EFFECTS OF PHYSICAL ACTIVITY LEVELS AND NUTRITIONAL INTAKE ON SKELETAL MUSCLE PROTEIN TURNOVER AND CELLULAR SIGNALING

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Dedicated to my parents, Van and Claydene Glynn. I would not be here without your never-ending support, love and encouragement.

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Loss of muscle mass is common in many clinical conditions such as cancer, AIDS, burns and paralysis as well as in aging. Decreased muscle mass can contribute to many other complications and co-morbidities related to diseases, trauma and aging including overall weakness, immobility, increased risk of falls, impaired stress response and metabolic dysfunction. Nutrition and resistance exercise are two readily available and extremely anabolic stimuli for skeletal muscle, though their specific cellular mechanisms remain largely unknown. Studies were designed to examine the mammalian target of rapamycin (mTOR) muscle hypertrophy pathway in conditions of differing physical activity levels, to determine the effects of low and high carbohydrate and insulin levels (combined with essential amino acids) on protein turnover and cellular signaling following resistance exercise, and to investigate similar parameters in response to various combinations of anabolic nutrients. Stable isotopic techniques with arterial/venous catheterization and muscle biopsies, immunoprecipitation and immunoblotting, quantitative real-time PCR and hormone (ELISA) assays were utilized to examine muscle protein turnover, cellular signaling pathways, mRNA expression related to proteins of interest and hormonal responses, respectively. The main findings from these studies were that increased physical activity downregulated the mTOR signaling pathway and decreased inhibitory phosphorylation of insulin receptor substrate 1 (IRS-1). In contrast, mTOR activity may play an important role in paraplegia-induced muscle atrophy as 10 weeks of paraplegia in rats significantly downregulated the mTOR pathway. In humans and compared to modest carbohydrate ingestion, higher amounts of carbohydrate and consequent increases in circulating insulin were unable to further reduce muscle protein breakdown, associated signaling or mRNA expression following a bout of resistance exercise. Similarly, increasing concentrations of leucine may not provide any additional benefit to net protein balance, as has been previously proposed. These studies further our understanding of muscle hypertrophy and atrophy, and begin to provide the scientific data necessary in order to establish evidence-based recommendations for the maintenance of skeletal muscle mass during conditions of muscle wasting.

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CHAPTER 1

Introduction[1]

Human skeletal muscle accounts for approximately 40% of body weight, contains 50-75% of all proteins in the body, and is imperative for locomotion (154, 228). It is also metabolically important, being responsible for up to seventy-five percent of insulin-dependent glucose disposal from the blood (189, 196). However, perhaps one of muscle's most important functions is to maintain circulating levels of amino acids in the blood in the postabsorptive state as well as instances of prolonged nutrient deprivation (44, 72). The combination of these important roles makes skeletal muscle crucial to maintaining health and sustaining life, though its contribution to these processes often goes unrecognized.

Whole body protein turnover is a combination of both protein synthesis and protein breakdown, and accounts for approximately 20% of resting energy expenditure (154, 228). It has been estimated that 1-2% of skeletal muscle is broken down and synthesized every 24 hours (94, 183, 230). This turnover rate is slow compared to rates of other tissues, however, because of the higher proportion of muscle to other tissues, skeletal muscle accounts for 30-50% of whole body protein turnover (depending on age, nutritional status and activity level) (228). Muscle anabolism occurs if the rate of muscle protein synthesis (MPS) is greater than the rate of muscle protein breakdown (MPB), resulting in a positive net balance (228). Conversely, catabolism occurs when the rate of breakdown is greater than that of synthesis (228). In healthy adults, amino acids are deposited or released from muscle mass depending on nutritional status, but there is generally no net growth or loss.

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[1] Excerpts from Drummond MJ, Dreyer HC, Fry CS, Glynn EL, Rasmussen BB. Nutritional and contractile regulation of human skeletal muscle protein synthesis and mTORC1 signaling. *J Appl Physiol*. 106(4): 1374-1384, 2009. ©American Physiological Society, reproduced with permission.

On the other hand, in states of disease or injury such as cancer, AIDS, burns, sepsis, and traumatic injury (228, 239), decreases in muscle mass are prevalent. In general, lower muscle mass correlates with decreased survival and overall poorer prognoses in such cases (4, 53, 176, 239). There is also a progressive decrease in muscle mass and function over time with aging (termed sarcopenia) (71, 142), which can lead to overall weakness, frailty, immobility and finally physical dependence (53, 221). In addition, muscle dysfunction is a key component of chronic life-style related diseases (i.e., heart disease and type II diabetes) that now account for over two-thirds of deaths in the United States (3, 115). It is clear that muscle is a central factor in whole-body protein metabolism, and thus in the body's stress response, making it a key component to consider when investigating methods aimed at improving outcomes in diseases and aging.

Nutrition and exercise are two interventions, perhaps the most universally available, that are extremely anabolic stimuli for skeletal muscle. Though recent research has made many important advances in this area, the physiological changes responsible for exercise- and nutrient-induced stimulation of muscle growth, and the cellular mechanisms producing these changes, are not completely understood. Historic stable isotope tracer studies in humans showed positive changes in net protein balance following feeding alone or in combination with resistance exercise but lacked mechanistic explanations for the alterations in protein metabolism. Recent evidence has implicated the mammalian target of rapamycin (mTOR) pathway, which is activated in response to anabolic stimuli such as resistance exercise (11, 26, 165) and amino acids (5, 128), as an important regulator of cell size (162, 244). Additionally, anabolic nutrients or stimuli can decrease MPB (1, 75, 90). Examining changes in muscle protein metabolism, cellular signaling and related gene expression in response to exercise- and nutrition-based interventions will help delineate the mechanisms regulating muscle growth and have translational value to the wide range of conditions affected by loss of muscle mass.

THE mTOR PATHWAY

In 1975, a new antifungal antibiotic named rapamycin was isolated from *Streptomyces hygroscopicus* bacteria in soil samples from Easter Island (219). Years later, TOR1 and TOR2 (target of rapamycin) were identified as novel products of mutant genes in yeast (102), and discovered to be critical regulators of cell growth and proliferation mediated by nutrients (13). TOR is also present in mammalian cells, termed mTOR, although there is only a single gene (FRAP1) encoding the mammalian form (37). The mTOR protein is found within two independently regulated and functionally distinct protein complexes: mTORC1 and mTORC2 (110) (Figure 1). The mTORC2 complex is comprised of mTOR, G-protein beta subunit-like protein (GβL), mSin, and rapamycin-insensitive companion of mTOR (rictor) (192). This complex is involved in cytoskeletal regulation and can signal to Akt/PKB (protein kinase B) of the insulin signaling cascade (110, 193), though it is not rapamycin sensitive nor does it appear to be involved in cellular hypertrophy.

Of greater interest in the context of muscle growth, and the complex mainly referred to hereafter, rapamycin-sensitive mTORC1 is activated in response to nutrients, growth factors and muscle contraction (104, 106, 123, 242). Thus, mTORC1 acts as a mediator of skeletal muscle cell size (27). Complex 1 is composed of mTOR, GβL, and regulatory associated protein of mTOR (raptor) (96, 123-124). Although there are numerous mTORC1 upstream regulators and downstream targets, the work presented herein will focus on a select group of proteins (Figure 1). Upstream, Akt can directly activate mTORC1 through phosphorylation (170) or indirectly by phosphorylating (and inhibiting) tuberous sclerosis complex 2 (TSC2) (108, 152). Also upstream, AMP-activated protein kinase (AMPK) acts as a negative regulator of mTORC1 by enhancing TSC2 activity (109) or directly phosphorylating raptor (91). Downstream, mTORC1 directly phosphorylates ribosomal p70 S6 kinase 1 (S6K1), which has at least nine different targets (187), including ribosomal protein S6 (rpS6) and eukaryotic elongation factor 2 (eEF2) kinase (185, 225). Phosphorylation of rpS6 is thought to enhance translation initiation, cell size, and proliferation (188), while phosphorylation of eEF2

kinase inhibits its action on eEF2 and results in enhanced translation elongation (38). mTORC1 phosphorylation of 4E-BP1 (eukaryotic initiation factor 4E binding protein 1) inhibits 4E-BP1 from binding to eIF4E and allows eIF4E to complex with eIF4G, a required step in formation of the translation initiation complex (226). While mTORC1 is not the only pathway involved in the hypertrophic response to anabolic stimuli, it does play an important role in the integration of multiple pathways that are explained in the following sections and illustrated in Figure 1.

BREAKDOWN SIGNALING

The four main classes of intracellular proteases are the calpains, caspases, cathepsins (lysosomal system) and the proteasome (ubiquitin-proteasome system) (204). The ubiquitin-proteasome system (UPS) is responsible for the majority of bulk protein degradation in the muscle (141), although all classes of proteases contribute. Within the UPS, the Forkhead box (FOX) family of transcription factors (FOXO1, FOXO3a, FOXO4) regulate the expression of E3 ubiquitin (Ub) ligases MAFbx (muscle atrophy F-box, also known as atrogin-1) and MuRF1 (Muscle RING Finger 1) (25, 140, 191). FOXO3a is inhibited via phosphorylation by Akt, and therefore insulin and other factors influencing Akt phosphorylation affect this pathway (40). AMPK activation also stimulates catabolic, energy producing pathways in response to low energy levels or cellular stress (97, 234) via the UPS (137, 169). In the lysosomal system, autophagy is the primary process by which cytoplasmic contents are delivered to the lysosome to be degraded. Several steps occur at the induction of autophagy that ultimately form the autophagosome, where cytosolic contents are sequestered and delivered to the lysosome. One step required for autophagosome formation is post-translational modification of microtubule-associated protein 1 light chain 3 (LC3B) from the cytosolic form LC3B-I, to the autophagosomal membrane-associated form, LC3B-II (114). Because this is a required step for autophagy induction, LC3B-II is used as a marker of enhanced

autophagy (114, 125, 131-132). A brief overview of breakdown-associated signaling proteins is presented in Figure 1.

PHYSICAL ACTIVITY[2]

An increase in physical activity or endurance exercise training can improve insulin sensitivity in skeletal muscle (48, 89, 246). More aerobic-type exercise stimulates insulin-independent GLUT4 (glucose transporter 4) translocation to the plasma membrane and thereby increases glucose uptake (39, 89). This most likely occurs via a pathway connecting AMPK, a cellular energy sensor, and AS160 (Akt-substrate of 160 kD), a key component for GLUT4 translocation to the plasma membrane (208, 211). Insulin stimulates GLUT4 translocation through a more clearly defined mechanism, first binding to the insulin receptor and enhancing IRS-1 (insulin receptor substrate-1) tyrosine phosphorylation. This in turn stimulates signaling to PI3K (phosphoinositide 3-kinase), Akt and AS160 (208). Disruption in this sequence of events has been shown to occur in insulin resistant states (115, 118, 189).

On the other hand, the mTOR signaling pathway is generally activated following more resistive-type exercise (11); however, there is a large amount of interplay between the mTOR pathway, AMPK and insulin-related signaling. Recent evidence suggests the mTOR pathway may have an inhibitory effect on insulin signaling, participating in a negative feedback loop which phosphorylates IRS-1 on specific serine residues. Phosphorylation of IRS-1 at $Ser^{636/639}$ prevents normal signal transduction through the PI3K/Akt pathway and is linked to the insulin resistance of type II diabetes and obesity (214, 217). Two major components of the mTOR pathway, mTORC1 and its downstream target S6K1, are both capable of phosphorylating IRS-1 on these residues (Fig. 1) (214). There is a growing body of evidence pointing to nutrient-overload, and perhaps then chronic activation of mTORC1, as a significant contributor to insulin resistance (122, 212-213).

[2] Physical activity is defined as increased energy expenditure that cannot be specifically classified as either aerobic or resistive exercise within this document.

Although overactivation of mTORC1 signaling may lead to impaired insulin signaling, normal function of the mTOR pathway is critically important since it is involved in the regulation of muscle protein synthesis, and consequently, of muscle mass. For example, in cases of extreme physical inactivity (i.e., spinal cord transection, hindlimb suspension), muscle atrophy occurs at elevated rates in a variety of animal models (12, 92-93, 105). Acute studies have found this atrophy is associated with increased rates of MPB (207), upregulation of the ubiquitin-proteasome protein degradation pathway (218), large decreases in MPS (207) and inhibition of multiple translation factors (105). While the mTOR pathway is downregulated in several models of acute muscle atrophy (<30 days) (26), there has been little investigation regarding this pathway in models of chronic atrophy. One report by Wang *et al*. showed resistance exercise could correct abnormalities in protein synthesis and mTOR signaling observed in chronic kidney disease-associated muscle atrophy (227). It is clear that a delicate balance must exist between the necessary activation of mTORC1 for maintenance of muscle mass, and overactivation of the pathway in instances of energy excess that may lead to metabolic dysfunction.

RESISTANCE EXERCISE

Resistance exercise (RE) has been shown to increase muscle cell size and MPS (47, 229). Previous studies have found the rate of MPS remains elevated for up to 24 hours in trained individuals (150), and 48 (177) and 72 hours in untrained individuals (160). Following resistance-type exercise, increases in MPS correspond to an increase in signal transduction through the mTOR pathway in both rodents and humans (11, 64). For example, Akt is involved in insulin signaling as previously discussed, but is also an upstream regulator of mTORC1 and is elevated following RE (64, 88). However, recent work suggests that the resistance-exercise induced activation of mTORC1 is independent of Akt and due primarily to mechanical production of phosphatidic acid which is a direct activator of mTORC1 (173). Activation of mTORC1 causes increased phosphorylation

of S6K1 in the first 1-6 hours following a bout of RE (64, 88). S6K1 can then phosphorylate and inactivate eukaryotic elongation factor 2 (eEF2) kinase (225), leading to reduced phosphorylation of eEF2 and ultimately enhanced translation elongation (38). Indeed, decreases in eEF2 phosphorylation are observed following a bout of RE, most likely due to increased S6K1 activation (64-65).

MPB is also elevated following a bout of RE (21), and despite the large increases in MPS and signal transduction through mTORC1, protein net balance will remain negative without the addition of nutrients during post-exercise recovery (209). AMPK is activated in response to alterations in cell-energy status (98). Energy-consuming RE activates AMPK in rodents (180, 235) and humans (64), logically acting to inhibit anabolic processes and stimulate energy-producing catabolism during cellular stress (234). Aside from AMPK, few studies have examined catabolic signaling or gene expression following RE, but it appears mRNA expression of the E3 ubiquitin ligase MURF1 (muscle RING finger 1) may be elevated (148, 181). Because the response of both MPS and MPB contribute to protein net balance following RE, it is important to further characterize the effects of RE on catabolic signaling and gene expression.

NUTRITION

Early studies in the 1980s using stable isotopic tracer techniques in humans showed that mixed nutrient ingestion or mixed amino acid infusion could significantly increase MPS (16, 182). About the same time, studies in rats showed the stimulatory effect of amino acids on MPS was due to the essential amino acids (EAA), and perhaps more specifically, the branched-chain amino acids (82). Studies in humans have confirmed that EAA stimulate MPS, but non-essential amino acids do not (197-198, 222). Increases in MPS occur in animals and humans within about 30 min of nutrient ingestion, and stay elevated for about 2 h before returning to basal levels (29, 233). The increase in MPS is associated with enhanced translation initiation via upregulation of the mTOR pathway, including increased phosphorylation of Akt, mTOR, and S6K1, and decreased

phosphorylation of eEF2 (57, 77, 233). 4E-BP1 phosphorylation does not appear to be affected by RE, but is increased following nutrient ingestion (77, 233), inhibiting 4E-BP1 from binding eIF4E and thereby enhancing translation initiation. To date, two proteins have been implicated in mediating signals from amino acids to mTORC1: a novel class 3 PI3K, human vacuolar protein sorting-34 (hVps34), and Ste20 family member mitogen activated protein kinase kinase kinase kinase-3 (MAP4K3) (42, 73). However, the specific mechanisms of hVps34 and MAP4K3 regulation of mTORC1 in response to amino acids are still unclear (Figure 1).

On the other hand, the role of CHO, or more specifically the CHO-induced rise in circulating insulin, relating to MPS has been a topic of debate in the field. Several studies have shown either local hyperinsulinemia (19, 80) or systemic rises in insulin in both animal and human models can increase MPS (78, 203). Others have found opposing results and suggest insulin has no significant effect on MPS (83, 90, 147, 206). It would seem logical that stimulation of insulin signaling and activation of Akt, which signals to mTORC1 may stimulate MPS. In any event, it is generally accepted that CHO and insulin exert greater effects on the response of MPB than that of MPS.

Nutrients can decrease MPB but this is dependent on the nutrient composition. For example, although EAA are not generally thought to affect MPB (22), several studies indicate the EAA leucine can reduce MPB (164, 166, 168). Carbohydrates ingested following RE or in catabolic states (33, 100), as well as increased circulating insulin levels at rest ($\geq$30 $\mu U \cdot mL^{-1}$) are able to reduce MPB (90). Ingestion of CHO stimulates insulin release, and thus enhances signaling through the insulin/PI3K/Akt pathway. Akt is not only involved in regulation of hypertrophy via the mTOR pathway, but can phosphorylate and inactivate FOXO3a (forkhead box subclass O), which regulates the expression of the E3 ubiquitin ligases MAFbx (muscle atrophy F-box) and MuRF1 (25, 140, 191). Several studies have reported decreases in the mRNA expression of components of the ubiquitin-proteasome system following nutrient ingestion (90, 159, 245), while others have shown insulin signaling through class I PI3K to Akt, and signal transduction through the mTOR pathway inhibit autophagy (112, 157, 224). However,

taken as whole, data obtained from human studies to date indicate that changes in MPB, associated signaling and mRNA expression are minor following nutrient ingestion.

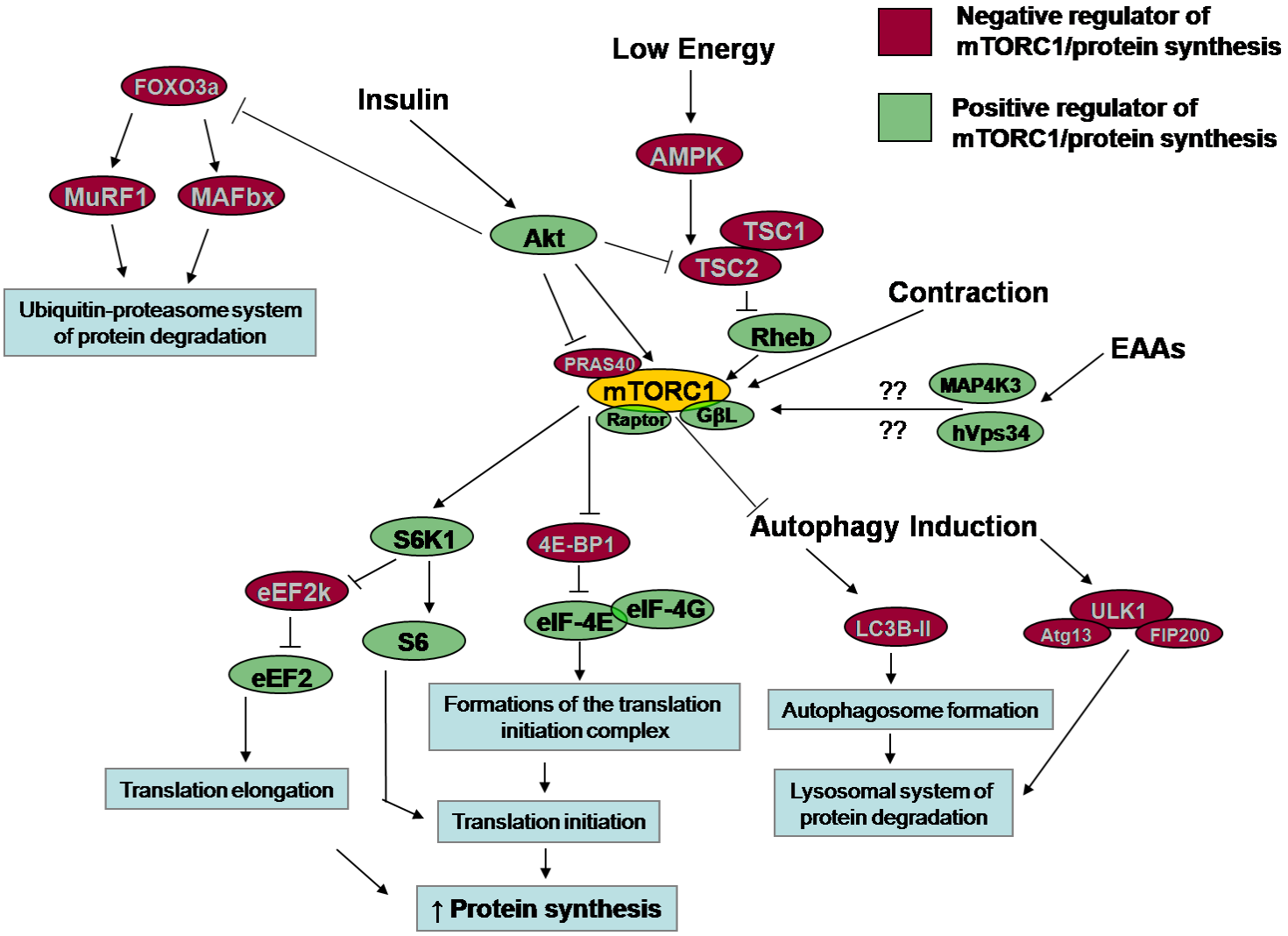
Leucine has received attention for its unique ability to increase MPS in skeletal muscle and activate mTORC1 without the addition of other EAA (5-6, 149, 197, 202). One mechanism by which leucine stimulates MPS is by enhancing translation initiation via mTOR signaling (5-6, 130, 143, 202). Reports indicate that the other branched chain amino acids (isoleucine and valine) do not cause the same increase in MPS (69, 81). Leucine also causes a small, transient increase in circulating insulin concentration in vivo (128), which may enhance the effect on MPS. However, it appears stimulation of mTOR signaling is driving leucine-induced increases in MPS because this effect is abolished with the administration of rapamycin, and carbohydrates alone do not stimulate MPS (6, 128, 202). In addition, leucine regulates MPB through both mTORC1-dependent and independent mechanisms (164, 166, 168). This may be due, in part, to leucine-stimulated insulin secretion since insulin reduces MPB, and studies confirm the effect of insulin on MPB is mTOR-dependent (116). Still, others report that the reduction in MPB due to decreased UPS and lysosomal system degradation is mTOR-independent (116, 164, 168), though these results are not inclusive of all proteolytic pathways. Additional studies are required to more clearly define the mechanisms regulating leucine's unique anabolic characteristics.

SUMMARY

Contractile and nutrient regulation of skeletal muscle protein turnover is the result of an incredibly complex network of cellular pathways, interacting to produce alterations in cellular conditions that ultimately result in observed changes. The mTOR hypertrophy signaling pathway, and the atrophy-inducing UPS and lysosomal pathways, are major contributors to the physiological response to exercise and nutrition. Therefore, the following studies seek to define the role of these components in response to varying treatments and conditions. First, insulin and mTOR pathways are examined following a

chronic model of increased daily energy expenditure. The second study focuses on alterations in mTOR signaling following chronic paraplegia-induced muscle atrophy. The goal of study 3 is to determine the effect of lower levels of carbohydrate ingestion versus higher levels following resistance exercise, and specifically the response of muscle protein breakdown to the carbohydrates and resulting insulin response. Finally, the last study sought to determine whether higher leucine content in an essential amino acid solution would enhance muscle protein synthesis, decrease muscle protein breakdown, or have no additional benefits when compared to a solution composed of essential amino acids at concentrations representative of high-quality protein sources. These studies will further our understanding regarding the regulation of muscle hypertrophy and atrophy, and contribute important data to the body of knowledge necessary for establishing evidence-based recommendations for the maintenance of skeletal muscle mass during conditions of muscle wasting.

Figure 1. Signaling Pathways.



CHAPTER 2

A chronic increase in physical activity inhibits fed-state mTOR/S6K1 signaling and reduces IRS-1 serine phosphorylation in rat skeletal muscle[3]

INTRODUCTION

There is a growing epidemic of insulin resistance and Type 2 diabetes in the United States and worldwide in part due to an increased prevalence of obesity and/or reduced physical activity levels (115). Skeletal muscle is a major regulator of insulin resistance, since it is responsible for up to 75% of insulin-dependent glucose disposal in human subjects (196). It is also clear that reduced caloric intake (156) and an increase in physical activity and/or endurance exercise training can improve insulin sensitivity in skeletal muscle (48, 89, 246). Therefore, because physical exercise and activity uses skeletal muscle for locomotion, it is likely that the improved insulin sensitivity following exercise training is due (in part) to cellular adaptations within skeletal muscle, which improve insulin signaling (111, 126).

The insulin signaling cascade has long been studied as a means to understand the molecular mechanisms of insulin resistance and Type 2 diabetes (49). Insulin binds to its receptor and initiates the intrinsic tyrosine kinase activity of the receptor, which further phosphorylates cellular substrates including IRS-1. IRS-1 is the predominant isoform of the insulin receptor substrate in skeletal muscle (8). Tyrosine phosphorylation of IRS-1 creates a scaffold for which the p85 regulatory subunit of PI3K can bind, further transducing the signal through the PI3K/Akt pathway. Insulin stimulation of PI3K is a

[3] Glynn EL, Lujan HL, Kramer VJ, Drummond MJ, DiCarlo SE, Rasmussen BB. A chronic increase in physical activity inhibits fed-state mTOR/S6K1 signaling and reduces IRS-1 serine phosphorylation in muscle. *Appl Physiol Nutr Metab*. 33(1): 93-101, 2008. ©NRC Research Press, reproduced with permission.

necessary step in the insulin signaling pathway that leads to the uptake of glucose from the blood (189). Therefore, signaling defects in this pathway disrupt normal insulin signaling and have been proposed as involved in the pathogenesis of insulin resistance (195).

Recent evidence implicates an inhibitory role in insulin signaling for the mTOR pathway via an increased serine phosphorylation of IRS-1 (214-215, 217). Serine or threonine phosphorylation of different sites on IRS-1 can have many consequences, including dissociation of IRS proteins from the insulin receptor, blockage of certain tyrosine phosphorylation sites of IRS, and inducing degradation of IRS proteins (118, 175). Phosphorylation of IRS-1 at $Ser^{636/639}$ has been shown to be involved in insulin resistance in cases involving obesity-linked insulin resistance and Type 2 diabetes (36, 122). Both mTORC1 and S6K1 appear to be involved in the $Ser^{636/639}$ phosphorylation of IRS-1 (36, 122, 213-214). In fact, S6K1 knockout mice are protected against diet-induced insulin resistance (217). Furthermore, mice lacking 4E-BP1 are highly susceptible to diet-induced insulin resistance, which implies that 4E-BP1 may be acting as a "metabolic brake" in the etiology of insulin resistance in skeletal muscle (139). These recent findings provide more evidence that overactivation or deregulation of the mTOR signaling pathway is an important regulator of insulin signaling. There have been numerous studies conducted in the area of insulin signaling and exercise over the past 30 years (210), however, the effect of chronic physical activity and/or exercise training on mTOR signaling and its role in regulating insulin signaling is not known.

Therefore, the purpose of the current study was to determine whether a chronic increase in physical activity alters expression and phosphorylation of regulatory proteins associated with both the insulin and mTOR signaling pathways. It was hypothesized that an increase in physical activity would inhibit or attenuate mTORC1/S6K1 signaling and reduce IRS-1 serine phosphorylation in rat skeletal muscle.

MATERIALS AND METHODS

Subjects and Design. All animal treatments were performed at Wayne State University School of Medicine in the laboratory of Dr. Stephen DiCarlo (Detroit, MI) and were reviewed and approved by the Institutional Animal Care and Use Committee at Wayne State University School of Medicine. Seventeen male Sprague-Dawley rats between 4-5 weeks old were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Weanling rats were assigned to either an Inactive group (N=8) or Active group (N=9). The Inactive rats were housed in standard polycarbonate rat cages and the Active rats were housed in cages with free access to running wheels (Nalgene; Rochester, NY). Daily voluntary running distance (i.e., total number of revolutions of the wheel) was monitored with an optical sensor attached to the side of the running wheel interfaced with a personal computer (activity wheel counter model 86060, Lafayette Instrument; Lafayette, IN) (52).

Both groups of rats were fed ad libitum throughout the study. Running wheels were removed from the exercise group cages 24 h before sacrifice of the animals to avoid any acute effects on muscle signaling proteins due to the prior exercise bout. Fed-state is defined as allowing the rats access to food (normal rodent diet) and water ad libitum. The rats were sacrificed at the same time of day (early morning) and thus large differences in food intake between groups was not expected. Rats were sacrificed by i.p. injection with sodium pentobarbital. Both groups were sacrificed at approximately 3 months of age and soleus muscles from both legs were removed and quickly frozen in liquid nitrogen. Samples were stored at -80^{o}C until analysis. To collect serum samples, a needle was inserted into the abdominal aorta and 3 mL of blood was withdrawn, centrifuged and stored at -80^{o}C until analysis.

Sample preparation. Soleus muscle specimens were dissected free from blood and connective tissue and homogenized (1:9, *w/v*) in ice-cold buffer (50 mmol·L^{-1} Tris-HCL, 250 mmol·L^{-1} mannitol, 50 mmol·L^{-1} NaF, 5 mmol·L^{-1} sodium pyrophosphate, 1 mmol·L^{-1} EDTA, 1 mmol·L^{-1} EGTA, 1% Triton X-100 1 (pH 7.4), 1 mmol·L^{-1} DTT (dithiolthreitol), 1 mmol·L^{-1} benzamidine, 0.1 mmol·L^{-1} PMSF (phenlymethylsulfonyl

fluoride), 5 $\mu g \cdot mL^{-1}$ soybean trypsin inhibitor (SBTI); DTT, benzamidine, PMSF, and SBTI were added to the buffer immediately prior to use). Supernatant was collected after centrifugation at 2000 *g* for 10 min at 4°C. A Bradford protein assay was performed to determine protein content of samples. Except for aliquots for 4E-BP1, samples were then combined with 2x sample buffer (SB) containing 125 $mmol \cdot L^{-1}$ Tris (pH 6.8), 25% glycerol; 2.5% sodium diodecyl sulfate (SDS), 2.5% β-mercaptoethanol, and 0.002% bromophenol blue. Aliquots used to detect 4E-BP1 were initially boiled at 100°C for 10 min and spun for 30 min at 5600 *g* before combining the supernatants with 2x SB.

SDS-PAGE and Immunoblotting. Samples containing 50 μg of total protein per lane were loaded in duplicate and separated by SDS-PAGE for 60 min at 150V using 7.5%, 10%, or 15% gels on Criterion electrophoresis cell. A molecular weight ladder (Precision Plus protein standard, BioRad; Hercules, CA) was also included on each gel. Following SDS-PAGE, proteins were transferred to polyvinylidene diflouride membranes (PVDF) (Hybond-P, Amersham Biosciences, Piscataway, NJ) at 50V for 1 h. Once transferred, PVDF membranes were placed in blocking buffer (5% non-fat dry milk (NFDM) in TBST (Tris-buffered saline and 0.1% Tween-20) for 1 h. Blots were then serially washed twice in deionized water and twice more in TBST before incubating with primary antibody in 5% bovine serum albumin (BSA) in TBST overnight at 4°C with constant agitation. The next morning, the blots were washed twice in TBST and incubated with secondary antibody (1:2000) for 1 h in 5% NFDM in TBST at room temperature with constant agitation. After secondary incubation the blots were washed for 15 min and then serially washed (4 x 5 min) with TBST. Blots were then incubated for 5 min with enhanced chemiluminescence reagent (ECL plus Western Blotting Detection System, Amersham Biosciences; Piscataway, NJ) to detect horseradish peroxidase activity. Optical density measurements were obtained with a CCD camera mounted in a ChemiDoc XRS imaging system (BioRad; Hercules, CA). Once the appropriate image was captured, densitometric analysis was performed using Quantity One 1-D analysis Software v. 4.5.2 (BioRad, Hercules, CA). Data are expressed as raw value of band minus a representative background sample from the membrane, divided by

a rat standard (50 μg/lane) that was loaded on every gel to ensure comparability across membranes.

The membranes described above were incubated in Restore™ Western blot stripping buffer (Pierce; Rockford, IL) for 20 min at 37°C and re-probed with appropriate polyclonal antibodies for detection of the total expression levels of each protein as needed. Equal loading of protein was determined spectrophotometrically.

Antibodies. The primary antibodies used were all purchased from Cell Signaling (Beverly, MA): phospho-mTOR (Ser^{2448}; 1:1000), phospho-p70 S6K1 (Thr^{389}; 1:500), phospho-IRS-1 ($Ser^{636/639}$; 1:500), phospho-PKB/Akt (Ser^{473}; 1:1000), total-mTOR (1:1000), total-p70 S6K1 (1:1000), total-IRS-1 (1:500), total-4E-BP1 (1:1000), total-eEF2 (1:1000), total-PKB/Akt (1:1000), and total-AMPKα (1:1000). Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was purchased from Amersham Bioscience (1:2000).

Insulin, Glucose, and Citrate Synthase. Plasma insulin concentrations were determined by rat-mouse insulin ELISA kit (Linco Research; St. Charles, MO) as directed by the manufacturer. Plasma glucose concentrations were measured using an automated glucose and lactate analyzer (YSI; Yellow Springs, OH). Citrate synthase activity was determined using adapted methods previously described by Srere (200).

Statistical Analysis. Data are presented as means ± SEM. An independent two sample Student's *t* test was used to assess differences in phosphorylation status and total protein content of each protein, muscle citrate synthase activity, body weight, soleus weight, and plasma insulin and glucose concentrations between groups. Significance was set at $P < 0.05$. Assumptions of the test included a normal distribution of the data, equal variances, and randomization of the independent sample groups.

RESULTS

Running distance and Weight. Peak running distance over the 9 weeks reached 5.4 ± 1.4 $km \cdot d^{-1}$ between weeks 4 and 5. The weights of the rats prior to sacrifice were

compared between groups. The Active group had a significantly lower body weight as compared to the Inactive group ($P < 0.05$; Table 1). However, there was no difference in soleus muscle weight between the two groups ($P > 0.05$; Table 1).

Insulin, Glucose, and Citrate Synthase. There was no difference in plasma insulin or glucose concentrations between the two groups ($P > 0.05$; Table 1). Soleus muscle citrate synthase activity was significantly higher in the Active group ($P < 0.05$; Table 1) as compared with the Inactive group.

Table 1. Rat Characteristics.

	Inactive	Active
Total body weight (g)	411 ± 10	338 ± 5*
Soleus weight (mg)	175 ± 5	164 ± 4
Plasma insulin ($\mu U \cdot mL^{-1}$)	23.2 ± 1.2	24.3 ± 3.8
Plasma glucose ($mmol \cdot L^{-1}$)	10.4 ± 0.5	10.6 ± 0.3
Citrate synthase activity ($\mu mol \cdot g^{-1} \cdot min^{-1}$)	29.9 ± 0.8	33.4 ± 0.9*

Values are mean ± SEM. *Significantly different from Inactive, $P < 0.05$.

Western Blot Analyses. The phosphorylation status of mTOR (Ser^{2448}) and S6K1 (Thr^{389}) was significantly reduced in the Active rats ($P < 0.05$; Figure 2A and 2C), whereas total protein content for both proteins remained unchanged ($P > 0.05$; Figure 2B and 2D). Total protein abundance of eEF2, 4E-BP1 and AMPKα were also not different between groups ($P > 0.05$; Figure 3).

The phosphorylation status of IRS-1 ($Ser^{636/639}$) was significantly reduced in the Active group compared with the Inactive group ($P < 0.05$, Figure 4A) with no change in total IRS-1 protein content ($P > 0.05$, Figure 4B). Phosphorylation status and total protein content did not change between groups for Akt ($P > 0.05$; Figure 4C and 4D).

Figure 2. Soleus muscle mTOR and S6K1.

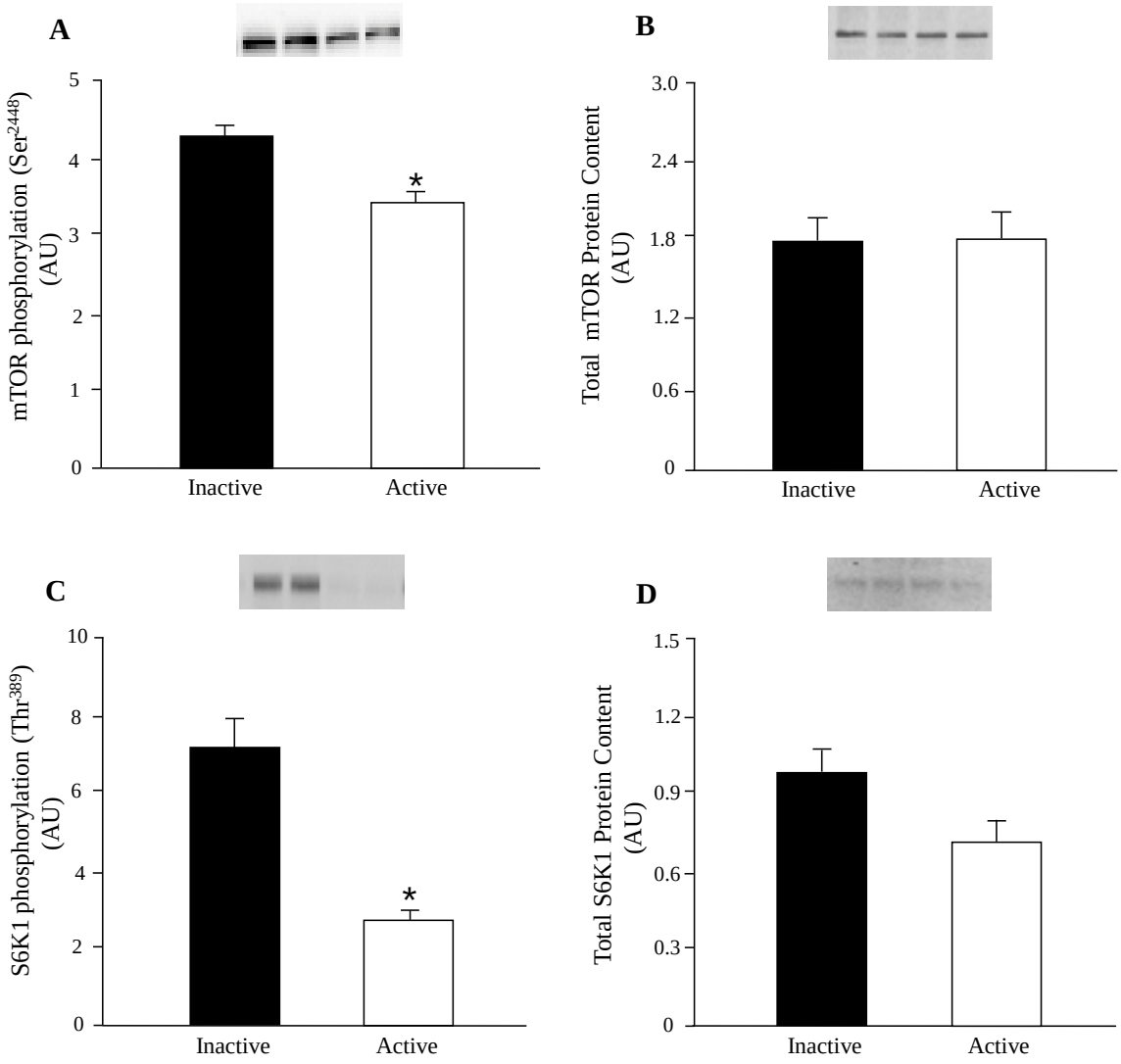


Fig. 2. Soleus muscle mTOR phosphorylation at Ser^{2448} (A), total mTOR protein content (B), S6K1 phosphorylation at Thr^{389} (C), and total S6K1 protein content (D). Data are expressed relative to a normalization control and as Mean ± SEM, N=8 per group. Insert shows representative Western Blot of duplicate samples for Inactive and Active rats. AU=arbitrary units. *Significantly different from Inactive, $P < 0.05$.

Figure 3. Soleus muscle total proteins.

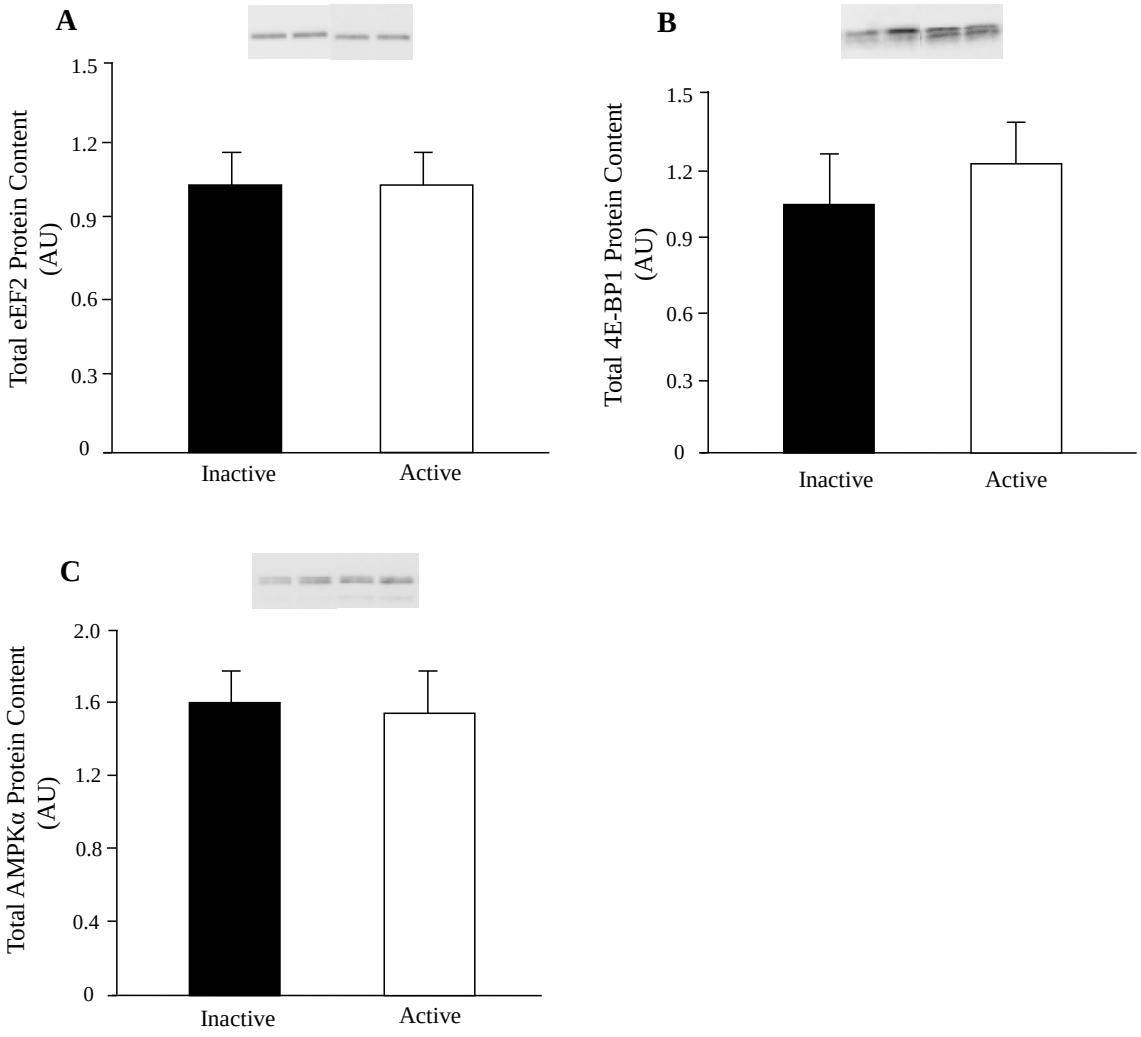


Fig. 3. Soleus muscle total eEF2 protein content (A), total 4E-BP1 protein content (B), and total AMPKα protein content (C). Data are expressed relative to a normalization control and as Mean ± SEM, N=8 per group. Insert shows representative Western Blot of duplicate samples for Inactive and Active rats. AU=arbitrary units. *Significantly different from Inactive, $P < 0.05$.

Figure 4. Soleus muscle insulin signaling.

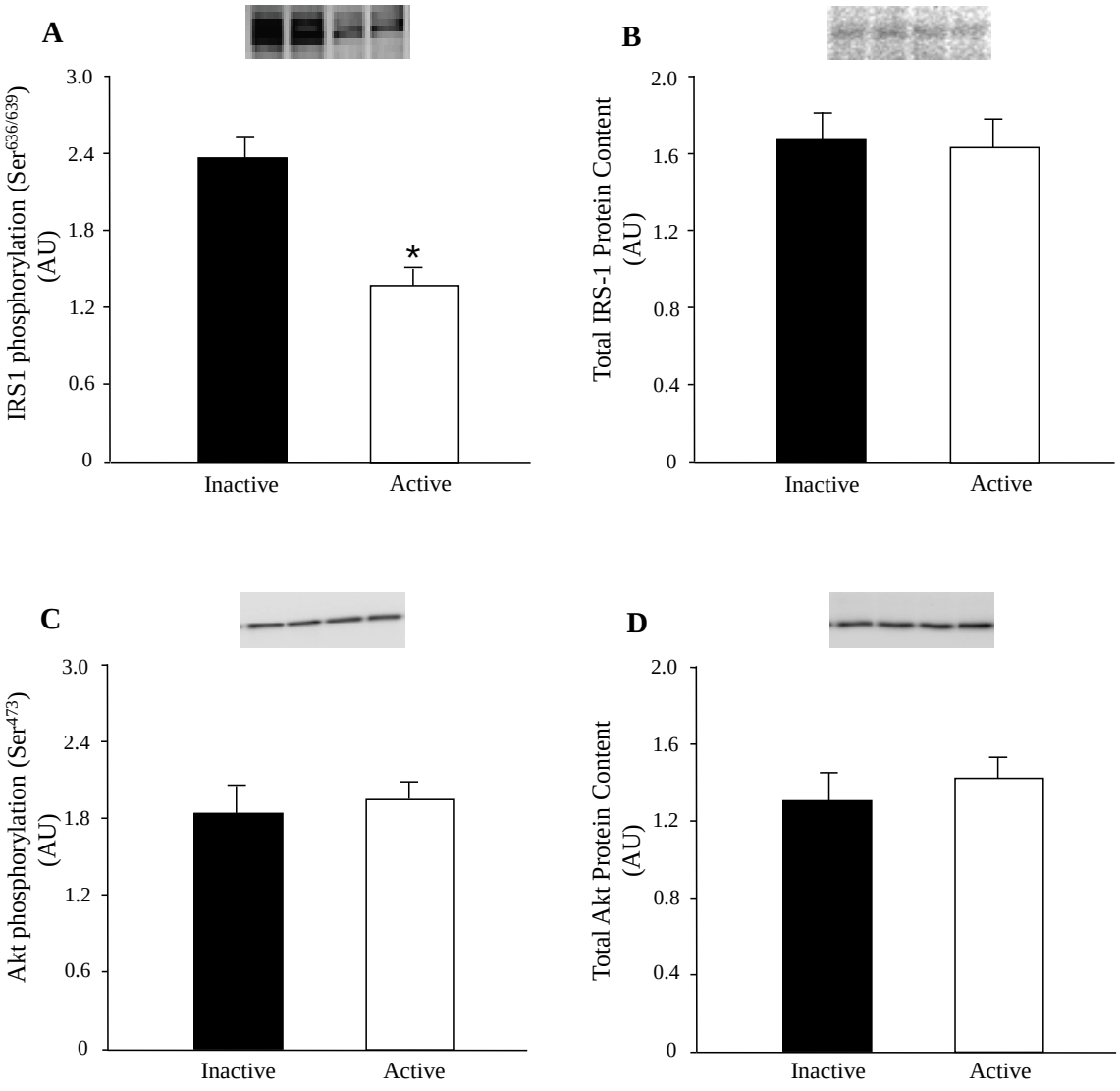


Fig. 4. Soleus muscle IRS-1 phosphorylation at $Ser^{636/639}$ (A), total IRS-1 protein content (B), Akt phosphorylation at Ser^{473} (C) and total Akt protein content (D). Data are expressed relative to a normalization control and as Mean ± SEM, N=8 per group. Insert shows representative Western Blot of duplicate samples for Inactive and Active rats. AU=arbitrary units. *Significantly different from Inactive, $P < 0.05$.

DISCUSSION

The primary and novel finding from this study was that an increase in physical activity was associated with a downregulation of the fed-state mTOR/S6K1 signaling pathway and a reduction in IRS-1 serine phosphorylation in rat skeletal muscle. Specifically, mTOR Ser^{2448}, S6K1 Thr^{389} and IRS-1 $Ser^{636/639}$ phosphorylation were significantly reduced in the Active rats that were exposed to 9 weeks of free access to a running wheel. Overactivation of the mTOR signaling pathway has been proposed as a mechanism by which insulin signaling can be inhibited because IRS-1 is a substrate for both mTORC1 and S6K1 (216). In fact, evidence is accumulating for a significant role of the mTOR pathway in nutrient overload or diet-induced insulin resistance in skeletal muscle (139, 214, 217). This study is the first to suggest a connection between chronic physical activity and improved insulin signaling by reduced mTOR signaling and thus reduced inhibitory IRS-1 serine phosphorylation in skeletal muscle.

It is well-known that exercise can stimulate insulin-independent GLUT4 translocation and glucose uptake within skeletal muscle (39, 89). Insulin promotes GLUT4 translocation by binding to its receptor, enhancing IRS-1 tyrosine phosphorylation, and stimulating signaling to PI3K and Akt (246). Exercise is proposed to enhance insulin sensitivity both by insulin-independent stimulation of GLUT4 translocation and by the interaction of many signaling pathways (111, 246). This study found that mTOR signaling and IRS-1 serine phosphorylation were reduced following a period of increased physical activity in rats. Therefore, in addition to the other well-known effects of exercise on enhanced insulin signaling and glucose uptake in skeletal muscle, reduced mTOR signaling may also play a role in decreasing IRS-1 $Ser^{636/639}$ phosphorylation in this model.

It has been demonstrated that the phosphorylation of serine residues 636/639 of IRS-1 are involved in insulin resistance (36). Other studies show serine residue phosphorylation on the C terminus of IRS-1 prevents insulin-stimulated tyrosine phosphorylation (118). It is the tyrosine phosphorylation on the C-terminus of IRS-1 that recruits PI3 kinase, a key step in insulin signal transduction. There are several recent

studies that support an important physiological role for mTOR signaling in the regulation of insulin resistance by the ability of mTORC1 and S6K1 to enhance IRS-1 serine phosphorylation (122, 174, 213-214). In a recent study by Um *et al.*, S6K1 deficient mice remained sensitive to insulin even with high levels of blood glucose and free fatty acids, and IRS-1 serine phosphorylation was reduced (217). In addition, LaBacquer *et al.* have shown that 4E-BP1 deficient mice were more sensitive to diet-induced insulin resistance, suggesting that 4E-BP1 interacts with and regulates S6K1 within muscle (139). The primary mechanisms for activating mTORC1 within skeletal muscle are most likely elevated insulin and amino acid concentrations (9, 77). Chronic exposure to nutrient overload would elevate circulating insulin levels and stimulate mTORC1 by upstream activation of Akt and hence reduce the suppression on mTORC1 by TSC2 (10, 178). However, inhibition of insulin signaling is the common characteristic of insulin resistance at which point it appears that amino acid signaling (i.e., from excess amino acid present during nutrient overload) can chronically activate mTORC1 and S6K1 in muscle and thus further inhibit insulin signaling via an increase in IRS-1 serine phosphorylation (122, 174, 213-214).

It was not possible with the current study design to measure insulin sensitivity with stable isotopic techniques or via a euglycemic-hyperinsulinemic clamp. There are, however, a few potential explanations for not seeing a difference in fed insulin and glucose levels. First, the Inactive group, although sedentary, may not have experienced a detectable insulin resistance. The fact that there was no detectable difference in the phosphorylation status or total Akt between groups supports this explanation. It has been hypothesized that the effect of exercise on insulin signaling and sensitivity is an acute effect and is rapidly reversed with the cessation of the exercise stimulus (41, 101). In fact, most control groups in rat physiological studies utilize sedentary rats with no detectable defects in insulin signaling. However, as recently suggested by Booth and Lees (31), the use of sedentary, inactive controls may introduce a bias into the interpretation of exercise studies because it is assumed that sedentary rats are healthy. Young, sedentary rats, although not showing outward signs of clinical hyperinsulinemia,

hyperlipidemia, or hyperglycemia, may not necessarily be considered healthy. Rats are normally very active animals and therefore maintaining rats in a small cage may be considered a type of inactivity model. These results are consistent with Booth and Lee's hypothesis (31), showing that the Inactive rats had higher phosphorylation levels of mTOR, S6K1, and IRS-1 serine residues. Thus, the reduction in phosphorylation in the Active rats may imply a return to a healthier overall status (i.e., improved insulin sensitivity) as would be predicted by the hypothesis presented by Booth and Lees (31). Secondly, the fed response between groups may have masked any basal differences in insulin and glucose concentrations. The fed state was chosen in lieu of a fasted state because it would likely be the best time to detect potential differences in mTOR signaling due to the stimulation of feeding on this pathway. It has previously been shown that active rats following a similar protocol actually consume a greater amount of food than inactive controls (55). Therefore, owing to lack of differences in either fed plasma insulin or glucose levels between groups, the feeding effect is likely negligible in analysis of the results.

Nine weeks of free access to a running wheel did not alter the total protein expression of mTOR, S6K1, 4E-BP1, Akt, AMPKα, eEF2, or IRS-1. These results are consistent with previous studies that have reported IRS-1 total protein expression does not change with exercise training (48) and that Akt concentrations were not altered (17), though results may have been different had Akt isoforms 1 and 2 been examined separately. Therefore, it appears that the increase in physical activity and overall energy expenditure was primarily responsible for the reduction in fed-state mTOR, S6K1, and IRS-1 phosphorylation.

It is well known that a chronic activation of AMPK is associated with an upregulation of citrate synthase and other mitochondrial oxidative enzymes in rat skeletal muscle (236). Endurance exercise and/or muscle contractions can activate AMPK activity in both rodent and human skeletal muscle (76, 180). Recently, it has been shown that AMPK activation (from both resistance exercise and endurance exercise protocols) in skeletal muscle is associated with a reduction in mTOR signaling (64, 232). AMPK is

an important cellular energy sensor within muscle, and it is therefore activated during cellular stress and increases in energy demand. The Active rats had a significantly reduced overall body weight as compared with the Inactive rats, suggesting that an increase in overall energy expenditure is an important component that could regulate mTOR signaling in muscle. Soleus muscle weight was not different between groups, implying that the reduced body weight in the Active rats was primarily due to a reduction in total body fat. Interestingly, citrate synthase activity was also significantly elevated (11%) in the Active rats. The modest increase in citrate synthase in the current study, as compared with the much larger increases in citrate synthase activity reported in exercise-training studies (205), suggests that a chronic increase in physical activity and energy expenditure (at an exercise intensity lower than commonly used induce an exercise-training effect) can have beneficial health effects on skeletal muscle mTOR signaling.

In summary, rats allowed free access to running wheels for 9 weeks increased their overall physical activity, reduced whole body weight, and lowered soleus muscle mTOR (Ser^{2448}), S6K1 (Thr^{389}), and IRS-1 ($Ser^{636/639}$) phosphorylation as compared with sedentary, inactive rats. Therefore, reduced mTORC1 and S6K1 activation during chronic increases in physical activity may play an important regulatory role in the serine phosphorylation status of skeletal muscle IRS-1 and may have important implications for reducing insulin resistance associated with this feedback pathway. Future studies are required to determine the specific role reduced mTOR signaling may play in regulating the well-known effect of enhanced insulin sensitivity associated with exercise-training.

ACKNOWLEDGEMENTS

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CHAPTER 3

Chronic paraplegia-induced muscle atrophy downregulates the mTOR/S6K1 signaling pathway[4]

INTRODUCTION

Skeletal muscle displays an incredible ability to respond to alterations in daily loading. For example, muscle atrophy occurs at an elevated rate in the initial few months following disuse in a variety of animal models such as spinal cord transection, hindlimb suspension, and spaceflight (12, 59, 62, 68, 93, 105, 231). The resulting muscle loss from these unweighting or denervation models appears to affect type I skeletal muscle fibers, such as those predominantly found in the soleus, to a greater extent (2). Both type I and type II muscle fiber size decrease following spinal cord injury (151, 155, 194). However, type II fibers are affected first followed by type I fiber atrophy, which becomes predominant during the later stages of paraplegia (194). Of the total soleus muscle weight ultimately lost due to atrophy, the majority (60%) is lost within the first 10 days (68), suggesting that alterations in protein synthesis and breakdown occur rapidly. Indeed, a decrease in protein synthesis has been measured after only 5 h of hindlimb suspension in rats (207) while in humans immobilized for only 48 h, mRNA for components of the ubiquitin-proteasome pathway were significantly upregulated (218).

Muscle protein synthesis is primarily regulated at the level of mRNA translation (119, 127). Alterations in skeletal muscle mass are controlled by the regulation of complex cell signaling pathways that govern muscle protein synthesis, breakdown, and cell proliferation (86). S6K1, a downstream component of the mTOR signaling pathway,

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[4] Dreyer HC*, Glynn EL*, Lujan HL, Fry CS, DiCarlo SE, Rasmussen BB. Chronic paraplegia-induced muscle atrophy downregulates the mTOR/S6K1 signaling pathway. *J Appl Physiol*. 104(1): 27-33, 2008. *Authors contributed equally to this manuscript.

is a key regulator of translation initiation and protein synthesis (119, 127). Phosphorylation of S6K1 is associated with increased muscle mass following hypertrophic stimuli (11), whereas muscle atrophy is associated with reduced S6K1 phosphorylation and overall protein abundance (26).

Phosphorylation of another downstream target of mTORC1, 4E-BP1, is a key event in translation initiation, releasing eIF4E to complex with eIF4G in the beginning steps of translation (87). On the other hand, activation of skeletal muscle AMPK during conditions of energetic stress is as an upstream negative regulator of mTOR signaling and protein synthesis (30, 46). Although there has been a considerable amount of work done assessing the role of muscle protein breakdown during muscle atrophy (117), less is known about the role of cell signaling pathways that regulate protein synthesis. In fact, it is not been determined whether mTOR or AMPK signaling is altered during paraplegia-induced muscle atrophy.

Before World War II, 80% of individuals with spinal cord injury died within 3 years of the injury primarily due to kidney and pulmonary infections (60). However, with the advent of antibiotic drugs and advancements in acute care and rehabilitation, the life expectancy of individuals with spinal cord injury has increased to near that for able-bodied individuals (61). Thus, the advantages of using a chronic muscle atrophy model (as done in the present study) may be useful in identifying the mechanisms of muscle protein synthesis and breakdown in the overall regulation of muscle mass. This could be clinically important in the rehabilitation of individuals with spinal cord injury because it may lead to the design of interventions targeting mTORC1, muscle protein synthesis, and cell growth. Therefore, the purpose of this study was to determine the effect of chronic paraplegia-induced muscle atrophy on components of the skeletal muscle mTOR signaling pathway.

MATERIALS AND METHODS

Study design. All surgical procedures and animal treatments were performed at Wayne State University School of Medicine in the laboratory of Dr. Stephen DiCarlo (Detroit, MI) and were reviewed and approved by the Institutional Animal Care and Use Committee at Wayne State University School of Medicine. Sixteen male Sprague Dawley rats (24 ± 0.2 wk of age at death) were separated into two groups: complete spinal cord transection between thoracic level 4 and 5 (T_4-T_5, Paraplegic, N=8) or sham spinal cord transection (Control, N=8). Following 10 wk of spinal cord transected-induced paralysis or sham-operated free living, the rats were killed and the soleus muscle was isolated, freeze clamped, immediately frozen in liquid nitrogen, and stored at -80ºC until analyzed.

Induced paraplegia. All surgical procedures were performed using aseptic techniques. Rats were anesthetized with pentobarbital sodium (45mg·kg^{-1} i.p. (intraperitoneal)) and supplemental doses (10-20 mg·kg^{-1} i.p.) were administered if rats regained the blink reflex or responded during the surgical procedure. After anesthesia, rats were positioned prone over a thoracic roll resulting in a moderately flexed spine. The T_4 and T_5 vertebrae were exposed via a midline dorsal incision. The underlying spinal cord between T_4 and T_5 was completely transected through the intervertebral space as described by Collins *et al*. (50-51). The control animals (sham operated) had the spinal cord exposed in an identical procedure; however the spinal cord was not transected. This approach allowed for minimal impact on the stability of the vertebral column. During the first week of recovery, all rats were handled several times daily. During the handling periods, visual inspection and physical manipulations were performed to prevent pressure ulcers. Additionally, bladder voiding was accomplished by manual compression after which all animals were weighed. After the first week of recovery, handling was reduced to one time each day and bladders no longer required manual compression for voiding. At day 7, rats underwent a motor activity score using previously described criteria (223). Briefly, the motor activity score was determined by placing the animal on a table and observing hindlimb movement for 1 min. Scores ranged from 0 to 5 with a 5 indicating

normal walking and a 0 indicating zero weight bearing or spontaneous movement specific to the hindlimbs. All paralyzed rats had a score of 0.

SDS-PAGE and Immunoblotting. Homogenization for immunoblotting, procedures and analyses was conducted in the same manner as previously described, and details of these procedures can be found in Chapter 2, Materials and Methods.

AMPK activity assay. The immunoprecipitation method and the enzyme activity measurements were completed according to methods described by Hardie *et al.* (99) with slight modifications. Briefly, ~70 mg of soleus muscle tissue was homogenized (1:9 *w/v*) in a buffer containing: 50mM Tris-HCl, 250 mM mannitol, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 (pH 7.4); 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF and 5 $\mu g \cdot mL^{-1}$ soybean trypsin inhibitor (STI). DTT, benzamidine, PMSF and STI were added to the buffer immediately prior to use. Supernatant was collected after centrifugation at 3500 *g* for 10 min at 4ºC. Total protein content determined using the Bradford Assay as described in Chapter 2. AMPKα2 was immunoprecipitated from homogenates by incubating 40 µL of supernatant with protein-G-Sepharose beads (Sigma; St. Louis, MO) complexed with anti-AMPKα2 antibody (Upstate Cell Signaling Solutions; Charlottesville, VA) overnight on a roller mixer at 4ºC. The suspension was then washed twice in a buffer containing 50 mM Tris-HCl, 1 M NaCl, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 5 $\mu g \cdot mL^{-1}$ STI (pH 7.4), and then once in a buffer containing 62.5 mM HEPES, 62.5 mM NaCl, 62.5 mM sodium pyrophosphate, 1.25 mM EDTA, 1.25 mM EGTA, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF and 1 $\mu g \cdot mL^{-1}$ STI. Immunoprecipitated AMPKα2 was then suspended in 30 µL of HEPES-Brij buffer containing 25 mM HEPES, 0.02% Brij, 1 mM DTT (pH 7.0). Activity was measured by adding 15 µL of working assay cocktail containing 40 mM HEPES, 8% glycerol, 80 mM NaCl, 0.8 mM EDTA, 0.8 mM $MgCl_2$ (pH 7.0), followed by addition of 0.2 mM AMP, 0.2 mM ATP, 0.8 mM DTT, 0.2 mM SAMS peptide (Zinsser Analytic; Maidenhead, Berkshire, UK) and 10 $mCi \cdot mL^{-1}$ [γ-^{32}P] ATP (MP Biomedicals; Aurora, OH). The mixture was incubated for 10 min in a water bath (30ºC, 60 $r \cdot min^{-1}$ agitation).

After incubation, a 15 μL aliquot was spotted onto a 1 cm^2 P81 Whatman filter paper (Whatman International Ltd; Maidstone, UK) and immediately placed in 200 mL of 1% phosphoric acid to stop the kinase reaction. Each filter paper was washed six times in 1% phosphoric acid for 5 min. After air drying, filter paper was added to 3 mL of Ecolite liquid scintillation fluid (MP Biomedicals; Solon, OH) and counted for 10 min in a LS 6500 multi-purpose scintillation counter (Beckman Coulter; Brea, CA). Activity is expressed as picomoles of phosphate incorporated per milligram of muscle protein subjected to immunoprecipitation per minute ($pmol \cdot mg^{-1} \cdot min^{-1}$). Each sample was run in duplicate and background activity (HEPES-Brij buffer + working assay cocktail) was subtracted.

S6K1 activity assay. The S6K1 activity assay was prepared as the AMPK activity described above with the following changes: Immunoprecipitation was achieved by incubating 40μl of supernatant with protein A-Sepharose beads (Sigma; St. Louis, MO) complexed with antibodies against rabbit anti-p70S6 kinase (C-18) (Santa Cruz; Santa Cruz, CA) for 2 h on a roller mixer at 4°C. The suspension was washed twice in a buffer containing 50 mM Tris·HCl, 50 mM β-glycerophosphate, 10 mM NaF, 2 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 50 mM β-mercaptoethanol (pH 8.0), and washed once in a kinase buffer containing 50 mM MOPS, 25 mM β-glycerophosphate, 2 mM EDTA, 5 mM EGTA, 20 mM $MgCl_2$, 1 mM DTT, 2 mM Na_3VO_4, and 10 mM NaF. Immunoprecipitated p70S6K1 was then suspended in 30μl of HEPES-Brij buffer. S6K1 activity was measured by adding 15μl of the working assay cocktail containing 0.1 mM ATP added to kinase buffer and 0.1 mM S6K1 substrate corresponding to *amino acids 231-239* of human 40S ribosomal protein S6 (sequence RRRLSSLRA, Santa Cruz Biotechnologies; Santa Cruz, CA) and 10 $mCi \cdot mL^{-1}$ [γ-^{32}P]ATP (MP Biomedicals; Aurora, OH). The final steps were performed as described above.

Antibodies. Most primary antibodies used were purchased from Cell Signaling (Beverly, MA): phospho-mTOR (Ser^{2448}; 1:1000), phospho-p70 S6K1 (Thr^{389}; 1:500), phospho-Akt/PKB (Ser^{473}; 1:500), phospho-4E-BP1 ($Thr^{37/46}$), phospho-eIF4G (Ser^{1108}), total-mTOR (1:1000), total-p70 S6K1 (1:500), total-Akt/PKB (1:1000), total-4E-BP1

(1:1000), and total-eIF4G (1:1000). The antibodies for eIF2 were purchased from BioSource (Camarillo, CA): phospho-eIF2α (Ser^{52}; 1:1000) and total eIF2α (1:1000). Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was purchased from Amersham Bioscience (1:2000).

Statistical Analysis. All values are expressed as mean ± SEM. All comparisons between control and paraplegic rats were performed with an independent Student's *t* test using SPSS statistical software v. 14.0. (SPSS; Chicago, Illinois). Significance was predetermined at $P < 0.05$.

RESULTS

Whole body and soleus muscle weight. Whole body weight was not different between Control and Paraplegic rats ($P > 0.05$; Figure 5A). Soleus muscle weight, however, was 28% lower in the Paraplegic rats compared with Control rats (174 ± 8 mg vs. 240 ± 13 mg, respectively; $P < 0.05$, Figure 5B).

Western blot analyses. There was no change in Akt phosphorylation (Ser^{473}); however, there was a significant decrease in total protein content of Akt in Paraplegic versus Control rats ($P < 0.05$, Figure 6A). There were significant decreases in the Paraplegic rats in mTOR phosphorylation (Ser^{2448}) as well as the total protein content of mTOR ($P < 0.05$, Figure 6B). Phosphorylation of eIF4G (Ser^{1108}) was significantly reduced in the Paraplegic rats ($P < 0.05$) with no change in phosphorylation of 4E-BP1 ($Thr^{37/46}$) or total protein content of either eIF4G or 4E-BP1 ($P > 0.05$; Figure 7). Phosphorylation (Thr^{389}) and total protein content of S6K1 were reduced in Paraplegic rats ($P < 0.05$, Figure 8A), mirroring a significant decrease in paraplegic soleus muscle S6K1 activity by 50% (2.3 ± 0.6 vs. 4.5 ± 0.3 $pmol \cdot mg^{-1} \cdot min^{-1}$ respectively; $P < 0.05$, Figure 8B). Phosphorylation (Ser^{52}) and phospho/total protein content of eIF2α were not different between groups ($P > 0.05$, Figure 9). However, total protein content of eIF2α was reduced in the Paraplegic rats ($P < 0.05$, Figure 9).

AMPKα2 activity. Soleus muscle AMPKα2 activity was not different between Control and Paraplegic rats, (3.5 ± 0.4 vs. 3.7 ± 0.5 pmol·mg^{-1}·min^{-1}, respectively; $P > 0.05$, Figure 10).

Figure 5. Rat whole body and soleus wet muscle weight.

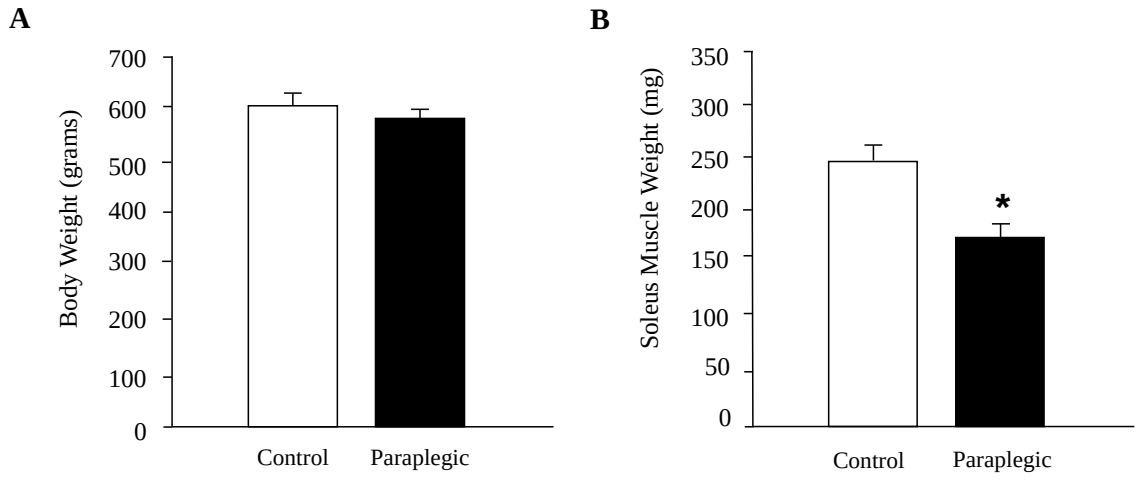

Fig. 5. Body weight is expressed in grams (A) and soleus weight in milligrams (B). Data are expressed as Mean ± SEM, N=8 per group. *Significantly different from Control, $P < 0.05$.

Figure 6. Soleus muscle Akt and mTOR.

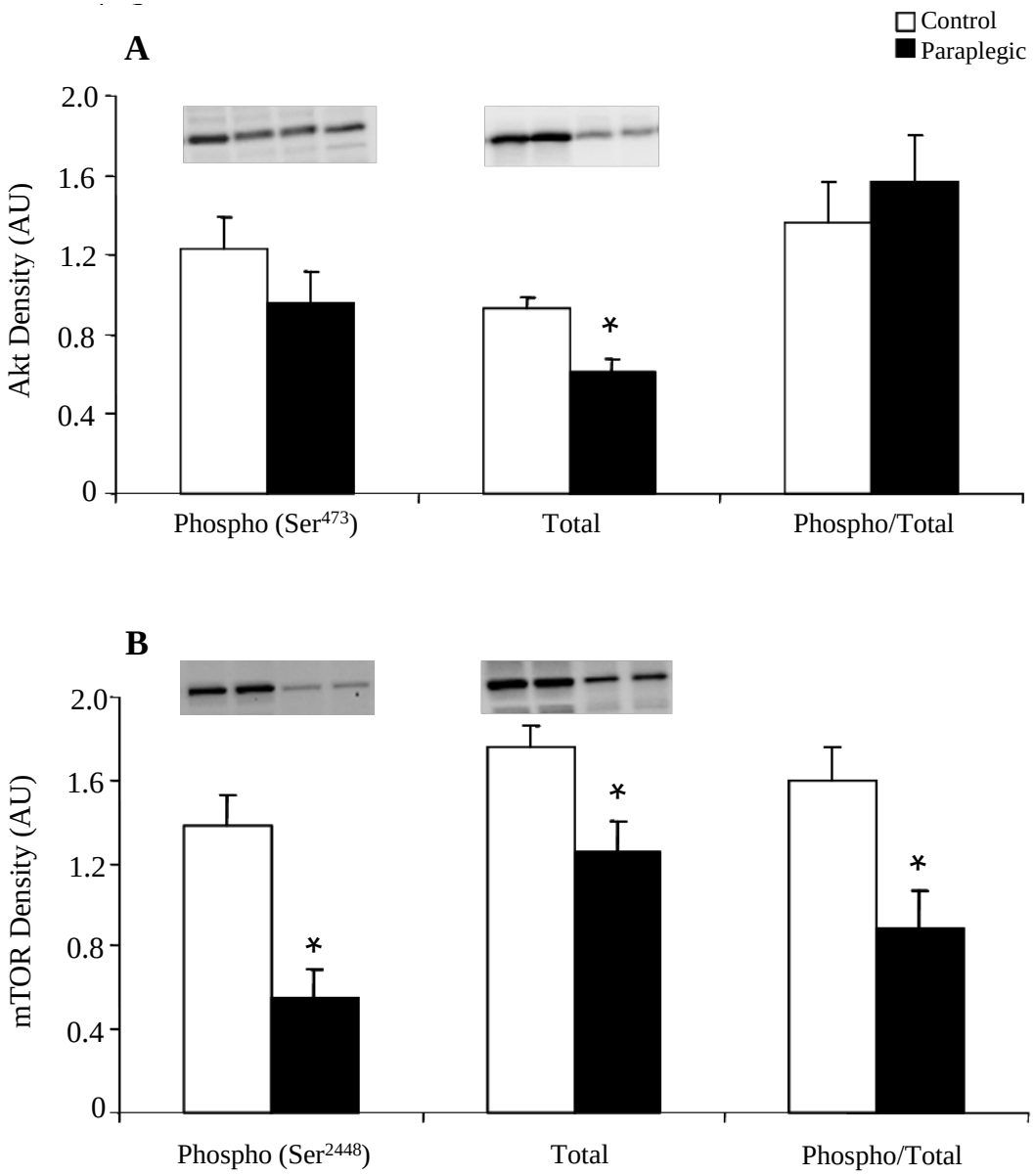

Fig. 6. Soleus muscle Akt phosphorylation (Ser^{473}) and total protein content (A), and mTOR phosphorylation (Ser^{2448}) and total mTOR protein (B). Data are expressed relative to a normalization control and as Mean ± SEM, N = 8 per group. Insert shows representative Western Blot of duplicate samples for Control and Paraplegic rats. AU=arbitrary units. *Significantly different from Control, $P < 0.05$.

Figure 7. Soleus muscle 4E-BP1 and eIF4G.

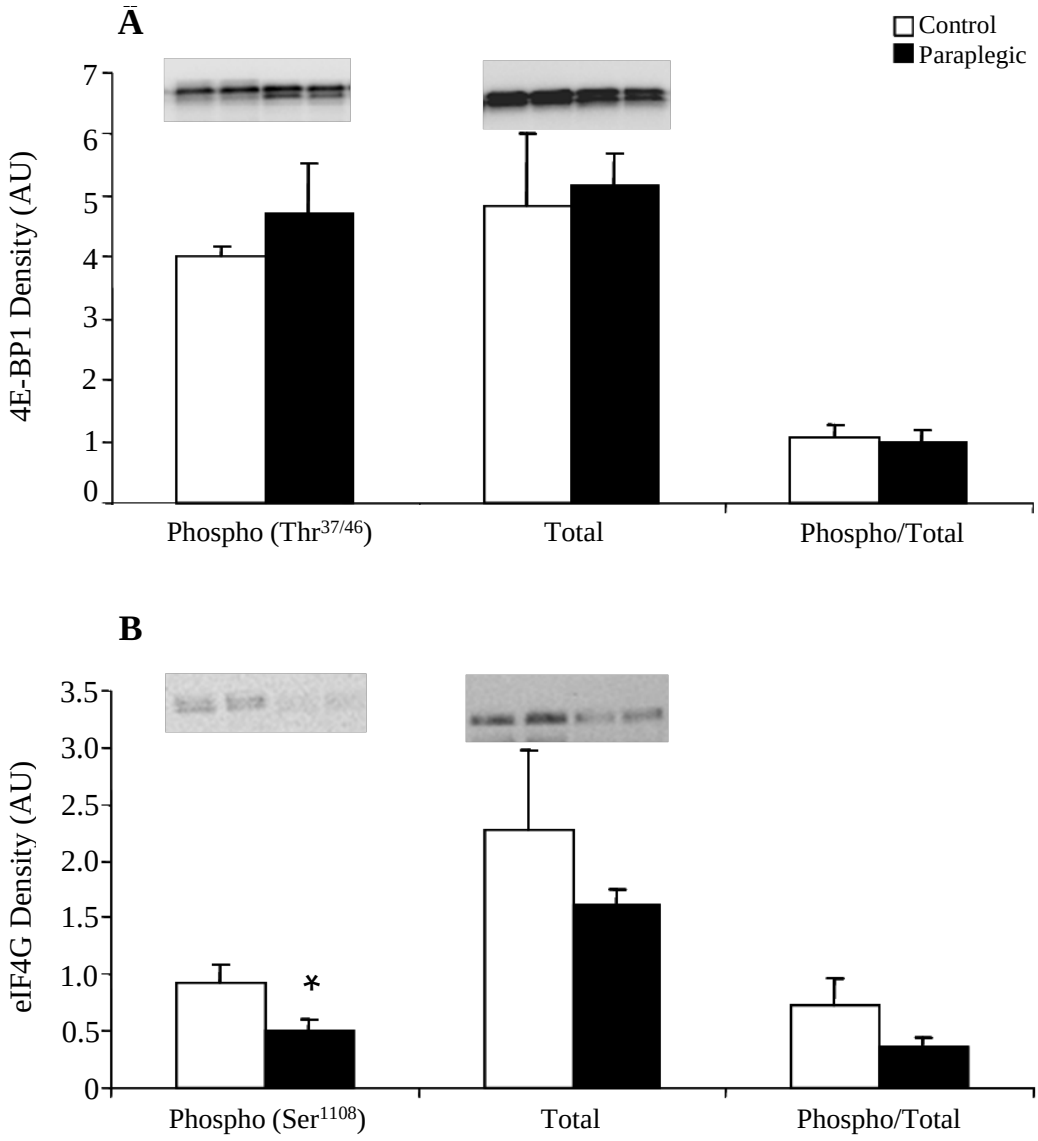

Fig. 7. Soleus muscle 4E-BP1 phosphorylation ($Thr^{37/46}$) and total protein content (A), and eIF4G phosphorylation (Ser^{1108}) and total protein content (B). Data are expressed relative to a normalization control and as Mean ± SEM, N=8 except total and phospho/total eIF4G, N=6. Insert shows representative Western Blot of duplicate samples for Control and Paraplegic rats. AU=arbitrary units. *Significantly different from Control, $P < 0.05$.

Figure 8. Soleus muscle S6K1.

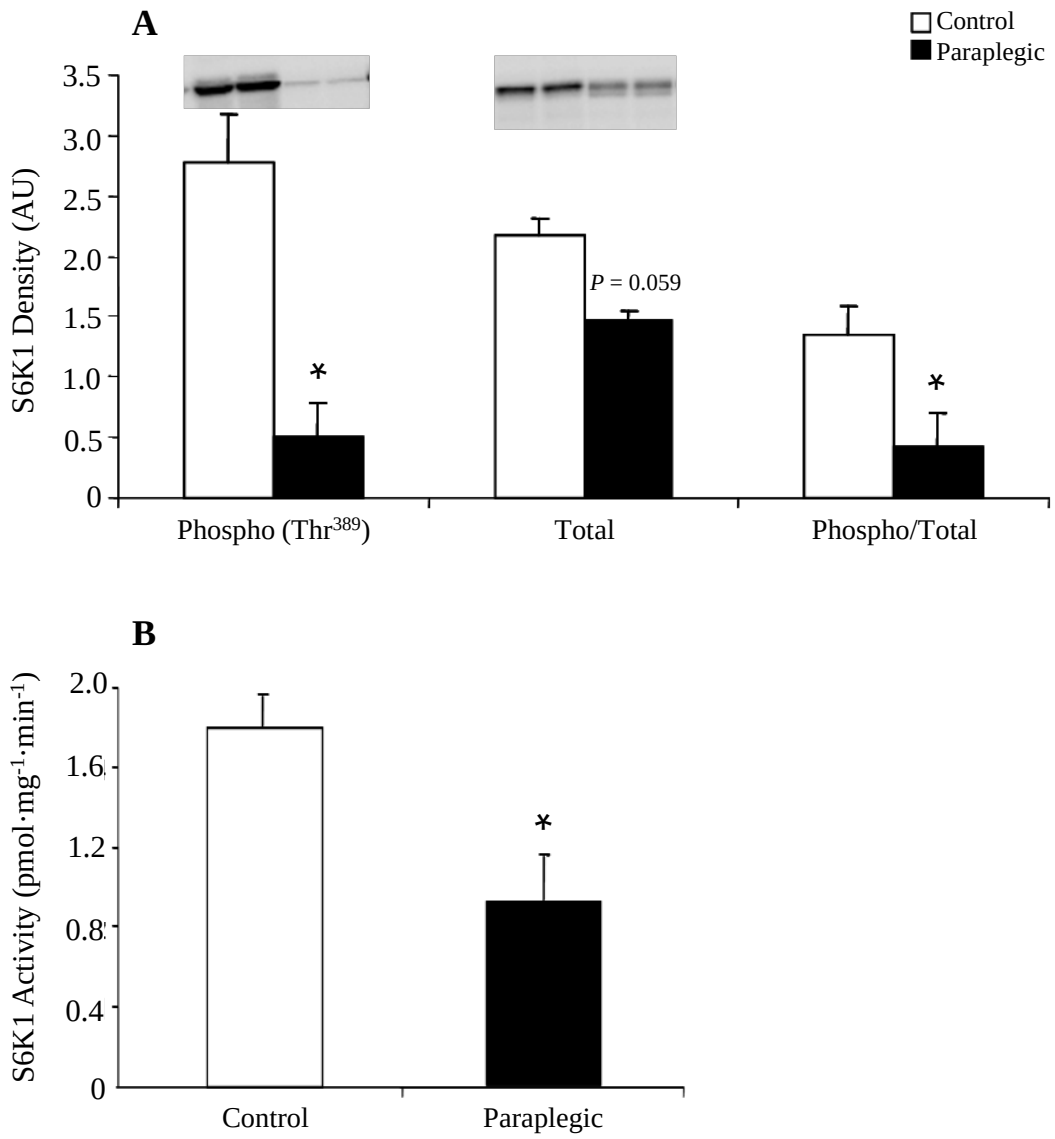

Fig. 8. Soleus muscle S6K1 phosphorylation (Thr^{389}) and total protein content (A). Data are expressed relative to a normalization control and as Mean ± SEM, N=8 per group. Insert shows representative Western Blot of duplicate samples for Control and Paraplegic rats. AU=arbitrary units. Soleus muscle S6K1 activity (B). Data are expressed as Mean ± SEM, N=4 per group. *Significantly different from Control, $P < 0.05$.

Figure 9. Soleus muscle eIF2α.

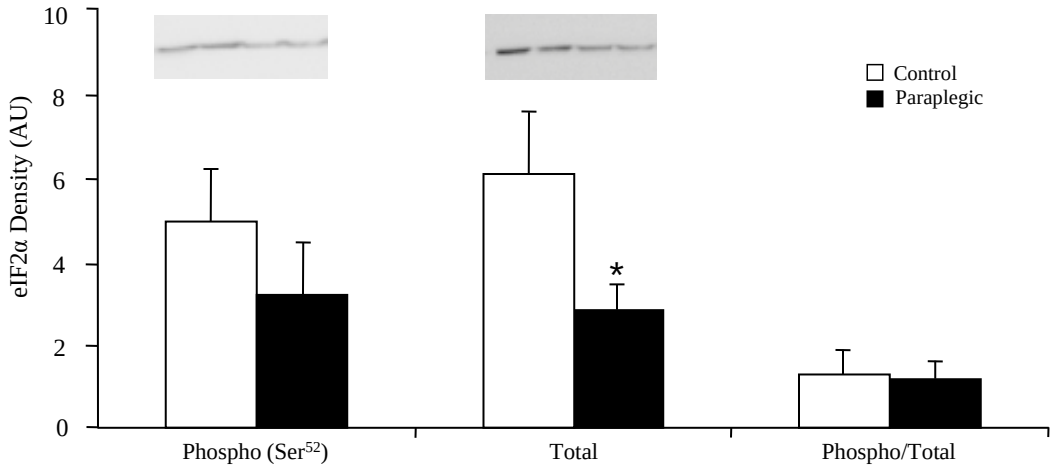

Fig. 9. Soleus muscle eIF2α phosphorylation (Ser^{52}) and total protein content. Data are expressed relative to a normalization control and as Mean ± SEM, N=8 per group. Insert shows representative Western Blot of duplicate samples for Control and Paraplegic rats. AU=arbitrary units. Significantly different from Control, $P < 0.05$.

Figure 10. Soleus muscle AMPKα2 activity.

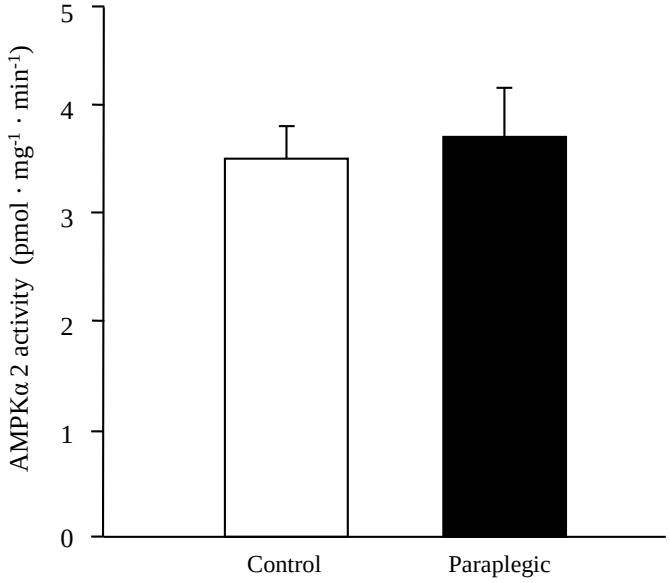

Fig. 10. Soleus muscle AMPKα2 activity. Data are expressed as Mean ± SEM, N=8 per group.

DISCUSSION

The literature concerning the acute effects (up to 15 days) of differing atrophy models on protein synthesis, morphology, gene expression, and certain signaling events is extensive. However, it is important to note that paraplegia, a chronic condition, should be studied as such. The primary finding from this study was that there are numerous proteins in the mTOR/S6K1 signaling pathway (mTOR, S6K1, eIF4G, Akt) that are significantly downregulated following muscle atrophy induced by chronic paraplegia in rats. Furthermore, AMPKα2 activity is unchanged in soleus muscle, suggesting that AMPK is not involved in the downregulation of mTOR signaling in long-term paraplegic muscle. However, S6K1 activity significantly decreased in the paraplegic group, reflecting what was measured in the immunoblots for S6K1 and suggesting that this kinase plays an important role in the regulation of initiation of protein synthesis and cell size. The abrupt reductions in the rate of soleus muscle protein synthesis during hindlimb unweighting are followed, albeit at a slower rate, by an increase in muscle protein breakdown resulting in a reduction of muscle mass (207). However, similar alterations in muscle morphology have also been identified in human paraplegic muscle (56). Muscle protein synthesis is primarily regulated through a complex signaling pathway involving mTORC1 (74) and is controlled by growth factors and nutrients such as insulin and amino acids (129, 226). The activation of mTORC1 enhances mRNA translation initiation and elongation resulting in an increase in muscle protein synthesis and growth (226). Accordingly, mTORC1 represents an important potential regulator of muscle growth and recovery following muscle stress or injury such as that brought on by paraplegia.

The purpose of this study was to determine the effects of 10 weeks of paraplegia on components of the mTOR signaling pathway. Specifically, the "potential" for muscle growth as indicated by the status of mTOR (phosphorylation and total protein content) as well as proteins associated with this important kinase was of interest. Ten weeks of paraplegia resulted in a 28% reduction in soleus muscle wet weight with no change in body weight. Accompanying the loss of soleus muscle mass was a significant reduction

of phosphorylated mTOR, total mTOR and S6K1 compared with sham-operated controls. These findings are novel as acute studies have not attempted to measure mTOR, and report varying results on the status of S6K1 in different atrophy models (26, 93, 105). Reduction in both the phosphorylation of mTOR and the total amount of mTOR and S6K1, along with a decreased activity of S6K1, suggest that the mTOR/S6K1 pathway may be an important regulator of muscle growth in this chronic model. This is supported by studies showing inhibition of mTORC1 by rapamycin blocks downstream targets S6K1 and 4E-BP1, and almost completely inhibits hypertrophy without causing atrophy (26). The combination of these results would suggest that both hypertrophy and atrophy pathways are at work in the chronic state of paraplegia and that the atrophied cells are not able to rebuild muscle, in part, due to downregulation of components of the mTOR/S6K1 pathway. It is also likely that regulators of both hypertrophy and atrophy are downregulated in this chronic paraplegia model, which is significantly different from other growth and atrophy models.

Many other proteins in the mTOR pathway were affected in the Paraplegic group compared with the Control group. Though there were no changes in the phosphorylation of Akt between groups, Paraplegic rats had a significantly reduced amount of total Akt. Initially, a reduction in total protein may be thought to explain the reduced mTOR activation, because Akt is an upstream activator of mTOR. However, in examining the phosphorylation data, it is clear that activation of Akt is similar between groups so this is not a likely explanation. Further downstream of mTOR, there was a decrease in the phosphorylation of eIF4G in Paraplegic rats. EIF4G acts as a scaffold protein for the assembly of eIF4E and eIF4A to form the eIF4F complex that mediates translation initiation (84). Decreased phosphorylation seems to indicate a reduced ability to initiate translation in the Paraplegic rats and could partially explain the atrophy in these rats.

The mTOR signaling pathway is most likely not the sole contributor to the reduction in muscle mass and/or changes in translation initiation. The eukaryotic initiation factor 2 (eIF2) pathway also has a regulatory role for translation initiation and protein synthesis in skeletal muscle. When eIF2α is phosphorylated, eIF2B is inhibited

from producing a complex with GTP and initiator methionyl tRNA. This mechanism prevents the complete assembly of the 28S preinitiation complex and, ultimately, mRNA translation (138). Therefore, the phosphorylation status of eIF2α Ser^{52} provides a marker as to the role of the eIF2 pathway in the control of protein synthesis. In this study, eIF2α total protein content was reduced in paraplegia; however, eIF2α Ser^{52} phosphorylation was unaffected. It appears that eIF2 regulation may not be affected to the same extent as the mTOR pathway following chronic paraplegia.

In addition to acting as a potential negative regulator of mTOR signaling, AMPK activation has been suggested to activate p38 MAPK (241), which has recently been shown to promote GLUT4 translocation (199) and glucose uptake into insulin-resistant denervated muscles (95). AMPK has also been shown to phosphorylate a novel protein called AS 160, which is involved in the upregulation of GLUT4 translocation to the sarcolemma (136, 211). Originally, it was hypothesized that AMPK activity would be elevated following 10 wk of paraplegia, partially explaining the reduced muscle protein synthesis known to occur with muscle atrophy (207) and the reduction in mTOR signaling (30, 46). However, AMPK activity was not different between Paraplegic and sham-operated Control rats. This data does not support a physiological role for AMPK in either the inhibition of mTORC1 signaling and protein synthesis, or glucose uptake following long-term paraplegia. However, the duration of this study (10 wk) may have been too long to have identified changes in AMPK activity. For example, a recent study reported an increase in AMPK activity at 4 and 8 wk of hindlimb suspension in rats (99). The majority of the loss of muscle mass almost certainly occurred sooner, within the first two wk of paraplegia, as previously demonstrated (68). Therefore, the possibility cannot be ruled out that AMPK may have been playing a key role in regulating mTOR signaling, protein synthesis, and glucose uptake in soleus muscle during the initial stages following the spinal cord transection procedure.

In addition to the general downregulation of the mTOR pathway, there was a decrease in S6K1 total protein content following 10 wk of paraplegia ($P = 0.059$). These findings extend upon the findings of Haddad *et al.* (93), who demonstrated that up to 15

days of spinal transection did not have significant effects on markers of cell signaling (4E-BP1, S6K1 and eIF2α), suggesting that in the paraplegic rat, changes in a few of the proteins downstream of mTORC1 may require more time for the differences to emerge. These results are in agreement with others that have shown the acute effects of 14 days of hindlimb suspension resulted in a significant decrease in total S6K1 and accompanied a 25-55% reduction in soleus muscle weight (26). S6K1 protein content and activity is important because it regulates translation of mRNAs that encode proteins necessary for elongation factors and ribosomal subunits (127). Moreover, in a subset of the rats, a significant decrease in the activity of S6K1 was measured (-48%). As with total mTOR content, these findings expand upon the acute affects of muscle atrophy on downstream components of the mTOR signaling pathway to include chronic (10 wk) changes in the paraplegic rat. In any event, 10 wk of paraplegia resulted in a downregulation of the mTOR/S6K1 signaling pathway, which may compromise the ability of the soleus muscle to respond to anabolic stimuli. Future studies are required to determine whether nutritional or contractile interventions in paraplegic muscle can attenuate or reverse the reduction in mTOR signaling.

These findings are in agreement with the acute muscle atrophy models showing a reduction in mTOR signaling (26, 105), expand on the reported alterations known to occur with an acute (14 day) response to muscle unloading, denervation, and spinal cord isolation, and are an initial characterization of a prolonged/chronic adaptation to reduced mechanical loading and/or the elimination of neural input. However, it is important to note that peripheral nerves are intact in the paraplegic rat (the paraplegic rat is not denervated but rather decentralized) and thus the muscle response to denervation and paraplegia may be different. Future studies are warranted to compare the differences between disuse models of muscle atrophy (e.g., hindlimb unloading, denervation, spaceflight, bed rest) and paraplegia (e.g., spinal cord transection/injury). Furthermore, the soleus (a slow twitch muscle) was examined in the present study because of the well-known atrophy this muscle experiences during unweighting atrophy models (2) and the observation that type I muscle fibers atrophy predominantly during the later stages of

spinal cord injury (194). However, it should be acknowledged that both type I and type II skeletal muscle fibers atrophy to a similar extent following paralysis/spinal cord injury (54, 151, 155, 194), and therefore these results should be taken in the context of having only examined a primarily slow-twitch muscle.

In conclusion, 10 wk of paraplegia-induced muscle atrophy (28% reduction in soleus muscle mass) is associated with a downregulation of the mTOR/S6K1 pathway, a key signaling pathway associated with muscle cell growth. Further investigation is necessary to determine the potential for upregulating the mTOR signaling pathway to promote muscle cell growth in paraplegic muscle and other muscle wasting conditions. Therefore, potential interventions such as the use of highly anabolic nutritional supplements/meal (e.g., essential amino acid mixtures) and/or muscle contractions (e.g., electrical stimulation, assistive exercise therapies) can be designed to activate the mTOR/S6K1 pathway, which may be useful for counteracting muscle atrophy in individuals with spinal cord injury.

ACKNOWLEDGEMENTS

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CHAPTER 4

Muscle protein breakdown has a minor role in the protein anabolic response to essential amino acid and carbohydrate intake following resistance exercise

INTRODUCTION

Muscle protein turnover is a continuous cellular process, and protein net balance is dictated by the equilibrium between muscle protein synthesis (MPS) and muscle protein breakdown (MPB). The effects of anabolic stimuli on MPS have been relatively well characterized, and it is established that resistance-type exercise alone, or followed by ingestion of essential amino acids (EAA) with or without carbohydrate (CHO), increases MPS in humans (21, 32, 150, 161, 243). The increase in MPS corresponds to an increase in signal transduction through the mTOR pathway in humans after resistance exercise (63-64) or after the ingestion of EAA + CHO (77). The combination of resistance exercise (RE) followed by ingestion of EAA or EAA + CHO elicits a greater increase in MPS and signaling through the mTOR pathway than to either stimulus alone (63-64, 77, 134, 163). However, additional CHO content added to protein ingested post-RE does not affect MPS (133).

On the other hand, there are multiple processes involved in skeletal muscle proteolysis including the Ub (ubiquitin)-proteasome system (UPS), lysosomal and calcium-activated systems, and caspases. Within the UPS, the Forkhead box (FOX) family of transcription factors (FOXO1, FOXO3a, FOXO4) regulate the expression of E3 Ub ligases MAFbx and MuRF1 (25, 140, 191). FOXO3a is inhibited via phosphorylation by Akt (40), which can be activated by high levels of circulating insulin. AMPK is activated in response to metabolic stress (64, 180, 234), inhibits anabolic processes and stimulates catabolic, energy producing pathways within the cell (97, 234) including MPB via the UPS (137, 169). In the lysosomal system, conversion of cytosolic LC3B-I to the

autophagosomal membrane-associated form, LC3B-II, is tightly correlated with the number of autophagosomes present and is therefore used as a marker of autophagy (114, 125, 131-132). Finally, caspases are a family of cysteine proteases generally activated in response to inflammation and/or cell injury. Caspase-3 is an effector caspase whose activation ultimately leads to increased proteolysis (171).

The effects of exercise and nutrient ingestion on these pathways and MPB have been less well defined, most likely due to the greater methodological difficulty of measuring MPB in vivo. However, MPB is elevated following an acute bout of RE (21, 23, 177), and it has been proposed that CHO ingestion can improve muscle protein anabolism when ingested following RE by reducing MPB (33, 161, 186). Additionally, postprandial concentrations of insulin ($\geq$ 30 μ U·mL^{-1}) can significantly reduce MPB at rest (90) suggesting that CHO intake sufficient to elicit a similar insulin response may also reduce MPB. While CHO and/or insulin release appear to affect MPB, EAA do not significantly alter MPB either alone or in combination with RE (22, 35, 209).

Studies examining the role of post-RE CHO ingestion vary, but indicate lower levels of CHO ingestion (< ~40g) are not sufficient to reduce post-RE whole body protein breakdown (133) or MPB (179), whereas much higher levels (100g CHO) are sufficient (33). Therefore, the purpose of this study was to determine if a combination of low CHO and EAA, or high CHO and EAA could effectively reduce the exercise-induced increase in MPB and improve net muscle protein balance. Specifically, it was of interest whether CHO content sufficient to elicit a low range postprandial insulin response (~30 μU·mL^{-1}, EAA + LCHO) or high range insulin response (~70 μU·mL^{-1}, EAA + HCHO) would inhibit MPB and associated signaling. The hypothesis was that higher circulating insulin concentrations (70 μU·mL^{-1} vs. 30 μU·mL^{-1}) would have a greater effect on inhibition of MPB and catabolic signaling, resulting in an augmented net muscle protein balance.

MATERIALS AND METHODS

Subjects. Thirteen young healthy males who reported that they were not currently engaged in a resistance exercise training program during the screening interview participated in the study. All volunteers were asked to refrain from performing vigorous physical activity for 24 hours prior to participating in the study. All subjects gave informed written consent before participating in the study, which was approved by the Institutional Review Board of the University of Texas Medical Branch (which is in compliance with the Declaration of Helsinki). Screening of subjects was performed with clinical history, physical exam, and laboratory tests including complete blood count with differential, liver and kidney function tests, coagulation profile, fasting blood glucose and oral glucose tolerance test (OGTT), hepatitis B and C screening, HIV test, TSH, lipid profile, urinalysis, drug screening, and ECG.

Study design. Each subject's 1 repetition maximum (1RM) was determined on a leg extension machine (Cybex-VR2, Medway, MA) which was located within the General Clinical Research Center's (GCRC) Exercise Laboratory and used for each study. The 1RM values obtained were used to determine the starting weight (70% of 1RM) for the RE portion of the study. A Dual-energy X-ray absorptiometry (DEXA) scan (Hologic QDR 4500W, Bedford, MA) was performed to measure body composition and lean mass (LM). Each subject was admitted to the Institute for Translational Sciences – Clinical Research Center (ITS-CRC) the day prior to the exercise study. The subjects were all fed a standardized meal (12 kcal·kg^{-1}of body weight; 60% carbohydrate, 20% fat, and 20% protein) prepared by the Bionutrition Division of the ITS-CRC. Each subject was also offered a snack at 2200 h and did not eat again until after the study the following day.

The morning of the study, polyethylene catheters were inserted into a forearm vein for tracer infusion, in the contra-lateral hand vein which was heated for arterialized blood sampling, and in the femoral artery and vein (retrograde placement) of the leg for blood sampling. The femoral lines were placed in the same leg from which muscle

biopsies were obtained. The arterial catheter was also used for the infusion of indocyanine green (ICG, Akorn, Inc., Buffalo Grove, IL) to determine blood flow.

After a background blood sample, a primed continuous infusion of either L-[ring-$^{2}H_5$] or L-[$^{13}C_6$] phenylalanine (Cambridge Isotope Laboratories, Andover, MA) was begun (time = 0) and maintained at a constant rate until the end of the experiment (Figure 11). The priming dose for the labeled phenylalanine was 2 μmol· kg^{-1} and the infusion rate was 0.05 μmol · kg^{-1}· min^{-1}.

Figure 11. Study Design.

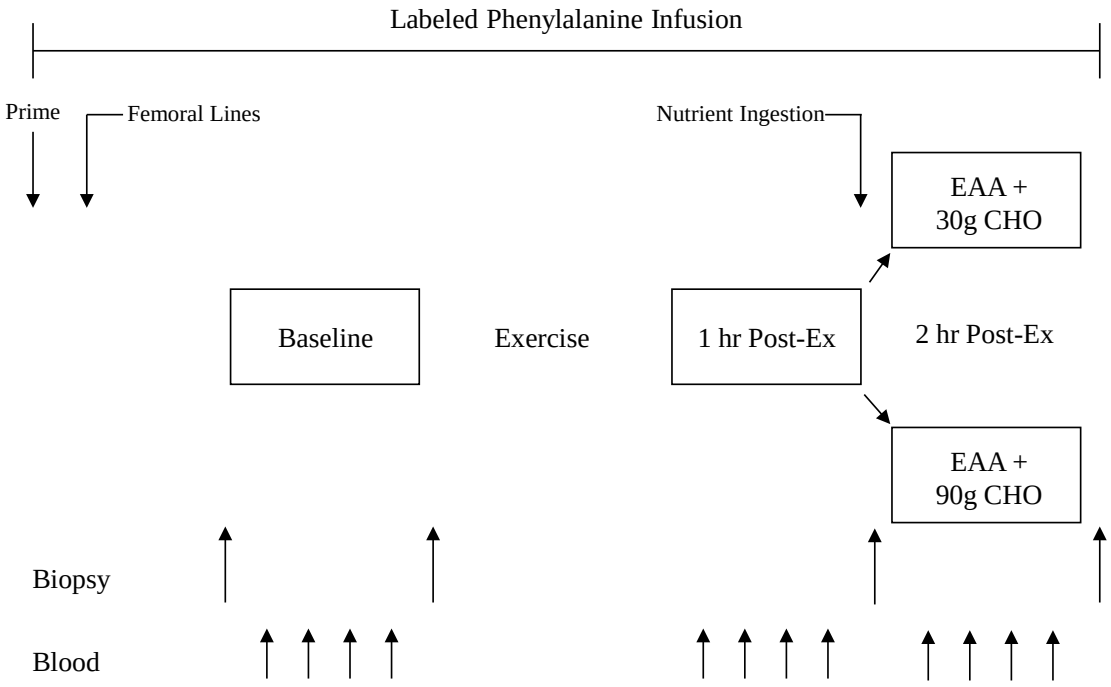

Fig. 11. The study consisted of a Baseline/basal period, a recovery period lasting 1 h (1h Post-Ex) and a nutrition period lasting 1 h (2 h Post-Ex). ICG, indocyanine green, was infused in each period to measure blood flow. Blood samples were collected to measure blood glucose, insulin and phenylalanine concentrations. Muscle biopsies were used to measure muscle protein breakdown, signaling proteins, and gene expression associated with protein breakdown.

Subjects in both groups were studied identically in three periods: a basal period (Baseline) which was the hour prior to exercise, one hour following exercise (1 h Post-Ex), and two hours following exercise (2 h Post-Ex) in which the groups were separated into those receiving either EAA + LCHO or EAA + HCHO. For each period, all subjects rested comfortably in the semi-recumbent position.

Marking the beginning of the basal period (Baseline), and two hours after starting the tracer infusion, the 1st muscle biopsy was obtained from the lateral portion of the vastus lateralis of the leg with the biopsy site between 15 and 25 cm from the mid-patella. The biopsy was performed using a 5 mm Bergström biopsy needle, under sterile procedure and local anesthesia (1% lidocaine). Once harvested the muscle tissue was immediately blotted and frozen in liquid nitrogen and stored at -80˚C until analysis. Immediately after the 1st biopsy, a continuous infusion of ICG was started in the femoral artery (0.5 $mg \cdot min^{-1}$) and maintained for 50 min. Blood samples were drawn 4 times, at 10 min intervals, from the femoral vein and the arterialized hand vein to measure ICG concentration (Fig. 1). In addition to the blood obtained for ICG measurement, blood samples were also taken from the femoral artery and vein and from the arterialized hand vein to measure glucose, phenylalanine and insulin concentrations. At the end of baseline, a 2nd biopsy was obtained; however, the biopsy needle was inclined at a different angle so that the second biopsy was taken approximately 5 cm apart from the first. The method of sequential biopsies from the same incision has been previously used by this lab (63-64, 77) and others (58, 120, 148).

Following the 2nd biopsy, the subjects were seated in a Cybex leg extension machine to perform the exercise protocol. After a brief warm up (23 kg x 10 reps), each subject performed 10 sets of 10 repetitions of bilateral leg extension exercises at 70% of their pre-determined 1RM. Each set was separated by 3 min.

During the period following exercise (1h Post-Ex), ICG was again infused continuously and blood was sampled as previously described. At the end of the first hour post-exercise, a 3rd muscle biopsy was obtained through a new incision site approximately 5 cm proximal to the first incision.

Marking the beginning of the final period (2h Post-Ex), subjects ingested either the EAA + LCHO solution or the EAA + HCHO solution (see below for solution composition). Blood samples were collected in the same manner as during the previous periods. At the end of the period (2h Post-Ex), a final muscle biopsy was collected from the second incision. Each biopsy was taken an average of 69 ± 1 min apart.

Composition of the EAA+CHO Solutions. The composition of the EAA+CHO solutions consisted of ~20g of the essential amino acids in the following proportions: histidine (8%), isoleucine (8%), leucine (35%), lysine (12%), methionine (3%), phenylalanine (14%), threonine (10%), and valine (10%) (Sigma-Aldrich, St. Louis, MO). Lean mass, determined by DEXA, was used to calculate the amount of EAA (0.35 $g \cdot kg\ LM^{-1}$) added to the nutrient solution. Similarly, carbohydrate was added at 0.5 $g \cdot kg\ LM^{-1}$ for the EAA + LCHO group and 1.4 $g \cdot kg\ LM^{-1}$ for the EAA + HCHO group. The EAA+CHO was mixed with a flavored, non-caloric beverage to aid in palatability (63, 77). To minimize the potential of tracer dilution with the addition of the amino acids, the appropriate phenylalanine tracer was added to the oral EAA solution at 6.5% of the total phenylalanine content. Average essential amino acid and carbohydrate content is shown in Table 2.

Blood flow, glucose uptake, blood glucose and insulin. Serum ICG concentration for the determination of leg blood flow was measured spectrophotometrically (Beckman Coulter, Fullerton, CA) at λ=805 nm (113). Plasma glucose concentration was measured using an automated glucose and lactate analyzer (YSI, Yellow Springs, OH). Leg glucose uptake was calculated as follows: leg glucose uptake = (CA – CV)·BF, where CA and CV are the blood glucose concentrations in the femoral artery and vein, respectively, and was expressed as micromoles of glucose utilized per minute per kilogram of fat free mass (FFM) of the leg ($\mu mol \cdot min^{-1} \cdot kg\ leg\ FFM^{-1}$). Plasma insulin concentrations were determined by ELISA (Linco Research, St. Charles, MO, USA).

Mass Spectrometry Analyses. Concentrations and enrichments of blood phenylalanine were determined on its *tert*-butyldimethylsilyl derivatives using L-[^{15}N]phenylalanine as an internal standard and gas chromatography/mass spectrometry

(GCMS; 6890 Plus GC, 5973N MSD/DS, 7683 autosampler; Agilent Technologies, Palo Alto, CA) according to methods published by Wolfe (237). Muscle tissue samples were ground, and intracellular free amino acids and muscle proteins were extracted as described by Wolfe (237). Intracellular free concentrations and enrichments of phenylalanine were determined by GCMS using L-[$^{13}C_6$]phenylalanine or L-[^{15}N]phenylalanine as an internal standard (237).

Calculations. Phenylalanine kinetics were calculated using both the two-pool and three-pool models of leg muscle amino acid kinetics and have been described in detail elsewhere (15, 20, 79). The two- and three-pool model parameters were calculated as follows:

Common parameters

- Delivery to the leg: $F_{in} = BF \cdot C_A$
- Release from the leg: $F_{out} = BF \cdot C_V$
- Net Balance across the leg: $NB = BF \cdot (C_A - C_V)$

Two-Pool

- Whole Body Leg Rate of Appearance: Total $R_a = BF \cdot C_A \cdot E_A / E_V$
- Release from proteolysis (Breakdown): Leg R_a = Total $R_a - F_{in} = BF \cdot C_A \cdot [(E_A / E_V) - 1]$
- Disappearance from blood (Synthesis): Leg R_d = Leg R_a + $NB = BF \cdot [(C_A \cdot E_A / E_V) - C_V)$

Three-pool

- Transport into muscle free pool: $F_{m,a} = \{[C_V \cdot (E_M - E_V) / (E_A - E_M)] + C_A\} \cdot BF$
- Transport from muscle free pool: $F_{v,m} = \{[C_V \cdot (E_M - E_V) / (E_A - E_M)] + C_V\} \cdot BF$
- Release from proteolysis (Breakdown): $F_{m,o} = F_{m,a} \cdot [(E_A \cdot E_M) - 1]$
- Utilization for protein synthesis: $F_{o,m} = F_{m,o} + NB$

C_A, C_V: plasma phenylalanine concentrations in the femoral artery and vein, respectively. E_A, E_V, E_M: free phenylalanine enrichments (tracer/tracee ratio) in femoral artery and vein, and in muscle, respectively. BF: leg blood flow. Data are expressed per 100 mL of leg volume. Use of these models allows for the determination of the rate of utilization of phenylalanine for muscle protein synthesis and appearance from breakdown, because phenylalanine is neither oxidized nor synthesized in muscle. Hourly averages for blood flow, blood and muscle phenylalanine concentrations, and enrichments were calculated from individual samples drawn within each hour.

Model assumptions. The calculation of intracellular phenylalanine utilization (protein synthesis) and appearance (protein breakdown) assumes that an isotopic steady state exists and there is no de novo tracee production or oxidation in the leg. Net plasma balance and the muscle biopsy data assume that the muscle accounts for the leg metabolism of amino acids. It is assumed that the tissue enrichment and amino acid concentrations are representative of the intracellular space, the intracellular free amino acid pool is homogenous and the free amino acid pool is the precursor for protein synthesis. Fujita *et al.* discusses general assumptions and assumptions specific to each model (79), and a more detailed account of these assumptions can be found elsewhere (18, 24).

SDS-PAGE and Immunoblotting. Details to the immunoblotting procedures have been previously described in Chapter 2 Materials and Methods. Total protein content for each phospho-protein was determined for each blot and did not change over the course of the experiment from baseline. Data are presented as phosphorylation status relative to a normalization control and as fold change from Baseline. Data for MuRF-1, MAFbx, LC3BI, and LC3BII are presented as total protein expression relative to the normalization control and as fold change from Baseline.

Antibodies. Primary antibodies were purchased from Cell Signaling (Beverly, MA): phospho-Akt (Ser^{473}; 1:1000), phospho-AMPKα (Thr^{172}; 1:500), total LC3B (1:500), phospho-FOXO3a (Ser^{253}; 1:250 and $Ser^{318/321}$; 1:1000), and Santa Cruz Biotechnology (Santa Cruz, CA): total MuRF1 (1:1000), and total MAFbx (1:1000).

Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was purchased from Amersham Bioscience (1:2000).

RNA Extraction. Total RNA was isolated by homogenizing 30-40 mg tissue with a homogenizing dispenser (T10 Basic Ultra Turrax, IKA; Wilmington, NC) in a solution containing 1.0 mL of Tri reagent. The RNA was separated into an aqueous phase using 0.2 mL of chloroform and precipitated from the aqueous phase using 0.50 mL of isopropanol. Extracted RNA was washed with 1 mL of 75% ethanol, dried, and then suspended in a known amount (1.5 $\mu l \cdot mg^{-1}$ tissue) of nuclease-free water. RNA concentration and integrity were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA). The 28S/18S ratio was 1.28 ± 0.02 and the RNA integrity number (RIN) was 8.27 ± 0.05 (1-10 scale: 10 best). RNA was DNase-treated using a commercially available kit (DNA-free, Ambion; Austin, TX).

cDNA Synthesis. One microgram of total RNA was reverse transcribed into cDNA according to the manufacturers' directions (iScript, BioRad; Hercules, CA). Briefly, a 20 µl reaction mixture consisting of 1 µg of total RNA, 4 µl of 5x iScript Reaction Mix, 1 µl of iScript Reverse Transcriptase, and a known amount of nuclease-free water and placed into the thermocycler (IQ5 Real-Time PCR cycler, BioRad; Hercules, CA) with the following temperature/time protocol: 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. All isolated RNA and cDNA samples were stored at -80°C until further analysis.

PCR Primers. Primer pairs were customized using Beacon Designer 5.0 software (Premier Biosoft Int.; Palo Alto, CA) in which they were designed to avoid homology (BLAST analysis) and secondary structures. Primers were purchased from Invitrogen (Carlsbad, CA). Primers were considered optimal if they produced: 1) primer efficiencies between ~90-100% and 2) a single DNA product of predicted size as identified with a melt analysis and DNA agarose gel. The custom designed DNA sequences and accession numbers are the following: MURF1 (NM_032588) Fwd: TGGACTTCTTTACTTTGGA TTTAG, Rev: CTTCCTCTTCCTGATCTTCTTC; MAFbx (NM_148177) Fwd: ACAGG CATCAAGCTCATTCG, Rev: ACAAAGGCACAGAAGTTACTCC; CASP3

(NM_004346) Fwd: TGTGAGAGAAAGTGTGAGC, Rev: GCATAGGGAAAAATCA GAAG.

Semi-quantitative Real-Time PCR. Determination of relative mRNA expression was performed by real-time PCR using the iQ5 Multicolor Real-Time PCR cycler (Bio-Rad; Hercules, CA). cDNA was analyzed using SYBR Green fluorescence (iQ SYBR Green Supermix, BioRad; Hercules, CA). Each reaction contained Sybr green, a mixture of forward and reverse primers, cDNA template and a known amount of sterile water. The total volume of the reaction tube was 25 μl. All samples were run in duplicate. An initial cycle for 5 min at 95ºC was used to denature the cDNA. This was followed with 40 PCR cycles consisting of denaturation at 95°C for 20s, and primer annealing and extension at 55°C for 30s. After all PCR runs, a melt analysis followed. The mean cycle threshold (Ct) from each sample was normalized to the internal control, GAPDH, after which relative fold changes were determined as described by Livak and Schmittgen (145). There were no differences across time for GAPDH.

Statistical Analysis. All values are expressed as mean ± SEM. Comparisons were performed using two-way analysis of variance with repeated measures (ANOVA), the effects being group (EAA + LCHO or EAA + HCHO) and time (Baseline, 1h Post-Ex and 2h Post-Ex periods). Post-hoc testing was performed using the Bonferroni's *t* test for multiple comparisons versus Baseline. If a test of normality and/or equal variance failed, simple transformations were performed. Significance was set at $P \leq 0.05$.

RESULTS

The subjects' physical characteristics are summarized in Table 2.

Table 2. Subject Characteristics.

	EAA + LCHO	**EAA + HCHO**
N	7	6
Age, yr	30 ± 2	32 ± 1
Height, cm	176 ± 2	179 ± 3
Weight, kg	79 ± 3	85 ± 5
Body Mass Index, $kg \cdot (m^2)^{-1}$	26 ± 1	27 ± 1
Lean body mass, kg	61 ± 2	65 ± 3
Body fat, %	19 ± 2	19 ± 2
Leg lean mass, kg	11 ± 0.4	11 ± 0.5
EAA content in solution, g	22 ± 1	23 ± 1
CHO content in solution, g	31 ± 1	91 ± 4
Total kcal consumed	210 ± 7	454 ± 20

Values are expressed as Mean ± SEM.

Blood flow, glucose and insulin. Blood flow increased during exercise (data not shown), but returned to baseline values during the first hour of post-exercise recovery (Table 3). No differences were observed between groups during the second hour post-exercise following the nutrient ingestion ($P > 0.05$, Table 3). Blood glucose concentrations were similar at Baseline and 1 h Post-Ex (5.3 ± 0.1 vs. 5.2 ± 0.1 $mmol \cdot L^{-1}$, $P > 0.05$). Glucose concentration was elevated at 2 h Post-Ex in both groups and significantly higher in EAA + HCHO (6.2 ± 0.3* (LCHO) vs. 7.8 ± 0.3*† $mmol \cdot L^{-1}$ (HCHO); *$P < 0.05$ vs. Baseline, †$P < 0.05$ vs. LCHO). Insulin levels were similar at Baseline and 1 h Post-Ex and increased significantly in both groups following nutrient ingestion, but to a much larger extent in the EAA + HCHO group (Figure 12A).

Plasma and intracellular phenylalanine concentrations. Arterial phenylalanine concentrations were not altered at 1 h Post-Ex ($P > 0.05$) but were significantly elevated to a similar extent in both groups 2 h Post-Ex ($P < 0.05$, Table 3). Muscle intracellular phenylalanine concentrations remained constant from Baseline to 1 h Post-Ex ($P > 0.05$), but increased similarly following nutrient ingestion at 2 h Post-Ex in both groups ($P < 0.05$, Table 3).

Leg phenylalanine kinetics. Leg phenylalanine kinetics are expressed per 100 mL leg volume and are reported in Table 3. There were no significant changes in any parameter at 1 h Post-Ex ($P > 0.05$). Common parameters of both kinetic models: Net balance across the leg was significantly increased in both groups following ingestion of either drink ($P < 0.05$). Phenylalanine delivery to and release from the leg increased to a similar extent following nutrient ingestion in both groups ($P < 0.05$). 2-pool model: Rate of disappearance of phenylalanine (R_d), which is indicative of protein synthesis, increased at 2 h Post-Ex in both groups ($P < 0.05$). Rate of appearance of phenylalanine (R_a), leg protein breakdown, tended to decrease in both groups similarly at 2 h Post-Ex (Figure 12D, $P < 0.1$). 3-pool model: Three pool model calculations are consistent with the results from the 2-pool model with a large and significant increase in protein synthesis ($F_{o,m}$) 2 h Post-Ex in both groups ($P < 0.05$). Release from proteolysis, or breakdown ($F_{m,o}$), tended to decrease in both groups (EAA + LCHO, $P = 0.15$; EAA + HCHO, $P = 0.12$). No significant changes were detected between groups for any kinetic parameter.

Table 3. Phenylalanine blood concentrations and kinetics across the leg.

	Baseline	1 h Post-Ex	2 h Post-Ex	
			EAA + LCHO	**EAA + HCHO**
Blood Flow, mL·min^{-1}·100mL leg^{-1}	3.4 ± 0.5	5.3 ± 0.8	4.0 ± 0.8	4.0 ± 0.7
Arterial Phe, μmol·L^{-1}	55 ± 2	54 ± 2	146 ± 13*	160 ± 4*
Intracellular Phe, μmol·L^{-1}	93 ± 17	78 ± 11	185 ± 9*	142 ± 13*
NB – net balance across the leg	-12 ± 3	-14 ± 5	107 ± 55*	100 ± 19*
F_{in} – delivery to the leg	189 ± 28	289 ± 47	561 ± 122*	646 ± 118*
F_{out} – release from the leg	201 ± 29	304 ± 49	454 ± 113*	547 ± 108*
R_d – rate of disappearance (synthesis)	64 ± 13	65 ± 11	157 ± 61*	154 ± 28*
$F_{m,o}$ – release from proteolysis	88 ± 17	83 ± 12	51 ± 12[a]	55 ± 16[b]
$F_{o,m}$ – utilization for protein synthesis	76 ± 16	68 ± 12	158 ± 61*	154 ± 30*

Data for Baseline are an hourly average of basal blood draws in the absence of nutrients and prior to performing exercise. Data for 1 h Post-Ex are an hourly average of blood draws in the absence of nutrients and during the first h following exercise. EAA + LCHO and EAA + HCHO values are an hourly average of blood draws during the second h post-exercise and 1 h following ingestion of either the EAA + LCHO or EAA + HCHO nutrient solution, respectively. Values for NB, F_{in}, F_{out}, R_d, $F_{m,o}$, and $F_{o,m}$, are expressed in nmol·min^{-1}·(100 mL leg volume)$^{-1}$. All data are mean ± SEM. * $P < 0.05$ vs. Baseline; [a] $P = 0.15$, [b] $P = 0.12$.

Cellular Signaling. Phosphorylation of Akt (Ser^{473}) significantly increased following exercise and remained elevated following nutrient ingestion in both groups ($P < 0.05$, Figure 12B*).* Phosphorylation of AMPKα at Thr^{172} also increased in both groups 1 h Post-Ex ($P < 0.05$) and remained significantly elevated in the EAA + LCHO group at 2 h Post-Ex ($P < 0.05$) while returning to basal values in the EAA + HCHO group ($P > 0.05$ vs. Baseline, $P < 0.05$ vs. EAA + LCHO; Figure 12C). Phosphorylation of FOXO3a (Ser^{253} and Ser$^{318/321}$) did not change significantly over time and was not different between groups ($P > 0.05$, data not shown). Total MuRF1 was significantly elevated at 1

h Post-Ex ($P < 0.05$) and there was a significant time effect at 2 h Post-Ex, however groups were not significant individually (Figure 13B). There were no changes in total MAFbx protein content (Figure 13D). LC3B-I (16kD) did not change significantly over time, however, LC3B-II (14kD) was decreased similarly in both groups following drink ingestion ($P < 0.05$ vs. Baseline, Figure 14).

mRNA Expression. MURF1 mRNA expression was significantly elevated at 1 h Post-Ex and remained similarly elevated in both groups following nutrient ingestion ($P < 0.05$, Figure 13A). MAFbx (Figure 13C) and CASP3 mRNA levels were unchanged across time and between groups ($P > 0.05$, CASP3 data not shown).

DISCUSSION

This study was designed to determine whether differing levels of CHO, combined with EAA, could effectively reduce the exercise-induced increase in MPB and improve net muscle protein balance. Specifically of interest was determining whether a postprandial insulin response (~30 $\mu U \cdot mL^{-1}$) was sufficient, or a higher physiological response (~70 $\mu U \cdot mL^{-1}$) was required to reduce MPB and associated signaling. The primary and novel finding from this study was that low and high levels of CHO added to an EAA solution in the post-RE recovery period, altered MPB, associated signaling and gene expression similarly. Concurrently with significant increases in circulating insulin levels and insulin signaling through Akt, measures of MPB were modestly improved following nutrient ingestion. These findings are consistent with other reports that indicate post-exercise MPS is not enhanced when large amounts of CHO (> 40g) are added to a protein solution (133), however, the data do show that a EAA + CHO nutrient solution is capable of attenuating and/or reducing MPB when ingested following RE regardless of CHO dose (LCHO or HCHO).

The current study was intended to elicit a maximal increase in MPS, in order to

Figure 12. Insulin, Akt and Protein Breakdown.

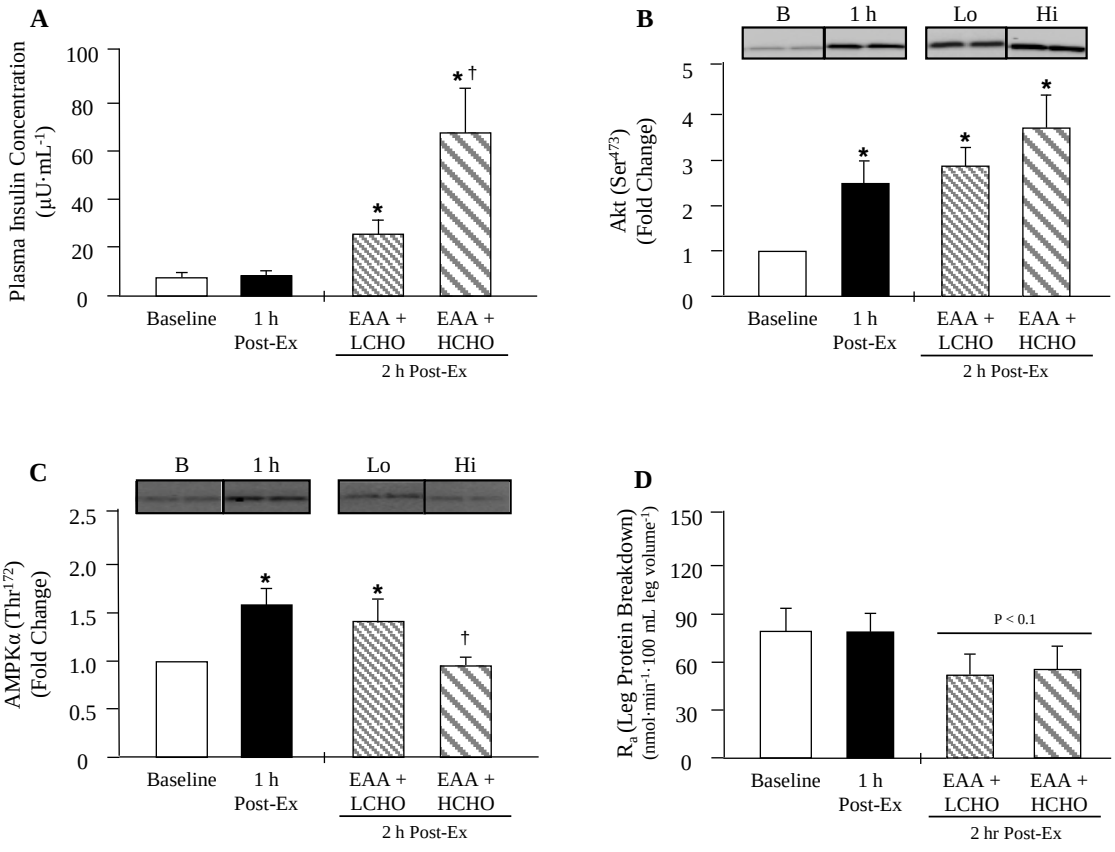

Fig. 12. Plasma insulin concentration (A), phosphorylation of Akt (B), phosphorylation of AMPKα (C), and leg protein breakdown represented as rate of appearance of phenylalanine across the leg (R_a)(D). Data for B and C are expressed relative to a normalization control and as Fold Change from Baseline ± SEM, N=6 per group (except N=5 EAA + LCHO AMPKα). Insert shows representative Western blot in duplicate for Baseline (B), 1 h Post-Ex (1h) and EAA + LCHO (Lo) and EAA + HCHO (Hi) groups. Line across groups in (D) indicates a time effect $P < 0.1$ at 2 h Post-Ex. *Significantly different from Baseline ($P < 0.05$), †Significant group difference ($P < 0.05$).

Figure 13. Breakdown mRNA and protein expression.

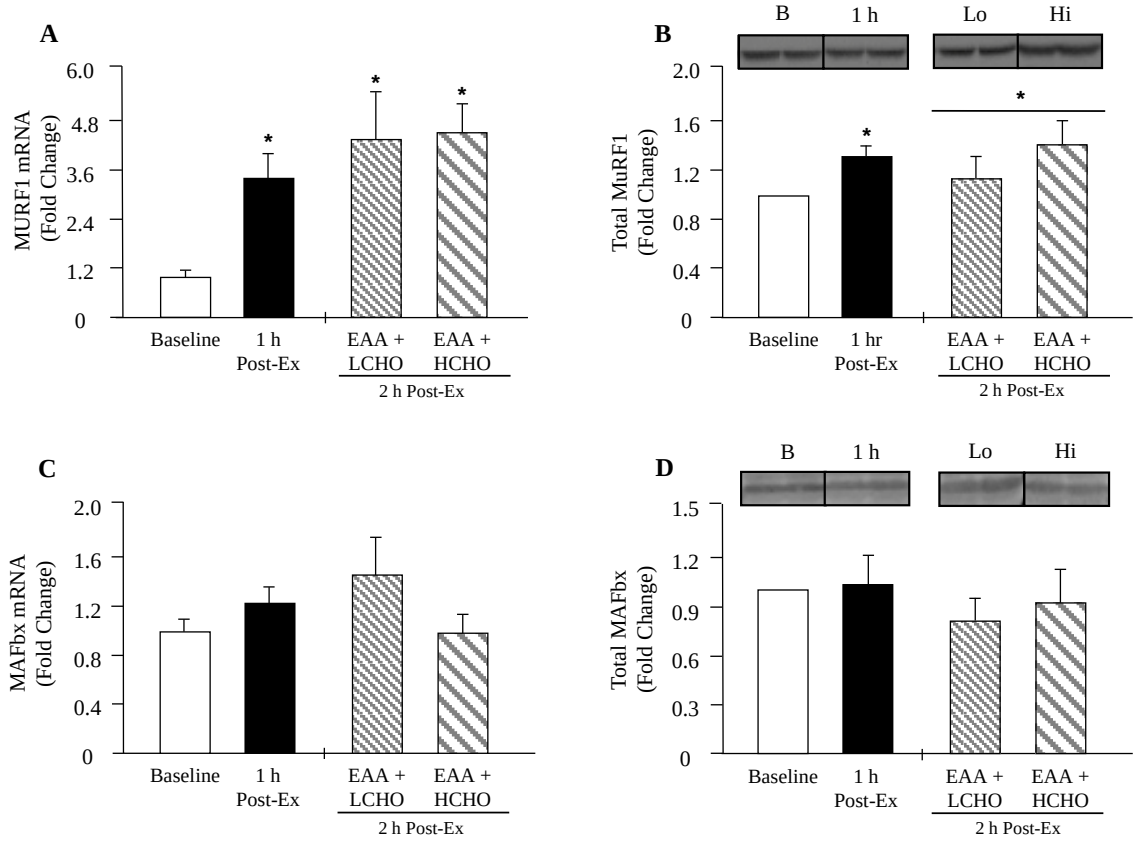

Fig. 13. MURF1 (A) and MAFbx (C) mRNA expression. Data are expressed as fold change after normalization to GAPDH as mean ± SEM, N=6 per group. Total protein content of MuRF1 (B), MAFbx (D). Data are expressed relative to a normalization control and as Fold Change from Baseline ± SEM, N=6 per group (except N=5 for EAA + HCHO MAFbx). Insert shows representative Western blot in duplicate for Baseline (B), 1 h Post-Ex (1h) and EAA + LCHO (Lo) and EAA + HCHO (Hi) groups. Line across groups in (A) indicates a significant time effect, $P < 0.05$ at 2 h Post-Ex. *Significantly different from Baseline ($P < 0.05$).

Figure 14. Autophagy Signaling.

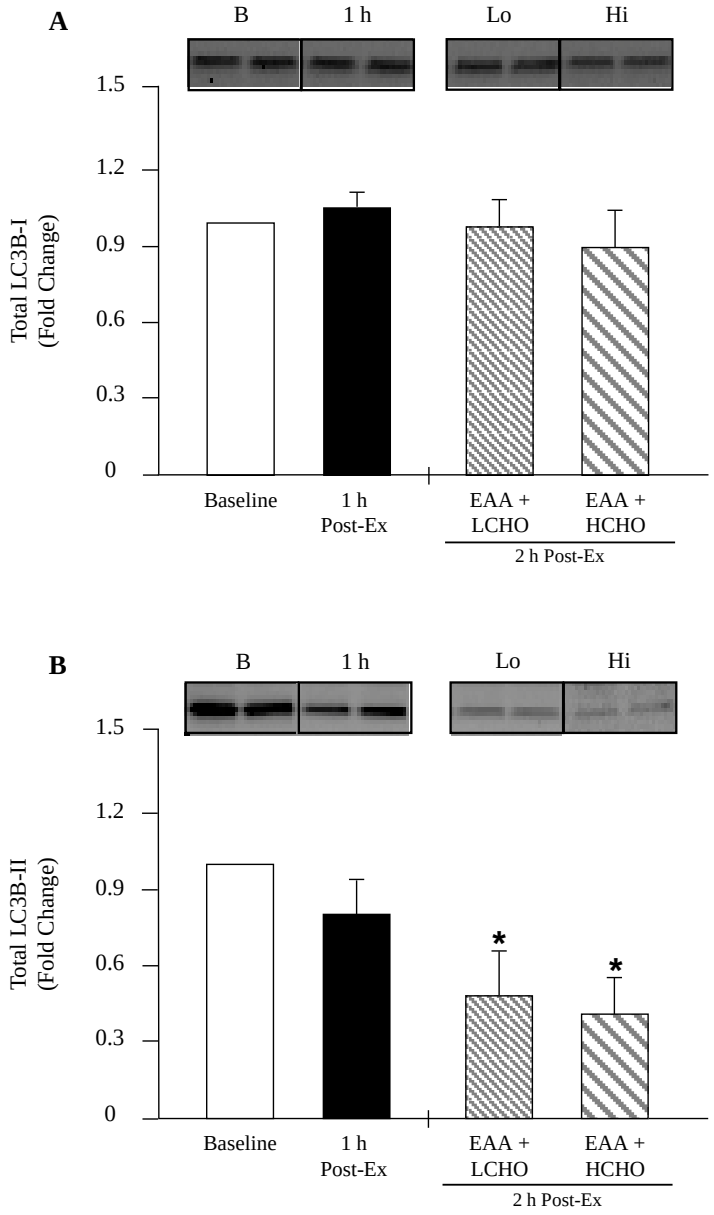

Fig. 14. Total protein content of LC3B-I (A) and LC3B-II (B). Data are expressed relative to a normalization control and as Fold Change from Baseline ± SEM, N=6 per group. Insert shows representative Western blot in duplicate for Baseline (B), 1 h Post-Ex (1h) and EAA + LCHO (Lo) and EAA + HCHO (Hi) groups. *Significantly different from Baseline ($P < 0.05$).

exclude even minor changes in synthesis between the groups as a confounding variable. Still, contrary to the hypothesis regarding breakdown, leg phenylalanine kinetics showed no significant differences between groups. Leg protein breakdown (LPB), represented as phenylalanine rate of appearance (R_a), decreased similarly in both groups though neither reached significance compared to basal values (time effect at 2 h Post-Ex, $P < 0.1$). Another measure of LPB, $F_{m,o}$, also declined following nutrient ingestion (time effect at 2 h Post-Ex, $P = 0.03$; EAA + LCHO, $P=0.15$; EAA + HCHO, $P = 0.12$). Based on previous findings by Biolo *et al*. which indicate a 50% increase in MPB over 3 h of post-RE recovery (21), these results, in which measures of MPB fall below basal levels following nutrient ingestion, suggest that the nutrients were able to alleviate the exercise-induced increase in breakdown. Nevertheless, MPB appears to be playing a minor role in the overall muscle protein anabolic response that occurs post-exercise following EAA + CHO ingestion, accounting for only about 30% of the improvement in Net Balance. It remains to be determined whether the addition of CHO to an EAA or protein solution ingested following resistance exercise (with the subsequent minor changes in MPB) is capable of enhancing protein accretion (as compared to EAA supplementation alone) during resistance exercise training.

Similar to previous findings (64), this study found an increase in AMPKα phosphorylation 1 h following exercise. AMPKα phosphorylation returned to baseline levels only when larger amounts of CHO were ingested (EAA + HCHO), implying the additional energy available in the HCHO solution was able to suppress the exercise-induced activation of AMPK. AMPK activation can stimulate autophagy (144, 153, 158, 224), however, there were not changes in the autophagy marker LC3B-II (132) at 1 h post-ex. This could be due to the timing of sample collection, such that the peak processing of LC3B-I to LC3B-II was missed. Nonetheless, LC3B-II was reduced similarly and significantly in both groups following nutrient ingestion. Insulin signaling through class I PI3K to Akt, and signal transduction through the mTOR pathway, are potent inhibitors of autophagy (112, 157, 224). The large increases in insulin and resulting Akt phosphorylation, as well as previous evidence from this lab showing large

increases in mTOR signaling following EAA+CHO ingestion post-ex (63), provide a potential mechanistic explanation for the observed reduction of LC3B-II. While the UPS is believed to be the main pathway for bulk protein degradation in skeletal muscle, these findings raise the possibility that the lysosomal system may begin to play more of a role in the regulation of MPB in response to nutrients.

Of the potential UPS markers examined (p-FOXO3a Ser^{253}, $Ser^{318/321}$; MAFbx and MuRF1 protein and mRNA expression), only MuRF1 showed any significant alterations. MuRF1 total protein and MURF1 mRNA expression levels were increased following exercise and remained elevated following ingestion of the nutrients. Similarly, the lack of changes to CASP3 mRNA expression connote there is little contribution from protease-dependent breakdown. These findings provide further evidence to support the hypothesis that the lysosomal system may play the most significant role, of the proteolytic pathways, in the regulation of MPB following nutrient ingestion. In fact, Mordier *et al.* has shown autophagy is an essential cellular response involved in increasing protein breakdown in muscle following food deprivation (164), which would support the role of the lysosomal system in regulation of the response to nutrients. However, this is mere conjecture at this point and future studies are required to determine whether the inhibition of autophagy following feeding is responsible for the minor reduction in the rate of MPB following exercise. In any event, collectively there were only small changes in the breakdown markers examined. This is in agreement with the relatively small variation observed in phenylalanine kinetic measures of MPB, and supports the idea that breakdown is playing a minor role in the overall protein anabolic response to EAA+CHO ingestion following RE.

There a few limitations with the current experiment. For example, a fasting exercise group was not included. This was because an acute bout of RE (without nutrients) has been previously shown to increase the rate of MPB by ~50% (21). A group that only ingested EAA following exercise was not included because only a high CHO ingestion following resistance exercise is capable of inhibiting MPB (33) and EAA ingestion (without CHO) results in minor increases in circulating insulin concentrations

and does not alter the rate of MPB (22, 35, 209). In addition, only a few of the most well known markers of proteolysis were examined and the signaling data is rather correlative in nature. However, since relatively little is known about how MPB is regulated in human skeletal muscle, this approach of combining tracer techniques (to assess breakdown rates) with the assessment of proteolytic markers does provide novel information about potential regulatory processes governing muscle protein breakdown in human skeletal muscle.

In summary, the ingestion of an anabolic nutrient solution (EAA + CHO) containing a higher amount of CHO (i.e., 90 vs. 30 g) decreased phosphorylation of AMPKα, but this did not further decrease MPB as similar decreases were observed in both groups. In addition, the E3 Ub-ligase MURF1 was significantly increased (both protein and mRNA expression) following exercise and remained elevated following drink ingestion, while the autophagy marker LC3B-II was not altered by exercise but decreased following nutrient ingestion in both groups. The observed improvement in MPB may be regulated largely by the lysosomal system, though additional studies are required to confirm the contribution of each proteolytic system in response to exercise and nutrition. In conclusion, the enhanced muscle protein anabolic response detected when EAA+CHO are ingested following resistance exercise is primarily due to enhanced protein synthesis with relatively minor changes in protein breakdown regardless of the level of CHO ingestion. Therefore, in the design of nutrient solutions to promote improved muscle recovery and growth following resistance exercise, it is not necessary to include large quantities of CHO since a nutrient solution containing a sufficient amount of EAA with modest amounts of carbohydrate is capable of producing a maximal protein anabolic response. However, future studies are needed to determine if the insulin release and reduction in muscle protein breakdown resulting from modest carbohydrate intake can generate significant improvements in net muscle protein accretion over time.

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CHAPTER 5

Higher leucine concentration in an essential amino acid solution decreases muscle protein breakdown but does not enhance the protein anabolic response in human skeletal muscle

INTRODUCTION

It is well established that essential amino acids (EAA) stimulate skeletal muscle protein synthesis (MPS) in animal and human models (82, 197-198, 222). Interestingly, the amino acid leucine stimulates MPS independently of all other amino acids in animal models, and is a potent stimulator of the mTOR pathway (5-6, 69, 81, 197). Additionally, leucine decreases muscle protein breakdown (MPB) and breakdown-associated cellular signaling and mRNA expression (164, 166-168). These unique qualities have made leucine a target of interest in recent years as a potential intervention to promote MPS and decrease MPB, thereby facilitating the maintenance of skeletal muscle mass. However, the mechanisms involved in leucine regulation of muscle protein metabolism are largely unknown.

Increases in MPS following mixed EAA or leucine ingestion are associated with enhanced translation initiation via activation of mTORC1 and downstream targets S6K1 and 4E-PB1 (6, 57, 77). It has recently been discovered that amino acids themselves can serve as nutrient signals to activate mTOR signaling, and novel proteins hVps34 (human vacuolar protein sorting-34), MAP4K3 (mitogen activated protein kinase kinase kinase kinase-3), and Rag GTPases (guanosine triphosphatase) have been identified as potential signal mediators of amino acids to mTORC1 (43, 73, 190). Upstream of mTORC1, Akt can directly activate mTORC1 through phosphorylation (170) or indirectly by inhibiting TSC2 (108, 152), while further downstream, S6K1 signals to eEF2 to enhance translation elongation (38). The mTOR pathway is a major contributor to the anabolic response following EAA or leucine ingestion, though multiple pathways are involved.

Similarly, regulation of MPB is a complex process controlled by multiple systems including the UPS, lysosomal degradation (autophagy), calpains and caspases. Although mixed amino acids or EAA do not reduce MPB in humans (22, 90), evidence indicates infusion of branched-chain amino acids or leucine alone can decrease MPB (146, 167). In chick skeletal muscle, leucine decreased MPB in association with decreased Ub and 20S proteasome C2 subunit mRNA expression (168). However, a study in rat skeletal muscle reported no changes in proteasome mRNA expression or expression of E3 Ub-ligases MAFbx and MuRF, but a decrease in autophagy marker LC3B-II expression (201). Overall, verification of the effects of leucine on muscle protein breakdown pathways with concurrent measures of muscle protein turnover in humans is lacking.

Despite strong evidence that leucine stimulates MPS in animal models, reports in humans vary with several studies indicating no improvements in MPS following leucine infusion (167), addition of leucine to an amino acid solution (135), or with increasing leucine concentrations in a mixture of EAA in healthy young adults (121). Similarly, there are conflicting results concerning the effects of leucine on MPB pathways in animal models and little information available in humans. Therefore, the purpose of this study was to determine the effects of different leucine concentrations in an isonitrogenous EAA mixture on muscle protein turnover (synthesis and breakdown) and associated cellular signaling. Specifically, it was hypothesized that a higher concentration of leucine would elicit an increase in MPS and mTOR signaling, and decrease MPB and UPS and/or lysosomal signaling.

MATERIALS AND METHODS

Subjects. Calculations were performed to determine the sample size required to detect a 50% difference between groups in our main outcome measure, FSR, with 80% power and $\alpha = 0.05$. The required sample size calculated was 7 subjects per group. The study enrolled 14 young subjects (6 males; 8 females) who reported that they were not currently engaged in any regular exercise training during the screening interview.

Screenings of subjects were performed with clinical history, physical exam, and laboratory tests including complete blood count with differential, liver and kidney function tests, coagulation profile, fasting blood glucose and oral glucose tolerance test, hepatitis B and C screening, HIV test, thyroid stimulating hormone, lipid profile, urinalysis, and drug screening. Volunteers were asked to refrain from performing vigorous physical activity for 48 hours prior to participating in the study. All subjects gave informed written consent before participating in the study, which was approved by the Institutional Review Board of the University of Texas Medical Branch (which is in compliance with the Declaration of Helsinki). A dual-energy X-ray absorptiometry (DEXA) scan (Hologic QDR 4500W, Bedford, MA) was performed to measure body composition and lean mass.

Study design. Subjects were admitted to the ITS-CRC the day prior to the study and fed a standard dinner (12 kcal·kg^{-1}of body weight; 60% carbohydrate, 20% fat, and 20% protein) and a snack at 2200h, prepared by the Bionutrition Division of the ITS-CRC. All subjects were studied following an overnight fast under basal conditions and were studied at the exact same time (between 0700 and 1600 h). The morning of the study, polyethylene catheters were inserted into a forearm vein for tracer infusion, in the contra-lateral hand vein which was heated for arterialized blood sampling, and in the femoral artery and vein (retrograde placement) of the leg for blood sampling. The femoral lines were placed in the same leg from which muscle biopsies were obtained. The arterial catheter was also used for the infusion of indocyanine green (ICG, Akorn, Inc., Buffalo Grove, IL) to determine blood flow. After drawing a background blood sample, a primed continuous infusion of L-[$^{13}C_6$] phenylalanine (priming dose = 2 μmol· kg^{-1}, infusion rate = 0.05 μmol · kg^{-1}· min^{-1}) and L-Leucine 1-^{13}C (priming dose = 4.8 μmol· kg^{-1}, infusion rate = 0.08 μmol · kg^{-1}· min^{-1}) was started and maintained at a constant rate until the end of the experiment (Isotec, Sigma-Aldrich; St. Louis, MO).

Two and a half hours after starting the tracer infusion, and marking the beginning of the Baseline period, the 1st muscle biopsy was obtained from the lateral portion of the *vastus lateralis* of the leg. The biopsy was performed using a 5 mm Bergström biopsy

needle, under sterile procedure and local anesthesia (1% lidocaine). The tissue was immediately blotted, dissected from any connective tissue and/or fat, frozen in liquid nitrogen and stored at -80^{o}C until analysis. After the 1st biopsy, infusion of ICG was begun in the femoral artery (0.5 mg·min^{-1}) and maintained for 10 minutes. Blood samples were obtained from the femoral vein and the arterialized hand vein to measure ICG concentration. Following collection of blood for ICG measurement, ICG infusion was stopped and blood was obtained from the femoral artery and vein and from the arterialized hand vein to measure amino acid enrichments, glucose and insulin concentrations. The ICG infusion was re-started for a minimum of 10 minutes between draws, and the sequence repeated for a total of 4 sets of blood draws per period approximately 15 min apart. Following the 4th set of blood draws, a 2nd biopsy was obtained from the same incision as the first; however, the biopsy needle was inclined at a different angle so that the second biopsy was taken approximately 5 cm apart from the first. This method has been previously used by this lab (63-64, 77, 220) and others (58, 120, 148).

After the second baseline biopsy, subjects ingested either the CTRL or LEU nutrient solution (composition below). Muscle biopsies were then sampled at 1, 2 and 3h following the ingestion of the nutrient solution. The final two muscle biopsies (2- and 3 h post-drink) were sampled from a separate incision on the same leg 7 cm proximal to the previous incision site, and the needle positioned at different angles as described previously, such that all biopsies were separated by approximately 5 cm. ICG infusion and blood sampling was performed in each of the periods as described previously.

Nutrient Solution Composition. Both CTRL and LEU solutions contained 10 grams of essential amino acids mixed in a non caloric, non caffeinated carbonated beverage (500 mL). The composition of the CTRL mixture was representative of high quality protein and contained essential amino acids in the following proportions: L-histidine (11%), L-isoleucine (10%), L-leucine (18%), L-lysine (16%), L-methionine (3%), L-phenylalanine (16%), L-threonine (14%), and L-valine (12%). The LEU mixture was enriched in leucine and composed as follows: L-histidine (8%), L-isoleucine (8%),

L-leucine (35%), L-lysine (12%), L-methionine (3%), L-phenylalanine (14%), L-threonine (10%), and L-valine (10%) (Sigma-Aldrich, St. Louis, MO).

Determination of blood flow, glucose and insulin concentrations. Serum ICG concentration for the determination of leg blood flow was measured spectrophotometrically (Beckman Coulter, Fullerton, CA) at λ=805 nm (113). Plasma glucose concentration was measured using an automated glucose and lactate analyzer (YSI, Yellow Springs, OH). Plasma insulin concentrations were determined by ELISA (Millipore; St. Charles, MO).

Amino Acid Enrichments and Concentrations. Enrichments of blood leucine and phenylalanine were determined on their *tert*-butyldimethylsilyl derivatives using L-Leucine 5,5,5-d_3 and L-[^{15}N]phenylalanine, respectively, as internal standards and gas chromatography/ mass spectrometry (GCMS; 6890 Plus GC, 5973N MSD/DS, 7683 autosampler; Agilent Technologies, Palo Alto, CA) and used to calculate amino acid concentrations as previously described (240). Muscle tissue samples were ground, and intracellular free amino acids and muscle proteins were extracted as previously described by Wolfe (237). Intracellular free enrichments of phenylalanine were determined by GCMS using L-[^{15}N]phenylalanine as an internal standard, and protein-bound phenylalanine enrichments were measured by GCMS (237).

Calculations. Kinetics were calculated using both the two-pool and three-pool models of leg muscle amino acid kinetics and were described in detail in Chapter 4 Materials and Methods (15, 20, 79). Kinetics calculated for leucine included delivery to the leg (F_{in}), transport into the muscle ($F_{m,a}$) and intracellular availability. Phenylalanine kinetics were calculated for Net Balance (NB), rate of appearance and disappearance of phenylalanine (R_a, R_d), release from proteolysis ($F_{m,o}$) and utilization for protein synthesis ($F_{o,m}$). Data are expressed per 100 mL of leg volume. The fractional synthetic rate (FSR) of mixed muscle proteins was calculated from phenylalanine as $(\Delta E_p / t) / [(E_{M(1)} + E_{M(2)}) / 2] \cdot 60 \cdot 100$, where ΔE_p is the increment in protein-bound phenylalanine enrichment between the two biopsies, t is the time between biopsies and $E_{M(1)}$ and $E_{M(2)}$

are the phenylalanine enrichments in the free intracellular pool in the two biopsies. Data are expressed as percent per hour.

SDS-PAGE and Immunoblotting. Details to the immunoblotting procedures have been previously described in Chapter 2 Materials and Methods. Total protein content for each phospho-protein was determined for each blot and did not change over the course of the experiment from baseline. Data are presented as phosphorylation status relative to a normalization control and as fold change from Baseline. Data for MuRF-1, MAFbx, and LC3BII are presented as total protein expression relative to the normalization control and as fold change from Baseline.

For quantification of the mTOR:ULK1 complex, mTOR protein was isolated in whole muscle homogenates using an immunoprecipitation technique previously characterized by Williamson *et al*. (232). Approximately 30 mg of tissue was homogenized in CHAPS buffer (1:9 *w/v*, pH 7.5). The buffer consisted of 40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM β-glycerophosphate, 40 mM NaF, 1.5 mM sodium vanadate, 0.3% CHAPS, microcystin and a protease inhibitor cocktail (Sigma-Aldrich, P8340; St. Louis, MO). Samples were centrifuged at 2500 *g* for 5 min at 4ºC and total protein content determined spectrophotometrically. Samples (700 μg protein) were combined with 2 μL total mTOR antibody and rocked over-night at 4ºC. Samples were combined with 500μL of BioMag goat anti-rabbit IgG beads (Quiagen; Valencia, CA) suspended in CHAPS buffer and 1% non-fat dry milk for 1 h at 4ºC, and isolated using a magnetic tube rack. Following two washes in CHAPS buffer, samples were washed with a second buffer (HEPES, NaCl (pH 7.5)). Sixty microliters of 2x sample buffer were added to each sample, boiled for 5 min at 100ºC, and separated from beads using the magnetic tube rack. Samples were loaded onto gels as described above and probed with ULK1 followed by total mTOR after stripping. Values of ULK1 are expressed relative to total mTOR in the precipitates.

Antibodies. The primary phospho- antibodies were purchased from Cell Signaling; Beverley, MA): Akt (Ser^{473}), mTOR (Ser^{2448}), S6K1 (Thr^{389}), 4E-BP1 ($Thr^{37/46}$), eEF2 (Thr^{56}), and PRAS40 (Thr^{246}). Total antibodies purchased from Cell

Signaling were Akt, mTOR, S6K1, 4E-BP1, eEF2, PRAS40, ULK1 and LC3B. MuRF1 and MAFbx were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was purchased from Amersham Bioscience (1:2000; Piscataway, NJ)

Statistical Methods. Values are expressed as mean ± SEM. Comparisons were performed using SigmaPlot v. 11.0 (Systat Software Inc.; Chicago, IL), and two-way repeated measures analysis of variance (ANOVA), the effects being group (CTRL, LEU) and time (Baseline, 1-, 2- and 3-h post periods; Baseline, 30 min, 1-, 2- and 3-h post for insulin; or 16 point time intervals for leucine kinetics). Post-hoc testing was performed using the Bonferroni's *t* test for multiple comparisons versus Baseline and CTRL. If a test of normality and/or equal variance failed, simple transformations were performed. An independent two sample Student's *t* test was used to assess differences in calculated area under the curve (AUC) for FSR and phenylalanine kinetics. All analyses were performed. Significance was set at $P \leq 0.05$.

RESULTS

The subjects' physical characteristics are summarized in Table 4.

Blood flow, glucose and insulin. Blood flow was not different across time or between groups ($P > 0.05$). Arterial glucose concentration decreased at 2 h post-nutrient ingestion in the LEU group versus Baseline (4.8 ± 0.1 vs. 5.0 ± 0.1 $mmol \cdot L^{-1}$, respectively; $P < 0.05$) and at 3 h post-ingestion in both groups versus Baseline (CTRL = 5.0 ± 0.1 vs. 5.1 ± 0.1 $mmol \cdot L^{-1}$; LEU = 4.8 ± 0.1 vs. 5.0 ± 0.1 $mmol \cdot L^{-1}$, respectively; $P < 0.05$) with no group differences. Plasma insulin concentrations were significantly elevated in both groups at 30 min following nutrient ingestion compared with Baseline (CTRL = 14.8 ± 2.6 vs. 4.3 ± 0.7 $\mu U \cdot mL^{-1}$; LEU = 13.2 ± 1.7 vs. 4.7 ± 0.9 $\mu U \cdot mL^{-1}$; $P < 0.05$), and remained elevated at 1 h only in the LEU group (8.0 ± 1.1 $\mu U \cdot mL^{-1}$; $P < 0.05$ vs. Baseline).

Table 4. Subject Characteristics.

	CTRL	**LEU**
N	3M, 4F	3M, 4F
Age, yr	32 ± 2	29 ± 2
Height, cm	170 ± 5	167 ± 6
Weight, kg	73 ± 3	71 ± 6
Body Mass Index, $kg \cdot m^2$	26 ± 1	25 ± 1
Lean body mass, kg	53 ± 4	50 ± 6
Body fat, %	25 ± 3	27 ± 4

Values are expressed as Mean ± SEM.

Leucine Kinetics. Arterial leucine concentration and leucine delivery to the leg (F_{in}) increased in both groups at all time points following drink ingestion ($P < 0.05$ vs. Baseline) and were significantly higher in the LEU group versus CTRL for 135 min following ingestion ($P < 0.05$, Figure 15A and B). Leucine transport into the muscle ($F_{m,a}$) significantly increased for 90 min post drink ingestion ($P < 0.05$, Figure 15C) with no group differences. Intracellular availability of leucine increased in both groups during the first hour following drink ingestion and was significantly elevated in LEU vs. CTRL 45 min following ingestion ($P < 0.05$, Figure 15D).

Muscle Protein Turnover. Mixed muscle fractional synthetic rate (FSR) was significantly elevated in both groups 1 h following nutrient ingestion ($P < 0.05$, Figure 16A). FSR calculated over the 3 h period following drink ingestion was significantly elevated above Baseline in the LEU group ($0.050\% \cdot h^{-1}$ vs. $0.076\% \cdot h^{-1}$, $P = 0.05$) but not in EAA ($0.053\% \cdot h^{-1}$ vs. $0.061\% \cdot h^{-1}$, $P = 0.52$). Areas under the curve (AUC) for FSR and the 16-point phenylalanine kinetic measures of $F_{o,m}$ and R_d were not different between groups (data not shown). However, similar to FSR results, hourly averages of $F_{o,m}$ and R_d were elevated in both groups 1 h post-ingestion ($P < 0.05$) with no group

differences (Figure 16B and C). Net Balance significantly increased 1 h following nutrient ingestion in both groups ($P < 0.05$) with no group differences (Figure 17A). Muscle protein breakdown ($F_{m,o}$ and R_a) was significantly decreased 1 h following nutrient ingestion only in the LEU group ($P < 0.05$ vs. Baseline, Figure 17B and C).

Cellular Signaling. Phosphorylation of Akt (Ser^{473}) was not different at any time point or between groups ($P > 0.05$; data not shown). Phosphorylation of mTOR (Ser^{2448}) increased from Baseline at 1 h in the LEU group, and at 2 and 3 h in both groups ($P < 0.05$, Figure 18A). S6K1 phosphorylation (Thr^{389}) significantly increased at all time points following drink ingestion in the LEU group, and was elevated at 1 and 3 h in the CTRL group ($P < 0.05$, Figure 18B). Phospho-4E-BP1 ($Thr^{37/46}$) increased significantly only in the LEU group ($P < 0.05$ vs. Baseline at 1 and 2h; $P < 0.05$ vs. CTRL at 1h, Figure 18C). Phosphorylation of eEF2 (Thr^{56}) decreased in both groups at 1 h ($P < 0.05$) and remained decreased at 2 h only in the LEU group ($P < 0.05$ vs. Baseline, Figure 18D). Total ULK1 complexed with mTOR was increased significantly at 3 h in LEU group ($P < 0.05$ vs. Baseline and CTRL, Figure 19A). Conversion of LC3B-I (no differences across time or between groups, data not shown) to LC3B-II tended to decrease 2 h following nutrient ingestion in LEU ($P = 0.1$), and significantly decreased at 3 h ($P < 0.05$), while tending to decrease at 3 h in the CTRL group ($P = 0.09$, Figure 19B). There were no changes across time or between groups for total protein of MuRF1 or MAFbx ($P > 0.05$, Figure 19C and D).

Figure 15. Leucine kinetics across the leg.

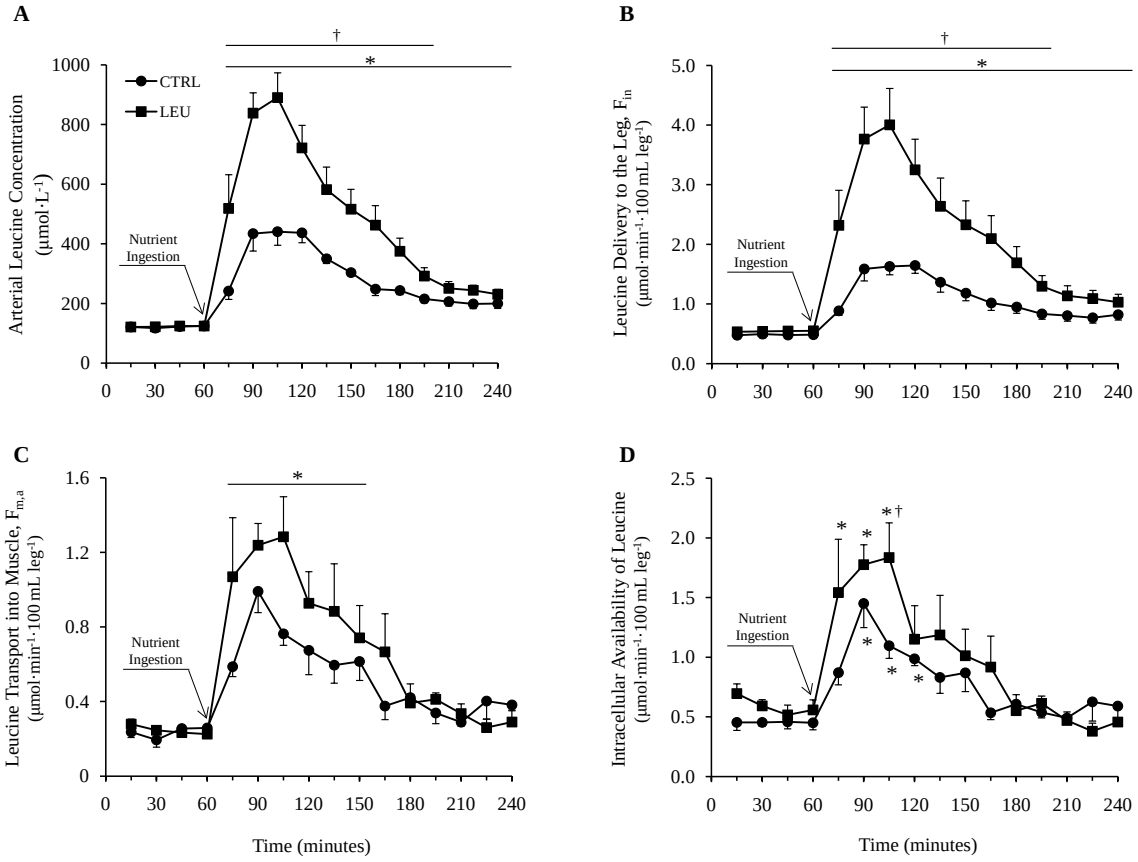

Fig. 15. Data calculated from L-Leucine 1-13C tracer every 15 min from time 0 to 240 min. Nutrients were ingested directly after 60 min time point. (A) Arterial leucine concentration, (B) Delivery to the leg (F_{in}), (C) Transport into the muscle ($F_{m,a}$) and (D) Intracellular availability. Data are expressed as (µmol·min-1·100 mL leg^{-1}) and as Mean ± SEM. *Significantly different from 15 min time point, $P < 0.05$; † Significantly different from CTRL at individual time point, $P < 0.05$.

Figure 16. Muscle protein synthesis.

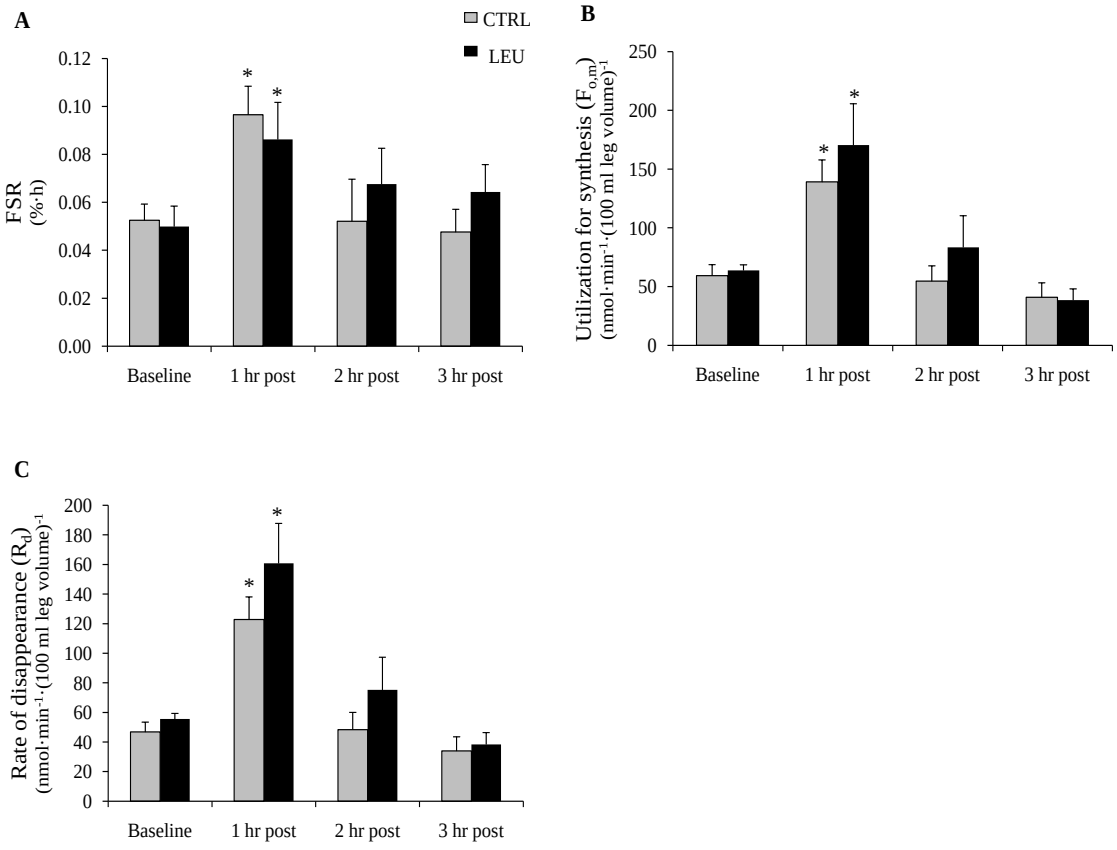

Fig. 16. Data calculated from L-[$^{13}C_6$] phenylalanine tracer. (A) Mixed muscle fractional synthetic rate (FSR) expressed as %·h^{-1}, (B) Utilization for protein synthesis and (C) Rate of disappearance from the blood. B and C are expressed as (μmol·min^{-1}·100 mL leg^{-1}) and as hourly averages of samples collected each period. All data are expressed as Mean ± SEM. *Significantly different from Baseline, $P < 0.05$.

Figure 17. Muscle protein balance and breakdown.

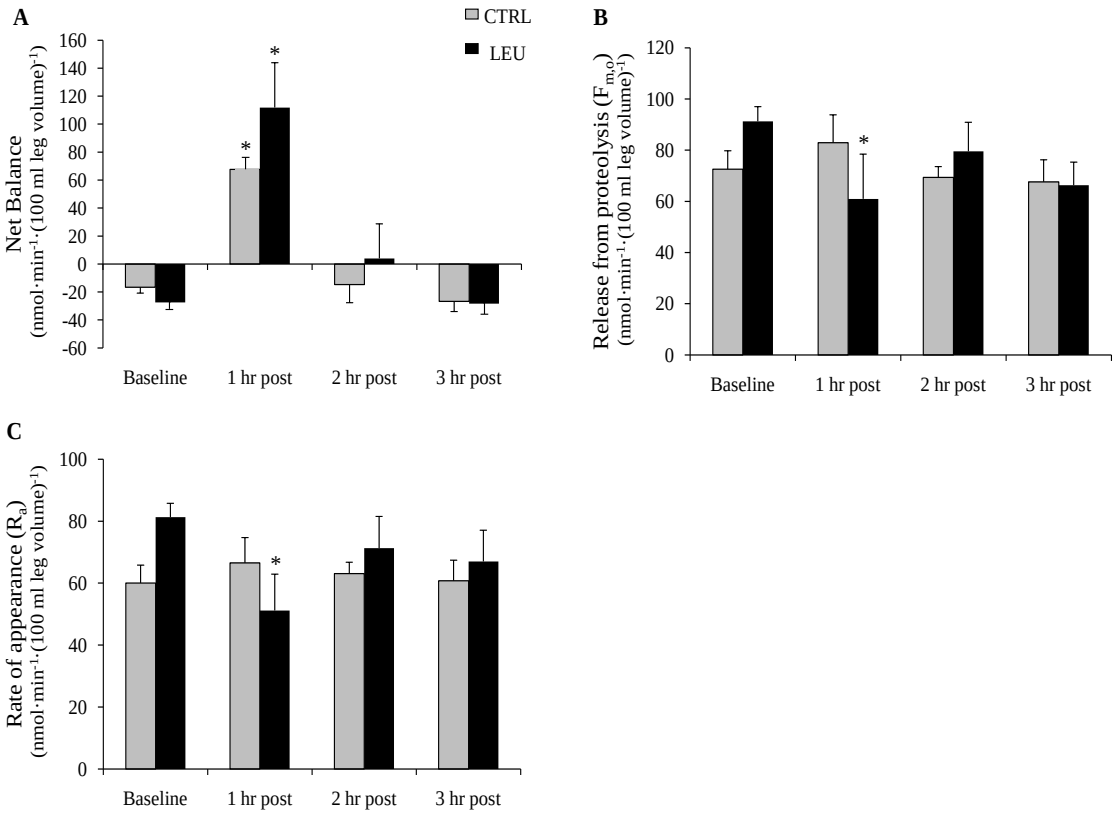

Fig. 17. Data calculated from L-[$^{13}C_6$] phenylalanine tracer. (A) Net balance across the leg, (B) Release from proteolysis, (C) Rate of appearance in the blood. Data are expressed as (μmol·min^{-1}·100 mL leg^{-1}), as hourly averages of samples collected each period and as Mean ± SEM. *Significantly different from Baseline, $P < 0.05$.

Figure 18. mTOR signaling.

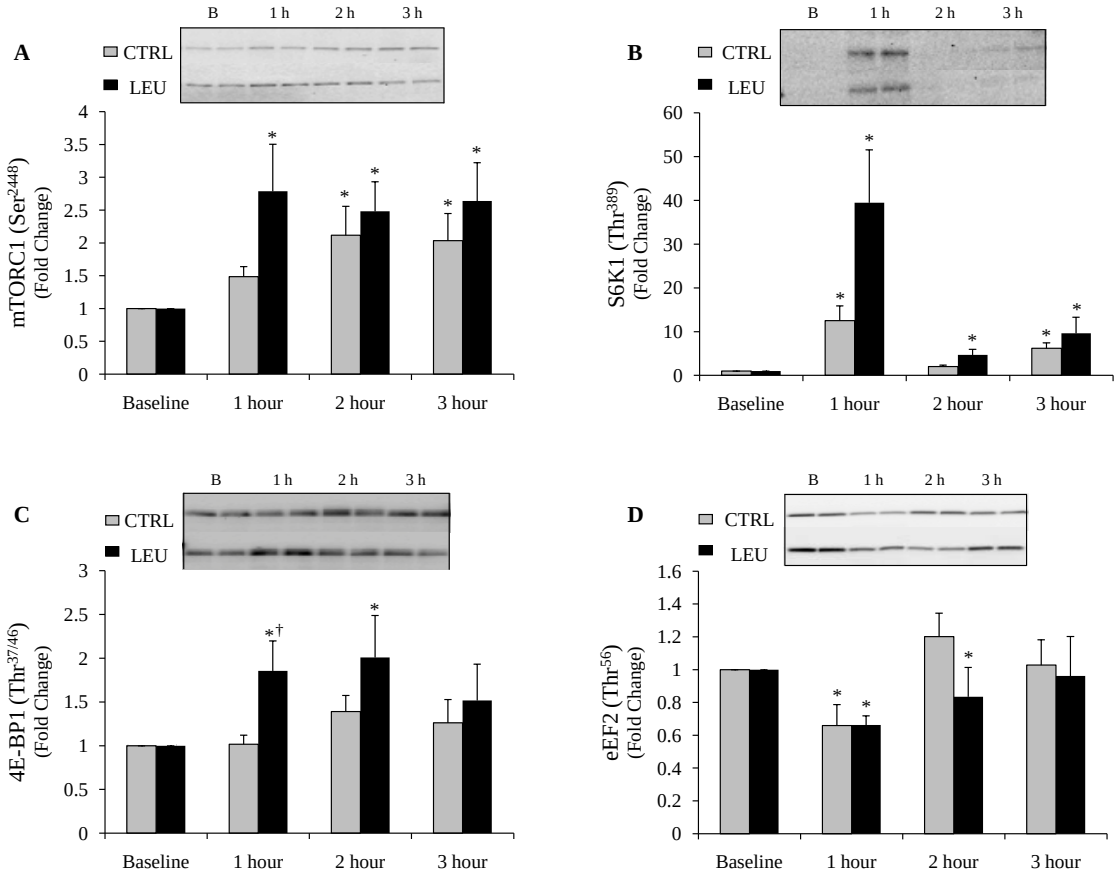

Fig. 18. Phosphorylation of (A) mTOR (Ser^{2448}), (B) S6K1 (Thr^{389}), (C) 4E-BP1 ($Thr^{37/46}$), and (D) eEF2 (Thr^{56}). Data are expressed relative to a normalization control and as Fold Change from Baseline ± SEM. Insert shows representative Western blot in duplicate for Baseline, 1-, 2- and 3-h post-nutrient ingestion. *Significantly different from Baseline, $P < 0.05$; † Significantly different from CTRL, $P < 0.05$.

Figure 19. Breakdown signaling.

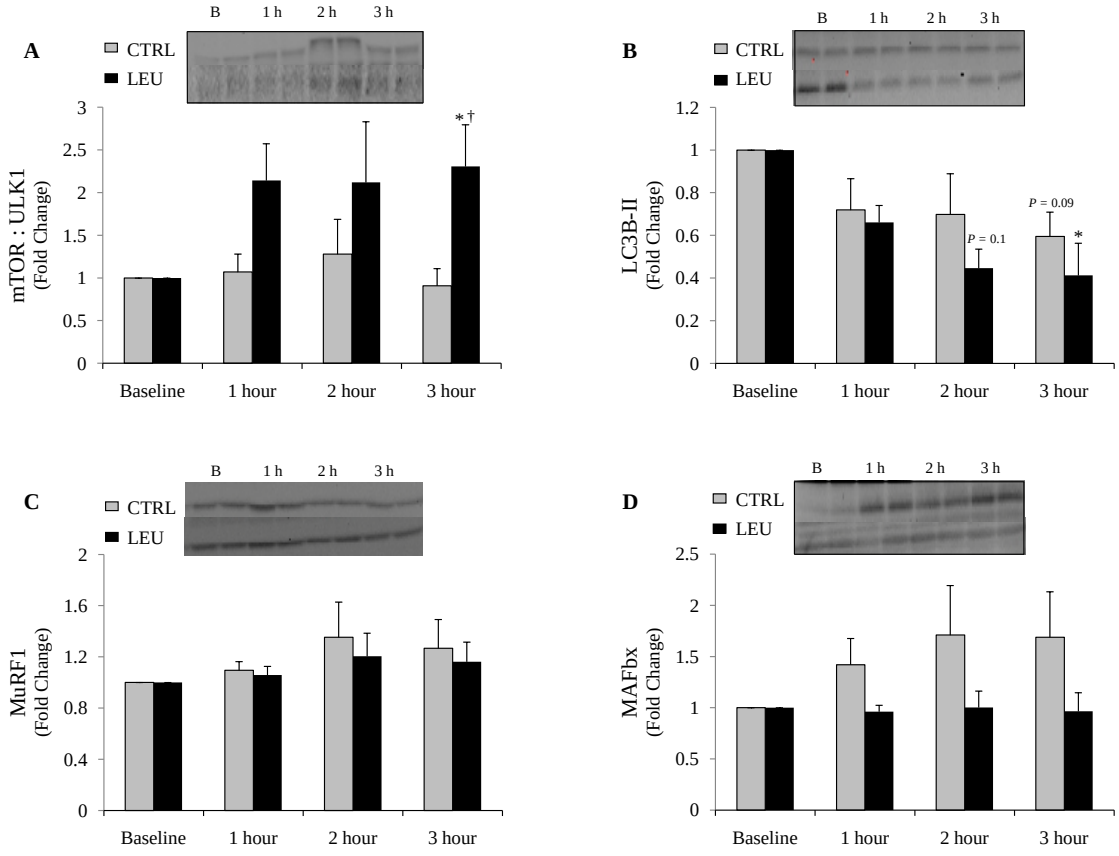

Fig. 19. (A) total ULK1 following mTOR immunoprecipitation, data expressed relative to total mTOR in the precipitates, to a normalization standard across gels and as Fold Change from Baseline ± SEM; total (B) LC3B-II, (C) MuRF1, and (D) MAFbx expressed relative to a normalization control and as Fold Change from Baseline ± SEM. Insert shows representative Western blot in duplicate for Baseline, 1-, 2- and 3-h post-nutrient ingestion. *Significantly different from Baseline, $P < 0.05$; † Significantly different from CTRL, $P < 0.05$.

DISCUSSION

Despite numerous studies in animal models concerning the positive effects of leucine infusion or ingestion on muscle protein turnover, conflicting results of investigations in humans make reaching a consensus on the effects of leucine in human skeletal muscle difficult. Therefore, this study was designed to determine the effects of 10g of EAA with a composition representative of high quality protein (CTRL) versus 10g of EAA with a higher concentration of leucine (LEU) on muscle protein turnover and associated cellular signaling. Specifically of interest was whether a higher concentration of leucine in an isonitrogenous EAA mixture would enhance MPS with increased mTOR signaling, and/or decrease MPB and activity of lysosomal or ubiquitin-proteasome system pathways. The primary and novel finding from this study was that higher leucine concentration in an EAA mixture decreased MPB with concurrent decreases in autophagy markers, and resulted in greater activation of the mTOR pathway, although greater activation of mTOR signaling did not translate to significant improvements in MPS versus a lower concentration of leucine.

Significant doses of leucine cause a small and transient increase in circulating insulin levels in animal models (5-6, 184), and this study found similar results of small increases in plasma insulin versus basal values in both groups 30 min following nutrient ingestion. This increase in insulin was sustained only in the LEU group at 1 h. Because insulin decreases muscle protein breakdown (83, 90, 147), the prolonged insulin response in the LEU group may contribute to the observed decreases in MPB at 1 h. However, despite the small increases in insulin levels in both groups, changes in the phosphorylation of Akt were not detected. This result is not completely unexpected as leucine has been reported to decrease insulin-stimulated phosphatidylinositol 3-kinase activity in rat skeletal muscle (14). Intriguingly, Herningtyas *et al*. found that leucine reduced MAFbx and MuRF1 mRNA expression in C2C12 cells, but only MAFbx expression was mTOR-dependent (103). While this lab observed no changes in MAFbx mRNA expression across time (personal observation, M.J. Drummond), total protein content of MAFbx slightly increased in the CTRL group while remaining at basal levels

in LEU (P = 0.24, 0.18 and 0.13 for 1-, 2- and 3 h group differences, respectively), suggesting the enhanced mTOR signaling in LEU may be suppressing increases in MAFbx. On the other hand, the pattern of MuRF1 protein content was not related to changes in mTOR signaling. In contrast to the UPS, there were significant changes in components of the lysosomal system. UNC-51-like kinase-1 (ULK1) is essential for autophagy (45), and is part of a complex (with autophagy factor Atg13 and focal adhesion kinase family-interacting protein of 200kDa (FIP200)) that is bound to, phosphorylated and inhibited by mTOR in nutrient-rich conditions, preventing autophagy (107). Though not significantly different until 3 h, ULK1 complexed to mTOR (autophagy inhibition) tended to be elevated at 1- and 2 h in the LEU group, consistent with greater decreases in MPB in this group. Similarly, autophagy marker LC3B-II decreased in both groups over the 3 h period, but to a greater extent in LEU. Overall, these results indicate subtle differences in insulin action and lysosomal signaling are associated with a small but significant decrease in measures of MPB with higher leucine concentration.

As expected, increases in arterial leucine concentration and delivery to the leg were observed following EAA ingestion in both groups, but were markedly increased in the LEU for 135 min post-ingestion. Similar to previous reports which found no changes in intracellular concentrations of amino acids despite higher circulating levels of amino acids (29), this study found that higher arterial concentrations of leucine in the LEU group did not translate to an increased rate of transport into the muscle compared to CTRL at any time point. Also, transport and intracellular availability were only increased for 90 and 60 min, respectively, following nutrient ingestion although leucine concentration and delivery to the leg were elevated for 180 min post-ingestion in both groups. Several previous reports have hypothesized and/or concluded that human MPS is modulated by extracellular amino acid availability (28, 34), which would suggest higher MPS may be expected in the LEU group due to higher extracellular leucine concentrations. However, no group differences were observed for any measure of MPS despite significantly higher extracellular concentrations of leucine. These data provide

support for the idea that muscle protein synthesis may not be solely regulated by extracellular amino acid availability but influenced by amino acid transport into the cell and intracellular availability as well.

The fact that higher arterial leucine concentrations did not result in increased leucine transport or intracellular availability suggests a rate limiting step at the level of leucine transport from blood into the cell, perhaps by lack of available amino acid transporters. Of particular interest, it was recently reported that inhibition of the L-glutamine transporter SNAT2 (a System A-type sodium-coupled neutral amino acid transporter) indirectly depleted other intracellular amino acids, including leucine, and impaired protein synthesis and mTOR signaling in L6 muscle cells (70). Recently, this lab found the mRNA expression of several amino acid transporters, including SNAT2, to be rapidly elevated following EAA ingestion (1 h), but immediately return to basal values by 2 h post-ingestion (67). This coincides almost precisely with the peak in intracellular availability of leucine (which occurs during 1 h post-ingestion) and the peak in MPS (30-60 min post-ingestion), further supporting the idea that MPS is driven by intracellular availability of amino acids. It is well established that a decrease in blood amino acid availability inhibits MPS (85, 172, 238), and we have recently reported that blood concentrations of the branched-chain amino acids leucine and valine remain elevated for at least 2 hours following the ingestion of 10 grams of EAA (66). Therefore, since blood amino acid availability was maintained but muscle protein synthesis increased only during the first hour following ingestion (i.e., when intracellular amino acid availability increased), further considerations about the role of extracellular versus intracellular amino acid availability in the regulation of MPS are warranted.

While there were no significant group differences in any measure of MPS (FSR, $F_{o,m}$, R_d) or mTOR signaling for the most part, the common theme throughout was a prolonged response with higher leucine concentration. AUC was not different between groups for any measure of synthesis, but FSR calculated over the entire 3 h postprandial period (biopsy 2 to biopsy 5) was significantly elevated versus Baseline only in the LEU group. In addition, Net Balance remained positive at 2 h in LEU but returned to basal

values in CTRL, similar to results reported by Katsanos *et al.* where NB remained elevated for a longer period of time with higher leucine concentration in young subjects (121). These results correspond to earlier and/or greater increases of mTOR and 4E-BP1 phosphorylation, and a prolonged dephosphorylation of eEF2 (which is indicative of enhanced translation elongation) in the LEU group. Although the findings support the idea that mTOR signaling is acutely responsive to differing concentrations of insulin and amino acids (specifically leucine) (7), the question of what level of changes in mTOR signaling translate to significant increases in MPS remains. Further studies are required to determine whether these small improvements in MPS and mTOR signaling can generate significant increases in net muscle protein accretion over time, while considering potential adverse effects that may occur with long term consumption of high quantities of leucine (81).

Despite the trends and visual appearance of slight improvements in measures of muscle protein turnover with greater leucine concentration, the fact is that no measures of turnover were significantly different between groups. One explanation for a lack of differences between groups is that the study was under-powered to detect differences. We based the estimations used in power calculations on data available from previous investigations, but the lack of a difference in FSR at 1 h post-ingestion, along with a larger than anticipated standard deviation in the current study, resulted in reduced statistical power. Still, using the results from the current study, with only small differences in the response following EAA ingestion and larger standard deviations, calculations reveal a sample size of 96 per group needed for 80% power at $\alpha = 0.05$. Sample sizes of this magnitude are not logistically feasible with the complexity and cost of these types of investigations, but also suggest that despite the fact that this study may be under-powered, our findings would be maintained with any reasonable increase in sample size. In support of this argument, 1 h FSR data from previous studies conducted by this lab (77) in which subjects consumed ~6.8 g of leucine in a 20 g EAA solution, are not statistically different from the groups herein (1.8 or 3.5 g leucine in 10 g EAA). The observation that there are no significant differences in the protein synthetic response

between concentrations of 1.8, 3.5 or 6.8 g of leucine indicates that leucine concentrations higher than those present in high quality protein do not significantly enhance MPS.

In summary, we report that consuming a higher concentration of leucine in 10g of EAA slightly prolongs the insulin response, which may contribute to the decrease in MPB observed in the LEU group. Despite increased circulating concentrations of leucine, there were no improvements in leucine transport into the muscle or intracellular availability with higher leucine content, suggesting both a rate limiting step at the level of leucine transport into the muscle, and that MPS is more dependent on intracellular, rather than extracellular, amino acid availability. Higher leucine content slightly enhanced mTOR signaling and decreased lysosomal system signaling, but did not affect UPS markers MAFbx and MuRF1. Despite small improvements in measures of muscle protein turnover and associated signaling, there do not appear to be significant group differences. We conclude that the effects of higher leucine concentration on measures of muscle protein turnover are small and most likely negligible. However, future studies are required to determine whether seemingly small improvements in net muscle protein balance translate into significant changes in skeletal muscle mass over time.

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CHAPTER 6

Summary and Conclusions

The preceding studies were designed to examine the mTOR pathway in conditions of differing physical activity levels, determine the effects of low versus high CHO in combination with EAA on muscle protein turnover and cellular signaling following resistance exercise, and to investigate the effect of leucine on similar parameters. The main findings from these studies were that increased physical activity downregulated the mTOR signaling pathway and decreased inhibitory phosphorylation of insulin receptor substrate 1 (IRS-1). In contrast, mTORC1 activity may play an important role in paraplegia-induced muscle atrophy as 10 weeks of paraplegia in rats significantly downregulated the mTOR pathway. In humans and compared to modest carbohydrate ingestion, higher amounts of carbohydrate and consequent increases in circulating insulin were unable to further reduce muscle protein breakdown, associated signaling or mRNA expression following a bout of RE. Similarly, higher concentrations of leucine may not provide advantages over a lower, but adequate amount since there were no detectable group differences in measures of MPB or MPS. Taken together, these studies provide insight into the effects of various stimuli on muscle hypertrophy and atrophy, which are important to characterize in order to ultimately elucidate the cellular mechanisms responsible for maintaining skeletal muscle mass.

In examining the effect of physical activity on mTOR signaling and mTOR/S6K1 inhibitory feedback on IRS-1, rats allowed free access to running wheels for 9 weeks reduced whole body mass and lowered soleus muscle mTOR, S6K1, and inhibitory IRS-1 serine phosphorylation compared with sedentary, inactive rats. Reduced mTORC1 and S6K1 activation during chronic increases in physical activity may play an important regulatory role in the inhibitory serine phosphorylation status of skeletal muscle IRS-1 ($Ser^{636/639}$), which may have important implications for reducing insulin resistance

associated with this feedback pathway. Future studies are required to determine whether reduced mTOR signaling is directly responsible for the reduction in IRS-1 inhibitory phosphorylation, and whether altered improvements in signaling correlate with improved insulin sensitivity. Answers to these questions will help to explain if these mechanisms are partly responsible for the known beneficial effects of exercise training on insulin sensitivity.

Emphasizing the need for fine control of pathways controlling protein synthesis and breakdown in the body, 10 weeks of paraplegia-induced muscle atrophy (28% reduction in soleus muscle mass) also downregulated the mTOR pathway. In the previous study, downregulation of mTOR and S6K1 following enhanced physical activity was beneficial for insulin signaling. However, this investigation suggests that in addition to the more characterized increases in atrophic signaling during conditions characterized by muscle loss, downregulation of mTOR signaling may also be an important contributor to the atrophic response. Further investigation is necessary to determine whether upregulating mTORC1 activity by known stimuli such as anabolic nutritional supplements, hormones, contractile stimulation and the like, would positively affect muscle cell growth in paraplegic muscle.

In humans, resistance exercise is a readily available method to maintain or increase skeletal muscle mass and quality. One goal of current research is to determine the most efficient training regimens and nutrient recommendations to maximize protein accretion, ultimately increasing muscle mass. Because protein accretion, or net balance, is determined by both protein synthesis and breakdown, the third study sought to examine whether carbohydrate ingestion could decrease muscle protein breakdown following resistance exercise, in order to improve the overall net balance in post-exercise recovery. Ingestion of an anabolic nutrient solution (EAA + CHO) containing a higher amount of CHO (i.e., 90 vs. 30 g) decreased phosphorylation of AMPKα, but this did not further decrease MPB as similar decreases were observed in both groups. In addition, MURF1 was significantly increased (both protein and mRNA expression) following exercise and remained elevated following drink ingestion, while autophagy marker LC3B-II was not

altered by exercise but decreased following nutrient ingestion in both groups. In conclusion, the enhanced muscle protein anabolic response detected when EAA + CHO are ingested following resistance exercise is primarily due to enhanced protein synthesis with relatively minor changes in protein breakdown regardless of the level of CHO ingestion. Therefore, in the design of nutrient solutions to promote improved muscle recovery and growth following resistance exercise, it is not necessary to include large quantities of CHO since a nutrient solution containing a sufficient amount of EAA with modest amounts of carbohydrate is capable of producing a maximal protein anabolic response. However, future studies are needed to determine if the insulin release and reduction in muscle protein breakdown resulting from modest carbohydrate intake can generate improvements in net muscle protein accretion over time that are significant enough to warrant the addition of CHO to such nutrient solutions.

The final project discovered that consuming a higher concentration of leucine in 10g of EAA slightly prolongs the insulin response, which may contribute to the decrease in MPB observed in the LEU group. Despite increased circulating concentrations of leucine, there were no improvements in leucine transport into the muscle or intracellular availability with higher leucine content, suggesting both a rate limiting step at the level of leucine transport into the muscle, and that MPS is more dependent on intracellular, rather than extracellular, amino acid availability. Higher leucine content slightly enhanced mTOR signaling and decreased lysosomal system signaling, but did not affect UPS markers MAFbx and MuRF1. Despite small improvements in measures of muscle protein turnover and associated signaling, there do not appear to be significant group differences. Therefore, the effects of higher leucine concentration on measures of muscle protein turnover are small and most likely negligible. However, future studies are required to determine whether seemingly small improvements in net muscle protein balance translate into significant changes in skeletal muscle mass over time.

These studies touch on the incredibly complex network of cellular pathways that interact to produce the observed alterations in protein turnover and muscle mass. The mTOR hypertrophy signaling pathway, and the atrophy-inducing UPS and lysosomal

pathways, are major contributors to the physiological response to exercise and nutrition. This work sought to define the response of these components to varying treatments and conditions in order to further our understanding of muscle hypertrophy and atrophy regulation at a cellular level. Collectively, these studies contribute new information to the body of knowledge required to provide evidence-based recommendations for the maintenance of skeletal muscle mass during conditions of muscle wasting.

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EDUCATION

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PUBLICATIONS

Manuscripts:

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