DIETARY PROTEIN AFFECTS BODY COMPOSITION, ENERGY PARTITIONING, AND CELLULAR ENERGETICS

BY

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DISSERTATION

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ABSTRACT

Skeletal muscle health is a key determinant of functional capacity in adults. It allows for locomotion and activities of daily living, and as a metabolically active tissue, it contributes substantially to energy expenditure. The amount of skeletal muscle is determined by the balance between anabolism (protein synthesis) and catabolism (protein degradation). As such, it is worthwhile to investigate any nutritional strategies that can maintain or promote net addition of muscle tissue, and can prevent age-related declines in skeletal muscle mass.

The importance of skeletal muscle is magnified in conditions of weight loss. A multitude of animal and clinical studies have shown that isocaloric substitution of dietary protein for carbohydrate within the Dietary Reference Intakes (DRI) promotes favorable outcomes during weight loss, including increased retention of lean body mass and improved insulin sensitivity. These results are typically attributed to satiety effects or to diet induced thermogenesis, which fail to consider the unique ability of dietary protein to affect metabolic signaling. The amount of carbohydrate and protein in a meal has a profound effect on the post-prandial metabolic activity of liver, skeletal muscle, and adipose. A study was conducted to evaluate the post-prandial responses of these tissues to a range of diets varied in the dietary carbohydrate / protein ratio. Consumption of a diet with a high carbohydrate / protein ratio induced a metabolic shift characterized by post-prandial hyperinsulinemia, reduced insulin sensitivity in skeletal muscle at the expense of adipose, an increased capacity for de novo lipogenesis, and activation of the integrated stress response in liver, all of which were associated with a decline in body composition quality. These data highlight an important, underappreciated aspect of dietary protein to favorably impact whole-body metabolic activity and body composition.

Numerous research groups have demonstrated the acute effects of dietary protein and / or leucine on muscle protein synthesis (MPS). However, it was unclear if this acute response would translate into net accretion of muscle mass and changes in body composition when applied over time. Rats were fed 3 meals per day of isocaloric,

isonitrogenous diets that varied in protein source with different leucine enrichments (WHEAT, 6.8% leucine; or WHEY, 10.9% leucine) for 11 weeks. At both 2 and 11 weeks, MPS was acutely stimulated by consumption of a standard 4 gram meal in WHEY but not WHEAT. Changes in MPS resulted in increased muscle mass, reduced fat mass, and greater diet-induced energy expenditure. At 11 weeks, gastrocnemius muscle weight was greater while body fat % was lower in WHEY-consuming animals. Interestingly, WHEAT-consuming animals had larger epididymal fat pad mass and greater total fat mass despite identical caloric input. Additionally, WHEAT preferentially partitioned dietary energy to fat while WHEY partitioned energy to lean tissue. These outcomes were associated with an induction of genes in skeletal muscle involved in mitochondrial biogenesis (e.g., PGC-1α, Tfam, cytochrome B) by WHEY. These data suggest that repeated consumption of leucine-rich meals stimulates MPS and presents the cell with dynamic and increased energy demands, provoking a response in the molecular machinery responsible for oxidation and energy production.

It has been well-established that the essential, branched-chain amino acid leucine has a unique ability to promote anabolism in skeletal muscle. Leucine stimulates mammalian target of rapamycin (mTOR), which subsequently activates a signaling cascade that increases muscle protein synthesis (MPS) through initiation factors such as p70 S6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein1 (4E-BP1). Our research group has shown that the leucine content of a meal predicts the acute response of MPS based on its ability to elevate the post-prandial plasma leucine concentration. Once stimulated, and yet despite continued elevations in plasma leucine and associated translation initiation factors (e.g., p70S6K and 4E-BP1), MPS returns to basal levels ~3 hours after a meal. However, administration of additional nutrients in the form of carbohydrate, leucine, or both ~2 hours after a meal was able to extend the elevation of MPS. This effect was associated with decreases in translation elongation activity as well as with increases in the AMP / ATP ratio and in the activity of 5' adenosine monophosphate-activated kinase (AMPK), a key cellular energy sensor. These data suggest that leucine-stimulated increases in MPS result in an acute cellular

energy deficit that in turn is likely responsible for the subsequent restoration of MPS activity to basal levels.

A study was designed to determine if AMPK directly responds to the MPS-induced deficit in cellular energy by inhibiting translation initiation, thusly inhibiting subsequent MPS activity. Rapamycin was administered to inhibit the leucine-induced stimulation of MPS, and separately compound C was administered to inhibit AMPK activity. Rapamycin appropriately inhibited mTOR signaling (Akt) and presumably MPS activity; however, compound C did not inhibit AMPK as anticipated. Further analysis of the experiment was abandoned due to failure of the key inhibitor compound C.

In total, this research demonstrates that consumption of a whole meal enriched in leucine acutely impacts cellular energy by activating protein synthesis in muscle, which compounded over time, promotes a repartitioning of energy and favorable body composition. These findings suggest that a substantial proportion of the observed benefits from increased protein consumption during meals is due to metabolic signaling effects and the associated energy costs, which are not appropriately captured in the traditional "diet as substrate" paradigm.

DEDICATION

This dissertation is dedicated to my grandfather, James Kord,

who cultivated my love for science at an early age.

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LIST OF ABBREVIATIONS

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

ACC – acetyl-CoA carboxylase

AICAR – 5-aminoimidazole-4-carboxamide 1-beta-d-ribonucleoside

AIN-93G – American Institute of Rodent Diets for growth, pregnancy, and lactation

AIN-93M – American Institute of Rodent Diets for adult maintenance

AMDR – acceptable macronutrient distribution ratio

AMP – adenosine monophosphate

AMPK – 5' adenosine monophosphate-activated protein kinase

ANOVA – analysis of variance

ATF3 – activating transcription factor 3

ATP – adenosine triphophate

BCAA – branched chain amino acid

BCAT – branched chain amino acid transferase

BMI – body mass index

cDNA – complementary deoxyribonucleic acid

CHO – carbohydrate

DASH – Dietary Approaches to Stop Hypertension

DIT – diet induced thermogenesis

DRI – Dietary Reference Intakes

DXA – dual-energy x-ray absorptiometry

EDTA – ethylene diamine tetraacetic acid

eEF2 – eukaryotic elongation factor 2

EGTA – ethylene glycol tetraacetic acid

ERRα – estrogen-related receptor alpha

FAS – fatty acid synthetase

FSR – fractional synthetic rate

GLUT4 – glucose transporter 4

GSK-3β – glycogen synthase kinase 3 beta

GTP – guanosine triphosphate

HbA1c – glycosylated hemoglobin

HCLP – high carbohydrate, low protein

HEPES – hydroxyethyl piperazineethanesulfonic acid

IRS-1 – insulin receptor substrate 1

kDa – kilodalton

LC – leucine + carbohydrate

LCHP – low carbohydrate, high protein

LEU – leucine

LSD – Fisher's least significant difference

MCMP – moderate carbohydrate, moderate protein

MPD – muscle protein degradation

MPS – muscle protein synthesis

mRNA – messenger ribonucleic acid

mtDNA – mitochondrial deoxyribonucleic acid

mTOR – mammalian target of rapamycin

NRF1/2 – nuclear respiratory factors 1 & 2

p70S6K – 70-kilodalton ribosomal protein S6 kinase

PGC-1α – peroxisome proliferator activated receptor γ coactivator 1α

PI3K – phosphoinositide 3-kinase

RIA -- radioimmunoassay

RNA – ribonucleic acid

RT-PCR – reverse transcriptase polymerase chain reaction

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – standard error of the mean

SOD1 – super oxide dismutase 1

SREBP-1c – sterol regulatory element-binding protein 1c

Tfam – transcription factor A, mitochondrial

USDA – United States Department of Agriculture

PREFACE

The aim of this dissertation is to better understand the positive influence of dietary protein and the essential amino acid leucine on body composition and energy partitioning. Studies in animals and humans indicate that moderate increases in dietary protein at the expense of carbohydrate support skeletal muscle health and favorably impact overall body composition. However, when characterizing this response, potentially unique metabolic signaling effects of dietary protein have not been accounted for. Consequently, Chapter II will evaluate the metabolic response of skeletal muscle, adipose, and liver, as well as the resultant body compositions to diets varied in the carbohydrate / protein ratio.

One aspect of dietary protein that is well understood is the ability of the component branched chain amino acid (BCAA) leucine to acutely activate mammalian target of rapamycin (mTOR), which subsequently stimulates muscle protein synthesis (MPS). It is yet unclear if repeated leucine-mediated stimulations of MPS ultimately result in net gain of lean muscle mass. Chapter III will explore this problem by chronically feeding discrete meals that are identical in total energy and macronutrient distribution, but vary in leucine density. Additionally, this chapter will address the potential for dietary leucine to influence tissues other than skeletal muscle as well as whole-body energy partitioning.

Separately, while the mechanisms directing the acute response of MPS activity to dietary protein have been well-characterized, the factors that determine the duration of the response are not well known. Therefore, Chapter IV will probe the ability of supplemental nutrients to influence the duration of protein-induced MPS activity when provided after a meal. Further consideration will be given to the energetic costs of MPS activity and the possibility that it accounts for a substantial proportion of diet induced thermogenesis (DIT).

Chapter V highlights the major findings of this dissertation, addresses certain limitations, and offers direction for future research in light of results herein.

CHAPTER I:
REVIEW OF LITERATURE

INTRODUCTION

This dissertation aims to improve understanding of the role of dietary protein and the branched-chain amino acid leucine on body composition, energy partitioning, and cellular energetics. The purpose of <u>Chapter I</u> is to present a brief review of the pertinent literature on these topics.

SKELETAL MUSCLE AND BODY COMPOSITION

Skeletal muscle contributes substantially to lean mass and overall body composition, as it makes up ~42% of the average adult male's body mass and ~36% of the average adult female's body mass (45). Muscle is essential for critical activities such as locomotion, food gathering, and reproduction in all mammals, as well as for discretionary human activities such as physical labor and athletics (83). Properly functioning muscle contributes to quality-of-life across all life stages (73), and it is particularly important for functional capacity in aging or elderly populations. Healthy skeletal muscle allows for performance of activities of daily living including cooking, shopping, use of transportation, house chores, and money management, which generally reflect the competence for independent living (38).

In healthy young adults, muscle protein synthesis and degradation are equally balanced, resulting in no net change in skeletal muscle mass (77). However, adults beyond the age of 50 (and perhaps earlier) suffer from an age-related loss of skeletal muscle mass at the rate of 1-2% per year (63). In 1989, this process was coined with the term sarcopenia – which comes from the Greek language – meaning "loss of flesh" (59). It has numerous contributors to its pathogenesis, including impaired muscle anabolism (17), lesser influence of growth and steroid hormones (13), as well as physical inactivity and poor nutrition (11). Sarcopenia limits the ability to complete activities-of-daily living in otherwise healthy older men and women (31), and low muscle mass is predictive of mortality in the elderly (70+ years) (10).

A potentially devastating condition involves an increase in fat mass concomitant with age-related muscle loss, which is known as sarcopenic obesity (60). Obesity in combination with low muscle strength leads to increased risk for poor mobility and functional decline, resulting in greater disability and health burden (75). Individuals with sarcopenic obesity are 2-3 times more likely to suffer disability of activities-of-daily living than those with lean sarcopenia (67). Recent research suggests that the inflammatory state associated with obesity can accelerate muscle catabolism, resulting in a vicious cycle that exacerbates sarcopenia, coupled with reduced physical activity and additional weight gain in the form of adipose (62). Consequently, there is clear need for approaches to attempt to prevent or combat the onset of sarcopenia and sarcopenic obesity.

SKELETAL MUSCLE AND ENERGY EXPENDITURE

The processes that consume energy in the human body can be divided into three components: basal metabolic rate, physical activity, and dietary thermogenesis. Basal metabolic rate, which includes all metabolic and transport activities required for bodily function at rest, makes up ~70% of total energy consumption. Physical activity consumes ~20% of total expended energy, while thermogenesis makes up the remaining ~10%. Skeletal muscle metabolism accounts for ~20% of the basal metabolic rate, or ~14% of total resting energy expenditure in the average adult (46). Energy expenditure varies among people independent of body size, and individuals with a "slow metabolism" are at an apparent greater risk for weight gain, which may be indicative of reduced energy expenditure by muscle. Early research demonstrated that approximately half of the variance in resting metabolic rate among individuals can be explained by resting metabolism in skeletal muscle (85). It is evident that modulation of skeletal muscle mass or its energy consumption can have an impact on total energy expenditure independent of physical activity or diet. Indeed, pharmacologic targeting of muscular energy expenditure has been proposed as a method to combat obesity (12).

Dulloo et al. have recently elaborated on a premise regarding skeletal muscle's fundamental contribution to whole-body thermogenesis and energy expenditure (19-21).

They have collected data indicating that, in conditions where skeletal muscle is appropriately sensitive to insulin, there exists a futile energy-dissipating cycle between de novo lipogenesis and lipid oxidation in muscle. In this scheme, synthesis of one molecule of palmitate from acetyl-CoA followed by its subsequent oxidation back to acetyl-CoA would consume 14 ATP molecules; therefore, repeated cycling of substrate in this manner would increase thermogenesis as well as provide a sink for glucose or lipid disposal in muscle. However, in conditions of insulin insensitivity and / or increased adiposity, this cycle is interrupted, thereby reducing muscular and whole-body energy expenditure. These studies suggest that optimizing skeletal muscle mass would not only increase overall thermogenesis, but could potentially draw energy substrate away from adipose storage and improve body composition.

DIETARY PROTEIN AND BODY COMPOSITION

It is widely held that total energy intake is the most important factor in determining body composition and weight management (56), but the independent influence of macronutrient proportions of the diet is controversial (22). Nonetheless, numerous clinical trials have provided evidence that increased dietary protein can positively influence body composition, promote retention of lean mass, and accelerate loss of fat mass during weight loss (37, 39, 41, 42, 44, 47). These effects are often attributed to the higher satiating effect of protein (28) or to the stronger influence that protein exerts on dietary thermogenesis (32). However, it has become evident that macronutrients not only provide energy, but that certain components can function as signaling molecules to regulate numerous physiological processes (81), including feeding behavior, efficiency of metabolism, overall energy balance (84), and in particular for the purposes of this dissertation – protein turnover in muscle.

LEUCINE AND MUSCLE PROTEIN SYNTHESIS

Leucine is one of the three branched-chain amino acids (with isoleucine and valine), and as an essential amino acid it must be consumed in the diet for good health. It is unique among all dietary amino acids in its ability to directly stimulate muscle

protein synthesis (MPS), and it seems to act as an indicator that there is an adequate supply of nutrients for protein synthesis to occur (24). Leucine increases activity of mammalian target of rapamycin (mTOR), a serine / threonine kinase, which activates downstream translation initiation factors such as 70-kDa ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E- binding protein1 (4E-BP1) (4). Activation of these initiation factors promotes assembly of the eiF4F complex, which is required for RNA binding to the ribosome and allows for protein synthesis to proceed (3).

Lynch et al. found that drinking water supplemented with leucine resulted in greater protein synthetic activity in skeletal muscle, adipose, and liver after 12 d in rats (43). Similarly, deletion of branched-chain amino acid transferase (BCAT), the first enzyme in leucine catabolism, resulted in a 10-fold elevation in the plasma leucine concentration of mice (64). This was associated with a significant elevation of protein synthetic activity in skeletal muscle, heart, kidney, and adipose. Although the capacity for leucine to stimulate protein synthesis in multiple tissues has been demonstrated, much attention has been directed toward skeletal muscle and the potential to affect muscle mass. It is evident that leucine administration activates MPS in both animals (35) and in humans (18). However, the acute response lasts < 3 hrs (5), and the chronic response to leucine-induced MPS is not known. As previously mentioned, numerous reports have shown that elevated dietary protein, which is generally enriched in leucine, promotes maintenance or addition of lean tissue over time. This effect is frequently attributed in part to increases in MPS, but the causal link has not been appropriately established.

DIETARY LEUCINE AND BODY COMPOSITION

The aforementioned BCAT null mice had 10-fold higher plasma leucine than wild-type mice, and exhibited a comparatively lean phenotype. This was associated with elevated energy expenditure, which the authors attributed to a futile cycle of protein turnover (64); however, these outcomes are not generalizable among normal physiologies.

Investigators have supplemented drinking water with leucine to evaluate potential effects on body composition. Mice that consumed twice the amount of leucine as control mice gained less fat and body weight when fed a high-fat diet for 15 wks (84), while rats provided supplemental leucine lost significantly more body fat when submitted to 50% caloric restriction for 6 wks (16). However, another research group found that supplemental leucine or mixed branched-chain amino acids (BCAAs) had no effect on the body composition of mice fed a high-fat diet for 14 wks. These studies are limited by the use of free access to leucine-containing drinking water, which introduces variance in dosing. As such, there is a need for dosing strategies that standardize leucine consumption in order to appropriately assess body compositional effects in animal models.

Few studies have directly investigated the effect of dietary leucine on body composition in humans. Competitive wrestlers undergoing moderate caloric restriction that consumed a BCAA-supplemented diet lost the most body weight and body fat over 19 d compared to those consuming a high-protein or low-protein diet (49). More broadly, a cross-sectional epidemiological study among 4400+ healthy, middle-aged adults from China, Japan, the United Kingdom, and the United States found that higher BCAA intake is associated with a lower prevalence of overweight and obesity (55). Future population-level analyses will be useful to better encapsulate the impact of dietary leucine on body composition.

Conversely, two observational studies have noted elevations of plasma BCAAs (including leucine) in obese conditions. Plasma leucine was restored from significantly elevated levels in obese individuals following gastric bypass surgery, which coincided with an increased capacity for leucine oxidation by adipose tissue (65). The second study evaluated metabolomic profiles of lean vs. obese individuals utilizing principal component analysis, which identified a correlation between insulin resistance and BCAA-associated metabolites (50). The authors suggested that in the context of overnutrition, dietary BCAAs contribute to insulin resistance; however, this hypothesis cannot be differentiated from the potential for BCAA metabolism to be deranged in a pathological state of obesity, particularly in light of a recent study suggesting that

adipose tissue is more active in BCAA oxidation than was previously believed (30). At present, there is not enough data to form a consensus view on how dietary leucine impacts body composition.

DIETARY PROTEIN AND THERMOGENESIS

Post-meal dietary thermogenesis, also known as diet-induced thermogenesis (DIT) or the thermic effect of food, consumes ~10% of total energy expenditure at rest. This obligatory component of energy expenditure is required for the proper absorption, digestion, and conversion of nutrients into their respective storage forms, such as glycogen or triglyceride (32), and ~90% of DIT occurs within 6 hrs of meal consumption (57). It has long been known that among macronutrients, protein contributes the greatest thermic effect of food (23); estimates based on analysis of metabolic pathways indicate that 20-30% of energy contained within a typical protein is lost to thermogenesis, while 5-10% and 0-3% of energy are lost from carbohydrate and fat, respectively (1). Once a dietary protein is digested into its component amino acids, the amino group must be removed by transamination or deamination, and disposed of via the urea cycle, which accounts for much of the additional energetic cost of catabolism.

There are numerous studies demonstrating that protein exerts the strongest influence on DIT among macronutrients. For example, post-prandial thermogenesis after consumption of a high-protein, low-fat meal was greater after consumption of a high-carbohydrate, low-fat meal in young, healthy women (33) and men (58). Post-prandial lipid oxidation and associated energy expenditure following a 30% protein meal was greater independent of BMI than that following a 15% protein meal (36), and similarly, consumption in a sealed metabolic chamber of a 30% protein diet resulted in greater DIT and 24 hr energy expenditure than consumption of a 10% protein diet (40). Conversely, reduced postprandial energy expenditure and increased exogenous fat oxidation was observed in young women after ingestion of test meals with low protein content compared to adequate protein content (52).

A regression analysis of 19 studies that compared the magnitude of DIT to the macronutrient composition of the test food found that a 1% increase in the protein

fraction resulted in a DIT increase of 0.2%, while no such influence was identified from the carbohydrate or fat components (78). Westerterp and others (79, 81) suggest that protein-induced thermogenesis can positively influence body temperature and lead to feelings of satiety independent of hormonal influence, thereby contributing to protein's greater satiating effect relative to fat and carbohydrate. This satiating effect is believed to contribute to protein's positive effect on energy balance (80).

A meta-analysis of studies comparing DIT among lean and obese adult populations found that in 22 of 29 trials, DIT was significantly lower in the obese, which the authors associated with the level of insulin resistance (15). This is in agreement with the previously mentioned work by Dulloo suggesting that insulin insensitivity contributes to a decline in thermogenesis by muscle (19). Therefore, it would be advantageous to utilize strategies that improve insulin sensitivity of muscle in combination with optimizing skeletal muscle mass in order to maximize the thermogenic capacity of muscle and to increase resting energy expenditure.

Recent research has evaluated the impact of different dietary proteins on thermogenesis. Tan et al. failed to find differential effects on 8 hr energy expenditure or lipid oxidation following consumption of isocaloric, 30% protein meals containing either meat, dairy, or soy protein (70), while another study found that consumption of isocaloric, isonitrogenous meals containing animal (pork) protein resulted in a 2% greater 24 hr energy expenditure when compared to meals containing vegetable (soy) protein (48). Most recently, energy expenditure in healthy adults was measured 5.5 hrs after consumption of an isocaloric meal containing 50% protein from soy, casein, or whey; the thermic effect from the whey protein was significantly greater than that of the soy or casein proteins despite equal protein content (2).

There is mounting evidence that protein's positive impact on thermogenesis may be due to its ability to stimulate protein synthesis. Giordano et al. evaluated infusions of increasing whole amino acid mixtures on DIT, finding that the absolute rise in energy expenditure was dose-responsive, and that it was positively associated with the ability of the amino acids to acutely stimulate protein synthesis (25). A similar study in rats found that intravenous administration of an amino acid mixture stimulated protein

synthesis in muscle and elevated core body temperature (82). These findings suggest that DIT may be determined in part by dietary protein's ability to stimulate protein synthesis, and furthermore by the ability of a particular protein source to stimulate protein synthesis. However, the mechanisms by which this might occur require further investigation.

LEUCINE AND MITOCHONDRIA

The mitochondrion is the primary energy-producing organelle of most tissues and cell types (76). Skeletal muscle has the ability to adapt to a variety of environmental stresses, such as cold exposure, caloric restriction, and exercise by modulating the size, number, and mass of mitochondria in a process known as mitochondrial biogenesis (74). In all cases, this process is mediated by the activity of peroxisome proliferator activated receptor γ coactivator 1α (PGC-1α), a "master co-regulator" that influences gene expression patterns involving multiple aspects of metabolism (54).

Cultured C2C12 myocytes incubated with leucine increased lipid oxidation in vitro (68), and subsequent leucine treatment at a concentration meant to mimic that of a high protein meal increased oxygen consumption and stimulated mitochondrial biogenesis, including induction of PGC-1α (69). In support of this finding, two animal studies indicate that dietary leucine can affect mitochondria in vivo. Guo et al. provided 1.5% leucine in drinking water to diabetic mice for 8 mos, which induced expression of a series of genes involved in effecting mitochondrial biogenesis (27). These animals expended 10% more energy than control animals, despite equal physical activity. D'antona et al. added BCAAs to drinking water of middle-aged mice at 1.5 mg / g body weight per day for the duration of the lifespan, which stimulated both the transcription and expression of numerous genes of mitochondrial biogenesis, and increased endurance capacity to treadmill running (14). These findings indicate that leucine may have a direct or indirect effect on muscle's ability to oxidize substrate and produce ATP, and it is reasonable to suspect that this is in response to an additional demand for energy to support leucine-induced protein synthetic activity.

Consumption of a meal supplemented with leucine was able to stimulate mitochondrial protein synthesis in addition to sarcoplasmic protein synthesis in both young and old rats (26), indicating that leucine-induced MPS activity has the potential to affect mitochondrial content. Mitochondrial function declines in the elderly due to cumulative oxidative damage of mitochondrial DNA (mtDNA), resulting in a reduction of the mitochondrial ATP production rate (66). These changes are likely determinants of the reduced oxidative capacity and functional decrement in aging muscle. An 8 hr infusion of BCAAs increased mitochondrial ATP production rate in young, but not elderly individuals (71). The authors did not measure MPS, but speculated its activity was perhaps related to the enhancement of mitochondrial ATP production in the young group. Elderly people become less sensitive to leucine-induced MPS activity than young adults (51), which suggests that Tatpati et al. may have observed an effect on mitochondrial ATP production in the elderly if a larger dose of leucine was utilized. It is similarly possible that the age-related decline in mitochondrial function is affected by a reduced ability of muscle to respond to leucine. Further research is required to determine how leucine can positively impact mitochondria in skeletal muscle.

CELLULAR ENERGETICS AND PROTEIN SYNTHESIS

In order for a cell to function properly, it requires a constant supply of biological energy to meet the demands of its most vital processes. This requirement is mediated by the actions of 5' adenosine monophosphate-activated protein kinase (AMPK), which is the key sensor of cellular energy in all tissues (34). AMPK is sensitive to the concentration of adenosine monophosphate (AMP), which is liberated from hydrolysis of adenosine triphosphate (ATP) during energy consumption. AMPK acts as a signaling kinase to coordinate the activity of anabolic (energy-consuming) and catabolic (energy-liberating) processes. In conditions where cellular energy availability is low, the AMP / ATP ratio becomes elevated, increasing AMPK activity and resulting in a shift towards catabolism. When a surfeit of cellular energy exists, the AMP / ATP ratio is relatively low and anabolism is favored (29).

Protein synthesis is an anabolic process that requires substantial amounts of energy; it has been estimated that 30-50% of all cellular energy is consumed by the process (7). The majority of this energy is utilized for translation elongation, which is the process of adding single amino acids to a nascent polypeptide. Four high-energy bonds (2 from ATP and 2 from GTP) are hydrolyzed for each amino acid added to a peptide chain (8). It is therefore logical that translation elongation activity would be dampened when cellular energy is limited. Translation elongation is regulated primarily via eukaryotic elongation factor 2 (eEF2), which inhibits elongation when it is in a phosphorylated state (8). eEF2 is phosphorylated by eEF2 kinase (9) and perhaps other signaling kinases, but its regulation is not well understood. Nonetheless, AMPK has been shown to activate eEF2 kinase and therefore activate eEF2 in vitro (7). This provides a potential link by which energy depletion could limit protein synthesis.

The AMP analogue 5-aminoimidazole-4-carboxamide 1-beta-d-ribonucleoside (AICAR) is frequently used to artificially stimulate AMPK activity. The first indication that AMPK could regulate protein synthesis came from injection of rats with AICAR, which resulted in a 50% decrement in MPS activity (6). This was elaborated upon by studies demonstrating that AICAR (and thus elevated AMPK activity) sufficiently abolished induction of MPS by leucine (53) and electrical stimulation (72). Most recently, incubation of rat muscle fibers with leucine reduced AMPK activity and stimulated MPS, while co-incubation with AICAR prevented the effect (61). These data suggest that AMPK functions to suppress MPS activity, thereby conserving ATP during conditions of energy deficit. Nonetheless, the threshold of the response and its timing in response to nutrients needs to be further elucidated.

SUMMARY

Improvements in the amount and quality of skeletal muscle provide clear benefits throughout adult life. Clinical trials indicate that elevated dietary protein promotes favorable body composition, including retention or accretion of muscle mass and reduction of fat mass. This effect may in part be attributed to leucine's effect on protein synthesis in muscle, although the association has not been appropriately established.

Sufficient dietary leucine acutely stimulates MPS, but the chronic effects of dietary leucine on body composition and energy are not well characterized. Additional investigation into the energetic demands of protein turnover in muscle may provide insight into the means by which increased dietary protein promotes favorable health outcomes.

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CHAPTER II:
METABOLIC RESPONSES TO DIETS REPRESENTING THE UPPER AND LOWER ENDS OF THE ACCEPTABLE MACRONUTRIENT DISTRIBUTION RANGE BECOME LESS FAVORABLE AS THE DIETARY CARBOHYDRATE / PROTEIN RATIO INCREASES

INTRODUCTION

The current Dietary Guidelines for Americans state that management of a healthy weight is a key factor in reducing risk for Type 2 Diabetes in adults (34), and further they endorse the USDA Food Patterns and the Dietary Approaches to Stop Hypertension (DASH) Eating Plan as model dietary patterns to accomplish this goal. These guidelines place emphasis on nutrient-rich food selections in part by limiting inclusion of added sugars, although they do not restrict amounts of non-sugar carbohydrate sources, per se. With regard to macronutrient composition, these patterns are constrained only to comply with the Acceptable Macronutrient Distribution Range (AMDR) of the Dietary Reference Intakes (DRI). The AMDR permits consumption of 45-65% of calories as carbohydrate, 10-35% of calories as protein, and 20-35% of calories as fat (33), which allows for myriad combinations of macronutrients within a dietary pattern that are compliant to the recommendation. However, with regards to weight management, the Dietary Guidelines state that the relative macronutrient proportions of a dietary pattern are not a critical factor. This approach disregards the potential for metabolic responses to vary among different diet patterns.

Recommendations from the USDA Food Patterns and the DASH diet typically result in consumption of carbohydrate and protein at the high and low ends of their AMDRs, respectively (9), which results in a dietary carbohydrate / protein ratio > 3. Conversely, diets with a reduced carbohydrate / protein ratio (≤ 1.5) have gained increasing approval, and have been shown in clinical trials to improve glycemic regulation, insulin homeostasis, body composition, and cardiovascular disease risk during weight loss (11, 22, 23, 25). There has been limited research focused on how metabolic responses to meals differ across the AMDR for macronutrient compositions.

Typical management for individuals with Type 2 Diabetes focuses on control of fasted glucose and chronic biomarkers such as glycated hemoglobin (HbA1c), but therapies targeting these measures may not positively affect mortality or incidence of co-morbidities (1). However, there is increasing evidence that post-prandial glucose and / or comparison to fasted glucose may better encapsulate the metabolic fitness of and individual and lead to better prevention and treatment strategies. A recent investigation suggests that post-prandial glucose may be a better index of glycemic regulation than fasting plasma glucose (13). Post-prandial hyperinsulinemia can induce or exacerbate post-prandial hypertriglyceridemia (17), and limiting the magnitude of post-prandial glucose excursions can reduce the risk of cardiovascular disease (12, 16). As such, dietary strategies that moderate carbohydrate intakes merit evaluation, since dietary carbohydrate is the major determinant of post-prandial glucose (2).

Separately, increases in post-prandial plasma amino acids (particularly the branched chain amino acid leucine) predict the degree of protein synthesis stimulation in skeletal muscle following consumption of a meal (27). This information supports the assertion that the macronutrient content of a diet influences the post-prandial plasma milieu and the subsequent physiologic responses. Previous work from our laboratory has shown that reduction of the dietary carbohydrate / protein ratio within the acceptable ranges of the DRI attenuates post-prandial glucose and insulin levels, reduces PI3K / Akt activation and subsequent glucose uptake by adipose (5), and increases insulin sensitivity by skeletal muscle at the expense of adipose (10) in rats. As such, there appears to be different metabolic regulation of glycemia in response to meals that differ in carbohydrate (and protein) content.

The purpose of this study was to further evaluate the metabolic and whole body responses to diets across three different carbohydrate / protein ratios in normal, non-diabetic adult rats. Previous results have shown that elevated carbohydrate diets exert a stronger influence on peripheral insulin signaling (3). It is of interest to determine if the related elevations in post-prandial glucose and insulin have any differential impact on metabolism in liver, as well as skeletal muscle and adipose. We hypothesized that as the carbohydrate / protein ratio increased, post-prandial hyperinsulinemia would dictate

a greater reliance on peripheral insulin signaling, and that enhanced glucose uptake by adipose would result in greater adiposity. The present study utilized a meal feeding model in normal, non-diabetic adult rats to determine chronic responses (25 d) to diets with different carbohydrate / protein ratios in order to evaluate responses in post-prandial insulin, peripheral insulin signaling, capacity for lipid synthesis, hepatic integrated stress response, and body composition.

METHODS

Animals. Forty-eight male Sprague-Dawley rats (Harlan-Teklad, Indianapolis, IN) with a mean body weight of 166 ± 7 g were housed individually in stainless steel wire-bottomed cages and maintained at 24°C with a 12 h reverse light cycle (light: 1900-0700 h) and free access to water. The animal protocol was approved by the University of Illinois Institutional Animal Care and Use Committee.

Rats were trained to consume three meals each day to mimic human eating patterns (4). The meal pattern consisted of a 3 g morning meal consumed between 0700-0720 h followed by free access to food between 1300-1400h and 1800-1900 h. The meals consisted of a powdered, modified AIN-93M diet (Table 2.1). The rats adapted to this pattern within two days, resuming normal food intake and growth.

On day three, rats were randomized to consume one of three isocaloric treatment diets (n = 16 per group): high carbohydrate-low protein (HCLP; 58% of energy from carbohydrate, 12% from protein, and 30% from fat), moderate carbohydrate-moderate protein (MCMP; 40% of energy from carbohydrate, 30% from protein, and 30% from fat), or low carbohydrate-high protein (LCHP; 25% energy from carbohydrate, 45% from protein, and 30% from fat). The carbohydrate / protein ratios of the diets were 4.8, 1.33, and 0.6, respectively. The net metabolizable glucose load of the diets was determined by adding ½ the sucrose weight to the maltodextrin weight. Diet compositions are presented in Table 2.1. Rats consumed their respective treatment diets for 25 d, while measurements of food intake and body weight recorded for the duration of the study.

On day twenty-five, rats from each treatment group (n = 8) were deprived from food for 12 h and then either euthanized by decapitation (food deprived) or fed their

normal 3 g morning meal. Ninety minutes after consumption of the meal, rats were euthanized by decapitation and blood and tissues were harvested. Trunk blood was collected in EDTA tubes to prevent clotting and immediately immersed in ice. Blood was centrifuged at 1800 x g for 20 min. at 4°C and plasma was separated and stored at -80°C until analysis. Tissues, including gastrocnemius, plantaris, and soleus muscle, renal and epididymal fat pads, and liver, were weighed prior to being snap frozen in liquid nitrogen and stored at -80°C for later analyses.

Plasma measurements. Plasma insulin concentrations were determined using a commercial RIA kit for rat insulin (Linco Research, MO). Plasma glucose was determined by glucose oxidase method (ThermoTrace, Victoria AUS).

Tissue homogenization. Gastrocnemius and epididymal fat were pulverized in liquid nitrogen prior to homogenization. 100 mg of muscle tissue was homogenized in 2 mL ice cold lysis buffer (20 mM HEPES ph 7.2, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1x HALT Protease Inhibitor (Pierce, Rockford, IL), 1x HALT Phosphatase Inhibitor (sodium fluoride, sodium orthovanadate, sodium pyrophosphate; Pierce, Rockford, IL), and 2mM phenylmethylsulfonyl fluoride. 200 mg adipose tissue was homogenized in 0.7 mL ice cold lysis buffer (40 mM HEPES pH 7.4, 4 mM EGTA, 100 mM NaF, 50 mM KCl, 0.4 mM EDTA, 100 mM B-glycerophosphate, 0.4g CHAPS/ 100 mL buffer, 1% Triton X-100, 1x HALT Protease Inhibitor, 1x HALT Phosphatase Inhibitor, and 2mM phenylmethylsulfonyl fluoride. Samples were centrifuged at 10,000 x g for 10 min at 4°C to concentrate protein in the supernatant. Total protein was determined using the bicinchoninic acid method.

Akt and p70S6 kinase activity. Whole cell lysates were separated by SDS-PAGE, and then transferred to nitrocellulose membranes for immunoblotting. After blocking in SuperBlock T20 (Pierce), membranes were incubated with either rabbit polyclonal Akt antibody (Cell Signaling, Danvers, MA) diluted 1:1000, rabbit polyclonal Ser[473] phospho-Akt antibody (Cell Signaling) diluted 1:1000, or affinity purified rabbit p70S6K antibody (Bethyl Laboratories, Montgomery, TX) diluted 1:1500 overnight at 4°C. Antigen was detected by enhanced chemiluminesence (GE, Piscataway, NJ), and exposure /

quantification was conducted using a Kodak Imaging Station 440 CF (Eastman Kodak Co., Rochester, NY).

Gene expression. Total RNA was isolated from ~50 mg frozen liver tissue or ~100mg frozen epididymal fat using TRI Reagent® (Sigma-Aldrich, St. Louis, MO). First-strand cDNA was synthesized using random hexamers on a thermal cycler. RT-PCR was run with 7.5 ng of cDNA template and oligonucleotide primers designed to amplify fatty acid synthetase or sterol regulatory element-binding protein 1c (SREBP-1c) in liver or adipose. All primers were obtained from IDT (Coralville, IA). The relative abundance of each gene was normalized to expression of the L7a control gene.

Statistical analysis. Data are expressed as mean ± SEM. All variables were evaluated for normality and homogeneity of variance by Levene's test. Percent change comparisons were made by subtracting the mean fasted value from post-prandial values. 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. Correlations were assessed by Pearson's linear regression. The level of significance was set at $P < 0.05$. All analyses were performed using SPSS Version 15.0 (Chicago, IL).

RESULTS

Food intake. Mean daily food intake was lower for the LCHP group (13.96 ± 0.26 g / d) than the MCMP (15.22 ± 0.21 g / d; $p < 0.01$) and HCLP (15.61 ± 0.33 g / d; $p < 0.01$) groups.

Body and tissue weights. After 25 d of feeding, the HCLP group had the lowest body weight, while the MCMP group was the heaviest, and the body weight of the LCHP group was intermediate between the two (Table 2.2). The LCHP and MCMP groups had heavier livers than the HCLP group. The HCLP had lower total (gastrocnemius + plantaris + soleus) muscle weight than the MCMP and LCHP groups, and the HCLP had greater total (renal + epididymal) adipose weight than the MCMP and LCHP groups.

Plasma measurements. Fasted plasma glucose and insulin were not different among treatments (Table 2.3). When expressed as a percent change from the fasted condition, the post-prandial insulin rise was greater in MCMP and HCLP than in LCHP (Figure 2.1).

Liver measurements. There were no effects of diet treatment on fasted hepatic FAS expression (Table 2.3), while post-prandial hepatic FAS was higher in MCMP and HCLP than LCHP (Figure 2.2). Overall, there were positive correlations of hepatic FAS expression with the carbohydrate content of the diet ($r = 0.377$; $p = 0.011$) and with the glucose load of the diet ($r = 0.339$; $p = 0.023$). In the fasted condition, hepatic SREBP1c expression was higher in the LCHP group than in the MCMP and HCLP groups (Table 2.3). When expressed as a percent change from the fasted condition, the increase in hepatic SREBP1c in the HCLP group was significantly greater than that of the LCHP group, while the respective increase in the MCMP group was intermediate (Figure 2.3).

Hepatic ATF3 expression in the fasted state was higher in the HCLP group than in the LCHP and MCMP groups (Table 2.3). Overall, there were positive associations of hepatic ATF3 expression with the carbohydrate content of the diet ($r = 0.380$; $p = 0.009$) and with the glucose load of the diet ($r = 0.373$; $p = 0.011$). Asparagine synthetase expression in liver was not different in the LCHP and MCMP groups in either the fasted or fed condition. However, expression levels in HCLP were ~50 times greater in the fasted state and ~15 times greater in the fed state than in the other two groups (Figure 2.4). Overall, there was a positive association of asparagine synthetase expression with the carbohydrate content of the diet ($r = 0.613$; $p < 0.001$) and with the glucose load of the diet ($r = 0.587$; $p < 0.001$).

Adipose measurements. In the fasted state, adipose expression of FAS in the LCHP group was higher than in the HCLP group, while the expression level of the MCMP group was intermediate between the two (Table 2.3). When expressed as a percent change from the fasted condition, the FAS expression increase in the HCLP group was greater than for the LCHP group, while the respective increase of the MCMP group was intermediate (Figure 2.5). No significant group differences were found in SREBP1c expression in adipose (Table 2.3), although there was trend for a significant

association of adipose SREBP1c expression with the glucose load of the diet ($r = 0.265$; $p = 0.090$).

There was no group treatment effect on Akt activity in adipose (Table 2.3). However, in the fasted condition, p70S6K activity was higher in the HCLP group than in the LCHP group, with the MCMP group intermediate (Table 2.3).

Skeletal muscle measurements. There were no group differences in fasted Akt activity (Table 2.3). However, when expressed as a percent change from the fasted condition, the rise in Akt activity in the LCHP group was significantly different from the decrease in Akt activity in the HCLP group, while the MCMP group was intermediate (Figure 2.6). Overall, there was a trend for a negative association of Akt activity with the carbohydrate content of the diet ($r = -0.268$; $p = 0.063$) and a significant negative association of Akt activity with the glucose content of the diet ($r = -0.315$; $p = 0.027$). There were no effects of diet treatment on p70S6K activity (Table 2.3).

DISCUSSION

The present study utilized a meal feeding protocol in normal, non-diabetic adult rats to determine chronic responses (25 d) to diets with different carbohydrate / protein ratios to evaluate metabolic responses in glycemia, peripheral insulin signaling, capacity for lipid synthesis, and body composition. The primary findings from this research were the post-prandial and metabolic responses to consumption of whole meals were least desirable in the high carbohydrate / low protein condition, including activation of the integrative stress response in liver that is indicative of marginal protein deficiency and increased endogenous fatty acid synthesis.

Use of a discrete meal feeding protocol versus conventional ad libitum feeding was employed to study post-prandial responses and examine metabolic flexibility when the body is displaced from metabolic homeostasis of the fasting period. Previous research using similar treatments has shown that high-carbohydrate diets demand an increased reliance on insulin-mediated glucose disposal (4). In the current study, across a wide range of carbohydrate intakes, plasma glucose was similar in all conditions

reflecting a substantial capacity to maintain glucose homeostasis. However, there were divergent metabolic responses related to achieving glycemic control.

The liver can respond to nutrient excess by converting carbohydrates into lipids via de novo lipogenesis. Physiological levels of carbohydrate and insulin are known to influence fatty acid synthetase (FAS) (32) and SREBP-1c (14), two key genes implicated in lipogenesis by liver and adipose. The capacity for hepatic lipid synthesis was evaluated by the relative expression of FAS and SREBP1c. FAS is responsive to insulin concentrations as well as dietary carbohydrate (21), while SREBP1c can be induced by insulin (28). As dietary carbohydrate (and dietary glucose load) increased, the hepatic capacity for lipid synthesis increased, as post-prandial FAS expression was greater in MCMP and HCLP than in LCHP. The rise in hepatic SREBP1c expression from fasted to fed conditions was greater with increasing carbohydrate and highest in the HCLP group.

While the meal response of FAS and SREBP1c was in proportion to the carbohydrate content of the meal, baseline SREBP1c expression in the fasted condition was highest in LCHP. It has been demonstrated that hepatic SREBP1c can be positively regulated by S6 kinase 1, which is a downstream effector of mammalian target of rapamycin (mTOR) (24). A similar high-protein diet was shown to activate hepatic mTOR (8), so it is possible that hepatic mTOR stimulation lead to increased basal SREBP1c expression. Nonetheless, the post-prandial response of SREBP1c was greater in magnitude with increasing carbohydrate and the corresponding rise in insulin, and hepatic SREBP1c is most responsive to refeeding and insulin levels (18). These results suggest that high dietary carbohydrate exceeds the amount of glucose that can be utilized as energy or stored as glycogen during the absorptive period, which requires shunting of the excess carbohydrate into lipid synthesis.

Similarly, in adipose high dietary carbohydrate resulted in a greater post-prandial elevation in expression of FAS, and there was a trend for SREBP1c expression to be greater with increasing dietary glucose load. This suggests that energy in the form of carbohydrate is increasingly taken up by adipose, which is subsequently utilized as substrate to synthesize lipids for energy storage.

Consistent with the response in the insulin-sensitive hepatic lipogenic gene expression, there was a differential post-prandial insulin effect of the diets. Plasma glucose is elevated in response to a meal, and pancreatic insulin release directs peripheral uptake of glucose to maintain glucose concentrations in the appropriate range. Post-prandial insulin levels were higher in the MCMP and HCHP groups than the LCHP group, and the 288% elevation in post-prandial insulin from the fasted level in LCHP was significantly larger than the 40% elevation in LCHP (with response of MCMP intermediate). This large excursion of insulin in the higher carbohydrate-containing groups indicates an increased reliance on insulin-mediated peripheral uptake of glucose to maintain glycemia. The relatively lower insulin response in the LCHP group suggests a lesser reliance on insulin-mediated glucose disposal, and an increased ability for hepatic glucose regulation (26).

The Dietary Reference Intakes (DRI) allow for consumption of protein as low as 10% of total energy as adequate for good nutrition. However, the HCLP diet provided protein at 12% of total energy and produced an integrated stress response in liver reflecting an early adaptation to amino acid deficiency. The integrated stress response is a pro-survival gene expression program that promotes cell survival (19). A variety of different stressors such as viral infection, hypoxia, or amino acid deprivation can activate the pathway by phosphorylating eukaryotic initiation factor-2 (eIF2), which subsequently depresses cellular protein synthetic activity (29). General Control Nondepressible 2 (GCN2) is one of at least 4 different mammalian kinases that can phosphorylate eIF2, and it does so in response to amino acid deprivation (6). Likewise, conditions of amino acid deficiency induce the transcription factor ATF3 via GCN2, which subsequently induces elevates expression of asparagine synthetase (7). In the present study, hepatic ATF3 expression was elevated in the post-prandial condition by the HCLP diet, and there was an overall association of ATF3 expression with the carbohydrate content and glucose load of the diets. Asparagine synthetase expression was drastically induced in HCLP, while no such changes were seen in either LCHP or MCMP. Although not responsive to dietary carbohydrate, this pathway is activated in

response to amino acid deprivation, which suggests that the 12% protein diet that is largely comprised of carbohydrate may be marginally inadequate in amino acid supply.

Circulating insulin activates a canonical signaling pathway in peripheral tissues, including skeletal muscle and adipose. Insulin interacts with a membrane-bound insulin receptor that autocatalyzes upon ligand binding, which in turn phosphorylates and activates insulin receptor substrate 1 (IRS-1). IRS-1 stimulates phosphoinositide 3-kinase (PI3K) activity, which subsequently activates Akt / protein kinase B, a serine / threonine kinase. Active Akt stimulates glucose transport via GLUT4 translocation, glycogen synthesis via GSK-3β inhibition, and protein synthesis via p70S6 kinase activation (31). In the present study, as dietary carbohydrate intake increased, the post-prandial plasma insulin concentration increased, but the insulin signaling response through Akt was depressed in skeletal muscle, and there was a trend for an inverse correlation between carbohydrate content of the diet and Akt activity reflecting insulin resistance with increasing carbohydrate exposure. These findings support the hypothesis that glucose mediates insulin resistance in muscle, but oppose the assertion that leucine is similarly implicated (30). Contrary to the response found in muscle, insulin signaling in adipose was elevated with increasing carbohydrate, as the HCLP group had the greatest basal p70S6K activity. These data suggest that as dietary carbohydrate increases, the insulin signal that induces skeletal muscle to take up glucose becomes weaker, while the insulin signal that induces adipose to take up glucose becomes stronger. The body composition results support the assertion that increased dietary carbohydrate would enhance the capacity for lipid synthesis and energy storage in adipose, as the HCLP had the largest fat pad weight and the lowest skeletal muscle weight. Conversely, the LCHP group had the smallest fat pad weight and the largest muscle weight. These data suggest that in high carbohydrate conditions, adipose increasingly becomes a depot for dietary carbohydrate, as de novo lipogenesis is stimulated in both liver and adipose.

The treatment diets were designed to vary the carbohydrate / protein ratio, and so when carbohydrate content of the diet was reduced, the protein content was accordingly increased. Dietary protein contributes carbon skeletons in the form of amino

acids for hepatic production of glucose, which contributes to liver-dependent regulation of glucose homeostasis (20). Additionally, dietary protein independently influences insulin signaling in skeletal muscle, without affecting glucose uptake (4). The contribution of the essential amino acid leucine from dietary protein has broad stimulatory effects on skeletal muscle protein synthesis (15), which will addressed in subsequent chapters.

This study evaluated metabolic responses to 25 d of feeding diets with varied carbohydrate / protein ratios. Across all of the diets, the adult rats were able to produce metabolic adaptations to maintain glucose homeostasis; however, the metabolic outcomes were least desirable in the high carbohydrate with low protein condition. The HCLP diet was associated with an enhanced capacity for de novo lipogenesis in both liver and adipose. The integrated stress response was activated liver, indicating at least a marginal deficiency in dietary amino acids. Skeletal muscle became less responsive to insulin while adipose became more responsive, reflecting an apparent increased reliance of insulin-mediated uptake of carbohydrate by adipose. Furthermore, the HCLP diet resulted in smaller skeletal muscle mass and relatively larger adipose mass. When dietary carbohydrate was exchanged for protein, the carbohydrate / protein ratio was reduced, and generally these responses were reversed. The low carbohydrate diet was characterized by enhanced insulin sensitivity in skeletal muscle but not in adipose, a reduced capacity for de novo lipogenesis, and more favorable body composition with larger skeletal muscle mass and reduced adipose mass. In summary, the array of diets with respect to carbohydrate and protein can result in significantly different metabolic outcomes, and these outcomes appear most favorable when the ratio of carbohydrate / protein ratio is reduced. The long-term implications of these metabolic adaptations to life-long health and disease risk need to be determined.

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Post-prandial Insulin
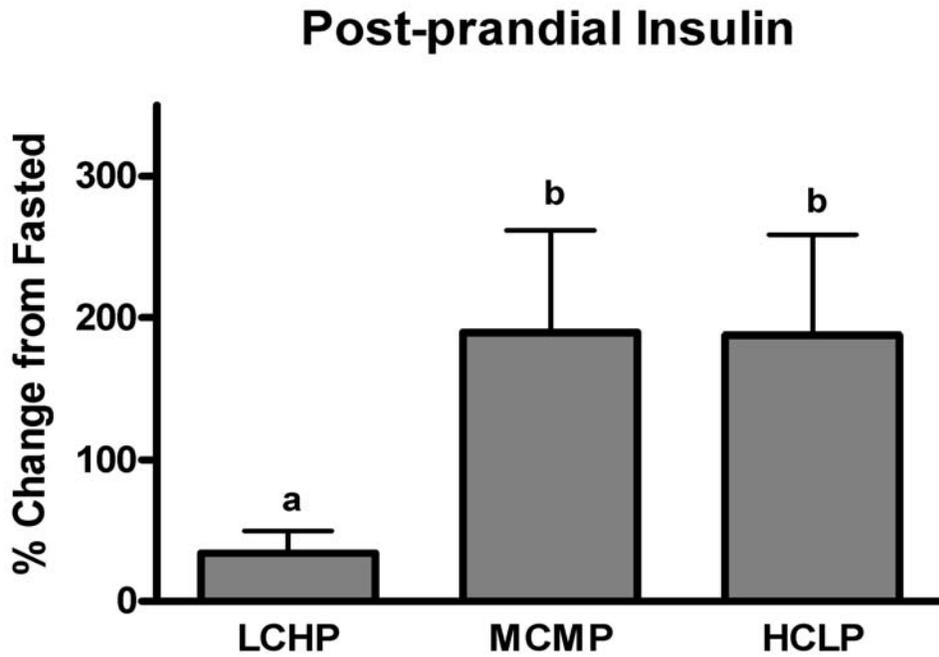
Figure 2.1. Percent change of post-prandial plasma insulin from the fasted state in rats fed diets of different carbohydrate / protein ratios for 25 d. Data are means ± SEM; n = 6-8. Labeled means without a common letter differ, P < 0.05.
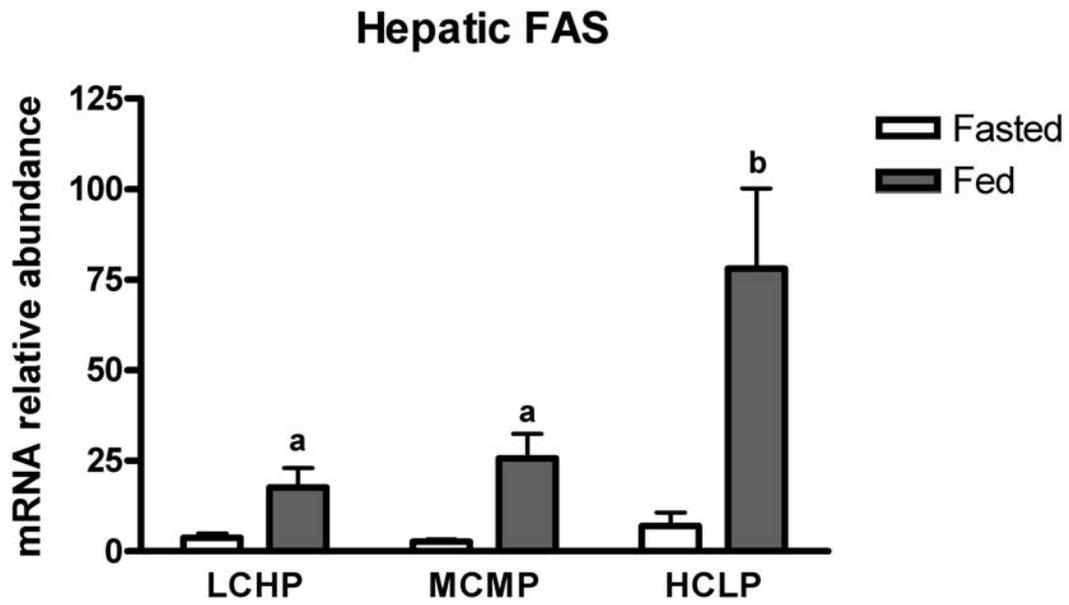
Figure 2.2. Hepatic FAS mRNA relative abundance in rats fed diets of different carbohydrate / protein ratios for 25 d. Data are means ± SEM; n = 6-8. Labeled means without a common letter differ, P < 0.05.

Figure 2.3. Percent change of post-prandial hepatic SREBP1c mRNA relative abundance from the fasted state in rats fed diets of different carbohydrate / protein ratios for 25 d. Data are means ± SEM; n = 6-8. Labeled means without a common letter differ, $P < 0.05$.

Hepatic Asparagine Synthetase
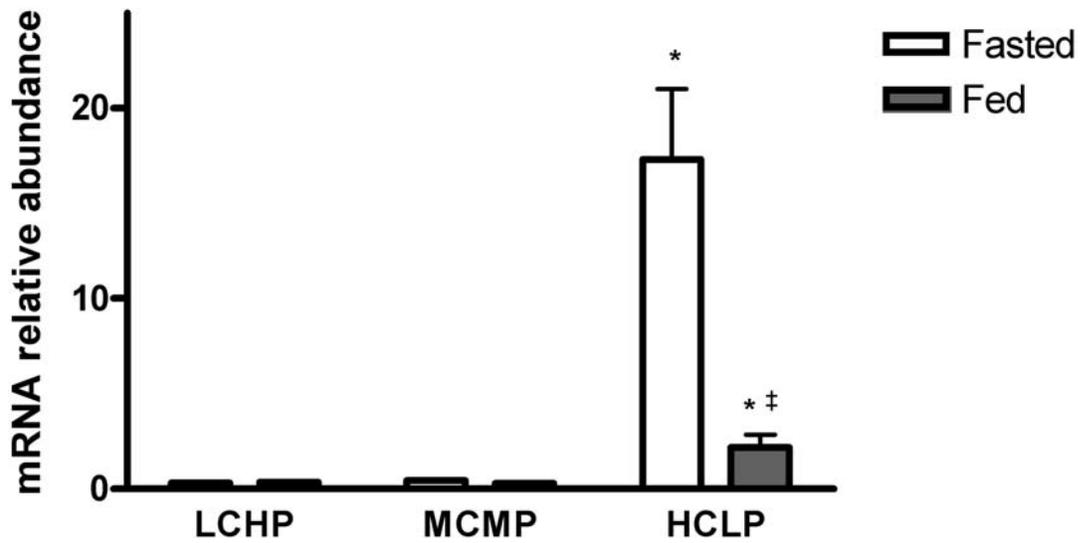
Figure 2.4. Hepatic asparagine synthetase mRNA relative abundance in rats fed diets of different carbohydrate / protein ratios for 25 d. Data are means ± SEM; n = 6-8. * indicates group difference among meal condition; ‡ indicates meal condition difference among group, P < 0.05.

Post-prandial Adipose FAS
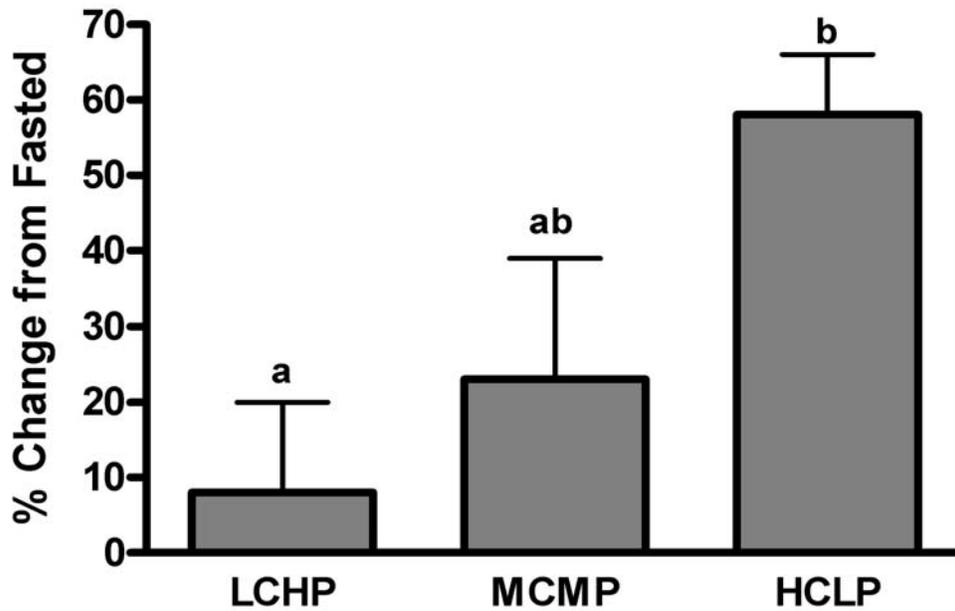
Figure 2.5. Percent change of post-prandial adipose FAS mRNA relative abundance from the fasted state in rats fed diets of different carbohydrate / protein ratios for 25 d. Data are means ± SEM; n = 6-8. Labeled means without a common letter differ, $P < 0.05$.
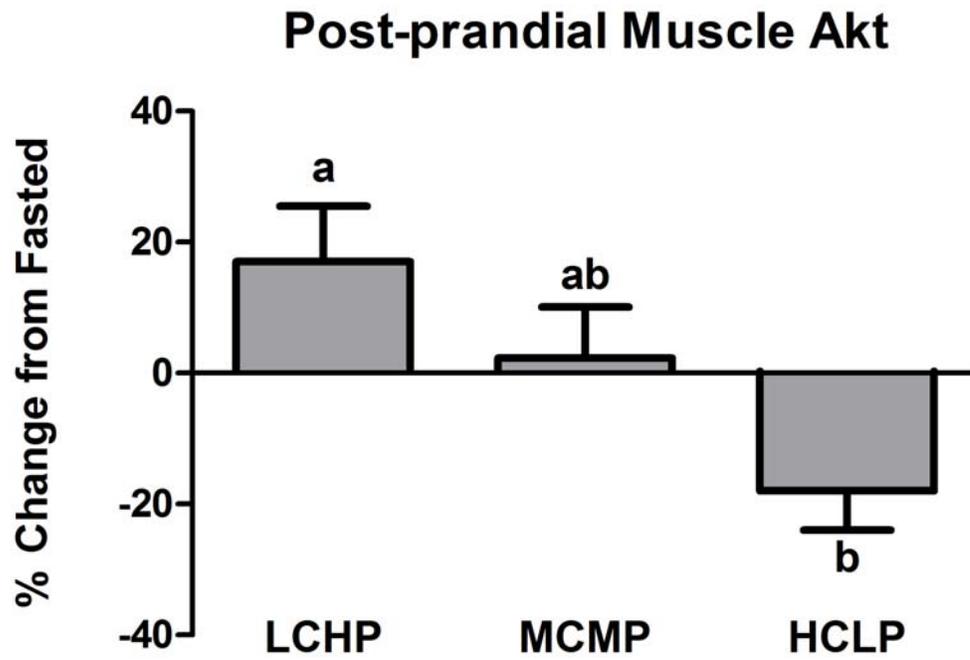
Figure 2.6. Percent change of skeletal muscle Akt % activation from the fasted state in rats fed diets of different carbohydrate / protein ratios for 25 d. Data are means ± SEM; n = 6-8. Labeled means without a common letter differ, P < 0.05.
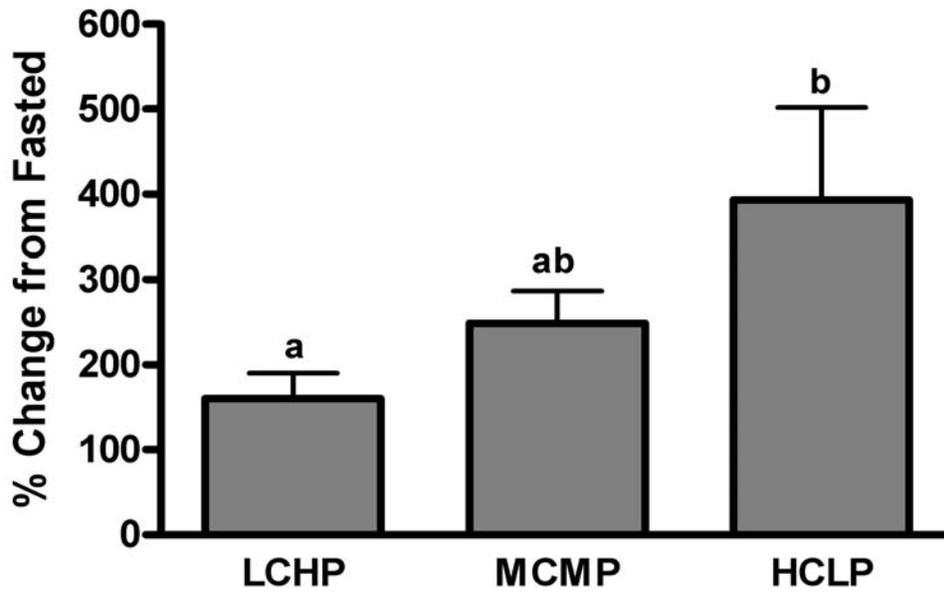
Table 2.1. *Diet composition for treatment groups*

Diet Component	LCHP	MCMP	HCLP
Casein	480.03	320.02	128.01
L-cysteine	7.31	4.87	1.95
Cornstarch	171.00	273.61	396.74
Sucrose	56.75	90.80	131.66
Maltodextrin	42.99	68.79	99.75
Cellulose	50	50	50
Soybean Oil	144.39	144.39	144.39
Choline bitartrate	2.5	2.5	2.5
Mineral mix	35	35	35
Vitamin mix	10	10	10
t-butylhydroquinone	0.014	0.014	0.014
Net metabolizable glucose	71.635	114.19	165.58

Grams per kg diet.

Table 2.2. *Body and tissue weights after 25 d of feeding diets with different carbohydrate / protein ratios*

	LCHP	MCMP	HCLP
Whole body (g)	297.5 ± 4.9[ab]	306.6 ± 3.6[a]	290.0 ± 3.7[b]
Liver (g)	10.43 ± 0.30[a]	10.45 ± 0.26[a]	9.25 ± 0.28[b]
Muscle total (g)	4.50 ± 0.07[a]	4.51 ± 0.08[a]	4.10 ± 0.4[b]
Adipose total (g)	3.32 ± 0.24[b]	4.24 ± 0.21[a]	4.46 ± 0.30[a]

Muscle total includes gastrocnemius, plantaris, and soleus. Adipose total includes epididymal and renal fat pads. Values represent means ± SEM; n=16. Different letters in a row indicate significant difference, $P < 0.05$.

Table 2.3. Fasted measurements after 25 d of feeding diets with different carbohydrate / protein ratios

	LCHP	MCMP	HCLP
Glucose (mmol / L)	10.62 ± 0.86	10.4 ± 0.34	12.17 ± 0.97
Insulin (pmol / L)	106.1 ± 11.1	103.1 ± 20.6	85.5 ± 12.2
Hepatic FAS (abundance)	3.76 ± 1.20	2.66 ± 0.69	7.08 ± 3.65
Hepatic SREBP-1c (abundance)	1.71 ± 0.20[a]	1.27 ± 0.15[ab]	1.15 ± 0.16[b]
Hepatic ATF3 (abundance)	0.91 ± 0.14[a]	0.98 ± 0.18[a]	2.48 ± 0.81[b]
Adipose FAS (abundance)	19.71 ± 2.48[a]	17.49 ± 1.54[ab]	13.37 ± 1.90[b]
Adipose SREBP-1c (abundance)	5.25 ± 0.85	4.80 ± 0.72	6.97 ± 1.06
Adipose Akt (% Phospho)	33.10 ± 7.60	38.10 ± 5.0	31.60 ± 4.90
Adipose p70S6K (% Phospho)	2.32 ± 0.64[a]	5.38 ± 1.12[ab]	8.91 ± 1.51[b]
Muscle Akt (% Phospho)	42.70 ± 4.11	43.95 ± 4.96	36.77 ±5.86
Muscle p70S6K (% Phospho)	50.80 ± 4.40	51.75 ± 3.90	50.80 ± 3.80

Values represent means ± SEM; n=6-8. Different letters in a row indicate significant difference, $P < 0.05$.

CHAPTER III:

DIETARY LEUCINE CONTENT OF ISOCALORIC, ISONITROGENOUS MEALS INFLUENCES BODY COMPOSITION, ENERGY PARTITIONING, AND MITOCHONDRIAL HOMEOSTASIS

INTRODUCTION

Chapter II demonstrated that the carbohydrate / protein ratio has a significant impact on post-prandial metabolic responses and that isocaloric exchange of protein at the expense of carbohydrate results in favorable metabolic and body composition outcomes. This chapter attempts to further describe the metabolic effects attributable to the protein component of the diet and examine long-term changes in body composition. The primary findings are that the meal-induced stimulation of muscle protein synthesis by dietary protein promotes skeletal muscle accretion, reduces dietary energy retention, elevates gene expression in muscle mitochondria, and ultimately reduces energy storage as body fat.

Skeletal muscle quality and health are key determinants of the functional capacity of an individual, particularly with regards to activities of daily living. Decreases in lean body mass are additionally associated with reduced resting energy expenditure and a lower functional capacity for physical activity, which contributes to reduced voluntary energy expenditure, and subsequently leads to energy storage and fat mass increase (20). This pattern compounds in the elderly, as the age-related loss of skeletal muscle mass, known as sarcopenia, can contribute to frailty, loss of strength, and it is associated with a high incidence of chronic disease (7). It is therefore important to identify dietary factors that promote skeletal muscle health and could potentially prevent or delay the onset of sarcopenia.

The continuous process of protein turnover in skeletal muscle involves anabolism in the form of muscle protein synthesis (MPS) and catabolism in the form of muscle protein breakdown (MPD); the balance of these processes ultimately determines the amount of skeletal muscle mass (8). The essential amino acid leucine has a unique ability to stimulate MPS, and it appears to act as a signal that an adequate supply of

amino acids is available for protein synthesis (9). Specifically, leucine stimulates the mammalian target of rapamycin (mTOR), a component of the insulin signaling pathway, which subsequently activates downstream translation initiation factors such as 70-kDa ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E- binding protein1 (4E-BP1) (2). The leucine content of dietary protein is proposed to be a major factor in directing peak MPS stimulation following consumption of a meal (16).

Our laboratory group has observed an apparent leucine threshold that promotes optimal MPS activity, but below which little or no MPS stimulation occurs. This threshold is governed by the ability of a meal to elevate plasma leucine concentrations to approximately twice the level of a food-deprived state. Specifically, consumption of a 4 g complete meal containing wheat protein (47 mg leucine) failed to stimulate MPS in adult rats, while consumption of a 4 g complete meal containing whey protein (64 mg leucine) sufficiently elevated plasma leucine, activated the mTOR signaling pathway, and stimulated MPS. These data indicate that the leucine content of a single meal has an acute effect on muscle protein metabolism, but it is not known if this effect compounded over time would result in a net accretion of skeletal muscle tissue. There is currently no research that supports the potential for chronic leucine supplementation to affect muscle mass or body composition long-term (3). Recent studies may be limited by the manner in which leucine is administered or consumed. For example, adult rats that were provided ad libitum access to chow diet supplemented with 4% leucine for 40 wks had no difference in skeletal muscle mass relative to control diet (25). Such approaches that gradually contribute additional leucine into the diet are unlikely to significantly elevate plasma leucine in comparison to bolus feeding of leucine (or protein), and are therefore unlikely to stimulate MPS. On the contrary, a dietary regimen designed to sufficiently elevate plasma leucine and optimize the leucine-related stimulation of MPS should promote beneficial outcomes with regards to skeletal muscle mass and body composition.

A second interest is the emerging effect of leucine on mitochondria. Cultured myocytes treated with leucine demonstrate increased lipid oxidation (22) and mitochondrial biogenesis (23), which entails the growth and division of pre-existing

mitochondria. Although not explicitly measured, this effect was attributed to increased levels of protein synthesis and associated energy demand. A recent study identified that addition of branched-chain amino acids (BCAAs) leucine, isoleucine, and valine as 1% of the diet significantly increased lifespan in mice, which was associated with an increase in skeletal muscle mitochondrial biogenesis (4). The authors suggested the mitochondrial effect was due to enhanced nitric oxide signaling, and although they made no consideration of protein turnover, they implied that nitric oxide may stimulate mTOR and its downstream targets.

A variety of environmental and energetic stresses can trigger mitochondrial biogenesis. These signals are mediated through peroxisome proliferator-activated receptor γ co-activator (PGC-1α), which coactivates a series of transcription factors (e.g. Tfam, NRF1/2, ERRα) that elevate mitochondrial protein expression and increase the capacity for ATP production (24). Aerobic exercise stimulates mitochondrial protein synthesis in response to increased demand for energy (19). Protein turnover requires substantial ATP consumption; therefore increases in protein turnover could potentially provoke a similar response in mitochondria to provide the required energy. This experiment was designed to determine if long-term feeding of meals containing protein sources that vary in leucine content has differential effects on skeletal muscle mass and body composition in relation to the previously observed post-prandial leucine-induced stimulation of MPS. In addition, the experiment will evaluate changes in mitochondrial status and development in skeletal muscle.

METHODS

Animals. Male Sprague-Dawley rats (Harlan-Teklad, Indianapolis, IN) with a mean body weight of 270 ± 10 g 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. The animal protocol was approved by the University of Illinois Institutional Animal Care and Use Committee.

Rats were trained to consume three meals / d using a modified AIN-93G diet (17) containing 54% of energy from carbohydrate, 16% of energy from protein, and 30% of

energy from fat. 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 3-4 d of adaptation, rats underwent body composition assessment by DXA (duel energy x-ray absorptiometry). Following adaptation, rats were randomized to consume a treatment diet containing either wheat gluten (WHEAT) or whey isolate (WHEY) as the protein source. 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.

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. Each of the diets was comprised of equivalent energy contributions from carbohydrate (54%), protein (16%), and fat (30%). As such, these two diets were considered to be isocaloric as well as isonitrogenous. The 4 g meals contained approximately 46 mg or 74 mg of leucine from the wheat and whey diets, while the 6 g meal contained approximately 69 mg or 111 mg of leucine, respectively. The carbohydrate / protein ratio of the diets was 3.375. The composition of the treatment diets is shown in Table 3-1.

Animals consumed the treatment diets for 2 wks, at which point a subgroup of animals (n = 8 per group) were deprived from food for 13 h and then either euthanized by decapitation (food deprived) or fed their normal 4 g morning meal. Ninety min after consumption of the meal rats were euthanized by decapitation and blood and tissues were harvested. Tissues were weighed prior to being snap frozen in liquid nitrogen, which included gastrocnemius, renal and epididymal fat pads, and liver. Tissues were then stored at -80°C until further analysis. The remaining animals (n = 12 per group) consumed the treatment diets for 9 additional wks (11 wks total), after which they underwent body composition assessment by DXA. After allowing for 3-4 d for recovery following DXA, the animals were euthanized in the same manner as at 2 wks.

Determination of protein synthesis. Protein synthesis was measured in skeletal muscle using the flooding dose method (10). A 100% enriched L-[^{2}H$_5$]-phenylalanine solution (150mmol / L; Cambridge Isotopes, Andover, MA) was administrated at 150 µmol / 100 g body weight and injected via tail vein (1 mL / 100g body weight). After 10 min animals were euthanized by decapitation and hind limbs were quickly removed and immersed in an ice-water mixture. Gastrocnemius muscles were removed from cooled hind limbs, frozen in liquid nitrogen and stored at -80°C.

Frozen muscle tissue was powdered in liquid nitrogen, and protein was precipitated with cold (4°C) perchloric acid (30 g / L, 1mL per 50 mg muscle tissue). The resulting supernatant and protein pellet were prepared for analysis by gas chromatography mass spectroscopy as described previously (6). 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 run under electron impact ionization and in splitless mode, and phenylethylamine ions at mass-to-charge ratio (m / z) 106 (m + 2) and 109 (m + 5) were monitored for enrichment.

The muscle supernatant was 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 ^{2}H$_5$-phenylalanine enrichment was determined using a propyl chloroformate derivative with GC-MS by monitoring the ions at m / z 206 (m) and 211 (m+5) (15).

Fractional rates of protein synthesis (FSR) were determined from the rate of incorporation of L-[^{2}H$_5$]-phenylalanine into total mixed muscle protein as described previously (14). The time from injection of the metabolic tracer until tissue cooling was recorded as the actual time for L-[^{2}H$_5$]-phenylalanine incorporation. FSR, defined as the percentage of tissue protein renewed each day, were calculated according to the formula: FSR = (E_b x 100) / (E_a x t) where t is the time interval between injection and cooling of sampled tissue expressed in day and E_b and E_a are the enrichments of [^{2}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 × g for 10 min at 4°C. Plasma insulin concentrations were determined using a commercial RIA kit for rat insulin (Linco Research, MO). Plasma leucine concentration was determined by HPLC using a Waters 2475 fluorescence detector (18).

Body composition and energy partitioning. Prior to the administration of the diet treatments and 11 wks after the diet treatments began, body fat percentage was assessed using dual energy X-ray absorptiometry (DXA) analysis. The DXA instrument (Hologic QDR 4500A, software version 11.1:3) was calibrated each day before measurement according to the manufacturer's guidelines. Rats were weighed following consumption of their normal morning 4 g meal, and then sedated by intraperitoneal injection of dexmedetomidine (0.3 mg / kg) prior to assessment. Rats were then placed on the DXA and 3 whole body scans were performed. All scans for a particular rat were performed in succession on the same day and analyzed by the same technician. Following the scans, rats were revived by intraperitoneal injection of atipamezole hydrochloride (1.5 mg / kg), and returned to their housing.

Total fat mass was obtained by multiplying % body fat as determined via DXA by the body weight of the rat on the morning of the assessment. Total lean mass was similarly determined by subtracting total fat mass from the body weight. The net fat mass gained was obtained by subtracting the total fat mass of the baseline DXA assessment (prior to diet assignment) from the total fat mass following 11 wks of feeding. The net lean mass gained was obtained similarly. In order to evaluate the body- and compartment-specific depositions of energy over the duration of the study, it was assumed that lean mass is composed of 20% protein and 80% water, and that fat mass is comprised of 80% lipid and 20% water (13). Thus, the net lean mass multiplied by a factor of 0.2 approximates the total protein accretion over the course of 11 wks; multiplying total protein accretion by 4 kcal / g protein provides an approximation of the net energy deposited into the lean compartment. Likewise, the net fat mass multiplied by a factor of 0.8 approximates the total lipid accretion over the course of 11 wks; multiplying total lipid accretion by 9 kcal / g lipid provides an approximation of the net energy deposited into the fat compartment. Finally, total energy deposition was obtained

by the sum of net energies deposited into the fat and lean compartments, and the percentages of energy deposited to the fat and lean compartments in relation to total energy deposited were calculated.

Gene expression. Total RNA was isolated from ~50 mg frozen muscle using TRI Reagent® (Sigma-Aldrich, St. Louis, MO). First-strand cDNA was synthesized using random hexamers on a thermal cycler. RT-PCR was run with 7.5 ng of cDNA template and oligonucleotide primers designed to amplify Tfam or cytochrome b in muscle. All primers were obtained from IDT (Coralville, IA). The relative abundance of each gene was normalized to expression of the 18S control gene.

Statistical analysis. Data are expressed as mean ± SEM. All variables were evaluated for normality and homogeneity of variance by Levene's test. Group differences were assessed by t-test. A one-way ANOVA was performed on MPS 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. The level of significance was set at $P < 0.05$. All analyses were performed using SPSS Version 15.0 (Chicago, IL).

RESULTS

Protein synthesis. After 2 wks of consuming the diets containing different protein sources, MPS was measured in rats in the fasted condition and 90 min after consumption of the standard 4 g morning meal. MPS was not stimulated above fasted levels in the WHEAT group, but was significantly elevated (189%) at 90 min after consuming the meal in the WHEY group. Similarly, after 11 wks of consuming the diets, post-prandial MPS was not different from fasted baseline in the WHEAT group, but MPS was significantly elevated (170%) at 90 min after the WHEY meal (Figure 3.1).

Plasma measurements. At 2 wks, post-prandial leucine in the WHEAT group was not different than the fasted condition; however, post-prandial leucine in the WHEY group was significantly elevated above the fasted condition (Table 3.2). This pattern was also seen at 11 wks, where post-prandial leucine was elevated following the meal in the WHEY group, but not in the WHEAT group. Plasma insulin was elevated above

fasted levels following the meal in both the WHEAT and WHEY groups at 2 and 11 wks (Table 3.2).

Body composition. Animals in the WHEAT group were significantly heavier than animals in the WHEY group (Table 3.3). Skeletal muscle (gastrocnemius) was larger in the WHEY group, while epididymal fat was heavier in the WHEAT group. There was no difference in liver weight between groups. Body fat percentage determined by DXA was higher in the WHEAT group (12.7%) than in the WHEY group (9.7%). When these percentages were multiplied by total body weight, the animals in the WHEAT group had approximately 26% more total fat mass than animals in the WHEY group after only 11 wks. Total lean mass was not difference between groups.

In relation to the baseline animals (i.e., wk 0) examined prior beginning the treatment diets, animals in the WHEAT group gained more than 3 times the fat during 11 wks of feeding than animals in the WHEY group (Table 3.3). Similarly, when accounting for the energetic equivalent of the accumulated fat and lean tissue, animals in the WHEAT group deposited more energy as fat than animals in the WHEY group, while the energy deposited as lean tissue was equal between groups. While consuming the same calories per day, animals in the WHEAT group deposited more total body energy than animals in the WHEY group (Table 3.3). Animals in the WHEY group partitioned 59% of deposited energy towards the fat compartment, which was significantly greater than the 29% of energy deposited towards fat by the animals of the WHEY group. Animals in the WHEY group deposited 71% of energy to the lean compartment, which was significantly greater than the 41% of energy that was deposited to the lean compartment by the WHEAT animals (Table 3-3).

Gene expression. PGC-1α, cytochrome B, and Tfam are a series of genes implicated in the progression of mitochondrial biogenesis (24)(24). The relative expression of these genes was elevated 49%, 103%, and 36%, respectively in animals from the WHEY group. SOD1 is not found in mitochondrial DNA but in somatic DNA, although its protein is localized within the mitochondria (12). The relative expression of SOD1 was not different between groups (Table 3-4).

DISCUSSION

This experiment examined if long-term feeding of isonitrogenous protein sources with varied leucine contents would produce differential skeletal muscle mass and body composition outcomes based on the potential to stimulate (or inability to stimulate) MPS following a meal. We found that feeding meals with equal energy and equal total protein but different leucine amounts for 11 wks produces differential effects on skeletal muscle weight, body composition, efficiency of energy deposition, and energy partitioning, as well as on expression of genes related to mitochondrial biogenesis. These findings were predicated on the ability of the leucine-rich WHEY protein to elevate post-prandial plasma leucine concentrations and to stimulate MPS. After 2 and 11 wks of meal feeding, a 4 g meal containing wheat gluten with 47 mg of leucine was unable to stimulate MPS, while a 4 g meal containing whey protein with 64 mg of leucine stimulated MPS beyond the fasted state. This response indicates that the WHEY group experienced more frequent post-prandial stimulations of MPS than WHEAT, which resulted in additional accretion of skeletal muscle mass. To our knowledge, these are the first data to demonstrate that variations in dietary leucine that produce differences in MPS also have a significant effect on skeletal muscle mass.

Interestingly, aspects of body composition beyond skeletal muscle mass were also impacted by the leucine content of the diet. The WHEY animals were leaner overall due to lower total fat mass, which was reflected in tissue weights, as the WHEY animals had lighter epididymal fat pads. Since the treatment diets were isocaloric, this suggests that the additional fat deposition in the WHEAT animals occurred in the absence of skeletal muscle accretion; the energy that was not utilized for MPS must be accounted for by other metabolic activity, perhaps in other tissues. However, unexpectedly the WHEAT animals were heavier overall, despite equal energy input. This is highlighted by the observation that the WHEAT animals deposited ~65% more energy over the course of 11 wks.

Post-prandial thermogenesis, also known as diet-induced thermogenesis (DIT), is the energy required for digestion, absorption, and metabolism of nutrients (11). Dietary protein has the largest associated DIT in comparison to fat or carbohydrate,

which is typically attributed to the energetic costs of catabolism and nitrogen disposal via the urea cycle (1). However, the WHEAT and WHEY diets were equal with respect to all macronutrients, and yet less energy was deposited by WHEY animals, which indicates that there is an additional component of DIT that is not accounted for solely by the macronutrient composition of a diet. Furthermore, >50% of the energy deposited by the WHEAT group was deposited in the fat compartment, while the WHEY group deposited only ~30% of dietary energy into fat tissue. These findings suggest that leucine-induced stimulations of MPS produced greater DIT and reduced retention of dietary energy, which resulted in differential partitioning of energy among bodily tissues producing a more desirable lean phenotype.

Similar results were recently identified in a knock-out mouse model, where deletion of the first step in leucine degradation produced chronically elevated levels of plasma leucine (~10 higher than wild type), and resulted in increased energy expenditure and obesity lean phenotype (21). The authors attributed the effects to the increased energy cost of protein turnover, which was elevated in numerous tissues including skeletal muscle, heart, liver, and kidney. Although it was not elevated to such a magnitude or duration in the present experiment, the WHEY meals sufficiently augmented plasma leucine to stimulate MPS. It is feasible that our results depict a similar, more moderate response to physiologically relevant plasma leucine concentrations in a wild type model.

Despite equal caloric input, we found that the WHEY group deposited less fat tissue over 11 wks of feeding. Studies have suggested that dietary leucine can influence fat mass in conditions of heavy caloric restriction (5) and diet-induced obesity (26), while other research under eucaloric conditions indicates little effect on fat mass (4). In vitro treatment with leucine has been shown to increase fatty acid oxidation in C2C12 myocytes and decrease expression of fatty acid synthetase in 3T3-L1 adipocytes, which suggests that leucine may have an effect on energy partitioning, whereby energy storage by adipocytes is reduced and fatty acid oxidation in muscle is enhanced (22). It is unclear if the differential energy efficiency and partitioning observed in the present

study is due to an unidentified direct effect on adipose, or due to a secondary effect related to the energy costs of protein turnover in skeletal muscle.

In support of the latter hypothesis, a group of genes involved in mitochondrial biogenesis were induced in skeletal muscle by the WHEY diet. This is perhaps indicative of a stress response in which muscle requires more cellular energy to support elevated protein synthesis rates. It follows that additional energy substrate could be liberated from adipose tissue to be oxidized in muscle for ATP production. In vitro treatment of C2C12 myocytes with leucine stimulated mitochondrial biogenesis and increased oxygen consumption (23), which suggests that leucine may either directly or indirectly signal muscle to enhance its capacity to produce energy. It is not yet clear if stimulation of MPS substantially depletes cellular energy to the extent that increased demand for ATP would promote mitochondrial biogenesis.

In conclusion, the present study demonstrates that the leucine content of a meal impacts post-prandial plasma leucine concentrations such that MPS is stimulated, and that these meal effects compounded over a period of 11 wks result in additional accretion of skeletal muscle, a lean phenotype, and favorable energy partitioning. Furthermore, these meal effects appear to have a positive impact on mitochondrial biogenesis in skeletal muscle, which may provide additional support for the additional associated energy demand. These findings merit further investigation into the demands of leucine-induced MPS stimulation on cellular energy.

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Figure 3.1. Post-prandial muscle protein synthesis expressed as % of fasted activity in rats fed diets of different protein sources for 2 wks. Data are means ± SEM; n = 5-8. * indicates significant difference from fasted condition, P < 0.05.
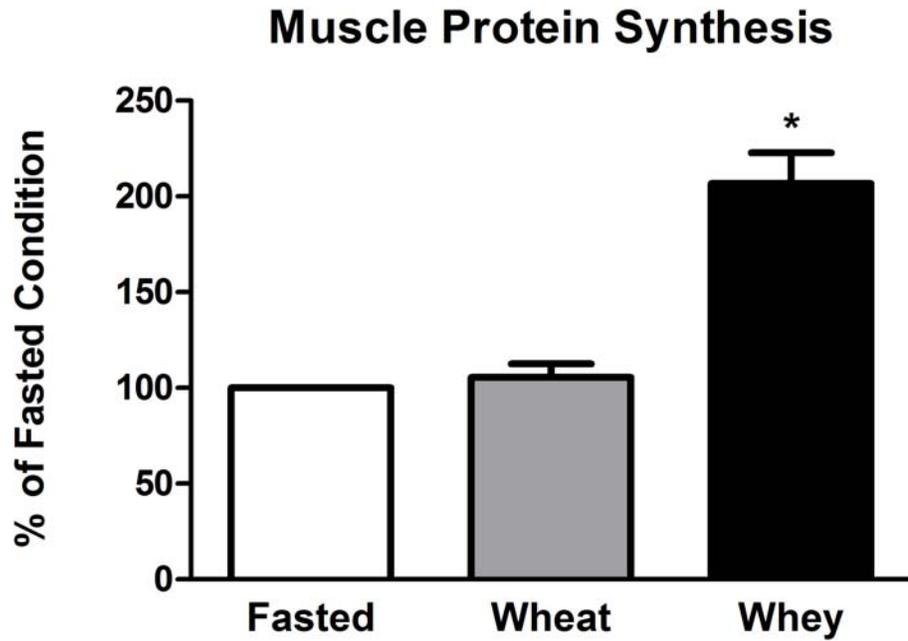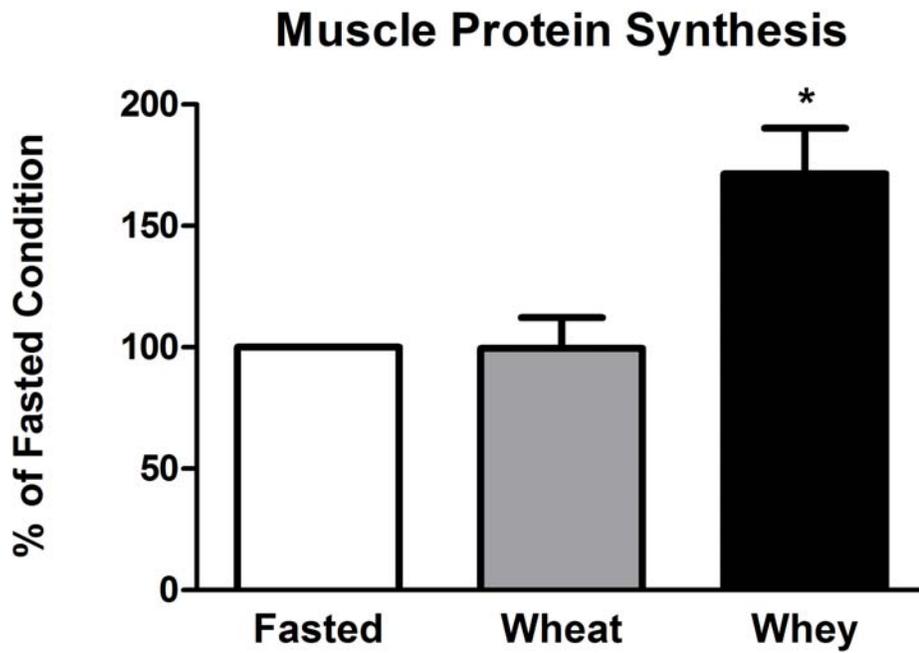
Muscle Protein Synthesis

Figure 3.2. Post-prandial muscle protein synthesis expressed as % of fasted activity in rats fed diets of different protein sources for 11 wks. Data are means ± SEM; n = 5-8. * indicates significant difference from fasted condition, P < 0.05.

Table 3.1. *Diet composition for treatment groups*

Diet component	WHEAT	WHEY
Wheat gluten	190.2	0
Whey protein isolate	0	188.8
L-lysine supplement	10.1	0
Cornstarch	316.7	328.2
Maltodextrin	134.1	134.1
Sucrose	101.5	101.5
Soybean Oil	140.9	140.9
Cellulose fiber	53.7	53.7
Mineral mix	37.6	37.6
Vitamin mix	10.7	10.7
Choline bitartrate	2.7	2.7
t-butylhydroquinone	0.014	0.014

Grams per kg diet.

Table 3.2. *Plasma leucine and insulin at 0 and 90 min after consumption a 4 g meal*

	Timepoint	Fasted	WHEAT	WHEY
Leucine (μmol / L)	2 wks	91 ± 4.4^a	78 ± 8.1^a	146 ± 28.4^b
	11 wks	84 ± 5.9^a	69 ± 3.8^a	143 ± 7.8^b
Insulin (pmol / L)	2 wks	140 ± 10.5^a	178 ± 9.0^b	170 ± 7.2^b
	11 wks	128 ± 15.4^a	190 ± 37.7^b	171 ± 20.2^b

Values represent mean ± SEM; n = 9 for each group at 2 wks, n = 12 for each group at 2 and 11 wks. Labeled means without a common letter differ, $P < 0.05$.

Table 3.3. *Body composition and energy partitioning measures following 11 wks of meal feeding with different protein sources*

	WHEAT	WHEY	p-value
Body weight (g)	394.2 ± 2.5	384.8 ± 2.4	0.012
Liver weight (g)	9.08 ± 0.24	9.08 ± 0.23	0.99
Gastrocnemius weight (g)	2.08 ± 0.04	2.20 ± 0.04	0.006
Epididymal fat weight (g)	2.82 ± 0.11	2.38 ± 0.14	0.023
% body fat	12.7 ± 0.5	9.7 ± 0.8	0.011
Total fat mass (g)	48.5 ± 2.2	38.5 ± 3.3	0.12
Total lean mass (g)	344.0 ± 3.3	343.3 ± 3.3	0.49
Net fat gain (g)	17.1 ± 1.7	5.5 ± 3.3	0.007
Net lean gain (g)	101.3 ± 3.5	101.1 ± 3.3	0.97
Fat compartment energy gain (kcal)	123.2 ± 12.3	39.7 ± 23.5	0.007
Lean compartment energy gain (kcal)	81.0 ± 2.8	80.9 ± 2.7	0.97
Total energy gain (kcal)	204.2 ± 10.6	123.1 ± 23.9	0.004
% total energy deposited to fat	58.5 ± 3.3	29.3 ± 8.0	0.004
% total energy deposited to lean	41.2 ± 3.3	70.7 ± 8.0	0.004

Values represent mean ± SEM. n = 12 for each group.

Table 3.4. *Relative mitochondrial gene expression in skeletal muscle following 11 wks of meal feeding with different protein sources*

Gene	WHEAT	WHEY	p-value
PGC-1α	1.00 ± 0.35	1.49 ± 0.53	0.038
Tfam	1.00 ± 0.35	2.03 ± 0.71	0.022
Cyt B	1.00 ± 0.17	1.36 ± 0.33	0.040
SOD1	1.00 ± 0.35	0.92 ± 0.32	0.72

Values represent mean ± SEM. n = 8-12 for each group.

CHAPTER IV:

THE POST-PRANDIAL MUSCLE PROTEIN SYNTHESIS RESPONSE IS EXTENDED WITH SUPPLEMENTAL LEUCINE OR CARBOHYDRATE BY DELAYING CELLULAR ENERGY DEPLETION

INTRODUCTION

Chapter III revealed that repeated stimulations of muscle protein synthesis (MPS) induced by consumption of leucine-rich meals leads to partitioning of dietary energy to accretion of skeletal muscle, away from fat storage, and results in more favorable body composition. An unexpected finding was that animals consuming the whey-containing diet had reduced energetic efficiency despite equal dietary energy input. Additionally, consumption of leucine-rich meals was associated with changes in mitochondrial gene expression that appear to be in support of the increased energy demand of MPS. However, it has not yet been shown that post-meal stimulation of MPS directly impacts cellular energy status. This chapter investigates the molecular regulation of MPS to determine if the post-prandial anabolic response has a measurable impact on cellular energy status.

Consumption of protein, and particularly consumption of the branched chain amino acid leucine, is able to stimulate muscle protein synthesis (MPS) beyond basal or fasted levels (6). Leucine stimulates mammalian target of rapamycin (mTOR), a signaling kinase and component of the insulin signaling pathway, resulting in activation of the downstream translation factors including p70S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). These factors enhance translation initiation and subsequent protein synthesis (7). Protein synthetic activity is estimated to consume 30-50% of cellular energy across all cell types (4). Due to the high energy demands of MPS, a logical assumption is that stimulation of protein synthesis beyond basal levels would elevate requisite cellular energy needs.

To our knowledge, there has been no research that has evaluated the ability of dietary leucine to directly affect cellular energy status. However, a study in mice that knocked out the first enzyme in leucine catabolism, the branched-chain amino acid

transferase (BCAT), resulted in continuously elevated plasma leucine and abnormally high protein synthesis rates in numerous tissues, including skeletal muscle, heart, and liver (14). These animals had improved glucose tolerance, resistance to diet-induced obesity, increased energy expenditure, and a lean phenotype in comparison to the wild-type. The authors attributed the lean phenotype to a "futile cycle" of energy expenditure associated with increased protein synthesis and degradation.

Separately, a pair of studies in C2C12 myocytes suggests that supplemental leucine may have an impact on cellular energy in vitro. The first found that addition of leucine to the culture medium stimulated fatty acid oxidation (16), while the second found that incubation with a physiologically relevant concentration of leucine increased mitochondrial mass and oxygen consumption (17). These effects suggest an adaptive response in the capacity to supply cellular ATP, and although it was not directly assessed in these experiments, the authors attributed the findings to the elevated energy demands of protein synthetic activity.

The direct effect of leucine on mTOR-stimulated initiation of translation is well characterized, and the subsequent increase in translation elongation is responsible for ~99% of the energy consumption during protein synthesis (4). Recent in vitro research suggests that translation elongation is a likely target for regulating the amount of energy consumed during protein synthesis. AMP kinase (AMPK) is the key energy sensor of the cell; it becomes more active when cellular energy levels are depressed and the AMP / ATP ratio is elevated (8). Activation of AMPK by the agonist AICAR inhibited translation elongation via increased phosphorylation of eukaryotic translation elongation factor 2 (eEF2), resulting in a depression of MPS activity (19). Taken together, these reports lead to the hypothesis that leucine-induced depletion of cellular energy by activation of MPS would be sensed by AMPK, which would depress translation elongation by inhibiting eEF2.

In evaluation of this hypothesis, it is of interest to determine: 1) if it is possible to extend the post-prandial period of MPS with supplemental energy, and 2) does leucine directly affect cellular energy status or is the effect indirectly related to triggering the initiation process? The following studies evaluated the effect of dietary leucine on

cellular energy and translation elongation activity following a meal. In order to determine if additional dietary energy can prolong MPS activity, we administered an oral gavage solution containing either leucine, carbohydrate, or a combination of leucine and carbohydrates to rats 135 min after consumption of a 4 g test meal. In order to determine if AMPK responds to MPS-induced reductions in cellular energy by inhibiting translation elongation, compounds were injected to inhibit either AMPK activity or MPS activity following consumption of a 4 g test meal.

METHODS

Animals. Sprague-Dawley rats (Harlan-Teklad, Indianapolis, IN) were housed individually in stainless steel wire-bottomed cages and maintained at 24°C with 12 h reversed light cycle (light: 1900-0700 h) and free access to water. Animal protocols were approved by the University of Illinois Institutional Animal Care and Use Committee.

Rats were trained to consume three meals / d using a modified AIN-93G diet (50% of energy from carbohydrate, 20% protein, and 30% fat; Table 4.1) (12). In order to normalize food intake and reduce variance in post-prandial metabolism, the treatment diets were fed at 80% of ad libitum intake (14 g / d). This was accomplished by feeding three daily meals with defined amounts and timing: 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.

Experiment 1 evaluated the responses of cellular AMP & ATP, AMPK activity, ACC activity (a putative downstream target of AMPK), and eEF2 activity following consumption of a leucine-rich meal and following post-meal supplements of leucine, carbohydrate, or the combination of leucine plus carbohydrate. After 7 d of meal training, rats (n = 34; 270 ± 7 g) were food deprived for 12 h (1900-0700h) then euthanized by decapitation, and blood and tissues were harvested (food deprived; n = 4) or fed the normal 4 g morning meal. Ninety min after consumption of the meal, rats (n = 6) were euthanized and blood and tissues were harvested. The remaining rats were fed the normal 4 g morning meal and then at 135 min post-meal received an intragastric oral gavage of a solution containing either water (sham), carbohydrate (CHO), leucine

(LEU), or a combination of carbohydrate and leucine (LC); n = 6 per group. Rats were euthanized 45 min later (180 min post-meal) and blood and gastrocnemius muscles were harvested. Timing for the gavage was based on previous published research that MPS returns to pre-meal rates 180 min following a meal (11). The compositions of the gavage supplements were based on previous research that the dose maximally stimulates MPS from the fasted state 45 min post-gavage (1). Blood was centrifuged at 1800 × g for 10 min at 4°C and the serum was decanted. All tissues and blood were stored at -80°C until later analyses.

The CHO gavage contained 2.63 g of carbohydrate (1.315 g glucose and 1.315 g sucrose. The LEU gavage contained 270 mg of leucine, which is equivalent to the daily leucine consumption of rats with free access to AIN-93 powdered diet (12). The LC gavage contained the same carbohydrate amounts as the CHO gavage, and contained the same leucine amount as the LEU gavage. All gavage solutions were prepared in distilled water, and all groups received a gavage volume of 5 mL. The sham gavage of distilled water was given to reproduce the stress response of the treatment gavages.

Experiment 2 utilized specific inhibitors of mTOR and AMPK to evaluate the potential for AMPK to inhibit MPS activity via depression of eEF2 activity. After 5 d of meal training, rats (n = 10; 250 ± 4 g) were food deprived for 12 h (1900-0700h) and then rapamycin (Sigma, St. Louis, MO) (0.75 mg / kg body weight; n = 3) or vehicle (n = 4) was injected intraperitoneally 120 min prior to consumption of the 4 g test meal. The rapamycin treatment was designed to block activation of MPS by specifically inhibiting mTOR (2). Compound C (6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine; Sigma, St. Louis, MO) was injected intraperitoneally (30 mg / kg body weight; n = 3) 60 min after introduction of the 4 g test meal. The compound C treatment was designed to inhibit AMPK activity (9, 15). Rats were killed 180 min after the meal, and tissues were collected and analyzed in a similar manner to Experiment 1.

Plasma measurements . Plasma was obtained from trunk blood by centrifugation at 1800 × g for 10 min at 4°C. Plasma leucine concentration was determined as previously described (Chapter III).

Determination of muscle protein synthesis. Protein synthesis was measured in skeletal muscle as previously described (Chapter III).

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 methanol 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 µL 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 the temperature set at 25°C. A single mobile phase was utilized at a flow rate of 1 mL / min with 25% acetonitrile / 10mM KH_2PO_4; pH 7.0 (pH was adjusted with potassium hydroxide / 5mM tetrabutylammonium chloride). Nucleotide peaks were measured by UV detection at a wavelength of 260 nm. Peaks were identified by comparison with external standards (Sigma-Aldrich, St. Louis, MO).

Western blot analysis. Muscle supernatants were subjected to protein immunoblot analysis as described previously (Chapters II & III). The activity of eEF2, AMPK, ACC, p70S6K, and Akt were determined by measuring the ratio of phosphorylated : total antigen. All primary antibodies and anti-rabbit IgG HRP-linked secondary antibody were purchased from Cell Signalling (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 meal time course (fasted, fed, sham, CHO gavage as control) or the gavage treatment as the independent variables. 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. Correlations were assessed by Pearson's linear regression. The level of significance was set at $P < 0.05$. All analyses were performed using SPSS Version 15.0 (Chicago, IL).

RESULTS

<u>Experiment 1</u>

      Plasma leucine. Plasma leucine increased 49% from the fasted condition to 90 min after the meal and remained elevated 180 min after the meal (Table 4.2). Plasma leucine was not different from sham following the CHO gavage, while both leucine-containing gavages significantly raised plasma leucine (Table 4.3). The Leu treatment increased plasma leucine concentration nearly 4-fold above the 180 min sham treatment while the LC treatment doubled plasma leucine (Table 4.3).

      Muscle protein synthesis. Relative to the fasted condition, MPS activity was doubled at 90 min following consumption of the 4 g morning meal (Figure 4.1). At 180 min following the meal, MPS in the sham control had returned to the fasted baseline level. However, all three gavage treatment groups provided 135 min following the meal maintained MPS at the 90 min post-meal level.

      Nucleotides. There was a trend for the AMP / ATP ratio to be reduced during the post-meal period at 90 min compared to the fasted baseline reflecting the increased energy available after the meal, but at 180 min after the meal without additional energy (i.e., the sham treatment) the ratio was 140% higher than at 90 min. All three gavage treatments depressed the ratio to 90 min post-prandial levels (Figure 4.2).

      AMPK and ACC activity. Activity of AMPK was determined by percent phosphorylation of the α-subunit at Thr^{172}, where higher phosphorylation indicates greater activity. AMPK activity was depressed from the fasted condition at 90 min, but returned to fasted baseline activity at 180 min after the meal. All gavage treatments depressed AMPK activity at 180 min compared to the sham treatment with values similar to 90 min post-meal activity (Figure 4.2). There was a significant inverse correlation across conditions between MPS and AMPK activity ($r = -0.581$).

      Similar to AMPK activity, phosphorylation of acetyl-CoA carboxylase (ACC) was depressed at 90 min compared with the fasted baseline, but returned to baseline levels 180 min after the meal (sham), while all three gavage conditions decreased phospho-ACC to that of the 90 min post-prandial levels (Figure 4.2).

eEF2 activity. As an indicator of translation elongation activity, phosphorylation of eEF2 was measured, where greater phosphorylation indicates less elongation activity. eEF2 was depressed 44% from the fasted condition at 90 min, but returned to basal activity at 180 min after the meal (sham). All gavage treatments depressed eEF2 activity from 180 min (sham), similar to 90 min (Figure 4.3). Across all gavage treatments, there was a significant inverse correlation between eEF2 activity and MPS activity at 180 minutes after the meal ($r = -0.500$; $p < 0.05$). Additionally, when the fasted group was excluded, there was a significant positive correlation between AMPK activity and eEF2 phosphorylation ($r = 0.495$; $p < 0.05$).

Experiment 2

p70S6K activity. The activity of p70S6K was measured to confirm the ability of rapamycin to inhibit mTOR signaling. p70S6K activity was significantly lower in the rapamycin group compared to vehicle, while the compound C group was intermediate (Figure 4.4).

AMPK activity. The activity of AMPK was measured to confirm the ability of compound C to inhibit AMPK. AMPK activity was not different between compound C and vehicle groups, indicating that compound C did not inhibit AMPK. AMPK activity was significantly greater in the rapamycin group compared to the compound C and vehicle groups (Figure 4.5).

DISCUSSION

Increased post-prandial plasma leucine stimulates MPS activity by triggering translation initiation via its effects on mTOR signaling. The subsequent increase in translation elongation activity results in assembly of the peptide chain and is responsible for the majority of energy consumed during MPS activity. These studies were designed to determine if cellular energy is depressed following leucine-induced stimulation of MPS, and to determine if AMPK responds to such an energy deficit by inhibiting translation elongation.

Plasma leucine was elevated at 90 min after consumption of the test meal, and remained elevated 180 min after the meal. MPS activity increased 90 min following consumption of the test meal, but had returned to basal levels at 180 min. There was a trend for AMP / ATP ratio to drop at 90 min after the meal, reflective of an influx of energy substrate. However, the AMP / ATP ratio was significantly higher at 180 min, indicating a decrease in available cellular energy. These findings are in disagreement with an experiment that exposed isolated muscle fibers from chow-fed rats to leucine, which stimulated MPS as anticipated (13). However, AMPK activity was reduced despite no change in the AMP / ATP ratio. The physiological relevance of this ex vivo technique and the accuracy of the nucleotide data is in doubt.

Both AMPK and ACC activity followed a similar pattern, indicating that AMPK responded to the energy depletion associated with an increase in MPS activity. Translation elongation activity was assessed by phosphorylation of eEF2, whereby increased phosphorylation indicates reduced elongation activity. Alteration in the phosphorylation state of eEF2 is the primary mechanism by which elongation activity is regulated (5). eEF2 activity at 90 min was reduced from fasted levels, which reflects greater translation elongation during peak MPS activity, but was restored at 180 min, indicative of basal MPS activity. The 180 min time point was associated with the largest energy deficit and the strongest inhibition of translation elongation. These findings are consistent with the hypothesis that energy-limited down-regulation of peptide elongation is a limiting factor in the duration of post-prandial MPS.

Previous studies in humans and animals indicate that post-prandial MPS activity returns to basal activity at 180 min despite continued elevations in plasma leucine and associated initiation factors (3, 11). In order to determine if supplemental nutrients can attenuate cellular energy depletion and prolong elevated MPS activity, carbohydrate, leucine, or a combination of carbohydrate and leucine were administered orally at the point of peak post-prandial MPS activity. Both leucine-containing gavage treatments further raised plasma leucine beyond the 180 min time point (sham), although the carbohydrate gavage did not. Despite equal leucine contents, the LEU gavage elevated plasma leucine to a greater extent than the LC gavage. The cause of different

responses to the LEU and LC treatments is unknown. The blunted increase in plasma leucine in the LC group may be associated with delayed gastric emptying related to the higher osmolality of this gavage with is reflected in lower plasma leucine (18).

All three treatments were able to extend the peak MPS activity to the 180 min time point. Since the carbohydrate gavage failed to further increase plasma leucine, the ability to extend MPS is not a leucine-specific effect. Therefore, while leucine acts as a unique initiation signal for activating MPS activity, the duration of the post-prandial response appears to be influenced by energy substrate. All three gavage conditions reduced the AMP / ATP ratio, AMPK activity, and ACC activity relative to sham control. This indicates that the supplemental nutrients were sufficient to prevent the MPS-associated depletion in cellular energy. Across all gavage conditions there was a significant negative association between MPS and AMPK activity, suggesting that AMPK is responsive to the energy consumption related to MPS activity. Similarly, eEF2 activity was depressed by each of the three gavage treatments relative to sham control. This indicates that the carbohydrate, leucine, and carbohydrate plus leucine gavages allowed for continued elevation of translation elongation activity. Consistent with these findings there was a significant negative association between eEF2 activity and MPS across all treatments. When the fasted group was excluded, an inverse association was identified between MPS activity and elongation activity. In total, all three supplemental conditions maintained cellular energy at 180 min and reduced eEF2 activity, which permitted an extension of peak MPS activity beyond 90 min.

It is logical that the magnitude of post-prandial MPS activity is regulated by translation elongation since 99% of the energy used to assemble a typical protein is consumed during elongation (4). In response to an energy deficit, cessation of translation elongation would conserve cellular energy while allowing polysomes to remain associated with the mRNA transcript. Thus, when cellular energy is restored to adequate levels, elongation could continue without the need to reassemble polysomes. As a robust cellular energy sensor, AMPK is an ideal candidate to mediate such a signal to the protein synthetic machinery. A proposed mechanism involves AMPK activating eEF2 kinase, which subsequently phosphorylates eEF2, thereby inhibiting peptide

elongation (10). Interestingly, the leucine gavage was equally capable as the carbohydrate-containing gavages of reducing the AMP / ATP ratio and related AMPK activity despite providing ~10 times less substrate for energy. No differences in plasma insulin could explain this phenomenon (data not shown). The differences in the energy content of the supplements suggests that supplemental leucine may exert a unique effect on maintaining cellular energy following peak activation of MPS, as plasma leucine was elevated 2- to 4-times higher than the sham control.

Results from experiment 1 suggest that cellular energy is a key determinant of post-prandial MPS activity, and that AMPK senses the MPS-associated energy depletion and subsequently inhibits the energetically costly process of translation elongation. In order to further elucidate this relationship, an experiment was designed to block post-prandial MPS to determine the effect on cellular energy, and separately, to block AMPK activity in order to remove its inhibition of translation elongation. We hypothesized that inhibition of MPS with rapamycin would prevent the post-prandial decrease in cellular energy at 180 min, and that inhibition of AMPK with compound C would relieve the inhibition of translation elongation, thus allowing MPS activity to continue at peak post-prandial levels. Rapamycin successfully inhibited mTOR signaling, as downstream initiation factor p70S6K was less active relative to vehicle. However, compound C failed to inhibit AMPK activity, which was no different between the compound C treatment and vehicle. As proper inhibition of AMPK was crucial to the experiment design, all further analysis of this experiment was abandoned. These results highlight a need for further research to identify the molecular mechanisms responsible for optimal utilization and protection of cellular energy in response to post-prandial activation of MPS.

In conclusion, the leucine-induced activation of MPS results in a cellular energy deficit that is sensed by AMPK. Furthermore, an association between AMPK activity and translation elongation activity suggests that AMPK responds to reductions in cellular energy by inhibiting the energetically expensive process of translation elongation, thereby inhibiting subsequent MPS activity. These findings warrant additional

investigation to determine if AMPK has a direct role in limiting post-prandial MPS activity when cellular energy is limited.

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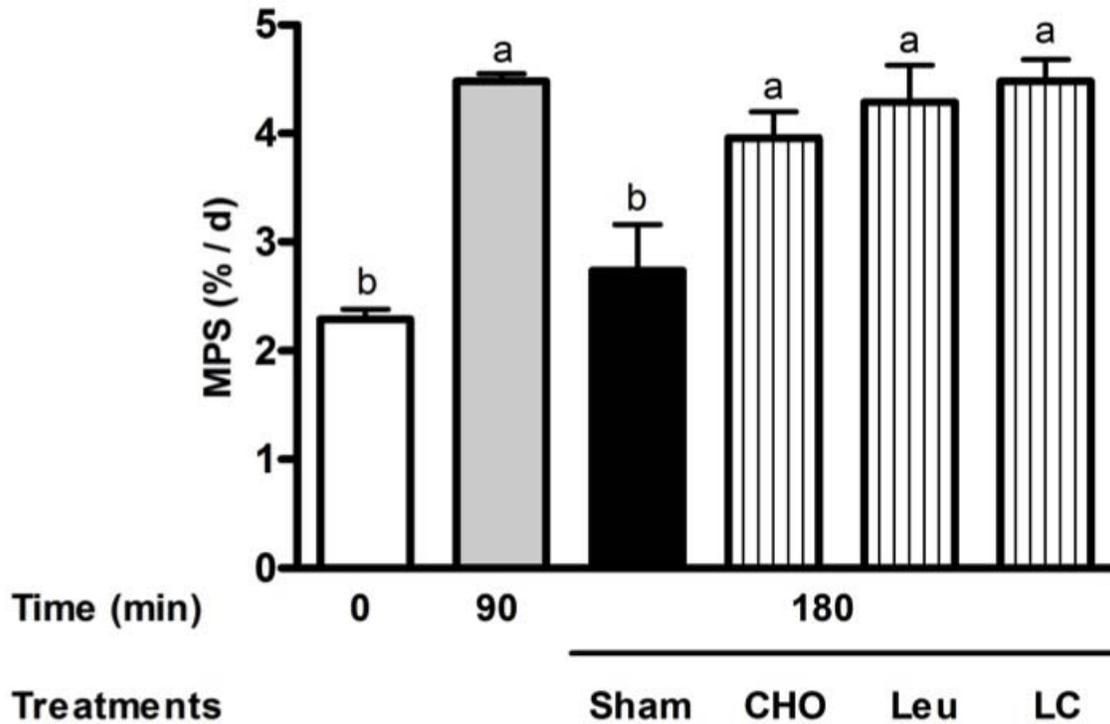
Figure 4.1. Muscle protein synthesis after 4g test meal; treatment groups were provided gavage 135 min after the meal as water (sham), carbohydrate (CHO), leucine (Leu), or a combination of leucine and carbohydrate (LC). Data are means ± SEM; n = 4-6. Means without a common letter differ, $P < 0.05$
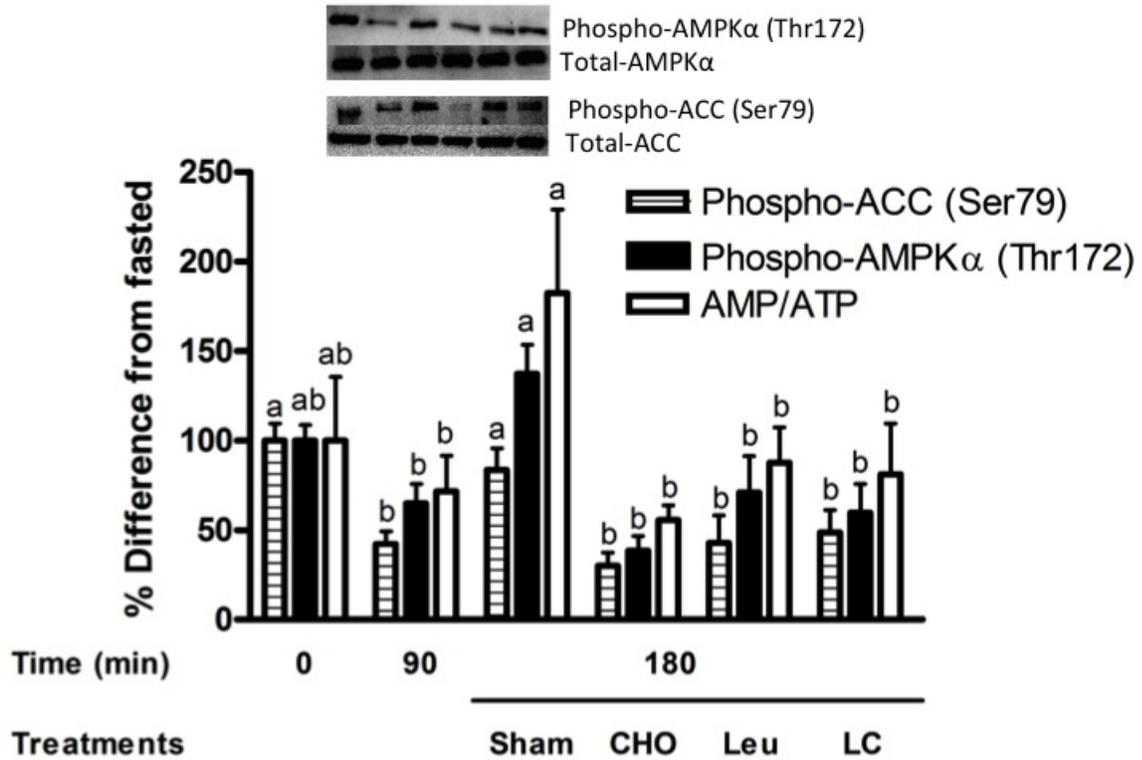
Figure 4.2. Post-prandial ACC activity, AMPK activity, and AMP / ATP ratio after 4g test meal; treatment groups were provided gavage 135 min after the meal as water (sham), carbohydrate (CHO), leucine (Leu), or a combination of leucine and carbohydrate (LC). Data are means ± SEM; n = 4-6. Means without a common letter differ, P < 0.05
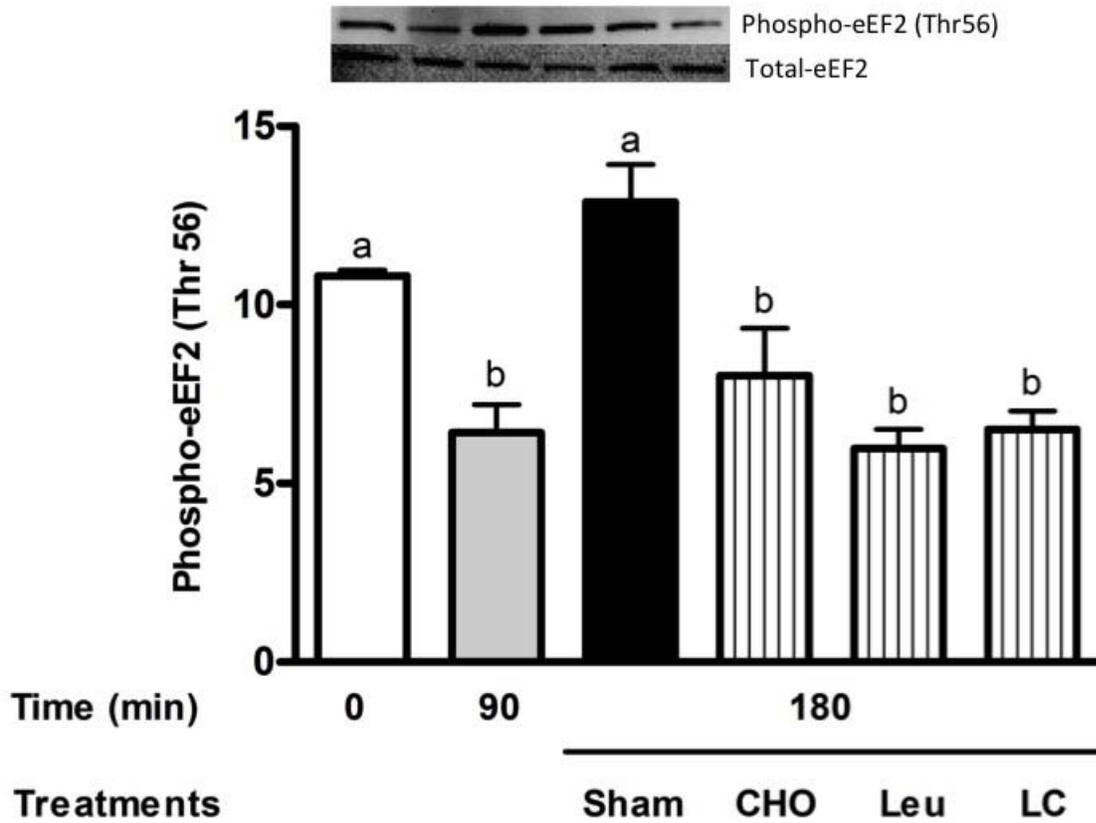
Figure 4.3. Post-prandial eEF2 activity after 4g test meal; treatment groups were provided gavage 135 min after the meal as water (sham), carbohydrate (CHO), leucine (Leu), or a combination of leucine and carbohydrate (LC). Data are means ± SEM; n = 4-6. Means without a common letter differ, P < 0.05
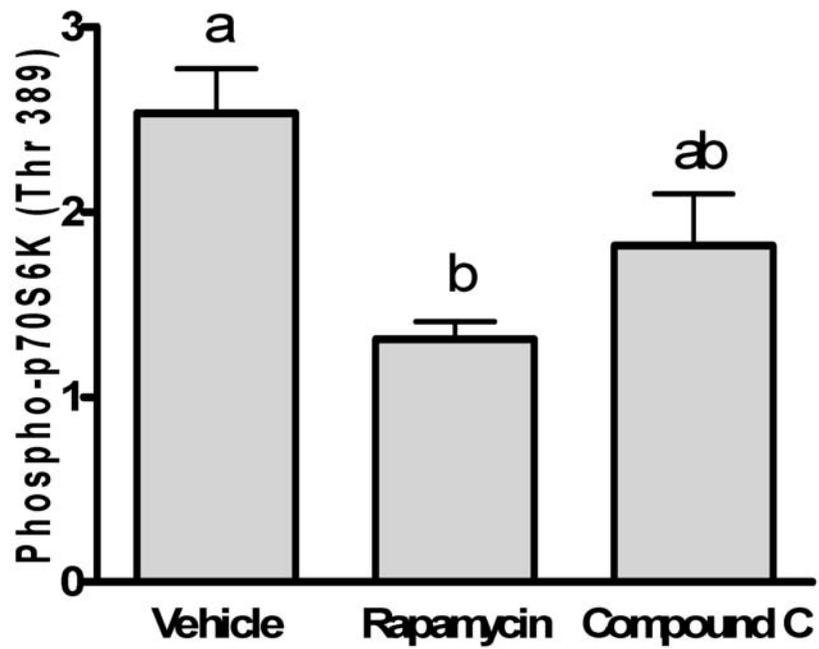
Figure 4.4. p70S6K activity 180 minutes after 4g test meal; treatment groups were injected intraperitoneally with 0.9% saline (vehicle) or rapamycin 120 min prior to test meal or with compound C 60 min after test meal. Data are means ± SEM; n = 3-4; labeled means without a common letter differ, P < 0.05
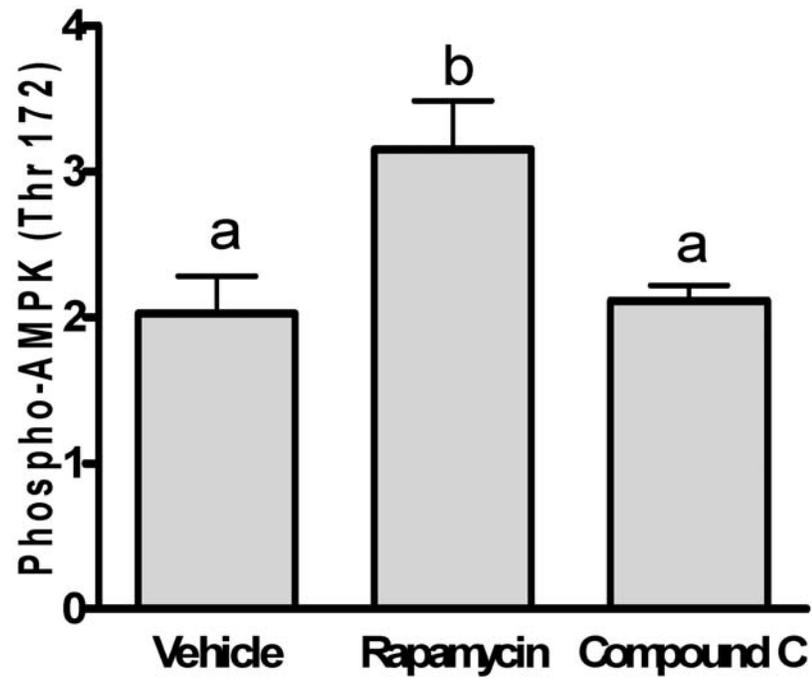
Figure 4.5. AMPK activity 180 minutes after 4g test meal; treatment groups were injected intraperitoneally with 0.9% saline (vehicle) or rapamycin 120 min prior to test meal or with compound C 60 min after test meal. Data are means ± SEM; n = 3-4; labeled means without a common letter differ, P < 0.05

Table 4.1. *Diet composition*

Diet Component	g / kg
Whey protein isolate	228.0
Cornstarch	290.0
Maltodextrin	134.1
Sucrose	101.5
Soybean Oil	140.9
Cellulose fiber	53.7
Mineral mix	37.6
Vitamin mix	10.7
Choline bitartrate	2.7
t-butylhydroquinone	0.014

Sources of whey, minerals, and vitamins

Table 4.2. *Time course changes in plasma leucine in rats fed a complete meal followed by leucine-free gavage*

	Fasted	Fed	Sham
Time after meal (min)	0	90	180
Leucine (umol / L)	200 ± 18^a	297 ± 31^b	312 ± 19^b

Values represent Means $\pm$ SEM, n=4-6. Labeled means without a common letter differ, $P < 0.05$.

Table 4.3. *Plasma leucine in rats following treatment gavage*

	Sham	CHO	Leu	LC
Time after meal (min)	180	180	180	180
Leucine	312 ± 19^a	224 ± 40^a	1375 ± 122^c	671 ± 74^b

Values represent Means $\pm$ SEM, n=4-6. Labeled means without a common letter differ, $P < 0.05$.

CHAPTER V:
SUMMARY AND SPECULATIONS

Protein is known to be essential for maintenance of skeletal muscle and other tissues, but recent research suggests that the quantity and quality of dietary protein may play an equally important role in the skeletal muscle health of adults. An expanding body of literature indicates that moderate substitution of dietary carbohydrate with protein leads to improvements in body composition, including maintenance of lean mass and reduction of fat mass (7). The benefits of increased dietary protein are frequently attributed to greater satiety (8) or to diet-induced thermogenesis (DIT) (12), but these mechanisms disregard changes in skeletal muscle and body composition that may be associated with the ability of dietary protein and / or carbohydrates to act as signaling molecules that regulate metabolism.

This dissertation sought to investigate the metabolically-active attributes of dietary protein and to determine the influence of these responses on body composition. In support of previous research, adjustments in the dietary carbohydrate / protein ratio altered peripheral insulin signaling and hepatic regulation of glycemia, which affected adipose mass and skeletal muscle mass. The impact of dietary protein was isolated by demonstrating that the leucine enrichment of a protein source determines its ability to promote addition of muscle mass and to increase energy expenditure. Finally, it was demonstrated that leucine-induced stimulations of muscle protein synthesis deplete cellular energy via translation elongation, and that daily repetitions of this response induce mitochondrial biogenesis in response to the increased energy demand. These results provide additional insight into the mechanisms by which dietary protein can improve body composition and overall health in adults.

DIETARY PROTEIN IMPROVES BODY COMPOSITION

Clinical trials have shown that moderate substitution of dietary protein for carbohydrate can positively influence body composition. These findings were confirmed in Chapter II. Healthy, adult rats were fed three complete meals under isocaloric

conditions for 25 d with 3 different carbohydrate / protein ratios: 4.8 (HCLP), 1.3 (MCMP), or 0.6 (LCHP). Animals in the LCHP group had significantly less epididymal fat and significantly larger gastrocnemius muscles than the other two groups. These body composition outcomes were associated with an increased capacity for adipogenesis in both liver and adipose in the HCLP group, which was coupled with insulin insensitivity by skeletal muscle. Conversely, the LCHP group had a reduced capacity for adipogenesis and improved insulin sensitivity by muscle. These results provide further evidence that exchange of protein for dietary carbohydrate can provoke favorable responses in peripheral metabolism, which can result in positive body composition outcomes within a relatively short period.

DIETARY LEUCINE IMPROVES BODY COMPOSITION

A potentially important aspect of dietary protein's capacity to improve body composition lies in its leucine content. The branched-chain amino acid (BCAA) leucine has a unique ability among dietary amino acids to stimulate muscle protein synthesis (MPS) (4). Our research group has shown that the leucine content of a meal at least in part determines the degree to which MPS is stimulated, based on the capacity of the meal to elevate post-prandial plasma leucine. The acute response to leucine has been well-characterized, but it is not known if repeated stimulations of MPS can have an impact on body composition over time by promoting accretion of muscle mass.

Adult rats were fed three complete meals under isocaloric conditions with diets of identical macronutrient components, but with different protein sources (wheat gluten, 6.8% leucine; or whey protein isolate, 10.9% leucine) for 11 wks (Chapter III). Previous results with this model indicated the existence of a "leucine threshold" of approximately 60 mg per meal beyond which optimal MPS is stimulated, but below which little or no MPS stimulation occurs (10). The meals were designed such that the WHEAT group would reach the leucine threshold once per day, while the WHEY group would reach the leucine threshold three times per day. As anticipated, after 11 wks of feeding the WHEY group had larger gastrocnemius muscles. Additionally, the WHEY animals had a significantly lower body fat % and larger epididymal fat pads. It is noteworthy that the

dietary differences in leucine had an effect on adipose tissue, which suggests that chronic responses in muscle provoked a secondary effect in adipose.

Further analysis revealed that the WHEY group retained 40% less dietary energy over the 11 wks compared to the WHEAT group, and additionally WHEY animals preferentially partitioned dietary energy to lean tissue, while WHEAT animals preferentially partitioned dietary energy to fat. This was surprising considering the equal energetic input among both groups, which leads to the conclusion that the WHEY diet induced a greater degree of DIT compared to the WHEAT diet.

DIETARY LEUCINE AFFECTS MITOCHONDRIA

In response to the dramatic differences in energy efficiency and partitioning in the WHEAT vs. WHEY experiment, it was hypothesized that the increased DIT in the WHEY group was due to increased energy consumption by MPS activity. In such a scenario, it would be logical that the cell might adapt over time in order to support the increased demand for energy. Leucine stimulates synthesis not only of sarcoplasmic proteins, but mitochondrial proteins as well (5), and leucine has been shown to stimulate the adaptive response of mitochondrial biogenesis in vitro (13) and in vivo (3). In Chapter III, selected genes related to mitochondrial biogenesis were analyzed, revealing that the WHEY diet induced PGC-1α, Tfam, and cytochrome B in muscle in comparison to the WHEAT diet. This apparent induction of mitochondrial biogenesis was likely in response to a greater demand for cellular ATP to support the additional stimulations of MPS. However, it was not yet known if dietary leucine could acutely deplete cellular energy to the extent that would provoke such a response.

POST-PRANDIAL NUTRIENTS IMPACT CELLULAR ENERGY

Protein synthesis is an energetically costly process, accounting for 30-50% of cellular energy expenditure (2). Based on previous results, it was hypothesized that leucine-induced MPS activity would substantially deplete cellular energy during peak activity. This response was evaluated in adult rats fed a 20% protein test meal that was known to optimally stimulate post-prandial MPS (Chapter IV). At 90 min following the

meal, the AMP / ATP ratio was slightly depressed, indicating a surfeit of energy substrate. However, 90 min later (180 min post-meal), the AMP / ATP ratio was 2.5- fold higher, indicating a depletion of cellular energy. This response was reflected by activity of AMPK kinase, which is a key energy sensor that is sensitive to the AMP / ATP ratio (6). MPS activity was elevated at 90 min post-meal, but after 180 min it had returned to baseline. This is in agreement with other studies demonstrating that leucine-induced elevations last < 3 hrs (1, 10).

Translation elongation has been reported to be a likely candidate for energy-sensitive regulation of protein synthesis, since it consumes 99% of the energy required by the process (2). The activity of translation elongation factor eEF2 was depressed at 180 min post-meal, corresponding with a high AMP / ATP ratio and AMPK activity. Evidence suggests that AMPK can inhibit translation elongation via AMPK (11). This data suggests that leucine-induced activation of MPS results in a temporary cellular energy deficit that is sensed by AMPK kinase, which then inhibits subsequent translation elongation in order to prevent over-depletion of cellular energy. This hypothesis was supported by the ability of post-meal supplemental nutrients in the form of carbohydrate or leucine to overcome the MPS-associated reduction of cellular energy, thusly providing more energy for translation elongation to continue.

LIMITATIONS AND FUTURE DIRECTIONS

The data presented in this dissertation are not without limitations. In Chapter III, it appears likely that DIT was truly different between the WHEAT and WHEY groups. However, resting energy expenditure and physical activity were not appropriately assessed to ensure that the different patterns of energy partitioning were truly a result of enhanced DIT in WHEY. Future experiments assessing the potential for dietary leucine to uniquely affect thermogenesis should consider these measurements. Additionally, the WHEAT vs. WHEY experiment was designed to assess the ability of dietary leucine to influence muscle tissue mass, and so it was unexpected to subsequently determine that mitochondrial function was influenced by the leucine content of the diet. Present results provide evidence that a series of genes related to mitochondrial biogenesis were

induced in muscle by WHEY, and indeed these are compulsory for the process (9), but in order to definitely identify that mitochondrial biogenesis has occurred, numerous data regarding initiation stimuli, gene transcription and expression, and physical measurements of the mitochondrial structure should be shown. An attempt to characterize induction of certain mitochondrial proteins was undertaken, but the tissues had been stored for ~2 years before these were determined to be useful measurements, and unfortunately the reduced integrity of the tissues yielded no results. Therefore, a need exists for future experiments to consider numerous measurements, including tissue microscopy, to confirm that dietary leucine truly affects the status of mitochondria.

CONCLUSION

In summary, this dissertation has shown that dietary protein and leucine favorably impact body composition. Repeated consumption of leucine-rich meals stimulates MPS and promotes accretion of new skeletal muscle. These leucine-induced activations of MPS result in a temporary deficit of cellular energy, which may regulate the duration of related translation elongation activity to spare energy for essential cellular processes. The repeated depletions of cellular energy following chronic consumption of leucine-rich whole meals appear to be sensed by an unknown mechanism, which initiates mitochondrial biogenesis to increase the capacity for ATP production in order to meet the additional energy demand of translation elongation. This expansive use of cellular energy may result in substantially greater energy consumption at the organism level, therefore comprising an as-of-yet unidentified component of dietary thermogenesis related to protein consumption, which is depicted in Figure 5.1. These findings and speculations provide additional insight into the means by which dietary protein positively influences body composition.

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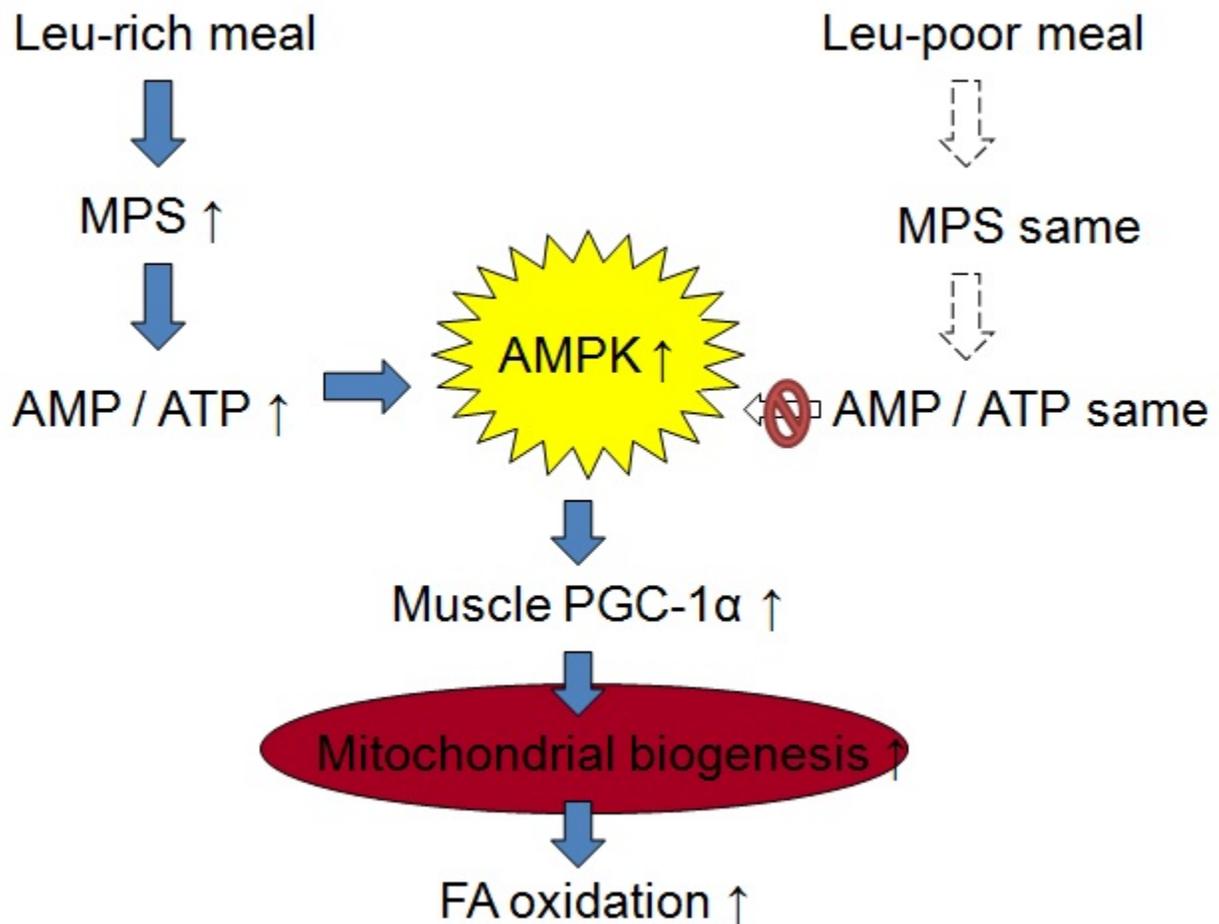
Figure 5.1. A proposed model accounting for the high relative diet-induced thermogenesis of protein; consumption of protein-containing meals that are appropriately enriched in leucine stimulates MPS, which temporarily depletes cellular energy and activates AMPK. When the process is repeated over time, the transcriptional activity of PGC-1α is elevated, which stimulates mitochondrial biogenesis. The capacity for fatty acid oxidation is thereby increased to provide the ATP required to support MPS activity. In addition to accretion of lean muscle mass, the MPS-associated energy demand affects whole body energy partitioning and improves body composition. Consumption of meals that are not adequately enriched in leucine fails to elevate MPS activity, such that cellular energy is not impacted. Therefore, the AMPK-PGC-1α-mitochondrial axis is not induced, and the positive effects on energetics and body composition are not realized.