HUMAN OBESITY AND ITS INFLUENCE ON MUSCLE PROTEIN SYNTHESIS

BY

JOSEPH WILLIAM BEALS

DISSERTATION

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Doctoral Committee:

Professor Kenneth R. Wilund, Chair
Assistant Professor Nicholas A. Burd, Director of Research
Professor Jeffrey A. Woods
Professor Stuart M. Phillips, McMaster University

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ABSTRACT

Improving skeletal muscle health is an important component of obesity treatment. Apart from locomotion, skeletal muscle tissue is fundamental for the regulation of macronutrient metabolism during the postprandial period, which is precisely where metabolic derangements are most often observed. In order for the skeletal muscle to adapt and retain its capacity for high throughput of macronutrients, damaged proteins must be degraded and replaced on a continual basis. Moreover, amino acids from meals are crucial for the muscle to replace those lost for other needs (e.g. gluconeogenesis and oxidation). Skeletal muscle appears to be more responsive to amino acid replacement in normal-weight than the muscle of obese individuals. However, no studies have assessed the impact of obesity on the muscle protein synthetic response to the fundamental anabolic stimuli (muscle contraction, protein ingestion) to human skeletal muscle tissue. Previous studies of obesity and muscle protein metabolism have employed intravenous amino acid infusions, which do not accurately reflect meal conditions. Therefore, this thesis details investigations that assessed muscle protein synthetic responses in both the myofibrillar and sarcoplasmic protein pools under a typical meal setting where a protein-dense food is consumed orally either at rest or after exercise. In study 1, we showed that the postprandial myofibrillar protein synthetic response to protein-dense food ingestion is blunted in overweight and obese compared with normal-weight adults. This finding was related to altered mTORC1 signaling in those groups. In study 2, we demonstrated that basal and postprandial mitochondrial protein synthesis rates are similar in young adults across a wide range of body mass indices. We also showed that muscle inflammatory protein content (e.g. TLR4 and MyD88) increases in response to protein-dense food ingestion in obese, but not normal-weight and overweight young adults. In study 3, we demonstrated that the resistance exercise-induced potentiation of postprandial

myofibrillar protein synthesis rates is diminished in obesity young compared with normal-weight adults. However, resistance exercise blunts the obesity-related increase in TLR protein after protein-dense food ingestion. The studies contained in this dissertation show an anabolic resistance to protein-dense food ingestion in obese adults that appears to be limited to the myofibrillar protein sub-fraction of skeletal muscle. Our findings suggest that contractile protein remodeling is a primary impairment in muscles of people with obesity and that exercise strategies to overcome this anabolic resistance are needed.

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CHAPTER I

GENERAL INTRODUCTION

1.1 INTRODUCTION

Presently, over one-third of Americans are obese (1). Recent predictions expect that number to rise to over 50% by 2030 if current trends continue (2). There are several chronic diseases associated with the rise in obesity [e.g. cancer, diabetes (3)], which may explain why estimates for these developments include an $860 billion price tag in health care costs (2). These numbers are staggering given that obesity is associated with increased mortality risk (4) and is likely somewhat modifiable based on simple behavior changes such as increased habitual physical activity and/or decreased physical inactivity (5).

1.1.1 Skeletal muscle and human health

Besides the clear role skeletal muscle plays in physical activity and activities of daily living, much of the muscle's contribution to health is with regard to macronutrient metabolism (6). Skeletal muscle is the primary determinant of meal-derived glucose uptake (7) and lipids (8). Further, skeletal muscle protein synthesis also utilizes a major portion of meal-derived amino acids (AA) released into systemic circulation (9,10). Changes in skeletal muscle macronutrient metabolism during the postprandial period can have profound effects on disease risk (11). Altogether the skeletal muscle health has widespread implications for whole-body health.

1.2 MUSCLE PROTEIN SYNTHESIS

Various methods have been used to estimate muscle protein synthesis rates *in vivo* in humans. The most common methods apply stable isotope tracers (e.g. amino acids or labeled water/deuterium oxide (D_2O)] to estimate muscle protein fractional synthetic rates, a direct measurement of muscle protein synthesis, and include: primed constant infusion methods (12,13), flooding dose methods (14), and D_2O methods (15). Each method strives to create an equilibrium in the precursor pool and subsequently measures the change in muscle bound tracer enrichment over time. Depending on the precursor pool used in the calculation will determine whether the upper or lower limit of muscle protein synthesis is determined. Our general understanding of the regulation of muscle protein synthesis rates *in vivo* in humans has been based on this methodology.

The pool of muscle protein is maintained by a delicate balance of synthesis vs. breakdown, however, protein synthesis appears to be more highly modifiable during periods of increased plasma amino acid availability as compared to protein breakdown (16–18). Proteins in the skeletal muscle are degraded for a variety of reasons including changes in tissue demands (19,20) or they become damaged (21–23). Moreover, as the body's largest pool of amino acids, the muscle provides gluconeogenic precursors to other tissues during a fast (24,25) and other more extreme catabolic situations (26).

Protein (amino acids) and exercise have been shown to be two primary anabolic stimuli to human skeletal muscle tissue. Of these, protein ingestion is particularly important as it also provides the necessary substrate [amino acid (AA)] for synthesizing new proteins. Muscle protein synthesis has been studied in a variety of settings and after consuming a variety of protein sources (27–33). Dose-response studies have shown that approximately a 20-40 g ingested dose of high quality protein is sufficient to maximize the muscle protein synthetic response during the

postprandial period, depending on the condition and population studied (17,34–37). Logically, it appears that the essential AAs, rather than the non-essential AAs, are primarily responsible for the stimulation of postprandial muscle protein synthesis rates (38).

The synthesis of the entire muscle proteome (i.e. mixed muscle protein synthesis), while useful, may not always provide a clear depiction of the muscle anabolic response relevant for certain phenotypic adaptations during the postprandial period. The measured rates of mixed muscle protein synthesis obtained are essentially a weighted average of each individual protein in the pool. To provide greater insight to how specific muscle protein pools are remodeling, numerous labs have investigated the stimulation of protein synthesis in sub-fractions of the skeletal muscle (e.g. myofibrillar, mitochondrial, sarcoplasmic) (39–41). In support of these methods, it has been shown that rates of protein synthesis are not equivalent across sub-fractions of the skeletal muscle (41,42). More importantly, muscle sub-fractional protein synthesis rates are differentially responsive to common stimuli [e.g. insulin, exercise (39,40,43)] and may be differentially impacted based on the population studied (44). When considering data involving the synthesis of specific muscle protein sub-fractions, it is important to recognize that these should not be considered purified and that each sub-fraction will likely contain small amounts of protein from one or more other protein pools; because of this, protein sub-fractions are often referred to as ‘enriched’.

1.2.1 Anabolic signaling mechanisms

The intracellular regulation and cascade of anabolic signaling is an extremely complex process and is an area of active research. For excellent reviews readers are directed to the following articles (45,46). The mechanisms relevant to studies contained within this dissertation are provided here (**Figure 1.1)**.

Insulin is the predominant hormone during the postprandial period and is secreted into circulation in response to protein ingestion in healthy individuals (47). In the skeletal muscle, insulin has an important role in stimulating the use of amino acids for protein synthesis via protein kinase B (Akt) activation and the subsequent phosphorylation and activation of mammalian target of rapamycin complex 1 (mTORC1) (48). The relationship between insulin and protein synthesis appears to mostly provocative rather modulatory for mixed muscle protein synthesis rates during an infusion of amino acids and insulin (43). This may be less true with regard to mitochondrial protein synthesis rates during hyperaminoacidemia, where high levels of insulin are potentially required to stimulate synthesis within this fraction (39,49). These discrepancies in the direct relationship between insulin and mTORC1 remains to be revealed. In addition, amino acids, and leucine in particular, are also involved in the activation of mTORC1 after protein ingestion (50–54). Moreover, hyperinsulinemia is unable to stimulate mTORC1 signaling without concurrent hyperaminoacidemia (54).

Elevated amino acids, primarily leucine, in the cell can facilitate aspects of mTORC1 activation by causing the dephosphorylation of the protein Sestrin2, through an unknown mechanism (50). This dephosphorylation allows the GTPase activator protein (GAP) activity toward Rags 2 (GATOR2) to complex with and activate mTORC1(50). Amino acids enter the cell through a coordination of several transporters (46). Because of the importance of leucine-mediated anabolic signaling, much of the research has focused on how this amino acid is transported. Leucine, among others, is transported by the large neutral amino acid transporter (LAT1) and CD98 (SLC3A2), which simultaneously antiport glutamine (46). The small neutral amino acid transporter (SNAT2) assists with maintenance of the glutamine gradient. These transporters increase in response to

essential AA ingestion (55). However, it is not known how this response is affected by excess fat mass.

The primary regulation of mTORC1 activation is through allosteric regulation by several proteins. For example, when the protein regulated in development and DNA damage response 1 (REDD1) allosterically binds mTORC1 it lowers its kinase activity (56,57). Other proteins, when co-localized with mTORC1, lead to increased kinase activity, such as Rheb (58) and Gator2 (50). Binding of these regulatory proteins coordinates the sub-cellular location of mTORC1 and therefore its activity (60–62). Activation of mTORC1 kinase activity is considered a central event in coordination of ribosomal assembly and synthesis of protein (62,63). Downstream targets of mTORC1 are numerous, however the most extensively studied is perhaps the 70 kDa Ribosomal protein S6 kinase (p70S6K), which facilitates activation of ribosomal protein S6 (64–68). Importantly, these anabolic pathways are activated by both amino acids and insulin (51,69,70). While this is an abridged discussion of anabolic signaling mechanisms above, it should be mentioned that each of the proteins described are regulated by multiple kinase reactions and have numerous residues that are phosphorylated during peak activation. With respect to mTORC1 it appears that phosphorylation of serine 2448 is regulated by p70S6K (71,72) as a potential feedback mechanism, but it is unclear if amplifies and dampens mTORC1 activity. Regardless, p70S6K phosphorylation at threonine 389 appears to be a nutrient-sensitive step for its activation (67). Moreover, it is still unknown how anabolic signaling events coordinate the synthesis of specific muscle protein fractions such as myofibrillar vs. sarcoplasmic proteins. In fact, recent evidence suggests the mTORC1 dynamic events are more relevant and specific towards coordinating the synthesis of myofibrillar proteins (59).

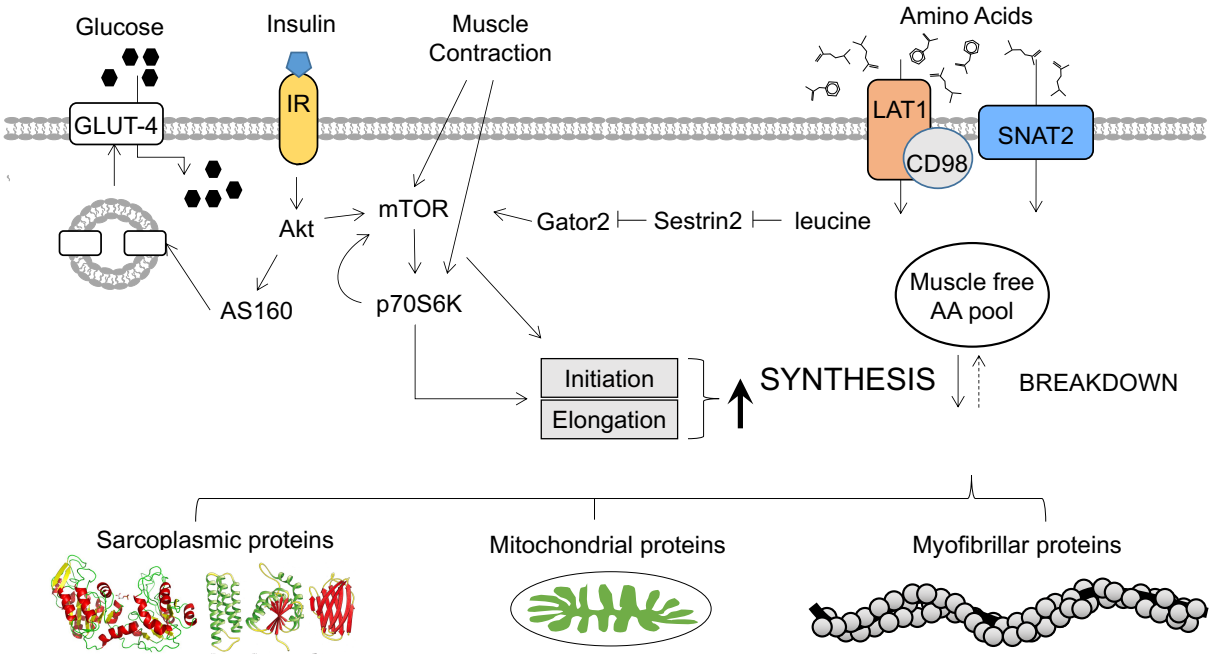

Figure 1.1 Anabolic signaling in the skeletal muscle. → indicates a stimulation. ⊥ indicates an inhibition.

1.3 MUSCLE PROTEIN SYNTHESIS IN OBESE HUMANS

Obesity is characterized by several well-known impairments in macronutrient metabolism in the skeletal muscle; specifically, altered regulation of blood glucose and lipids (73). However, stimulation of skeletal sub-fractional muscle protein synthesis rates during the postprandial period also appear to be altered in obese individuals as compared to controls, but not all studies are in agreement (74–77). Discrepancies between studies may be related to a lack of standardized participant grouping, muscle protein sub-fractional analysis, and/or simulated postprandial clamp conditions (i.e. hyperinsulinemic, hyperaminacidemic) among study designs.

Basal rates of muscle protein synthesis do not appear to be affected by obesity. Basal muscle protein synthesis has been assessed in obese individuals in mixed muscle (76,77) and within the myofibrillar (74,75), sarcoplasmic (75), and mitochondrial (76) protein sub-fractions.

Of these reports, only Guillet *et. al.* (76) reported differences in basal muscle protein synthesis rates in both mixed muscle and the mitochondrial protein sub-fractions as compared to non-obese individuals, which may be related to the inclusion of overweight individuals in the non-obese group.

How the stimulation of muscle protein synthesis rates responds to oral protein-dense food ingestion in obese in comparison to normal-weight adults is currently unknown. One study to date fed obese participants isolated soy or whey proteins and demonstrated a stimulation of myofibrillar protein synthesis rates above basal, but these data were not compared to those of lean individuals (78). However, data from clamp studies yields good insight as to how this comparison might look. During hyperinsulinemic, hyperaminoacidemic clamp conditions, it has been shown that obese men exhibit lower rates of muscle protein synthesis [mixed muscle: (76), myofibrillar: (74), mitochondrial: (76)] when compared to controls. However, another group showed no differences between obese and normal-weight men during similar clamp conditions with respect to both myofibrillar and sarcoplasmic protein synthesis rates (75).

Currently, we are aware of two studies that directly compared rates of *myofibrillar* protein synthesis rates between normal-weight and obese humans (74,75). The results of these studies differed in that one showed no differences between groups during hyperinsulinemic, hyperaminoacidemic conditions (75) and the other showed that obesity is associated with reduced myofibrillar protein synthesis rates (74). The latter study also demonstrated that during simulated postprandial conditions obese individuals have reduced rates of leg muscle protein breakdown. However, it is important to consider that in both of these experiments, obesity is overlaid with an aging component, which may complicate interpretation.

Altogether, these studies indicate that human obesity is associated with impaired muscle protein anabolism during the postprandial period, but most likely not during basal, fasted conditions. When a protein-dense meal is ingested, it is rapidly digested by proteolytic enzymes contained in the pancreatic juice secreted into the duodenum (79). Amino acids are transported to the liver and the AAs that survive first pass splanchnic extraction become available in systemic circulation (9). These actions lead to a time course of amino acids in systemic circulation whereby AA concentrations increase during the early postprandial period (30-180 min) and fall off as the meal becomes fully digested. This same trend is witnessed when observing insulin concentrations after protein ingestion in healthy adults (47). By contrast, during clamp conditions the concentrations of AA and insulin are 'clamped' at either supraphysiological or hyperphysiological levels over an extended period of time, which may have unintended consequences on muscle protein synthesis rates. In fact, it has been shown that the plasma amino acid profile during the postprandial period may be an important determinant of postprandial muscle protein synthesis (80). Clamp conditions also bypass the digestive tract, which plays several important roles in stimulating uptake of nutrients from a meal into peripheral tissues (81–83). Nevertheless, several differences in postprandial protein metabolism in the skeletal muscle of obese compared with non-obese individuals have been uncovered in the studies discussed above. However, the impact of obesity on the muscle anabolic response to protein-rich food ingestion remains to be explored. These needs are addressed in both **Chapter 2 and 3**. Below we will discuss some commonly observed metabolic disturbances in obesity and how these mechanisms may be related to impaired muscle protein synthesis during the postprandial period in these individuals.

1.4 INSULIN AND ANABOLIC RESISTANCE OF MUSCLE PROTEIN SYNTHESIS

In the fasted state, obese individuals display hyperinsulinemia, hyperglycemia, and hyperlipidemia (84). Extended hyperinsulinemia, due to insulin resistance, is a well-established consequence of meal ingestion in obese individuals as compared to non-obese individuals (85). Resistance to the insulin is historically uncommon in the literature, initially represented by case studies (86). However, by the 1950s it was recognized that some diabetics were less responsive to insulin and these individuals also have several other chronic disease risk factors such as atherosclerosis and hypertension (87). This study also represents the first time that insulin resistance was associated with being overweight or obese (87). Most available data concerning insulin resistance is with regard to glucose and lipid metabolism, but as noted earlier, insulin is pivotal for the stimulation of protein anabolism as well.

While insulin resistance is not entirely understood, many of the key steps in its pathophysiology have been characterized. Much of the alterations of the insulin signaling cascade appear to be related to changes in intracellular lipid metabolites (88,89). Lipid-induced insulin resistance is largely due to the increased formation of the sphingolipid, ceramide, is a well-known contributor to insulin resistance (90). With respect to protein metabolism, muscle cells *in vitro* treated with ceramide have diminished amino acid transport and reduced muscle protein synthesis; these effects are potentially related to reduced phosphorylation of Akt and p70s6k in those cells compared with those treated with vehicle (91). This may be mediated in part through TLR4 signaling, discussed below in 1.5 and shown in **Figure 1.2**.

Infusions of lipids with heparin, which increases non-esterified fatty acids in plasma, can blunt skeletal muscle glucose uptake even in healthy, insulin sensitive subjects (92). A similar effect is observed when myofibrillar protein synthesis is measured during lipid infusions (93).

Under hyperinsulinemic, euglycemic conditions, a bolus of protein did not stimulate myofibrillar protein synthesis rates in healthy male volunteers during an infusion of lipids, compared with an approximate 2-fold increase when no lipids were infused (93). During this experiment lipid infusion resulted in changes in anabolic signaling necessary for ribosomal biogenesis, specifically Akt and 4EBP1 phosphorylation. However, another similar experiment conducted earlier did not demonstrate these differences in mixed muscle protein synthesis rates when participants ingested a small bolus of essential AAs (94). These differences may be related to muscle protein sub-fractional analysis (i.e. myofibrillar vs mixed muscle protein). The impact of chronically elevated plasma NEFA with obesity on muscle protein synthesis has not been examined in humans.

Intriguingly, rodent obesity has shown to be associated with increased mTORC1 signaling in the fasted-state (57,95,96). The reason for this is not known, but human obesity is associated with increased anabolic stimuli (e.g. insulin and leucine) in the fasted-state (97), both of which could lead to greater mTORC1 signaling. In addition, it is possible that elevated NEFA, specifically palmitate, are contributing to greater basal levels of anabolic signaling with obesity in these animals as well (68). These findings suggest an additional mechanism for insulin resistance could be feedback inhibition of insulin signaling (95). Unfortunately, the rates of muscle protein synthesis in these animal studies were not measured. Increased basal mTORC1 activity has yet to be confirmed by current studies in obese humans, where no baseline differences from controls have been observed (74,75). Downstream of mTORC1, p70S6K phosphorylation may also be altered in participants with insulin resistance (67), where the phosphorylation of p70S6K at thr389 is less responsive to protein-rich meal ingestion as compared to healthy controls.

1.5 MUSCLE INFLAMMATION AND MUSCLE PROTEIN SYNTHESIS

Obesity is associated with chronic low-grade inflammation (98). This inflamed state has been linked to impaired glucose tolerance (99) and lipid metabolism (100). Because of this, inflammation in the skeletal muscle has been suggested to contribute to impaired protein anabolism in obese individuals (101). Rodent studies of inflammation are centered on the increased inflammation associated with aging, but discussion of these animals provides relevant insight as they have similar inflammatory milieu as witnessed with obesity [e.g. elevated interleukin-6 (IL-6), tumor necrosis factor α (TNFα) (102,103)]. Old rats with chronic low-grade inflammation have an impaired muscle protein synthetic response to protein ingestion as compared with non-inflamed rats (103). Another study demonstrated that in old rats with chronic inflammation, ibuprofen administration restores the normal increase in muscle protein synthesis after protein ingestion compared with inflamed control rats (104). Taken together, these studies suggest that inflammation may be a promising target for addressing the postprandial anabolic impairments with obesity. Data in humans with inflammation thus far is limited and apparently absent in obese humans.

Relatively few studies have attempted to describe the muscle protein synthetic response to protein ingestion in humans with low-grade inflammation (105,106). In healthy older men stratified by plasma C-reactive protein (CRP) concentrations, postprandial mixed muscle protein synthetic rates were not different between groups (105). Another group in a placebo-controlled manner tested the effect of acute ibuprofen administration on basal and postprandial myofibrillar protein responses to whey protein ingestion in older men with elevated CRP and compared these responses to those of healthy non-inflamed older men (106). In that study, basal and postprandial myofibrillar protein synthesis were not different between ibuprofen and placebo groups. Moreover, rates of myofibrillar protein synthesis were similar between the placebo group with elevated CRP

and the non-inflamed controls (106). Others have sought to isolate the effects of acute administration of purported inflammatory markers in healthy adults (107,108). In one experiment, fasted rates of mixed muscle protein synthesis were determined in healthy young men before and after participants received an infusion of tumor necrosis factor α (TNFα); no differences in protein synthesis rates were observed. More data is needed before any conclusions can be made on the impact of low-grade inflammation on the muscle anabolic response to protein ingestion, particularly in obese adults.

Within skeletal muscle, toll-like receptor 4 (TLR4) protein correlates with body fat percentage in older adults (109) and has been implicated in the development of muscle insulin resistance (110,111). This receptor is part of innate immunity and binds NEFA in circulation (110). TLR4 recruits several intracellular proteins for signal transduction, among these is myeloid differentiation factor 88 (MyD88), which appears to be involved in the development of TLR4-induced insulin resistance (110,111). Moreover, this receptor has been shown to be responsible for the generation of ceramides, which may interfere with anabolic signaling in the skeletal muscle (**Figure 1.2**). However, the above data is from cell and rodent models and therefore needs confirmed by human studies.

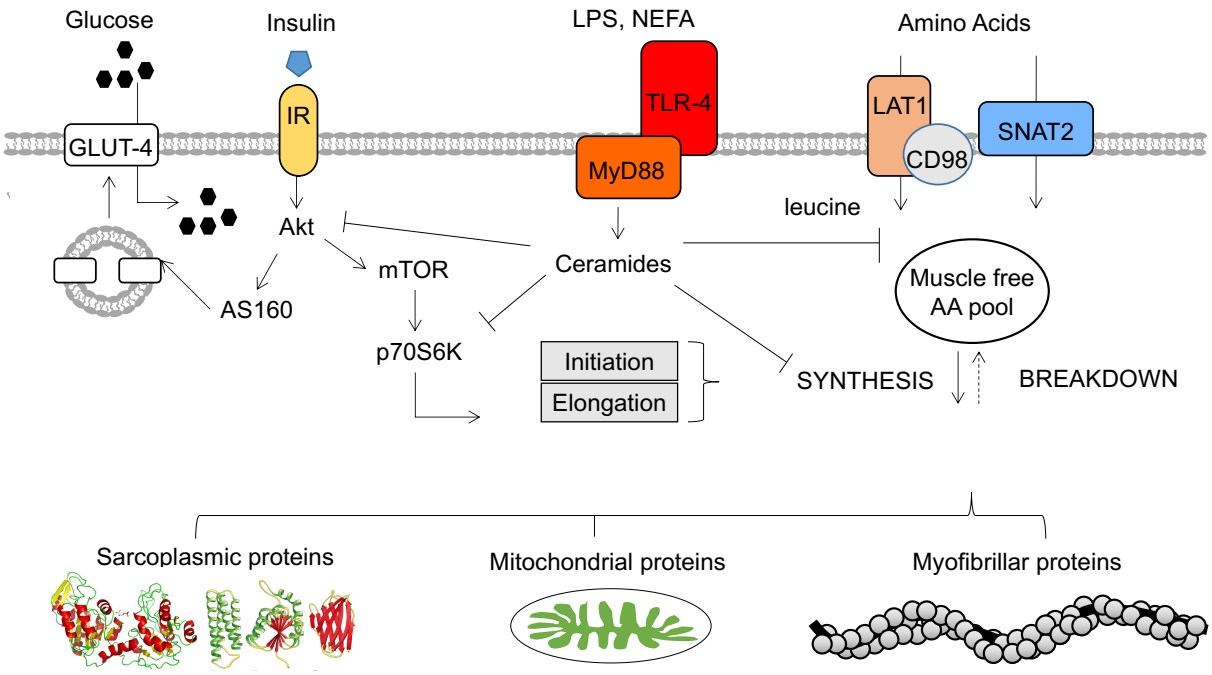

Figure 1.2 The potential role of inflammation on anabolic signaling in the skeletal muscle. → indicates a stimulation. ⊥ indicates an inhibition.

1.6 ACUTE RESISTANCE EXERCISE AND MUSCLE PROTEIN SYNTHESIS

Resistance exercise is a strong stimulus that results in dramatic alterations of macronutrient metabolism. For instance, a single bout of resistance exercise is capable of improving glucose tolerance (112) and postprandial lipemia (113) in healthy young men. Further, acute resistance exercise enhances muscle protein synthesis with (32) or without (27) protein ingestion in young adults. Moreover, the consumption of protein immediately after resistance exercise further enhances mixed muscle protein synthesis rates compared with feeding alone in healthy-weight young and older men (32). It appears that the enhancement of the muscle protein synthetic response to protein ingestion after resistance exercise affects the myofibrillar more than the sarcoplasmic protein sub-fraction during recovery (42).

Resistance exercise enhances the amino acid sensitivity of skeletal muscle tissue to food-derived amino acids during recovery; an effect that persists for at least 1-2 days (28,114). What is noteworthy is that there appears to be redundant methods (manipulation of volume and load) to maximize the post-exercise myofibrillar protein synthetic response. In particular, it has been established the bulk muscle fiber recruitment during the acute exercise bout is an important factor to robustly stimulate myofibrillar protein synthesis, and this can be achieved with all sorts of contraction manipulations (114). Despite the available evidence, the efficacy of resistance exercise for stimulating postprandial muscle protein synthesis with obesity has yet to be determined.

Resistance exercise has been studied in order to assess its potential to repair several aspects of the obesity-related metabolic signature. The effect of acute resistance exercise on the myofibrillar protein synthetic response to whey protein ingestion in older adults with low-grade inflammation (elevated CRP) has been previously determined (106). In this study, low-grade inflammation did not modulate post-exercise myofibrillar protein synthesis rates in older men in comparison to non-inflamed controls. Acute resistance exercise has also been shown to improve insulin sensitivity during intravenous insulin (115) and glucose (112) tolerance tests.

Anabolic signaling is profoundly affected by resistance exercise. Mechanical stretch of muscle fibers *in vitro* induces phosphorylation of p70S6K at threonine 389 in an mTORC1-dependent manner (116). Acute resistance exercise augments the stimulation of anabolic signaling (i.e. mTORC1, p70S6K) induced by protein ingestion in young men (66). In support of these data, phosphorylation of p70S6K at threonine 389 in response to a single bout of resistance exercise can predict the training-induced increase in strength and muscle hypertrophy (117) but not always (118).

1.7 STUDIES AND HYPOTHESES TESTED

The purpose of the studies outline in this dissertation was to assess the impact of excess fat mass on the regulation of postprandial muscle protein synthesis rates within the myofibrillar and sarcoplasmic protein fractions. In the following chapters we describe how muscle sub-fractional protein synthesis rates are affected in response to a single bolus of protein-dense food ingestion at rest and after an acute bout of resistance exercise in obese as compared with normal-weight individuals. In addition, we studied the activation of the anabolic signaling mechanisms phosphorylation of mTORC1and p70S6K along the inflammatory pathways related to TLR4/MyD88 within the skeletal muscle.

1.7.1 **Chapter 2:** STUDY 1 – Anabolic sensitivity of postprandial muscle protein synthesis to the ingestion of a protein-dense food is reduced in overweight and obese young adults

Muscle protein synthetic rates in response to consumption of a single bolus of oral protein in obese adults has never been established. Therefore, the purpose of this study (**Ch. 2**) was to determine the muscle protein synthetic response to protein-dense food ingestion across a wide range of body mass indices (BMI). We hypothesized that ingestion of a protein-dense food in an amount representative of a typical American dinner [170 g of lean pork (119)] would be an effective stimulus for anabolic signaling and the postprandial muscle protein synthetic response across the BMI range. We also assessed relevant anabolic signaling mechanisms before and after protein ingestion. This chapter serves as a baseline of understanding for the impact of fat mass on the muscle protein synthetic response to bolus food ingestion.

1.7.2 **Chapter 3:** STUDY 2 – Protein-rich food ingestion stimulates mitochondrial protein synthesis in sedentary young adults of different BMIs

Mitochondrial protein synthesis rates have never been examined in response to the ingestion of a single bolus of protein. The purpose of this work is to examine the impact of protein-rich food ingestion on mitochondrial protein synthesis rates in adults of varying BMIs. We hypothesized that protein ingestion would provide a strong stimulus for mitochondrial protein synthesis in normal- and overweight, but not in obese adults. We also sought to determine the relationship between mitochondrial protein synthesis rates and muscle insulin and inflammatory signaling mechanisms at basal and throughout the postprandial period.

1.7.3 **Chapter 4:** STUDY 3 – Divergent stimulation of muscle protein synthesis and muscle inflammatory responses to feeding and resistance exercise in people with obesity

The effectiveness of resistance exercise, a potent anabolic stimulus, to stimulate postprandial muscle protein rates in obese adults has yet to be examined. In **Chapter 4**, we challenged the hypothesis that an acute bout of resistance exercise prior to protein-dense food ingestion would provide a strong signal to the muscle protein synthetic machinery to stimulate a robust muscle protein synthetic response in obese adults. For this study we used a unilateral resistance exercise model to assess muscle protein synthesis rates at basal and after protein ingestion in both, a non-exercised control (CON), and an exercised (EX) leg in normal-weight and obese young adults. This allowed us to assess the effect of protein ingestion with and without an acute bout of exercise. We also determined anabolic and inflammatory signaling mechanisms before and after protein ingestion in both the CON and EX legs.

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CHAPTER II: STUDY 1

ANABOLIC SENSITIVITY OF POSTPRANDIAL MUSCLE PROTEIN SYNTHESIS TO THE INGESTION OF A PROTEIN-DENSE FOOD IS REDUCED IN OVERWEIGHT AND OBESE YOUNG ADULTS [1]

Key words: insulin resistance, leucine, muscle mass, inflammation, amino acid transporters, mTORC1

Abbreviations used: FSR, fractional synthetic rate, LC/MS/MS, liquid chromatography-tandem mass spectrometry, GC/MS, gas chromatography-mass spectrometry, TTR, tracer-to-tracee ratio, mTORC1, mammalian target of rapamycin complex 1, p70S6K, 70 kDa S6 protein kinase,

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2.1 ABSTRACT

Background: Excess body fat leads to diminished muscle protein synthesis rates in response to a hyperinsulinemic hyperaminoacidemic clamp. To our knowledge, no studies have compared the postprandial muscle protein synthetic response after the ingestion of a single meal containing a protein dense food source across a range of body mass indices and fat masses.

Objective: We aimed to compare the myofibrillar protein synthetic (MPS) response and underlying nutrient sensing mechanisms after the ingestion of lean pork loin between obese, overweight, and healthy-weight adults.

Design: 10 healthy-weight (HW; Age 24±1 y, BMI 22.7±0.4 kg/m^2, HOMA-IR 1.4±0.2), 10 overweight (OW; Age 26±2 y, BMI 27.1±0.5 kg/m^2, HOMA-IR 1.25±0.11), and 10 obese males and females (OB; Age 27±3 y, BMI 35.9±1.3 kg/m^2, HOMA-IR 5.8±0.8) received primed continuous L-[*ring*-$^{13}C_6$]phenylalanine infusions. Blood and muscle biopsy samples were collected before and after ingestion of 170 g of pork (36 g protein and 3 g fat) to assess skeletal muscle anabolic signaling, amino acid transporters (LAT1, CD98, SNAT2), and MPS.

Results: At baseline, OW and OB showed greater relative amounts of mTORC1 protein compared to the HW group. However, pork ingestion only increased phosphorylation of mTORC1 in the HW group (P=0.001). LAT1 and SNAT2 protein content increased during the postprandial period in all groups (Time effect: P<0.05). Basal MPS were not different between groups (P=0.43). However, postprandial MPS (0-300 min) was greater in the HW group (1.6-fold; P=0.005) after pork ingestion when compared with the OW and OB groups.

Conclusions: There is diminished responsiveness of postprandial MPS to the ingestion of a protein dense food in overweight and obese adults as compared to healthy-weight controls. These data

indicate that impaired postprandial MPS may be an early defect with increasing fat mass and may be dependent on altered anabolic signals leading to poor sensitivity to protein ingestion.

2.2 INTRODUCTION

An estimated 35% of U.S. adults are obese with a BMI >30 $kg \cdot m^{-2}$ (1)**.** Obesity and excess fat mass have been linked to metabolic alterations in various tissues (2). This impaired metabolism is prominent in skeletal muscle despite the greater lean body mass generally observed in obese vs. healthy weight adults (3). Skeletal muscle metabolism has a prominent role in the regulation of blood glucose and blood lipids and is a primary contributor to basal metabolic rate (4). These metabolic functions may be impaired in obese individuals due to reduced protein turnover, which leads to reduced quality/composition of the muscle proteome (5).

Few studies have assessed the effects of adiposity on skeletal muscle protein metabolism *in vivo* in humans. The available evidence indicates that increased adiposity, and its associated metabolic perturbations, may lead to altered regulation of muscle protein synthesis rates in response to elevated plasma amino acid availability during insulin clamped conditions (5–7). In other studies where an impaired postprandial muscle protein synthetic response was observed, the obese participants were older adults with no comparison to healthy-weight controls (8,9). Thus, it is difficult to distinguish between the independent effects of obesity versus aging on the regulation of muscle protein synthesis rates in these studies. For example, aging muscle appears to be 'anabolically resistant' to dietary protein derived amino acids independent of the potential negative effects of increased adiposity (10).

To date, studies that have examined protein metabolism with obesity have been performed in a setting where intravenous amino acid infusions are applied under insulin clamped conditions (5–7) or small intermittent feeding patterns (8,9) as opposed to a setting that more closely reflects a normal eating pattern of US adults such as bolus protein ingestion. Moreover, we are unaware of any studies assessing the postprandial muscle protein synthetic response to the ingestion of a

protein dense food source, across a continuum of body mass indices and fat percentages in young adults. Therefore, the purpose of the current study was to compare the postprandial myofibrillar protein synthetic response to the ingestion of a single protein-dense meal in young healthy weight, overweight, and obese males and females. We hypothesized that ingesting 6 oz of lean pork loin (36 g protein, ~3.3 g leucine, 2.8 g fat), representing a typical portion for an American dinner (11), would provide a strong stimulatory signal to skeletal muscle tissue and, as such, result in a similar stimulation of postprandial muscle protein synthesis rates across all conditions.

2.3 PARTICIPANTS AND METHODS

Participants and ethical approval

Ten healthy-weight (HW, BMI: 22.7±0.4 $kg \cdot m^{-2}$), 10 overweight (OW, BMI: 27.1±0.5 $kg \cdot m^{-2}$), and 10 obese adults (OB, BMI: 35.9±1.3 $kg \cdot m^{-2}$) volunteered to participate in this study. The participants were counterbalanced for age and sex. Participants were not involved in a regular exercise-training program and were considered sedentary. Participant characteristics are presented in **Table 1**. All participants were deemed healthy based on responses to a routine medical screening questionnaire and had no prior history of participating in stable isotope amino acid tracer experiments. Each participant was informed of the purpose of the study, experimental procedures, and all its potential risks prior to providing written consent to participate. The study was approved by the University of Illinois Institutional Review Board and conformed to standards for the use of human participants in research as outlined in the sixth Declaration of Helsinki.

Experimental design

A parallel group design was used for this study. Prior to the infusion trial, participants reported to the laboratory in the morning after a 10 h fast for the determination of oral glucose tolerance and resting blood pressure. Blood glucose and plasma insulin concentrations were determined before and after consumption of 75 g glucose dissolved in 500 mL of water. In addition, body weight and height were measured as well as body composition by dual-energy X-ray absorptiometry (Hologic QDR 4500A, Bedford, MA, USA). Participants waist-to-hip ratio was also measured using the minimum waist-maximum hip method (12). Participants were instructed to refrain from vigorous physical activity and alcohol for three days prior to the tracer infusion. All participants consumed a standardized meal of the same composition (~30% estimated total daily energy expenditure, providing 50 energy% (En%) carbohydrate, 25 En% fat, and 25 En% of protein) the evening prior to each tracer infusion trial.

Infusion protocol

On the day of the infusion trial, participants reported to the laboratory at ~0700 h after an overnight fast. A Teflon catheter was inserted into an antecubital vein for baseline blood sample collection (t=-195 min), after which the plasma phenylalanine pool was primed with a single intravenous dose of L-[*ring*-$^{13}C_6$]phenylalanine (2 μmol·kg^{-1}). Subsequently, an intravenous infusion of L-[*ring*-$^{13}C_6$]phenylalanine (0.05 μmol·kg^{-1}·min^{-1}) was initiated (*t*=-180 min) and maintained until the end of the trial. A second Teflon catheter was placed in a contralateral dorsal hand vein and placed in a heated blanket for repeated arterialized blood sampling. In the post-absorptive state, muscle biopsies of the *vastus lateralis* were collected at *t*=-120 and 0 min of infusion. Subsequently, the participants consumed 170 g ground pork. Participants were also given 300 mL of water enriched to 4% with L-[*ring*-$^{13}C_6$] phenylalanine according to the phenylalanine content

of pork to minimize disturbances in isotopic equilibrium during the infusion. Additional muscle biopsies were collected at 120 and 300 min after pork ingestion. Arterialized blood samples were drawn every 30 or 60 min during the post-absorptive and postprandial-states. The biopsies were collected from the middle region of the *vastus lateralis* (15 cm above the patella) through separate incisions with a Bergström needle under local anesthesia. The resting muscle biopsies were obtained from one leg and the postprandial biopsies from the contralateral leg (randomized). All muscle biopsy samples were freed from any visible adipose, connective tissue and blood, immediately frozen in liquid nitrogen, and stored at –80˚C until subsequent analysis.

Meal composition

Lean center-cut pork loins were homogenized, ground, and individually packaged and stored at –20°C until each experimental trial. Prior to the infusion trial, the pork was thawed overnight at 4°C and grilled until the inner temperature reached 65°C. Proximate analysis of center cut pork loin was performed as previously described (13). In brief, four sub-samples of the master block of pork were analyzed for fat and protein content. Samples were dried at 110°C for 24 h and extracted in an azeotropic mixture of warm chloroform and methanol. Protein concentrations were determined by measuring nitrogen content using the combustion method (method 990.03; AOAC International, 2000; TruMac; LECO Corp., USA). Moisture, protein, and extractible lipid analyses were performed in duplicate. The variability between sub-samples was within the acceptable range (CV <5%). The 170 g ground pork patty provided 36 g protein and 5 g of fat. This amount of pork was selected since others have shown that the ingestion of 170 g lean beef (36 g protein) was required to stimulate the postprandial muscle protein synthetic response in healthy adults (14) and it represents a typical amount of protein consumed with dinner by US adults.

Blood analyses

Glucose concentrations were analyzed in whole blood using an automated glucose analyzer (YSI 2300 Stat Plus, Yellow Springs Instruments, USA). Plasma insulin and C-reactive protein (CRP) concentrations were determined using a commercially available high sensitivity enzyme-linked immunosorbent assays (Alpco diagnostics; USA). Plasma amino acid concentrations and enrichments were determined by GC/MS analysis (Agilent 7890A GC/5975C; MSD, USA). Plasma samples were prepared for amino acid analysis using a mixture of isopropanol:acetonitrile:water (3:3:2, v/v) and centrifuged for 10 min at 4ºC. Subsequently, the supernatant was dried and the amino acids converted into tert-butyldimethylsilyl (*t*-BDMS) derivatives prior to GC/MS analysis. The plasma L-[*ring*-$^{13}C_6$]phenylalanine enrichments were determined using electron impact ionization by ion monitoring at mass/charge (*m/z*) 336 (*m*+0) and 342 (*m*+6) for unlabeled and labeled phenylalanine, respectively, using an internal standard (DL-p-Chlorophenylalanine, 10 $\mu g \cdot mL^{-1}$). Amino acid concentrations were quantified using the AMDIS software package (v. 2.71, NISTTM, USA) and standards with known concentrations.

Myofibrillar protein synthesis

Myofibrillar protein-enriched fractions were extracted from ~50 mg of wet muscle as described previously (15). Myofibrillar protein pellets were hydrolyzed overnight in 6 M HCL at 110°C. The resultant free amino acids were purified using cation exchange chromatography (Dowex 50W-X8-200 resin; Acros Organics, Geel, Belgium) and dried under vacuum. Free amino acids were re-suspended in 60% methanol and centrifuged before analysis by 5500 QTRAP LC/MS/MS. The samples were dried and re-suspended in 100 µL 60% methanol and centrifuged. Subsequently, samples were injected (5 µL) and the LC separation was performed on a Thermo Hypercarb

column (4.6 × 100 mm, 5 μm) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 0.4 mL/min. Mass spectra were acquired under positive electrospray ionization (ESI) with the ion spray voltage at +5500 V. The source temperature was 450°C. The curtain gas, ion source gas 1, and ion source gas 2 were 35, 65, and 55 psi, respectively. The L-[*ring*-$^{13}C_6$]phenylalanine enrichments were determined by multiple reaction monitoring (MRM) at *m/z* 166.0 → 103.0 and 172.0 → 109.0 for unlabeled and labeled L-[*ring*-$^{13}C_6$]phenylalanine, respectively. Software Analyst 1.6.2 was used for data acquisition and analysis.

Western blotting

A portion of whole muscle homogenates isolated during the myofibrillar protein extractions was used for Western blotting analysis. Protein content of the homogenates was determined by Bradford Assay (Bio-Rad) and then equal amounts of protein were separated by SDS-PAGE before being transferred to polyvinyl difluoride membranes. After blocking, membranes were incubated in primary antibodies overnight at 4°C to determine the phosphorylation status and total protein content of protein kinase B (Akt) at Ser473, mammalian target of rapamycin complex 1 (mTORC1) at Ser2448, 70 kDa S6 protein kinase 1 (p70S6K) at Thr389, and adenosine monophosphate dependent protein kinase α (AMPK-α) at Thr172 using antibodies from Cell Signaling Technology (Danvers, MA, USA). The antibodies used for detection of skeletal muscle amino acid transporters were as follows: large neutral amino acid transporter (LAT1 SLC7A5 Bioss, USA), small neutral amino acid transporter (SNAT2 SLC38A2, Abcam, USA), and CD98 (SLC3A2, Abcam, USA). Membranes from the respective proteins were then incubated with appropriate secondary antibodies and protein content was detected using West Femto Maximum

Sensitivity substrate (SuperSignal, Thermo Scientific, USA) and the ChemiDoc-It^2 Imaging System (UVP, USA). After detection of phosphorylated proteins, membranes were stripped with western blot stripping buffer (Restore, Thermo Scientific, USA) and re-incubated with antibodies against total protein (Cell Signaling Technology, USA). Western blot data were normalized to an internal control (α-tubulin). Bands were quantified using ImageJ software (NIH), normalized to a control sample run on each blot to account for inter-blot variability, then expressed as fold change from HW basal.

Calculations

Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the fasting glucose and insulin values from the OGTT ($Glucose_{fast}$ x $Insulin_{basal}$ /22.5 (16)). The fractional synthetic rates (FSR) of myofibrillar protein were calculated using standard precursor-product methods by dividing the increment in tracer enrichment in the myofibrillar protein by the enrichment of the plasma free precursor pool over time.

Statistics

A parallel group repeated measures design was used for this study. A power analysis based on previous research (6,8,14) revealed that an n=9 per group was sufficient to detect differences in postprandial muscle protein synthesis between groups when using a two sided statistical test (P<0.05, 80% power, f=0.7; G*power version 3.1.9.2). Considering a potential dropout rate of 10% during the protocol, the final number of participants recruited was 10 per group. Differences in myofibrillar protein synthesis, muscle anabolic signaling, blood glucose, and plasma insulin were tested by two-factor (group × time) repeated measures analysis of variance (ANOVA). Group

comparisons were performed when indicated by a significant interaction. Body composition, HOMA-IR, plasma CRP, demographics, and net area under the blood glucose, plasma insulin, and AA curves (AUC) were analyzed using one-factor ANOVA. Group comparisons were performed when indicated by a significant group effect. Tukey's post-hoc tests were performed to locate group differences. Pearson's *r* product-moment correlation was used to examine the relationship between fat mass and postprandial myofibrillar protein synthesis rates. For all analyses, differences were considered significant at $P<0.05$. All calculations were performed using IBM SPSS Statistics Version 20. All data are expressed as means ± SEMs.

2.4 RESULTS

Oral Glucose Tolerance testing

During the oral glucose tolerance test, fasting blood glucose was similar across groups ($P=0.94$). At 120 min post-beverage consumption, blood glucose was greater in OB (99.2 ± 7.7 mg/dL) as compared with the OW (80.6 ± 4.2 mg/dL) and HW (78.5 ± 6.1 mg/dL) groups (all $P<0.05$). Fasting insulin was greater ($P<0.001$) in the OB group (25.2 ± 3.8 μIU/mL) compared with the OW (6.5 ± 0.5 μIU/mL) and HW (7.1 ± 0.9 μIU/mL) groups. HOMA-IR was greater ($P<0.001$) in the OB group (5.82 ± 0.81) than OW (1.25 ± 0.11) and HW groups (1.36 ± 0.17) (**Table 1**).

Blood variables during the infusion trial

Fasting plasma CRP concentrations were elevated (-180 min, $P<0.001$) in the OB group (6.55 ± 1.19 mg/L) compared with the OW (0.84 ± 0.18 mg/L) and HW groups (0.82 ± 0.29 mg/L; **Table 1**). Similarly, blood glucose concentrations were higher in the OB group at -180 min of the infusion trial ($P<0.05$) when compared with OW and HW group. The blood glucose values remained stable

over the duration of the infusion trial in all groups (all $P>0.05$; **Figure 1A**). Plasma insulin concentrations increased to a greater extent after protein ingestion in the OB group. As such, plasma insulin concentrations reached higher peak values ($P<0.001$) in the OB group (56.7 ± 8.0 μIU/mL) when compared with the OW (14.3 ± 1.7 μIU/mL) and HW groups (11.9 ± 1.6 μIU/mL; **Figure 1B**). Plasma essential amino acid (EAA) concentrations increased after pork ingestion with no differences between groups (Time effect: $P<0.001$ **Figure 2A**). Moreover, the net AUC for EAA were similar between all groups ($P=0.11$). Similarly, plasma branched chain amino acid concentrations also increased after pork ingestion with no differences between groups (Time effect: $P<0.001$; **Figure 2B**). Plasma L-[*ring*-$^{13}C_6$]phenylalanine enrichments are shown in **Figure 3**. Plasma L-[*ring*-$^{13}C_6$]phenylalanine enrichments were different between groups (Group effect: $P=0.05$). However, plasma L-[*ring*-$^{13}C_6$]phenylalanine enrichments were stable for each respective group throughout the infusion protocol indicating a tracer steady-state was achieved (Time effect: $P=0.20$).

Anabolic signaling and amino acid transporters

In the OB and OW groups, the relative concentrations of total mTORC1 protein were significantly greater ($P<0.001$) at baseline compared to the HW group. In contrast, total p70S6K protein was similar in all groups ($P=0.14$; **Figure 4A**). In the postabsorptive-state, mTORC1 phosphorylation was significantly higher in the OB and OW groups compared to the HW group ($P=0.001$; **Figure 4B)**. During the postprandial period, mTORC1 phosphorylation increased at 300 min in the HW group ($P<0.05$) after pork ingestion with no changes in the OB and OW groups. Phosphorylation of p70S6K was increased in the OB group at 300 min after pork ingestion ($P=0.02$; **Figure 4C**), but no changes were observed in the OW and HW groups. There were no observed differences in

total protein or the phosphorylation-status at any time points for AKT and AMPK (data not shown). There were no differences in skeletal muscle LAT1, CD98, or SNAT2 protein content at baseline or after pork ingestion between the three groups (P>0.05; **Figure 5**). During the postprandial phase, pork ingestion resulted in increased LAT1 protein content above baseline values (Time effect: P=0.003; **Figure 5A**). Similarly, pork ingestion increased SNAT2 protein content during postprandial period (Time effect: P<0.001 **Figure 5C**). CD98 protein content remained unaltered after pork ingestion in all groups (P>0.05; **Figure 5B**). Representative western blots for anabolic signaling proteins and skeletal muscle amino acid transporters are shown in **Supplementary Figure 1**.

Myofibrillar protein synthesis

Basal myofibrillar protein synthesis rates did not differ between the groups (P=0.43). Pork ingestion increased cumulative myofibrillar protein synthetic rates calculated over the entire 300 min postprandial period by 1.6-fold in the HW group versus basal (P=0.005), but no increases were observed in the OW or OB groups (**Figure 6**).

Myofibrillar protein synthesis rates calculated during the early (0-120 min) and late (120-300 min) postprandial period revealed differences in the temporal pattern of change in the stimulation of the postprandial myofibrillar protein synthetic response. During the early postprandial phase (0-120 min), myofibrillar protein synthesis did not differ from basal values in the OB, OW, or HW groups *(P*=0.95). However, myofibrillar protein synthetic rates were increased (P<0.001) in the HW group (absolute change from basal: 0.067 ± 0.092 % · hr^{-1}) but not the OW group (0.015 ± 0.011 % · hr^{-1}) or OB group (0.005 ± 0.011 % · hr^{-1}) in the late (120-300 min) postprandial phase. There was a trend for a relationship between total body fat mass and the postprandial muscle protein

synthetic response value at 0-300 min (r= -0.35, *P*=0.06). Further, total body fat mass was significantly correlated with postprandial myofibrillar protein synthesis rates in the late postprandial period (120-300 min; r= -0.45, *P*=0.02).

2.5 DISCUSSION

In this study, we compare the nutrient sensing mechanisms and subsequent postprandial myofibrillar protein synthetic response to the ingestion of a protein-dense food source across a wide-range of body fat percentages in humans. We showed that excessive fat mass does not impair the basal myofibrillar protein synthetic response. However, we observed a poor responsiveness of postprandial muscle protein synthesis rates to the ingestion of a meaningful amount of high quality animal-based protein (36 g) in the OB and OW group as compared to their HW counterparts. These differences were driven by the responsiveness of the postprandial myofibrillar protein synthesis rates in the late postprandial phase (2-5 h) in the HW group. Interestingly, the OW group did not demonstrate overt differences in whole body glucose tolerance or systemic inflammation versus the HW group, which may illustrate that skeletal muscle anabolic insensitivity is an early impairment associated with increased adiposity that occurs prior to glucose intolerance (OGTT), chronic low-grade systemic inflammation (e.g., elevated plasma CRP concentrations), and hyperinsulinemia. Moreover, the net exposure of amino acids (total, EAA, BCAA, and leucine) during the postprandial period was similar between all groups and further supports that intrinsic defects within skeletal muscle tissue may precede dysregulated metabolism in other tissues. What is noteworthy is that our data demonstrate that consuming a protein quantity that is similar to that provided during an average American meal (11) is incapable of overcoming the poor anabolic

sensitivity of skeletal muscle tissue to dietary amino acids with increasing adiposity in young adults.

Only a handful of previous studies have examined the regulation of muscle protein synthesis in obese individuals (5–9). Both basal and postprandial muscle protein synthesis rates have an important role in the maintenance of skeletal muscle mass (e.g., contractile function and metabolic health). Our findings are in agreement with several previous studies that have shown similar basal rates of muscle protein synthesis with obesity when compared to their healthy weight counterparts (5,7,17). In a different manner, exogenous amino acid administration has been shown to be both effective (8) and ineffective (5,9) at stimulating postprandial muscle protein synthesis in obese, older adults. Moreover, it has been shown that obese men exhibited a 'normal' muscle protein synthetic response during euglycemic, hyperinsulinemic, hyperaminoacidemic clamp (6,7). While our data conflicts with these findings, the clamped conditions used in these studies were not reflective of the postprandial conditions seen in the present study (i.e. profoundly greater aminoacidemia and insulinemia (6,7)) and likely created a more anabolic environment for muscle. Here, we show deficits in the responsiveness of postprandial muscle protein synthesis rates to dietary protein derived amino acids in both OW and OB groups when applied under conditions more reflective of a typical meal setting. This defect in the postprandial muscle protein synthetic response to food ingestion with increasing adiposity does not negatively impact overall lean mass (**Table 1**), but is likely contributing to poor metabolic quality of skeletal muscle. For example, impairments in protein turnover/renewal lead to greater protein half-life. These 'older' proteins are more susceptible to damage than newly synthesized proteins, which can contribute to poor composition and impaired metabolic function of the muscle proteome (18). Alternatively, the lean body mass in the OW group was not different from the HW group despite the elevated fat mass.

These findings suggest that reduced postprandial muscle protein synthesis rates in overweight individuals, as evidence from the blunted myofibrillar protein synthesis to protein ingestion, is a potentially contributing process to the development of metabolic impairments commonly observed with obesity. Murton et al. (5) described similar observations where a reduced muscle protein synthetic response occurred to amino acid provision in obese older adults with no impact on muscle strength and muscle fatigue when compared to healthy weight controls. Moreover, these workers observed a reduced proteolytic response in the obese older adults (5), which is consistent with the notion that protein synthesis and breakdown respond in a coordinated manner (19). Given all this, our data, and others (5), supports the notion that our data is more reflective of poor skeletal muscle remodeling and not related to reduced accretion or maintenance of the myofibrillar protein pool with greater adiposity. Essential amino acids are direct activators of muscle anabolic signaling pathways (20) with a pronounced leucinemia being exceptionally important to optimize the postprandial muscle protein synthetic response (21). We observed no differences in plasma EAA or BCAA concentrations or net exposure to amino acids (AUC) between the 3 groups. These findings indicate that the defect in postprandial muscle protein synthesis rates in the OW and OB groups are likely not due to reduced plasma amino acid availability since postprandial release of dietary protein-derived amino acids into circulation are a relevant driver of changes in plasma amino acid concentrations (22). Previous research has shown that obesity is associated with elevated postabsorptive plasma BCAA concentrations (23). However, the association between obesity and circulating BCAAs becomes less clear when obese participants are stratified by insulin sensitivity (24). We specifically recruited obese participants without known health concerns, which may explain the discrepancy seen in the present study.

We examined relevant nutrient sensing mechanisms in muscle in an effort to discern what factor(s) is at the root of the reduced postprandial muscle protein synthetic response with increased adiposity as it appeared not to be driven by systemic factors. Interestingly, we show that basal mTORC1 protein concentrations and the phosphorylation of mTORC1 are elevated in both OW and OB groups compared to the HW group. This may represent a compensatory mechanism to maintain a 'normal' basal muscle protein synthetic response with increasing adiposity. However, this hyperactivation of mTORC1 in the basal-state may diminish the 'sensing' capacity of skeletal muscle tissue to protein-derived amino acids during the postprandial period. Our findings are supported by rodent studies, which have shown that mTORC1 is hyperactive with obesity in the fasting-state (25–27). We also measured skeletal muscle LAT1, CD98, and SNAT2 protein content for insight into amino acid transport/sensing capacity in muscle. Both LAT1 and SNAT2 protein content increased at 5 h of the postprandial phase in all 3 groups (**Figure 5**). This suggests that amino acid transport capacity is not impaired with greater adiposity. Overall, these findings intimate that intrinsic anomalies in skeletal muscle anabolic signaling are present and may partly explain the poor sensitivity of postprandial muscle protein synthesis rates to dietary protein derived amino acids with increasing fat mass.

From a practical perspective, the current findings suggest that simply instructing people with overweight/obesity to eat more protein in a meal to overcome the defect in postprandial muscle protein synthesis would be unproductive. This notion is based on the generous amount of protein (36 g) provided in the present study that mirrors the portion of protein commonly contained in a typical American dinner (11), which was effective in elevating plasma amino acid concentrations, and has been shown to be satiating (28). Instead, strategies aimed at improving the amino acid

sensitivity of muscle, such as increasing habitual physical activity or incorporation of exercise regimes into the daily lifestyle routine (29), is likely more prudent.

In conclusion, greater adiposity abolishes the increase in postprandial myofibrillar protein synthesis rates seen in HW after ingestion of a protein-dense meal in apparently "healthy" young overweight and obese adults. In the case of the OW group, this finding was observed independent of many of the typically associated problems that occur with weight gain (e.g., reduced glucose tolerance and/or chronic inflammation). However, key muscle anabolic signaling pathways are clearly disrupted in overweight and obese adults and may underpin the defect in postprandial muscle protein synthesis rates. The focus of future work should aim to increase the anabolic sensitivity of muscle protein synthesis rates, with exercise prior to food ingestion representing a logical next step.

2.6 TABLES AND FIGURES

Table 2.1. Participant characteristics

Variable	Healthy-weight	Overweight	Obese
Sex (females)	10 (5)	10 (5)	10 (5)
Age (y)	24 ± 1	26 ± 2	27 ± 3
Ht (m)	1.73 ± 0.03	1.70 ± 0.02	1.71 ± 0.03
Wt (kg)	68.5 ± 3.5	78.6 ± 2.2	106.0 ± 5.0*†
BMI (kg/m^2)	22.7 ± 0.4	27.1 ± 0.5	35.9 ± 1.3
Waist:Hip	0.79 ± 0.02	0.83 ± 0.02	0.92 ± 0.01*†
% Body fat	22.2 ± 1.8	29.1 ± 1.4*	35.3 ± 1.8*†
Lean mass (kg)	51.3 ± 3.7	53.8 ± 1.3	65.5 ± 3.2*†
Systolic BP (mmHg)	120.4 ± 3.0	125.9 ± 2.2	131.5 ± 3.0*
Diastolic BP (mmHg)	75.2 ± 1.7	79.4 ± 1.8	87.2 ± 1.5*†
C-reactive protein (mg/L)	0.82 ± 0.29	0.84 ± 0.18	6.55 ± 1.19*†
Fasting glucose (mg/dL)	78.0 ± 2.2	78.3 ± 1.2	82.0 ± 2.1
120 min glucose (mg/dL)	78.5 ± 6.1	80.6 ± 4.2	99.2 ± 7.7*†
Fasting insulin (μIU/mL)	7.1 ± 0.9	6.5 ± 0.5	25.2 ± 3.8*†
HOMA-IR	1.36 ± 0.17	1.25 ± 0.11	5.82 ± 0.81*†

Data are mean ± SEM. Glucose values were obtained from the oral glucose tolerance test. * Indicates a difference from the Healthy-weight group, † Indicates a difference from the Overweight group, (P<0.05). Demographic, body composition, blood pressure, C-reactive protein, and HOMA-IR data were analyzed with one factor ANOVA. Fasting glucose and 120 min glucose data were analyzed using a two factor repeated measures ANOVA. A Tukey's post hoc test was used to locate differences between group means when indicated by significant group effects or Group × Time interactions. Glucose: Group effect P=0.59, Time effect P<0.001, Group × Time P<0.001.

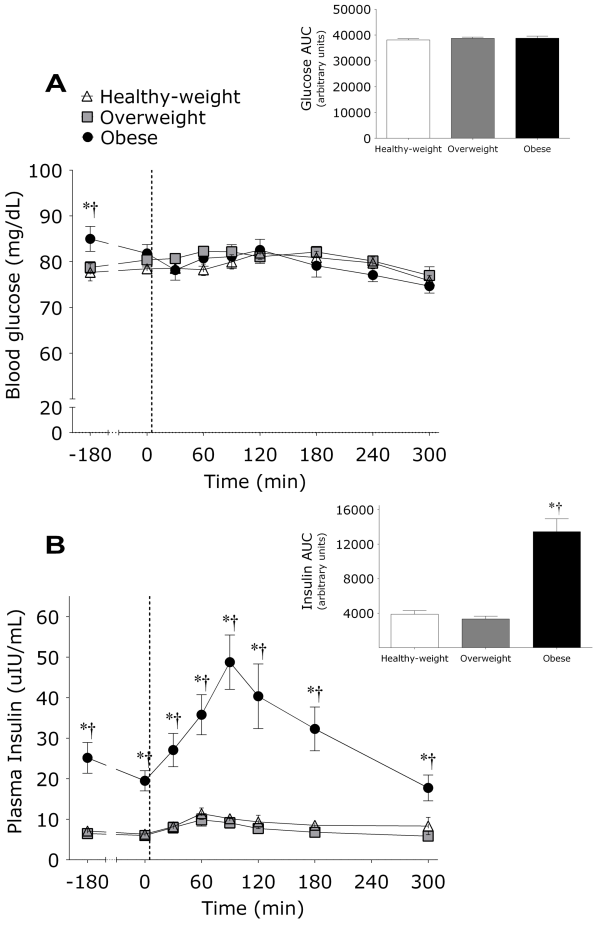


Figure 2.1. Blood glucose (**A**) and plasma insulin concentrations (**B**) (mg·dL^{-1} and μIU·mL^{-1}, respectively) in the basal state and after pork ingestion (n=10 per group). Inset are the area under the glucose and insulin curves. Dashed vertical line refers to pork ingestion. Blood glucose and plasma insulin data were analyzed with a two-factor ANOVA with repeated measures on time. The area under the glucose and insulin curves were analyzed using one factor ANOVA. A Tukey's post hoc test was used to locate differences between means for all significant interactions. * Indicates a difference from HW. † Indicates a difference from OW. Glucose: Group × Time P=0.012. Glucose AUC: Group Effect P=0.65. Insulin: Time effect P<0.001, Group × Time P<0.001. Insulin AUC: Group Effect P<0.001. Data are Mean ± SEM.

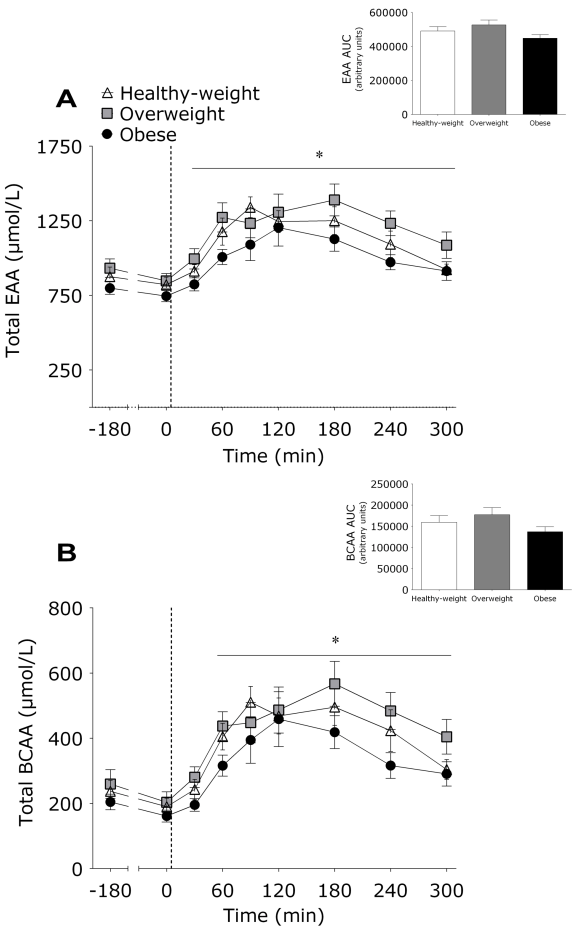


Figure 2.2. Plasma essential amino acid (EAA; **A**) and branched chain amino acid concentrations (BCAA; **B**) (µmol·L^{-1}) in the basal state and after consumption of pork (n=10 per group). Inset are the area under the EAA and BCAA × time curves. Dashed vertical line refers to pork ingestion. Data were analyzed with a two-factor ANOVA with repeated measures on time. The area under the EAA and BCAA curves were analyzed using one factor ANOVA. A Tukey's post hoc test was used to locate differences between means for all significant interactions. * Indicates a difference from -180 min. Significant time effects are indicated by a line above points that differ from -180 min. EAA: Time effect: P<0.001, Group × Time: P=0.68. EAA AUC: Group Effect P=0.11. BCAA: Time effect: P<0.001, Group × Time: P=0.76. BCAA AUC: Group Effect P=0.26. Mean ± SEM.

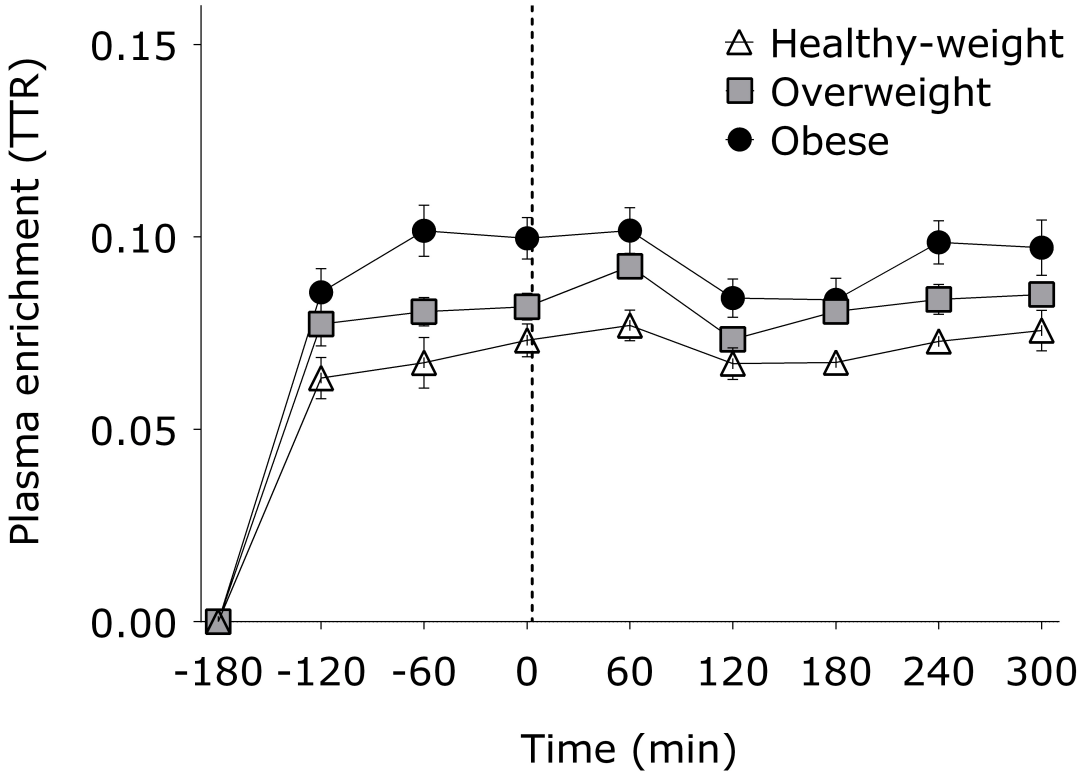


Figure 2.3. Plasma L-[*ring*-$^{13}C_6$]phenylalanine enrichments (tracer-to-tracee ratio [TTR]) in the basal state and after pork ingestion (n=10 per group). Dashed vertical line refers to pork ingestion. Data were analyzed with a two-factor ANOVA with repeated measures on time. A Tukey's post hoc test was used to locate differences between means for all significant interactions. L-[*ring*-$^{13}C_6$]phenylalanine: Group effect P=0.05, Group × Time: P=0.19. Mean ± SEM.

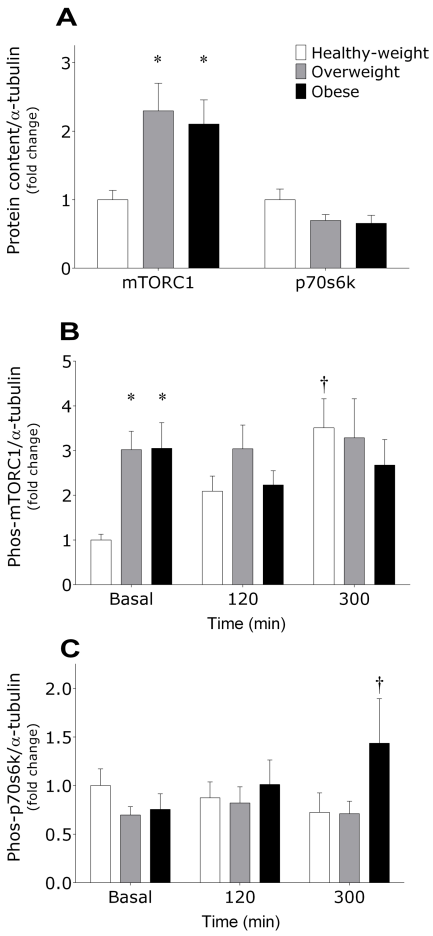


Figure 2.4. Protein content for mammalian target of rapamycin complex 1 (mTORC1) and 70 kDa S6 protein kinase (p70S6K) in the basal-state (**A**). Phosphorylation of mTORC1 at Ser2448 (**B**), and p70S6K at Thr389 (**C**) at basal and after the ingestion of pork (n=10 per group). Data were analyzed with a two-factor ANOVA with repeated measures on time. A Tukey's post hoc test was used to locate differences between means for all significant interactions. * Indicates a difference from HW. † Indicates a difference from basal. mTORC1 protein: Group effect: P<0.001. p70S6K protein: Group effect: P=0.14. Phos-mTORC1: Group effect P=0.48, Time effect: P=0.11, Group × Time: P=0.04. Phos-p70S6K: Group effect P=0.53, Time effect: P=0.64, Group × Time: P=0.03. Mean ± SEM. Data are expressed as fold change from HW basal.

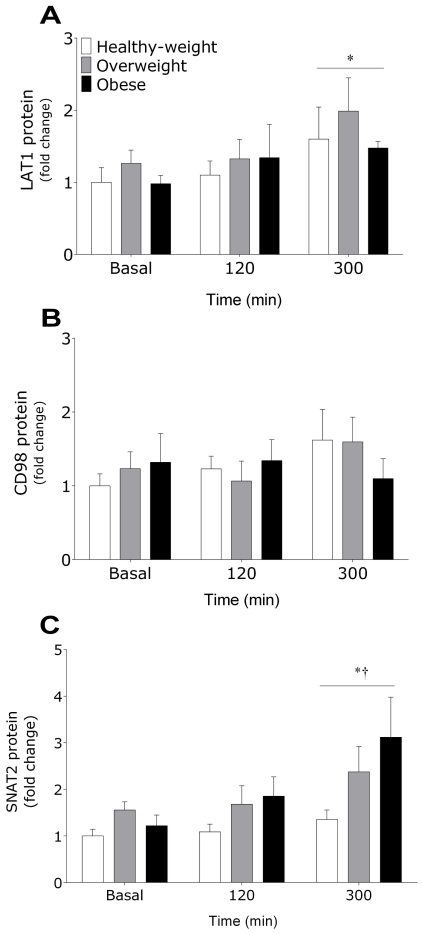


Figure 2.5. Protein content of large neutral amino acid (LAT1, **A**) and small neutral amino acid transporters (CD98, **B** and SNAT2, **C**) at basal and after the ingestion of pork (n=10 per group). Data were analyzed with a two-factor ANOVA with repeated measures on time. A Tukey's post hoc test was used to locate differences between means for all significant interactions. Significant time effects are indicated by a line above the points that differ. ***** Indicates a difference from basal in all groups, † Indicates a difference from 120 min in all groups. LAT1: Time effect: P=0.003, Group × Time: P=0.64. CD98 Time effect: P=0.13, Group × Time: P=0.30. SNAT2: Time effect: P<0.001, Group × Time: P=0.25. Mean ± SEM. Data are expressed as fold change from HW basal.

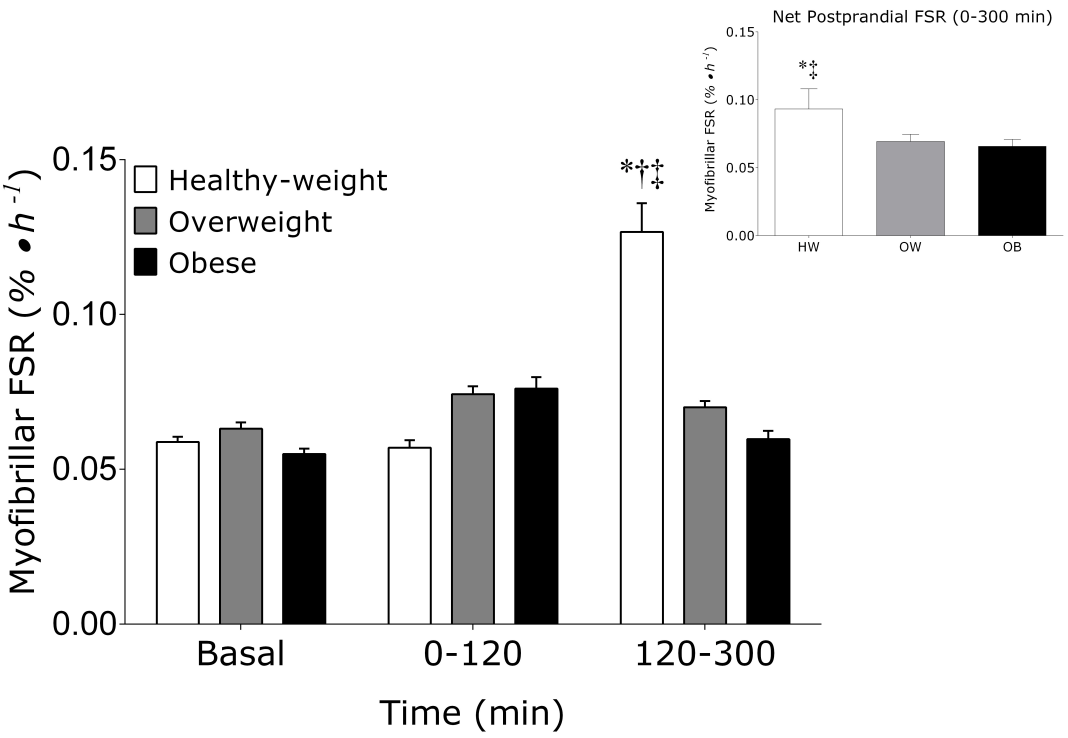
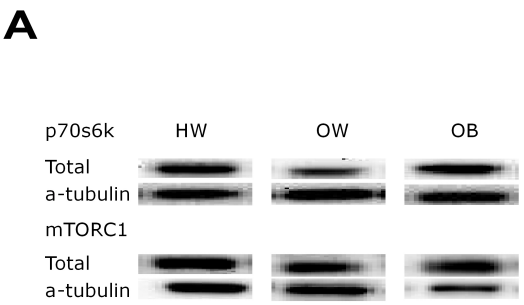
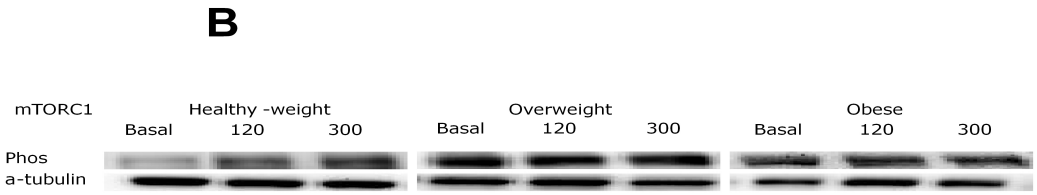


Figure 2.6. Myofibrillar fractional synthesis rates (FSR) at basal and after (0-120 min and 120-300 min) the ingestion of pork (n=10 per group). Inset is the cumulative rates of myofibrillar protein synthesis during the postprandial period (0-300 min). Data were analyzed with a two-factor ANOVA with repeated measures on time. A Tukey's post hoc test was used to locate differences between means for all significant interactions. * Indicates a difference from basal, † Indicates a difference from 0-120 min. ‡ Indicates a difference from the OW and OB groups. FSR: Group × Time: P=0.002; Inset FSR: Group × Time: P=0.005. Mean ± SEM.





Supplementary figure 2.1. Western blots for protein content for mammalian target of rapamycin complex 1 (mTORC1) and 70 kDa S6 protein kinase (p70S6K) in the basal-state (**A**). Phosphorylation of mTORC1 at Ser2448 (**B**), and p70S6K at Thr389 (**C**) at basal and after the ingestion of pork (*n*=10 per group).

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CHAPTER III: STUDY 2

PROTEIN-RICH FOOD INGESTION STIMULATES MITOCHONDRIAL PROTEIN SYNTHESIS IN SEDENTARY YOUNG ADULTS OF DIFFERENT BMIs[2]

Abbreviations used: FSR, fractional synthetic rate; LC/MS/MS, liquid chromatography-tandem mass spectrometry; GC/MS, gas chromatography-mass spectrometry; TTR, tracer-to-tracee ratio; TLR4, toll-like receptor 4; MyD88, myeloid differentiation primary response protein 88; TNFα, tumor necrosis factor α; IL-6, interleukin 6.

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3.1 ABSTRACT

Context:

Excess fat mass may diminish the anabolic potency of protein-rich food ingestion to stimulate muscle protein sub-fractional synthetic responses. However, the impact of adiposity on mitochondrial protein synthesis rates (MPS) after protein-rich food ingestion has not been thoroughly examined *in vivo* in humans.

Objective:

We compared basal and postprandial MPS and markers of muscle inflammation (Toll-like receptor 4 [TLR4] and myeloid differentiation primary response protein 88 [MyD88] protein content) in young adults with different BMIs.

Methods:

10 normal-weight (NW; BMI 22.7±0.4 kg/m^2), 10 overweight (OW; BMI 27.1±0.5 kg/m^2), and 10 obese (OB; BMI 35.9±1.3 kg/m^2) adults received primed continuous L-[*ring*-$^{13}C_6$]phenylalanine infusions, blood sampling, and skeletal muscle biopsies before and after the ingestion of 170 g of pork.

Results:

Pork ingestion increased muscle TLR4 and MyD88 protein content in the OB group ($P<0.05$), but not in the NW or OW groups. Basal MPS were similar between groups (P>0.05). Pork ingestion stimulated MPS (P<0.001) (0-300 min) in the NW (2.5±0.6-fold above baseline values), OW (1.7±0.3-fold), and OB groups (2.4±0.5-fold) with no group differences (P>0.05).

Conclusions:

Protein-dense food ingestion promotes muscle inflammatory signaling only in obese adults. However, the consumption of a dinner-sized amount of protein strongly stimulated a postprandial

MPS response irrespective of BMI. Our data suggest that alterations in postprandial mitochondrial protein synthesis are unlikely to contribute to compromised muscle macronutrient metabolism witnessed with obesity.

Key terms: insulin resistance, muscle mass, obesity, protein metabolism, inflammation

3.2 INTRODUCTION

Obesity is a disease associated with systemic metabolic complications characterized by impaired postprandial macronutrient metabolism (1,2). Specifically, obese individuals have elevated plasma inflammatory mediators [e.g. tumor necrosis factor α (TNFα), Interleukin 6 (IL-6), non-esterified fatty acids (NEFA)] (3–5), which have been linked to metabolic abnormalities in the skeletal muscle including insulin resistance (4–7). Moreover, muscle inflammation as indicated by modulations in toll-like receptor 4 (TLR4) signaling has been implicated in impairing mitochondrial function and inducing insulin resistance in rodents (8,9). Currently, there are little data in humans that describe the interaction between inflammation, adiposity, and the stimulation of postprandial mitochondrial protein synthesis in response to food ingestion. Impairments in the feeding-induced synthesis of specific muscle protein fractions, such as mitochondrial proteins, may diminish muscle quality or metabolic function and ultimately limit quality of life.

Amino acid administration either orally or intravenously has been shown to stimulate mitochondrial protein synthesis rates in healthy, normal weight adults (10,11). However, the stimulation of mitochondrial protein synthesis in response to intravenous amino acid administration during hyperinsulinemic clamps is impaired in young obese adults (11). It is currently unknown if obesity-related differences in the stimulation of the mitochondrial protein synthetic response to amino acid administration persist after the ingestion of a meal-like amount of high quality protein in young adults. Previous work has shown that there is an interactive effect between insulin and plasma amino acid availability on the stimulation of mitochondrial protein synthesis rates in humans (12,13). For example, higher plasma insulin concentrations are required to maximize the anabolic effect of increased plasma amino acid availability on the stimulation of mitochondrial protein synthesis rates in healthy, normal weight adults (13). As such, increased

adiposity, and consequently impaired whole body (and muscle) insulin sensitivity (14), could potentially cause an anabolic inflexibility of postprandial mitochondrial protein synthesis to dietary amino acids in otherwise healthy adults.

The purpose of this investigation was to assess the basal and postprandial inflammatory milieu and skeletal muscle mitochondrial protein synthesis rates in response to the ingestion of a protein-rich food source in wide range of BMIs (and fat masses) in young men and women. We hypothesized that elevated systemic and muscle inflammation related to alterations in TLR4 signaling underpins defects in the stimulation of postprandial mitochondrial protein synthesis rates after the ingestion of 6 oz lean pork (36 g protein and 2.8 g fat) in obese adults when compared with normal- and overweight adults. Our work is the first to characterize the responsiveness of postprandial mitochondrial protein synthesis rates to a meal-like amount of high quality food protein in sedentary young adults of different BMIs.

3.3 PARTICIPANTS AND METHODS

Participants and ethical approval

Ten normal-weight (NW), 10 overweight (OW), and 10 young obese volunteers (OB) were recruited to participate in this study. The groups were counterbalanced for age and sex. The characteristics of these participants have been described in detail elsewhere (2). This study represented an extension of our previous work (2) to include measurements of the systemic and muscle inflammatory responses and mitochondrial protein synthesis measurements before and after food ingestion *in vivo* in humans. Participant characteristics are reported in **Table 1**. Participants were classified as *insufficiently active* according to a Godin Leisure-Time Exercise Questionnaire (GLTEQ: < 14 units (15)) and deemed healthy based on responses to a routine

medical screening questionnaire. Each participant was informed of the study purpose, experimental procedures, and all of its potential risks prior to providing written consent to participate. The study was approved by the Institutional Review Board at University of Illinois at Urbana-Champaign and conformed to standards for the use of human participants in research as outlined in the seventh revision of the Declaration of Helsinki.

Pretesting

Participants reported to the laboratory on two separate occasions for screening sessions to assess height, weight, and body composition by dual-energy X-ray absorptiometry (Hologic QDR 4500A, Bedford, MA, USA). Participants were also screened for diabetes risk using an oral glucose tolerance test.

Infusion protocol

Participants were instructed to refrain from physical activity, analgesic drugs, and alcohol for three days prior to the experimental infusion trial. The evening prior to the trial, all participants consumed a standardized meal of the same composition (providing ~30% of estimated total daily energy expenditure and containing 50% of energy of carbohydrate, 25% energy of fat, and 25% energy of protein). On the trial days, participants reported to the laboratory in the morning after an overnight fast and a Teflon catheter was inserted in an antecubital vein for baseline blood sample collection. Subsequently, a primed (2 $\mu mol \cdot kg^{-1}$) continuous infusion of L-[*ring*-$^{13}C_6$]phenylalanine (0.05 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) was initiated (*t*=-180 min), which was passed through a 0.2 μmol filter, and maintained until the end of the trial. A second Teflon catheter was inserted in a contralateral dorsal hand vein and kept patent with a 0.9% saline drip for repeated arterialized

blood sampling using a heated blanket. Biopsy samples of the *vastus lateralis* were collected in the postabsorptive-state at *t*=-120 and 0 min of the infusion trials. Subsequently, participants consumed 170 g ground lean pork loin (containing 36 g protein, ~3 g leucine, and 3 g fat) and 300 mL of water enriched to 4% with L-[*ring*-$^{13}C_6$]phenylalanine according to the phenylalanine content of pork to minimize disturbances in isotopic equilibrium during the infusion (*t*=0). Additional muscle biopsies were collected at 120 and 300 min after pork ingestion. Arterialized blood samples were drawn every 30 or 60 min during the post-absorptive and postprandial-states. Blood samples (8 ml) were collected in EDTA-containing tubes and centrifuged at 3000×g at 4˚C for 10 min. Aliquots of plasma were frozen and stored at –80˚C until subsequent analysis. Biopsies were collected from the *vastus lateralis* (15 cm above the patella) with a Bergström needle under local anesthesia (2% lidocaine). The postabsorptive muscle biopsies were randomly obtained from one leg and the postprandial biopsies from the contralateral leg. All muscle biopsy samples were freed of any visible adipose, connective tissue and blood tissue, frozen in liquid nitrogen, and stored at –80˚C until subsequent analysis.

Plasma analyses

Plasma NEFA, IL-6, TNFα, and C-reactive protein (CRP) concentrations were determined according to manufacturer's instructions using a commercially available enzyme-linked immunosorbent assays (NEFA: Abcam; IL-6: R&D systems; TNFα, insulin, and CRP: Alpco diagnostics; USA). Plasma leucine and phenylalanine concentrations and L-[*ring*-$^{13}C_6$]phenylalanine enrichments were measured by GC/MS analysis using electron impact ionization (Agilent 7890A GC/5975C; MSD, USA) as previously described (2). Amino acid

concentrations were quantified using the AMDIS software package (v. 2.71, NISTTM, USA) and standards with known concentrations.

Mitochondrial protein synthesis measurements

Mitochondrial protein-enriched fractions were extracted from ~100 mg of wet muscle tissue using a Dounce glass homogenizer on ice in ice-cold homogenizing buffers supplemented with a Complete Mini, protease inhibitor and phosphatase cocktail tablets (PhosSTOP, Roche Applied Science, Germany) and differential centrifugation method as described in detail previously (16). Mitochondrial-enriched protein pellets were hydrolyzed overnight in 6 M HCL at 110°C. The resultant free amino acids were purified using cation exchange chromatography (Dowex 50W-X8-200 resin; Acros Organics, Geel, Belgium) and dried under vacuum. Free amino acids were re-suspended in 60% methanol and centrifuged before the mitochondrial protein-bound enrichments were determined by LC/MS/MS analysis (5500 QTRAP, Sciex, USA) as described previously (2). The L-[*ring*-$^{13}C_6$]phenylalanine mitochondrial protein-bound enrichments were determined by multiple reaction monitoring (MRM) at m/z 166.0 → 103.0 and 172.0 → 109.0 for unlabeled and labeled L-[*ring*-$^{13}C_6$]phenylalanine, respectively. Software Analyst 1.6.2 was used for data acquisition and analysis.

Western blotting analysis

An aliquot of muscle homogenate representing the sarcoplasmic fraction, which was isolated during the mitochondrial protein extractions, was used for Western Blot analysis. Total protein concentrations of each sample were determined by Bradford assay (Bio-Rad), and then equal amounts of protein (80 μg) were separated by SDS-PAGE before being transferred to polyvinyl difluoride membranes. After blocking, membranes were incubated in primary antibodies overnight

at 4°C to determine the total protein content of Toll-like receptor 4 (TLR4: R&D systems, USA) and myeloid differentiation factor 88 (MyD88: Cell Signaling, USA). In addition, total protein content and phosphorylation status of Akt at Thr308 (Akt/PKB: Cell Signaling, USA), and the 160 kDa Akt Substrate (AS160: Cell Signaling, USA) were also determined. Membranes were then incubated with appropriate secondary antibodies, and protein content was detected using West Femto Maximum Sensitivity substrate (SuperSignal, Thermo Scientific, USA) and the ChemiDoc-It^2 Imaging System (UVP, USA). Bands were quantified using ImageJ software (NIH) and then normalized to either α-tubulin (Abcam, USA; TLR4, MyD88) or total protein (Akt, AS160).

Calculations

The fractional synthetic rates (FSR) of mitochondrial protein were calculated using standard precursor-product methods by dividing the increment in tracer enrichment in the mitochondrial protein by the enrichment of the plasma free precursor pool over time.

Statistics

Differences in plasma insulin, NEFA, IL-6, TNFα and CRP, muscle TLR4 and MyD88, and mitochondrial protein synthesis rates were tested by two-factor (group × time) repeated measures analysis of variance (ANOVA). Demographics, body composition, level of habitual physical activity (GLTEQ), and net area under the time curve (AUC) for plasma insulin, NEFA, IL-6, TNFα and CRP were analyzed using one-factor (group) ANOVA. Any data not conforming to ANOVA assumptions were transformed prior to statistical analysis. In addition, Mauchly's test of sphericity was also performed and Greenhouse-Geisser or Hunyh-Feldt corrections were applied as appropriate. When significant effects were detected in the ANOVA, Tukey's post-hoc tests were

performed to locate the differences between means for all significant main effects and interactions. For all analyses, differences were considered significant at $P<0.05$. All calculations were performed using IBM SPSS Statistics Version 20. All data are expressed as means ± SEMs.

3.4 RESULTS

Plasma variables

Plasma leucine (**Figure 1A**) and phenylalanine (**Figure 1B**) concentrations increased after pork ingestion ($P<0.001$) and did not differ between groups (both, $P>0.05$). Plasma NEFA concentrations were not different between groups at basal (both, $P>0.05$) and decreased after pork ingestion in all groups (**Figure 1C**; $P<0.05$). However, plasma NEFAs concentrations (**Figure 1C**) decreased earlier in the NW group (t=60 min; $P=0.02$), whereas the plasma NEFAs concentrations decreased later in the OW and OB groups of the postprandial period (120 min; both $P<0.05$). Moreover, plasma NEFA concentrations increase above basal values after pork ingestion at t=300 min in the NW group ($P<0.001$) and tended to increase in the OW group ($P=0.06$) but not in the OB group ($P=0.23$). Plasma insulin concentrations (**Figure 1D**) increased after pork ingestion ($P<0.05$) with higher peak values in the OB group (2.7-Fold; $P<0.05$)) when compared to the NW (1.9-Fold) and OW groups (1.7-Fold).

Plasma IL-6 concentrations (**Figure 2A**) were elevated throughout the basal and postprandial period in the OB group ($P=0.03$) and tended to be elevated in the OW ($P=0.16$) as compared with the NW group. However, pork ingestion increased plasma IL-6 concentrations in all groups (all, $P<0.05$). Plasma TNFα concentrations (**Figure 2B**) were not different among groups at basal ($P>0.05$). At 300 min, plasma TNFα was greater in the OB ($P=0.04$) but not the OW group

(P=0.98) as compared to NW group. Plasma CRP concentrations (**Figure 2C**) were greater at all time points in the OB group (P<0.001) when compared with the NW and OW groups (all, P>0.05).

Muscle insulin signaling

Phosphorylation of Akt (**Figure 3A**) was similar among groups at baseline (all P>0.05). Phosphorylation of Akt increased similarly above basal at 300 min after pork ingestion in all groups (P=0.01). By contrast, AS160 phosphorylation (**Figure 3B**) was greater in the OW group at basal when compared to the NW and OB groups (both, P<0.05). After pork ingestion, AS160 phosphorylation decreased from basal values at 120 and 300 min in the OW group (both, P<0.05), but no changes in phosphorylation were observed in the NW or OB groups (all, P>0.05).

Muscle inflammation

The relative concentrations of total muscle TLR4 protein were greater in the OB as compared with the NW and OW groups at basal (both, P=0.05; **Figure 3C**). After pork ingestion, total muscle TLR4 protein was greater in the OB group at 120 and 300 min of the postprandial period when compared to the NW and OW groups (all, P<0.05). In addition, there was a trend for increased TLR4 protein content after pork ingestion in the OB group at 300 min as compared to the NW group (P=0.14). No changes we observed in total muscle TLR4 protein content in the NW or OW groups (both P>0.05). At baseline, total muscle MyD88 protein content (**Figure 3D**) was greater in the OW and OB compared to the NW group (both P<0.05). Total MyD88 protein content increased above basal values after pork ingestion in the OB group at 300 min (P=0.001) of the postprandial phase with no observed differences in total MyD88 protein in either the NW or the OW groups (all P>0.05).

Mitochondrial protein synthesis

Plasma and mitochondrial protein L-[*ring*-$^{13}C_6$]phenylalanine enrichments are shown in **Table 2**. Plasma L-[*ring*-$^{13}C_6$]phenylalanine enrichments over time were not significantly different during the infusion trials indicating a tracer steady-state (time effect: P=0.20). Plasma L-[*ring*-$^{13}C_6$]phenylalanine enrichments were greater in the OB group than the NW group (group effect: P=0.05). Mitochondrial protein-bound L-[*ring*-$^{13}C_6$]phenylalanine enrichments increased over time in all groups (P<0.001). Mitochondrial protein L-[*ring*-$^{13}C_6$]phenylalanine enrichments were greater in the OB group when compared to the NW and OW group (P=0.04, and 0.02, respectively). Basal mitochondrial protein synthesis rates (**Figure 6**) were not different among the NW, OW, and OB groups (P=0.91) Pork ingestion increased cumulative postprandial mitochondrial protein synthesis rates measured over the 0-300 min postprandial period (**Figure 6 inset**) in the NW (2.5±0.6-fold above baseline values), OW (1.7±0.3-fold), and OB groups (2.4±0.5-fold; all P<0.05). However, the postprandial mitochondrial protein synthetic response determined during the early postprandial period (0-120 min) increased in the NW (2.6±0.4-fold above basal; P=0.01) and OB groups (2.3±0.6 fold above basal; P=0.03) but not in the OW group (1.4±0.3, P=0.62) after pork ingestion. There was no significant feeding-induced stimulation of mitochondrial protein synthesis rates during the late postprandial period (120-300 min) in any of the groups (all P>0.05).

3.5 DISCUSSION

To our knowledge, this study was the first to assess basal and postprandial muscle inflammation and mitochondrial protein synthesis rates after the ingestion of a protein-rich food source across a

wide range of young adults of various BMIs. Here, we show that protein-rich food ingestion stimulates postprandial mitochondrial protein synthesis, irrespective of BMI, in young, sedentary men and women. Moreover, the systemic and muscle inflammatory response related to TLR4/Myd88 signaling is modulated after the ingestion of protein-rich food in obese but not in normal-weight or overweight adults.

Systemic and muscle inflammation has been shown to be prevalent in people with obesity (17–19) and has been proposed as a potential contributing factor to anabolic impairments in protein and amino acid metabolism in obese adults (20). However, in participants stratified solely by their chronic low-grade inflammatory status, it has been shown that basal and postprandial muscle protein synthesis rates are not modulated by elevated systemic inflammatory markers such as plasma CRP (2,21) or TNFα concentrations (22). In agreement with previous reports (17), the OB group displays low-grade inflammation as indicated by greater plasma IL-6 and CRP concentrations in the postabsorptive state when compared to the NW and OW groups. Of these biomarkers, plasma IL-6 concentrations were found to be nutritionally responsive as this cytokine increased similarly after pork ingestion in all groups (**Figure 3**). By contrast, plasma TNFα concentrations were found to be responsive to pork ingestion only in the OB group but not in the NW or OW groups.

Elevated plasma NEFA concentrations are also often used as a biomarker for disease risks, such as insulin resistance (23–26). Moreover, previous studies where circulating NEFAs were elevated experimentally have shown that the stimulation of postprandial muscle protein synthesis rates in response to free amino acid ingestion during high plasma NEFA availability is either blunted (27) or unchanged (28) in young normal weight men. Since elevated plasma NEFA concentrations are commonly connected to obesity (1), we assessed how protein-rich food

ingestion modulated the temporal pattern of change in circulating NEFAs during the postprandial period. Here, we show that differences in postprandial plasma NEFA concentrations among the groups were relatively subtle (**Figure 1**), which may partly explain why observed differences in the postprandial mitochondrial protein synthetic response between the groups were also subtle. Postprandial NEFA concentrations appeared to be less 'responsive' to pork ingestion in the OW and OB groups as noted by a more mild suppression during the immediate postprandial period as compared to the NW group. These findings may indicate mild adipose tissue insulin resistance in the OW and OB groups.

When viewed from a muscle inflammation perspective, total muscle TLR4 protein content has been shown to be related to increased adiposity (18) and likely plays a role in the development of insulin resistance (3) and altered mitochondrial function (8). Here, we observed greater muscle TLR4 protein content in the OB group at baseline when compared to the NW and OW groups. These differences were maintained throughout the postprandial period. Moreover, we observed an obesity-specific trend for increased total muscle TLR4 protein content throughout the postprandial period, which may partly relate to the greater plasma IL-6 concentrations in the OB group (29). Upon ligand binding, TLR4 recruits several adaptor proteins to potentiate intramuscular signals. Among these, MyD88 is linked to the progression of insulin resistance and intramuscular inflammation induced by inactivity in rodents (9). At baseline, muscle MyD88 protein content was greater in the OW and OB groups when compared to the NW group. However, similar to the muscle TLR4 response, total MyD88 protein concentrations increased after pork ingestion only in obese muscles. The increased total muscle TLR4 protein, and its intracellular counterpart MyD88, in response to food ingestion may perpetuate the chronic low-grade inflammatory- and insulin resistant-state by increasing ligand binding (e.g., lipopolysaccharide or NEFA) and intracellular

signaling capacity in people with obesity (19). However, the observed effects of food ingestion on modulating TLR4-driven signaling may be short-lived as indicated by the similarities in total muscle TLR4 and MyD88 protein content in the postabsorptive-state among the groups.

Despite the upregulation of TLR4 signaling in obese muscles, the postprandial mitochondrial protein synthetic response to protein-rich food ingestion is maintained when compared to the NW and OW groups (**Figure 6**). Interestingly, the stimulation of postprandial mitochondrial protein synthesis rates was temporally less responsive during the early postprandial period (0-120 min) to pork ingestion in the OW group when compared to the NW and OB groups. The apparent earlier stimulation of postprandial mitochondrial protein synthesis rates in the OB group as compared with the OW group is perhaps related to the exaggerated insulinemia after pork ingestion in the OB group, which may be a compensatory mechanism that accompanies excess fat gain. For example, previous studies have demonstrated that stimulation of muscle mitochondrial protein synthesis rates are strongly dependent on plasma insulin concentrations with or without elevated plasma amino acid availability in healthy weight adults (13,30). To determine the extent to which plasma insulin availability may have contributed to the differential temporal regulation of the postprandial mitochondrial protein synthetic response between the groups, we assessed the phosphorylation-state of Akt signaling. This pathway has been shown to be dose-related to plasma insulin concentrations in humans (31). Moreover, previous studies have linked Akt phosphorylation at Thr308 to the stimulation of mitochondrial protein synthesis rates, but other anabolic signaling molecules such as mTOR, p70S6K were not related to the response (12). Here, we did not observe group differences in Akt phosphorylation with all groups being greater at 300 min of the postprandial period in response to pork ingestion. We also probed AS160, which is a downstream target of AKT related to glucose uptake, to identify if defects were existent in

alternative targets of this pathway. Interestingly, we showed a greater AS160 phosphorylation at basal in the OW group with a subsequent decline in the phosphorylated-state after pork ingestion as compared with the NW and OB groups (**Figure 3**). As insulin-induced Akt-AS160 phosphorylation is involved with GLUT-4 translocation, perhaps there is a link between insulin-stimulated glucose metabolism and postprandial rates of mitochondrial protein synthesis. However, more work is required to better understand the commonalities in the signaling pathways involved in glucose metabolism and the synthesis of specific protein fractions such as mitochondrial proteins in humans.

Although we show that excess fat mass does not impair basal or the stimulation postprandial mitochondrial protein synthetic responses, we cannot make conclusions with regards to other aspects of mitochondrial physiology (e.g. function, capacity) in these volunteers. For example, it has been recently shown that rates of mitochondrial protein synthesis do not reflect measurements of respiratory capacity in both young and older adults (32). What is noteworthy, however, is that we have previously shown that the stimulation of the postprandial myofibrillar protein synthetic response to pork ingestion is diminished in the OW and OB groups when compared to the NW group (2). Thus, greater adiposity may differentially alter the stimulation of muscle protein sub-fractional synthetic responses to protein-rich food ingestion in human skeletal muscle with contractile remodeling being more negatively affected. More work is needed to determine the mechanisms responsible for these discrepancies.

In conclusion, we show that increased adiposity (and associated chronic low-grade inflammation) does not impair mitochondrial protein synthesis in the postabsorptive state or in response to protein-rich food ingestion in sedentary young adults. In addition, we show obese participants have increased muscle TLR4 signaling proteins in response to protein-rich food

ingestion, which may perpetuate their systemic inflammation. However, our data do not establish a mechanistic link between this muscle inflammatory response and the stimulation of postprandial mitochondrial protein synthesis rates. Thus, the ability to synthesize muscle mitochondrial proteins, which largely consist of enzymes involved in oxidative energy production, is unlikely to contribute to the metabolic derangements commonly witnessed with obesity.

3.6 TABLES AND FIGURES

Table 3.1. Participant characteristics

Variable	Normal-weight	Overweight	Obese
n (females)	10 (5)	10 (5)	10 (5)
Age (y)	24 ± 1	26 ± 2	27 ± 3
Ht (m)	1.73 ± 0.03	1.70 ± 0.02	1.71 ± 0.03
Wt (kg)	68.5 ± 3.5	78.6 ± 2.2	106.0 ± 5.0†‡
BMI (kg/m^2)	22.7 ± 0.4	27.1 ± 0.5	35.9 ± 1.3
% Body fat	22.2 ± 1.8	29.1 ± 1.4†	35.3 ± 1.8†‡
Waist Circumference (cm)	77.2 ± 2.5	89.9 ± 2.5†	110.6 ± 3.8†‡
Physical Activity (GLTEQ score)	7.7 ± 3.0	2.5 ± 1.7	2.8 ± 2.0
HOMA-IR	1.36 ± 0.17	1.25 ± 0.11	5.82 ± 0.81†‡

† $P<0.05$ *vs.* NW. ‡ $P<0.05$ *vs.* OW. Data are Mean ± SEM.

Table 3.2. Plasma and mitochondrial-protein bound L-[*ring*-$^{13}C_6$]phenylalanine enrichments as expressed as mole percent excess (MPE) in the basal-state and after pork ingestion (*n*=10 per group).

	Time (min)			
Group	-120	0	120	300
Normal-weight				
Plasma enrichment (MPE)	5.96 ± 0.54	6.82 ± 0.42	6.29 ± 0.41	7.03 ± 0.53
Mitochondrial enrichment (MPE) **	0.0083 ± 0.0006	0.0147 ± 0.0010	0.0316 ± 0.0027	0.0474 ± 0.0025
Overweight				
Plasma enrichment (MPE)	7.09 ± 0.54	7.67 ± 0.35	7.23 ± 0.52	7.93 ± 0.33
Mitochondrial enrichment (MPE) **	0.0077 ± 0.0013	0.0154 ± 0.0009	0.0278 ± 0.0016	0.0474 ± 0.0029
Obese				
Plasma enrichment (MPE) **†‡	7.88 ± 0.61	9.06 ± 0.53	7.76 ± 0.50	8.86 ± 0.71
Mitochondrial enrichment (MPE) **†‡	0.0110 ± 0.0020	0.0209 ± 0.0023	0.0410 ± 0.0039	0.0577 ± 0.0054

† $P<0.05$ *vs.* NW. ‡ $P<0.05$ *vs.* OW. ** Time effect $P<0.05$. Data are Mean ± SEM.

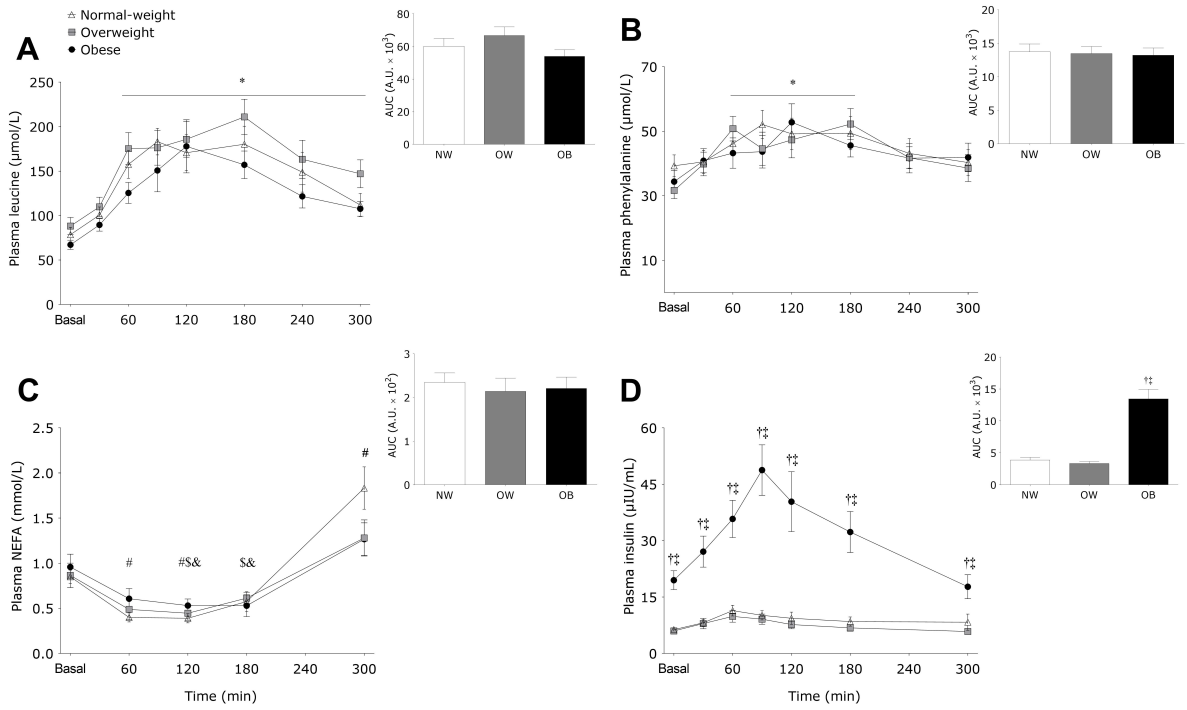


Figure 3.1. Plasma leucine (**A**), phenylalanine (**B**), non-esterified fatty acid (NEFA; **C**), and insulin [adapted from (2) **D**] concentrations in the basal-state and after pork ingestion (n=10 per group). Insets show the areas under the time curves (arbitrary units). * P<0.05 *vs.* basal in all groups. # P<0.05 *vs.* basal in NW. $ P<0.05 *vs.* basal in OW. & P<0.05 *vs.* basal in OB. † P<0.05 *vs.* NW. ‡ P<0.05 *vs.* OW. NW, normal weight; OW, overweight; OB, obese. Data are mean ± SEM.

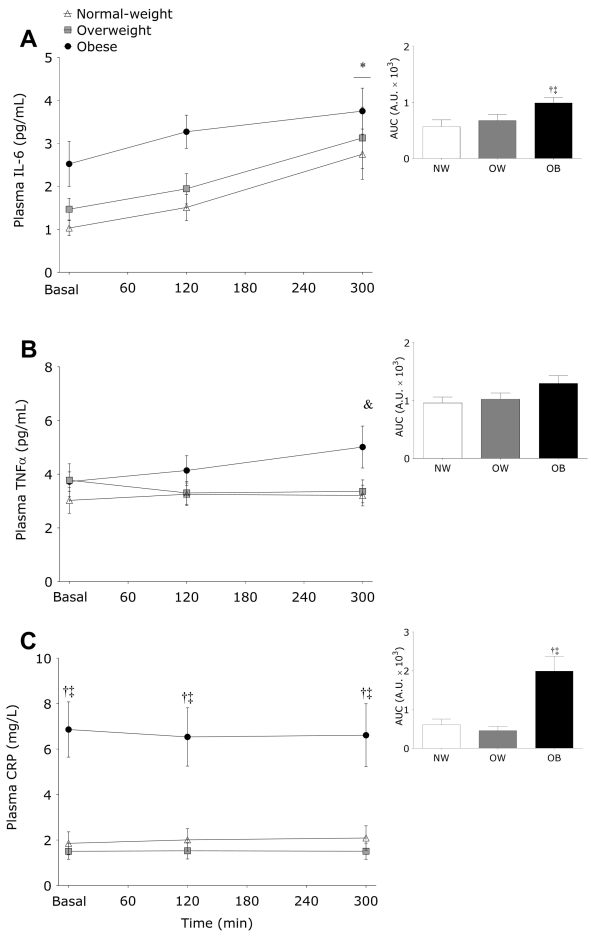


Figure 3.2. Plasma Interleukin 6 (IL-6; **A**), Tumor Necrosis Factor α (TNFα; **B**), and C-reactive protein (CRP; **C**) concentrations in the basal state and after consumption of pork (n=10 per group). Inset shows the area under the time curves (arbitrary units). * P<0.05 *vs.* basal. & P<0.05 *vs.* basal in OB. † P<0.05 *vs.* NW. ‡ P<0.05 *vs.* OW. NW, normal weight; OW, overweight; OB, obese. Data are mean ± SEM.

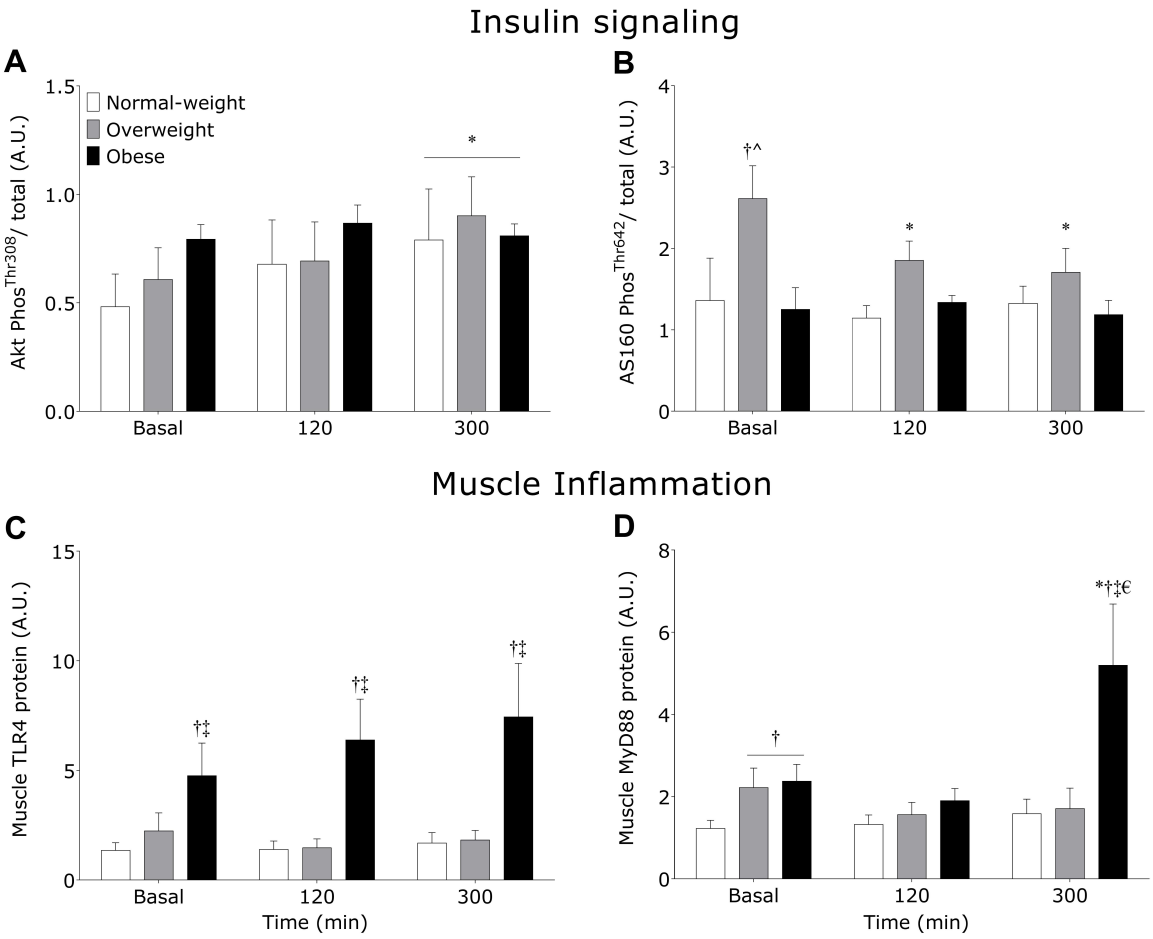


Figure 3.3. Phosphorylation of Akt at Thr308 (**A**), AS160 at Thr642 (**B**) and protein content for toll-like receptor 4 (TLR4; **C**), myeloid differentiation factor 88 (MyD88; **D**) at basal and after the ingestion of pork (n=10 per group). * P<0.05 *vs.* basal. † P<0.05 *vs.* NW. ‡ P<0.05 *vs.* OW. ^ P<0.05 *vs.* OB. € P<0.05 *vs.* 120 min. NW, normal weight; OW, overweight; OB, obese. Data are mean ± SEM.

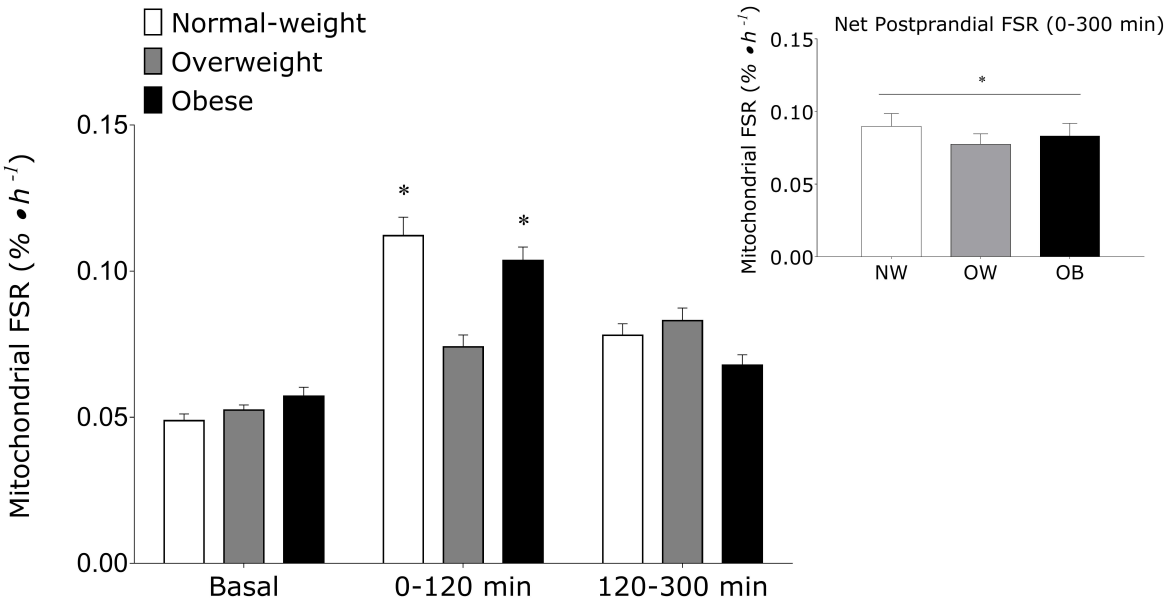
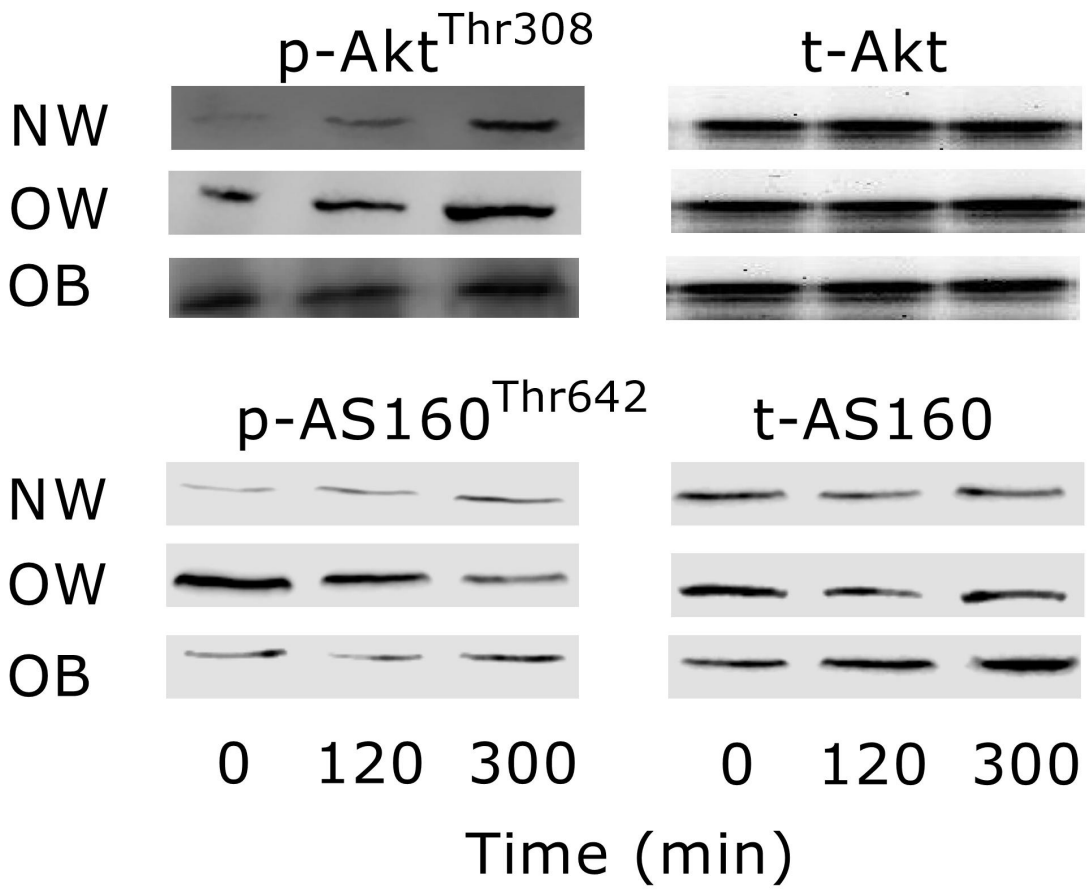
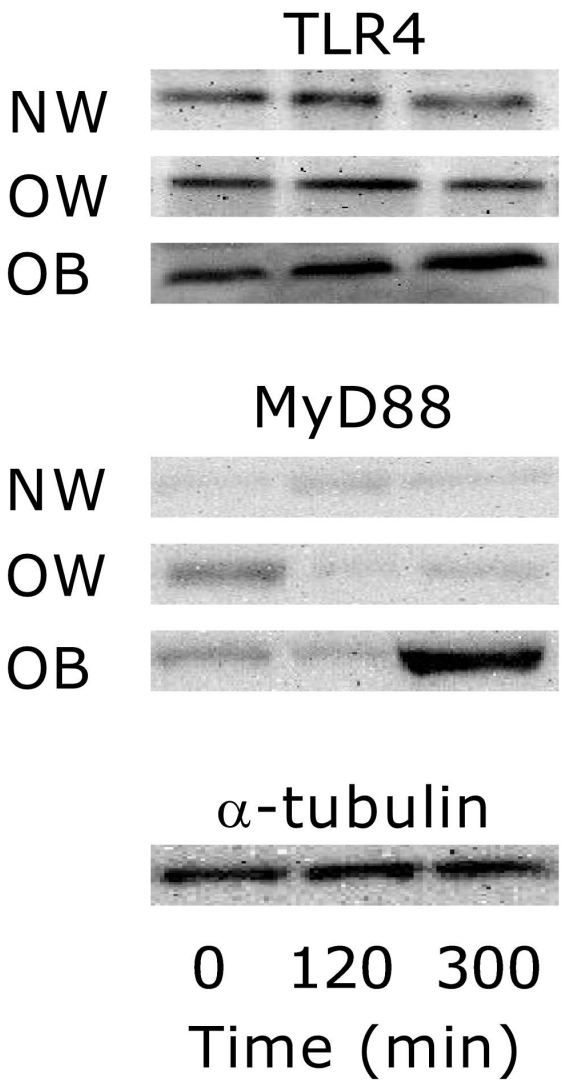


Figure 3.4. Skeletal muscle mitochondrial protein fractional synthesis rates (FSR) in the basal-state and after (0-120 and 120-300 min) pork ingestion (n=10 per group). Inset shows the cumulative postprandial (0-300 min) mitochondrial protein synthetic response. * P<0.05 *vs.* basal. NW, normal weight; OW, overweight; OB, obese. Data are mean ± SEM.



Supplemental Fig 3.1. Western blots for total and phosphorylated Akt (Thr308) and total and phosphorylated AS160 (Thr642) proteins in the basal-state (0 min) and after the ingestion of pork (120 and 300 min).



Supplemental Fig 3.2. Western blots for total Toll-like receptor 4 (TLR4), myeloid differentiation factor 88 (MyD88), and α tubulin proteins in the basal-state (0 min) and after the ingestion of pork (120 and 300 min).

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CHAPTER IV: STUDY 3

DIVERGENT STIMULATION OF MUSCLE PROTEIN SYNTHESIS AND MUSCLE INFLAMMATORY RESPONSES TO FEEDING AND RESISTANCE EXERCISE IN PEOPLE WITH OBESITY[3]

Running head: Obesity and exercise-induced muscle protein synthesis

Corresponding author: Nicholas A. Burd, PhD, Department of Kinesiology and Community Health, University of Illinois at Urbana-Champaign, 352 Louise Freer Hall, 906 S. Goodwin Avenue, Urbana, IL 61801, USA, Email : naburd@illinois.edu, Phone : +1 (217) 244-0970

Key words: strength training, leucine, muscle mass, mTORC1, inflammation, TLR4

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4.1 ABSTRACT

We aimed to determine if obesity alters anabolic signaling phosphorylation, muscle inflammatory protein content, and the subsequent stimulation of muscle protein synthesis within the myofibrillar (MYO) and sarcoplasmic (SARC) protein fractions after resistance exercise. Nine normal weight (NW) (21±1y, BMI 22±1 kg/m^2) and nine obese (OB) (22±1y, BMI 36±2 kg/m^2) adults received L-[*ring*-$^{13}C_6$]phenylalanine infusions with blood and muscle sampling at basal and fed-state of the exercise (EX) and non-exercise (CON) legs. Participants performed unilateral leg extensions and consumed pork (36 g protein) immediately after exercise. Basal muscle TLR4 protein were similar between OB and NW groups (P>0.05), but increased at 300 min after pork ingestion only in OB group (P=0.03). Resistance exercise reduced TLR4 protein in the OB group at 300 min (EX vs. CON leg in OB: P=0.04). Pork ingestion increased p70S6K phosphorylation at 300 min in CON and EX of the OB and NW groups (P>0.05), but the response was lower in the EX leg of OB vs. NW at 300 min (P=0.05). Basal MYO was similar between NW and OB groups (P>0.05) and was stimulated by pork ingestion in the EX and CON legs in both groups (Δ from basal NW: CON 0.04±0.01%/h; EX 0.10±0.02%/h; OB: CON 0.06±0.01%/h; EX 0.06±0.01%/h; P<0.05). MYO was more strongly stimulated in the EX vs. CON legs in NW (P=0.02) but not OB (P=0.26). SARC was feeding sensitive but not further potentiated by resistance exercise in both groups. Our results suggest that obesity may attenuate the effectiveness of resistance exercise to augment fed-state MYO.

4.2 INTRODUCTION

Obesity is associated with metabolic alterations during the postprandial period (Golay *et al.*, 1986), many of which stem from impaired skeletal muscle metabolism (e.g. insulin resistance (Bajpeyi *et al.*, 2011)). Specifically, several studies have documented a reduced stimulation of the muscle protein synthetic response to amino acid provision in obese adults when compared to normal weight controls (Beals *et al.*, 2016; Chevalier *et al.*, 2015; Guillet *et al.*, 2009; Murton *et al.*, 2015; Smeuninx *et al.*, 2017). Interestingly, it seems that this anabolic resistance of postprandial muscle protein synthesis rates in response to exogenous amino acid administration may be confined to distinct muscle protein sub-fractions (Guillet *et al.*, 2009; Chevalier *et al.*, 2015; Murton *et al.*, 2015; Beals *et al.*, 2016, 2017). For instance, we have recently demonstrated an impairment in the stimulation of postprandial myofibrillar protein synthesis rates (Beals *et al.*, 2016), but not mitochondrial protein synthesis rates (Beals *et al.*, 2017), after ingestion of protein-dense food in sedentary obese versus normal-weight adults. The apparent lack of a stimulation of postprandial myofibrillar protein synthesis rates with obesity may be related to alterations in anabolic signaling and/or muscle inflammatory proteins related to TLR4 signaling in response to increased dietary amino acid availability in circulation (Beals *et al.*, 2016). This obesity-related anabolic resistance of myofibrillar proteins to protein nutrition is concerning as this protein pool is the most abundant and is the primary storage depot of amino acids within skeletal muscles. Hence, the maintenance of the myofibrillar protein fraction is especially relevant to maintain overall muscle mass and quality throughout adult life.

Resistance exercise is widely recognized as a fundamental anabolic stimulus by increasing mTORC1 phosphorylation and subsequently facilitating more effective use of circulating dietary amino acids for the stimulation of postprandial myofibrillar protein synthesis rates, but not the

sarcoplasmic protein fraction (Moore *et al.*, 2009; Burd *et al.*, 2011*b*), when compared to the resting-state in healthy young adults (Moore *et al.*, 2009; Pennings *et al.*, 2011). Importantly, the performance of acute resistance exercise prior to eating a protein dense meal has been shown to maximize the postprandial myofibrillar protein synthetic response in other populations with anabolic resistance such as the elderly (Yang *et al.*, 2012). Despite this, the efficacy of resistance exercise to augment postprandial muscle protein sub-fractional synthetic rates has yet to be examined in adults with obesity.

Therefore, the purpose of this investigation was to gain an understanding into how human skeletal muscle tissue remodels within the myofibrillar and sarcoplasmic protein fractions in response to the main anabolic stimuli to skeletal muscle tissue, protein ingestion and exercise, in obese and normal weight adults. In addition, we wished to assess the impact of obesity, exercise, and their relationship to muscle inflammatory responses during post-exercise recovery. We used a unilateral exercise model to allow us to simultaneously assess the effects of food ingestion in the exercise and non-exercise states. We hypothesized that resistance exercise prior to protein dense food ingestion (36 g protein and 4 g fat) would potentiate the phosphorylation of mTORC1 signaling and myofibrillar protein synthesis rates, but not the sarcoplasmic fraction, when compared to the non-exercise leg in both obese and normal weight adults. Moreover, we hypothesized that muscle inflammatory proteins related to TLR4/MyD88 signaling would be elevated with obesity (Beals *et al.*, 2017) and remain unresponsive, irrespective of fat mass, to resistance exercise prescribed to maximize myofibrillar protein synthesis (i.e., 4 sets × 10-12 repetitions performed to volitional fatigue) (Burd *et al.*, 2010*a*).

4.3 PARTICIPANTS AND METHODS

Participants and ethical approval

Nine normal-weight (NW, 21 ± 1 y, BMI: 21.9±0.5 kg•m^{-2}) and nine obese (OB, 22 ± 1 y, BMI: 35.7±2.3 kg•m^{-2}) men and women were enrolled to participate in this study. Participants were not involved in a regular exercise-training program and were considered *insufficiently active* based a Godin leisure-time exercise questionnaire (GLTEQ <14 units (Godin & Shephard, 1997)). Participant characteristics are presented in **Table 1**. All participants were deemed healthy based on responses to a routine medical screening questionnaire and had no prior history of participating in stable isotope amino acid tracer experiments. Each participant was informed of the purpose of the study, experimental procedures, and all its potential risks prior to providing written consent to participate. The study was approved by the University of Illinois Institutional Review Board and conformed to standards for the use of human participants in research as outlined in the Declaration of Helsinki.

Baseline procedures

Prior to the infusion trial, participants reported to the laboratory on two separate occasions in the morning. On the first visit, participants' body weight and height were measured as well as body composition by dual-energy X-ray absorptiometry (Hologic QDR 4500A, USA). Participants' waist-to-hip ratio was also measured using the minimum waist-maximum hip method (Shetterly *et al.*, 1993). Finally, participants were familiarized with unilateral leg extension and the ten-repetition maximum (10RM) procedure. On a separate occasion, each subject arrived at the lab after an overnight fast for the determination of oral glucose tolerance. Blood glucose concentrations were determined before and after consumption of 75 g glucose dissolved in 500

mL of water. At the end of this visit, participant's unilateral 10RM for leg extension was assessed and used to determine exercise loads for the infusion protocol. The leg selected for exercise was randomized and counterbalanced for leg dominance across groups and sex. Obtained 10RMs were then used to estimate the participants' single repetition maximum (Brzycki, 1993; Nascimento *et al.*, 2007). In addition, participants were instructed to refrain from vigorous physical activity, alcohol, and to maintain their normal dietary intakes. Food records were obtained during the three days prior to the infusion using the Automated Self-Administered Recall System (ASA24 version 2016, National Cancer Institute, USA). Dietary macronutrient intakes are reported in **Table 2**.

Infusion protocol

The protocol for the infusion trials is presented in **Figure 1**. On the day of the infusion trial, participants reported to the laboratory at ~0700 h after a 10 h fast. An intravenous catheter was inserted into an antecubital vein for baseline blood sample collection, after which a primed (2 μmol•kg lean body mass $(LBM)^{-1}$) continuous infusion (0.05 μmol•kg $LBM^{-1} \cdot min^{-1}$) of L-[*ring*-$^{13}C_6$]phenylalanine was initiated (t =-180 min) and maintained until the end of the trial. A second intravenous catheter was placed in a contralateral dorsal hand vein and placed in a heated blanket for repeated arterialized blood sampling. In the post-absorptive state, muscle biopsies of the *vastus lateralis* were collected at 0 min of infusion from the non-exercised control leg (CON). Subsequently, participants performed unilateral leg extension exercise (4 × 10-12 at 65-70% one repetition maximum (1RM))to volitional failure. Upon completion, the participants consumed 170 g of lean ground pork (36 g protein, ~3.3 g leucine, 4 g fat). Additional muscle biopsies were collected from both the exercised (EX) and CON legs at 120 and 300 min after pork ingestion. The biopsies were collected from the middle region of the *vastus lateralis* (~15 cm above the patella)

with a Bergström needle under local anesthesia. All muscle biopsy samples were freed from any visible adipose, connective tissue and blood, immediately frozen in liquid nitrogen, and stored at –80˚C until subsequent analysis. Arterialized blood samples were drawn every 30 or 60 min during the post-absorptive and postprandial-states and placed in pre-chilled EDTA tubes. Blood samples (8 ml) were collected in EDTA-containing tubes and centrifuged at 3000×g at 4˚C for 10 min. Aliquots of plasma were frozen and stored at –80˚C until subsequent analysis.

Meal composition

Lean center-cut pork loins were homogenized, ground, and individually packaged and then stored at –20°C until each experimental trial. Prior to the infusion trial, the pork was thawed overnight at 4°C and grilled until the inner temperature reached 65°C. Proximate analysis of center cut pork loin was performed as previously described (Novakofski *et al.*, 1989; Beals *et al.*, 2016). The 170 g ground pork patty provided 36 g protein and 4 g of fat. This amount of pork was selected since it represents a typical amount of protein consumed with dinner by US adults (USDA Agricultural Research Service, 2016).

Blood analyses

Glucose and lactate concentrations were analyzed in whole blood using an automated biochemical analyzer (YSI 2300 Stat Plus, Yellow Springs Instruments, USA). Plasma insulin and C-reactive protein (CRP) concentrations were determined using commercially available enzyme-linked immunosorbent assays (ALPCO; USA). Plasma amino acid enrichments and concentrations were determined by GC/MS analysis (Agilent 7890A GC/5975C; MSD, USA) as previously described (Beals *et al.*, 2016). Briefly, plasma samples were deproteinized and converted into tert-

butyldimethylsilyl (*t*-BDMS) derivatives prior to GC/MS analysis. Plasma L-[*ring*-$^{13}C_6$]phenylalanine enrichments were determined using electron impact ionization by ion monitoring at mass/charge (*m/z*) 336 (*m*+0) and 342 (*m*+6) for unlabeled and labeled phenylalanine, respectively. Amino acid concentrations were quantified using the AMDIS software package (v. 2.71, NISTTM, USA) and standards with known concentrations.

Muscle protein synthesis

Muscle intracellular (IC) amino acids were extracted from ~15 mg of wet muscle in 0.6 M perchloric acid. Myofibrillar and sarcoplasmic protein-enriched fractions were extracted from ~50 mg of wet muscle as described previously (Burd *et al.*, 2015). A portion (100 μL) of the resultant muscle homogenate was reserved for sarcoplasmic protein synthesis measurements. The remaining muscle homogenate was stored at –80°C for subsequent western blot analyses. Myofibrillar and sarcoplasmic protein fractions were hydrolyzed overnight in 6 M HCl at 110°C. The resultant free amino acids (IC, myofibrillar, sarcoplasmic protein fractions) were purified using cation exchange chromatography (Dowex 50W-X8-200 resin; Acros Organics, Belgium) and dried under vacuum. Free amino acids were re-suspended in 60% methanol and centrifuged before analysis by 5500 QTRAP liquid chromatography–tandem mass spectrometry using previously reported methodology (Beals *et al.*, 2016, 2017). The L-[*ring*-$^{13}C_6$]phenylalanine enrichments were determined by multiple reaction monitoring (MRM) at *m/z* 166.0 → 103.0 and 172.0 → 109.0 for unlabeled and labeled L-[*ring*-$^{13}C_6$]phenylalanine, respectively. Software Analyst 1.6.2 (Sciex, USA) was used for data acquisition and analysis.

Western blotting

A portion of muscle homogenates isolated during the myofibrillar protein extractions was used for Western blotting analysis. Phosphorylation status and total protein content of mammalian target of rapamycin complex 1 (mTORC1) at Ser2448 and 70 kDa S6 protein kinase 1 (p70S6K) at Thr389 were determined using antibodies purchased from Cell Signaling Technology (Cell Signaling Technology, USA). Muscle protein content was also determined for Toll-like receptor 4 (TLR4: Abcam, USA) and myeloid differentiation factor 88 (MyD88: Cell Signaling, USA). Protein content of the homogenate was determined by Bradford Assay (Bio-Rad) and then equal amounts of protein were separated by SDS-PAGE before being transferred to either nitrocellulose (mTORC1 and TLR4) or polyvinyl difluoride membranes (p70S6K and MyD88). After blocking, membranes were incubated in primary antibodies overnight at 4°C. Membranes from the respective proteins were then incubated with appropriate secondary antibodies and protein content was detected using West Femto Maximum Sensitivity substrate (SuperSignal, Thermo Scientific, USA) and the ChemiDoc XRS+ Imaging System (Bio-rad, USA). After detection of phosphorylated proteins, membranes were stripped with western blot stripping buffer (Restore, Thermo Scientific, USA) and re-incubated with antibodies against total protein. Western blot data were normalized to an internal control (α-tubulin, Abcam, USA). Bands were quantified using ImageJ software (NIH), normalized to a control sample run on each blot to account for inter-blot variability.

Calculations

Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the fasting glucose and insulin values from the OGTT ($Glucose_{fast} \times Insulin_{fast}/22.5$ (Matthews *et al.*, 1985)). The fractional synthetic rates (FSR) of myofibrillar and sarcoplasmic proteins were calculated

using standard precursor-product methods by dividing the increment in tracer enrichment in the protein fractions by the enrichment of the muscle intracellular free precursor pool over time. The baseline muscle-bound enrichment for the calculation of resting muscle protein synthesis rates were estimated using mixed proteins in plasma collected prior to initiation of the infusion as described in detail elsewhere (Burd *et al.*, 2011*a*, 2012).

Statistics

Differences in myofibrillar and sarcoplasmic protein synthesis, plasma and intracellular enrichments, muscle signaling proteins, blood glucose and lactate, and plasma insulin were determined using factorial analysis of variance (ANOVA) with repeated measures on time. Bonferroni post-hoc tests were used to compare means whenever significant interactions were observed. Body composition, demographics, exercise variables, dietary intake, HOMA-IR, plasma CRP, and net area under the blood glucose, plasma insulin, and AA curves (AUC) were analyzed using unpaired Students T-tests. For all analyses, differences were considered significant at $P<0.05$. All calculations were performed using IBM SPSS Statistics Version 20. Data are expressed as means ± SEMs.

4.4 RESULTS

Oral Glucose Tolerance testing

Data obtained during the oral glucose tolerance testing is presented in **Table 1**. Blood glucose was similar among groups at baseline (P=0.33). However, at 120 min post-beverage consumption, blood glucose was greater in OB (110.3 ± 8.3 mg/dL) compared with the NW group (81.6 ± 3.9 mg/dL; P=0.008). Fasting insulin was greater (P=0.01) in the OB group (19.0 ± 3.2 μIU/mL) compared with the NW (8.5 ± 1.1 μIU/mL) group. Similarly, HOMA-IR was greater (P=0.01) in the OB group (3.88 ± 0.70) than the NW group (1.73 ± 0.25). Fasting CRP was not different between groups (P=0.19).

Resistance exercise

Exercise performance variables are presented in **Table 3**. Total external work (repetitions × load (kg)) performed during resistance exercise was similar between groups (NW: 1610±164 kg; OB: 1888±154 kg, P=0.23). However, total external work relative to the lean mass of the EX leg (leg LM) was greater in NW (196.4±3.1 kg•kg leg LM^{-1}) compared with OB group (176.0±7.7 kg•kg leg LM^{-1}; P=0.03). Resistance exercise increased blood lactate concentrations similarly between the NW and OB groups (Absolute change from pre-exercise NW: 3.80±0.93 mM; OB: 3.40±0.52 mM, Exercise effect: P<0.001; Group effect: P=0.30).

Blood variables

Blood glucose concentrations did not change after pork ingestion (P<0.05) with no differences between the NW and OB groups (P<0.05; **Figure 2A**). Basal insulin concentrations were approximately 3.2-fold greater in OB group (19.0±3.4 μIU/mL) when compared with the NW

group (8.2±0.9 μIU/mL; **Figure 2B**). Plasma insulin concentrations increased similarly above basal in both groups (Time effect: P=0.02; Group × time: P=0.36). However, plasma insulin concentrations were greater in the OB group throughout the infusion trial compared with the NW group (Group effect: P=0.002). Plasma essential amino acid concentrations (EAA) were not different at baseline (P=0.89) and increased similarly after pork ingestion in the NW and OB groups (Time effect: P<0.001) with peak values at 90 min of the postprandial period (**Figure 2C**). Moreover, the net area under the time × curve (AUC) for EAA were similar between groups (P=0.92). Similarly, plasma branched chain amino acid (BCAA) concentrations increased after pork ingestion with no differences between groups (Time effect: P<0.001; **Figure 2D**).

Muscle inflammatory proteins

Relative total muscle protein concentrations for TLR4 and MyD88 are presented in **Figure 3**. Basal muscle TLR4 protein content was similar among groups (P=0.22). Pork ingestion did not affect muscle TLR4 protein concentrations in either the CON or EX legs of NW (All, P>0.05). By contrast, muscle TLR4 protein content in the muscle of the OB group was elevated above basal in both the CON and EX legs at 300 min (P<0.05), however, muscle TLR4 protein content was reduced in the EX when compared to the CON leg of the OB group (P=0.04). Total MyD88 protein contents were stable with no effect of pork ingestion or exercise in the CON or EX legs of the NW and OB group (All, P>0.05). Representative western blots for TLR4 and MyD88 proteins are shown in **Supplementary Figure 1A**.

Muscle anabolic signaling

Relative total protein concentrations for mTORC1 was stable after pork ingestion (P=0.21) or exercise (P=0.93) in both the NW and OB groups. However, total mTORC1 protein content was 2.3-fold greater at basal in the OB group compared to the NW group (Group Effect: P=0.03). Phosphorylation of mTORC1 at Ser2448 was also unaffected by pork ingestion (P=0.84) or exercise (P=0.66) in the NW and OB groups throughout the 0-300 min postprandial period. However, there was a trend for increased mTORC1 phosphorylation in NW as compared to the OB group (Group Effect: P=0.08; **Figure 4A**).

Total p70S6K protein concentration was 1.6-fold greater at basal in the OB group when compared to the NW group (Group Effect: P=0.02). However, protein ingestion (P=0.27) or resistance exercise (P=0.26) did not alter total p70S6K protein content in either group throughout the 0-300 min postprandial period. At basal, p70S6K phosphorylation at Thr389 was lower in OB group when compared to the NW group (P=0.04; **Figure 4B**). Phosphorylation of p70S6K was increased above basal at 120 and 300 min in the CON and EX legs of the NW group (Both, P<0.05) with no differences between CON and EX (All, P>0.05). By contrast, at 120 min of the postprandial period, p70S6K phosphorylation was only increased above basal in the EX leg of OB group (P=0.01). At 300 min, p70S6K phosphorylation was increased in both the CON and EX legs (Both, P>0.05). However, in the EX leg there was lower p70S6K phosphorylation in the OB group as compared with the NW group (P=0.05). Representative western blots for anabolic signaling proteins are shown in **Supplementary Figure 1B**.

Plasma and intracellular precursor enrichments

Plasma and muscle intracellular (IC) free L-[*ring*-$^{13}C_6$]phenylalanine enrichments are shown in **Figure 5**. Plasma L-[*ring*-$^{13}C_6$]phenylalanine enrichments were steady during the postabsorptive

period, but decreased after pork ingestion at 90 and 120 min of the postprandial period (Time effect: P=0.002) with no group differences (P=0.29). IC L-[*ring*-$^{13}C_6$]phenylalanine enrichments also decreased at 120 min of the postprandial period (Time effect: P=0.01) with no differences observed between groups (group effect: P=0.22). In addition, IC enrichments were also greater in EX as compared with CON legs (exercise effect: P=0.03). However, due to the differences in IC enrichments between EX and CON legs (described above), only protein synthesis rates using this precursor are presented.

Muscle protein synthesis

Basal sarcoplasmic protein synthesis rates did not differ between the NW and OB groups (P=0.72; **Figure 6A**). Postprandial sarcoplasmic protein synthesis rates increased similarly in CON leg after pork ingestion throughout the 0-300 min postprandial period in the NW and OB groups (P=0.45). Moreover, there were no differences observed in the EX *vs.* CON legs throughout the 0-300 min postprandial period in the NW or OB groups (P=0.52). The time course of stimulation of sarcoplasmic protein synthesis rates after pork ingestion is shown in **Table 4**. Sarcoplasmic protein synthesis rates increased in the CON leg at 0-120 min and 120-300 min (P<0.05) with no group differences (P=0.30). However, postprandial sarcoplasmic protein synthesis rates only increased in the EX leg of the OB group at 0-120 min and were greater than the response observed in the NW group (P=0.03). Sarcoplasmic protein synthesis rates were similar at 120-300 min after pork ingestion in the EX leg in the NW and OB groups (P=0.31).

Basal myofibrillar protein synthesis rates were similar between NW and OB groups (P=0.42, **Figure 6B**). Cumulative (0-300 min) myofibrillar protein synthesis rates were stimulated in the EX and CON legs after pork ingestion in both the NW and OB groups. However, the

postprandial myofibrillar protein synthetic response was stimulated to a greater extent in the EX *vs.* CON legs in NW group (absolute difference from CON: 0.052±0.016 %•h^{-1}; P=0.03) but not OB group (absolute difference from CON: -0.001±0.015 %•h^{-1}; P=1.00). The time course of stimulation of the myofibrillar protein synthetic responses are shown in **Table 4**. Pork ingestion stimulated postprandial myofibrillar protein synthesis rates above basal values in the CON legs at 0-120 and 120-300 min of the postprandial period with no group differences (All, P>0.05). Postprandial myofibrillar protein synthesis rates were greater in EX than CON leg at 0-120 and 120-300 min after pork ingestion in the NW group (P<0.05). However, postprandial myofibrillar protein synthesis rates in the EX-state were not stimulated above those of CON (P>0.05) and were lower in the OB when compared to the NW group (P<0.05). Sarcoplasmic and myofibrillar protein synthesis rates based on the plasma and muscle intracellular free L-[*ring*-$^{13}C_6$]phenylalanine enrichments as precursors were highly correlated (Pearson r: 0.92 P<0.001; data not shown).

4.5 DISCUSSION

To our knowledge this is the first assessment of muscle anabolic signaling, inflammatory protein content, and changes in myofibrillar and sarcoplasmic protein synthesis rates after protein dense food ingestion in the non-exercise and exercise-states in people with obesity. Here, we show that myofibrillar and sarcoplasmic protein synthesis rates are similarly enhanced with pork ingestion in the non-exercise leg in both the NW and OB groups. However, resistance exercise potentiated the fed-state synthetic response only within the myofibrillar protein fraction in the NW group, but not in the OB group. The stimulation of sarcoplasmic protein synthesis rates was namely feeding responsive with no additive effect of prior resistance exercise on this protein fraction irrespective of BMI score. What is noteworthy is that this blunted post-exercise myofibrillar

protein synthetic response with obesity occurred independently of overt differences in habitual physical activity levels or systemic inflammation between the groups (**Table 1**). As such, our results demonstrate that excess adiposity attenuated the effectiveness of resistance exercise to potentiate the fed-state myofibrillar protein synthetic response in otherwise healthy young adults.

Previous reports have emphasized the importance of resistance exercise volume (Burd *et al.*, 2010*a*) and the achievement of bulk muscle fiber recruitment (Burd *et al.*, 2010*b*) as important anabolic factors to maximize the postprandial myofibrillar protein synthetic response during recovery from resistance exercise in healthy young adults. Based on this, we provided (or what we believed to be) a potent resistance exercise prescription to maximize fed-state myofibrillar protein synthesis rates. Specifically, the volunteers performed resistance exercise at 4 sets × 10-12 repetitions with each set performed to volitional failure, which generally provides a proxy indicator that a strong stimulus was provided to maximize muscle fiber recruitment patterns in both the NW and OB groups (Potvin & Fuglevand, 2017). Moreover, both the NW and OB groups performed similar amounts of external total work (repetitions × load [kg]) in both absolute terms and when expressed relative to maximal strength (**Table 3**), which resulted in similar blood lactate concentrations immediately after resistance exercise. The amount of external work performed during resistance exercise has been shown to directly influence both the amplitude and the duration of the stimulation of post-exercise myofibrillar protein synthesis rates (Burd *et al.*, 2010*b*). Hence, it would seem that a sufficient and equivalent resistance exercise stimulus was provided to both the NW and OB groups. What is noteworthy, however, is that obesity is associated with greater total lean mass when compared to normal weight individuals in this data set and others (Forbes & Welle, 1983; Beals *et al.*, 2016). Therefore, we compared the amount of external work performed relative to the lean mass of the exercised leg. We observed that the NW group performed more

total work per leg lean mass basis despite both groups exercising at a high level of effort and until voluntary failure. Thus, it could be speculated that the 'quality' of the overall contractile stimulus on skeletal muscle tissue was reduced in the OB when compared to the NW group. We did not assess electromyography during exercise and, as such, it is not possible to decipher if muscle fiber recruitment patterns were different between the OB and NW groups. Notwithstanding, studies in aging populations, who also display anabolic resistance (Wall *et al.*, 2015), have shown that more resistance exercise volume is required to elicit a robust post-exercise myofibrillar protein synthetic response when compared to their younger-counterparts (Kumar *et al.*, 2012). Therefore, it is possible that we under-prescribed resistance exercise and subsequently missed an opportunity to maximize the anabolic potential of resistance exercise to counteract the negative consequences of obesity on myofibrillar protein remodeling despite providing exercise prescription in far excess of current exercise recommendations for novice weight lifters (Donnelly *et al.*, 2009; ACSM, 2009; CSEP, 2011).

Similar to past efforts (Moore *et al.*, 2009; Burd *et al.*, 2011*b*), we show that resistance exercise had no additional effect on the stimulation of postprandial sarcoplasmic protein synthesis rates in the OB or NW groups. Interestingly, the time-course in the stimulation of sarcoplasmic protein synthesis rates was different at 2 h after protein dense food ingestion in the EX-state between NW and OB groups. Specifically, the postprandial sarcoplasmic protein response in the EX-state was greater at 0-2 h in the OB group when compared to the NW group. Given that the synthesis rates of myofibrillar protein fraction were robustly stimulated in the NW group at 0-2 h when compared to the OB group, it appears that perhaps with resistance exercise the dietary amino acids remained confined to the more rapidly turning over sarcoplasmic pool in obese muscles as opposed to being used for post-exercise myofibrillar protein accretion.

The cellular mechanisms that regulate myofibrillar protein synthesis rates are thought to be centered on anabolic pathways such as mTORC1 signaling (Abou Sawan *et al.*, 2018). We previously showed dysregulated mTORC1 signaling in muscles of obese vs. normal weight adults (Beals *et al.*, 2016). In particular, muscles of individuals with obesity demonstrated increased relative concentrations of total mTORC1 protein, and increased phosphorylation, when compared to the NW adults. Here, we also observed elevated mTORC1 protein concentrations and phosphorylation in the basal and fed-states in the OB group when compared to the NW group. Indeed, when assessing the ratio of phosphorylation to total mTORC1 protein there were no observed differences between the OB and NW groups (**Figure 4**), but these normalized data mask that aberrant mTORC1 signaling occurred in obese vs. healthy-weight muscles. This notion is further underlined by an obesity-related decrease in p70S6K phosphorylation in the basal-state and at 300 min of the exercise leg when compared to the NW group (**Figure 4**). Previously, it has been shown that the amplitude of increase in post-exercise p70S6K phosphorylation at Thr389 is sensitive to resistance exercise volume in normal weight adults (Terzis *et al.*, 2010). Moreover, it has been demonstrated that doubling the resistance exercise volume (6 sets > 3 sets at 75% of 1RM) in older adults induces a more robust increase in p70S6K phosphorylation during post-exercise recovery (Kumar *et al.*, 2012). Thus, we speculate that anabolic signaling mechanisms may be desensitized to exercise in obese muscles such that a higher threshold of volume may be required to restore the anabolic sensitivity of the muscle protein synthetic machinery.

Our lab (Beals *et al.*, 2017) and others (Reyna *et al.*, 2008; Timmerman *et al.*, 2016) have previously shown that individuals with obesity have elevated muscle TLR4 protein content. However, we did not observe this in the present study. In agreement with our previous report, we observed an obesity-specific increase in muscle TLR4 protein content after protein-dense food

ingestion. We have also recently demonstrated that people with obesity have greater MyD88 protein, an adaptor protein downstream of TLR4 signaling, at baseline and after eating protein-dense food when compared to their normal-weight counterparts (Beals *et al.*, 2017). Similar to TLR4 protein content, these findings of elevated MyD88 protein with obesity were not recapitulated in the present study, which may be related to the fact that the obese volunteers in the present study were less inflamed as noted by the lower basal muscle TLR4 protein content when compared to our past efforts (Beals *et al.*, 2017).

Interestingly, we demonstrated that an acute bout of resistance exercise suppresses the feeding-induced increase in muscle TLR4 protein content in people with obesity. This receptor has been implicated in the development of insulin resistance (Shi *et al.*, 2006) and reductions in TLR4 protein may be a mechanism for resistance exercise-induced increases in insulin sensitivity (Croymans *et al.*, 2013). Relatively few studies have attempted to capture the acute or prolonged effect of exercise on muscle TLR4 protein (Lambert *et al.*, 2008; Li *et al.*, 2015; Ghosh *et al.*, 2015). However, it has been shown that 12 weeks of combined endurance and resistance exercise training appears to be more effective than diet-induced weight loss at reducing total muscle TLR4 protein content in older adults with obesity (Lambert *et al.*, 2008). In support, it has been shown that 16 weeks of aerobic exercise reduces muscle TLR4 protein in non-obese older adults to amounts that are similar to younger adults (Ghosh *et al.*, 2015). Here, we show that acute resistance exercise can modify and reduce total muscle TLR4 protein content in obese muscles. Certainly, more work is ultimately needed to determine how acute vs. chronic exercise modulates muscle inflammation in people with obesity and the associated phenotypic consequence.

In conclusion, we show that excess fat mass diminishes the interactive nature of feeding and resistance exercise on the stimulation of myofibrillar protein synthesis in sedentary young

adults. We observed no defect in the stimulation of sarcoplasmic protein synthesis rates between obese and normal weight adults. In addition, we show that muscle anabolic signaling mechanisms are also reduced in obese vs. normal weight adults. The focus of future research should aim to re-sensitize the anabolic machinery and myofibrillar protein synthesis rates by prescribing a more robust resistance exercise bout via manipulation of volume and/or load. Of course, reducing fat mass would also be beneficial to elicit a robust exercise-induced myofibrillar protein synthetic response in people with obesity.

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Disclosure statement

The authors declare no conflicts of interest, financial or otherwise.

Author contributions

JWB and NAB contributed to the conception and the design of the experiment. JWB, SKS, EGP, SAF, SvV, IGM, AVU, ZL, SAP, and NAB contributed to collection, analysis, and interpretation of data. JWB and NAB contributed to drafting or revising intellectual content of the manuscript. JWB and NAB had primary responsibility for final content. JWB, SKS, EGP, SAF, SvV, IGM, AVU, ZL, SAP, and NAB read, edited, and approved the final version of the manuscript. This research was supported by an ACSM Foundation Doctoral Student Research Grant from the American College of Sports Medicine Foundation. The researchers were responsible for the study

design, data collection and analysis, decision to publish, and preparation of the manuscript. The American College of Sports Medicine Foundation approved the study design.

4.6 TABLES AND FIGURES

Table 4.1. Participant characteristics

Variable	NW	OB
n (females)	9 (5)	9 (4)
Age (y)	21 ± 1	22 ± 1
Ht (m)	1.70 ± 0.04	1.71 ± 0.02
Wt (kg)	63.9 ± 3.8	104.0 ± 5.9‡
BMI ($kg \cdot m^2$)	21.9 ± 0.5	35.7 ± 2.3
% Body fat	23.3 ± 2.7	36.8 ± 2.7‡
Lean mass (kg)	47.8 ± 4.3	63.2 ± 3.5‡
Waist-to-hip ratio	0.78 ± 0.02	0.89 ± 0.02‡
Physical Activity (GLTEQ score)	6.7 ± 2.3	11.2 ± 1.5
Fasting blood glucose (mg/dL)	79.7 ± 2.7	84.4 ± 3.5
Fasting insulin (μIU/mL)	8.5 ± 1.1	19.0 ± 3.5‡
HOMA-IR	1.7 ± 0.3	3.9 ± 0.7‡
120 min blood glucose (mg/dL)	81.6 ± 3.9	110.3 ± 8.3‡
C-reactive protein (mg/L)	0.95 ± 0.31	2.31 ± 0.73

Data are Mean ± SEM. ‡ $P<0.05$ *vs.* NW.

Table 4.2. Macronutrient intake

Variable	NW	OB
Energy intake		
Total (kcal • d^{-1})	2021 ± 323	2288 ±333
Relative (g • kg^{-1} • d^{-1})	31 ± 4	22 ± 2
Protein intake		
Total (g • d^{-1})	69.3 ± 14.3	114.0 ± 18.8 ‡
Relative (g • kg^{-1} • d^{-1})	1.1 ± 0.1	1.03 ± 0.2
Energy from protein (%kcal • d^{-1})	10 ± 3	21 ± 3 ‡
Carbohydrate (CHO) intake		
Total (g • d^{-1})	246.4 ± 50.3	221.9 ± 62.3
Relative (g • kg^{-1} • d^{-1})	3.3 ± 0.6	1.8 ± 0.5
Energy from CHO (%kcal • d^{-1})	47 ± 3	39 ± 6
Fiber (g • d^{-1})	14.3 ± 1.6	14.7 ± 2.7
Fat intake		
Total (g • d^{-1})	79.9 ± 11.8	106.5 ± 8.4
Energy from fat (%kcal • d^{-1})	35 ± 2	44 ± 4 ‡
Saturated fat (g • d^{-1})	22.8 ± 2.9	37.8 ± 2.1 ‡
Cholesterol (mg • d^{-1})	291.0 ± 68.5	443.5 ± 68.0

Data are Mean ± SEM. ‡ $P<0.05$ *vs.* NW.

Table 4.3. Exercise variables

Variable	NW	OB
Unilateral leg extension 10 RM (kg)	31.8 ± 3.0	37.8 ± 2.8
Pre-exercise blood lactate (mM)	0.99 ± 0.09	1.22 ± 0.20
Post-exercise blood lactate (mM)	4.79 ± 1.00	4.61 ± 0.66
Total work (kg)	1610 ± 164	1888 ± 154
Relative work (kg•kg 1 RM^{-1})	38.1 ± 1.3	37.3 ± 0.9
Work: leg LM ratio (kg•kg leg LM^{-1})	196.4 ± 3.1	176.0 ± 7.7‡

1RM: one repetition maximum. LM: lean mass. Data are mean±SEM. * $P<0.05$ *vs.* NW.

Table 4.4. Temporal myofibrillar and sarcoplasmic protein synthetic responses in the exercised (EX) and non-exercised control (CON) legs before and after pork ingestion (*n*=9 per group).

			Time (min)			
			0-120		120-300	
MPS (%•h^{-1})	Group	Basal	CON	EX	CON	EX
Myofibrillar	NW	0.06 ± 0.01	0.12 ± 0.02*	0.18 ± 0.02*†	0.09 ± 0.01*	0.14 ± 0.03*†
	OB	0.05 ± 0.01	0.12 ± 0.02*	0.12 ± 0.01*‡	0.11 ± 0.01*	0.11 ± 0.02*
Sarcoplasmic	NW	0.10 ± 0.01	0.18 ± 0.03*	0.10 ± 0.04	0.20 ± 0.05*	0.22 ± 0.04*#
	OB	0.11 ± 0.02	0.18 ± 0.04*	0.20 ± 0.02*‡	0.25 ± 0.05*	0.20 ± 0.03*

Data are Mean ± SEM. * P<0.05 *vs.* basal, #P<0.05 *vs.* 0-120, † P<0.05 *vs.* CON leg, ‡ P<0.05 *vs.* NW.

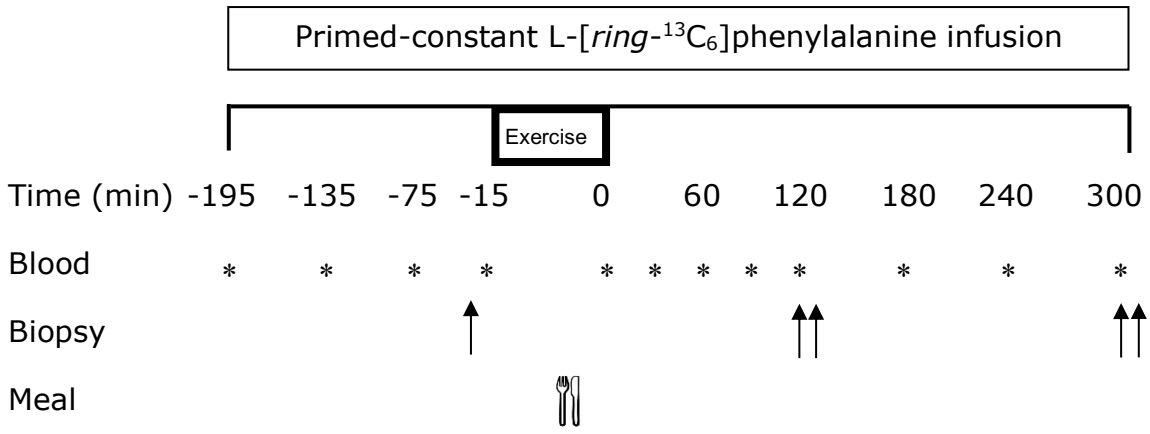


Figure 4.1: Study timeline. The top bar indicates intravenous infusion of labeled amino acids throughout the day. At t = 0 subjects received a pork meal. Blood samples are indicated with an asterisk (*) and muscle biopsy samples are indicated with an arrow (↑).

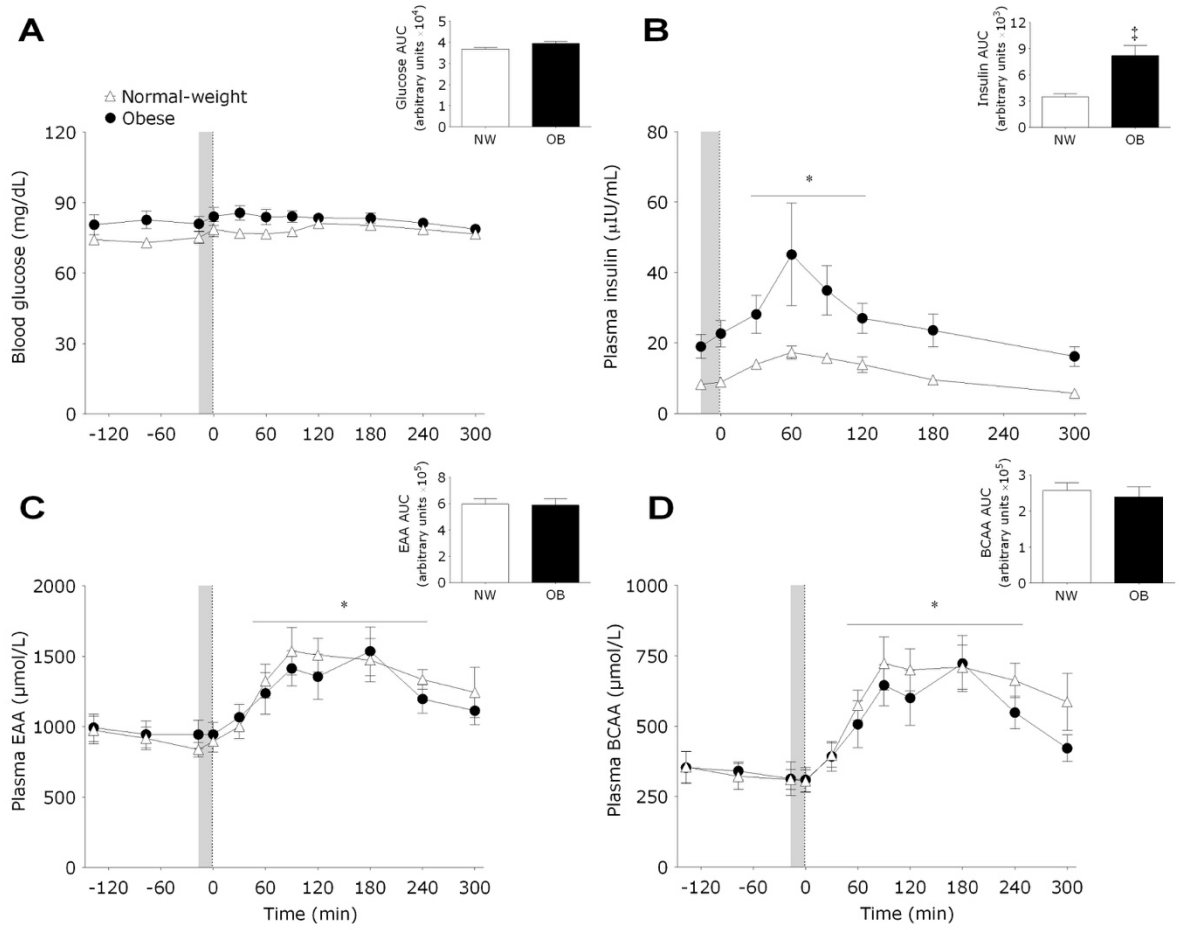


Figure 4.2. Blood glucose (**A**) and plasma insulin (**B**) essential amino acids (EAA, **C**) and branched chain amino acids (BCAA, **D**) concentrations in the basal state and after pork ingestion (n=9 per group). Inset are the respective area under the time curves. Gray bar indicates a bout of unilateral leg extension exercise. Dashed vertical line refers to pork ingestion. Data are Mean ± SEM. Insulin concentrations – Group effect: P=0.002. * P<0.05 *vs.* basal. ‡ P<0.05 *vs.* NW.

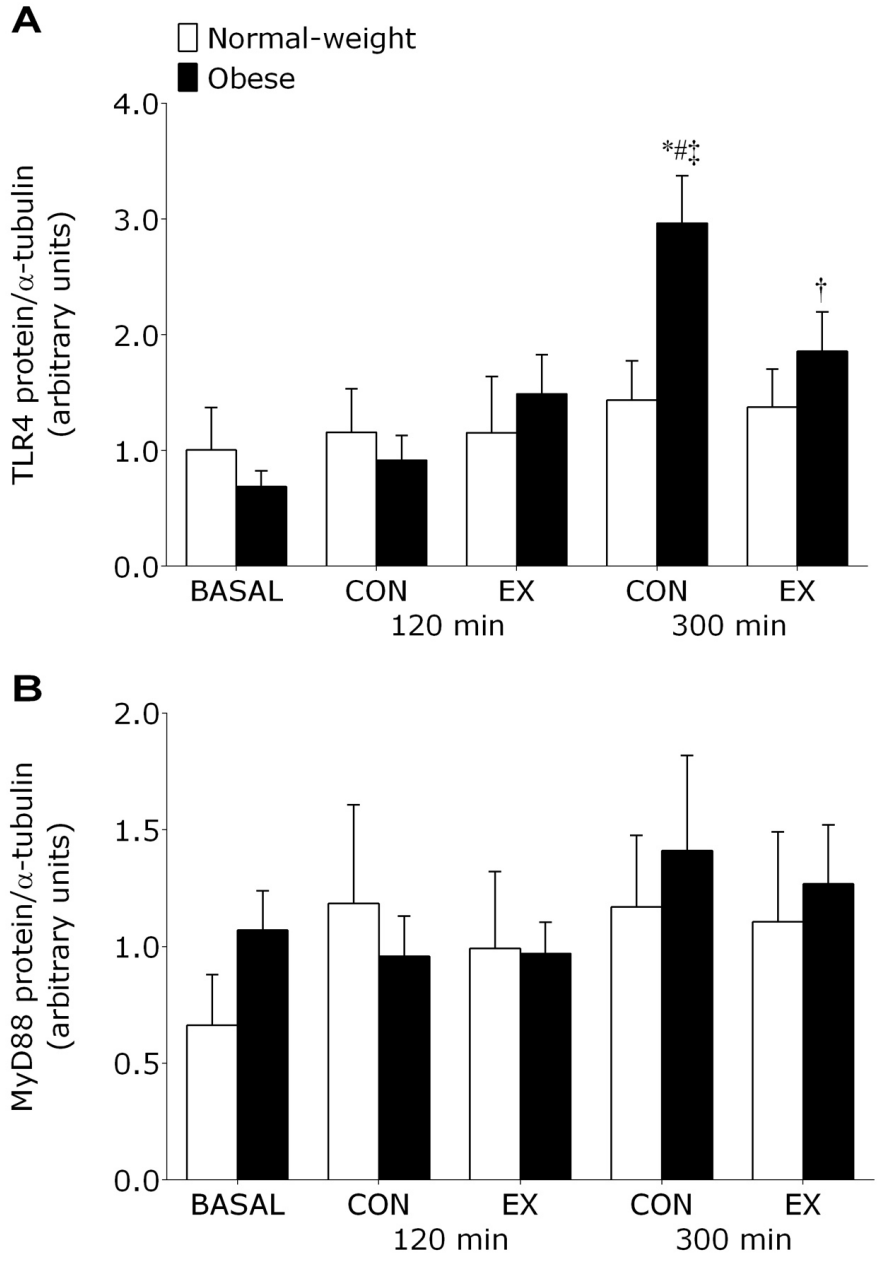


Figure 4.3. Muscle protein content for toll-like receptor 4 (TLR4; **A**), myeloid differentiation factor 88 (MyD88; **B**) at basal and after the ingestion of pork (*n*=9 per group) in both the non-exercised (CON) and exercised (EX) leg. Immediately prior to pork ingestion participants performed a bout of unilateral leg extension exercise. Data are Mean ± SEM. * $P<0.05$ *vs.* basal, # $P<0.05$ *vs.* 120 min, † $P<0.05$ *vs.* CON leg, ‡ $P<0.05$ *vs.* NW.

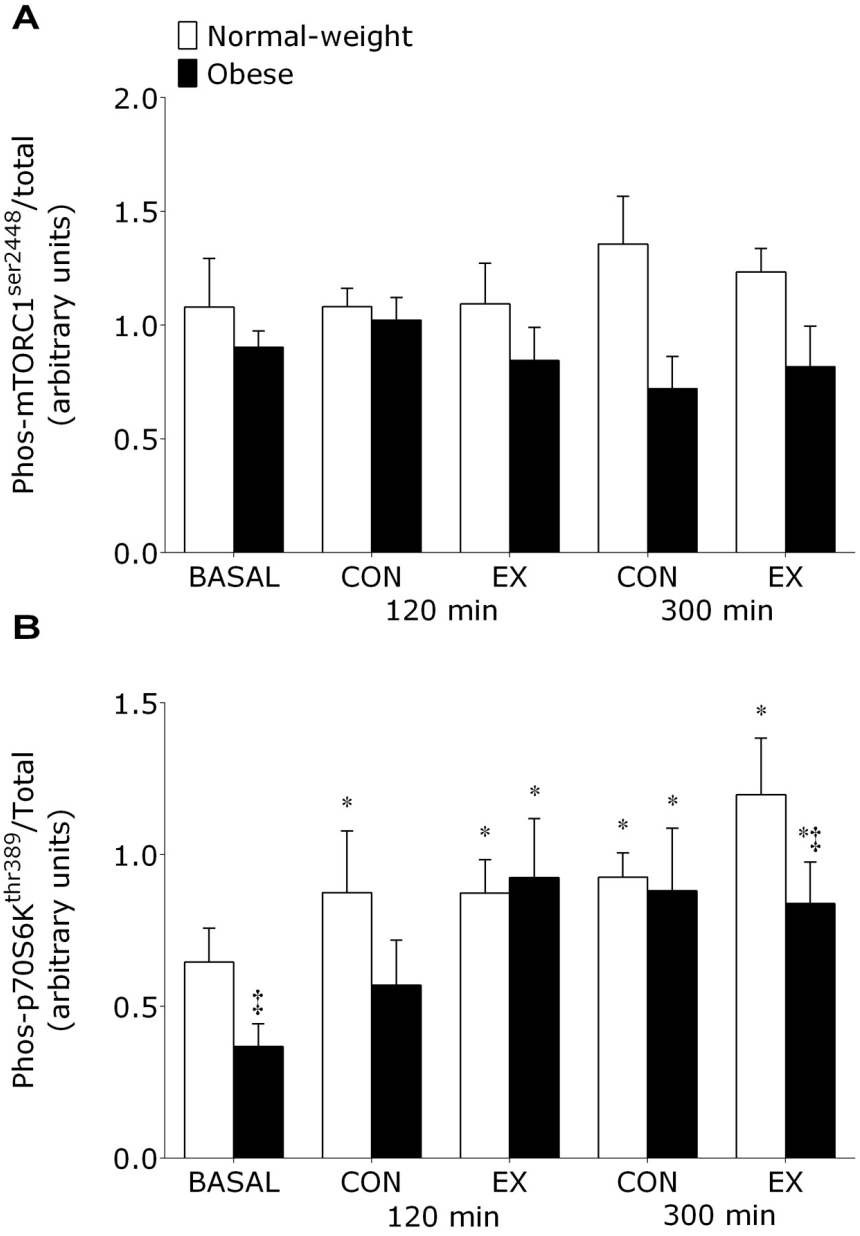


Figure 4.4. Phosphorylation of Mammalian target of rapamycin complex 1 (mTORC1) at Ser2448 (**A**), and 70 kDa S6 protein kinase (p70S6K) at Thr389 (**B**) at basal and after the ingestion of pork (*n*=9 per group) in both the non-exercised (CON) and exercised (EX) leg. Immediately prior to pork ingestion participants performed a bout of unilateral leg extension exercise. Data are Mean ± SEM. * $P<0.05$ *vs.* basal, ‡ $P<0.05$ *vs.* NW.

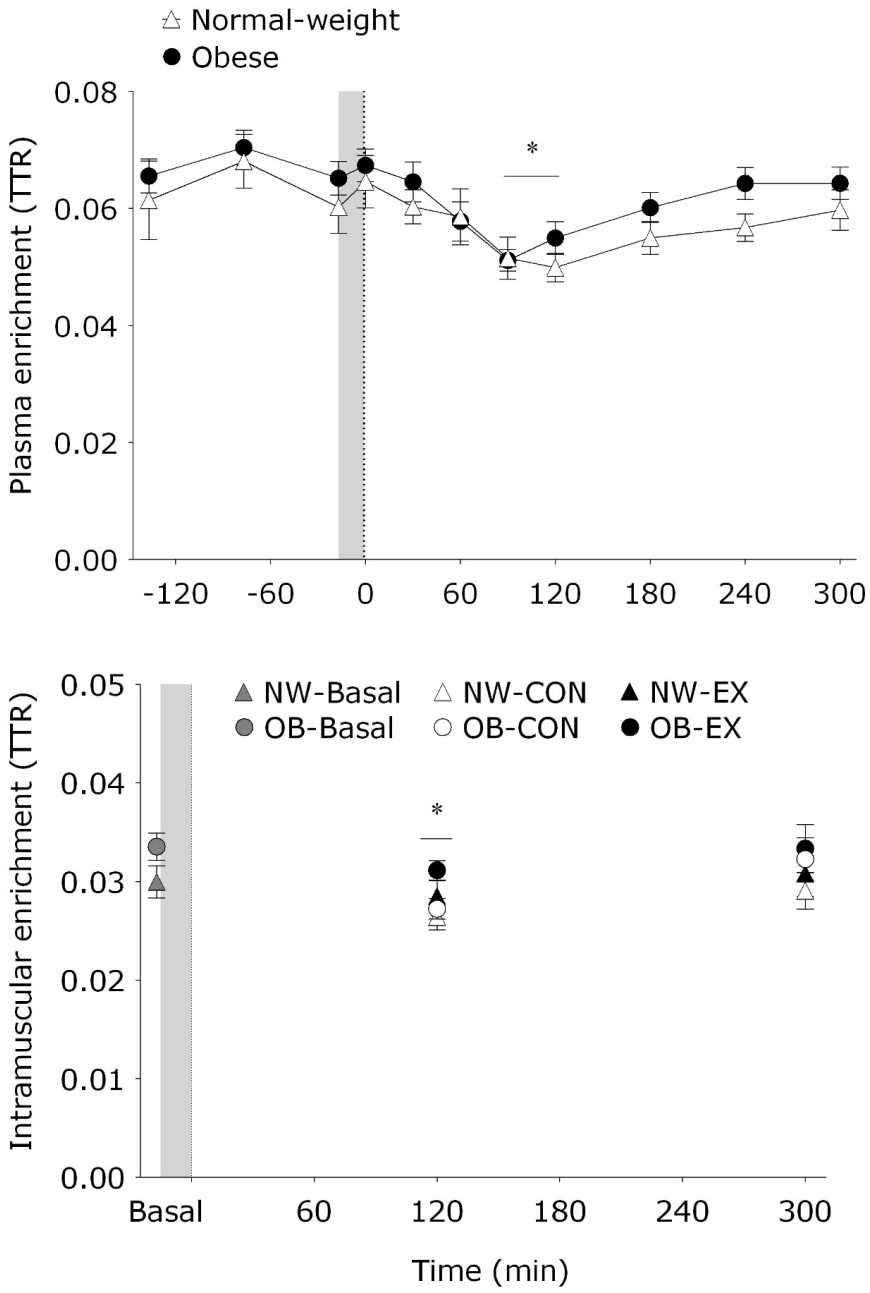


Figure 4.5. Plasma (**A**) and muscle-free (**B**) L-[*ring*-$^{13}C_6$]phenylalanine enrichments (tracer-to-tracee ratio [TTR]) in the basal state and after pork ingestion (n=9 per group). Dashed vertical line refers to pork ingestion. Gray bar indicates a bout of unilateral leg extension exercise. Data are Mean ± SEM. IC enrichments - EX effect: P=0.03. * P<0.05 *vs.* basal.

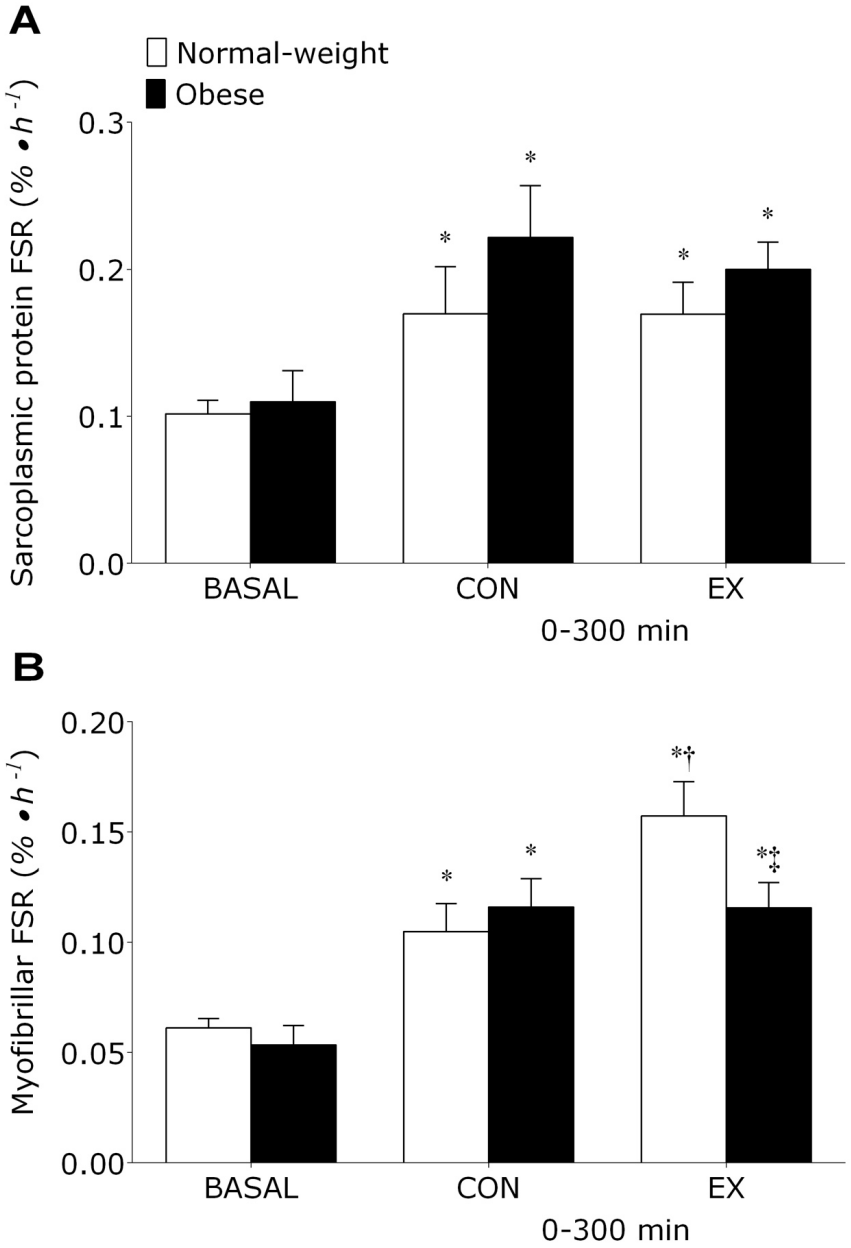
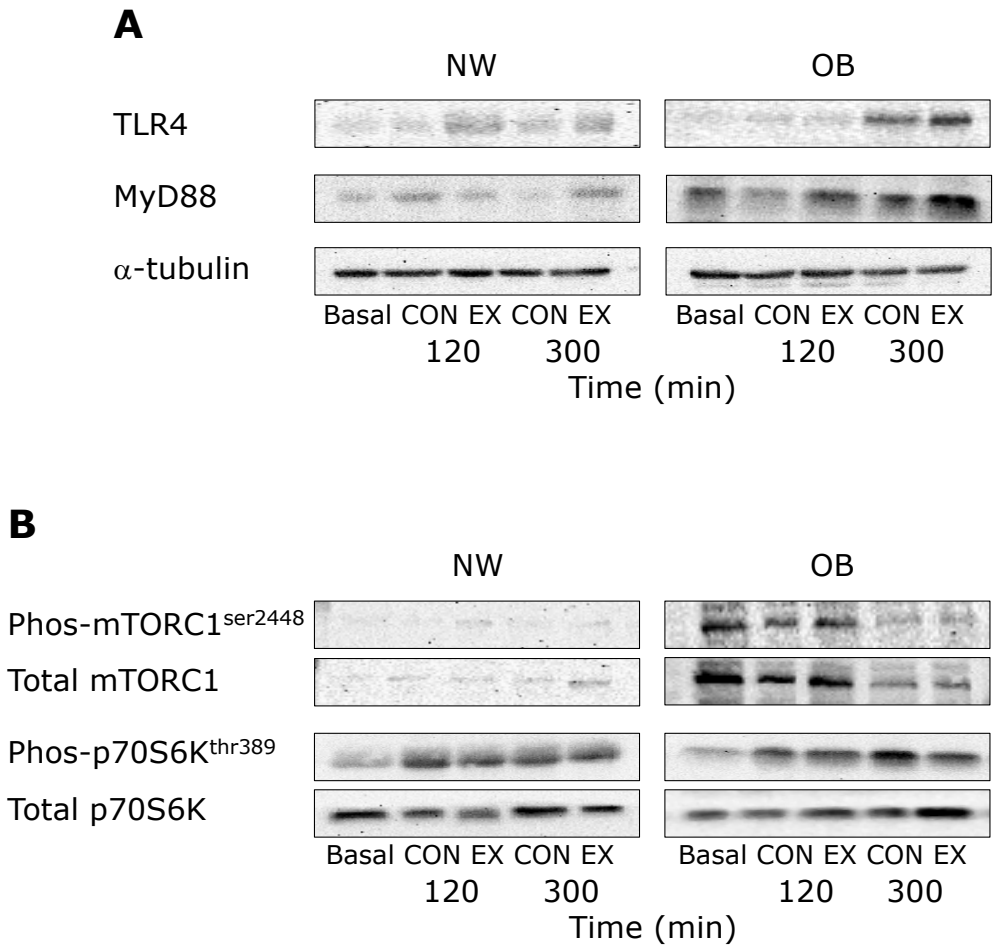


Figure 4.6. Sarcoplasmic (**A**) and Myofibrillar (**B**) fractional synthesis rates (FSR) at basal and after (0-300 min) the ingestion of pork (n=9 per group) in both the non-exercised (CON) and exercised (EX) leg. Immediately prior to pork ingestion participants performed a bout of unilateral leg extension exercise. Data are Mean ± SEM. * P<0.05 *vs.* basal, † P<0.05 *vs.* CON leg, ‡ P<0.05 *vs.* NW group.



Supplementary Figure 4.1 Representative western blots for total protein content of toll-like receptor 4 (TLR4), myeloid differentiation factor 88 (MyD88), and α-tubulin (**A**) and total and phosphorylated mammalian target of rapamycin complex 1 (mTORC1) at serine 2448 and 70 kDa S6 protein kinase (p70S6K) at Thr389 (**B**) at basal and after the ingestion of pork (*n*=9 per group) in both the non-exercised (CON) and exercised (EX) leg. Immediately prior to pork ingestion participants performed a bout of unilateral leg extension exercise.

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CHAPTER V: GENERAL DISCUSSION

The primary aim of the works included in this dissertation was to determine the impact of obesity on postprandial muscle sub-fractional protein synthesis rates in response to the main anabolic stimuli to skeletal muscle, muscle contraction and protein ingestion. This chapter will summarize the key findings from the studies described in the previous chapters and will outline recommendations for further research on how exercise and eating protein regulates muscle protein synthesis rates with obesity.

As highlighted in **Chapter 1**, the available literature regarding how postprandial muscle protein synthetic responses in people with obesity compare with normal-weight was limited, methodologically, to simulated clamped postprandial conditions (*i.e.* hyperinsulinemia, hyperaminoacidemia (1–4)]. While these studies can help to reveal mechanisms, this is not representative of a meal setting, where meals are ingested orally as opposed to being intravenously delivered. Therefore, the studies is this dissertation extends the literature regarding muscle protein metabolism in obesity to include the ingestion of a bolus of protein-dense food that represents the typical protein portion contained in an American dinner meal. In addition, we sought to define if resistance exercise could be used as an adjunct strategy to improve the use of protein in the diet for the stimulation of muscle protein synthesis rates.

5.1 PROTEIN-DENSE FOOD INGESTION AND MUSCLE PROTEIN SYNTHESIS

In **Chapters 2 & 3**, we assessed the effect of protein-dense food ingestion on muscle sub-fractional protein synthesis rates (e.g. myofibrillar, mitochondrial) in normal-weight, overweight, and obese men and women. In addition to rates of muscle protein synthesis, we also assessed relevant nutrient sensing mechanisms and muscle inflammatory protein content in these participants.

5.1.1 Muscle sub-fractional protein synthesis

In agreement with previous reports (2,4) we observed that basal rates of myofibrillar protein synthesis were similar among BMI categories. However, contrary to our hypothesis, the ingestion of a protein-dense food stimulated net postprandial myofibrillar protein synthesis rates (0-300 min) in only the normal-weight group, where the overweight and obese groups were not different from basal. The defects found in the synthesis of the myofibrillar sub-fraction, did not extend to the synthesis of mitochondrial proteins.

In contrast to the findings of Guillet *et. al.* (3) we found that mitochondrial protein synthesis rates did not differ among groups at basal. Mitochondrial protein synthesis rates in response to protein-dense food ingestion increased in all groups when calculated for a 300 min postprandial period. However, mitochondrial protein synthesis rates were temporally less responsive in the overweight group when compared with the normal-weight and obese groups, as illustrated by a lack of stimulation to rates calculated from 0-120 min of the postprandial period.

Altogether our findings indicate that defects in muscle protein synthesis rates in response to ingestion of protein foods are may be limited to the myofibrillar sub-fractions during the postprandial period. These findings are in agreement with those of Murton *et. al.* (4), but directly contrast with those of Chevalier and colleagues (2). The latter study included a hyperglycemic infusion to their clamp protocol, where the former maintained euglycemia. Previous studies investigating the effect of co-ingestion of carbohydrate with dietary protein showed no effect of the inclusion of carbohydrate on postprandial mixed muscle protein synthesis in healthy subjects (5–7). However, the impact of carbohydrate co-ingestion on the postprandial myofibrillar protein synthetic response has yet to be determined, particularly in people with obesity, but it is likely

permissive and not modulatory for this fraction as well. In **Chapter 1** we discussed the potential mechanisms potentially related to alterations in postprandial muscle protein synthesis with obesity. Below we discuss several aspects of the mechanisms and how they may relate to myofibrillar protein synthesis.

5.1.2 Systemic anabolic and inflammatory milieu

In healthy, young individuals the influence of insulin, and amino acid (AA) availability (esp. leucine) on the myofibrillar protein synthetic response is a central theme in the literature (8–10). Therefore, we measured blood glucose, plasma insulin, and essential AA concentrations in an attempt to capture what factors may be related to the differential stimulation of postprandial myofibrillar protein synthesis rates between normal-weight, overweight, and obese men and women. Of these, blood glucose and essential AA were not different among groups at any time point. This finding that there were no differences between groups in AA concentrations, particularly the branched chain AAs, was surprising given that several studies have indicated that this subset of AAs relate to insulin resistance in obese populations (11–13). While we did not observe differences in blood glucose, plasma insulin concentrations appear to be hyper-responsive to protein ingestion in people with obesity when compared to either normal-weight or overweight people. We also assessed postprandial plasma non-esterified fatty acids (NEFA), which are capable of suppressing insulin signaling (14) and postprandial mixed muscle protein synthesis (15). In **Chapter** 3, we observed that overweight and obese individuals included in the study may have subtle insulin resistance in the adipose tissue resulting in a slower suppression of plasma NEFA concentrations than the normal-weight group. However, during the late postprandial period

(120-300 min) plasma NEFA concentrations increased to values above basal in the normal-weight group to a greater extent than the overweight and obese groups.

Because systemic low-grade inflammation is associated with an obese phenotype (16), we also measured postprandial concentrations of several potential plasma biomarkers of low-grade inflammation, including C-reactive protein (CRP), interleukin-6 (IL-6) and tumor necrosis factor α (TNFα). Of these biomarkers, none appeared to be related to the impaired postprandial myofibrillar protein synthetic rates in overweight and obese individuals. We did show, however, that plasma IL-6 concentrations increases after protein-dense food ingestion in all groups, which may be, in part, responsible for the degradation of insulin in circulation (17).

5.1.3 Muscle anabolic signaling

Because amino acids (esp. leucine) are an important mediator of the postprandial muscle protein synthetic response (9,10), we also determined the level of AA transporters in the skeletal muscle. Other groups have outlined a model where AA transport is coordinated by interplay between several transport mechanisms (18). Studies in healthy individuals have shown that AA transporters increase in response to essential AA ingestion (19). Importantly, AA transport is an important regulatory step in the postprandial activation of mTORC1 signaling *in vitro* (20). We found that AA transporters [large neutral amino acid transporter (LAT1), small neutral amino acid transporter (SNAT2), and CD98 (SLC3A2)] increase after protein-dense food ingestion, however, no group differences existed.

In order to determine how anabolic signaling mechanisms respond to protein-dense food ingestion, we measured the phosphorylation of several proteins stimulated by either insulin or AA as proxies for the activation of these pathways. For insulin signaling we determined

phosphorylation of protein kinase B (Akt) at Serine 473 and Threonine 308, and the 160 kDa Akt substrate (AS160), but we did not observe any differences between groups in Akt signaling. These findings suggest that insulin resistance is not a factor contributing to our findings regarding myofibrillar or mitochondrial protein synthesis. However, the plasma insulin concentrations in the obese group were exceptionally high, suggesting that some participants were resistant to insulin.

In support of data from rodent studies (21,22), in **Chapter 2** we learned that overweight and obese have profoundly altered mTORC1 protein content and phosphorylation at basal compared with normal-weight individuals. However, mTORC1 phosphorylation increased above basal in response to protein ingestion in only the normal-weight group. p70S6K, the downstream target of mTORC1, was similarly phosphorylated in all groups. However, since p70S6K is responsible for mTORC1^{ser2448} phosphorylation (23) it seems clear that this kinase was also activated after protein-dense food ingestion.

5.1.4 Muscle inflammatory proteins

Muscle toll-like receptor 4 (TLR4) protein is tightly correlated with body fat % in older adults (24) and insulin resistance (25). This receptor is involved in the innate immune response and is primarily known for responding to lipopolysaccharide from the cell wall of Gram-negative bacteria, but is also responsive to NEFA in circulation (25). TLR4 signaling involves docking with several intracellular proteins, among these is myeloid differentiation factor 88 (MyD88), which appears to potentiate the intracellular signaling of TLR4-induced insulin resistance (25,26). For these reasons we were interested in determining the muscle content of these proteins of the participants for **Chapter 3**. As expected, at basal the obese group had greater protein content of both TLR4 and MyD88 in their muscles compared with the normal-weight group. However, the

overweight group also had greater MyD88 than the normal-weight group. We observed an increase in both TLR4 and MyD88 proteins in the obese groups in response to protein-dense food ingestion, but no changes in either the normal-weight or overweight groups. CRP has been demonstrated in rodent smooth muscle cells to regulate TLR4/MyD88 signaling (27,28) and may be related to the postprandial increases in these immune receptors in the obese group due to their ~5-fold greater CRP concentrations compared with the normal-weight and overweight groups. Moreover, the increases in TLR4 and MyD88 protein content in the muscle of participants with obesity, is likely a contributor to the elevated postprandial TNFα concentrations in this group.

5.1.5 Plasma proteomic profiling

While separate mechanisms may be responsible for the anabolic resistance seen in the overweight and obese groups, we believe that similar impairments exist in both groups causing a poor stimulation of myofibrillar protein synthesis. However, we were unable to establish a clear relationship between any systemic factors and the anabolic resistance witnessed with greater fat mass.

Above we highlight distinct similarities in mTORC1 signaling between the overweight and obese groups that differentiate them from the normal-weight participants. However, we were unable to reveal a potential direct link to any upstream factors related to the mTORC1 pathway. Therefore, in an attempt to identify any potential plasma biomarkers of protein-dense food ingestion and subsequent muscle anabolic responses, we assessed the postprandial plasma proteome at basal, 120 min and 300 min after protein-rich food ingestion in normal-weight, overweight, and obese individuals. Samples for this analysis were taken from participants included in **Chapters 2 & 3**. This sample represents a cross-section of the American populous where

approximately one-third of people fit into each of those three BMI categories. Plasma proteomic profiles are presented in **Figures 5.1 and 5.2**. Though this approach was promising, the circulating plasma proteomic profile was unresponsive to protein ingestion across the BMI range. Moreover, there were no group differences in the plasma proteome. We are aware of only one study that has captured the impact of food ingestion on the postprandial plasma proteome (29). In that study overweight participants were fed a high fat mixed meal, which resulted in only minimal effects on the circulating proteomic signature. Potentially, the broad range of participants used for our study masked the effects of food ingestion in this study.

The plasma proteome is technically challenging to quantify due to the vast range of protein concentrations and that many tissues contribute to its makeup (30). Because of this, it is common to deplete the most abundant proteins (e.g. albumin) in order to increase the signal of less abundant proteins prior to proteomic analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS). We selected spin columns that removed 12 of the commonly most abundant proteins, but it may have been prudent to remove more. Because of this, we did not deplete many of the highly abundant immunoglobulins and lipoproteins (**Table 5.1)**, which likely reduced the signal of many other, perhaps more interesting proteins.

5.2 PROTEIN-DENSE FOOD INGESTION AFTER ACUTE RESISTANCE EXERCISE AND MUSCLE PROTEIN SYNTHESIS

In **Chapter 4**, we tested the hypothesis that acute resistance exercise would provide a robust signal to improve the postprandial myofibrillar synthetic responses to protein-dense food ingestion in obese individuals. In this work we prescribed a unilateral resistance exercise protocol known to provide a robust signal to stimulate myofibrillar protein synthesis in young men (31).

5.2.1 Postprandial muscle sub-fractional protein synthesis after acute resistance exercise

Rates of sarcoplasmic protein synthesis were similar among groups at all time points during the infusion trial. The ingestion of a protein-dense food robustly stimulated postprandial sarcoplasmic protein synthesis in both groups. Resistance exercise did not impact sarcoplasmic rates of protein synthesis.

Similar to the findings in **Chapter 2** we did not observe basal differences in myofibrillar protein synthesis rates between normal-weight and obese groups. Protein-dense food ingestion stimulated rates of myofibrillar protein synthesis in both the exercised and non-exercised states in both the normal-weight and obese groups. However, we saw that rates of myofibrillar protein synthesis were stimulated by protein-dense food ingestion to a much greater extent in the exercised-state the normal-weight group, which agrees with previous findings (32). Unfortunately, this finding was not witnessed in the obese group; rates of myofibrillar protein synthesis from 0-300 min of the postprandial period were similar in both the exercised and non-exercised states in this group.

5.2.2 Muscle anabolic and inflammatory signaling

In **Chapter 2** we described impaired anabolic signaling in people with obesity. In **Chapter 4,** we once again pursued these signaling mechanisms to see how resistance exercise affects these pathways with obesity. In agreement with the previous study, we found that obesity was associated with greater activation of the mTORC1 signaling pathway compared to the normal-weight adults. However, despite greater overall phosphorylated p70S6K protein in the obese group, the activation of p70S6K was not augmented by resistance exercise in this group.

As in **Chapter 3**, we showed that protein ingestion increases muscle TLR4 protein content in obese, but not normal-weight individuals. However, resistance exercise effectively prevents the postprandial increase in muscle TLR4, which may in part mediate the effects of resistance exercise on improving insulin sensitivity (33).

In contrast with some (4,34,35), but not all (2,36) previous reports, in the present study we showed that postprandial myofibrillar protein synthesis rates are stimulated in individuals with obesity when in the fed, non-exercised state. Several noteworthy differences exist between the obese group used here and those included in the previous investigation, specifically a lack of elevated low-grade systemic and muscle inflammatory proteins (fasting plasma CRP and muscle TLR4) and branched-chain amino acids, the latter of which has been shown to differentiate between metabolically healthy and unhealthy obese phenotypes (37). Altogether, these studies underscore the notion that metabolic health varies within obese populations (38) and extend previous discussions to muscle protein metabolism. Moreover, because these participants performed exercise with one leg, we cannot fully exclude that subtle effects of walking to the exercise equipment and/or contralateral exercise are present as CON does not represent a truly rested condition. More importantly, resistance exercise failed to augment postprandial myofibrillar protein synthesis rates in individuals with obesity; a defect that presented in participants with seemingly normal fed-state myofibrillar protein synthesis rates.

5.2.3 Resistance exercise

In **Chapter 4**, we prescribed the participants a bout of resistance exercise designed to elicit a strong postprandial myofibrillar protein synthetic response. This prescription was in excess of most recommendations for untrained weightlifters (39–41). However, this was an insufficient stimulus

to potentiate myofibrillar protein synthesis rates in people with obesity. This is supported by works in an aging population (also anabolically resistant) where there is an altered dose-response between resistance exercise and the stimulation myofibrillar protein synthesis compared with young adults (42), where older adults require greater resistance exercise volume elicit a strong postprandial myofibrillar protein synthetic response (43). Our finding suggests that it may be necessary to revise exercise prescriptions for those with obesity. Potentially, more volume and/or different exercise intensities are required to fully stimulate the muscle in this population.

5.3 LIMITATIONS AND FUTURE DIRECTIONS

A primary limitation to the above studies was that we were only able to partially describe the defects in anabolic signaling in the skeletal muscle and how they relate to the myofibrillar protein synthetic responses to food ingestion, at rest or post-exercise, with obesity. For these studies we were interested in studying previously well-characterized mechanisms in healthy adults and potential factors related to the postprandial muscle protein synthetic response in obese vs lean individuals. However, there are numerous other factors that could play a role in the regulation of muscle protein synthesis in obese adults. We were able to confirm rodent data that mTORC1 is hyperactive with human obesity, but we did not study the patterns of mTORC1 colocalization within the cell that also affect its activity. In terms of inflammation, for example, there are many cytokines (e.g. IL-1β, interferon-γ), some of which are anti-inflammatory (IL-10) that could play a role in how the muscle anabolic response is mediated in response to either exercise or protein-dense food ingestion. In addition, other inflammatory pathways are present in the skeletal muscle, for instance TNFα has its own receptor and does not signal through TLR4. We were able to establish a pattern of TLR4 upregulation in the skeletal muscle, but we did not measure ceramide

biosynthesis, of which there are numerous species. Future directions should investigate the impact of exercise strategies, such as either higher volume resistance exercise prescriptions to attempt to overcome the anabolic resistance of the myofibrillar protein synthetic response to food ingestion with obesity.

5.4 CONCLUSION

This dissertation provides compelling evidence for anabolic resistance of the myofibrillar protein sub-fraction of the skeletal muscle to protein-dense food ingestion at rest and after an acute bout of resistance exercise. This anabolic resistance is related to altered activation of the mTORC1/p70S6K signaling axis. In our view, suggesting that people with obesity consume greater amounts of protein at each meal is unwarranted. Though the exercise prescribed in **Chapter 4** was unsuccessful at overcoming obesity-related anabolic resistance, we believe that further studies should pursue alternative exercise strategies. Moreover, interventions that combine exercise with weight loss are likely to be particularly useful for people with obesity.

5.5 TABLES AND FIGURES

Table 5.1. Top 10 GO protein classifications (44).

Protein categories	Term	Representative protein(s)	*P-value*
Biological processes			
	Platelet degranulation	Platelet basic protein	2.56×10^{-53}
	Protein activation cascade	Complement C1 subunits	2.78×10^{-52}
	Acute inflammatory response	Complement factors	1.34×10^{-45}
	Defense response	LPS-binding protein	1.16×10^{-38}
	Regulation of proteolysis	Carboxypeptidase B2	4.67×10^{-38}
	Complement activation	Immunoglobulin	1.56×10^{-37}
	Proteolysis	Plasma serine protease inhibitor	9.36×10^{-37}
	Vesicle-mediated transport	Apolipoprotein D	2.77×10^{-36}
	Response to stress	Galectin-3-binding protein	2.46×10^{-35}
	Inflammatory response	Serum amyloid A	2.72×10^{-35}
Molecular function			
	Endopeptidase inhibitor activity	Kallistatin	1.37×10^{-31}
	Endopeptidase regulator activity	Protein AMBP	3.94×10^{-31}
	Peptidase inhibitor activity	Inter-alpha-trypsin inhibitor	6.59×10^{-31}
	Peptidase regulator activity	Kininogen-1	6.85×10^{-30}
	Enzyme inhibitor activity	Alpha-1-antichymotrypsin	4.55×10^{-27}
	Serine-type endopeptidase inhibitor activity	Protein Z-dependent protease inhibitor	1.27×10^{-25}
	Enzyme regulator activity	Angiotensinogen	2.23×10^{-18}
	Serine-type endopeptidase activity	Prothrombin	1.53×10^{-16}
	Glycosaminoglycan binding	Thrombospondin	5.25×10^{-16}
	Serine-type peptidase activity	Plasminogen	8.45×10^{-16}

*LPS; lipopolysaccharide

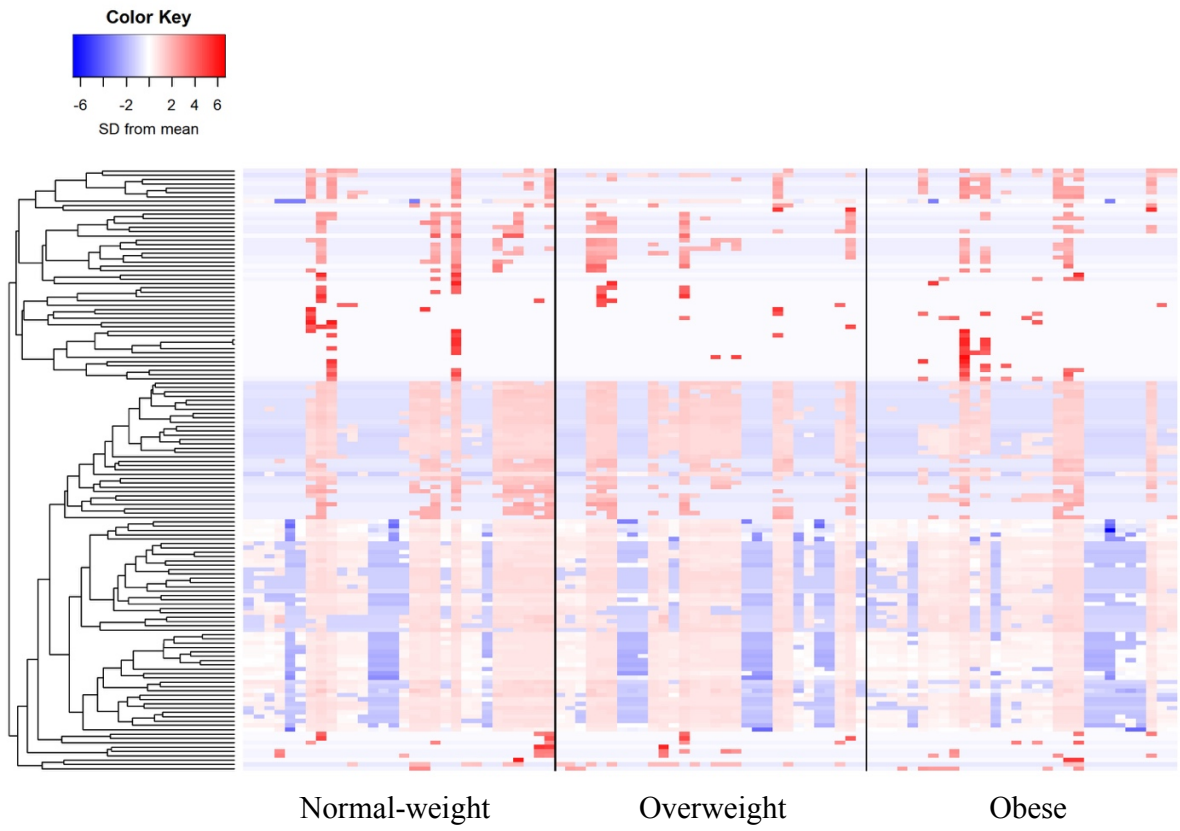

Figure 5.1. Plasma proteomic signature organized by BMI category.

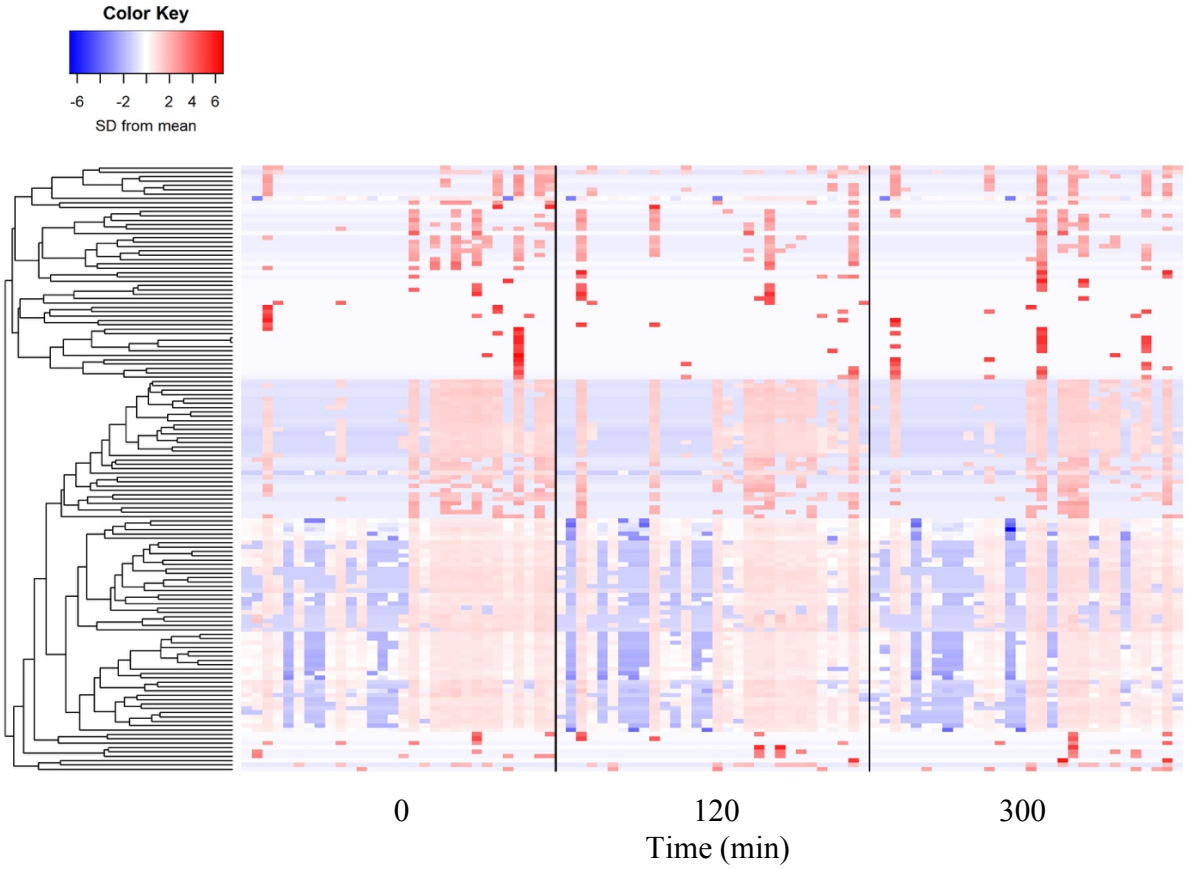

Figure 5.2. Plasma proteomic signature in response to protein-dense food ingestion.

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APPENDIX A: PLASMA AMINO ACID EXTRACTIONS

For amino acid quantification and enrichment analysis. This section describes the extraction procedures and analysis performed in Chapter 2-4 to determine the amino acid concentrations in the plasma of participants.

SUPPLIES NEEDED:

1. 1.5 ml Eppendorf Tubes
2. 2-200µL pipette
3. 200-1000µL pipette

REAGENTS NEEDED:

1. MIX A (3:3:2: Acetonitrile:Isopropanol:ddH2O)
2. INTERNAL STANDARD – IS (p-chlorophenylalanine at 1 mg/mL in 0.1 M HCl)

** use $^{13}C_9$-phenylalanine or $^{13}C_6$-leucine as IS when calculating plasma kinetics

NOTES:

Do not use these tracers in samples for enrichments analysis.

Use p-chlorophenylalanine for simultaneous enrichment and AA quant on GC-MS.

No INTERNAL STANDARD needed for enrichment analysis

EXTRACTION PROTOCOL:

1. Put 1 mL of MIX A in an empty Eppendorf tube
2. Add 200µL of plasma sample to Eppendorf tube
3. Add10µL of INTERNAL STANDARD to Eppendorf tube
4. VORTEX
5. Place the mixed sample in freezer (- 20°C) for ≥30 min
6. Spin at 20800 × *g* for 10 min (4°C)
7. Transfer 200µL of supernatant to new labeled Eppendorf
8. Dry in speedvac prior to derivatization and amino acid analysis on GC-MS.

QUANTIFICATION PROTOCOL:

1. Open Amdis_32 (chemdata.nist.gov)
2. Go to FILE → BATCH JOB → CREATE AND RUN JOB)
3. Go to ADD → select GC/MS files to analyze (labeled x.D; e.g., 1.D, 2.D … *n*.D etc)
4. Select SIMPLE as ANALYSIS TYPE
5. Open TXT file with excel and quantify each amino acids relative to IS (i.e. IS is always 1).
6. Plot this ratio to the generated standard curve to find the corresponding amino acid concentration in µmol/l

ENRICHMENT ANALYSIS PROTOCOL:

1. Open RTR data analysis (MSD ChemStation)
2. Go to CHROMATOGRAM → EXTRACT ION CHROMATOGRAM and select the *m/z* IONS to monitor (e.g, 336 for *m*+0, unlabeled and 342 for *m*+6 labeled L-[$^{13}C_6$]phenylalanine)
3. Click A/B tab next to mouse cursor icon and check MANUAL INTEGRATION box.
4. Zoom in on the peak and the correct retention time (RT; see AMDIS analysis above to get this for the AA of interest)
5. Select the entire area below the peak of interest (e.g., leucine) and go to CHROMATOGRAM→PERCENT REPORT
6. Find the value of corresponding corrected area as the integrated peak.
7. Express the peaks as a TTR (tracer-to-tracee) for each sample (with subtraction of the 'baseline' sample).

APPENDIX B: MUSCLE SUB-FRACTION PROTEIN EXTRACTION

This section describes the extraction procedures utilized in Chapters 2-4 to isolate myofibrillar mitochondrial, and sarcoplasmic proteins for tracer analysis and generate whole muscle homogenate to perform Western Blot analysis.

TISSUE NEEDED:
Mitochondrial protein extractions require ~80-100 mg of frozen wet muscle (**Chapters 2 & 3**). Myofibrillar and sarcoplasmic protein-bound tracer enrichments can reliably obtained from ~20 mg of muscle, however, to provide for enough whole muscle homogenate for Western Blot analysis a larger piece of ~50 mg of muscle was used in **Chapter 4**.

SUPPLIES NEEDED:
1. Glass Dounce homogenizer
2. 1.5 ml eppendorf Tube
3. 4 ml Glass Top Vial + lid

REAGENTS NEEDED:
1. 0.3 M Sodium Hydroxide (NaOH)
2. 1 M Perchloric Acid (PCA)
3. 70 % Ethanol (EtOH):
4. 6 M HCL
5. Isolation buffer 1 (recipe below)
6. Isolation buffer 2 (recipe below)

Isolation buffer 1 - IB1: **this recipe is sufficient for ~10 mito protein extractions**
1. 670 µL 1 M Sucrose
2. 500 µL 1 M TrisHCl
3. 500 µL 1 M KCl
4. 170 µL 1 M EDTA
5. Adjust pH to 7.4 with NaOH or HCl
6. Bring to 10 mL with DI water
7. Add 1 cOmplete tablet (protease inhibitor) and 1 PhoStop (phosphatase inhibitor)

Isolation buffer 2 - IB2:
1. 2.5 mL 1 M Sucrose
2. 300 µL 0.1 M EGTA/TrisHCl
3. 100 µL 1 M TrisHCl
4. Adjust pH to 7.4 with NaOH or HCl
5. Bring to 10 mL with DI water

NOTE: Buffers can be stored for 1-2 months at -20°C, otherwise make fresh.

PREPARATION:
1. Label Eppendorfs: MYO, MITO, SARC, WEST and BRAD prior to protocol depending on analysis.

MUSCLE HOMOGENIZATION PROCEDURE

1. Add 10 µL/mg of IB1 and muscle sample to glass homogenizer
2. Homogenize thoroughly on ice (several minutes)
3. Transfer to eppendorf labeled MYO
4. Spin at 700 × *g* for 15 min at 4°C
5. Transfer supernatant to new labeled eppendorf
   MITO – *if mito analysis is being performed*)
   SARC – *if NOT conducting mito analysis*
   NEEDS ~100 µL HOMOGENATE
   WEST – *if NEITHER mito of sarc are being measured*
   transfer 5 µL from the tube labeled WEST to BRAD (use small RNA tubes)
6. Freeze MYO, WEST and BRAD at -80°C to process later.

MITOCHONDRIAL ISOLATION used in **Chapter 3**

1. Spin MITO tube at 14,000 × *g* for 20 min at 4°C
2. Transfer supernatant to new labeled eppendorf
   SARC – *if conducting this analysis*
   NEEDS ~100 µL HOMOGENATE
   WEST – *remaining supernatant*
   transfer 5 µL from the tube labeled WEST to BRAD (use small RNA tubes)
3. Freeze SARC, WEST and BRAD at -80°C to process later
4. Re-suspend pellet in 500 µL IB2 do NOT vortex
5. Spin MITO tube at 14,000 × *g* for 15 min at 4°C
6. Discard supernatant
7. Re-suspend pellet in 500 µL IB2 do NOT vortex
8. Spin MITO tube at 14,000 × *g* for 15 min at 4°C
9. Discard supernatant
10. Add 500 µL 95% EtOH do NOT vortex
11. Spin MITO tube at 14,000 × *g* for 5 min at 4°C
12. Discard supernatant
13. Transfer pellet to 4-ml screw top glass vials
14. Add 2.0 ml of 6 M HCL to sample
15. Hydrolyze overnight at 110°C
16. In the morning, add 2 mL of DI H_2O to samples

SARCOPLASMIC ISOLATION (with or without MITO removed) used in **Chapter 4**

1. Add 1000 µL 1 M PCA to SARC tube vortex
2. Spin SARC tube at 10,000 × *g* for 5 min at 4°C
3. Discard supernatant
4. Add 1000 µL 70% EtOH vortex
5. Spin SARC tube at 10,000 × *g* for 5 min at 4°C
6. Discard supernatant
7. Add 1000 µL 70% EtOH vortex

8. Spin SARC tube at 10,000 × *g* for 5 min at 4°C
9. Discard supernatant
10. Transfer pellet to 4-ml screw top glass vials
11. Add 2.0 ml of 6 M HCL to sample
12. Hydrolyze overnight at 110°C
13. In the morning, add 2 mL of DI H_2O to samples

MYOFIBRILLAR ISOLATION used in **Chapters 2 &4**

1. Add 500 µL of ddH_2O to MYO tube vortex
2. Spin MYO tube at 700 × *g* for 10 min at (4°C)
3. Discard supernatant
4. Add 1 mL of 0.3 M NaOH to the pellet vortex
5. Heat at 50°C for 30 min (vortex every 10 min)
6. Spin MYO tube at 10000 × *g* for 5 min
7. Transfer supernatant to 4-ml screw top glass vials
8. Add 1 mL of 0.3 M NaOH to the remaining pellet in MYO tube vortex
9. Spin MYO tube at 10000 × *g* for 5 min (4°C).
10. Transfer supernatant from MYO tube to 4-ml screw top glass vials (2nd transfer)
11. Discard pellet if PHE tracer.
    Otherwise, pellet can now be frozen at -80°C as COLLAGEN.
12. Add 1 mL of 1 M PCA to 4-ml glass vials (should see snow globe effect)
13. Spin 15 min at 3000 × *g* (4°C). (this is the max the glass vials can be spun at or else they are at risk of breaking)

NOTE: if samples appear cloudy add 1mL of PCA and re-centrifuge

14. Discard supernatant
15. Add 1 mL of 70 % EtOH to sample (DO NOT VORTEX OR ADD ETOH DIRECTLY TO PELLET; RATHER PIPET IT DOWN SIDE OF GLASS)
16. Spin for 15 min at 3000 × *g* at 4°C
17. Remove supernatant (EtOH) and discard
18. Add 2.0 ml of 6 M HCL to sample
19. Hydrolyze overnight at 110°C
20. In the morning, add 2 mL of DI H_2O to samples

APPENDIX C: INTRACELLULAR MUSCLE FREE AMINO ACID EXTRACTION

This section describes the extraction procedures utilized in Chapter 4 to obtain the intracellular (IC) muscle free amino acids, which can subsequently be used to determine IC muscle free tracer enrichment. IC muscle free tracer enrichments can be reliably obtained from ~10 mg of muscle.

Mixed muscle enrichment analysis were not conducted within this dissertation

SUPPLIES NEEDED:

1. Teflon Pestle
2. 2.0 ml Eppendorf Tube
3. 4.0 ml Glass Top Vial and Cap

REAGENTS NEEDED:

1. 0.6 M Perchloric Acid (PCA)
2. 70 % Ethanol (ETOH)
3. 6 M Hydrochloric Acid (HCL)
4. 0.1 M HCL

MUSCLE HOMOGENIZATION PROCEDURE:

1. Place 10-15 mg of sample in 1.5 mL Eppendorf tube and add 500 µL of 0.6 M PCA.
2. Homogenize with Teflon Pestle on ice vortex
3. Spin at 3600 × *g* for 5 min (4°C).
4. Remove supernatant (1st shot) and place in IC tube
5. Add 400 µL of 0.6 M PCA to pellet vortex
6. Spin at 3600 × *g* for 5 min (4°C).
7. Remove supernatant (2nd shot) and place in IC tube
8. Leave IC at room temp if going to column the next day (See 'Dowex Purification Protocol' below); otherwise freeze at -80°C.
9. Remaining pellet is Mixed muscle – Freeze at -80°C for future analysis.

APPENDIX D: AMINO ACID PURIFICATION

This section describes the cation-exchange procedures utilized **in Chapters 2-4** to purify the IC, myofibrillar, mitochondrial, and sarcoplasmic free amino acids prior to analysis by LC-MS/MS.

SUPPLIES NEEDED:

1. pH Paper
2. 5 mL syringes
3. Glass wool

REAGENTS NEEDED:

1. Dowex 50WX8-200 ion-exchange resin
2. 4 M NH_4OH (FOR DOWEX PREPARATION)
3. 2 M NH_4OH (FOR COLUMN CLEAN UP)
4. 1 M HCL
5. 0.1 M HCL

DOWEX PREPARATION:

1. Add 4 M NH_4OH to Dowex container to cover all resin
2. Replace cap and gently agitate container
3. Let Resin settle to bottom and discard NH_4OH (check PH, should be very basic)
4. Add DI H_2O to Resin
5. Replace cap and gently agitate container
6. Let Resin settle to bottom and discard H_2O (check pH)
7. Repeat steps 4-6 until ~neutral pH
8. Add 0.1 M HCL to cover resin and store in Dowex container at 4°C

COLUMN PREPARATION:

1. Remove plunger of 5 mL syringe
2. Add small amount of glass wool to syringe (~1 mL mark). Push the wool firmly to the bottom, but do not "pack"
3. Replace plunger

COLUMN CLEAN UP:

NOTE: Place *n* +1 columns into rack (+1 for test column). Treat ALL columns identically throughout procedure (including test column)

1. Set columns up over collection container (old coolers work well)
2. Add 1 mL of DI H_2O to column
3. Replace any columns that do not flow smoothly
4. Add 1.5 mL of Dowex Resin to each column (keep it consistent across all columns)
5. Add 1 mL of DI H_2O to column
6. Replace any columns that do not flow smoothly
7. Add 1.5 mL of 2 M NH_4OH to each column to elute contaminants
8. Test pH, must be VERY basic
9. Repeat step 8 if necessary

10. Add DI H_2O to each column (keep it consistent across all columns)
11. repeat until neutral (takes ~10 mL)
12. Add 1.5 mL of 1 M HCL to charge column
13. Test pH, must be VERY acidic
14. Repeat step 13, if necessary
15. Add samples to columns
16. Add 4 mL 3 M HCL to test column
17. Add DI H_2O to each column (keep it consistent across all columns)
18. repeat until neutral (takes ~10 mL)

WORK WITH YOUR TEST COLUMN FOR THE NEXT STEPS

NOTE: play it safe with the below steps, you could lose sample if you add too much NH4OH

19. Add 500 µL 2 M NH4OH to your test column
20. Test pH
21. IF pH > ~10, STOP
22. IF pH <10, add 500 µL 2 M NH4OH to your test column
23. Repeat steps 20-22 as necessary
24. Record volume of 2 M NH4OH added to test column
25. Subtract 500 µL from the volume recorded in step 24
26. Record this new volume
27. Add volume of 2 M NH4OH (from step 26) to all columns
28. Discard test column

WORK WITH ALL SAMPLES FOR THE NEXT STEPS

29. Place columns over labeled 4-ml glass vials to collect samples
30. Add 4 mL 2 M NH4OH to each column to elute purified AA
31. Dry in speedvac overnight
32. Reconstitute dried sample in 1 mL 0.1 M HCL and transfer to labeled eppendorf
33. Store in Freezer (-80°C).
34. Submit 20 µL for LC-MS/MS analysis

APPENDIX E: WESTERN BLOTTING PROTEIN CONDITIONS

This section describes the conditions used to determine protein content for western blotting analysis used in **Chapter 4**.

Protein	Gel	1°	2°	Run	Membrane	transfer
mTORC1 Ser 2448	7%	1:500 BSA Cell signal	1:5000 abcam	200V	Nitrocell	150 min @100 V
mTORC1 total	7%	1:500 BSA Cell signal	1:5000 abcam		Reprobe	
TLR4	10%	1:500 Milk abcam	1:5000 abcam	150V	PVDF	120 min @100 V
p70 Thr389	10%	1:500 Milk Cell signal	1:5000 abcam	150V	PVDF	120 min @100 V
p70 total	10%	1:500 BSA Cell signal	1:5000 abcam		Reprobe	
MyD88	10%	1:1000 Cell signal	1:10000 abcam	150V	PVDF	120 min @100 V
α-tubulin	10%	1:5000 BSA abcam	1:50000 abcam	150V	PVDF	120 min @100 V

NOTES: for mTORC1 transfer reduce transfer buffer methanol from 20% to 15%. Also add 0.10% SDS to transfer buffer.