were no group differences.

There was an overall main effect for plasma threonine to be decreased by the Leu80 treatment, relative to the CHO1.0 treatment and Sham control (two-way ANOVA $P < 0.05$ main effects for treatment and time, with no interaction); however, post hoc analysis did not detect differences among treatments, within time-points (Table 5.4).

**DISCUSSION**

Consistent with previous findings (29), this study demonstrates that Leu or carbohydrate supplements provided ~2 h after a meal can extend the postprandial anabolic period of MPS (Fig. 5.1). Further, the incongruity between MPS and mTORC1 signaling reported in both humans (7) and rats (25) ~3 h after a meal is associated with activation of AMPK and reduced translation elongation activity (Table 5.1 and Table 5.3) suggesting that the duration of the anabolic period after a meal relates, at least in part, to muscle energy status. The current research extends previous findings by further characterizing dose and time-dependent responses to Leu or carbohydrate supplements.
MPS peaked 90 min after the meal and declined by 180 min (Fig. 5.1a). While supplementation with Leu270 or CHO2.6 at 135 min after the meal increased MPS at 180 min above Sham controls and equal to the control group at 90 min (Fig. 5.1a). Supplementation with Leu80 or CHO1.0 at 135 min post-meal increased MPS at 180 min above Sham rats; however, MPS was still lower than the 90 min peak value (Fig. 5.1a). Lastly, the ability of Leu80 and CHO1.0 to extend post-prandial MPS occurred 20-45 min after administration, but returned to sham values by 120 min post gavage (Fig. 5.1b).

Results showed an inverse association with measures of energy status (phospho-AMPKα, ACC, and AMP / ATP) and MPS. Leu and carbohydrates are both energy substrates in skeletal muscle but would be expected to have different impacts on cellular energy and signaling. Yet, this research demonstrated that both substrates were effective at increasing energy and extending post-prandial MPS. When considering that CHO2.63 and CHO1.0 supplements provided ~10-15 and 5-6 times more energy than Leu270 and Leu80 supplements, respectively, it is surprising that similar energetic responses (phospho-AMPKα, ACC, and AMP / ATP) were found in skeletal muscle. These findings suggest that the BCAA are a more efficient source of energy for maintaining MPS than carbohydrates. Indeed, while carbohydrates are used as an energy source in tissues and cells throughout the body, the BCAA have unique and preferential utilization in skeletal muscles (24, 26).

Catabolism of most amino acids occurs in the liver facilitating disposal of the amino-nitrogen group in the urea cycle. However, the liver lacks the branched-chain aminotransferase (BCAT) enzyme required to initiate BCAA degradation. After a meal, amino acid degradation accelerates in the liver and enterocytes, blunting post-prandial swings in amino acid concentrations. Nearly 99% of glutamine, 75% of threonine and 50% of phenylalanine are lost to degradation or protein synthesis on the first pass through the visceral bed (12, 21). However, absence of the BCAT in liver results in over 70% of Leu and the other two BCAA, valine and isoleucine, reaching systemic circulation. This is a striking metabolic difference for these amino acids which becomes even more
remarkable with the realization that the three BCAA account for over 20% of total dietary protein.

Increased BCAA oxidation is triggered by transamination of Leu to ketoisocaproate (KIC), mediated by BCAT. Increased KIC levels in skeletal muscle have been shown to increase oxidation of all three BCAA (Leu, isoleucine, and valine) by allosteric activation of branched-chain α-keto-acid dehydrogenase (BCKDH) – which is responsible for the non-reversible oxidation of all three BCAA into their respective keto acids (1, 14). While all three keto-acids of Leu, isoleucine, and valine can increase BCKDH activity, research shows that KIC is a much stronger stimulus (1). In support of the hypothesis that Leu provided ATP to muscle by increasing BCAA oxidation, Leu270 reduced isoleucine and valine concentrations by ~50%, while other plasma EAAs remained unchanged relative to sham control (Table 5.2).

Beyond increased BCAA oxidation in skeletal muscle, Leu can also provide energy by providing substrate for synthesis of glutamine and alanine, thereby facilitating the glucose-alanine cycle (2, 19). Previous research has also demonstrated that higher protein diets increase the “flux” of glucose into skeletal muscle, without increasing plasma glucose concentrations, suggesting that protein makes muscle more efficient at "re-cycling" existing glucose (8). The collective result of Leu metabolism in skeletal muscle is increased TCA cycle intermediates (anaplerosis) (28), increased ATP production (11), and increased MPS (3).

The ability of the Leu supplements to decrease AMPKα phosphorylation (Table 5.1) is consistent with the work of Du et al. (11), who showed that Leu treatment in C2C12 myoblast cells resulted in a 36% decrease in the AMP / ATP ratio, a 28% reduction in phospho-AMPKα, and a 43% reduction in AMPK activity.

One apparent deviation between the association of energy status and MPS, was that the while the Leu80 supplement increased MPS for 45 min, it was only able to depress phospho-AMPKα, and AMP / ATP for 20 min. However, phospho-ACC remained depressed 45 min after the Leu80 gavage, suggesting
that AMPK activity was not peaked, yet; perhaps, explaining why MPS still remained elevated 45 min after the Leu80 supplement (Fig. 5.1a), in spite of elevated AMPK at that time-point. Corresponding with the drop in MPS, Phospho-ACC rose to sham values 120 min after the Leu80 gavage, indicating that AMPK had reached its peak activity.

It is interesting that the Leu80 supplement, which contained a balance BCAA formula, was not as effective as Leu270 at decreasing phospho-AMPK (Table 5.1). While all three keto-acids of Leu, isoleucine, and valine can increase BCKDH activity, research shows that the Leu metabolite KIC is the strongest stimulus (1). Thus, in the present experiment, Leu270 likely served as a greater stimulus for BCAA oxidation, and therefore, ATP production, than the more balanced Leu80 formula. This dose effect of Leu supplements to improve cellular energy could also explain why cellular energy decreased 180 min post meal, despite the fact that plasma BCAA were approximately 50% higher than baseline values (Table 5.2). Collectively, these findings suggest that robust increases in plasma BCAA are required to maximally stimulate these energetic pathways, and provide adequate energy to maintain MPS (Fig. 5.3).

As with previous findings (29), phosphorylation of eEF2 was associated with MPS responses, indicating that translation elongation becomes limiting in the postprandial state. Proud and colleagues (9, 20) have shown that inhibition of eEF2 and protein synthesis in response to mild energy deficits, is associated with activation of AMPK, which stimulates the regulatory kinase eEF2K to subsequently phosphorylate (inhibit) eEF2. Likewise, the results of this experiment suggests that temporal changes in skeletal muscle energy state following meal feeding influence the period of muscle anabolism, particularly through inhibition of translation elongation. Further, the energy status of muscle and period of muscle anabolism can be enhanced by post-meal nutrition supplements.

MPS declined 180 min post-meal (sham) in spite of continued elevations in phosphorylation of mTORC1 signaling target 4E-BP1 (Table 5.1) and plasma amino acids (Table 5.2). Likewise, insulin does not appear to be a critical factor
in extending MPS (Table 5.1 and Table 5.3). Plasma insulin rose at 90 min post-meal and returned to food-deprived (time 0) values at 180 min (Sham) (Table 5.1). The CHO2.6 and CHO1.0 supplements restored insulin to 90 min peak insulin values. Furthermore, plasma insulin was increased by the CHO1.0 supplement 45 and 120 min post-gavage, relative to Sham control (Table 5.3). However, Leu270 and Leu80 supplements had no effect on insulin relative to sham control, at any time-point (Table 5.3).

It is noteworthy that both Leu270 and Leu80 supplements had no effect on plasma insulin, as previous research has shown that Leu increases plasma insulin when given in the fasted state (5). In the present scenario, where Leu is administrated post meal, it is possible that the test meal depleted insulin vesicle storage and that Leu has no direct effect on protein kinase B (PKB) stimulation of insulin synthesis. Regardless, it appears that Leu270 and Leu80 supplements given after a meal are able to extend protein synthesis without significantly elevating plasma insulin.

Previous research has demonstrated that administering carbohydrates in the fasted state is ineffective at elevating MPS (4). Thus, the finding in this experiment that consumption of carbohydrates alone is sufficient to increase MPS is a novelty. The likely explanation for this discrepancy is that in these experiments, carbohydrates are administered shortly after consumption of a mixed meal. In this scenario, amino acids are still elevated and translation initiation has already been triggered. The “system” is simply limited by energy; thus, administration of an energy source (carbohydrates) appears to be satisfactory to prolong MPS.

In summary, intake of a Leu or carbohydrate supplement ~2 h after consumption of a complete meal can extend of the postprandial rise in MPS, and this response is associated with enhanced energy and translation elongation activity. However, the novel finding of this research is that the extent to which Leu or carbohydrate supplements are able to enhance energy status and prolong the period of muscle anabolism, are dose and time-dependent. In practical terms, this would suggest that a “snack” between meals would extend the postprandial
anabolic period. But this effect is transient, and another meal must be consumed shortly thereafter to prolong muscle anabolism. Improved understanding of factors that regulate the duration of MPS may have implications for maintenance of lean tissue during weight loss or aging, for treatment of patients suffering trauma or prolonged bed rest, as well as for acceleration of muscle development in athletes. As such, the roles of eEF2 and AMPK in regulation of postprandial MPS warrant further investigation.
REFERENCES


Fig. 5.1. Postprandial changes for muscle protein synthesis (MPS). Values are mean ± S.E.M, n = 5-8 per group. Rats were fed a 4 g breakfast meal containing 20% whey protein, and received supplements at 135 min post-meal. Panel A (experiment 1). Rats were gavaged with water (Sham), 270 mg of leucine (Leu270), 80 mg leucine, 40 mg isoleucine, and 40 mg valine (Leu80), 2.63 g carbohydrates (CHO2.6), or 1 g carbohydrates (CHO1.0). Means not sharing a common letter are different (One-way ANOVA P < 0.05). Panel B (experiment 2). Rats were gavaged with water (Sham), 80 mg leucine, 40 mg isoleucine, and 40 mg valine (Leu80), or 1 g carbohydrates (CHO1.0). Means not sharing a common letter are different within time-points (P < 0.05). Two-way ANOVA P < 0.05 indicated significant main effects for treatment and time, with no interaction (P = 0.160).
Fig. 5.2. Postprandial changes for nucleotide ratios of AMP / ATP (adenosine 5'-monophosphate / adenosine 5'-triphosphate). Values are mean ± S.E.M, n = 5-8 per group. Rats were fed a 4 g breakfast meal containing 20% whey protein, and gavaged 135 min post-meal. Panel A (experiment 1). Rats were gavaged with water (Sham), 270 mg of leucine (Leu270), 80 mg leucine, 40 mg isoleucine, and 40 mg valine (Leu80), 2.63 g carbohydrates (CHO2.6), or 1 g carbohydrates (CHO1.0). Means not sharing a common letter are different (One-way ANOVA P <0.05). Panel B (experiment 2). Rats were gavaged with water (Sham), 80 mg leucine, 40 mg isoleucine, and 40 mg valine (Leu80), or 1 g carbohydrates (CHO1.0). Means not sharing a common letter are different within time-points (P <0.05). Two-way ANOVA P <0.05 indicated significant main effect for treatment.
Leucine (Leu) metabolism and regulation of muscle protein synthesis (MPS). Leu is able to pass through the liver without being oxidized due to the lack of BCAT (branched-chain amino-acid transferase). In muscle, Leu activates the mTOR (mammalian target of rapamycin) pathway, resulting in improved translation initiation and MPS. The Leu metabolite KIC (keto-isocaproate) induces allosteric activation of BCKDH (branched-chain keto acid dehydrogenase), resulting in increased oxidation of the BCAA (branched-chain amino-acids). Increased energy from BCAA oxidation (or a decrease in AMP:ATP ratio, adenosine 5'-monophosphate / adenosine 5'-triphosphate) reduces activity of AMPK (AMP activated protein kinase) attenuating AMPK induced activation of eEF2K (eukaryotic elongation factor 2 kinase), the regulatory kinase of eEF2. TCA (tricarboxylic acid cycle); ETC (electron transport chain).
Table 5.1. Post-meal signaling\(^1\) and plasma insulin\(^2\) results for experiment one\(^3\)

<table>
<thead>
<tr>
<th>Time(^4)</th>
<th>AMPK</th>
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<th>eEF2</th>
<th>4E-BP1</th>
<th>Akt</th>
<th>Insulin</th>
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<td>51±3.3(^ab)</td>
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\(^1\)Western blot results indicate relative changes in phosphorylation of adenosine monophosphate-activated protein kinase alpha (phospho-AMPK\(\alpha\) (Thr172) / total AMPK\(\alpha\)), acetyl-CoA carboxylase (phospho-ACC (Ser79) / total ACC), eukaryotic elongation factor 2 (phospho-eEF2 (Thr56) / total eEF2), eIF4E binding protein-1 (phospho-4E-BP1 (Thr37/46) / total 4E-BP1), and phospho-Akt (Ser473) / total Akt.

\(^2\)Plasma insulin expressed as pmol/L.

\(^3\)Values are means ± S.E.M, \(n = 5-8\) per group. Means not sharing a common letter are different (One-way ANOVA \(P < 0.05\)).

\(^4\)Time expressed as min post-meal.

\(^5\)Control animals were fed a 4 g breakfast meal containing 20% whey protein, and administered a sham (water) gavage.

\(^6\)Rats were gavaged with 270 mg of leucine (Leu270).

\(^7\)Rats were gavaged with 80 mg leucine, 40 mg isoleucine, and 40 mg valine (Leu80).

\(^8\)Rats were gavaged with 2.63 g carbohydrates (CHO2.6).

\(^9\)Rats were gavaged with 1 g carbohydrates (CHO1.0)
Table 5.2. *Post-meal plasma amino acid responses for experiment one* ¹¿

<table>
<thead>
<tr>
<th>Time³</th>
<th>Leu</th>
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<th>Lys</th>
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<td>122±13 ²</td>
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<td>256±10 ²bc</td>
<td>797±33 ²ab</td>
<td>98±7 ²ab</td>
<td>780±28 ²ab</td>
</tr>
</tbody>
</table>

Sham⁴ | 1375±113²111±11 ²b | 172±16 ² | 925±68 ²a | 88±5 ²b | 743±47 ²b |

Leu²⁷⁰ ⁵ | 523±51 ²b | 301±28 ² | 591±40 ²b | 767±34 ²b | 73±5 ²b | 640±71 ²b |

Leu⁸⁰ ⁶ | 224±36 ²cd | 128±18 ² | 200±37 ² | 623±117 ² | 92±11 ² | 737±51 ² |

CHO².6 ⁷ | 188±22 ²d | 154±20 ² | 231±27 ² | 779±80 ²b | 88±13 ²b | 747±42 ² |

CHO¹.0 ⁸ | 220±9 ² | 163±7 ² | 256±10 ² | 797±33 ² | 98±7 ² | 780±28 ² |

¹Plasma amino acids expressed as µmol/L.
²Values are means ± S.E.M, n = 5-8 per group. Means not sharing a common letter are different (One-way ANOVA P < 0.05).
³Time expressed as min post-meal.
⁴Control animals were fed a 4 g breakfast meal containing 20% whey protein, and administered a sham (water) gavage.
⁵Rats were gavaged with 270 mg of leucine (Leu²⁷⁰).
⁶Rats were gavaged with 80 mg leucine, 40 mg isoleucine, and 40 mg valine (Leu⁸⁰).
⁷Rats were gavaged with 2.63 g carbohydrates (CHO².6).
⁸Rats were gavaged with 1 g carbohydrates (CHO¹.0).
Table 5.3. Post-gavage signaling\(^1\) and plasma insulin\(^2\) results for experiment two\(^3\)

<table>
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<th>eEF2(^-)</th>
<th>4E-BP1(^-)</th>
<th>Akt</th>
<th>Insulin(^-)</th>
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**Leu80\(^6\)**

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**CHO1.0\(^7\)**

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\(^1\)Western blot results indicate relative changes in phosphorylation of adenosine monophosphate-activated protein kinase alpha (phospho-AMPK\(\alpha\) (Thr172) / total AMPK\(\alpha\)), acetyl-CoA carboxylase (phospho-ACC (Ser79) / total ACC), eukaryotic elongation factor 2 (phospho-eEF2 (Thr56) / total eEF2), eIF4E binding protein-1 (phospho-4E-BP1 (Thr37/46) / total 4E-BP1), and phospho-Akt (Ser473) / total Akt.  
\(^2\)Plasma insulin expressed as pmol/L.  
\(^3\)Values are means ± S.E.M, \(n=5-8\) per group. Means not sharing a common letter are different within time-points (\(P<0.05\)). Two-way ANOVA \(P<0.05\) indicated significant main effects for treatment (\(\ast\)), time (\(#\)), and significant interaction (\(\#\)).  
\(^4\)Time expressed as min post-gavage.  
\(^5\)Control animals were fed a 4 g breakfast meal containing 20% whey protein, and administered a sham (water) gavage at indicated times.  
\(^6\)Rats were gavaged with 80 mg leucine, 40 mg isoleucine, and 40 mg valine (Leu80).  
\(^7\)Rats were gavaged with 1 g carbohydrates (CHO1.0).
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<tr>
<td>45</td>
<td>188±22⁶</td>
<td>153±20⁶</td>
<td>230±26⁶</td>
<td>778±80</td>
<td>88±13</td>
<td>746±42</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>170±7⁶</td>
<td>134±8</td>
<td>205±10⁶</td>
<td>783±50</td>
<td>84±7</td>
<td>708±76</td>
<td></td>
</tr>
</tbody>
</table>

¹Plasma amino acids expressed as µmol/L.
²Values are means ± S.E.M, n = 5-8 per group. Means not sharing a common letter are different within time-points (P < 0.05). Two-way ANOVA P < 0.05 indicated significant main effects for treatment (‘), time (‘), and significant interaction (‘).
³Time expressed as min post-gavage.
⁴Control animals were fed a 4 g breakfast meal containing 20% whey protein, and administered a sham (water) gavage at indicated times.
⁵Rats were gavaged with 80 mg leucine, 40 mg isoleucine, and 40 mg valine (Leu80).
⁶Rats were gavaged with 1 g carbohydrates (CHO1.0).
CHAPTER VI:
SUMMARY AND SPECULATIONS

DIETARY LEUCINE DETERMINES MUSCLE ANABOLISM

Studies have demonstrated that leucine (Leu) is a potent stimulator of mammalian target of rapamycin (mTORC1) kinase activity, resulting in improved translation initiation and muscle protein synthesis (MPS) (2, 8). However, most of this research has been done with purified Leu formulas, or in vitro experiments, and is untested with whole foods using in vivo models. Accordingly, this dissertation sought to better define the role of dietary Leu in regulation of skeletal muscle protein balance using whole food feeding models.

Research from this dissertation demonstrates that the peak activation of MPS following a small breakfast meal is proportional to the Leu content of a meal and its ability to elevate plasma Leu. Furthermore, consuming higher Leu diets results in greater increases in muscle mass over ~3 months of meal feeding. Healthy adult rats were fed three complete meals daily with identical macronutrient compositions (16% of energy from protein, 54% carbohydrates, 30% fats), which only differed in the source of protein (Chapter II). Four proteins (wheat gluten, soy isolate, egg white, and whey protein isolate) were selected to provide a range of Leu concentrations from 6.8% to 10.9% (w/w). Results demonstrated that consumption of animal proteins with higher Leu contents (whey and egg) increased MPS, mTORC1 signaling, and plasma Leu; while consumption of plant proteins (wheat and soy) were unable to increase MPS or plasma Leu. Moreover, supplementing wheat with Leu to match the Leu content of the meal to that of whey also equalized rates of MPS. To my knowledge, this is the first study to show the significance of Leu as a factor of protein quality within meals containing complete macronutrient and fiber mixtures. These findings are consistent with the concept of a “minimum threshold” for Leu to stimulate mTOR and MPS (2).

Additional research from this dissertation showed minimal changes in muscle protein degradation (MPD) pathways, regardless of the Leu content of
the diets. Suggesting that MPS is the primary determinant of protein turnover in healthy adult rat feeding models.

Previous MPD research has focused on regulation of the ATP-dependent ubiquitin-proteosome-proteolytic pathway, which is the primary mode of intracellular protein degradation (6). While there is evidence that the ubiquitin-proteosome pathway may be a major factor in protein turnover during aging and in states of muscle wasting (1), nearly all research on the effects of protein or amino acid supplementation on protein turnover has focused on MPS. Therefore, this research extends the current understanding of how nutrition impacts MPD. Chapter III examined ubiquitin-proteosome outcomes after feeding rats a range of proteins varying in Leu density (whey, egg, and wheat) over a period of 11 weeks. The primary finding was that total PSMA2 (a portion of the outer ring of the 20S proteasome) significantly decreased in rats fed egg and whey compared to animals fed wheat, suggesting decreased total proteasome degradative capacity in rats consuming higher Leu diets. However, there was no effect of diet treatment on any of the regulatory ubiquitin ligases. These results suggest that protein quality within accepted dietary ranges has minimal regulatory effects on MPD in healthy adult rats. Thus, the changes in muscle mass observed overtime in this dissertation are likely due to the differences in MPS, rather than MPD.

After an overnight fast, muscle is in a state of catabolism, and nutrients must be consumed to halt this response. Indeed, research has demonstrated that the breakfast meal is an important determinant of metabolism and satiety outcomes for the remainder of the day, with higher protein, lower carbohydrate breakfast meals improving these outcomes (5, 7). However, Americans typically consume small breakfast meals (20% of daily energy) with a mixture of macronutrients that are limited in protein quantity (16% of energy from protein, 54% carbohydrates, 30% fats). Accordingly, the research presented in this dissertation was designed to mimic the typical American breakfast meal, to determine how the quality of proteins determines muscle anabolism and muscle mass outcomes.
Results from this dissertation showed that the Leu density of proteins is an important determinant of protein quality, and determines the post meal anabolic response. Thus, when consuming a small breakfast meal with limited protein quantity, the quality of the protein (Leu content) becomes absolutely critical. In practical terms, this means that consuming plant proteins such as a bagel and orange juice that are limited in both protein quantity and quality for breakfast is ineffective at halting muscle catabolism. Rather, protein sources that are rich in Leu, such as animal proteins including milk and eggs, must be consumed to begin muscle repair.

These results call into question current recommendations of protein quality. For the proteins used in this study only the wheat glutens lysine content was below the National Research Council (NRC) requirement for the adult rat (10). This specific requirement was corrected by supplementing the wheat gluten diet with lysine to the level of the concentration in whey protein. All other EAA exceeded NRC guidelines. The unique regulatory role of Leu may in part explain the inconsistencies in comparison of optimal needs for Leu compared with current measures of minimum protein requirements for nitrogen balance or indispensable amino acid oxidation (IAAO) measurements. These standard measurements for protein requirement interpret postprandial increases in plasma amino acids as inefficient and excessive protein intake. While increased amino acid oxidation may reflect reduced efficiency of nitrogen retention of dietary protein and may provide an accurate representation of minimum requirements for an EAA such as lysine with no known metabolic signaling or regulatory roles, the extrapolation that protein intakes above the minimum amount for short-term nitrogen balance are physiologically meaningless is not consistent with the regulatory role of Leu in MPS.

**CELLULAR ENERGY DETERMINES MUSCLE ANABOLISM**

Chapter IV and V investigated the postprandial duration of MPS and whether a second dose of nutritional supplements could extend the duration of peak MPS, as well as the mechanisms underlying these responses. Results
showed that MPS increased 90 min post meal, but returned to fasted values 180 min post meal, in spite of maintained elevations in mTORC1 signaling and plasma Leu. Rather, an inverse association was found between MPS, markers of translation elongation (eukaryotic elongation factor 2 (eEF2)), and cellular energy (AMP/ATP, adenosine monophosphate-activated protein kinase (AMPK), and acetyl CoA carboxylase (ACC)). However, supplementation with a second dose of Leu or carbohydrates 135 min post meal was able to prevent the drop in cellular energy, translation elongation, and MPS in a dose and time-dependent manner.

Leu and carbohydrates are both energy substrates in skeletal muscle but would be expected to have different impacts on cellular energy and signaling. Yet, this research demonstrated that both substrates were effective at increasing energy and extending post-prandial MPS. When considering that the carbohydrate supplements provided ~10-15 and 5-6 times more energy than the Leu supplements, it is surprising that similar energetic responses (phospho-AMPKα, ACC, and AMP / ATP) were found in skeletal muscle. These findings suggest that the BCAA are a more efficient source of energy for maintaining MPS than carbohydrates. Indeed, while carbohydrates are used as an energy source in tissues and cells throughout the body, previous research has shown that the BCAA have unique and preferential utilization in skeletal muscles (24, 26).

To my knowledge, this is the first demonstration of limitations in translation elongation and cellular energy determining MPS outcomes, in a physiological feeding model. Muscle has a number of energy substrates to protect ATP levels (creatine-phosphate, lipids, glycogen, etc.); however, this research suggests that the metabolically costly process of MPS can exceed the muscles ability to maintain cellular energy.

Proud and colleagues have shown inhibition of eEF2 and protein synthesis in response to mild energy deficits is associated with activation of AMPK, which stimulates the regulatory kinase eukaryotic elongation factor 2 kinase (eEF2K) to subsequently phosphorylate (inhibit) eEF2 (9). The ability of AMPK to respond to changes in AMP/ATP – with AMP stimulating AMPK, and
ATP inhibiting it – allows this kinase to be an effective cellular energy sensor (4). Collectively, these results suggest that energy is a likely determinate of the duration of postprandial MPS, and that eEF2 is the regulatory step responding to energy status.

Considering that protein turnover is a metabolically costly process, accounting for approximately one quarter of resting energy expenditure (11), it is logical that a cell would suppress protein synthesis activity during an energy deficit to preserve critical functions. Furthermore, when considering that translation elongation accounts for the majority (> 99%) of the energy used for polypeptide assembly (9), it appears logical that the cell would regulate translation elongation to protect energy status. These findings may also explain why consuming dietary proteins results in a greater thermic effect than either carbohydrates or fats, via stimulation of MPS (3).

In practical terms, these results suggest that a “snack” between meals would extend the postprandial anabolic period. But this effect is transient, and another meal must be consumed shortly thereafter to prolong muscle anabolism. Improved understanding of factors that regulate the duration of MPS may have implications for maintenance of lean tissue during weight loss or aging, for treatment of patients suffering trauma or prolonged bed rest, as well as for acceleration of muscle development in athletes.

CONCLUSION

In conclusion, this dissertation has shown that the Leu content of dietary proteins is an important determinate of protein quality, and determines the post meal anabolic response and chronic changes in muscle mass. MPD outcomes were less responsive than MPS to the Leu content of dietary proteins. Thus, the changes in muscle mass observed overtime are likely due to the differences in MPS, rather than MPD. Results showed that Leu works as both an anabolic signal via stimulation of the mTORC1 pathway, and as an energy substrate for muscle, thereby providing ATP, and inhibiting AMPK, to support the metabolically costly process of MPS.
This dissertation demonstrates the importance of protein quality at the breakfast meal, where small meals with limited protein are typically consumed. In practical terms, this means that consuming plant proteins such as a bagel and orange juice for breakfast is ineffective at halting muscle catabolism. Rather, high quality animal proteins such as milk and eggs must be consumed to begin muscle repair.

Protein synthesis is an oscillating process, increasing after consumption of food, and decreasing in fasting conditions. This research demonstrated that MPS declined 180 min after a complete meal, in spite of maintained elevations in mTORC1 signaling and plasma Leu. Rather, an inverse association was found between markers of translation elongation, cellular energy, and MPS outcomes. However, supplementation with a second dose of Leu or carbohydrates 135 min post meal was able to prevent the drop in cellular energy, translation elongation, and MPS in a dose and time-dependent manner. In practical terms, these results suggest that a “snack” between meals would extend the postprandial anabolic period. But this effect is transient, and another meal must be consumed shortly thereafter to prolong muscle anabolism.

This research has implications for maintenance of muscle health and functionality. Future studies should seek to extend these results in differing populations such as in muscle wasting models, the elderly, and with exercise.
REFERENCES