Abstract

Paired Effects of Dietary Leucine Supplementation and Overload on Protein Translational Signaling and Hypertrophy in Aged Rat Skeletal Muscle

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Sarcopenia is an age-associated disorder that causes loss of skeletal muscle mass, particularly in type II (fast-twitch) muscle fibers. This loss in muscle mass can cause disability, reductions in the quality of life, and can contribute to the development of other more life-threatening morbidities and even death. Researchers have utilized muscle overloading and ergogenic aids, such as whey protein and essential amino acids (specifically leucine), in rats and humans in attempts to reduce or attenuate these losses as part of a primary prevention strategy. Unfortunately, there is also a loss of overload-induced growth capacity in aged fast-twitch skeletal muscle. However, no studies have explored the potential synergistic effect of leucine supplementation on overload-induced skeletal muscle growth in aged animals. To that end, the purpose of this study was to examine the effects of dietary leucine supplementation on protein translational signaling and hypertrophy in the overloaded fast-twitch skeletal muscles of aged animals. It was hypothesized that supplementing a standard chow diet with 5% leucine would enhance muscle hypertrophy in overloaded fast-twitch plantaris muscles of aged (33-month old) rats to levels observed in young adult (8-month old) rats. It was also hypothesized that 5% dietary leucine supplementation would enhance protein translational [70 kDa ribosomal protein S6 kinase (p70s6k), ribosomal protein S6 (rpS6), eukaryotic elongation factor 2 kinase (eEF2k),

and eEF2] signaling in the overloaded fast-twitch plantaris muscles of aged rats to levels observed in young adult rats. Young adult and old male Fisher$^{344}$ x Brown Norway F1 Hybrid (FBN) rats underwent a 1-week unilateral plantaris muscle overload via tenotomy of the synergistic gastrocnemius muscle. Within each age group, animals were matched for body weight and separated into either a dietary leucine supplementation group (additional 5% leucine content in place of 5% of the carbohydrate content in normal rat chow starting 2 days prior to, and throughout, the overload intervention; n = 7/age group) or placebo group (normal rat chow; n = 6/age group). No differences in daily calorie consumption were observed between the placebo vs. leucine groups within each age group. Plantaris muscles were harvested at the end of the overload period. Dietary leucine enrichment significantly ($p \leq 0.05$) enhanced overload-induced fast-twitch plantaris muscle hypertrophy in old, but not in young adult, animals. Additionally, western blotting analyses revealed that phospho-p70S6k (Thr389) and phospho-rpS6 (Ser235/Ser236) were significantly lower in old vs. young overloaded muscles under placebo conditions, but leucine partially restored both phospho-p70S6k and phospho-rpS6 in old overloaded muscles to that of young adult overloaded muscles. Overload significantly increased eEF2k phosphorylation in young, but not in old animals, and leucine supplementation had no affect on eEF2k phosphorylation in either age group. Overload significantly increased total eEF2 content and decreased inhibitory eEF2 phosphorylation (Thr56; normalized to total eEF2) in young adult muscles regardless of leucine supplementation. Total eEF2 content was unaffected by overload in old placebo muscles, but leucine supplementation in old animals non-significantly ($p = 0.09$) restored the overload-induced increase in total eEF2 content. These novel findings indicate that a leucine-enriched diet may potentially enhance overload-induced

growth of aged fast-twitch muscle, in part by enhancing pathways known to stimulate protein synthesis.

# Paired Effects of Dietary Leucine Supplementation and Overload on Protein Translational Signaling and Hypertrophy in Aged Rat Skeletal Muscle

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# Dedications

I could not have completed this literary work without the unwavering support of my friends and family. I am forever grateful. As a token of my gratitude, I wish to dedicate this work to you all.

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# Chapter I: Introduction

## *Sarcopenia*

As humans age, one of the many physiological processes that leads to disease development and disability is the loss of skeletal muscle. This age-associated decline in skeletal muscle is sarcopenia (Evans, 1995). Sarcopenia has been linked to a reduction in muscle fiber size and number (Welle, 2002), particularly in type II, fast-twitch fibers (reviewed in Lexell, 1995). Muscle fiber degeneration takes place primarily in type II muscle fiber size, with very little, if any, reduction in type I fiber size (reviewed in Lexell, 1995, Thomson and Gordon, 2006). Animal models have demonstrated reductions in total muscle mass and cross sectional area, as well as, declines in individual muscle fiber cross sectional area (Blough, 2000).

A decrease in muscle mass has a positive correlation with decreases in strength (Frontera, 2000) and contractile quality (Blough, 2000). This loss in muscle quality generates functional limitations (Hairi, 2010) and physical disability (Dorrens, 2003), specifically in normal activities of daily living among elderly individuals (Dorrens, 2003; Hairi, 2010). An increased risk of falling among elderly individuals has also been correlated with excessive losses in muscle strength (Wickham, 1989). An estimated ~1.5% of the total national healthcare expenditure in the US has been directly attributed to sarcopenia. This seemingly minuscule percentage equates to an approximate monetary value of 18.5 billion US dollars (Janssen, 2004). Although this chronic process has been defined, it is difficult to diagnose sarcopenia because no absolute values have been established for losses of fat free mass, cell mass, or total muscle mass (Roubenoff, 2000).

Muscle fiber degeneration can be seen in both genders, although it can affect males and females at different rates and times throughout their middle and late adult lives. Males lose an

estimated 1.9 kg/decade, while females are slightly less affected with losses of 1.1 kg/decade (Janssen, 2000). Over the span of a human life, losses in muscle area up to 40% have been documented in participants between 20 and 80 years of age (reviewed in Lexell, 1995).

Lean body mass can play a role in the survival rate for individuals suffering from life-threatening illnesses (Tellado, 1988). There is a strong correlation between muscle mass and strength. As stated previously, this loss in strength can become debilitating even for carrying out normal activities of daily living. However, a loss of muscle mass is not always accompanied by a reduction in strength (Roubenoff, 2000). In order to determine what causes the loss of muscle fibers with age, a closer examination must be performed on muscle protein synthesis and degradation pathways at the molecular level.

### *Protein Synthesis*

Growth within, and maintenance of, existing muscle fibers is a complex process incorporating several different pathways. The muscle protein synthesis translational pathway consists of three separate stages (initiation, elongation, and termination) that work together to form specific proteins corresponding to the blueprint laid out by DNA in the cell (Nadar, 2002; Wang, 2006). The main areas of focus for this investigation were protein translation initiation and elongation.

One pathway that has been recognized for stimulating protein synthesis is the mammalian target of rapamycin (mTOR) pathway (Figure 2.1). Cell size regulation has been attributed partly to this pathway (Bodine, 2001). Activation and deactivation of the specific signaling proteins depends on phosphorylation or dephosphorylation status. The protein mTOR controls two important downstream signaling proteins involved in the initiation process. Eukaryotic initiation factor 4E binding protein (4E-BP1) acts to inhibit protein translational signaling by

binding with eIF4E. When phosphorylated by mTOR, 4E-BP1 loses its affinity for eIF4E, allowing eIF4E to participate in the formation of the ribosomal complex eIF4F. The second signaling protein controlled by mTOR is p70 ribosomal protein S6 kinase, or p70s6k. P70s6k acts to phosphorylate the S6 subunit of the 40S ribosome involved in the translation initiation (Nadar, 2002).

The second step in this translational pathway involves elongation of the protein chain. Eukaryotic elongation factor 2 (eEF2) is one of two important factors required for this process to successfully occur (Wang, 2006). EEF2 can only participate in the elongation process when it has been dephosphorylated. Activation of eEF2 is controlled by its respective kinase, eEF2k. Phosphorylation of eEF2k allows for eEF2 to become active. Researchers have postulated that p70s6k has the ability to phosphorylate eEF2k on one of its inhibitory sites. This could be indicative of a relationship between signaling proteins found in translation initiation and elongation pathways (Fick, 2007).

*Aging and Protein Synthesis*

Aging may inhibit certain mechanisms within the pathways described above and create a decline in protein synthesis (Welle, 1993; Welle, 1994; Parkington 2004; Bagalopal, 1997; Paturi, 2010). This lower protein synthesis, particularly in type II muscle fibers, is believed to be one of the main contributors for the development of sarcopenia in the elderly (Parkington, 2004; Thomson and Gordon, 2005; Thomson and Gordon, 2006; Paturi, 2010). Moreover, normal stimulation of muscle protein synthesis with muscle overloading declines with age making it difficult for individuals to counteract the effects of sarcopenia (Blough, 2000; Thomson and Gordon, 2005; Thomson and Gordon, 2006). Our investigation will analyze signaling proteins (located downstream from mTOR) involved in the Akt-mTOR pathway (p70s6k, rpS6, eEF2K

and eEF2). An assessment of phosphorylation and abundance status of these signaling proteins, and changes in hypertrophy, will be performed to evaluate the effectiveness of possible intervention strategies (chronic muscle overload and leucine supplementation) used to attenuate sarcopenia.

### *Muscle Overloading and Protein Synthesis*

Overloading skeletal muscle with an external force has been shown to activate skeletal muscle protein synthesis and enhance the development of mixed muscle and myofibrillar proteins (Yarasheski, 1999; Hasten, 2000). Translational proteins demonstrate increased phosphorylation/activation in the initiation stage, particularly with mTOR, 4E-BP1, p70s6k, and rpS6, and dephosphorylation/activation of eEF2k and eEF2 in the elongation stage (Cuthbertson, 2005; Parkington, 2004; Thomson and Gordon, 2006; Kumar, 2009). Increases in muscle cross sectional area, lean muscle mass, and strength are also positive outcomes of chronic muscle overload (Parkington, 2004).

Suppression of hypertrophic responses to chronic resistance training has been demonstrated in aging rats (Blough, 2000; Thomson and Gordon, 2005; Thomson and Gordon, 2006) and humans (Kumar, 2009). Also, measurably smaller activations of mTOR, 4E-BP1, and p70s6k1 have been observed in older muscle when compared to muscles in younger counterparts. Yet these increases do still occur in response to resistance training (Kumar, 2009; Cuthbertson, 2005). It has been suggested that increasing the amount of mechanical overload in skeletal muscle could be used as an intervention to delay, attenuate, or perhaps even reserve the effects of sarcopenia (Kosek, 2006). The effects of muscle overloading on muscle hypertrophy and protein translational signaling in aging muscle will be evaluated in the current study. The effects on aged muscle will be compared to the responses found in adult rats.

### *Supplementation and Protein Synthesis*

Researchers believe that amino acid availability is an important factor for protein synthesis (Hara, 1998; Fujita, 2007). Various ergogenic aids, such as whey protein, essential amino acids, and leucine, have been supplemented to determine if enhancements in muscle protein synthesis can be made above that of normal homeostatic responses to overload. The addition of essential amino acids (EAA) to a normal diet has generated improvements in skeletal muscle protein synthesis (Katsanos, 2008; Paddon-Jones, 2006). Signaling within the synthesis pathway causes mTOR and certain downstream components of translation initiation and elongation (mentioned previously) to become activated (Hara, 1998; Crozier, 2005; Du, 2007). Chronic supplementation of EAA also enhances lean body mass and basal protein synthesis. Specifically, leucine, one key essential amino acid, has proven to alter phosphorylation and activation status of mTOR (Suryawan, 2008). Whole body protein degradation also becomes suppressed with the supplementation of leucine (Koopman, 2006 #2).

Leucine supplementation has exhibited an enhanced effect on muscle protein signaling, specifically in the Akt-mTOR pathway, and fractional protein synthesis rates in the muscles of elderly individuals. Increases in the amount of leucine administered to aged participants have generated positive gains in protein signaling and muscle protein synthesis reaching similar values observed in young participants (Katsanos, 2006). Using this information, we postulate that the addition of leucine to a normal diet will increase the stimulation of muscle protein synthesis in the overloaded muscles of rats.

### *Aims*

To the best of our knowledge, there have been no studies observing the combined effects of leucine supplementation and muscle overloading on translational signaling and protein

synthesis in elderly rodents or human beings. Therefore, the purpose of this study was to examine the effects of dietary leucine supplementation on protein translational signaling and hypertrophy in the overloaded fast-twitch skeletal muscles of aged animals.

*Hypothesis*

It is hypothesized that supplementing a standard chow diet with 5% leucine will enhance muscle hypertrophy in overloaded fast-twitch plantaris muscles of aged (33-month old) rats to levels observed in young adult (8-month old) rats. It is also hypothesized that 5% dietary leucine supplementation will enhance p70s6k, rpS6, eEF2k, and eEF2 signaling in the overloaded fast-twitch plantaris muscles of aged rats to levels observed in young adult rats. If this intervention proves to be successful, it will support the possibility of supplementing leucine during chronic resistance training to enhance gains in muscle mass and strength in elderly humans. This could lead to an improvement in quality of life with age and a decline in progressive ailments associated with age.

# Chapter II: Review of Literature

Sarcopenia is a debilitating disorder caused by an age-associated loss of skeletal muscle (Evans, 1995), primarily in type II muscle fibers (reviewed in Lexell, 1995). Losses in skeletal muscle area reach values as high as 40% for individuals ranging between 20 to 80 years of age (reviewed in Lexell, 1995). Reductions in muscle strength (Frontera, 2000) and contraction quality (Blough, 2000) accompany this disorder and can lead to a reduced ability to function (Dorrens, 2003; Hairi, 2010). Furthermore, a decline in skeletal muscle can lead to a reduced survival rate among individuals suffering from life-threatening illness (Tellado, 1988)

Skeletal muscle fiber atrophy has been attributed to an imbalance in the ratio of muscle protein synthesis to protein degradation (Kimball, 2010). Recognized methods of stimulating hypertrophy, such as muscle overloading, have demonstrated a limited ability to increase protein translational signaling and muscle mass with age (Blough, 2000; Thomson and Gordon, 2005; Chale-Rush, 2009). The signaling protein mTOR and downstream signaling markers (p70s6k, rps6, eEF2k, and eEF2), which are involved in muscle protein translation, have been correlated with the reduced ability to stimulate muscle hypertrophy with age (Thomson and Gordon, 2005; Chale-Rush, 2009).

Leucine, a branched-chain amino acid, has shown promising effects on protein anabolism and translational signaling marker phosphorylation enhancement among young adult rats and humans when combined with EAA (essential amino acids) and other nutrients (Anthony, 2000; Fujita, 2007) or administered independently (Anthony, 2000 #2; Anthony, 2002 #2; Crozier, 2005 Suryawan, 2008). Enhancements in muscle protein synthesis and translational signaling have also been observed in old rats and humans when dosages of amino acids are increased (Dardevet, 2002; Paddon-Jones, 2006). In the current study, we chose to analyze the combined

effects of dietary leucine-enrichment and muscle overloading in an attempt to discover a possible intervention for reversing the age-associated reduction in muscle hypertrophy and muscle translational signaling. To our knowledge, the combined effects of a leucine-enriched diet and chronic muscle overload on muscle hypertrophy and muscle protein translation in old rats have yet to be measured.

### *Muscle Protein Translation Initiation and Elongation Signaling*

One particular protein that plays a significant role in translational signaling is mammalian target of rapamycin (mTOR). In addition to its involvement in protein translation, mTOR has a multitude of functional roles including participation in cell proliferation, apoptosis, and autophagy, (reviewed in Miyazaki, 2009). mTOR is comprised of two distinct isoforms: mTORC1 and mTORC2. A specific binding partner has been identified for each isoform: raptor and rictor, respectively (Wang, 2006). Rapamycin is a specific pharmaceutical inhibitor of raptor binding mTORC1 and, when administered, almost completely prevents any in vivo muscle hypertrophic response to overload (Bodine, 2001). Rapamycin has been utilized to determine whether certain stimuli could control mTOR signaling (Bodine, 2001; Ionki, 2003; Ionki, 2003 #2) and which downstream signaling proteins from mTOR were affected (Redpath, 1996; Wang, 2001; Fingar, 2002; Browne, 2004).

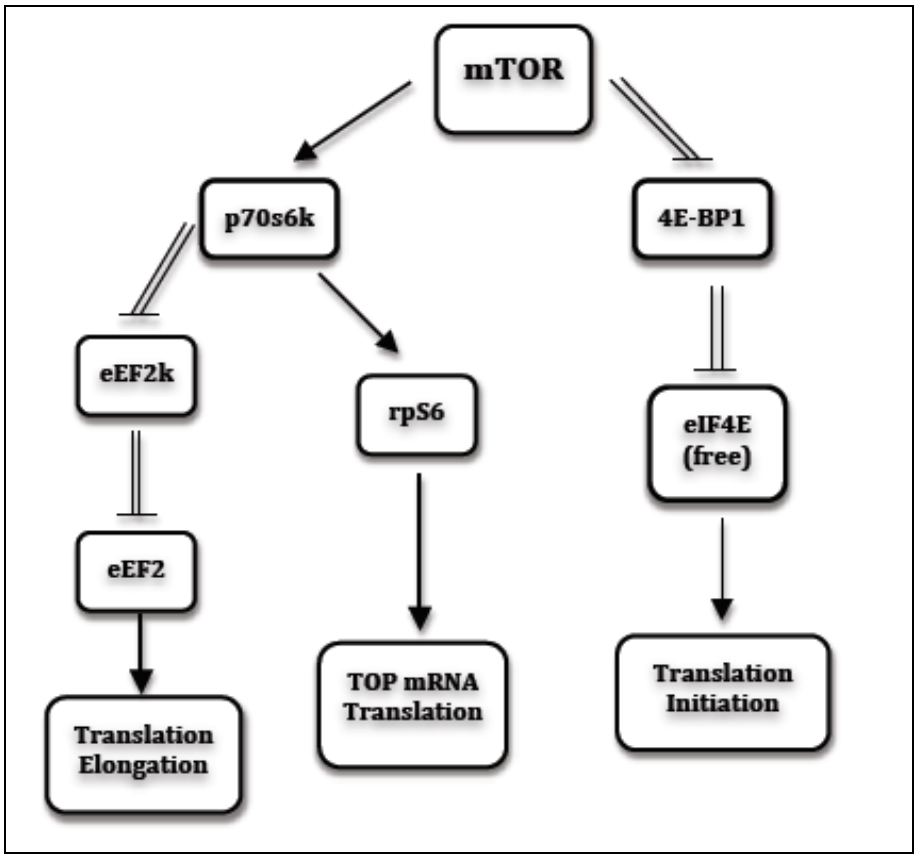


Figure 2.1: mTOR and signaling proteins.

One specific pathway that leads to the activation of mTOR and its downstream signaling proteins is the insulin growth factor-1 (IGF-1) pathway (Figure 2.1). Hormones or growth factors, such as insulin or IGF-1, have been shown to stimulate this pathway. Once activated, phosphoinositide-3-kinase (PI(3)k) phosphorylates and activates Akt (also known as protein kinase B (PKB)) (Rommel, 2001). Akt controls a direct mTOR-inhibiting complex known as tuberous sclerosis protein 1 and 2 (TSC1/TSC2) (Ionki, 2003). From there, TSC1/TSC2 facilitates the hydrolysis of GTP to GDP on Rheb and allows for activation of mTOR (Ionki, 2003 #2). The increased availability of nutrients, such as amino acids, has also been associated with increased phosphorylation of signaling proteins (4E-BP1 and p70s6k) controlled by mTOR (Anthony, 2000; Anthony, 2000 #2; Anthony, 2002 #2; Fujita, 2007; Atherton, 2010). However, nutrient signaling is believed to occur through separate pathways than those controlled by

hormones or growth factors (Hara, 1998; Drummond, 2010). Specific upstream complexes known as Rag A/B and Rag C/D GTPases are believed to contribute to the altered phosphorylation status of 70 kDa ribosomal protein S6 kinase (p70s6k) after amino acids are administered (Kim, 2008). Cellular amino acid transporters LAT1/CD98 (L-type amino acid transporter with and a glycoprotein) and SNAT2 (sodium-coupled neutral amino acid transporter) have also demonstrated an involvement in mRNA expression and mTOR activity (Drummond, 2010). Additionally, human vacuolar protein sorting 34 (hVps34) increased phosphorylation of proteins downstream from mTOR (rpS6 and p70s6k1), without having any effect on proteins immediately upstream (TSC2, PKB/Akt), after amino acids were administered (Nobukuni, 2005).

As mentioned previously, mTOR controls the activity of two signaling proteins involved in the translational initiation and elongation stages of protein synthesis (Figure 2.1). The first of which is eukaryotic initiation factor 4E binding protein 1 (4E-BP1). This protein plays a pivotal role in the formation of the eIF4F complex (composed of eIF4E, eIF4G, and eIF4A) by either preventing or allowing eIF4E to interact with the other two components. mTOR phosphorylates 4E-BP1 and releases the inhibitory bond between 4E-BP1 and eIF4E (reviewed in Kimball, 2010). Although this protein and the processes involved in protein translation initiation are important in synthesizing new proteins, we have chosen to focus our attention on the other protein that is regulated by mTOR; ribosomal protein S6 kinase p70 (p70s6k) (Nadar, 2002). mTOR's control of p70s6k has been observed in vivo (Sprague-Dawley rats) and in vitro (C2C12 myoblasts). Following a treatment of rapamycin, in vivo increases in the phosphorylation of p70s6k in response to exercise (Bodine, 2001), as well as in vitro in C2C12 myoblasts (Rommel, 2001) were attenuated.

P70s6k is phosphorylated and activated by mTOR on residue Thr389 (Nadar, 2002). Although p70s6k controls many aspects of cellular activity (reviewed in Magnuson, 2012), our focus is specifically on the two components p70s6k regulates for cellular growth: ribosomal protein S6, rpS6 (Ruvinsky, 2005), and eukaryotic elongation factor 2 kinase, eEF2k (Browne, 2002). Similar to mTOR, increases in phosphorylation of p70s6k occur in vivo (Parkington, 2004; Thomson and Gordon, 2005) in the overloaded muscles of rats. Increases in p70s6k phosphorylation were also observed following an acute bout(s) of resistance training in humans (Eliasson, 2006; Dreyer, 2006; Dreyer, 2010; Terzis, 2010) and increases in response to resistance training stimuli were dose-dependent (Terzis, 2010). mTOR has facilitated the phosphorylation of a downstream signaling protein, 40S ribosomal protein S6 through p70s6k (Nadar, 2002; Wang, 2006). The exact roles of ribosomal protein S6 are still unclear. One function that is still up for debate is the possibility that rpS6 assists in upregulating 5'-terminal oligopyrimidine (5'-TOP) sequences, which participate in coding for translational machinery (Jefferies, 1997; Magnuson, 2005). However, it has been postulated that p70s6k-rpS6 pathway is not the only means of upregulating 5' TOP mRNA (Jefferies, 1997; Ruvinsky, 2005). Regardless, it is clear that rpS6 does participate in the regulation of cell size (Ruvinsky, 2005), which is why rpS6 was chosen as one of the signaling markers of protein translation to analyze in this study.

The intermediate stage of muscle protein translation is elongation. This process involves two eukaryotic elongation factors: eEF1 and eEF2 (reviewed in Wang, 2006). The elongation process actually generates the bulk of the protein chain. eEF2 is a monomer and will be the focus of this study. eEF2 is active when bound to GTP, and in the absence of phosphorylation, catalyzes the translocation process necessary for protein elongation (Jorgensen, 2006).

Hydrolyzation of the inorganic phosphate on GTP yields GDP and provides the energy necessary for this process to occur (Proud, 2007). eEF2 kinase is a calcium/calmodulin-dependent, regulatory kinase that controls eEF2 activity by phosphorylating eEF2 at Thr56 and thus inhibiting eEF2 (Browne, 2002). With regard to translational elongation, mTOR controls the phosphorylation and activity of eEF2 kinase at three separate residues (Ser78, Ser359, Ser366) (Proud, 2007). Phosphorylation of eEF2k on Ser78 is directly attributed to the hormone stimulated mTOR pathway, but independent of p70s6k (Browne, 2004). eEF2 kinase is phosphorylated (in vitro) by p70s6k on residue Ser366. This phosphorylation results in the inactivation of eEF2 kinase (Wang, 2001; Browne, 2002). General inhibition of elongation occurs when eEF2 kinase phosphorylates eEF2 at Thr56 (Redpath, 1993). The addition of insulin has caused a decrease in eEF2k activity, decline in eEF2 phosphorylation to very low values immediately, and increased the transient time of elongation in vitro in Chinese hamster ovary cells. These resulting effects were suppressed following rapamycin treatment signifying a controlling effect over eEF2k from the mTOR pathway (Redpath, 1996). Acute resistance bouts in human participants also led to a decline in eEF2 phosphorylation 1-2 hours post-exercise (Dreyer, 2010). Chronic overload in rats also results in a decline in signaling status (phosphorylation-to-total concentration) for eEF2 as well (Thomson and Gordon, 2005). These data indicate that muscle overload likely affects elongation, in part by eEF2 activation.

The following sections will analyze how translational signaling proteins previously discussed are affected by muscle overloading and/or amino acid supplementation. Furthermore, the effects of aging on signaling responses to these stimuli will also be assessed.

### *Muscle Overloading and Resistance Exercise*

Skeletal muscle metabolism can be affected in a variety of ways when an external force (i.e. muscle overloading models and resistance exercise) is applied to the body. In order to quantify homeostatic responses in muscle protein synthesis and translational initiation and elongation signaling to these external forces, one must assess variables such as frequency (acute vs. chronic), duration (time), intensity (percentage of 1RM, VO2max, etc.), type and percentage of contraction (isometric vs. concentric vs. eccentric; maximal vs. submaximal, respectively), etc. This section will focus on differing modes of muscle overloading and resistance exercise in rodent and human subjects of various ages. The primary foci will be: 1) muscle protein synthesis rates, and 2) quantification of the phosphorylation status/total abundance of signaling markers associated with the Akt/mTOR pathway.

Translational signaling markers found along the Akt/mTOR pathway have shown dramatic changes in response to acute resistance exercise. During 1- and 2- hours post-exercise, young adult participants increased in phosphorylation of Akt/PKB$^{Ser473}$, mTOR$^{Ser2448,}$ p70s6k$^{Thr389}$, and decreased in phosphorylation for eEF2$^{Thr56}$ (Dreyer, 2006). The pharmaceutical inhibitor rapamycin has been utilized to verify mTOR's association with muscle protein synthesis in response to an acute bout of resistance exercise in young humans (Drummond, 2009). Increases in p70s6k$^{Thr421/Ser424}$ and decreases in eEF2$^{Thr56}$ phosphorylation attributed to resistance exercise were attenuated following rapamycin administration and corresponded with a ~40% reduction in muscle protein synthesis (Drummond, 2009). These data validated the correlation between skeletal muscle protein synthesis and mTOR signaling. An important factor to consider was the possibility of gender differences in response to resistance exercise. Among male and female participants performing acute resistance exercise (10 sets of

10 reps at 70% 1RM), no variability was observed in mixed muscle protein synthesis (males, 52%; females, 47% increase at 2-hr post-exercise above baseline values). Furthermore, increases in phosphorylation of mTOR$^{Ser2448,}$ p70s6k$^{Thr389}$ and decreases in phosphorylation for eEF2$^{Thr56}$ were not different at 1- and 2-hrs post-exercise between males and females, indicating that there were no acute gender differences among young humans (Dreyer, 2010).

The incorporation of progressively increasing load volumes during acute resistance exercise has demonstrated variable effects on signaling protein phosphorylation and potential activation (Terzis, 2010). Additional sets of repetitions of leg presses revealed increased phosphorylation of p70s6k$^{Thr389}$ and rpS6 $^{Ser235/236}$ at 30 minutes post-exercise for 3 and 5 sets of 6 maximum repetitions (3-fold, 5-fold; 30-fold, 55-fold, respectively)(Terzis, 2010). Interestingly, no alterations occurred to the phosphorylation status of mTOR$^{Ser2448}$, possibly signifying that increasing load volume may not alter p70s6k and rps6 phosphorylation specifically through mTOR activation. Acute resistance exercise also presented inconsistencies in the degree of phosphorylation for translational signaling markers between muscle fibers. Koopman et al (2006) demonstrated that type I and II muscle fibers responded to a bout of upper and lower body resistance exercise, with a larger increase in type II fibers for the phosphorylation of p70s6k$^{Thr421/Ser424}$ within the first 30 minutes post-exercise (Koopman, 2006). During that time frame, rpS6 showed no significant increases correlating with those of p70s6k; however, the phosphorylation of rpS6 via mTOR is controlled on by p70s6k$^{Thr389}$ (Ruvinsky, 2005). Important to note were the effects that various types of muscle contractions had on protein synthesis and translational signaling. Maximal eccentric contractions demonstrated the most promising effects, with the greatest increases in phosphorylation of p70s6k$^{Thr389}$, p70s6k$^{Thr421/Ser424}$, and rp$^{S6Ser235/236}$ between 1- and 2-hr post-exercise. Maximal concentric and

submaximal eccentric contractions had very little, if any, effect on acute signaling markers mentioned previously (Eliasson, 2006). These data suggested the additional possible involvement of a signaling pathway that bypass mTOR when resisted contractions are presented as a stimulus. Muscle protein synthesis and translational signaling can also respond differently to different intensities and modes of exercise. Anaerobic cycling for 120 seconds at 110% of young male participant's $VO_2$max resulted in an increased ratio of phosphorylated-to-total eEF2 (2.3-fold) above rest. This response was accompanied by a shift in eEF2. At rest, phosphorylation of eEF2 was 55% greater in type I muscle fibers. Following exhaustive exercise, type II fibers exhibited 55% higher phosphorylation of eEF2 (Rose, 2008). Recall that eEF2 participates in the elongation stage of translation when dephosphorylated (Proud, 2007). With high intensities, type II fiber eEF2 phosphorylation was suppressed immediately post-exercise. However, acute low intensity, aerobic cycling (35%, 60%, and 85% of $VO_2$max) did not show significant differences in elongation signaling (Rose, 2008).

Previous research has been performed to analyze age- (Paturi, 2010) and gender-related (Smith, 2008) differences in phosphorylation and abundance of signaling markers at rest. Paturi et al (2010) measured resting phosphorylation and abundance status in extensor digitorum longus (EDL) muscles of adult, aged, and very aged male and female (6, 30, 36 month; 6, 26, 30 month, respectively) Fischer$^{344}$ x Brown Norway rats (Paturi, 2010). Values for Akt, mTOR, and eEF2 total abundance were higher (38%, 182%, 34%) and rpS6 was lower (14%) in very aged rats when compared to adult rats. However, phosphorylation was significantly lower among signaling proteins downstream from mTOR (p-p70s6k$^{Thr389}$, 14%; p-rpS6$^{Ser235/236}$, 21%; p-4E-BP1$^{Thr37/46}$, 24/25%; p-eEF2$^{Thr56}$, 75%) in very aged rats compared to adult rats (Paturi, 2010). A comparison between very aged males and females revealed a significantly higher

phosphorylation status in all signaling markers previously mentioned for females at rest (Paturi, 2010). In a similar study among aged humans (65-80 yr), resting values for muscle protein synthesis and the phosphorylation status of specific signaling markers (p70s6k$^{Thr389}$ and eEF2$^{Thr56}$) favored fasted females (MPS, 30% greater, eEF2$^{Thr56}$, ~40% less) (Smith, 2008). However, following the consumption of a standard meal (15% protein, 55% carbohydrate, 30% fat), aged males solely demonstrated an increase in muscle protein synthesis (and previously mentioned signaling markers) equivalent to the values observed in their female counterparts (Smith, 2008). These data suggested that a decline in phosphorylation, not expression, of signaling markers of the Akt/mTOR pathway existed during resting condition in aged rats, specifically in males (Paturi, 2010), and that feeding can restore protein synthesis in males to the level observed in aged-matched females (Smith, 2008). This lower level of resting muscle protein synthesis may account for the swifter progression of sarcopenia among males when compared to females (Janssen, 2000).

Researchers have developed and implemented various overload models in rodents that mimic acute and chronic resistance training in humans. These methods have been utilized to study abundance and phosphorylation of translational signaling markers and muscle protein synthesis, as well as, to identify differences in responses to these stimuli between young and old rodents. High frequency electrical stimulation (HLES) is a common model of muscle overload performed in vitro. Adult (6 mo) and old (30 mo) Fischer344 x Brown Norway rats were subjected to a single session of 10 sets of 10 repetitions of HLES to determine the impact that acute in vitro muscle stimulation (via neuromuscular innervation) had on translational signaling markers mTOR and p70s6k (Parkington, 2004). Of the muscles analyzed (tibialis anterior, TA; plantaris, PLT), neither showed age-related differences in mTOR or p70s6k total abundance.

However, a muscle-specific (TA only) increase in mTOR phosphorylation was reported with age (Parkington, 2004). Furthermore, an observed decline of 50% in post-HFES maximal phosphorylation of mTOR and p70s6k occurred during the 6 hours following the end of stimulation in aged rats. It appeared that an acute post-stimulation diminishment exists in the ability to phosphorylate signaling markers (Parkington, 2004).

Ablation of the synergist muscle(s) of the hind limbs of rats (i.e. gastrocnemious) is another commonly used model for chronic overload (Thomson and Gordon, 2005; Thomson and Gordon, 2006; Chale-Rush, 2009). Chale-Rush et al incorporated a bilateral synergist ablation or sham surgery on young (6 mo) and old (33 mo) Fischer$^{344}$ x Brown Norway rats for 28 days. Following normalization for body weight, the overloaded plantaris muscles of young and old rats that underwent synergist ablation were 35% and 20% heavier than the plantaris muscles of control rats. Despite increases in muscle weight, a 15% greater amount of hypertrophy was achieved in the young rats when compared to the old. At the end of the study (28 days), expression of mTOR, p70s6k, rpS6 and 4E-BP1 was unaffected by age or overload. However, mTOR and rpS6 phosphorylation was greater (44%, 35%; 114%, 24%) in overloaded young and old plantaris muscles over the controls, respectively (Chale-Rush, 2009). Interestingly, age-related differences in signaling protein phosphorylation within the overloaded group were no longer significant after 28 days (significant differences were present after 7 days) (Chale-Rush, 2009).

In a similar study, young adult (8 mo) and old (30 mo) Fischer$^{344}$ x Brown Norway rats underwent 1-week unilateral synergist ablation of the gastrocnemious muscle to determine the effects of aging on muscle hypertrophy (Thomson and Gordon, 2005). Again, increased muscle wet weight for both young adult and old rats were above that of the intra-specimen sham

(control) muscles. Nevertheless, old rat plantaris muscles still exhibited hypertrophy to a lesser extent when compared to control muscles than did their younger counterparts (Thomson and Gordon, 2005). After only 7 days of overload, mTOR phosphorylation was substantially higher in young vs. old adults (292% vs, 88%, respectively) (Thomson and Gordon, 2005). Thomson and Gordon utilized the same experimental protocol previously discussed (Thomson and Gordon, 2005) to quantify the phosphorylation-to-total concentrations (labeled signaling status) of signaling proteins Akt, mTOR, p70s6k, rpS6, eEF2, and 4E-BP1 in young and old rats (Thomson and Gordon, 2006). Signaling statuses for mTOR, p70s6k, rps6 and 4E-BP1 showed increases in both young adult and old overloaded rats when compared to their age matched controls. Also, Akt (old rats only) and eEF2 statuses showed marked decreases. Important to note is that these increased and decreased phosphorylation statuses were significantly greater in young adult rats, indicating that there was a signaling deficit in with age in response to muscle overload. Additionally, a correlation between absolute p70s6k phosphorylation and muscle hypertrophy was established from this study (Thomson and Gordon, 2006). When the duration of muscle overloading was increased, Blough et al observed greater gains in contractility, whole muscle fiber (plantaris) and muscle fiber cross sectional area (CSA) over an 8-week time period in adult (8.5 month) rats over the age matched controls (Blough, 2000). On the other hand, overload alone was not able to rescue the hypertrophic response of whole plantaris muscle or individual muscle fiber cross-sectional areas in old (38 month) rats, and actually showed declines in the CSA of type I, IIA, and IIX/IIB (31, 35, 39%) muscle fibers, respectively (Blough, 2000). There is an apparent inability to stimulate muscle protein synthesis and the phosphorylation of specific translational signaling markers in old rats to the extent produced in young/adult rats (Parkington, 2004; Thomson and Gordon, 2005, Thomson and Gordon, 2006; Chale-Rush, 2009). Whether

the deficiencies observed in these elements solely contribute to the inability to stimulate an anabolic response, or whether they work in conjunction with other mechanisms, has yet to be determined.

In terms of exercise intensity, different acute dosages can cause variance in signaling marker phosphorylation in humans, especially when age is a variable. Twenty-five young and 25 old fasted men performed an acute bout of leg extension and flexion exercise at intensities ranging from 20% to 90% of the participant's 1RM with corresponding sets and repetitions (i.e. 20% 1RM, 3 sets x 27 reps; 75% 1RM, 3 sets x 8 reps; etc.) (Kumar, 2009). A relationship was established between the intensity of resistance training and myofibrillar muscle protein synthesis (MPS), where gradual increases were generated from 20% to 60%, followed by a plateau at ≥75%. Gains in MPS were 30% greater among young subjects than in old (Kumar, 2009). Maximal phosphorylation of 4E-BP1 and p70s6k was observed during 1-hr post-exercise and these increases were correlated with the increase in MPS, although only among young participants. eEF2 phosphorylation was unaffected by any variability in age or intensity (Kumar, 2009). It was apparent that acute in vivo stimulation of MPS and the correlating phosphorylation of muscle protein translation initiation proteins were depressed or blunted in aged humans.

Chronic resistance training has demonstrated variability in its stimulatory effects on muscle hypertrophy amongst young and old humans. Kosek et al (2006) examined the effects of a chronic 16-weeks resistance-training regimen performed 3 days per week using 3 knee extensor exercises at 3 sets for 8-12 reps per set (Kosek, 2006). The chronic cycle of resistance training generated increases in type I (18%) and II (32%) fiber cross-sectional area (CSA) among young males and females (20-35 yrs). Type I fibers showed no increases in CSA, while type II fibers exhibited limited increases (23%) in CSA among older subjects (60-75 yrs) (Kosek, 2006).

Furthermore, a transition from type IIx fibers to type IIa fibers was observed in all groups (females 47.4+/-1/7 to 60+/-2.3%; and males 49.8 +/-2.3 to 65.3 +/- 2.5%). One positive result is that older individuals were able to stimulate growth in type II fiber CSA after 16-weeks of exercise to the baseline level of younger subjects indicating that there is the potential for muscle growth. However, the hypertrophic response was still not as dramatic as that observed in younger humans at the end of the training period (Kosek, 2006). These data contradict those of Blough, 2000 stating that declines were actually observed in muscle fiber CSA follow 8-weeks of muscle overloading in aged rats (Blough, 2000). Longer duration of resistance training and variability in the mode used to cause physical stress on the muscles may account for these differences. From the data discussed, it is clear that aging produces a desensitization to muscle overloading as a stimulus for skeletal muscle hypertrophy and translational signaling. Researchers have examined nutritional supplementation as a possible intervention for sarcopenia. The findings will be discussed at length in the following section.

*Supplementation of Whey Protein and Amino Acids*

Nutritional supplementation has been shown to act as a catalyst by enhancing muscle protein synthesis above the stimulatory effects observed in the fasted state (Burd, 2010). Recent focus has been centered around the addition of dietary whey protein (Paddon-Jones, 2006; Rieu, 2006; Burd, 2010,) and the amino acids that comprise this ergogenic aid (essential and non-essential amino acids)(Dardevet, 2000; Dardevet, 2002; Guillet, 2003, Paddon-Jones, 2004; Katsanos, 2006; Paddon-Jones, 2006; Fujita, 2007; Atherton, 2010; Katsanos, 2008; Dickinson, 2011) in an attempt to determine which specific component(s) contributed to the stimulatory effect on muscle protein synthesis.

An instrumental experiment performed by Volpi et al quantified protein synthesis and degradation kinetics among differing age groups (Volpi, 2001). Kinetic markers for protein synthesis and breakdown were examined in the vastus lateralis of fasted young (28+/-2yr) and elderly (70+/-1yr) male humans without dietary manipulation. Among participants, net muscle protein balance was equal. Remarkably, muscle protein synthesis was higher in elderly males. Researchers speculated that equal values in net protein balance, rather than values favoring the elderly group, were a product of the equally high degradation levels among elderly participants (Volpi, 2001). In a separate study, gender differences where quantified in the fasted- and fed-states among aged humans (Smith, 2008). Aged females in the postabsorptive state exhibit a greater percentage of basal (fasted) muscle protein synthesis (~30%) than those values observed in aged males (Smith, 2008). These differences are not observed in p70s6k$^{Thr389}$ phosphorylation at basal levels. Following the consumption of 15 small liquid meals (15% protein, 55% carbohydrate, 30% fat) over 150 minutes, increases in p70s6k phosphorylation occurred to the same extent in both gender groups. However, increases in MPS were only found to exist in males (Smith, 2008). From these data, it was evident that the consumption of a meal acted to increase muscle protein synthesis and exhibited greater values for muscle protein synthesis measured in the fasting state. It is important determine whether one, or a combination of components, found in a standard meal generated these observed increases in muscle protein synthesis and translational signaling.

Proteins are comprised of amino acids, which are classified as either essential (EAA) or non-essential amino acids (NEAA), and can be supplemented together (i.e. in whey protein) or separately (Katsanos, 2008). Amino acids administered via intravenous infusion to young human participants in the postabsorptive state have demonstrated an initial delay or latency

period of ~30 minutes, followed by a dramatic increase (2.8-fold; 0.08+/- 0.01 (basal) to 0.21+/- 0.07 (30-60min) to 0.24+/-0.04%/hr (60-120 min)) in mixed muscle protein synthesis between 30 and 120 min (Bohe, 2001). These data suggested that amino acids administered in vivo could facilitate increases in muscle protein synthesis, although there was a delay in the initiation of protein synthesis. To further discern which specific component(s) possess anabolic properties, amino acids were separated into their respective EAA and NEAA groups and measured in vitro and in vivo in order to determine their anabolic properties on protein synthesis and accrual (Atherton, 2010; Katsanos, 2008). Thirty minutes of in vitro incubation of specific amino acids to C2C12 myocytes revealed that neither a combination of nonessential amino acids (NEAA), nor the branched-chain amino acids (BCAA) valine or isoleucine, acted as stimuli for phosphorylating initiation signaling markers and muscle protein synthesis (Atherton, 2010**).** On the contrary, in vitro incubation with essential amino acids (EAA) and leucine independently caused dramatic increases in the phosphorylation of $mTOR^{Ser2448}$, $4E\text{-}^{BP1Thr37/46}$, $rpS6^{Thr235/236}$ and especially $p70s6k^{Thr389}$ (p-p70s6k: EAA, 1.6-2.0 fold increase; leucine, 5.9+/-0.5 fold increase) (Atherton, 2010). Administration of NEAA (7.57g) in vivo has shown no affect on protein accumulation at 3.5 hr post-ingestion among elderly humans (Katsanos, 2008). Although EAA (6.72 g) demonstrated an increase in protein accrual, these values could not compare to the increases observed when whey protein was supplemented (15 g) (Katsanos, 2008).

The exact signaling pathway(s) by which EAA elicits such effects remains unclear, but stimulation of either mTOR pathway signaling marker phosphorylation or muscle protein synthesis has been observed following the administration of EAA (Atherton, 2010). Cellular transport allows for essential components of protein synthesis, such as amino acids, to cross the cell membrane and become incorporated in the production of proteins. Skeletal muscle amino

acid transporters LAT1/CD98 and SNAT2 have been associated with acute mTOR pathway stimulation (via phosphorylation of rpS6$^{Ser240/244}$) at 1-hr post-administration of 10 g of a mixture of essential amino acids (Drummond, 2010). Acute resistance exercise has caused equivalent expression and upregulation of the fore-mentioned transporters along with others (LAT1/SLC7A5, SNAT2/SLC38A2, CD98/SLC3A2, PAT1/SLC36A1, and CAT1/SLC7A1), in young (28+/-2yr) and old (68+/-2 yr) humans (Drummond, 2011). Although neither expression nor upregulation of these amino acid transporters was significantly different between age group, mTOR associated stimulation previously observed (Drummond, 2010) in the form of phosphorylation of rpS6$^{Ser240/244}$ was substantially lower in older humans (Drummond, 2011). Desensitivity to amino acid stimulation with age (Paddon-Jones, 2004) could be associated with cellular transporters lacking the ability excite activity associated with translational signaling and muscle protein synthesis. For that reason, we chose to also evaluate the effectiveness of nutrients (specifically, the amino acid leucine) on stimulating increases in phosphorylation and abundance of downstream mTOR signaling markers, as well as muscle hypertrophy. Lack of excitation by nutrients, combined with a reduced ability of muscle overload/resistance exercise to generate anabolic responses in translational signaling and muscle protein synthesis in old rats and humans, equivalent to that of young, could indeed be the major cause for the decrements in muscle mass and quality with age.

A key study by Dickinson et al also helped to provide clarity on this issue. The specificity of EAA on mTOR signaling was demonstrated in fasted young human participants following an acute administration of an essential amino acid solution (Dickinson, 2011). As expected, muscle protein synthesis and phosphorylation of mTOR$^{Ser2448}$, 4E-$^{BP1Thr37/46}$, and p70s6k$^{Thr389}$ increased dramatically at 1-hr post-administration. These effects on muscle protein synthesis and the

phosphorylation of mTOR$^{Ser2448}$ and p70s6k$^{Thr389}$ were completely attenuated following consumption of 16 mg of rapamycin. Elongation signaling marker eEF2 was unaffected by either EAA or rapamycin treatment (Dickinson, 2011). EAA have been combined with other nutrients such as carbohydrates (CHO) to determine whether any additional benefits could be produced. Fujita et al combined a CHO mixture (0.5g/kg·fat free mass) with a mixture of leucine-enriched EAA (0.35 g/kg·FFM; 35% leucine) and measured the metabolic changes in young fasted males consuming the mixture over the control (did not consume any nutrients) (Fujita, 2007). Translational signaling markers upstream from mTOR (Akt/PKB$^{Ser473}$, TSC2$^{Thr1462}$) showed no differentiation between groups. Phosphorylation of mTOR$^{Ser2448}$, 4E-BP1$^{Thr37/46}$, and p70s6k$^{Thr389}$ demonstrated a remarkable increase, while eEF2$^{Thr56}$ decreased in phosphorylation from the control group. The combination of carbohydrates and leucine-enriched amino acids proved to have a positive impact on stimulating translation initiation signaling (Fujita, 2007). The lack of stimulation of signaling markers upstream from mTOR may signify that amino acids utilize an alternate pathway to phosphorylate mTOR other than the Akt/mTOR pathway.

The amino acid leucine has proven to act as a catalyst for protein anabolism and translational signaling marker phosphorylation enhancement among young adult rats and humans when combined with EAA and other nutrients (Anthony, 2000; Fujita, 2007) or administered independently (Anthony, 2000 #2; Anthony, 2002; Crozier, 2005 Suryawan, 2008). An acute study by Anthony et al demonstrated the anabolic effects of leucine (both alone and in combination with other nutrients)(Anthony, 2000). Food-deprived Sprague-Dawley rats were given 24 hr access to either saline, 100% CHO (235.5 g/L glucose and 235.5 g/L sucrose), 100% leucine (54.0 g/L), or a combination of CHO and leucine (Anthony, 2000). Leucine alone

generated a 16-fold increase in serum leucine concentration compared to the 5.5-fold increase gained by combining CHO and leucine. Furthermore, increased phosphorylation of 4E-BP1 and p70s6k occurred in both the leucine and CHO + leucine groups above the control group, while increased association of eIF4E with eIF4G was only attributed to leucine supplementation. Interestingly, neither the consumption of leucine alone nor CHO + leucine diet further enhanced protein synthesis above values measured in control rats (leucine, 1.62+/-0.11 mg/hr; CHO+leucine, 1.64+/-0.11 mg/hr; control rats, 1.83+/-0.11 mg/hr) (Anthony, 2000). In an attempt to solidify the theory that leucine has the most profound effect on muscle protein synthesis and translational signaling, all three BCAA were evaluated for their effects on mTOR signaling. Four groups of male Sprague-Dawley rats were given either 1) saline (control), 2) valine (1.35 g/kg·bw), 3) isoleucine (1.35 g/kg·bw), or 4) leucine (1.35 g/kg·bw) after 18 hours of food deprivation (Anthony 2000). Researchers concluded the leucine alone promoted translational initiation by enhancing 4E-BP1 phosphorylation (5-fold increase), 4E-BP1-eIF4E complex dissociation (17%), eIF4G-eIF4E interaction (4-fold increase), and p70s6k$^{Thr389}$ phosphorylation. Clearly, the addition of leucine stimulated translational initiation signaling in vivo in rat specimens beyond any other BCAA. Variations in supplementation dosage had a similar effect to variations in training volume, as discussed previously. Crozier et al examined the efficacy of dosages ranging from 5% (0.068 g L-leucine/L H2O) to 100% (1.35g L-leucine/L H2O) leucine in young male Sprague-Dawley rats and compared them to control rats consuming 0.155 mol/L NaCl at 2.5 mL/100g·bw (Crozier, 2005). Increases in total mixed muscle protein synthesis rate correlated with rising dosages 30 min post-administration in the gastrocnemius and plantaris muscles (10, 25, 50, 100%; 31, 30, 37, 43, respectively). Amplification of phosphorylation for some signaling proteins was observed with concentrations as low as 5% (4E-

BP1), 10% (eIF4E-eIF4G association), while p70s6k phosphorylation occurred at all dosages (Crozier, 2005).

To insure that these effects on mTOR signaling are a true product of leucine, rapamycin was given in addition to the amino acid in vivo to rats (Anthony, 2000 #2) and neonatal pigs (Suryawan, 2008). The infusion of rapamycin with leucine did not affect signaling proteins upstream from mTOR. However, rapamycin did cause destabilization of the raptor-mTOR complex and inhibition of $mTOR^{Ser2448}$, $4E\text{-}^{BP1Thr37/46}$, and $p70s6k^{Thr389}$ phosphorylation that was originally enhanced by leucine. Also worth mentioning is that total abundance of each of these signaling marker was not affected by rapamycin (Suryawan, 2008). Among rats, rapamycin plus leucine caused muscle protein synthesis stimulation to be suppressed when compared to leucine alone. Additionally, leucine-dependent signaling activity was greatly diminished with the inclusion of rapamycin (Anthony, 2000 #2). Once again, these data demonstrated the involvement of mTOR in EAA stimulation of muscle protein synthesis.

It was evident that leucine had the largest effect on mTOR signaling among amino acids. However, the question arose as to whether increases in leucine coincided with increases in hormone levels, such as insulin, which could play a part in the increased activation of signaling marker, which lie upstream from mTOR along the IGF-1/PI3/Akt/mTOR pathway, and muscle protein synthesis. To determine if leucine indeed acts alone in the stimulation of muscle protein synthesis apart from increases in serum insulin, Anthony et al administered either 1) saline, 2) leucine, or 3) leucine and somatostatin (an insulin inhibitor) to young male Sprague-Dawley rats (Anthony 2002). Leucine did generate increases in muscle protein synthesis (at 30 and 60 min post-administration) along with increased translation initiation signaling marker phosphorylation (4E-BP1, $p70s6k^{Thr389}$, rpS6) above baseline values. However, the affects of leucine were

partially (4E-BP1) or fully (p70s6k$^{Thr389}$, rpS6) attenuated in the presence of somatostatin (Anthony, 2002). These reduced in vivo responses revealed that insulin may be required to work in conjunction with amino acids, particularly leucine to generate increases in muscle protein synthesis. Other studies have produced conflicting results. One study showed that insulin increases proportionally to the dosages of leucine being administered (Crozier, 2005), while others have demonstrated that increases in leucine dosages (in addition to EAA) were independent of increases in serum insulin (Katsanos, 2006). Insulin may have been required to stimulate translational signaling and muscle protein synthesis, although proportional increases that correlate with increased amino acids administered may not be necessary.

Desensitization to nutrient supplementation has been observed with increasing age in regards to translational signaling (Dardevet, 2000), muscle (Dardevet, 2000; Dardevet, 2002; Paddon-Jones, 2004) and myofibrillar protein synthesis rates (Guillet, 2003), muscle fiber composition (CSA), and whole muscle strength (1RM) (Verhoeven, 2009). Age-ranged comparative analyses were conducted on rats and humans supplementing nutrients in vitro and in vivo in an effort to determine the cause(s) and possible interventions for these decrements. A study performed by Dardevet et al examined age differences in response to in vitro leucine administration. The epitrochlearis muscles of young (4-5 weeks), adult (6-8 mo) and old (20 mo) Wistar rats were incubated in a medium representing 1) arterial postasbsorptive amino acid status, or 2) postprandial amino acid status for 2 hr. Stimulatory effects of these two mediums on muscle protein synthesis were compared to epitrochlearis muscle incubated in dose-dependent leucine medium (Dardevet, 2000). In young and adult rats, leucine (200μmol/L) maximally stimulated in vitro protein synthesis to the same extent as the medium containing postprandial quantities of all amino acids. Older rats required twice the concentration of leucine (~400

μmol/L) for maximal in vitro protein synthesis stimulation and p70s6k phosphorylation (Dardevet, 2000). The addition of inhibitors LY294002 and rapamycin were utilized to measure the pathways involved in amino acid signaling. LY294002 (inhibitor of PI(3)K) significantly decreased basal protein synthesis and attenuated amino acid-induced muscle protein synthesis. Rapamycin (inhibitor of mTOR) had no affected on basal protein synthesis, but attenuated both in vitro amino acid- and leucine-induced muscle protein synthesis indicating the involvement of an alternate stimulatory pathway for mTOR other than through IGF-1/PI(3)K/Akt (Dardevet, 2000). When these data are compared with that of studies previously discussed, it is apparent that leucine controls the stimulation of muscle protein synthesis through either direct or indirect association with mTOR regardless of age (Anthony, 2000 #2; Dardevet; Suryawan, 2008) although a larger quantity of leucine must be supplemented (at least in vitro) with advanced aging (Dardevet, 2000).

In an attempt to rectify possible in vivo age-related deficits that exist in muscle protein synthesis, two studies incorporated amino acid-enriched diets of variable dosages in rats and evaluated the effects (Dardevet, 2002; Guillet, 2003). Adult (8 mo) and old (22) Wistar rats were given 1-hr access to either an alanine- or leucine-enriched diet (twice the normal postprandial concentration) administered in meal form and compared to rats in the postabsorptive state. Although leucine concentrations in the plasma increased in the leucine-enriched diet over that of the postprandial alanine-enriched diet, no further increases were observed in muscle fractional synthesis rate (FSR) or total synthesis rate (ASR) in adult rats. Comparing the effects of the postprandial alanine- and leucine-enriched diets, old rats exhibited substantial increases in both FSR and ASR (FSR values: gastrocnemius post-absorption (PA) 4.15+/-0.11 %/day vs. postprandial (PP)+Leu 4.94+/-0.22%/day; and soleus PA 7.75+/-0.23%/day vs PP+Leu 8.89 +/-

0.21%/day) from only the leucine-rich diet above that of the control diet between 90-120 min post-feeding (Dardevet, 2002). In a similar experiment (Guillet, 2003), adult (8 mo) and old (22 mo) Wistar rats consumed either an alanine-enriched (44.5 g/kg DM of alanine) or a leucine-enriched (44.5 g/kg DM of leucine) diet for 1 hr, and the effects were compared to those found in rats in the postabsorptive state (following a 17 hr fast) (Guillet, 2003). Measurements of mitochondrial, sarcoplasmic, and myosin heavy chain FSR revealed no variability between age groups in the postabsorptive state. However, between 90-120 min post-feeding, the old rats in the leucine diet group showed a lesser ability to synthesize MHC proteins compared to adult rats in the same group. Researchers attributed the lack of stimulation in producing these contractile elements to the declines in muscle function and ultimately muscle atrophy (Guillet, 2003).

Increased dosages of EAA (~15 g) have demonstrated enhanced muscle protein synthesis among elderly males and females (Paddon-Jones, 2006), above the values produced from supplementing 6.72 g of EAA normally found in 15 g of whey protein (Paddon-Jones, 2006; Katsanos, 2008). To simulate the acute in vivo effects of a single meal, healthy young and elderly male and female human participants consumed a single 15 g bolus of EAAs following a 12 hr fast. Although both groups demonstrated an increase in mixed muscle FSR, postprandial increases for elderly participants were much more gradual as determined by a positive net phenylalanine balance in comparison to values observed in young (Paddon-Jones, 2004). Net phenylalanine uptake peaked at 30 min in young (102.0 +/-6.0 mg Phe/leg) with a dramatic decline thereafter. This peak did not occur until 60 min post-EAA for elderly (155.3+/-19.4 mg Phe/leg), but declined at a muscle slower rate and remained higher than their young counterparts (Paddon-Jones, 2004). However, when analyzing the quantities of phenylalanine in the intracellular pool, elderly also are incorporating much less for protein anabolism than young

(150.2+/-19.4 nmol/mL vs. 115 +/- 5.4 nmol/ml, intracellular, respectively). From these data, researchers believed that a time frame of 60 to 120 min existed post-EAA administration in which elevated plasma levels can stimulate protein synthesis. Clear evidence indicated that higher dosages of leucine were required to stimulate muscle protein synthesis and translation signaling marker phosphorylation (Dardevet, 2000). Along with this, the greatest effects on muscle protein fractional synthesis rates are observed in the postprandial stage (Guillet, 2003). Finally, in addition to larger requirements for amino acid dosages, aging causes a slower response to peak muscle protein synthesis, followed by a much more gradual decline (Paddon-Jones 2004).

*Nutritional Supplementation Paired with Muscle Overload and Resistance Exercise*

In an effort to maximally reduce, or attenuate the loss of muscle mass and quality associated with age, researchers have studied the combined effects of muscle overloading/resistance exercise regimens with various forms of supplementation to assess whether additional benefits and possible advanced interventions existed. Recall that the ability to stimulate muscle protein translational signaling (Parkington, 2004; Thomson and Gordon, 2006; Kumar, 2009; Chale-Rush, 2009) and muscle protein synthesis (Kumar, 2009), as well as improve muscle contraction quality (Blough, 2000; Frontera, 2000) and increase muscle fiber and overall hypertrophy (Kosek, 2006) in response to muscle overloading or resistance exercise is reduced with age in rats and humans. Furthermore, variability in the anabolic properties associated with supplemental amino acids make it difficult to discern which combination of nutrients may generate the most effective intervention when paired with muscle overloading or resistance exercise. A review of the combined effects of amino acid, protein, and carbohydrate

supplementation, with muscle overloading and resistance training in animals and humans can be found in the following section.

Acute (Hulmi, 2009) and chronic (Hulmi, 2009; Coburn, 2006) supplementation of whey protein and resistance exercise have generated positive translational signaling stimulation (Hulmi, 2009) and increased maximal muscle strength in young untrained human participants (Coburn, 2006). Whey protein (15 g) supplemented before and immediately after an acute bout of resistance exercise has demonstrated increased phosphorylation of mTOR at 1-hr and 48-hr post-exercise (Hulmi, 2009). Gains in muscle cross sectional area (CSA) have been observed in young humans chronically (8 week) supplementing leucine-enriched (8 g) whey protein (20g) along with unilateral leg extension exercise to a greater extent than those supplementing a placebo (7.31%, 4.58%, respectively). Interestingly, gains in CSA and 1RM were also observed in the untrained limb of the supplementation group (Coburn, 2006). In a similar study, untrained, young participants supplementing leucine+whey protein in conjunction an acute bout of resistance exercise could not further enhance positive net muscle protein synthesis that was produced by participants consuming whey protein only, despite blood leucine concentrations remaining elevated for 215 minutes post-administration (Tipton, 2007).

The acute addition of leucine and/or protein to CHO has produced remarkable increases in the metabolic responses to resistance exercise. An intra-participant study was conducted over three separate 1-day sessions to determine whether post-exercise co-ingesting leucine and protein with carbohydrates (CHO) enhanced muscle anabolism over CHO+PRO or CHO alone (Koopman, 2005). Untrained, young (22.3+/-0.9 yr) male participants were given the initial bolus (3 mL/kg) immediately post-exercise, followed by repeated boluses every 30 minutes until 330 minutes post-exercise to ensure a continuous supply of nutrients. CHO+PRO+LEU

significantly increased whole body net protein balance and muscle protein synthesis rates to a greater extent over CHO alone in young males. Whole body protein breakdown was significantly suppressed in CHO+PRO and CHO+PRO+LEU (reduced by 50+/-2 and 62 +/-2). Additionally, whole body protein synthesis was increased in CHO+PRO and CHO+PRO+LEU (54+/-5 and 45+/-5%, respectively). Mixed muscle FSR was also significantly greater with CHO+PRO+LEU than CHO alone. Plasma insulin response was ~250% greater in individuals consuming CHO+PRO+LEU than those consuming CHO alone. Mixed muscle FSR did not positively correlate with plasma insulin response, but did correlate with the amount of leucine that was ingested (Koopman, 2005)

The specific timing of certain supplements in relation to an exercise bout has been shown to have a profound effect on muscle metabolism. In two separate studies, young humans consumed either no nutrients (control) or leucine-enriched (35%) EAA+CHO 0.35 g/kg·LM of EAA + 0.5 g/kg·LM of CHO (experimental) at: 1) 1-hr pre-exercise (Fujita, 2009), or 2) 1-hr post-exercise (Dreyer, 2008) (10 sets of 10 repetitions of bilateral leg extensions) (Dreyer, 2008; Fujita, 2009). In the pre-exercise supplementation study, the experimental group showed increased intracellular leucine concentrations above baseline values. Contrary to participants supplementing EAA+CHO, intracellular leucine in the exercise only group declined during recovery. Remarkably, EAA+CHO caused mixed muscle FSR to increase above basal values observed in the fasting group during the pre-exercise period. Mixed muscle FSR returned to baseline during exercise, remained unchanged during 1-hr post-exercise, then increased significantly at 2 hr post-exercise in the EAA+CHO group. $mTOR^{Ser2448}$ phosphorylation increased prior to exercise and maintained basal levels during exercise in the supplement group. No differences were observed in FSR between groups during 1-hr and 2-hr post-exercise (Fujita,

2009). P70s6k$^{Thr389}$ phosphorylation also showed an increase prior to exercise, and remained elevated during 1-hr and 2-hr post-exercise in the EAA+CHO group. Elevated phosphorylation of p70s6k$^{Thr389}$ in the fasting group was seen at 2-hr post-exercise, although not to as great of an extent as in the supplementation group (Fujita, 2009). Phosphorylation of eEF2 was significantly reduced prior to exercise, followed by a return to baseline during exercise, and an additional reduction at 1-hr and 2-hr post-exercise in the supplementation group. A pre-exercise reduction in eEF2 phosphorylation was not observed in the fasting group (Fujita, 2009). In participants consuming EAA+CHO supplementation post-exercise, serum insulin levels rose equally with the control group until 1 hr post-exercise, where a dramatic increase occurred (Dreyer, 2008). Arterial and intramuscular leucine concentrations at 2 hrs post-exercise were significantly higher in EAA+CHO group than the control (564+/-31 vs 142+/-16μM, respectively). For both groups, mixed muscle FSR decreased immediately following exercise, then increased at 1 hr and 2 hr post-exercise, with substantially higher values seen in the EAA+CHO group (145% vs 41%, respectively) (Dreyer, 2008). Most notable were the increases in phosphorylation of signaling markers found in the Akt/mTOR translation initiation pathway. mTOR$^{Ser2448}$ and p70s6k$^{Thr389}$ increased immediately post-exercise and remained elevated in both groups through 2 hr, with greater increases in participants consuming EAA+CHO. 4E-BP1$^{Thr37/46}$ significantly increased only at 2 hr post-exercise in the EAA+CHO group. eEF2$^{Thr56}$ phosphorylation reduced from baseline at 1 hr and 2 hr post-exercise with no significant differences between groups (Dreyer, 2008).

Apparent benefits were exhibited from supplementing amino acid mixtures with other nutrients before (Fujita, 2009) and after (Koopman, 2005; Dreyer, 2008) resistance exercise. In an attempt to evaluate the combined effect of acute pre- and post-exercise supplementation,

Karlsson et al administered 150 mL of BCAA (45% leucine, 30% valine, 25% isoleucine) to young human participants before warming up, immediately before resistance exercise, and during recovery (at 15, 30, 60, 90, and 120 min post-exercise). Significantly greater increases in the phosphorylation of signaling markers located downstream of mTOR (p70s6k and rpS6) were noted (Karlsson, 2004). P70s6k$^{Ser421/Thr424}$ phosphorylation increased immediately following exercise in both the BCAA and control groups, but only continued to remain elevated for the entirety of recovery (2 hr) in the BCAA group. Contrary to residue Ser421/Thr424, phosphorylation of p70s6k at residue Thr389 only showed marked increases in participants supplementing BCAA during the recovery period. Chronic supplementation of amino acids, in addition to aerobic and anaerobic training regimens, has also assisted in improving exercise tolerance and markers of performance. Pre- and post-, long-term (6 weeks) leucine supplementation (45 mg/kg·d) was performed on young outrigger canoeists during the competitive season (Crowe, 2006). At the end of the 6-week period, post-supplement upper body peak power had increased from baseline in both the placebo and leucine groups, although increases were substantially higher in the leucine group. Similarly, an overall increase was observed in exhaustive row time (72.2+/-4.4 min to 76.3+/-5.8 min). Finally, pre-supplemental RPE values did not differ between groups. However, a significant decline in RPE occurred in the leucine group during post-supplementation (12.9+/-1.4 vs. 15.0+/-1.4) (Crowe, 2006)

Two intra-participant studies were conducted to determine whether adding protein or protein and leucine to a nutrient regimen could enhance markers of protein metabolism. On two separate days, young (20+/-1 yr) and elderly (75+/-1 yr) consumed repeated boluses every 30 minutes (for 330 min) of either 1) a placebo CHO beverage (1.33 mL/kg/hr volume; 0.49 g/kg/hr CHO) or, 2) a CHO+PRO+LEU (1.33mL/kg volume; 0.49 CHO, 0.16 g/kg whey protein, 0.03

g/kg leucine) in a double blind manner immediately following a resistance exercise bout resembling activities of daily living (~650 kJ/30 min) (Koopman, 2006 #2). Following a 7-day period, each participant repeated the protocol and consumed the alternate combination of nutrients. Regardless of age, whole body protein breakdown was suppressed and synthesis was amplified when adding whey protein and leucine to CHO supplements resulting in an overall net increase of 47+/-3% and 44+/-4% for young and elderly participants. Although both age groups showed significant increases in muscle fractional synthesis rates when consuming CHO+PRO+LEU over CHO alone, the overall synthesis values were ~30% lower among elderly participants (Koopman, 2006 #2). Among elderly participants, additional leucine facilitated higher phenylalanine uptake into the cell (measured by rate of disappearance) and greater net protein balance above that of CHO+PRO (Koopman, 2007). However, no significant differences were found in whole body protein breakdown synthesis, mixed muscle protein fractional synthesis rates (FSR) per hour between CHO+PRO+LEU and CHO+PRO groups (Koopman, 2007). Pennington et al (2011) utilized the same resistance exercise protocol discussed previously (Koopman, 2007) to evaluate effects of supplemental protein and resistance exercise between age groups (Pennings, 2011). Immediately post-exercise, young (21+/-1 yr) and elderly (74+/-1 yr) consumed a 250 mL bolus of casein protein (20g) containing labeled phenylalanine. Interestingly, postprandial muscle protein synthesis rates were increased above baseline in both age groups (Pennings, 2011). Although both groups showed similar increases in postprandial plasma amino acid concentrations and plasma insulin responses, rises in both variables were to a greater extent in elderly participants (Pennings, 2011)

Most essential to the current study was the data produced by Drummond et al, which demonstrated the delayed and suppressed responses to the combination of acute resistance

exercise and nutritional supplementation (Drummond, 2008 #2). Elderly (70.0+/-1.7 yr) and young (29.7+/-2.1 yr) male participants performed an acute resistance exercise bout (8 sets of 10 repetitions) of bilateral leg extensions and consumed leucine-enriched (35% leucine, 20 g total EAA) EAA post-exercise. An equivalent increase in muscle intracellular leucine concentration at 3-hr post-exercise was observed in young participants only, which remained elevated in both young and elderly through 6-hr post-exercise (Drummond, 2008 #2). Interestingly, mixed muscle protein fractional synthesis rate was only elevated in the young group between 1-3 hr post-exercise. Similar increases of FSR were not achieved between groups until reaching 3-6 hrs post-exercise. Delivery of amino acids accounts for this delay. With regards to mTOR and p70s6k phosphorylation, increases were apparent at all time points following the exercise bout. However, young participants exhibited an increase phosphorylation of p70s6k before EAA administration and at 6-hrs post-exercise. Phosphorylation of elongation protein, eEF2, progressively decreased from 1-hr to 3- and 6-hrs post-exercise. This delay in muscle protein synthesis cannot be attributed to amino acid delivery nor lack of phosphorylation, and presumed activation of mTOR and mTOR regulated signaling markers (Drummond, 2008 #2).

Pharmaceutical inhibitors of specific translational signaling proteins that have been incorporated into recent studies in an effort to verify the relationship between 1) muscle overloading/resistance exercise and mTOR pathway signaling, and 2) amino acid supplementation and mTOR pathway signaling. These inhibitors also helped to pinpoint were along the pathway each stimulant affected signaling and ruled out the involvement of other pathways/stimuli in muscle protein signaling and/or synthesis. Although pharmaceutical inhibitors were not incorporated into the current study, previous research was analyzed and helped to further solidify the direct effect that leucine had on mTOR signaling. Administration

of rapamycin, a known inhibitor of mTOR, blunted increases in muscle protein synthesis that were observed in the control group following by an acute bout of resistance exercise in humans. Also, p70s6k$^{Thr421/Ser424}$ and eEF2$^{Thr56}$ remained unchanged from baseline following treatment with rapamycin (Drummond, 2009). The partially diminished increase in MPS (~40%) and blunted phosphorylation of signaling proteins located downstream from mTOR indicated that signaling through the mTOR pathway is involved in exercise-stimulated MPS (Drummond, 2009). Additionally, leucine-dependent signaling activity was greatly diminished with the inclusion of rapamycin, especially at residue Thr389 on p70s6k (Anthony, 2000 #2). Other inhibitors, such as LY294002 (inhibitor of PI(3)K; a signaling protein found upstream from mTOR), have been used in conjunction with rapamycin to solidify the theory that amino acid stimulation occurs directly through mTOR and does not occur through upstream signaling proteins. LY294002 did significantly decrease basal protein synthesis and attenuated amino acid-induced muscle protein synthesis. On the other hand, rapamycin had no affected on basal protein synthesis, but attenuated both in vitro amino acid- and leucine-induced muscle protein synthesis indicating the involvement of an alternate stimulatory pathway for mTOR other than through IGF-1/PI(3)K/Akt upstream signaling (Dardevet, 2000).

Recall that muscle overloading is an affective stimulus for skeletal muscle hypertrophy and protein translational signaling in young rats (Thomson and Gordon, 2005; Thomson and Gordon, 2006) and humans (Eliasson, 2006; Dreyer, 2008). A desensitization to muscle overloading develops with age (Chale-Rush, 2009; Thomson and Gordon, 2005; Thomson and Gordon, 2006; Kosek, 2006). Nutritional supplementation has been shown to act as a catalyst by enhancing muscle protein synthesis above the stimulatory effects observed in the fasted state (Burd, 2010). The amino acid leucine has proven to act as a catalyst for protein anabolism and

translational signaling marker phosphorylation enhancement among young adult rats and humans when combined with EAA and other nutrients (Anthony, 2000; Fujita, 2007) or administered independently (Anthony, 2000 #2; Anthony, 2002; Crozier, 2005 Suryawan, 2008). Desensitization to nutrient supplementation has been observed with increasing age in regards to translational signaling (Dardevet, 2000), muscle (Dardevet, 2000; Dardevet, 2002; Paddon-Jones, 2004) and myofibrillar protein synthesis rates (Guillet, 2003), muscle fiber composition (CSA), and whole muscle strength (1RM) (Verhoeven, 2009). Clear evidence indicated that higher dosages of leucine were required to stimulate muscle protein synthesis and translation signaling marker phosphorylation in old rats (Dardevet, 2000). Along with this, the greatest effects on muscle protein fractional synthesis rates are observed in the postprandial stage (Guillet, 2003). Finally, in addition to larger requirements for amino acid dosages, aging causes a slower response to peak muscle protein synthesis, followed by a much more gradual decline (Paddon-Jones 2004). It is best to consume pre- and post-exercise in order to maximize the benefits of supplementation (Dreyer, 2008; Fujita, 2009). Limited data was available for analysis on the paired effects of chronic muscle overloading with continuous leucine supplementation in aged animals and humans. For that reason, the purpose of this study was to examine the effects of dietary leucine supplementation on protein translational signaling and muscle hypertrophy in the overloaded fast-twitch skeletal muscles of aged animals. It is hypothesized that supplementing a standard chow diet with 5% leucine will enhance muscle hypertrophy in overloaded fast-twitch plantaris muscles of aged (33-month old) rats to levels observed in young adult (8-month old) rats. It is also hypothesized that 5% dietary leucine supplementation will enhance p70s6k, rpS6, eEF2k, and eEF2 signaling in the overloaded fast-twitch plantaris muscles of aged rats to levels observed in young adult rats. If the application of

this intervention method proves to be beneficial for stimulating protein translation in aged rats, additional chronic supplementation studies with human subjects could be warranted. This combination could be a pivotal intervention strategy to delay, or perhaps even cease, muscle fiber degeneration and reduce the affects of aging on normal functioning and disease progression.

# Chapter III: Methods

## *Experimental Animals*

Specimens chosen for the present study were young adult (YA; 8 mo; n = 13) and old (O; 33 mo; n = 13) Fischer$^{344}$ X Brown Norway F1 hybrid (FBN) male rats. FBN rats were housed at the East Carolina University Brody School of Medicine animal care facility. The specimens were acclimated to a 12 hr light/dark cycle with continuous ad libitum access to water and normal rodent chow prior to initiation of dietary intervention. Experimental groups were formed from each age category. Specimens were divided into a placebo group (n=6) or a leucine supplementation group (n=7). A 1-week comparison of overloaded vs. non-overloaded muscle was achieved in the plantaris muscle of each rat following a unilateral tenotomy of the left hind limb. All procedures performed in this study were approved by the East Carolina University Animal Care and Use Committee prior to the initiation of the experiment (see appendix A).

## *Rationale for Experimental Animals*

Fischer$^{344}$ x Brown Norway (FBN) rats were the primary choice for this investigation due to the similarities they share with humans with regards to aging. In previous research, FBN rats have demonstrated reductions in muscle mass, CSA and contractile properties of the plantaris muscles as age increases. The greatest losses in these areas have been observed during the latest stages of life (38 mo) (Blough, 2000). Similar losses of muscle strength (Vandervoort, 2002) are apparent in human participants in their seventh and eighth decades. Overloading has been shown to improve overall muscle functioning and mass for humans even into their tenth decade (Fiatarone, 1994). Although the responses to overloading stimuli are to a lesser degree in elderly rats than with young, there is evidence that improvement in strength and hypertrophy can be made, and these improvement are analogous those seen in humans (Blough, 2000; Fiatarone,

1994). In aged FBN rats, proteins that inhibit synthesis initiation (AMPK) increase and have demonstrated a negative correlation with hypertrophy generated through overloading, particularly in muscles that are predominately composed of fast-twitch fibers (Thomson and Gordon, 2005). FBN rats have been shown to express the same progressive aging trends as both male and female humans (Thomson and Gordon, 2005; Thomson and Gordon, 2006; Thomson, 2009). Because there are few gender differences in muscle atrophy and diminished hypertrophy with age, and male FBN rats are a good model to represent both genders, we chose to use only male FBN rats.

*Dietary Intervention*

Upon arrival to the East Carolina University Brody School of Medicine animal care facility, animal specimens participated in a 2 day acclimation period where they consumed an ad libitum diet of standard rodent chow (placebo diet found in Table 3.1) and water. Following the acclimation period, the specimens were separated into different age categories. Animals between age groups were paired for their body weight and fed either a standard rodent chow diet (placebo) or a 5% supplemental leucine-enriched chow (provided by Research Diets, Inc., New Brunswick, NJ) during the 2 days before surgery. Composition of the placebo and leucine diets can be found below in Table 3.1. The addition of 5% leucine to the standard rat chow was chosen for this study due to its previous success in decreasing postprandial protein degradation over a 10-day period in aged skeletal muscle (Combaret, 2005). Composition of the placebo diet was 20% protein, 65% carbohydrate, and 15% fat. The leucine diet was composed of 20% protein, 5% free leucine, 59% cabohydrate, and 15% fat.

Following the initial tenotomy surgery (described below), the control group continued to receive standard rat while the supplementation group continued to consume the 5% leucine diet.

Chow consumption was monitored daily to assess caloric and leucine intake. The experimental supplementation lasted 9 days (2 days pre-surgery plus 7 days post-surgery) and rats continued on with their assigned diets ad libitum until the time of sacrifice.

Table 3.1: Dietary Compositions

| **Diet** | **Placebo** | | **Leucine** | |
|---|---|---|---|---|
| Product # | D09051102 | | D11020301 | |
| | **gm%** | **kcal%** | **gm%** | **kcal%** |
| Protein | 19 | 20 | 24 | 25 |
| Carbohydrate | 63 | 65 | 58 | 59 |
| Fat | 7 | 15 | 7 | 15 |
| Total | | 100 | | 100 |
| kcal/g | 3.8 | | 3.8 | |
| | | | | |
| **Ingredient** | **Gm** | **kcal** | **gm** | **kcal** |
| Casein | 200 | 800 | 200 | 800 |
| L-Leucine | 0 | 0 | 54 | 216 |
| L-Cystine | 3 | 12 | 3 | 12 |
| | | | | |
| Corn Starch | 346 | 1384 | 292 | 1168 |
| Maltodextrin 10 | 45 | 180 | 45 | 180 |
| Dextrose | 250 | 1000 | 250 | 1000 |
| | | | | |
| Cellulose, BW200 | 75 | 0 | 75 | 0 |
| Inulin | 25 | 25 | 25 | 25 |
| | | | | |
| Soybean Oil | 70 | 630 | 70 | 630 |
| | | | | |
| Mineral Mix S10026 | 10 | 0 | 10 | 0 |
| Dicalcim Phosphate | 13 | 0 | 13 | 0 |
| Calcium Carbonate | 5.5 | 0 | 5.5 | 0 |
| Potassium Citrate, 1 H2O | 16.5 | 0 | 16.5 | 0 |
| | | | | |
| Vitamin Mix V10001 | 10 | 40 | 10 | 40 |
| Choline Bitartrate | 2 | 0 | 2 | 0 |
| | | | | |
| Yellow Dye #5 FD&C | 0.025 | 0 | 0.025 | 0 |
| Red Dye #40, FD&C | 0 | 0 | 0.25 | 0 |
| Blue Dye #1, FD&C | 0.025 | 0 | 0 | 0 |
| | | | | |
| **Total** | **1071.05** | **4071** | **1071.5** | **4071** |

*Synergist Tenotomy Procedure*

The 1-week overloading model that all animals were subjected to was achieved through surgical tenotomy of the Achilles tendon. Unilateral tenotomy was selected for this study to allow for a more accurate comparison of muscle hypertrophy and pathways controlling protein synthesis within animals and to eliminate the risk of bias between animals.

The Achilles tendon acts as the connective tissue for the major synergist muscle (the gastrocnemious) of the lower leg. Tenotomy of this synergist muscle allowed for the plantaris muscle of each hind limb to become overloaded. The procedure began by weighing each animal. A general anesthetic (2-3% isoflurane and supplemental oxygen) was administered prior to surgery. It was ensured that aseptic conditions were achieved before beginning any invasive procedures. The distal portion of the Achilles tendon of the left hind limb was located and cut using a surgical scalpel. Once the gastrocnemious was completely free, the exposed tissue was closed using surgical staples. The right hind limb was used for the sham or control limb. The gastrocnemious was exposed and the Achilles tendon was isolated in the sham limb. However, no surgical tenotomy was performed on the control limb, which left the plantaris and soleus under normal muscular strain during the overload period. Again, the exposed tissues were closed using surgical staples. A subcutaneous injection of an analgesic (Buprenex, 0.03 mg/kg·bw) was administered following the procedure.

*Tissue Harvesting and Euthanasia*

On the day of muscle extraction and sacrifice (7 days post-surgery), animals were allowed free access to their designated chow until terminal anesthesia. This decision was based on previous research, which indicated that the postprandial state was the period in which the effects of leucine are most prevalent (Katsanos, 2006; Fujita, 2007). Animal sacrifice order was

randomized and counterbalanced, but kept to within ~2 hrs of the end of the dark cycle (during which the animals are feeding more than during the light cycle).

Animals were weighed and anesthetized with an intraperitoneal injection of ketamine and xylazine (90 and 10 mg/kg body weight, respectively), and the plantaris muscle of each hind limb was extracted. Following extraction, excess connective tissue and fat were removed and each muscle was weighed using an analytical balance. Samples were then flash frozen in liquid nitrogen and stored in a freezer at -80˚C. Animals were sacrificed by cardiectomy while still anesthetized.

*Western Blot Analysis*

Plantaris muscle protein content and phosphorylation states of p70S6k, rpS6, eEF2k and eEF2 were measured using western blotting analysis methods. Muscle samples were placed in a test tube and homogenized in a buffer (composition consisted of 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7 \bullet 10H_2O$, 100 mM ß-glycerophosphate, 25 mM NaF, 50 µg/ml leupeptin, 50 µg/ml pepstatin, and 33 µg/ml aprotinin) using a ground glass homogenizer. To prevent excess heat build up and protein denaturation, all homogenations were performed with the test tube placed in a beaker of ice.

The total protein concentration of each homogenate was determined in triplicate using a modified Lowry procedure (DC Protein Assay, Bio-Rad, Hercules, CA, USA). Protein homogenates were then mixed in a loading buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 2% ß-mercaptoethanol, 0.1% bromophenol blue) with a dilution ratio of 1 mg protein per mL. The homogenate mixture was then heated to a boil for 5 minutes. Protein separation was achieved using 4-15% gradient sodium doecyl sulfate-polyacrylamide gel electrophoesis (SDS-PAGE). The blotting transfer spanned 1.5 hrs at a temperature of 4˚C onto a PVDF membrane at

100V in a transfer buffer (25 mM Tris-base pH~8.3, 192 mM glycine, and 20% methanol). All groups were equally represented on all gels/membranes, and the membranes were stained using Ponceau S to assess protein loading of the lanes. Once the staining was complete, membranes were allowed to dry. Membranes were scanned to form digital images. These images were later analyzed by software (NIH Image, National Institute of Health, Bethesda, MD), which uses gray scale optical density to determine relative total protein loaded into each lane over the full length of the individual lanes. Membranes were placed into a blocking buffer composed of 5% nonfat dry milk in TBS-T (20 nM Tri-base, 150 mM NaCl, 0.1 % Tween-20) pH 7.5 for 1 hr at room temperature. Once the blocking procedure was completed, the membranes were incubated in the primary antibody diluted in 1% bovine serum albumin in TBS-T at 4˚C over night. All primary antibodies were commercially available from Cell Signaling Technology, Inc. (Danvers, MA); all were rabbit polyclonal antibodies except for Phospho-p70s6k, which was a mouse monoclonal antibody. Four separate washes in TSB-T were performed for 5 minutes on each membrane, followed by incubation in horseradish peroxidase (HRP)-linked anti-rabbit or anti-mouse secondary antibodies solution (GE Healthcare, Piscataway, NJ) in a blocking buffer for 1 hr at room temperature. Four additional 5-minute washes were then performed in TBS-T.

After the last wash, HRP activity was quantified by exposure to an enhanced chemiluminescence solution (GE Healthcare) and exposure to autoradiographic film (Classic Blue Sensitive; Midwest Scientific, St. Louis, MO, USA). The densitometry method via Gel Pro Analyser software (Media Cybernetics, Silver Springs, MD, USA) was used to measure integrated optical densities (IODs) for each band. IOD calculations performed were normalized to relative total muscle protein initially loaded on each gel (as quantified by ponceau staining).

*Statistical Analysis*

Statistical analysis of each variable was performed using a 2x2x2 factorial ANOVA (age, dietary intervention, and overload) with repeated measures for overload. Percent changes with overload were quantified using a 2x2 ANOVA. Fischer's LSD Post-Hoc was used to determine significance. The level of significance will be set at $p \leq 0.05$.

## Chapter IV: Results

### *Body Weight*

The main finding for this section was that animal body weight was significantly different between age groups regardless of time points or dietary conditions (Table 4.1). A diet containing 5% leucine proved to have no significant effect on body weight over that of the placebo chow for either age group. Animal weight did not significantly change throughout the experiment.

| | Body Weight (g) | | | |
|---|---|---|---|---|
| | Placebo Diet Initiation | Split Leucine/Placebo | Surgery | Sacrifice |
| Young Placebo | 374.4 (12.2) | 378.4 (11.8) | 377.9 (12.1) | 365.4 (11.3) |
| Young Leucine | 374.4 (10.2) | 378.1 (9.5) | 379.8 (8.8) | 363.6 (8.3) |
| Old Placebo | 549.7 (28.7)* | 551.5 (27.9)* | 559.0 (29.0)* | 535.6 (22.6)* |
| Old Leucine | 544.9 (19.3)* | 548.1. (17.4)* | 557.2 (17.9)* | 531.2 (14.6)* |

Table 4.1. Mean ±SEM body weights (g) for young (8 mo.) vs. old (33 mo.) rats. Animals were fed normal rodent chow (placebo) or chow with 5% leucine supplementation (leucine). *Significant ($p \leq 0.05$) main effect of age regardless of time points or dietary conditions.

*Food Intake*

Table 4.2 indicates that there were no significant differences observed in food consumption between the diet initiation period and the surgical procedure within age groups. Food consumption did differ between age groups during the overloading period (days 5-11) indicating that older animal food intake was significantly lower than that of young animals.

| | Food Intake (g/kg bw/d) | | |
|---|---|---|---|
| | Days 1-2 | Days 3-4 | Days 5-11 (overload) |
| Young Placebo | 47.03 (1.73) | 40.44 (2.28) | 35.00 (1.67) |
| Young Leucine | 43.93 (1.93) | 41.43 (1.92) | 34.48 (1.13) |
| Old Placebo | 44.29 (2.35) | 39.73 (2.04) | 25.55 (3.14)* |
| Old Leucine | 46.91 (2.29) | 42.09 (2.31) | 27.99 (2.32)* |

Table 4.2. Mean ±SEM food intake (g/kg bw/d) for young (8 mo.) vs. old (33 mo.) rats. All animals were fed normal chow on days 1-2. Animals were split into normal rodent chow (placebo) or chow with 5% leucine supplementation (leucine) for days 3-4 (prior to surgery), and these diets continued post-surgery during the overload period (days 5-11). * Significantly ($p \leq 0.05$) different than young animals during overload period regardless of dietary condition.

## *Muscle Wet Weight*

### *Plantaris*

The plantaris muscles of young rats showed several significant differences. Figure 4.1 showed that overloaded plantaris muscles for both the placebo and the leucine groups of young rats were significantly heavier than the opposing sham muscles of the opposite limb in the same animals. In old rats however, only those consuming leucine demonstrated an increase in plantaris weight over that of the muscle in the sham limb. Between age groups, old rats had significantly lighter muscles in the sham and overloaded limbs regardless of the diet consumed.

Analysis of data in Figure 4.2 indicated that, within age groups, young placebo and young leucine rats had no significant difference in the percentage of hypertrophy. Old leucine rats did express a significantly higher percentage of hypertrophy than old placebo rats. Between age groups, old rats consuming the placebo diet had significantly less hypertrophy than rats in the young placebo group.

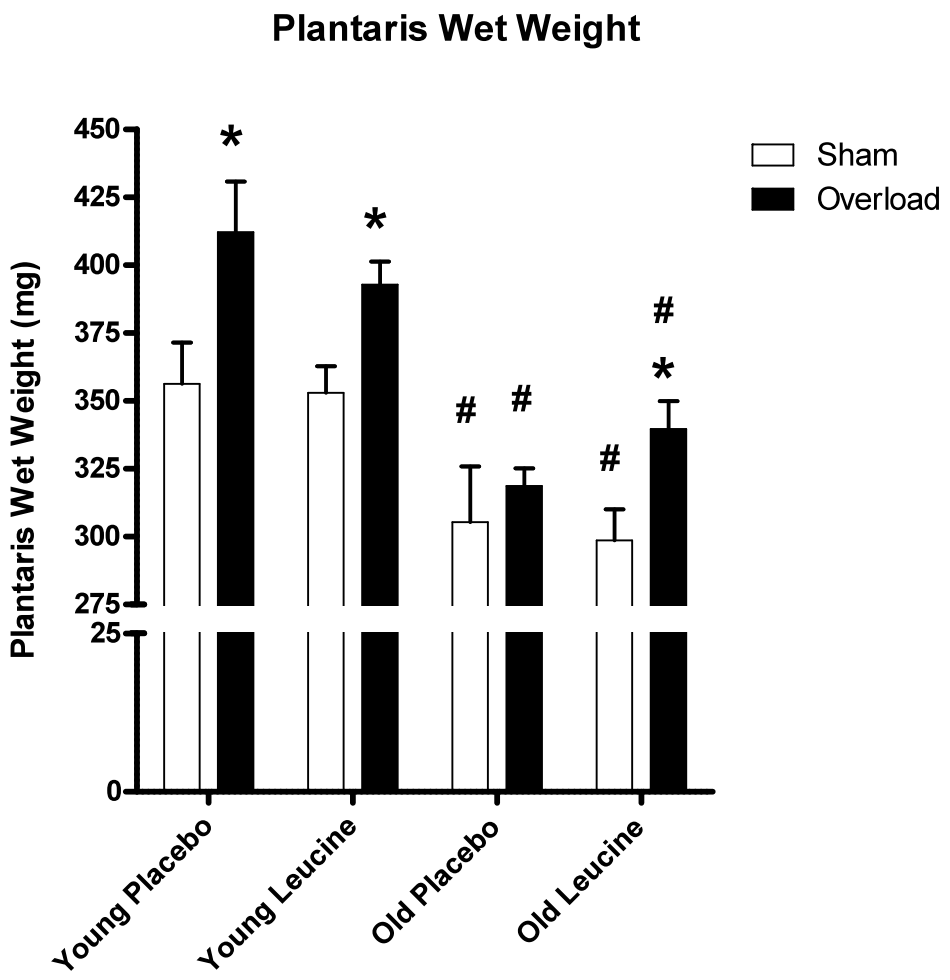


Figure 4.1. Mean ±SEM wet weights of sham-operated vs. 7-day overloaded plantaris muscles in young adult (8 mo.) vs. old (33 mo.) rats. Animals were fed normal rodent chow (placebo) or chow with 5% leucine supplementation (leucine).
* Significantly ($p \leq 0.05$) different than sham-operated muscle within specified age group and dietary condition. # Significant main effect of age regardless of dietary or loading condition.

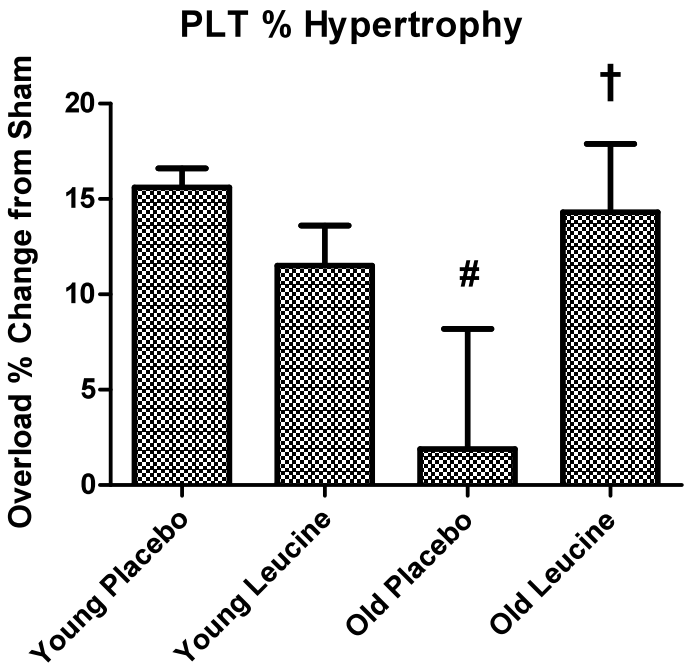


Figure 4.2. Mean ±SEM percent change in wet weights of 7-day overloaded vs. sham-operated plantaris (PLT) muscles in young adult (8 mo.) vs. old (33 mo.) FBN rats. FBN rats. Animals were fed normal rodent chow (placebo) or chow with 5% leucine supplementation (leucine). # Significantly ($p \leq 0.05$) different than young placebo group. † Significantly different than old placebo group.

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## *Protein Content*

### *P70s6 kinase*

In young rats, p70S6k phosphorylation at Thr389 was significantly greater in the overloaded muscle of the placebo and the leucine groups (Figure 4.3). On the contrary, phosphorylated p70S6k at Thr389 was not significantly different between the sham and overloaded muscle in either the placebo or the leucine groups of old rats. Phosphorylation of p70S6k at Thr389 did show significant differences between young and old overloaded muscle of the placebo groups. However, there was no significant difference in p70S6k phosphorylation of the overloaded muscle in the leucine groups of young and old rats.

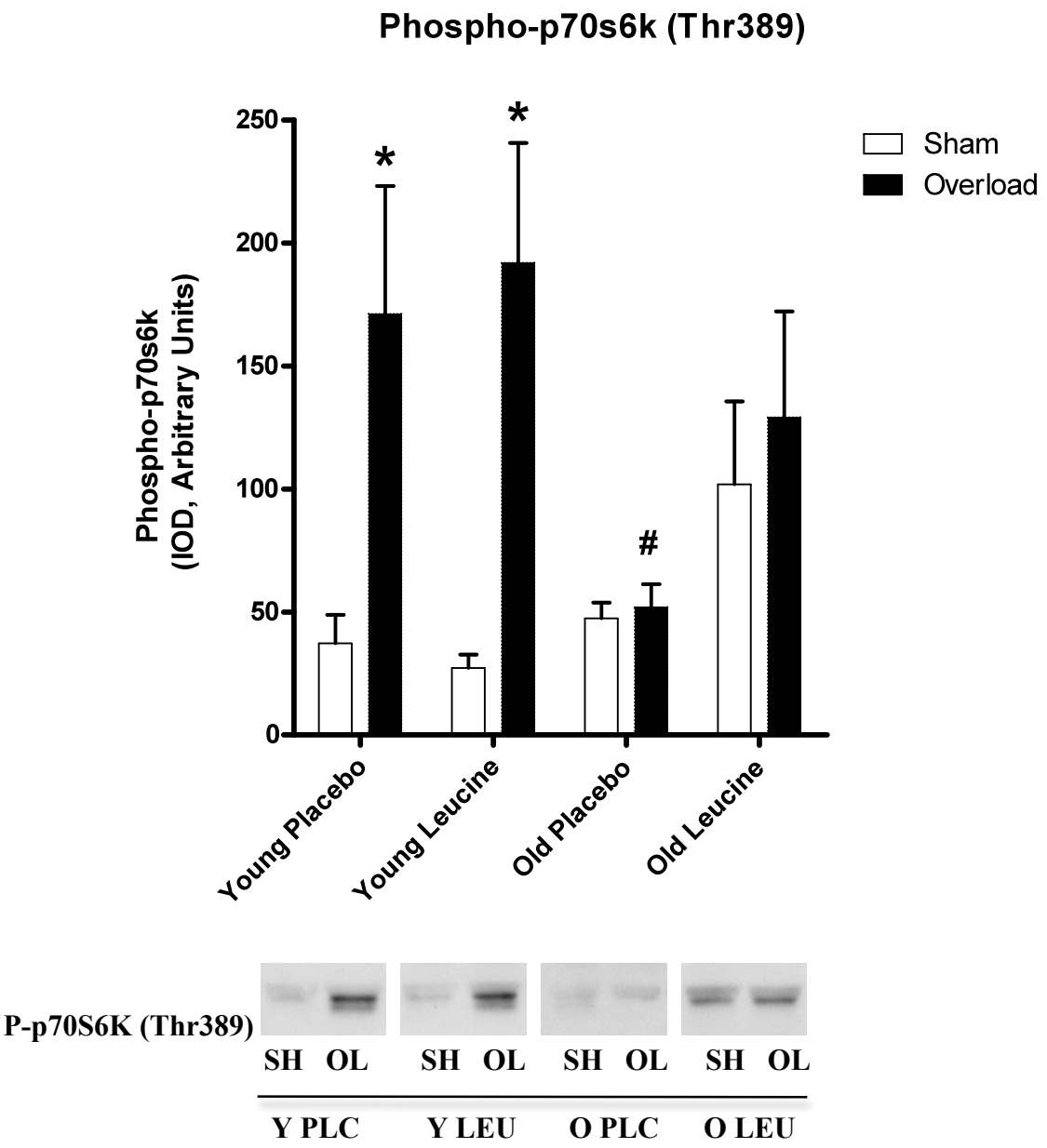


Figure 4.3. Mean ±SEM phospho-70 kDa ribosomal protein S6 kinase (p70S6k; Thr389) content and representative blots of sham-operated (sham) vs. 7-day overloaded (overload) plantaris muscle in young (8 mo.) vs old (33 mo.) rats fed either normal chow (placebo) or chow with 5% leucine supplement (leucine). * Significant ($p \leq 0.05$) effect of overload within young age group regardless of dietary condition. # Significantly lower than young overload group within placebo condition.

Figure 4.4 shows that, in young rats, the total amount of p70s6k was significantly higher in the overloaded muscles of both the placebo and leucine chow groups than in sham muscles. There was no significant difference in total p70s6k content between sham and overloaded muscles in either group of old rats. There was no significant difference between young and old groups for the total p70s6k protein content.

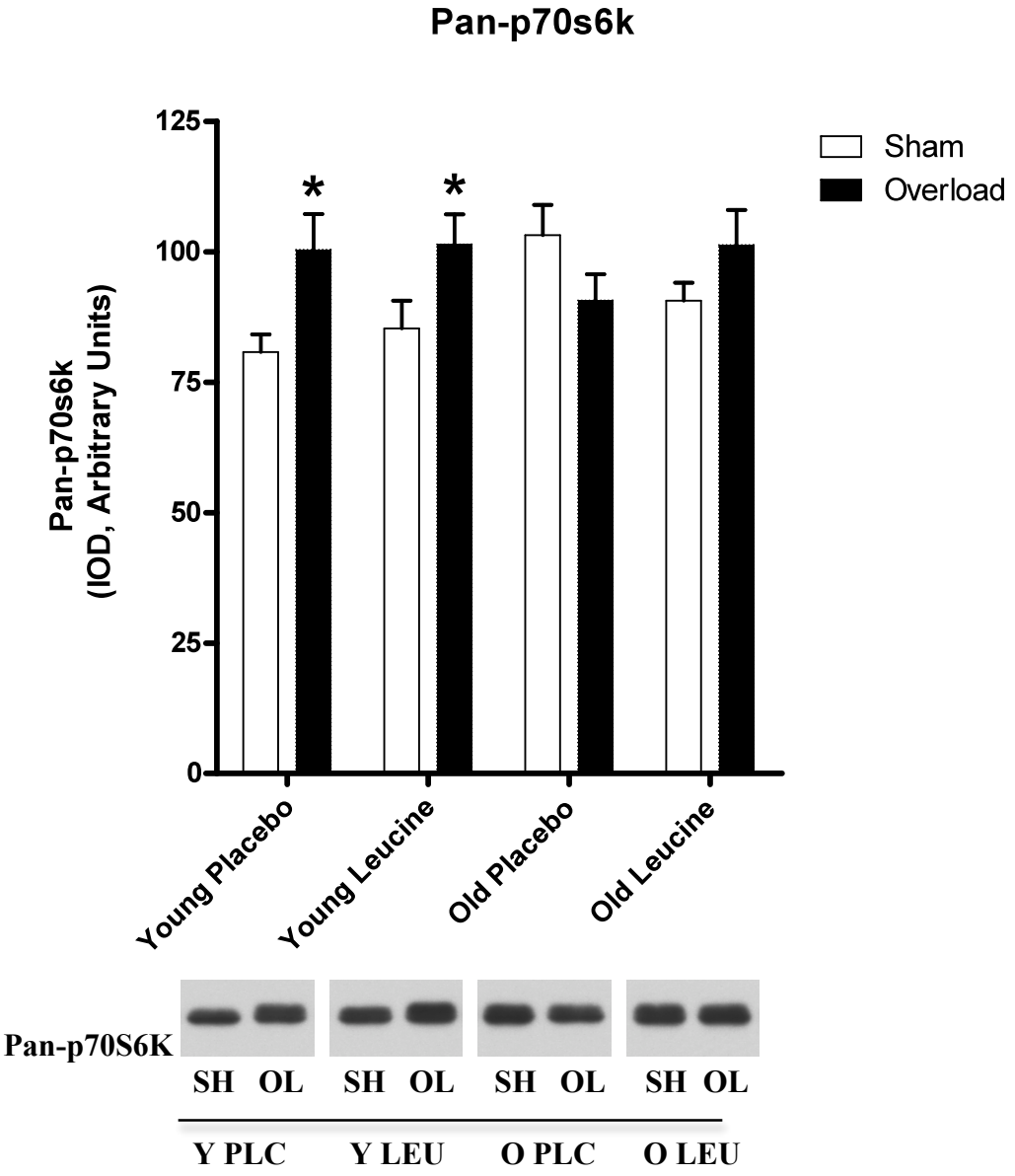


Figure 4.4. Mean ±SEM total 70 kDa ribosomal protein S6 kinase (p70S6k) content and representative blots of sham-operated (sham) vs. 7-day overloaded (overload) plantaris muscle in young (8 mo.) vs old (33 mo.) rats fed either normal chow (placebo) or chow with 5% leucine supplement (leucine). * Significant ($p \leq 0.05$) effect of overload within young age group regardless of dietary condition.

The phosphorylated-to-total p70S6k ratio demonstrates the quantity of total protein available that was activated. In young rats, a significantly higher activation ratio was observed in the overloaded muscle of the placebo and leucine groups (Figure 4.5). In old rats, neither the placebo group nor the leucine group exhibited a significant difference between the sham and overloaded muscle for phosphorylated-to-total p70S6k ratio. The old leucine sham group had a greater baseline for the phosphorylated-to-total p70S6k ratio. This may have contributed to the lack of significance.

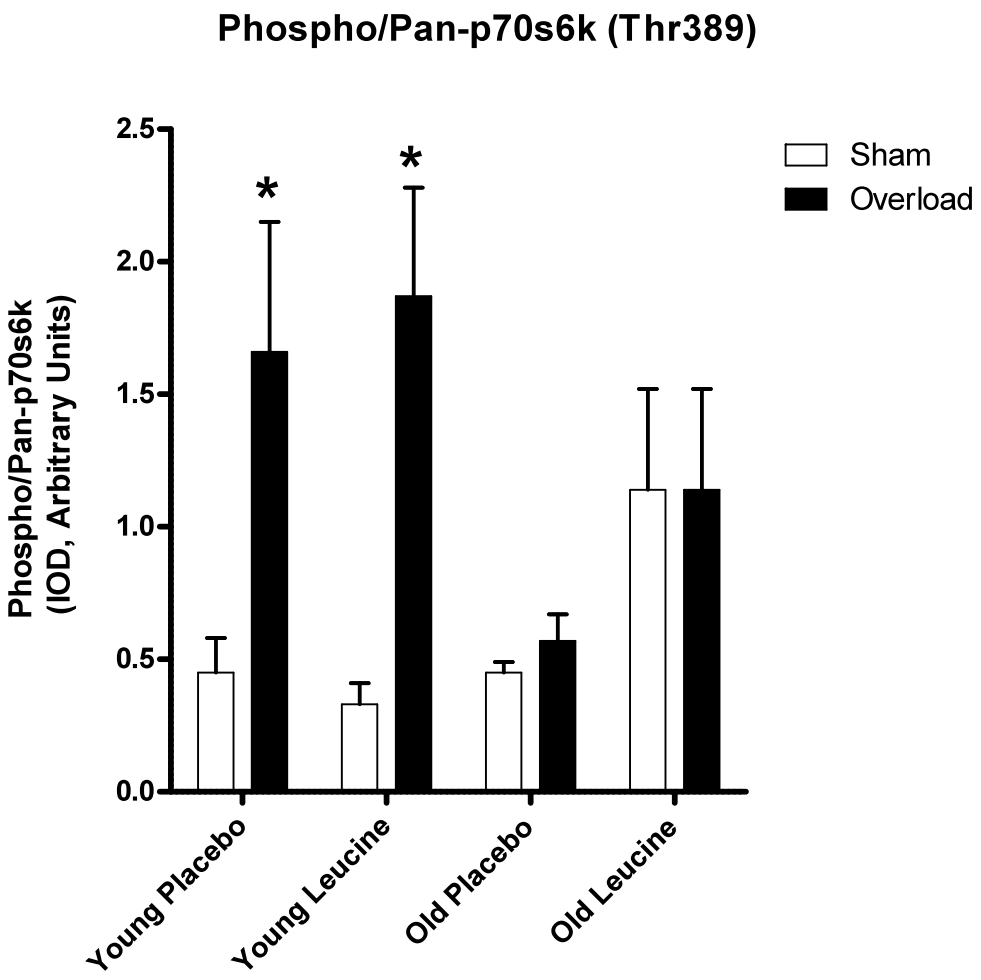


Figure 4.5. Mean ±SEM phospho (Thr389) – 70 kDa ribosomal protein S6 kinase (p70S6k) content / total p70S6 kinase content of sham-operated (sham) vs. 7-day overloaded (overload) plantaris muscle in young (8 mo.) vs old (33 mo.) rats fed either normal chow (placebo) or chow with 5%leucine supplement (leucine). * Significant ($p \leq 0.05$) effect of overload within young age group regardless of dietary condition.

*rpS6*

Data expressed in figure 4.6 indicates that, in young rats, both the placebo and leucine groups showed significant difference in rpS6 phosphorylation at Ser235/236 between the sham and the overloaded muscles. In old rats, there was no significant difference observed between the sham and the overloaded muscle in either the placebo or leucine groups. When comparing the young and old groups, rpS6 in young overloaded muscle showed a much greater phosphorylation status than that of old overloaded muscle.

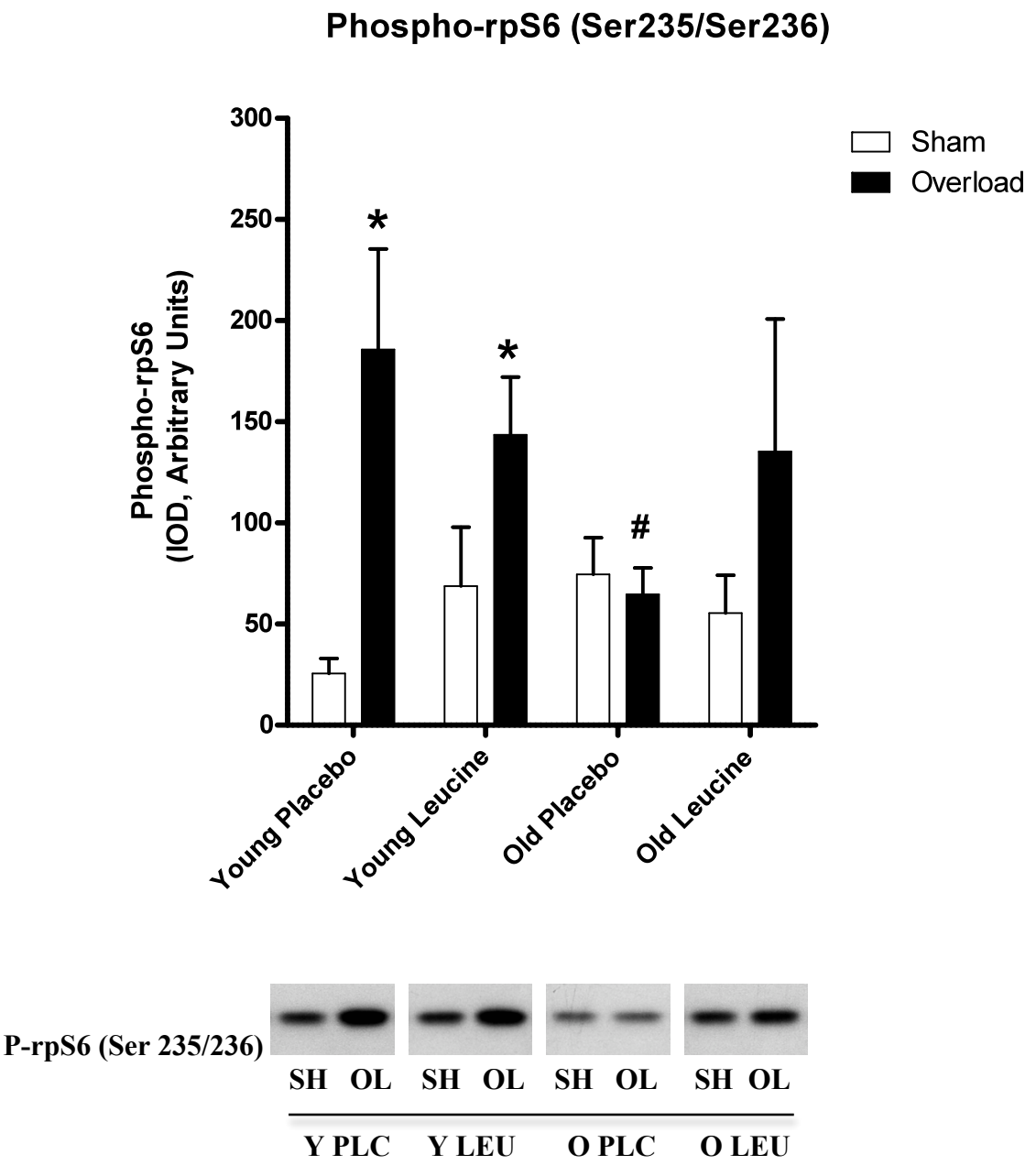


Figure 4.6. Mean ±SEM phospho-ribosomal protein S6 (rpS6k; Ser235/236) and representative blots of sham-operated (sham) vs. 7-day overloaded (overload) plantaris muscle in young (8 mo.) vs old (33 mo.) rats fed either normal chow (placebo) or chow with 5% leucine supplement (leucine). * Significant ($p \leq 0.05$) effect of overload within young age group regardless of dietary condition. # Significantly lower than young overload group within placebo condition.

The total amount of rpS6 protein available in the muscle of young rats was significantly different in the placebo group only between sham and overloaded muscle (Figure 4.7). In old rats, total rpS6 showed significant difference in the leucine group only with greater quantities in the overloaded versus the sham muscle. Leucine supplementation also had variable effects between age groups, exhibiting significantly greater quantities of total rpS6 in old overloaded muscle over that of young overloaded muscle.

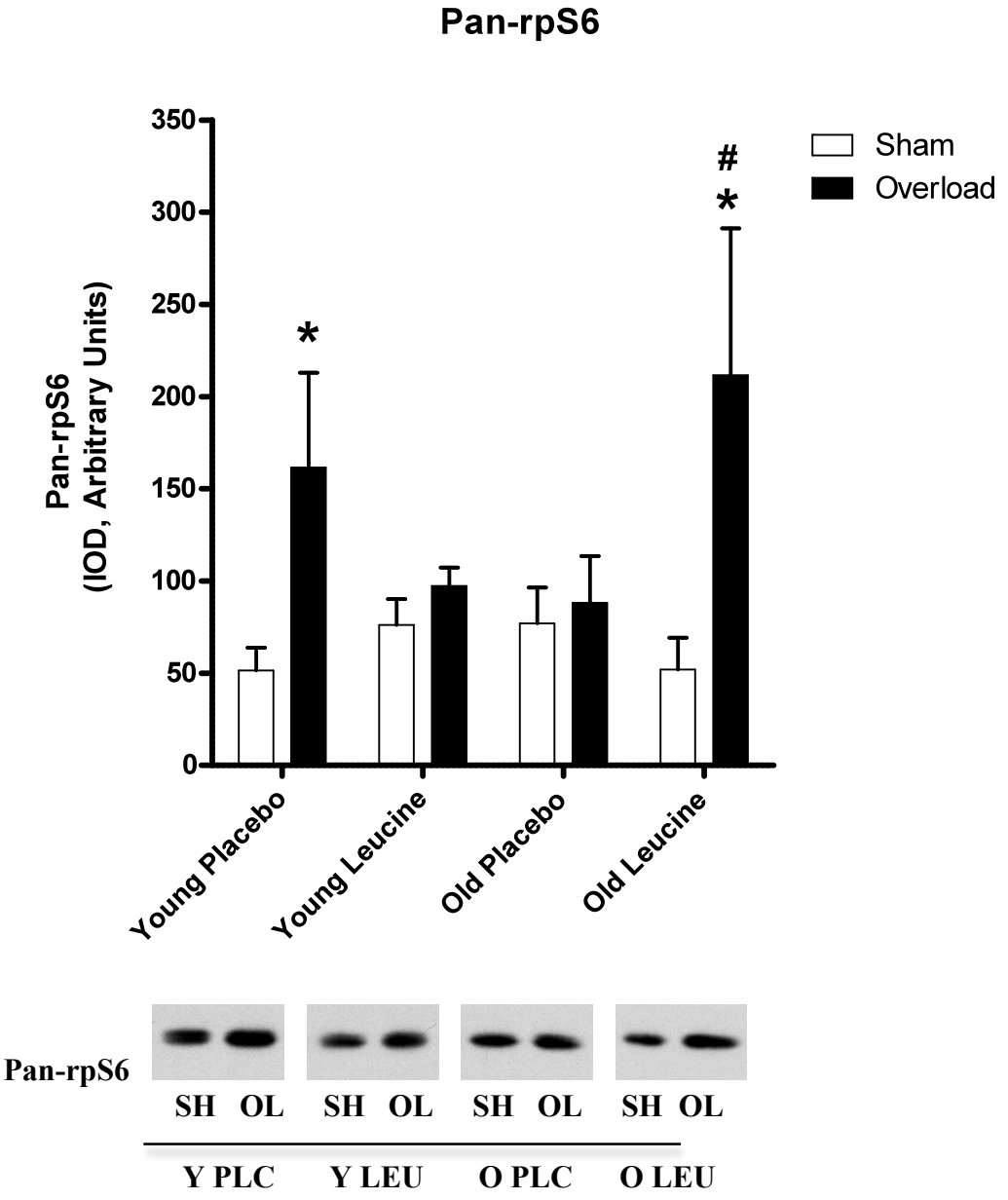


Figure 4.7. Mean ±SEM total ribosomal protein S6 (rpS6k) content and representative blots of sham-operated (sham) vs. 7-day overloaded (overload) plantaris muscle in young (8 mo.) vs old (33 mo.) rats fed either normal chow (placebo) or chow with 5% leucine supplement (leucine). * Significantly ($p \leq 0.05$) different than sham within specified age group and dietary condition of age or dietary conditions. # Significantly different than young leucine overload group.

The ratio of phosphorylated-to-total rpS6 (Figure 4.8) was significantly greater in the overloaded muscle of the placebo and leucine group in young rats. In old rats, there was no significant difference in the ratio of phosphorylated-to-total rpS6 between sham and overloaded muscle for neither the placebo nor the leucine groups. No significant difference was observed between young and old groups in the ratio of phosphorylated-to-total rpS6.

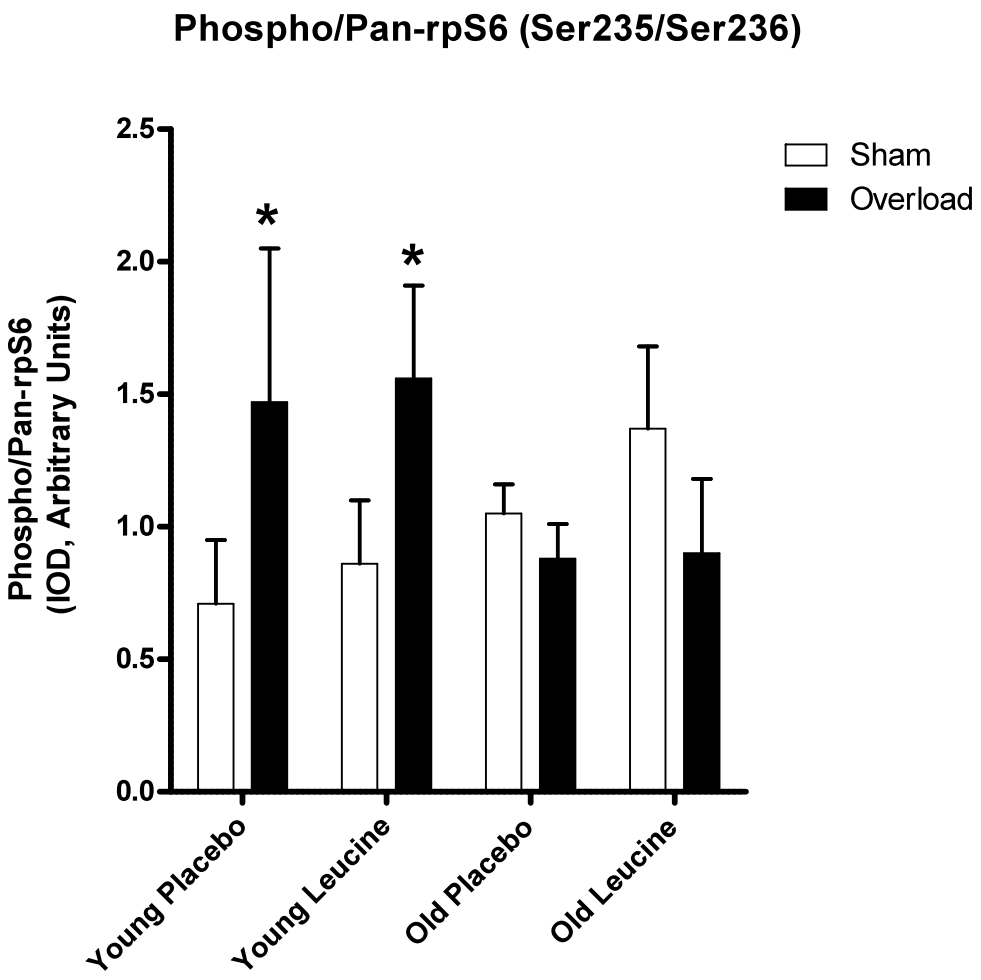


Figure 4.8. Mean ±SEM phospho (Ser235/236) – ribosomal protein S6 (rpS6 / total rpS6 content of sham-operated (sham) vs. 7-day overloaded (overload) plantaris muscle in young (8 mo.) vs old (33 mo.) rats fed either normal chow (placebo) or chow with 5% leucine supplement (leucine). * Significant ($p \leq 0.05$) effect of overload within young age group regardless of dietary condition.

*eEF2 kinase*

In young rats, there was a significant increase in phosphorylated eEF2k with overload in both the placebo and leucine groups (Figure 4.9). No significant difference was observed between sham and overloaded muscle of old rats in either the placebo or leucine groups. Phosphorylated eEF2k did not differ significantly between young and old rats in either the placebo or leucine groups.

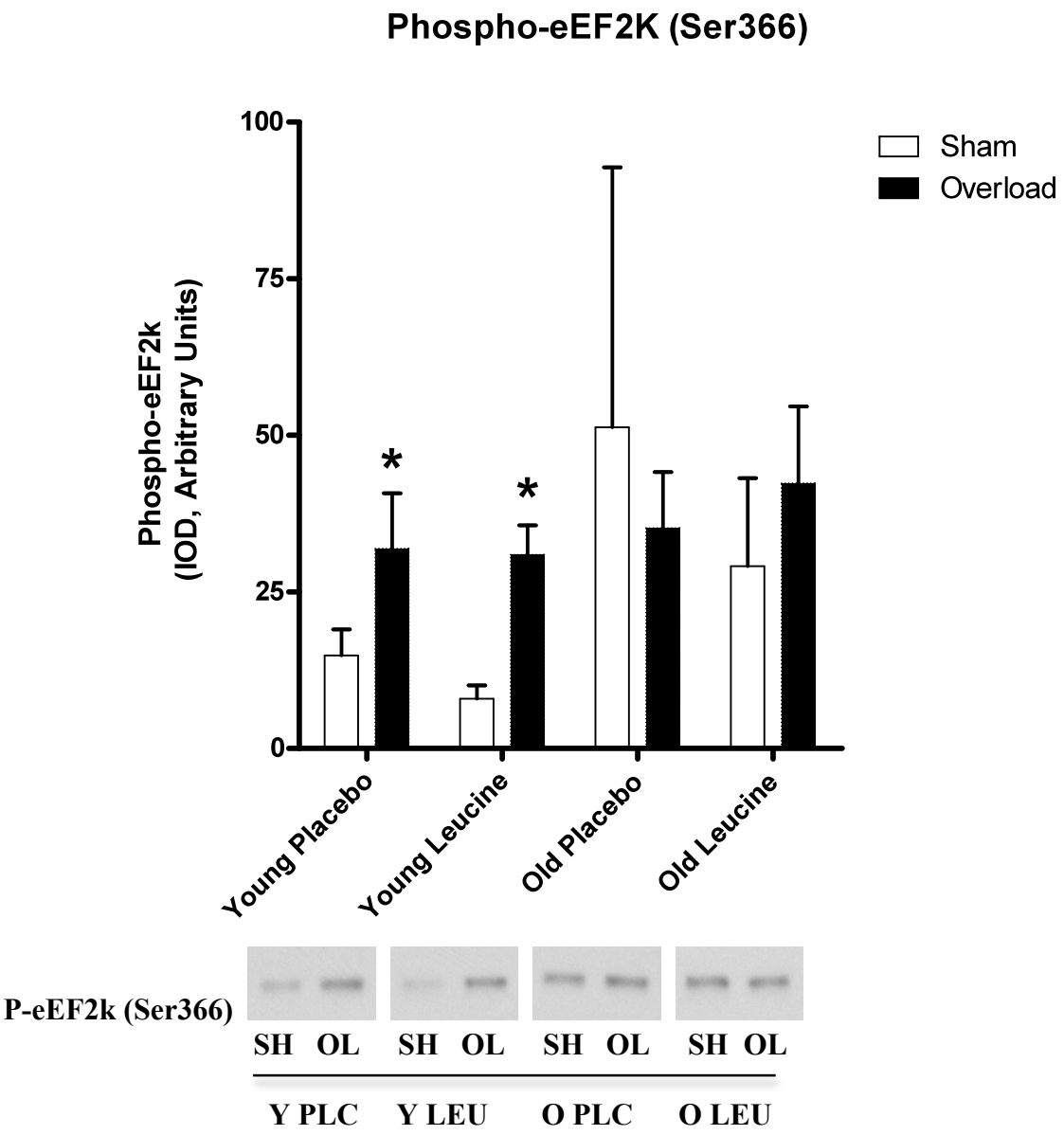


Figure 4.9. Mean ±SEM phospho-eukaryotic elongation factor 2 kinase (eEF2k; Ser366) content and representative blots of sham-operated (sham) vs. 7-day overloaded (overload) plantaris muscle in young (8 mo.) vs old (33 mo.) rats fed either normal chow (placebo) or chow with 5% leucine supplement (leucine). * Significant ($p \leq 0.05$) effect of overload within young age group regardless of dietary condition.

Total eEF2k available for phosphorylation (Figure 4.10) was not significantly different in young rats for sham and overloaded muscles in either the placebo or leucine supplemented groups. Similar results were observed between sham and overloaded muscled of old rats for both the placebo and leucine groups. There was a significantly higher quantity of eEF2 available in old leucine overloaded muscle when compared to the overloaded muscle of young rats supplementing leucine.

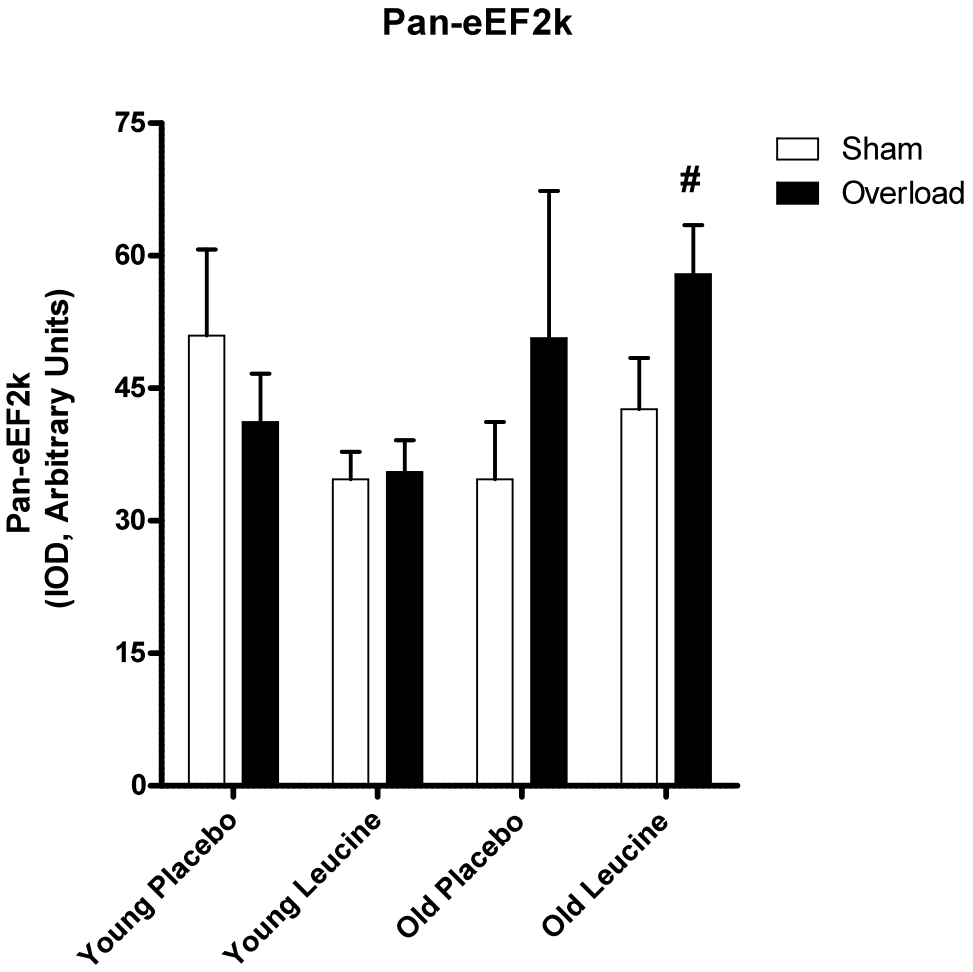


Figure 4.10. Mean ±SEM total eukaryotic elongation factor 2 kinase (eEF2k) content and representative blots of sham-operated (sham) vs. 7-day overloaded (overload) plantaris muscle in young (8 mo.) vs old (33 mo.) rats fed either normal chow (placebo) or chow with 5% leucine supplement (leucine). # Significantly different than young leucine overload group.

Analysis of data expressed in Figure 4.11 indicated that, in young rats, the ratio of phosphorylated-to-total eEF2k showed significant increases in overloaded muscle when compared to sham muscle in both the placebo and leucine groups. No significant differences were observed in either the placebo or the leucine supplemented groups. There was no significant difference in the phosphorylated-to-total eEF2k ratio between young and old rats in either the placebo or leucine groups.

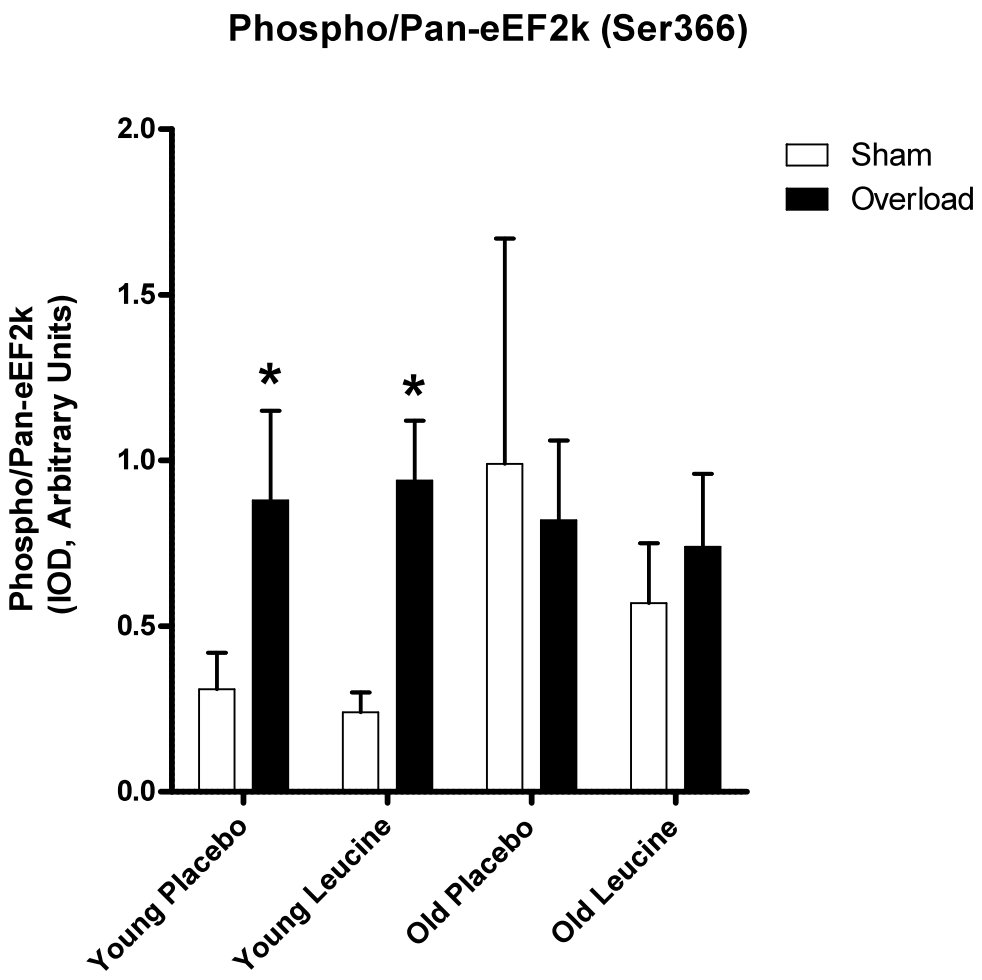


Figure 4.11. Mean ±SEM phospho (Ser366) – eukaryotic elongation factor 2 kinase (eEF2k) / total eEF2k content of sham-operated (sham) vs. 7-day overloaded (overload) plantaris muscle in young (8 mo.) vs old (33 mo.) rats fed either normal chow (placebo) or chow with 5% leucine supplement (leucine). * Significant ($p \leq 0.05$) effect of overload within young age group regardless of dietary condition.

*eEF2*

No significant difference was observed in phosphorylated (Thr56) eEF2 between young and old rats, regardless of age differences (Figure 4.12). There was also no significant difference in the phosphorylation status of eEF2 between rats consuming normal rat chow (placebo) and rats consuming chow with 5% leucine supplement (leucine).

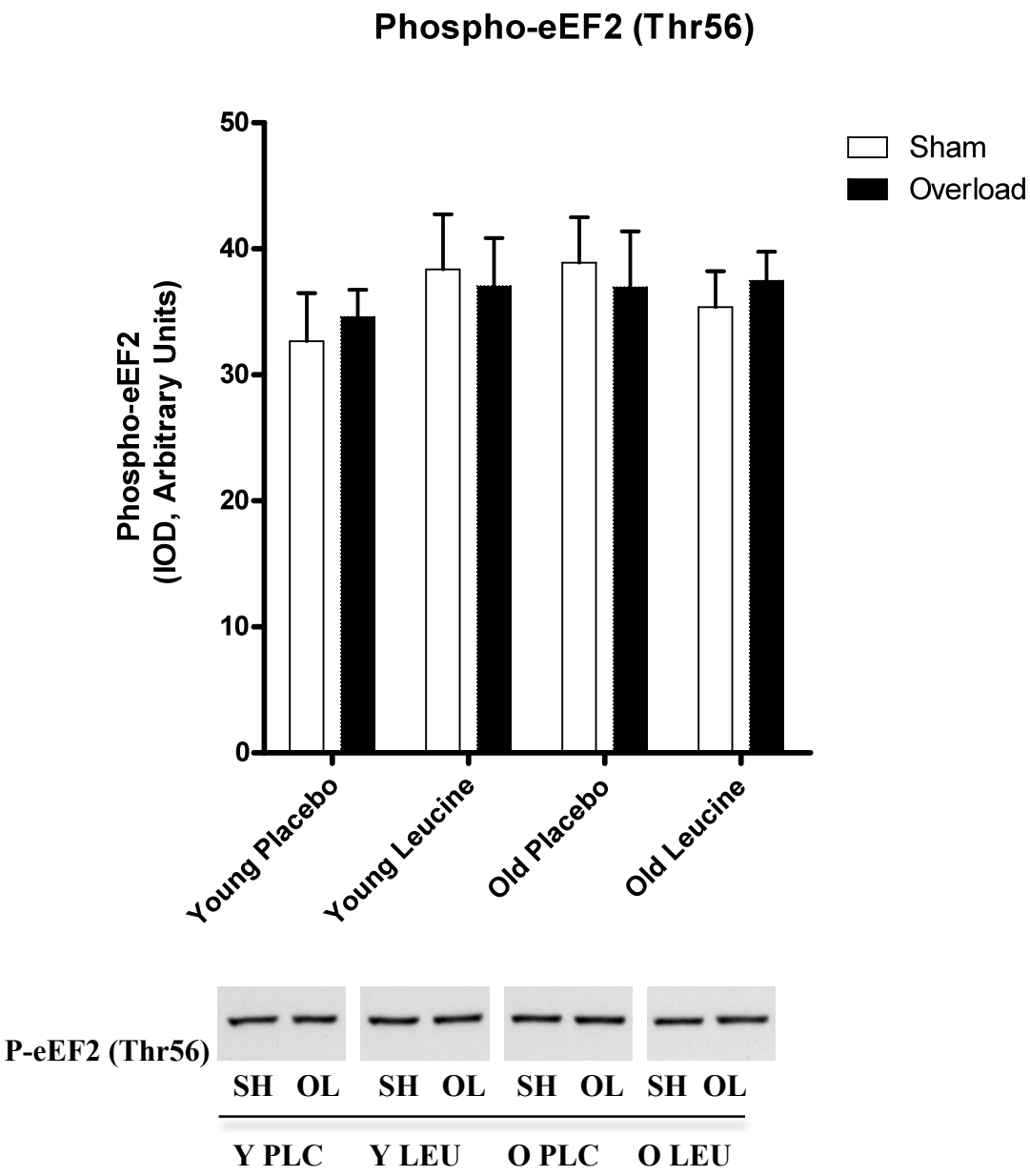


Figure 4.12. Mean ±SEM phospho-eukaryotic elongation factor 2 (eEF2; Thr56) content and representative blots of sham-operated (sham) vs. 7-day overloaded (overload) plantaris muscle in young (8 mo.) vs old (33 mo.) rats fed either normal chow (placebo) or chow with 5%leucine supplement (leucine). No significant differences.

Figure 4.13 shows that young rats in the placebo and leucine groups were significantly different in total eEF2 between sham and overloaded muscles. No significant difference in total eEF2 was observed between sham and overloaded muscle of old rats in either the placebo or the leucine groups when the significance level was set at 0.05. Old rats demonstrated a greater total eEF2 in the sham muscles of both the placebo and the leucine groups than the sham muscle of the young placebo or leucine groups.

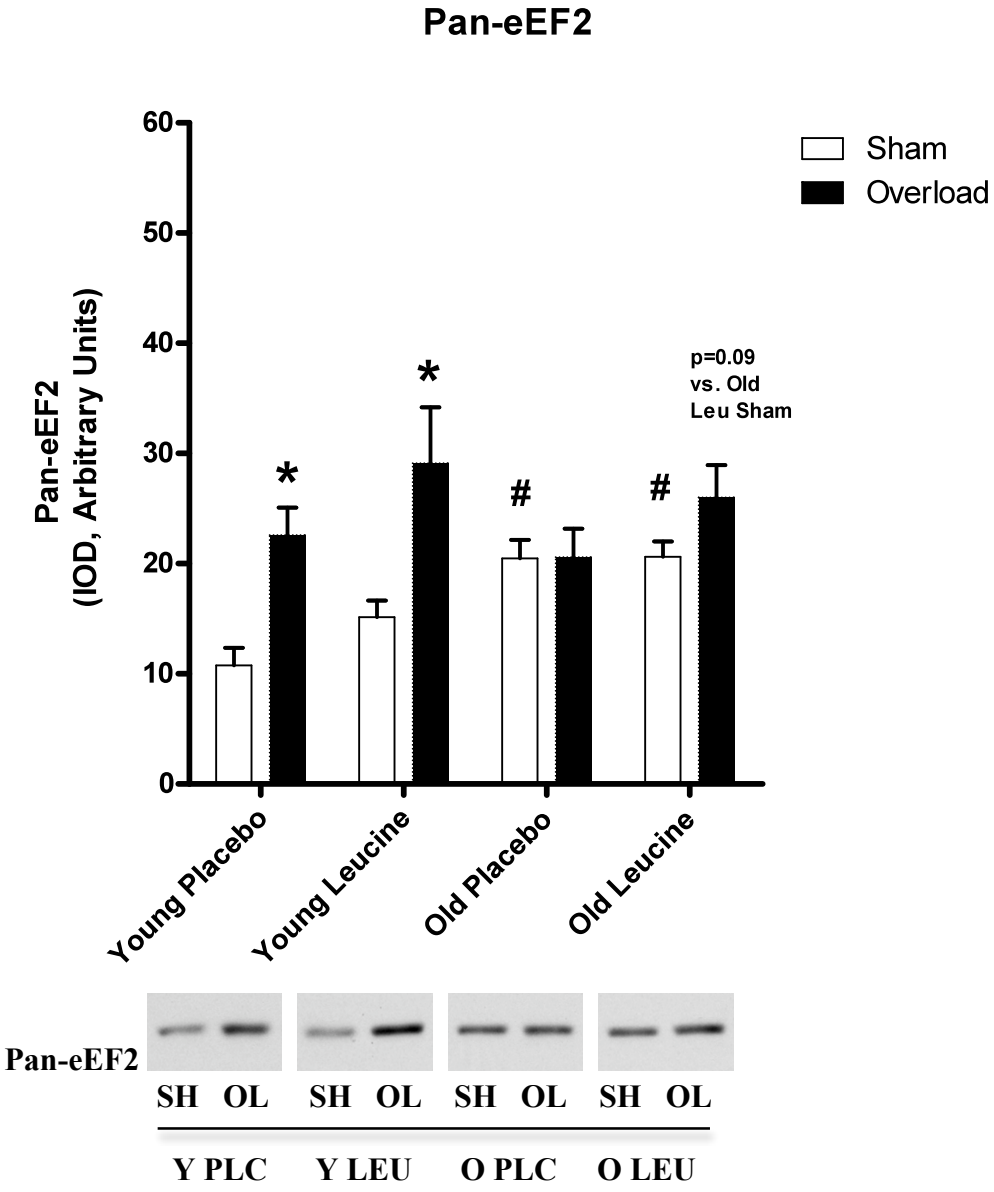


Figure 4.13. Mean ±SEM total eukaryotic elongation factor 2 (eEF2) content and representative blots of sham-operated (sham) vs. 7-day overloaded (overload) plantaris muscle in young (8 mo.) vs old (33 mo.) rats fed either normal chow (placebo) or chow with 5% leucine supplement (leucine). ). * Significant ($p \leq 0.05$) effect of overload within young age group regardless of dietary condition. # Significant effect of age within sham muscles regardless of dietary condition. Overload resulted in a non-significant ($p = 0.09$) increase vs. sham in the old leucine group.

Figure 4.14 indicates the ratio of phosphorylated-to-total eEF2. For both the young placebo and young leucine groups, there was a significant decrease in the ratio of phosphorylated-to-total eEF2 in overloaded muscle when compared to the sham muscle. No significant differences were observed in this ratio between the sham and overloaded muscle of either the old placebo or old leucine groups. In old rat sham muscle of both the placebo and the leucine groups, there was a significantly lower ratio for phosphorylated-to-total eEF2 when compared to the young rat sham muscle.

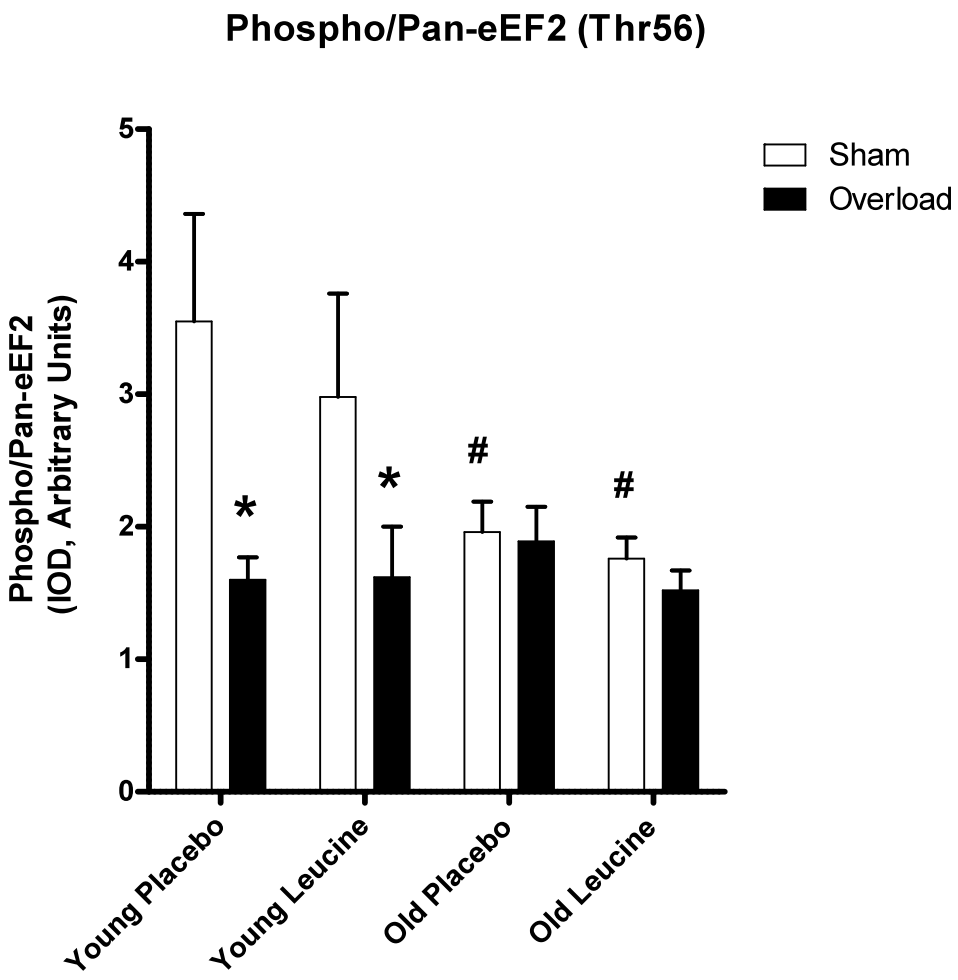


Figure 4.14. Mean ±SEM phospho- (Ser366) eukaryotic elongation factor 2 (eEF2) / total eEF2 content and representative blots of sham-operated (sham) vs. 7-day overloaded (overload) plantaris muscle in young (8 mo.) vs old (33 mo.) rats fed either normal chow (placebo) or chow with 5% leucine supplement (leucine). * Significant ($p \leq 0.05$) effect of overload within young age group regardless of dietary condition. # Significant effect of age within sham muscles regardless of dietary condition.

# Chapter V: Discussion

The age-associated loss of skeletal muscle mass continues to be both detrimental to an individual's functionality (Wickham, 1989; Dorrens, 2003; Hairi, 2010) and overall health (Tellado, 1988), as well as a financial burden for both the individual affected and the healthcare system as a whole (Janssen, 2004). Therefore, it is imperative to determine the causes of this condition and establish the most effective method for improving the diminished hypertrophic response associated with increasing age. The purpose of the current study was to examine the effects of dietary leucine supplementation on specific markers of protein translational signaling and muscle hypertrophy in the overloaded fast-twitch plantaris muscles of young and aged Fischer$^{344}$ x Brown Norway rats. It was hypothesized that supplementing a standard chow diet with 5% leucine would enhance muscle hypertrophy in overloaded fast-twitch plantaris muscles of old (33 mo) rats to levels observed in young adult (8-month old) rats. It was also hypothesized that 5% dietary leucine supplementation would enhance p70s6k, rpS6, eEF2k, and eEF2 signaling in the overloaded fast-twitch plantaris muscles of old rats to levels observed in young adult (8-month old) rats. The most significant findings of the current study were that additional 5% dietary leucine during chronic muscle overload: 1) enhanced muscle hypertrophy in old rat plantaris muscles to levels observed in young control rats 2) increased phosphorylation of signaling proteins p70s6k and rpS6 in old overloaded rat plantaris muscle equivalent to that observed in young rat plantaris muscles. Additionally, supplementing a diet with 5% leucine enhanced total abundance of rpS6 and eEF2k in old overloaded plantaris muscle over the sham muscles of the leucine group.

The current study demonstrated the effects of dietary 5% leucine and chronic muscle overloading on muscle hypertrophy with age. As hypothesized, the addition of 5% leucine to

standard rat chow helped to rescue the hypertrophic response to muscle overloading in old rats. Other studies have documented a diminished hypertrophic response to muscle overloading with age in the absence of leucine supplementation. When muscle overloading was used in an attempt to increase muscle mass in old rats and humans, hypertrophic gains were not to the same extent as in young rats (Blough, 2000; Chale-Rush, 2009; Paturi, 2010) and human participants (Kosek, 2006). Of the rats that consumed the placebo chow in the current investigation, older rats had a significantly lower percent hypertrophy in overloaded plantaris muscles. As hypothesized, old rats consuming a leucine-enriched diet showed higher increases in muscle hypertrophy compared to old rats consuming the placebo chow. These data indicate that the combination of a standard rat chow with 5% leucine and muscle overloading can be used to rescue the hypertrophic responses in old rats to the levels observed in their younger counterparts.

It is important to note that young rats did not show any additional increases in muscle hypertrophy from overload when supplemental leucine was administered in the current study. Previous studies have shown that young and adult rats (Thomson and Gordon, 2005; Thomson and Gordon, 2006; Chale-Rush, 2009) and humans (Kosek, 2006) performing muscle overloading were able to generate a much greater hypertrophic response than older rats and humans. It is unclear why leacine supplementation did not enhance hypertrophy in the young animals in the current study. However, the rat synergist tenotomy model is an extremely robust model of muscle growth, in this case resulting in over 15% hypertrophy in one week. It may be possible that young rat plantaris muscles had reached a maximized hypertrophic response for the given time period of 7 days, and 5% leucine supplementation could not further elicit additional hypertrophy that was observed in old rats.

In order to determine whether the enhancements in muscle hypertrophy were associated with increased translation initiation and elongation signaling, we further analyzed the phosphorylation and total abundance of signaling markers downstream from mTOR in old rat plantaris muscles. We then compared our findings with the values observed in their younger counterparts. In the current study, muscle overload alone could not stimulate an increase in the phosphorylation of p70s6k, rpS6, or eEF2k over that observed in the sham control muscles in old rats as with young rats. Again, this may partly account for the age-associated reduction in responsiveness to muscle overloading and resistance exercise (Thomson and Gordon, 2005; Kosek, 2006; Thomson and Gordon, 2006; Chale-Rush, 2009). As hypothesized, similar levels of phosphorylation for p70s6k and rpS6 were observed when comparing old overloaded plantaris muscles in the leucine group vs. young placebo animals. Since all conditions of the study design were the same except for the additive 5% leucine in the experimental diet groups, one can attribute the enhanced phosphorylation of these signaling markers to leucine. These findings support previous findings showing a positive relationship between p70s6k phosphorylation and muscle hypertrophy (Thomson and Gordon, 2006). Recall that changes in the phosphorylation status of p70s6k at residue Thr389 are attributed to changes in mTOR signaling (Nadar, 2002). Concurrent positive changes in rpS6 (Ser235/236) (and theoretical activation) and eEF2k (Ser366) phosphorylation (and theoretical deactivation) were expected in response to increased phosphorylation of $p70s6k^{Thr389}$ following stimulation by muscle overload (Brown, 2002; Ruvinsky, 2005). Interestingly, phosphorylation of eEF2 did not differ between age groups. Furthermore, neither muscle overloading nor the addition of 5% leucine altered eEF2 phosphorylation when compared to the phosphorylation observed in the old control rats. Thus, eEF2k Ser366 phosphorylation by p70s6k in the young overloaded muscles apparently did not

affect eEF2$^{Thr56}$ phosphorylation status, regardless of the fact that this kinase facilitates eEF2's participation in the elongation process (Browne, 2002; Proud, 2007).

Previous research by Chale-Rush et al (2009) found that chronic (28 day) muscle overloading had no beneficial effects on increasing the total abundance of the signaling proteins mTOR and p70s6k in young (6 mo) or old (30 mo) FBN rat plantaris muscles (Chale-Rush, 2009). Contrary to these findings, the current study demonstrated that muscle overloading and dietary 5% leucine intervention had profound effects on total abundance for the signaling proteins analyzed between age groups. Old overloaded rat plantaris muscles in the leucine group demonstrated increases in total rpS6 and eEF2k over that observed in the sham muscles. Also, as mentioned previously, total eEF2 was substantially higher in the overloaded muscles of old leucine rats compared to sham muscles (albeit at a p=0.09 level). The increase in availability of eEF2 could theoretically improve eEF2's participation in translation elongation and potentially enhance muscle protein synthesis. Most notably, the total abundance of rpS6 was significantly higher in overloaded muscles of old rats in the leucine group than the quantity found in their younger counterparts. Regulation of cell size is one of the many roles that have been designated for rpS6 (Ruvinsky, 2005). As previously discussed, rpS6 may assist in upregulating 5'-terminal oligopyrimidine (5'-TOP) sequences, which participates in coding for translational machinery as well as for eEF2 (Jefferies, 1997; Magnuson, 2005). It is possible that an increased abundance of rpS6 could have contributed, either partially or fully, to the restoration of muscle hypertrophy in aged rats. In order to determine whether changes in phosphorylated signaling proteins were a result of changes in total protein abundance, we chose to quantify the ratio of phosphorylated-to-total protein for each translational initiation signaling marker analyzed. In a similar study to the present, the Gordon laboratory determined the phosphorylation-to-total ratios of signaling

proteins Akt, mTOR, p70s6k, rpS6, eEF2, and 4E-BP1 generated by 1-week of unilateral synergist muscle overload (without any dietary intervention) in young adult (8 mo) and old (30 mo) Fischer$^{344}$ x Brown Norway rats (Thomson and Gordon, 2005). Signaling statuses for mTOR, p70s6k, rps6 and 4E-BP1 showed marked increases in both young adult and old overloaded rats when compared to their age matched controls. Also, Akt (old rats only) and eEF2 statuses showed marked decreases. However, the enhancements in signaling statuses were significantly greater in young adult rats, indicating that there was a signaling deficit in with age in response to muscle overload. Additionally, a correlation between absolute p70s6k phosphorylation and muscle hypertrophy was established in this study (Thomson and Gordon, 2006). Among young rats in the current study, ratios of phosphorylated-to-total for p70s6k, rpS6, and eEF2k were all greater in overloaded muscle when compared to ratios found in sham muscles regardless of the diet consumed indicating that leucine had no beneficial effects. Additionally, the ratio of phosphorylation-to-total eEF2 was reduced in response to overload in young rats consuming both the placebo and leucine below values observed in the sham muscles, as would be expected due to the inhibitory nature of eEF2 phosphorylation. Contrary to young muscle, old rat muscles demonstrated no differences in phosphorylated-to-total ratios for p70s6k, rpS6, eEF2k or eEF2 between sham and overloaded muscle or between dietary conditions.

In the current study, profound improvements were observed in muscle hypertrophy in old rats with the addition of 5% leucine to the diet. However, the variability in signaling marker phosphorylation, abundance, and the phosphorylation-to-total ratios indicate that another potential mechanism affected by leucine may also be aiding the enhanced hypertrophy in aged rats. Recall that dietary leucine enrichment in quantities as low as 5% has been shown to stimulate downstream mTOR signaling phosphorylation (Crozier, 2005), and also inhibit

proteolysis (Combaret, 2005). It is also apparent from previous research, which incorporated pharmaceutical inhibitors of specific translational signaling proteins, that there are relationships between 1) muscle overloading/resistance exercise and mTOR pathway signaling, and 2) amino acid supplementation and mTOR pathway signaling (Anthony, 2000 #2; Dardevet, 2000; Drummond, 2009). Of the data reviewed from previous studies, and the data collected in the current study, we can deduce two possible mechanisms for the improvements in hypertrophy: 1) muscle overload and 5% leucine in aged rats had a potentially positive effect on improving protein synthesis via mTOR signaling and stimulation of its downstream signaling markers, p70s6k and rpS6, with a possible additional effect on total eEF2k and eEF2; and/or, 2) muscle overload and 5% leucine in aged rats had positive effects on reducing protein degradation pathway activation. Both of these mechanisms combined could cause a positive shift in the synthesis-to-degradation ratio, which would ultimately lead to the enhanced muscle hypertrophy in aged rats with leucine supplementation.

In the current study, we chose Fischer$^{344}$ x Brown Norway rats due to the distinct similarity that they shared with humans in regards to aging (Rice, 2008). In order to validate that 5% dietary leucine does indeed improve the hypertrophic response, and possibly slow or attenuate the progression of sarcopenia, further testing in human participants is warranted. The time frame for muscle overloading was selected based on data from previous studies, which indicated clear changes in protein translational signaling marker phosphorylation and abundance, as well as muscle hypertrophy after 7 days (Thomson and Gordon, 2005; Thomson and Gordon, 2006; Chale-Rush, 2009). Further improvements in signaling and hypertrophy may have been generated if the duration of the overload period had been extended. In addition to the time frame, it is important to emphasize how substantially robust the stimulus was from the

overloading model itself. Young rats may have had varying effects from overload and/or leucine supplementation if a different overloading model had been incorporated.

Along with the duration of overloading, dietary content and administration were also important in designing the experiment. Recall that dietary leucine enrichment in quantities as low as 5% has been shown to stimulate downstream mTOR signaling phosphorylation (Crozier, 2005), and inhibit proteolysis (Combaret, 2005). Due to the age-associated desensitization to amino acids (Paddon-Jones, 2004), larger quantities of leucine may be required to further enhance the effectiveness of the amino acid in older animals. We provided the rats ad libitum access to chow rather than administering it at different time points throughout the experiment. Reasoning for this decision was because it was important to keep each animal's amino acid availability as high as possible within the body. Visual inspections of the animal cages were conducted each day to ensure that no food spillage had occurred (the chow was colored bright pink and bright green for the leucine-supplemented and placebo diets, respectively). Any visible particles of chow were accounted for when remaining chow was weighed. An analysis of chow consumption at different time points throughout the experiment revealed a significant difference between age groups in chow weight following the tenotomy. This indicated that old rats consumed less chow (per body weight) than their younger counterparts after surgery. This may be due to a greater general effect of anesthesia and surgery in older animals. Nevertheless, the old rats demonstrated marked improvement in muscle hypertrophy responses and with select signaling responses. Thus, any decrease in food intake in older animals due to surgery apparently did not negate the positive effect of leucine supplementation on muscle growth.

## *Conclusion*

To our knowledge, this is the first study to analyze the effects of supplemental leucine on muscle hypertrophy and muscle protein translational signaling proteins in aged muscle under conditions of chronic muscle overload. The addition of muscle overload and 5% supplementation leucine in the current study showed a remarkable potential benefit in terms of enhancing the overload-induced fast-twitch muscle growth that is typically reduced with age. Plantaris wet weight hypertrophy among old overloaded muscles was less than young in rats consuming normal chow. However, leucine supplementation was able to recover overload-induced hypertrophy, presumably by restoring the balance between protein synthesis and degradation, in old rats. The addition of leucine facilitated concomitant increases in phosphorylation of p70s6k and rpS6 in young and old overloaded plantaris muscles. Old rats consuming leucine also showed significantly enhanced total abundance of rpS6, and a non-significant increase in total eEF2 ($p=0.09$), indicating that leucine may have some ability to reduce the loss of translation initiation signaling with age in overloaded muscles. These novel findings indicate that a leucine-enriched diet may potentially enhance overload-induced growth of aged fast-twitch muscle, in part by enhancing pathways known to stimulate protein synthesis.

## *Practical Applications*

The administration of leucine in conjunction with muscle loading activities, particularly for elderly individuals, may help to rescue the diminished hypertrophic response of fast-twitch muscles with age. However, further research is required to determine whether interventions such as resistance training paired with increased dietary leucine intake can restore overload-induced hypertrophy in human participants. In addition, further studies are also warranted to determine

whether therapeutic leucine supplementation may be implemented to delay or prevent the loss of basal muscle mass with age, or sarcopenia.

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APPENDIX A: East Carolina University Use Protocol Approval Document

East Carolina University.

**Animal Care and Use Commitee**
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

**252-744-2436** office
**252-744-2355** fax

December 16, 2010

Scott Gordon, Ph.D.
Department of EXSS/Physiology
Ward Sports Medicine Bldg.
ECU Brody School of Medicine

Dear Dr. Gordon:

Your Animal Use Protocol entitled, "Leucine Supplementation and Skeletal Muscle Growth in Aged Animals" (AUP #P064) was reviewed by this institution's Animal Care and Use Committee on 12/16/10. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure