Abstract

Effects of Dietary Leucine Supplementation on Muscle Mass and Markers of Protein Degradation in Overloaded Skeletal Muscles of Young Adult and Aged Rats

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The hypertrophic response to overload in fast-twitch skeletal muscle is impaired in aged humans and rats, and upregulation of protein degradation pathways are hypothesized to be a contributing factor. Muscle growth occurs when protein synthesis is greater than protein degradation. Dietary supplementation of the essential amino acid leucine has been shown to reduce protein degradation in both young and aged skeletal muscle. Specifically, leucine acts in part by attenuating 5'-AMP-activated protein kinase (AMPK) activation as well as the translocation of the forkhead box transcription factor 3A (FoxO3, known to promote transcription of mRNAs encoding degradation pathway proteins) to the nucleus. Akt (a promoter of muscle growth) prevents translocation of FoxO3 into the nucleus by phosphorylating FoxO3 phosphorylation at $Ser^{318/321}$. However, AMPK, inhibits Akt's phosphorylation of FoxO3, allowing it to enter the nucleus and increase transcription of protein degradation pathway genes encoding ubiquitin ligase proteins such as muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx, or Atrogin-1). During the aging process, AMPK Thr^{172} phosphorylation (and thus its activation) is increased, purportedly inhibiting gains in muscle mass and strength.

Although dietary leucine supplementation has been shown to enhance muscle hypertrophy in response to resistance training in young humans, the potential for leucine supplementation to enhance overload-induced muscle hypertrophy in aged humans or animal models has not been examined. Thus, the aim of this study was to determine whether dietary leucine supplementation can attenuate markers of protein degradation and rescue hypertrophy during overload in the fast-twitch skeletal muscles of aged rats to levels comparable to their younger counterparts. It was hypothesized that dietary leucine supplementation during 7 days of fast-twitch plantaris muscle overload would enhance plantaris muscle hypertrophy in aged rats to levels observed in young adult rats not receiving leucine. It was also hypothesized that dietary leucine supplementation during the overload period would alter markers of protein degradation (enhance FoxO3 phosphorylation and reduce the levels of AMPK phosphorylation, Atrogin-1 protein content, and MuRF1 protein content) in the overloaded fast-twitch plantaris muscles of the aged rats to levels observed in young adult rats not receiving leucine. Young adult (8 mo.) and old (33 mo.) male Fisher 344 x Brown Norway F1 Hybrid (FBN) rats underwent a 1-week unilateral overload of the fast-twitch plantaris muscles 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 (normal rat chow supplemented by an additional 5% leucine content in place of 5% of the carbohydrate content; n = 7/age group) or placebo group (normal rat chow; n = 6/age group). The leucine groups started the leucine-enriched diet 2 days prior to, and throughout, the overload intervention. All animals had ad libitum access to water and chow during the entire experiment; no differences in daily calorie consumption were observed between the placebo vs. leucine groups within each age group. At the end of the overload period, sham-operated and overloaded plantaris muscles were harvested and analyzed via western blotting for

the phosphorylations of AMPK and FoxO3 as well as total levels of Atrogin-1 and MuRF1. Dietary leucine enrichment significantly ($p \leq 0.05$) enhanced overload-induced plantaris muscle hypertrophy in old, but not in young adult, animals. Sham and overloaded plantaris muscle AMPK phosphorylation was significantly higher in aged animals receiving normal chow compared to young adult animals; however, leucine supplementation in old animals reduced this AMPK phosphorylation to levels similar to young adult animals. Compared to placebo, leucine also non-significantly ($p = 0.07$) enhanced FoxO3 phosphorylation in the overloaded muscles of both young adult and old animals (thus theoretically reducing FoxO3 translocation to the nucleus). Accordingly, leucine also non-significantly ($p = 0.07$) reversed the overload-induced increase (from a 22.8% increase to a 17.0% decrease) in Atrogin-1 content in aged muscles and non-significantly ($p = 0.14$) enhanced the overload-induced decrease in MuRF1 content in the muscles of both age groups. These findings indicate that a leucine-enriched diet may potentially enhance overload-induced growth of aged fast-twitch muscle, in part by suppressing pathways known to stimulate protein degradation.

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Protein Degradation in Overloaded Skeletal Muscles of Young Adult and Aged Rats

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Chapter I: Introduction

Sarcopenia

Aging is an inevitable part of the human life and is usually accompanied by the loss of muscle mass and strength. This gradual loss of muscle mass is known as sarcopenia. The muscle atrophy attributed to sarcopenia is often targeted at fast-twitch fibers and is accompanied by changes in neural activation, which leads to decrease in muscle function (Macaluso & De Vito, 2003). This targeting of fast-twitch muscle fiber not only leads to atrophy but also a decrease in total muscle fibers (Lexell, 1995). Even though there is a decrease in fast-twitch fibers, slow-twitch fibers remain relatively unchanged, but are, however, less conducive for gaining strength (Lexell, 1995).

Muscle mass is fairly stable between 25 and 50 years of age, however there is an approximate decrease of 25% from 50 to 70 years of age (Baumgartner et al., 1998). Per year there is an average rate of muscle loss of 1-2% per year, past the age of 50 (Rice et al., 2005). Although this imposes a certain amount of stress on the health care system, this economic burden can be modified (Janssen et al., 2004). There are two main criterion that impact this phenomenon: initial muscle mass and the rate in decline of lean body mass with age. Although there are many theories behind sarcopenia (Rosenberg 1997), two factors play an immense role on this change in lean body mass: regulation of protein synthesis and degradation. Decreases in muscle mass with age is predominantly due to a loss of total muscle fibers and a decrease in fiber cross-sectional area(Lexell, 1995). This loss of fiber size predominantly occurs in type II fibers, with the greatest occurrence in type IIb fibers (Grimby et al., 1982; Lexell, 1995). The type II

fibers are responsible for total strength and power of a muscle group (Lexell, Taylor, & Sjostrom, 1988).

Resistance Training

Resistance training during advancing age can help postpone the effects of sarcopenia. Some studies have shown a significant decrease in fast-twitch fiber distribution in the elderly (Kirkendall & Garrett, 1998), which can detract from the effectiveness of a resistance training program. Fast-twitch fibers are the main fibers focused on hypertrophying through resistance training because of their ability to increase in size and strength as compared to the more oxidative slow-twitch fibers (Lexell, Taylor, & Sjostrom, 1988). The elderly show a maintenance or increase in slow-twitch muscle fiber number and decrease in total fast-twitch fibers (Grimby et al., 1982; Lexell, 1995; Macaluso & De Vito, 2003), this change in distribution can significantly decrease the degree to which an elderly individual can hypertrophy and strengthen muscle. However, strength gains can still be seen from even a single bout of resistance training. Frontera et al. (Frontera et al., 1988) has shown that up to 5% strength gains can be obtained when training elderly men at 80% of their 1 rep maximum. These studies as well as others (Kosek et al. 2006; Thomson & Gordon, 2005) show that there is a blunted response in the elderly to resistance training induced fast-twitch muscle hypertrophy. This inhibition of muscle hypertrophy has also been shown in certain breeds of rats (Thomson & Gordon, 2005).

Roles of Protein Synthesis & Degradation

Throughout any given day, both protein synthesis and degradation will climax and bottom in a constant undulating cycle (Patton, Willems, & Tyers**,** 1998). Both of these pathways

share many upstream regulators (Zhang et al., 2007). Most of the observed changes in protein mass are facilitated by changes in protein synthesis (Phillips, Glover, and Rennie, 2009). Protein degradation is much less studied and analyzed as compared to protein synthesis. The changes in degradation are much less clear than those in synthesis with aging.

Response to Resistance Training

While there are many pathways affecting both protein degradation and protein synthesis, protein synthesis must outweigh degradation for skeletal muscle to hypertrophy. Without an overall increase or decrease in one or the other, muscle mass will remain the same. Following resistance training, both synthesis and degradation are at increased levels (Biolo et al., 1995). Even though protein synthesis has been shown to decrease during exercise, the post exercise period shows a dramatic rise in synthesis (Dreyer et al., 2006). This post exercise rise in protein synthesis has been shown to remain at elevated levels for as long as 48 hours in untrained subjects (Phillips et al., 1997) and up to 16 hours in trained subjects (Tang et al., 2008). Untrained subjects show between 2 and 5 fold increases in protein synthesis during this time (Phillips et al., 1997). Markers of protein degradation, specifically Atrogin-1, have also been shown to be increased pre- and post-exercise in older individuals as compared to their younger counterparts (Raue et al., 2007).

Regulation of Protein Synthesis & Degradation

At Rest & Following Overload

There are two main pathways responsible for catabolic effects on skeletal muscle: the ubiquitin-proteasomal and autophagy-lysosome pathways (Mammucari, Schiaffino, & Sandri, 2008). The regulator responsible for most of the turnover of most soluble and myofibrillar

muscle protein is ubiquitin-proteasomal proteolysis (Lecker, Goldberg, & Mitch,2006). 5'-adenosine monophosphate (AMP)-activated protein kinase (AMPK) causes an increase in the activation of the atrophy-related forkhead box (FoxO) transcription factors (Tong et al., 2009), and thus the AMPK hyperphosphorylation observed in aging fast-twitch muscle has been postulated to lead to an increase in both muscle proteasomal and lysosomal proteolysis with age (Gordon et al., 2008). In atrophied mouse skeletal muscle, FoxO3 activation has been shown to increase mRNA expression of the ubiquitin-ligase proteins muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx, or Atrogin-1) (Sandri et al., 2004). FoxO3, Atrogin-1, and MuRF1 have all been shown to be at increased levels in aged muscle following resistance training (Raue et al., 2007; Nakashima & Yakabe, 2007). However, both of these proteolytic pathways can be suppressed through the anabolic affects of feeding (Katsanos et al., 2006) and increasing insulin (58). Both of these anti-proteolytic mechanisms are blunted with age (Katsanos et al., 2006; Welle et al., 1993).

One of the primary upstream regulators of protein synthesis is mammalian Target of Rapamycin. mTOR activation up regulates protein synthesis which will in turn lead to muscle hypertrophy (Bodine & Stitt et al., 2001). Mechanical overload will cause an increase in mTOR activation in the young (Drummond et al., 2008) however this increase is blunted in the elderly (Thomson & Gordon, 2005). Possible explanation for this decreased mTOR activation with age is due to increased 5'-AMP-activated protein kinase, or AMPK, activation with age (Thomson & Gordon, 2005). AMPK has been termed an energy sensing switch because it is activated in times of elevated AMP:ATP ratios (Hardie & Sakamoto, 2006). AMPK down regulates protein synthesis and increases protein degradation, which in turn will lead to increased muscle atrophy

(Nakashima & Yakabe, 2007). An intervention able to decrease or inhibit hyperphosphorylation of AMPK in overloaded aged skeletal muscle could help improve overall muscle hypertrophy.

Dietary Intervention

Whey protein supplements have long been used in the world of athletics and aesthetics to improve performance and body composition. One hypothesis behind whey protein's higher anabolic, as compared to casein or soy protein, is its higher concentration of the essential amino acid leucine (Kadawoki & Kanazawa, 2003; Norton et al., 2009). The addition of leucine, increased from 26% to 41%, in whey protein has even been shown to stimulate protein synthesis in the elderly to the same extent as their younger counterparts (Katsanos et al., 2006). Leucine acts on protein synthesis and degradation through activation of the Akt/mTOR pathway, an inhibitor of both lysosomal and proteasomal proteolysis (Zhang et al., 2007). A study (Combaret et al., 2005) showed that supplementation of 5% leucine to a meal can completely reverse the increase in proteasome activities seen with aging. An intervention with leucine supplementation may decrease the chances of sarcopenia or able to decrease or block in the decline of lean body mass in individuals already suffering from the disorder. With leucine added to the diet we hope to decrease the overactive degradation pathways and improve the effectiveness of overload induced hypertrophy.

Specific Aim

The hypertrophic response to overload in fast-twitch muscle is impaired in aged humans and rats. Muscle growth occurs when protein synthesis is greater than protein degradation. Leucine has been shown to reduce protein degradation in both young and aged skeletal muscle. Both leucine supplementation and resistance training have been analyzed in many different

aspects over the past few decades, used on many different populations and species, but rarely used in combination with each other. Although dietary leucine supplementation has been shown to enhance muscle hypertrophy in response to resistance training in young humans, the potential for leucine supplementation to enhance overload-induced muscle hypertrophy in aged humans or animal models has not been examined. Thus, the aim of this study was to determine whether dietary leucine supplementation can attenuate markers of protein degradation and rescue hypertrophy during overload in the fast-twitch skeletal muscles of aged rats to levels comparable to their younger counterparts. It was hypothesized that dietary leucine supplementation during 7 days of fast-twitch plantaris muscle overload would enhance plantaris muscle hypertrophy in aged rats to levels observed in young adult rats not receiving leucine. It was also hypothesized that dietary leucine supplementation during the overload period would alter markers of protein degradation (enhance FoxO3 phosphorylation and reduce the levels of AMPK phosphorylation, Atrogin-1 protein content, and MuRF1 protein content) in the overloaded fast-twitch plantaris muscles of the aged rats to levels observed in young adult rats not receiving leucine.

Chapter II: Review of Literature

Sarcopenia

It seems that an inevitable part of the aging process is the loss of lean muscle mass, increase in fat mass, and decrease in independence. However, there may be methods of intervention that may postpone or eliminate this event. The loss of muscle with aging is known as sarcopenia, literally translated as flesh loss (Rosenberg, 1997). With an increase in the number of elderly people a similar increase in the number of cases of sarcopenia are expected, especially with the aging baby-boomer population. In the year 2000, the estimated cost of the direct impact sarcopenia places on the national healthcare was around $18.5 billion (Janssen et al., 2004). Even with a mild intervention, there could be an ease of the impact this phenomenon plays on healthcare costs in the U.S. It is estimated that in men and women aged 65-70, there is between 13% and 24% who suffer from disabling sarcopenia. By the age of 80 years, the percentage is estimated at over 50% (Baumgartner et al., 1998).

There are two criterion that impact sarcopenia: initial lean body mass and the rate of decline in lean body mass through the years. An individual with higher initial amounts of lean body mass would be at less of a risk for developing sarcopenia compared to an individual with lower lean body mass. Due to the fact that one has a greater amount of lean body mass, it would take a greater amount of muscle loss to impact one's activities of daily living. Past the age of 50 there is a consistent rate of muscle loss, approximately 10%-20% per decade of life (Rosenberg, 1997), which is equivalent to 1.1kg-1.9kg of skeletal muscle loss in women and men, respectively (Jenssen et al., 2000). With this decrease in skeletal muscle mass, a similar decrease

in strength would be expected. The relationship between loss of muscle strength and increase in nursing home admissions is clear (Gillick, 2001). However, the loss of muscle mass and strength is not similar across the fiber type spectrum. Some theories have been proposed to explain sarcopenia: impaired muscle capacity to regenerate (deficiency in satellite cells and protein turnover), an increase in oxidative stress, loss of motor neurons and reorganization of neuromuscular junctions, deterioration in immune system, and development of chronic inflammation (Marcell, 2003).

The age-related skeletal muscle fiber atrophy occurs because of a loss in muscle fibers and decrease in fiber cross-sectional area (Lexell, 1995). This loss of fiber size predominantly occurs in type II fibers, with the greatest occurrence in type IIb fibers (Grimby et al., 1982; Lexell, 1995). There also seems to be a relative maintenance of type I fibers (Kimball et al., 2004). Not only is there a maintenance of type I fibers, but it has been observed that the number of type I fibers increases with age, through re-innervation of motor units (Grimby et al., 1982; Macaluso & De Vito, 2003). Decrease in muscle fiber numbers can range from 61%-75% of the original number by the age of 80 years (Lexell, 1995; Lexell, Taylor, & Sjostrom, 1988).

This fiber type death is currently held under the notion that there is a progressive loss of type II motornuerons from the spinal cord. Denervation of type II muscle fibers is accompanied by a re-innervation of close proximity type I motorneurons, which results in a decrease in type II fibers with an increase in type I fibers (Macaluso & De Vito, 2003). With the decrease in type II fiber number and atrophy selective to type II fibers, there is a significant decrease in the total area of type II fibers in skeletal muscle. This is imperative because muscle fiber distribution is correlated to muscular strength (Häkkinen et al., 2001). Specifically targeting the prevention of

type II muscle fibers may be the key to intervention and maintaining muscle mass and strength with age.

Resistance Training

Resistance training is a very popular intervention for much of the population to increase strength and muscle hypertrophy. Resistance training may help postpone the effects of sarcopenia with advancing age. However, there can be some drawbacks when applying this to the already elderly population. Some studies have shown a significant decrease in fast-twitch fiber distribution in the elderly (Kirkendall & Garrett, 1998). Fast-twitch fibers are the main fibers focused on hypertrophying through resistance training because of their ability to increase in size and strength as compared to the more oxidative slow-twitch fibers (Lexell, Taylor, & Sjostrom, 1988). As stated previously, the elderly show a maintenance or increase in slow-twitch muscle fiber number and decrease in total fast-twitch fibers (Grimby et al., 1982; Lexell, 1995; Macaluso & De Vito, 2003), this change in distribution can significantly decrease the degree to which an elderly individual can hypertrophy and strengthen muscle. However, strength gains can still be seen from even a single bout of resistance training. Frontera et al. (Frontera et al., 1988) has shown that up to 5% strength gains can be obtained when training elderly men at 80% of their 1 rep maximum. Even in an 8 week period, frail women have shown strength increases up to 175% and a 15% increase in muscle cross-sectional area (Fiatarone et al., 1990). This same lab has shown however that even after 10 weeks of resistance training thigh muscle cross-sectional area can increase less than 3%. The discrepancy between strength increases and the amount of hypertrophy suggest that neural adaptations rather than hypertrophic responses are responsible for strength increases. These studies as well as others (Kosek et al., 2006; Thomson & Gordon, 2005) show that there is a blunted response in aged animals to

resistance training induced fast-twitch muscle hypertrophy. This inhibition of muscle hypertrophy has also been shown in certain breeds of rats (Thomson & Gordon, 2005), particularly the Fisher 344 x Brown Norway F1 Hybrid.

Roles of Protein Synthesis and Degradation

Muscle protein synthesis and degradation are in a constant undulating cycle throughout the day, resulting in neither a net loss or gain in protein turnover rates (Phillips, Glover, & Rennie**,** 2009). Even an insignificant increase in protein degradation over a brief period, the accumulated effect over a number of years can lead to a significant increase in muscle atrophy. The same can be said for muscle protein synthesis. Even without an increase in protein degradation, minute decreases in protein synthesis can lead to noticeable increases in atrophy. The most likely explanation for muscle atrophy in the aging population is a combination of both protein degradation and synthesis, influenced by physical inactivity, changes in diet, and alterations in hormones (Janssen et al., 2000). A study by Welle et al. (Welle et al., 1993) showed that myofibrillar protein synthesis rate is decreased in older compared to young men. Although it is common that the decrease in protein synthesis increases muscle atrophy, a study by Kimball et al. (Kimball et al., 2004) showed a presence of sarcopenia even with elevated protein synthesis. Although protein synthesis rates were elevated, the attempt seems futile due to the greater increase in protein degradation. There is an association of increased protein degradation with increased age, this process is less studied that that of decreased protein synthesis. It is apparent that any intervention associated with increased protein synthesis, decreased protein degradation, or both would be promising in delaying, preventing, or possibly even reversing the effects of sarcopenia.

Response to Resistance Training

While there are many pathways affecting both protein degradation and protein synthesis, protein synthesis must outweigh degradation for skeletal muscle to hypertrophy. Without an overall increase or decrease in protein synthesis or degradation, muscle mass will remain the same. Following resistance training, both synthesis and degradation are at increased levels (Biolo et al., 1995). Even though protein synthesis has been shown to decrease during exercise, the post exercise period shows a dramatic rise in synthesis (Dreyer et al., 2006). This post exercise rise in protein synthesis has been shown to remain at elevated levels for as long as 48 hours in untrained subjects (Phillips et al., 1997) and up to 16 hours in trained subjects (Tang et al., 2008). Untrained subjects show between 2 and 5 fold increases in protein synthesis during this time (Phillips et al., 1997).

Even with such a robust increase in protein synthesis, some measures of protein degradation, such as fractional breakdown rate, can increase as much as 50% in a 3 hour period following resistance training (Phillips et al., 1997). Protein degradation levels have also been shown to be increased pre- and post-exercise in older individuals as compared to their younger counterparts (Tamaki et al., 2000). This alteration in levels of protein degradation has been seen in humans and rats (Kumar et al., 2009).

Regulation of Protein Degradation & Synthesis

At Rest and Following Overload

There are two major catabolic systems responsible for muscle loss: the ubiquitin-proteasome and autophagy-lysosome pathways (Mammucari, Schiaffino, & Sandri, 2008). Autophagy/lysosomal proteolysis is mediated by a series of signaling proteins, which include

many members of the autophagy-specific gene family of kinases (Rajawa, Hiliot, & Bossis, 2009). Lysosomal proteolysis is also stimulated by forkhead box o (FoxO), which itself can cause atrophy of muscles and myotubes (Zhao et al., 2007). The ubiquitin-proteasome pathway is responsible for most of the turnover of most soluble and myofibrillar muscle protein (Lecker, Goldberg, & Mitch, 2006).

The ubiquitination of specific proteins is the cause of proteasome proteolysis. The ubiquitin proteasome pathway operates in an ATP-dependent manner in charge of regulating processes in eukaryotic cells (Pickart & Eddins, 2004), with one of these processes being protein degradation (Hershko & Ciechnavor, 1998). The ubiquitin proteasome pathway is unable to degrade intact myofibrils (Solomon & Goldberg, 1996). The process begins with a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and catalyzed by the action of a ubiquitin-ligase enzyme (E3) (Murton, Constantin, & Greenhaff, 2000). Ubiquitin is first bound by E1 through an ATP-dependent process and high-energy bond, leading to the formation of a new thioester linkage between ubiquitin and E2. The final step is the ubiquitin monomer is catalyzed by E3 and is conjugated to the target protein through the 26s proteasome. Several dozen ubiquitin-conjugating enzymes are also present in humans, along with hundreds of ubiquitin ligases (Patton, Willems, & Tyers, 1998).

There are two muscle-specific ubiquitin ligases found to be elevated in atrophied muscle (Sandri et al., 2004), these 2 ligases are Atrogin-1 and MuRF1. The afore mentioned FoxO family of transcription factors is responsible for the transcription of Atrogin-1 and MuRF1 genes (Murton, Constantin, & Greenhaff, 2000). Activation of the FoxO transcription factors is essential for fiber atrophy and Atrogin-1 induction upon denervation, fasting, and glucocorticoid treatment (Sandri et al., 2004). However, Akt, an important factor in muscle protein synthesis,

renders the FoxO transcription factors inactive (Mammucari, Schiaffino, & Sandri, 2008) and in turn, inhibits the expression of Atrogin-1 and MuRF1 (Stitt et al., 2004). Even in the blockade of the proteasome or loss of Atrogin-1 or MuRF1 genes, autophagy in skeletal muscle is not impaired (Bodine & Latres et al., 2001). This process shows that FoxO3 regulates both the ubiquitin-proteasome and autophagy-lysosomal systems during atrophy (Mammucari, Schiaffino, & Sandri, 2008; Zhao et al., 2007). Following resistance training Atrogin-1 as well as MuRF1 mRNA levels have been shown to be increased in the elderly compared to the young (Raue et al., 2007). A study by Zhao et al (Zhao et al., 2007) showed that FoxO3 caused transcription of 7 autophagic related genes. Aging rat skeletal muscle has shown an increase in postprandial ubiquitin-proteasome dependent proteolysis (Combaret et al., 2005), which is extremely unusual considering that normally there is a decrease in proteolysis and an increase in protein synthesis following a complete meal (Phillips, Glover, & Rennie**,** 2009).

Both protein synthesis and protein degradation share many common upstream regulators. Insulin-like growth factor-1 is a known stimulator of protein synthesis. IGF-1 stimulates protein synthesis through activation of the Akt/mammalian Target of Rapamycin pathway (Bodine et al., 2001). Insulin also promotes muscle accretion by inhibition of proteolysis in an apparent dose-dependent fashion (Chow et al., 2006), and proteolysis is maximally inhibited with plasma insulin concentrations around 30 μIU/mL (Pozefsky et al., 1969; Wilkes et al., 2009). When insulin levels are raised to great than 5μIU/mL, leg protein breakdown was suppressed by 47% in young adults (Wilkes et al., 2009). With aging, this normal suppression of proteolysis becomes impaired. Another aspect of increased muscle loss with aging may be due to a relative insensitivity of the antiproteolytic effects of insulin.

The Akt/mTOR pathway is known to not only inhibit the ubiquitin-proteasome proteolysis but also inhibit autophagy through lysosomal proteolysis (Zhao et al., 2007). The main mediator for mTORs inhibition of protein degradation is through the TORC2 complex (Mammucari, Schiaffino, & Sandri, 2008). Translocation of FoxO3 to the nucleus is inhibited by mTOR activity through the positive feedback loop of Akt (Latres et al., 2005).

On the flipside of the synthesis/degradation coin, is 5'-AMP-activated protein kinase (AMPK), an inhibitor of mTOR. AMPK is activated during times of reduced cellular energy, seen as a decrease in the ATP/AMP ratio (Hardie & Sakamoto, 2006). AMPK also regulates homeostatic balance between growth and cell atrophy, allowing the cell to either focus on ATP production to support metabolic needs or ATP use for maintenance and growth of cell size depending on energy state (Gordon et al., 2008). Decreases in muscle glycogen as well as endurance training can alter the ratio of ATP/AMP (Hardie & Sakamoto, 2006). AMPK's ability to inhibit mTOR helps provide an explanation for the lack of muscle hypertrophy seen by endurance training.

The FoxO transcription factors, responsible for expression of atrophy-related ubiquitin-proteasome ligases, are stimulated through AMPK activation (Nakashima & Yakabe, 2007). The FoxO family of transcription factors are all expressed in skeletal muscle, and their expression is increased during times of caloric restriction (Murton, Constantin, & Greenhaff, 2000). An important aspect of muscle atrophy stimulated by AMPK activation is its up regulation in fast-twitch fibers (Gordon et al., 2008). In young adult versus old rats, the old rats saw a 5-fold increase in AMPK activity in fast-twitch muscle but not in slow twitch muscle (Thomson & Gordon, 2005). Gordon et al. (Gordon et al., 2008) showed how constant stimulation of AMPK in resting muscle even showed a high frequency of fiber death. Although significant decreases in

protein synthesis would lead to severe atrophy, protein degradation would have to be increased to show this magnitude of atrophy and fiber death. Not only is this process detrimental in muscle wasting, it is also noted as being increased in aging muscle (Gordon et al., 2008). So, not only does AMPK decrease protein synthesis and increase protein degradation, but there is a particular focus on fast-twitch fibers, the fibers that contribute the most debilitating effects when lost during sarcopenia.

Pathways leading to muscle hypertrophy and atrophy overlap in many aspects. It is conceivable that the implement of a protocol acting on AMPK inhibition could stimulate mTOR or that the stimulation of the Akt pathway could decrease FoxO transcription phosphorylation. Either route could prove beneficial to those in muscle wasting situations.

Dietary Intervention

Whey protein supplementation has long been used as an ergogenic aide for increasing muscle hypertrophy by athletes and those wanting to improve body composition. Whey protein supplementation has been shown to increase protein synthesis and decrease protein degradation (Kadawoki & Kanazawa, 2003; Tang & Phillips, 2009). A common theory behind the mechanisms of this phenomenon is whey protein's high content of the essential branched-chain amino leucine (Katsanos et al., 2006; Rieu et al., 2006; Vandervoot, 2002). Some studies have shown leucine to stimulate protein synthesis despite an increase in other amino acids (Crozier et al., 2005; Rieu et al., 2006). Leucine is responsible for increased protein synthesis through the mTOR pathway (Crozier et al., 2005; Drummond & Rasmussen, 2008). Although leucine content of a complete meal cannot dictate duration of protein synthesis, it can direct peak activation through the mTOR pathway (Norton et al., 2009).

Leucine is a branched chain amino acid, named so for its aliphatic side chain. This amino acid cannot be synthesized by animals and must be obtained through nutritional means. Unlike other amino acids, branched-chain amino acids cannot be degraded in the liver due to absence of branched-chain amino acid aminotransferase. However, skeletal muscle can degrade these amino acids due to the presense of branched-chain amino acid aminotransferase (Sweatt et al., 2004). Since leucine can only be metabolized in the muscle, it has been hypothesized that once degraded, leucine metabolites such as beta-hydroxy-beta-methylbutyrate could decrease AMPK activity and increase mTOR activation (Wilson, Wilson, & Manninen, 2008). Du et al. (Du et al., 2007) has shown that leucine is able to increase ATP content, decrease the AMP/ATP ratio, and thereby inhibit a rise in AMPK levels.

The antiproteolytic effects of leucine have not been studied as much as its anabolic effects. A study by Nakashima and Yakabe (Nakashima & Yakabe, 2007) showed that leucine down-regulated the ubiquitin-proteasome pathway in chick-skeletal muscle without the involvement of the mTOR pathway but achieved it through the protein kinase c and PI3K pathways. Nakashima et al. (Nakashima et al., 2005) have also shown that leucine inhibits myofibrillar proteolysis through decreases in N^{τ} -methylhistidine concentration in chick skeletal muscles. N^{τ} -methylhistidine is a byproduct of actin and myosin degradation but is not reused for protein synthesis, which is why it has been used as a marker of myofibrillar proteolysis.

As mentioned before, the ubiquitin-proteasome pathway has a major role in skeletal muscle proteolysis. Following a complete meal, there should be a decrease in pathway's activity, however it has shown to still be increased in older animals (Combaret et al., 2005). The addition of leucine to the meal is able to completely restore the postprandial effects of eating in 22-month-old rats (Combaret et al., 2005). Sugawara et al. (Sugawara et al., 2007 & 2008) has

shown in multiple studies that in protein deficient diets, leucine has the ability to suppress myofibrillar degradation and fast-twitch muscle atrophy. Increases in proteasome activity have also been evident in diets deficient in protein (Sugawara et al., 2008). Even in as little as a week, a protein deficient diet can cause decreases in overall muscle mass. Without the addition of complete proteins, a leucine-supplemented diet in rats can attenuate this loss of muscle mass (Sugawara et al., 2008).

The elderly show a blunted response to exercise (Kim, Cross, & Bamman, 2005), decreased postprandial anabolic effect from feeding (Dardevet et al., 2002; Katsanos et al., 2006), and increased protein degradation (Blough & Linderman, 2000; Guillet et al., 2004). Part of these responses is thought to stem from a decrease in leucine sensitivity; however Katsano et al. (Katsanos et al., 2006) showed that when the leucine content of whey protein is increased from the typical 26% to 41%, fractional synthesis rates can be restored in the old to levels observed in the young. The addition of leucine to a meal has been shown to restore levels of postprandial protein synthesis in the elderly to that of their younger counterparts (Dardevet et al., 2002; Koopman et al., 2006).

Leucine has also been shown to direct Akt signaling (Drummond & Rasmussen, 2008). Although the mechanisms are not completely understood, leucine appears to affect pathways of protein synthesis and degradation through signaling of mTOR, AMPK, and Akt, leading to an overall net protein synthesis. These significant findings show how leucine, or whey protein, supplementation can plan a key role in the process of muscle atrophy during aging. Along with the aforementioned anabolic and antiproteolytic effects of leucine, this essential amino acid has also been shown to improve glucose metabolism, reduce diet-induced insulin resistance, as well as reduce diet-induced hypercholesterolemia independent of adiposity (Zhang et al., 2007). An

intervention with leucine supplemented to the diet, gives a way to possibly attenuate the negative aged-induced effects on skeletal muscle overload. Figure 2.1 diagrams the hypothesized pathways through which leucine may affect the ability to hypertrophy muscle.

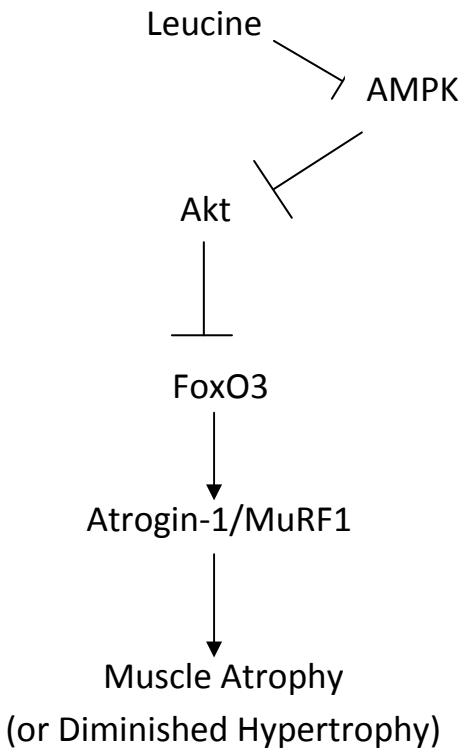

Figure 2.1 The hypothesized role of leucine and 5'-AMP-activated protein kinase (AMPK) signaling. AMPK is activated during periods of low cellular energy, which in turn prevents Akt from phosphorylating forkhead box transcription factor 3A (FoxO3) at $Ser^{318/321}$, allowing FoxO3 translocation into the nucleus. Upon entering the nucleus, FoxO3 increases transcription of protein degradation pathway gene encoding ubiquitin ligase proteins such as muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx, or Atrogin-1). Leucine is hypothesized to inhibit the rise in AMPK activity, thereby preventing translocation of FoxO3 into the nucleus and increasing MuRF1 and Atrogin-1 transcription.

Specific Aim

Muscle fibers are shown to atrophy with age (Lexell, 1995), and muscle fiber hypertrophy from exercise induced overload is decreased with advancing age (Kosek et al., 2006; Thomson & Gordon 2005). Partially responsible for this diminished overload-induced hypertrophy is the increase in protein degradation (Dreyer et al., 2006). Downstream regulators

of protein degradation such as Atrogin-1 and MuRF1 are shown to be increased in old compared to young (Nakashima & Yakabe, 2007) even following resistance training (Raue et al., 2007). Leucine supplementation is able to blunt these atrophy related genes (Sugawara et al., 2007 & 2008) as well as restore postprandial protein synthesis in the old to levels seen in younger populations (Dardevet et al., 2002).

Both leucine supplementation and overload have been analyzed in many different aspects over the past few decades, used on many different populations and species, but rarely used in combination with each other. The aim of this study was to determine whether dietary leucine supplementation can attenuate markers of protein degradation and rescue hypertrophy during overload in the fast-twitch skeletal muscles of aged rats to levels comparable to their younger counterparts. It was hypothesized that dietary leucine supplementation during 7 days of fast-twitch plantaris muscle overload would enhance plantaris muscle hypertrophy in aged rats to levels observed in young adult rats not receiving leucine. It was also hypothesized that dietary leucine supplementation during the overload period would alter markers of protein degradation (enhance FoxO3 phosphorylation and reduce the levels of AMPK phosphorylation, Atrogin-1 protein content, and MuRF1 protein content) in the overloaded fast-twitch plantaris muscles of the aged rats to levels observed in young adult rats not receiving leucine.

Chapter III: Methods

Experimental Animals

The subjects in this study consisted of 13 young adult (8 months) and 13 old (33 months) male Fisher 344 x Brown Norway F1 Hybrid (FBN) rats. The subjects were housed at the East Carolina University Brody School of Medicine animal care facility and kept on a 12-hour light-dark cycle. This project were approved by the East Carolina University Animal Care and use Committee. Each age group of 13 animals was broken down into groups of 6 (placebo) or 7 (leucine supplementation), matched for body weights within ages. In all rats, there was a unilateral 1-week plantaris and soleus muscle overload process, allowing the observation of overloaded vs. non-overloaded within each animal. The East Carolina University Animal Care and Use Committee approved all procedures before this investigation (see appendix A).

Rationale for Experimental Animals

FBN rats have been established to show similar fast-twitch fiber-specific atrophy as seen in young adults (Fick & Gordon, 2007; Thomson & Gordon, 2005) as well as been found to upregulate AMPK phosphorylation in aging FBN rat fast-twitch plantaris muscle (Fick & Gordon, 2007; Thomson & Gordon, 2005). Studies by Blough and Linderman (Blough & Linderman, 2000) along with Rice et al. (Rice et al., 2005) have found that FBN rats are a better model for human skeletal muscle aging and sarcopenia as compared to other rat models.

In humans, whole skeletal muscle and fast-twitch muscle fiber-specific atrophy begin in adulthood (around the ages of 40-50 in men and women) (Guillet et al., 2004; Lecker, Goldberg, & Mitch, 2006). This threshold effect is seen in humans and also in the FBN rat. The lab at

ECU has shown that fast-twitch muscle is relatively stable or growing in young adult FBN rats between the ages of 6 and 9 months, and possibly out to 18 months, yet greatly declines by 25 months and continues to decline to 36 months (Blough & Linderman, 2000; Fick & Gordon, 2007; Thomson & Gordon, 2005). So, for this study we used 8 month old and 33 month old FBN rats. This ensured that the old-aged animals will experience significant age-related atrophy prior to intervention (Gomes & Booth, 1998). With age, humans show an attenuated response to overload induced hypertrophy specifically in fast-twitch muscle fibers (Fiatarone et al., 1990). The FBN rats are an excellent comparison with aged humans due also to the lack of fast-twitch overload induced muscle hypertrophy with age (Thomson & Gordon, 2005).

Only male rats were used for this study. The goal of the proposed research is not to delineate potential gender-related differences in fast-twitch skeletal muscle with age, because the vast majority of findings in humans demonstrate predominant fast-twitch fiber atrophy in both men and women (Vandervoot, 2002). The results observed to date indicate that the aging FBN male rat is an excellent model for humans of both genders with respect to predominantly fast-twitch-specific atrophy with age (Sehl et al., 2001; Thomson & Gordon, 2005). Analysis of the difference between male and female FBN rats would be the subject of further studies.

Dietary Intervention

The rats in the study were fed ad libitum standard rodent chow for 2 days after arriving at ECU. Animals were then divided into their determined groups, paired for body weight between dietary conditions within age groups, and were fed either the standard rodent chow or a 5% supplemental leucine-enriched chow (specially ordered from Research Diets, Inc., New Brunswick, NJ) for 2 days prior to surgeries. The 5% mark was selected because of the findings

made by Combaret et al. (Combaret et al., 2005) showing an optimal inhibition proteolysis at this set point. As a percentage of total calories, the placebo diet (normal chow) was composed of 20% protein, 65% carbohydrate, and 15% fat, while the leucine diet was composed of 20% protein, 5% free leucine, 60% carbohydrate, and 15% fat. The "protein" component of both diets was comprised of the dairy protein casein, which itself is ~8.4% leucine by weight (Ellinger & Boyne, 1965). Thus, the placebo diet consisted of ~1.7% total leucine, while the leucine diet consisted of ~6.7% total leucine. Animals were housed individually and food consumption was measured daily to assess total caloric and leucine consumption. All animals were given ad libitum access to water and chow during the entire experiment. The leucine groups were started on the leucine-enriched diet 2 days prior to, and throughout, the overload intervention. Placebo groups were maintained on a normal chow rodent diet throughout. Although the older groups consumed more chow (due to their larger body weights) than the young adult groups, there were no differences in daily chow or calorie consumption between the placebo vs. leucine groups in young adult or old animals (Table 4.2).

Synergist Tenotomy Protocol

In this study, all rats were subject to 1-week of overload of the plantaris and soleus mscles induced by tenotomy of the Achilles tendon of the synergistic gastrocnemius muscle. Both fast- and slow-twitch muscles were harvested. The plantaris, which is made up of approximately 93% fast-twitch muscle fibers (Armstrong & Phelps, 1984), was of primary interest. Rats were weighed and anesthetized with 2-3% isoflurane and supplemental oxygen. Under aseptic conditions, the distal tendon of the gastrocnemius muscle was surgically cut in the left hind limb. A control sham operation was performed on the right hind limb and consisted of an incision through the skin and isolation of the Achilles tendon but without disruption of the

gastrocnemius muscle. The right limb synergist muscles, the soleus and plantaris, served as controls. The incision was closed with stainless steel surgical clips following the procedure, after which the animals received a one-time subcutaneous injection of an analgesic (Buprenex, 0.03 mg/kg body weight). This procedure has been proven successful in previous literature (Thomson & Gordon, 2005).

Unilateral tenotomy was chosen over bilateral tenotomy because it allows for within-subject comparisons between overloaded and control muscles. This set up eliminates the bias due to systematic differences between groups of animals and allows for more precise measurements of muscle hypertrophy of each animal.

Tissue Harvesting and Animal Sacrifice

Animals were not fasted prior to sacrifice, because the effects of leucine are much more evident in the postprandial than the postabsorptive state in aged muscle (Combaret et al., 2005; Fujita et al., 2007; Katsanos et al., 2006; Solomon & Goldberg, 1996). To resemble a real-life situation, the animals were not fed a specific meal, and were allowed to feed ad libitum until sacrifice. Animals were sacrificed in a randomized but counterbalanced order with the 1-2 hours before their dark cycle ended.

The muscles excised were quickly trimmed of excess fat and connective tissue, weighed on an analytical balance, flash-frozen in liquid nitrogen, and stored at -80°C until further processing. Animals were then euthanized via heart excision while still under anesthesia.

Western Blot Analyses

FoxO3 phosphorylation, AMPK phosphorylation, MuRF1 protein, and Atrogin-1 protein measurements were made using western blot analysis. A small piece of each frozen muscle sample was homogenized using a buffer that 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. All homogenizations were performed on ice to prevent excessive heat build-up. Proteins will denature when a buildup of heat is present. Additionally, all homogenizations were performed using a ground glass homogenizer that utilizes a variable speed motor.

Assessment of the homogenates for protein concentration was carried out in triplicate using a modification of the Lowry procedure (DC Protein Assay, Bio-Rad, Hercules, CA, USA). Total muscle protein homogenates were mixed in a loading buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 2% ß-mercaptoethanol, 0.1% bromophenol blue) at a dilution of 1 mg per ml. The mixture was then boiled for 5 minutes. Proteins were separated by a 4-7.5% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Blotting occurred for 1.5 hours at 4˚C onto a PVDF membrane at 100V in a transfer buffer. The buffer contained 25 mM Tris-base pH~8.3, 192 mM glycine, and 20% methanol. Ponceau S was used to stain the membranes. Following staining, they were dried and scanned into a digital image. This image allows measurement of the relative total protein loaded into each label through the gray scale integrated optical density of the full length of each individual lane. Membranes were then blocked for one hour at room temperature in blocking buffer, consisting of 5% nonfat dry milk in TBS-T (20 nM Tri-base, 150 mM NaCL, 0.1% Tween-20) pH 7.5. Following that step, the membranes were incubated in the primary antibody diluted in 1% bovine serum albumin in

TBS-T overnight at 4˚C. All rabbit primary antibodies were commercially available: anti-MuRF1 [Cell Signaling Technology (CST), Danvers, MA, Cat # 4305]; 1:2000 dilution; anti-atrogin-1 (ECM Biosciences, Versailles, KY, Cat. # AP2041; 1:1000); anti-phospho-FoxO3 (Ser318/321; CST, Cat # 9465; 1:1000), anti-AMPK (CST, Cat. # 2532; 1:1000), and anti-phospho-AMPK (Thr172; CST, Cat. # 4188; 1:4000)). Membranes were then washed 4 times for 5 minutes each in TBS-T, incubated in a horseradish peroxidase (HRP)-linked anti-rabbit secondary antibody in blocking buffer for an hour while at room temperature. There was then another round of 4 x 5-minute wash periods in TBS-T.

Following the last wash, detection of the HRP activity occurred using enhanced chemiluminescence reagent and exposure to autoradiographic film (Classic Blue Sensitive; Midwest Scientific, St Louis, MO, USA). The integrated optical densities (IODs) were quantified by densitometry using Gel Pro Analyser software (Media Cybernetics, Silver Spring, MD, USA) and calculation of the concentration of the antigen present in each muscle as the IOD was normalized to units of total muscle protein initially loaded on the gel. Correction for the grayscale IOD of each total lane was evaluated on the image of the Ponceau stain that was previously captured. The HRP-conjugated anti-rabbit secondary antibody was acquired from Amersham.

Statistics

A 2x2x2 factorial ANOVA with repeated measures was used for analyses of the effects of age, dietary intervention, and overload (the repeated measure) on muscle hypertrophy (increase in muscle mass). A 2x2 ANOVA with repeated measures was used to measure the percent changes in hypertrophy and western blot analyses. Post-hoc comparisons were

accomplished via a Fisher's Least Significant Difference test, with statistical significance being set at a level of $P \leq 0.05$.

Chaprter IV: Results

Animal Body Weight

A significant main effect of age was seen regardless of timepoint or dietary condition or time period (Table 4.1). Body weight of animals in the same age group did not differ when leucine was added to the diet. There was no significant change in body weight of the animals from beginning to end of the investigation.

Body Weight

	Start Placebo Chow	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 animal body weights (grams) in young adult (8 mo.) vs old (33 mo.) rats fed normal chow (placebo) or chow with 5% dietary leucine supplementation.
* Significant ($p \leq 0.05$) main effect of age regardless of time point or dietary condition.

Food Intake

Food intake for the young adult animals was not affected by the surgery. However, during the overload period, the aged rats consumed significantly less food than the young adults during the overload period (Table 4.2). Prior to the surgery, food intake did not differ between groups. Leucine supplementation did not have an effect on food intake.

Food Intake (g/kg BW/day)

	Days 1-2	Days 3-4	Days 5-11 (overload period)
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 chow consumed (g/kg BW/day) in young adult (8 mo.) vs old (33 mo.) rats fed normal chow (placebo) or chow with 5% dietary leucine supplementation.
* Significantly ($p \leq 0.05$) different than young adult groups during overload period.

Plantaris & Soleus Hypertrophy

A significant increase was seen in the amount of hypertrophy of the plantaris from sham to overload in both the young adult groups and in the old leucine group (Figure 4.1). There was a significant main effect of age through an overall decrease in muscle weight in the plantaris, regardless of dietary or loading condition. For the percent hypertrophy from overload, the aged placebo group had significantly less hypertrophy compared to the young adult placebo group (Figure 4.2). There was also a significant increase seen from the old placebo to old leucine groups in the plantaris. A significant increase in the amount of hypertrophy for the soleus was seen from sham to overload in both the young adult groups and the aged leucine group (Figure 4.3). There was a significant decrease in wet weight from young to old regardless of dietary or loading protocol. For the percent hypertrophy of the soleus, a significant decrease was seen in the young adult placebo vs. the old placebo groups (Figure 4.4).

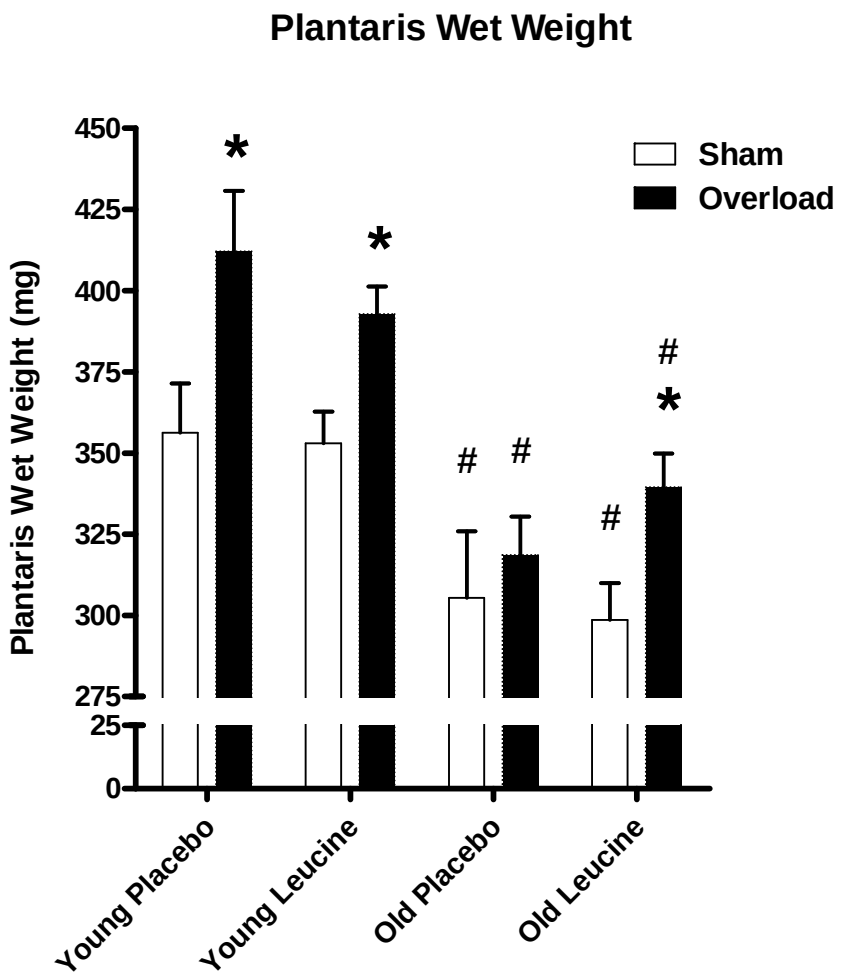

Figure 4.1. Mean + SEM wet weights of sham-operated vs. 7-day overloaded plantaris muscles in young adult (8 mo.) vs. old (33 mo.) rats fed normal chow (placebo) or chow with 5% dietary leucine supplementation. * Significantly ($p \leq 0.05$) different than sham-operated muscle within specified age group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

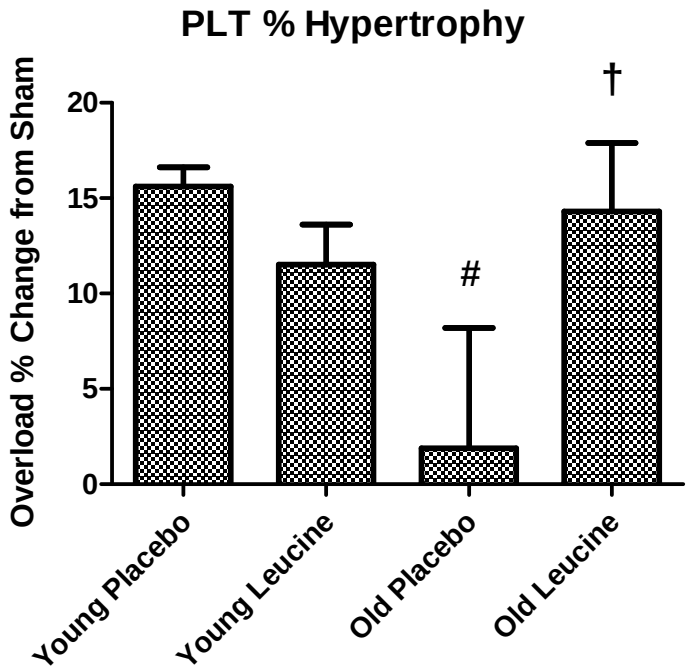

Figure 4.2. Mean + SEM percent change in wet weights of 7-day overloaded vs. sham-operated plantaris (PLT) muscles in young adult (8 mo.) vs. old (33 mo.) rats fed normal chow (placebo) or chow with 5% dietary leucine supplementation. # Significantly ($p \leq 0.05$) different than young placebo group.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

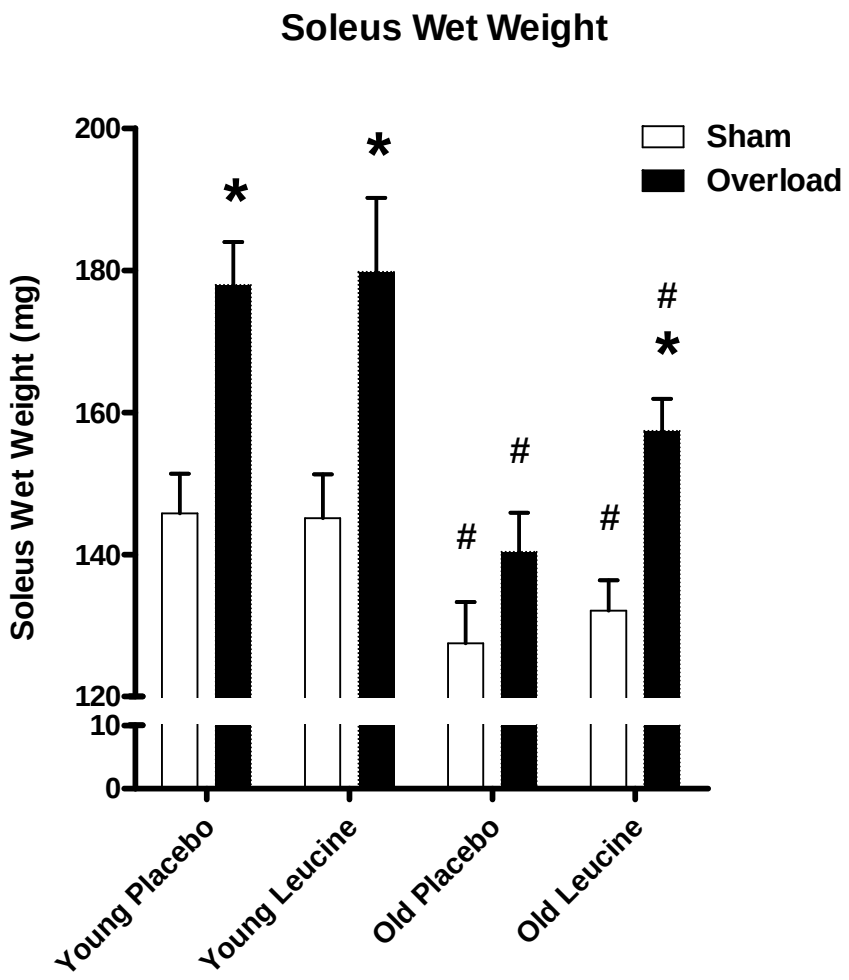

Figure 4.3. Mean + SEM wet weights of sham-operated vs. 7-day overloaded soleus muscles in young adult (8 mo.) vs. old (33 mo.) rats fed normal chow (placebo) or chow with 5% dietary leucine supplementation. * Significantly ($p \leq 0.05$) different than sham-operated muscle within specified age group and dietary condition. # Significant main effect of age regardless of dietary or loading condition.

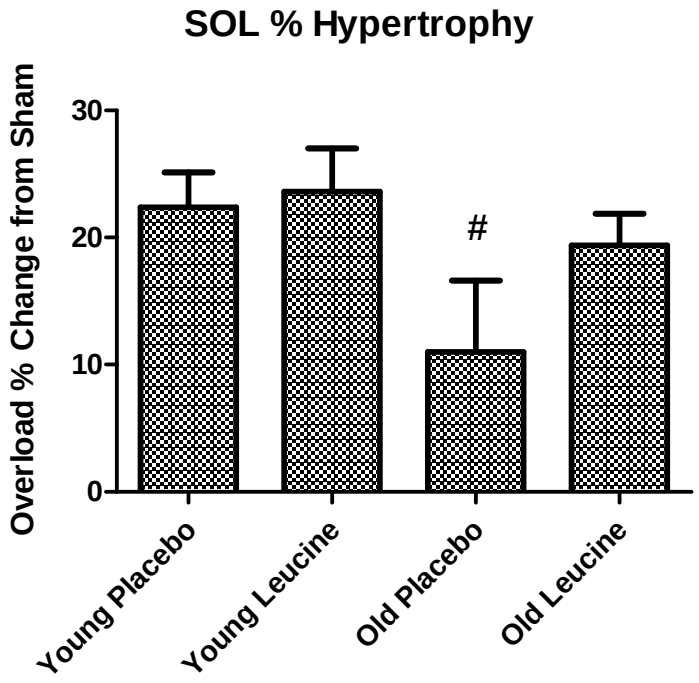

Figure 4.4. Mean + SEM percent change in wet weights of 7-day overloaded vs. sham-operated soleus (SOL) muscles in young adult (8 mo.) vs. old (33 mo.) rats fed normal chow (placebo) or chow with 5% dietary leucine supplementation. # Significantly ($p \leq 0.05$) different than young placebo group.

AMPK Western Blotting (Plantaris)

A significant main effect of overload was seen for AMPK phosphorylation at Thr^{172} in the fast-twitch plantaris regardless of age or dietary protocol (Figure 4.5). A significant interaction of increased AMPK phosphorylation was also seen with age from the young adult placebo to old placebo group within the specified loading conditions. A significant effect of leucine was seen as a decrease in AMPK phosphorylation from the old placebo to old leucine group within both the sham and overload conditions. There was no significant effect seen in percent change of AMPK phosphorylation in any group (Figure 4.6). A main effect of age was seen for total AMPK levels however it did not reach significance ($p = 0.065$) (Figure 4.7). A significant effect for the increase in the phospho-/total AMPK ratio from sham to overload was seen in all groups, regardless of age or dietary condition (Figure 4.8). The old placebo group had a significantly higher phospho-/total AMPK ratio than the young adult group with overload. The old leucine group had a significantly lower phospho-/total AMPK ratio than the old placebo group in the overload protocol. There was no significant effect seen in the phospho-/total AMPK ratio increase from overload in any group (Figure 4.9).

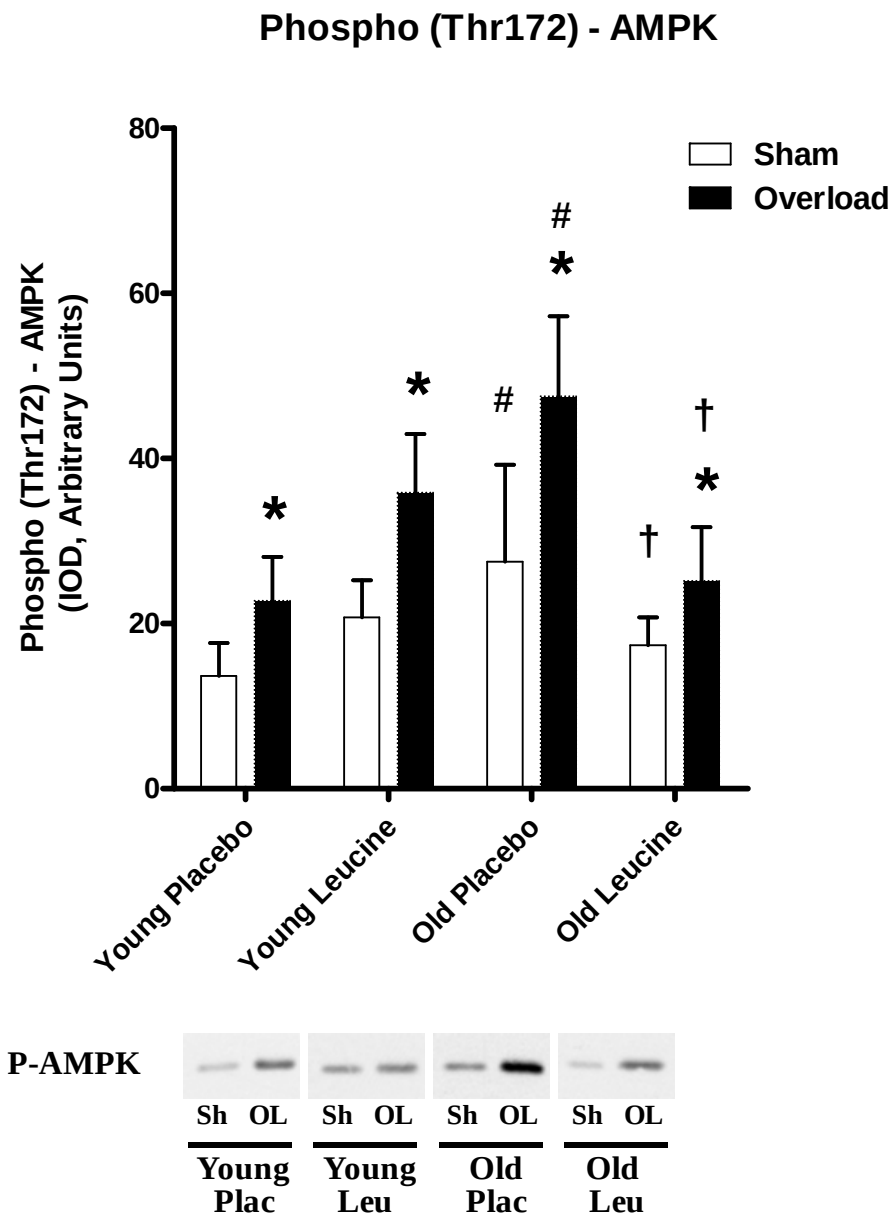

Figure 4.5. Mean + SEM phospho (Thr^{172}) - 5'-adenosine monophosphate (AMP)-activated protein kinase (AMPK) contents and representative blots of sham-operated (Sh) vs. 7-day overloaded (OL) plantaris muscles in young adult (8 mo.) vs. old (33 mo.) rats fed normal chow (placebo; Plac) or chow with 5% dietary leucine supplementation (Leu). * Significant ($p \leq 0.05$) main effect of overload regardless of age group and dietary condition. # Significantly different than young placebo group within specified loading condition. † Significantly different than old placebo group within specified loading condition.

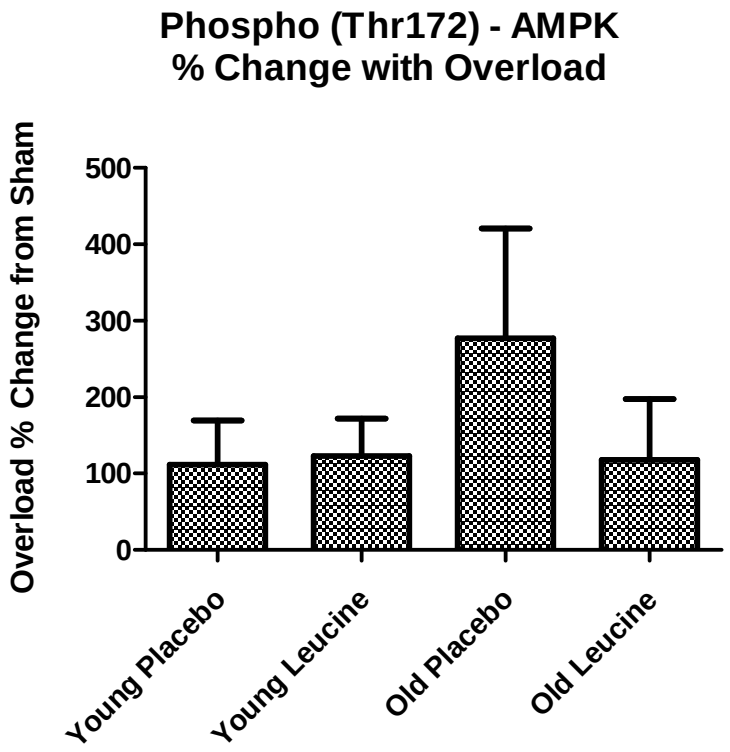

Figure 4.6. Mean + SEM percent change in phospho (Thr^{172}) - 5'-adenosine monophosphate (AMP)-activated protein kinase (AMPK) contents of 7-day overloaded vs. sham-operated plantaris muscles in young adult (8 mo.) vs. old (33 mo.) rats fed normal chow (placebo) or chow with 5% dietary leucine supplementation. No significant differences were found in % change for any group.

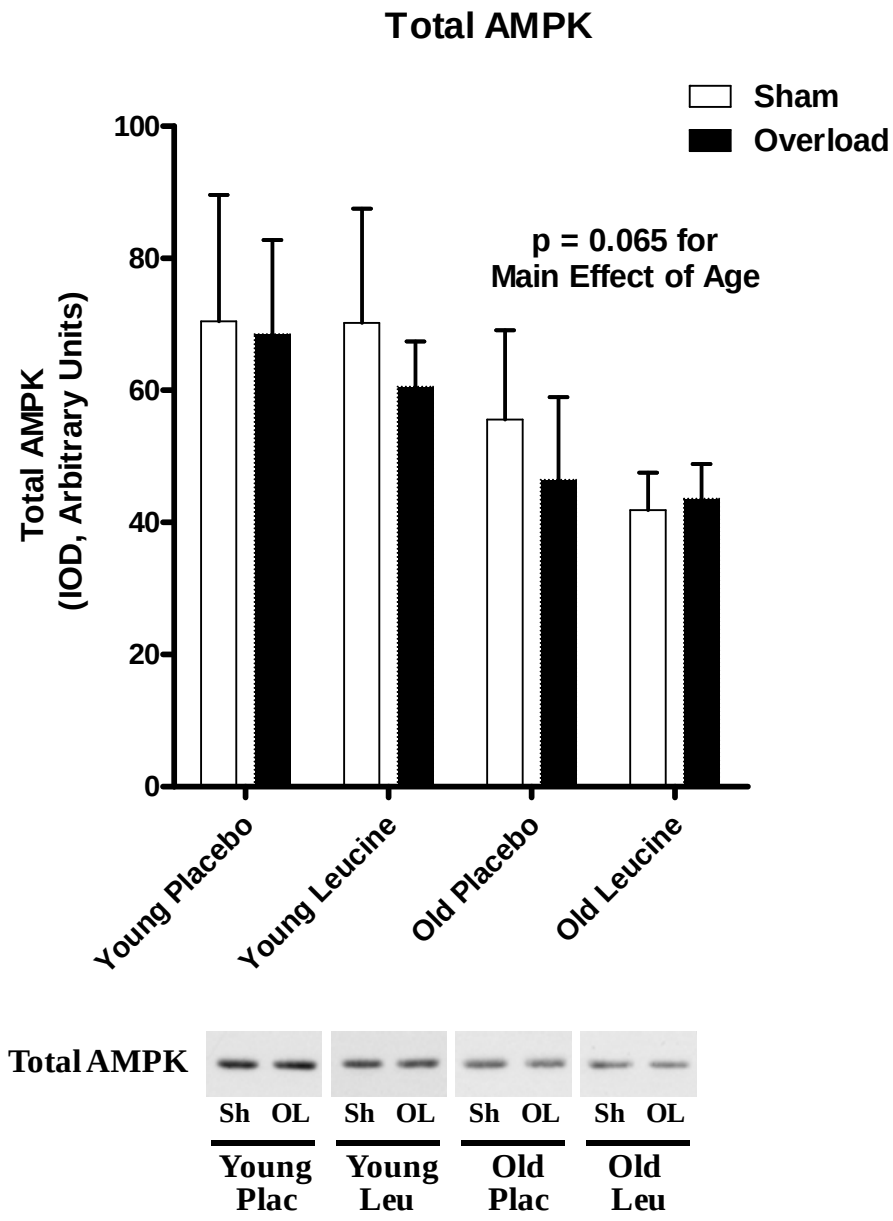

Figure 4.7. Mean + SEM total 5'-adenosine monophosphate (AMP)-activated protein kinase (AMPK) contents and representative blots of sham-operated (Sh) vs. 7-day overloaded (OL) plantaris muscles in young adult (8 mo.) vs. old (33 mo.) rats fed normal chow (placebo; Plac) or chow with 5% dietary leucine supplementation (Leu).

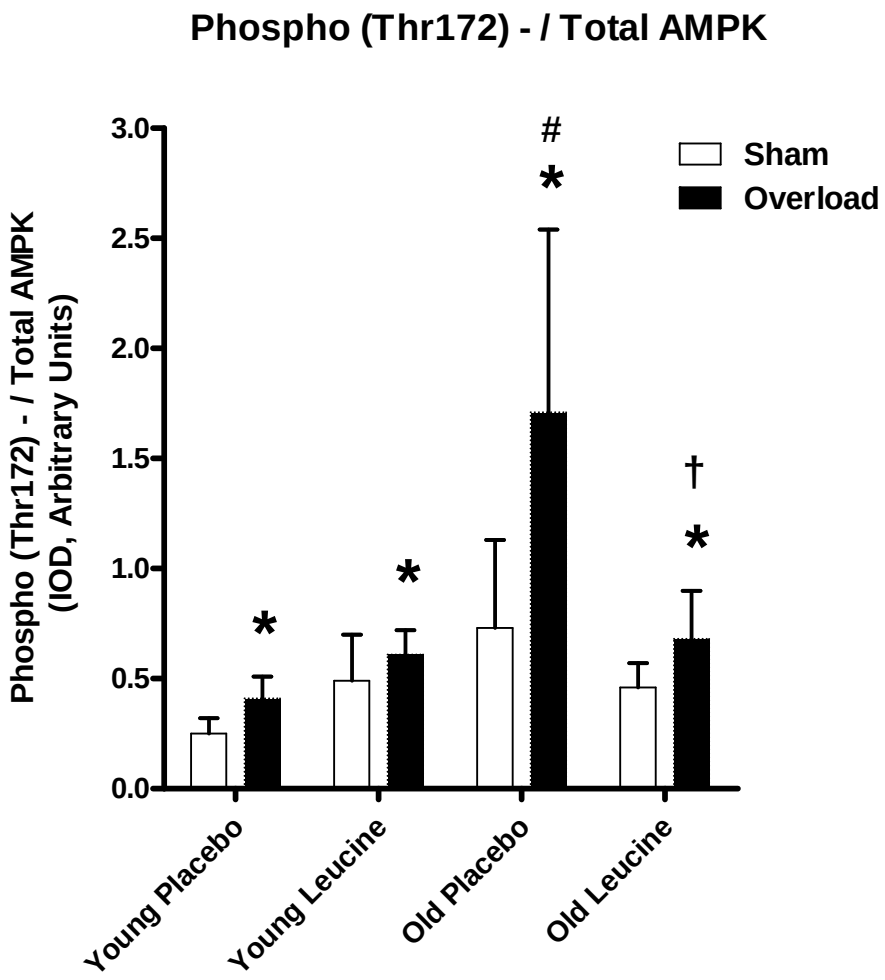

Figure 4.8. Mean + SEM ratio of phospho (Thr^{172}) - 5'-adenosine monophosphate (AMP)-activated protein kinase (AMPK) contents / total AMPK contents of sham-operated vs. 7-day overloaded plantaris muscles in young adult (8 mo.) vs. old (33 mo.) rats fed normal chow (placebo) or chow with 5% dietary leucine supplementation.
* Significant ($p \leq 0.05$) main effect of overload regardless of age group and dietary condition.
# Significantly different than young placebo group within specified loading condition.
† Significantly different than old placebo group within specified loading condition.

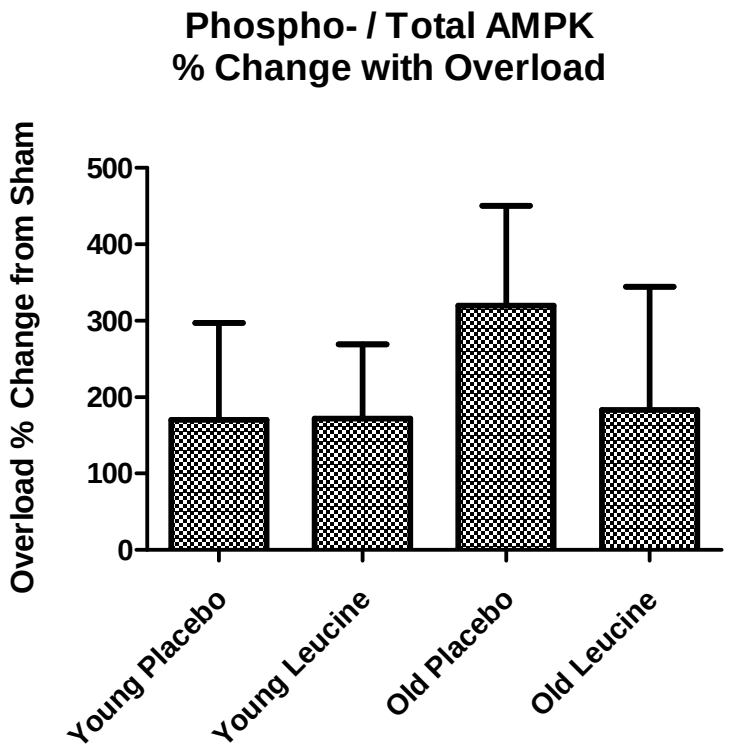

Figure 4.9. Mean + SEM percent change in ratio of phospho (Thr^{172}) - 5'-adenosine monophosphate (AMP)-activated protein kinase (AMPK) contents / total AMPK contents of 7-day overloaded vs. sham-operated plantaris muscles in young adult (8 mo.) vs. old (33 mo.) rats fed normal chow (placebo) or chow with 5% dietary leucine supplementation.

FoxO3 Western Blotting (Plantaris)

There was a significant interaction between dietary condition and overload for FoxO3 phosphorylation at $Ser^{318/321}$ in the fast-twitch plantaris (Figure 4.10). There was only an interaction when leucine was combined with overload for an increase in FoxO3 phosphorylation status. There was also a main effect of age with a p-value of 0.07 that did not reach significance.

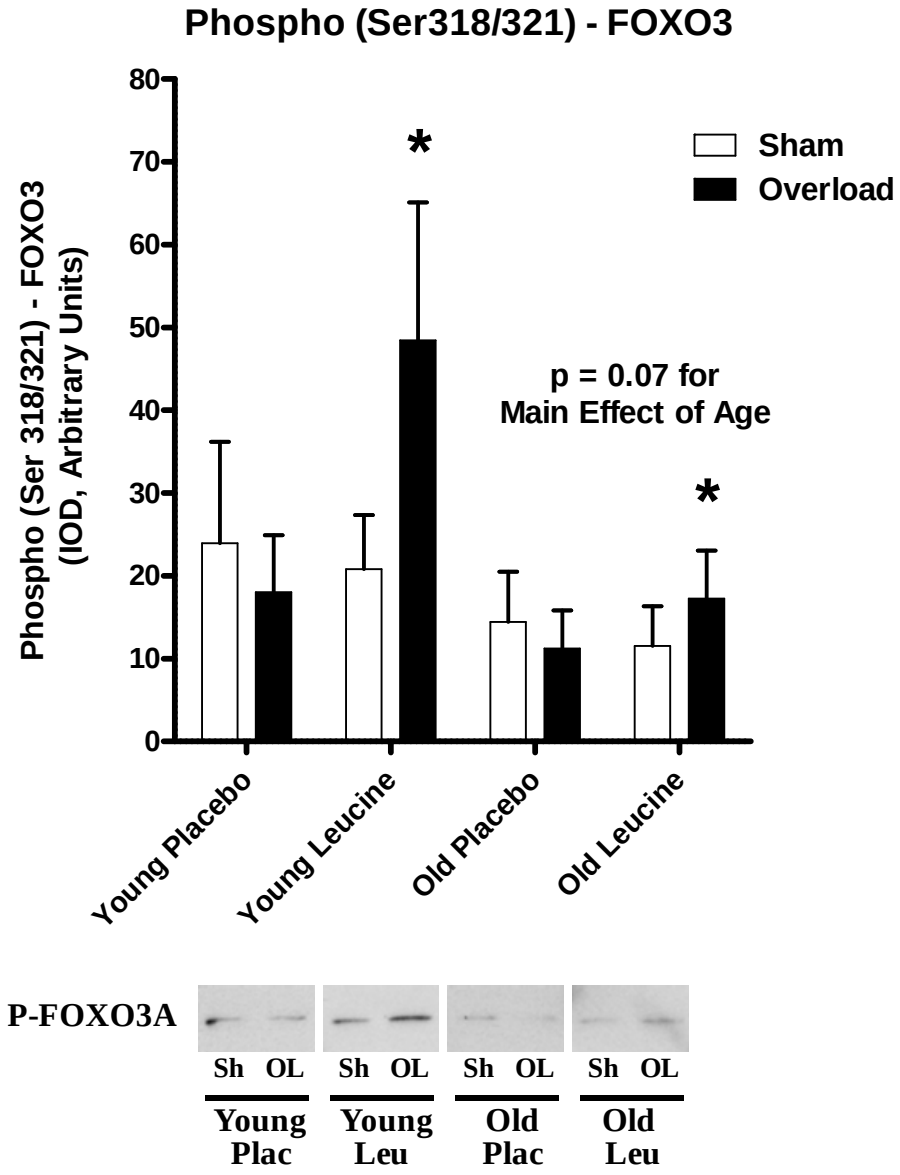

Figure 4.10. Mean + SEM phospho ($Ser^{318/321}$) - atrophy-related forkhead box 3A (FoxO3) contents and representative blots of sham-operated (Sh) vs. 7-day overloaded (OL) plantaris muscles in young adult (8 mo.) vs. old (33 mo.) rats fed normal chow (placebo; Plac) or chow with 5% dietary leucine supplementation (Leu). * Significant ($p \leq 0.05$) interaction between dietary condition and overload (overload-induced increase with leucine supplementation only, regardless of age).

Atrogin-1 Western Blotting (Plantaris)

A significant main effect of age was seen through a decrease in total Atrogin-1 from young adult to old, levels regardless of dietary or loading protocol (Figure 4.11). There were no significant changes for percent increase with overload (Figure 4.12). However, there was a trend in the increase in total levels with age in the old placebo vs. young adult placebo ($p = 0.11$). There was also a trend in the decrease of total Atrogin-1 levels in the old placebo vs. old leucine group ($p = 0.07$).

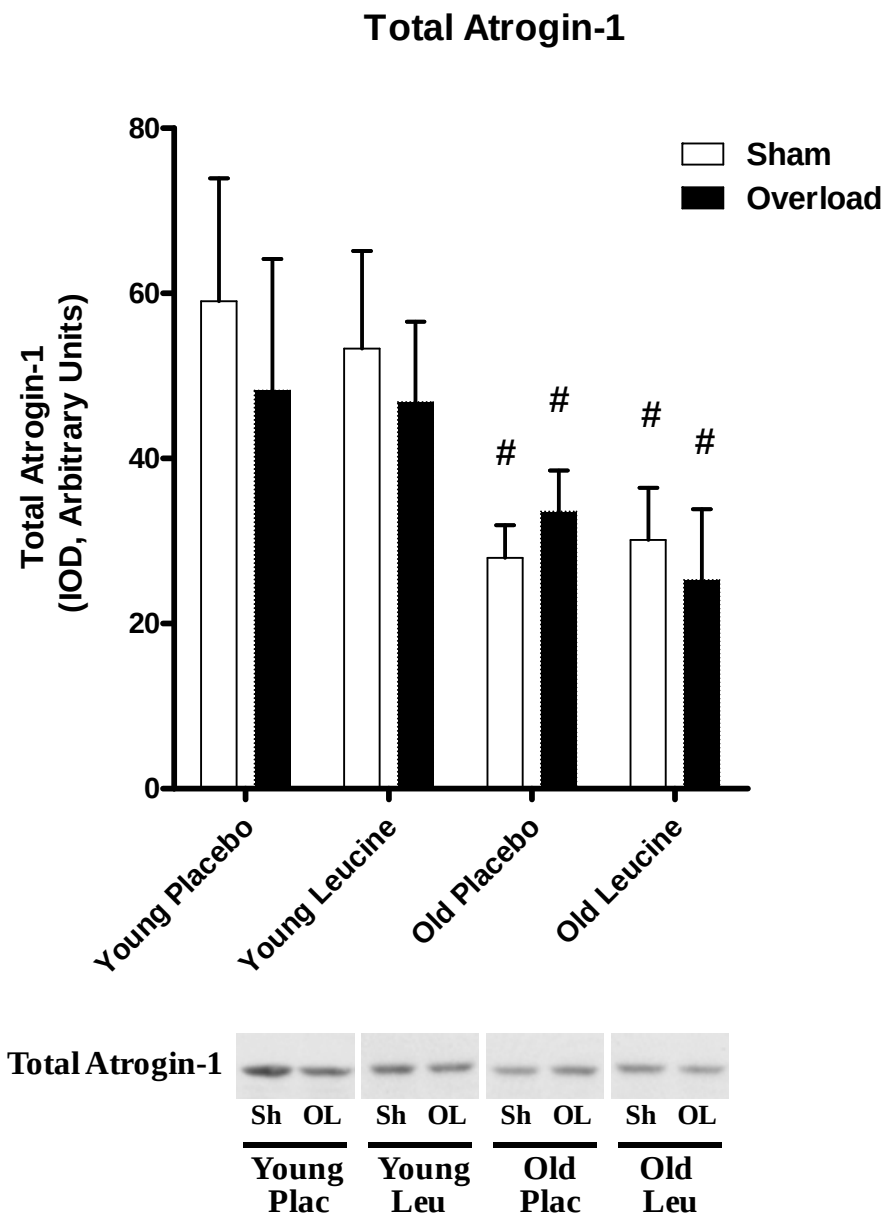

Figure 4.11. Mean + SEM total atrogin-1 contents and representative blots of sham-operated (Sh) vs. 7-day overloaded (OL) plantaris muscles in young adult (8 mo.) vs. old (33 mo.) rats fed normal chow (placebo; Plac) or chow with 5% dietary leucine supplementation (Leu).

Significant ($p \leq 0.05$) main effect of age regardless of dietary or loading condition.

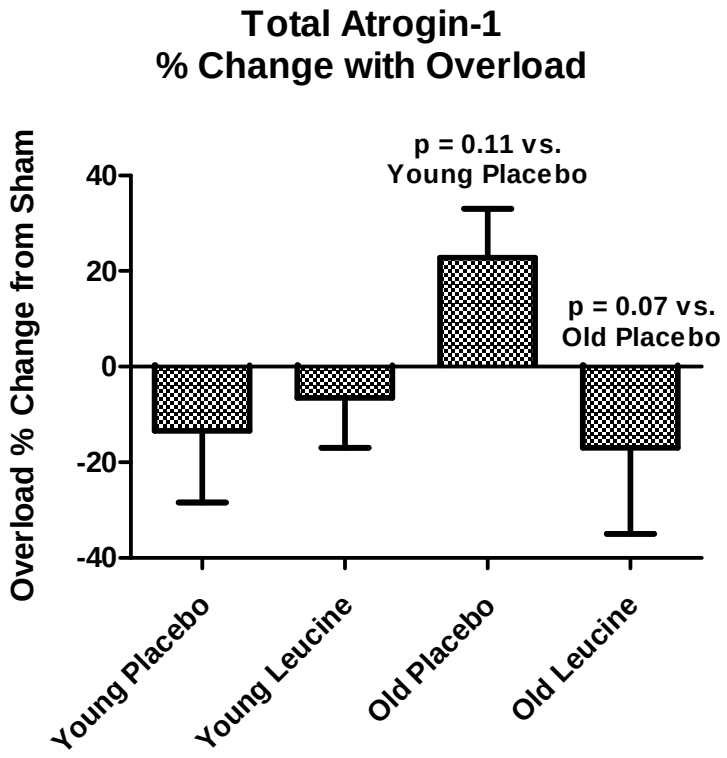

Figure 4.12. Mean + SEM percent change in total atrogin-1 contents of 7-day overloaded vs. sham-operated plantaris muscles in young adult (8 mo.) vs. old (33 mo.) rats fed normal chow (placebo) or chow with 5% dietary leucine supplementation.

MuRF1 Western Blotting (Plantaris)

A significant main effect of overload was seen regardless of age of dietary protocol (Figure 4.13). There was also a significant main effect of age regardless of the dietary or loading condition. There was no significant interaction seen for percent decrease from overload, however the p-value did reach 0.14 in regards to a decrease in levels with the addition of leucine (Figure 4.14).

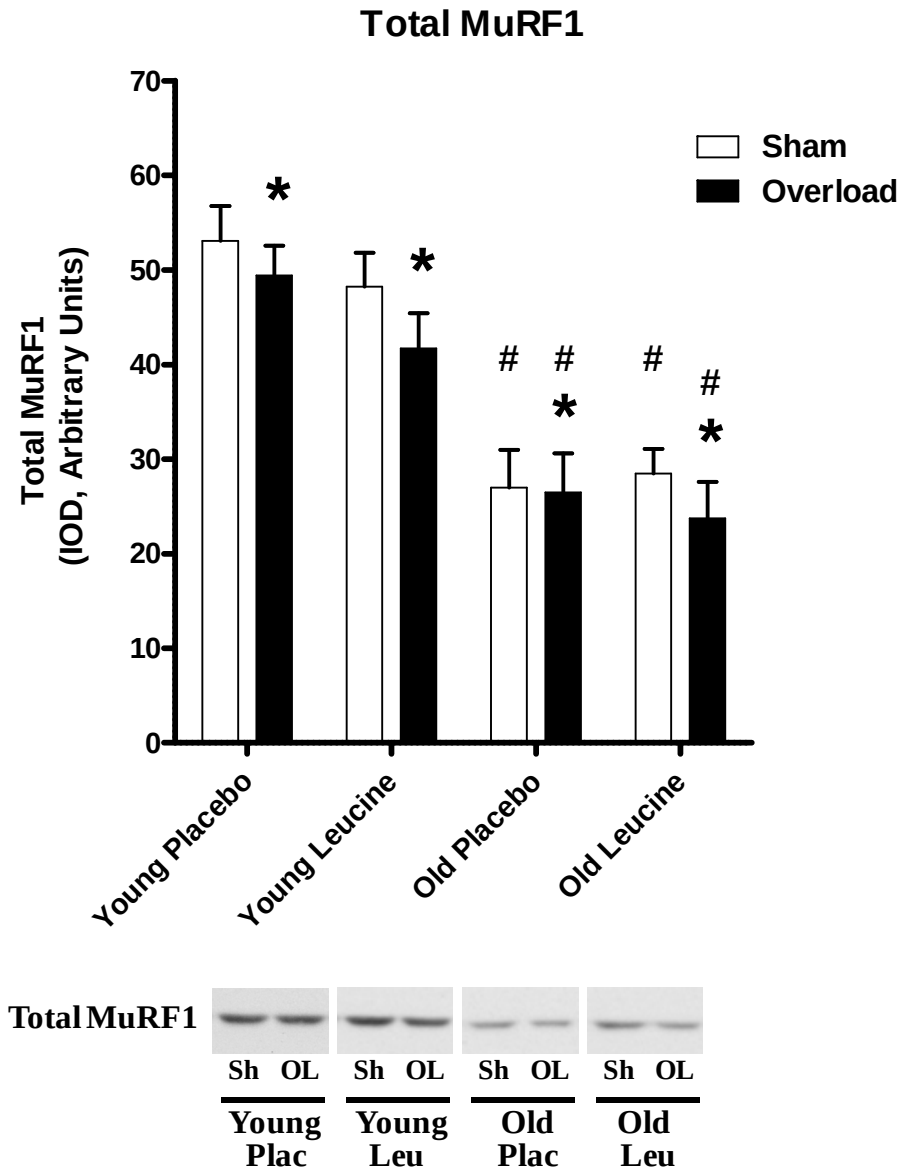

Figure 4.13. Mean + SEM total muscle RING finger 1 (MuRF1) contents and representative blots of sham-operated (Sh) vs. 7-day overloaded (OL) plantaris muscles in young adult (8 mo.) vs. old (33 mo.) rats fed normal chow (placebo; Plac) or chow with 5% dietary leucine supplementation (Leu). * Significant ($p \leq 0.05$) main effect of overload regardless of age or dietary condition. # Significant main effect of age regardless of dietary or loading condition.

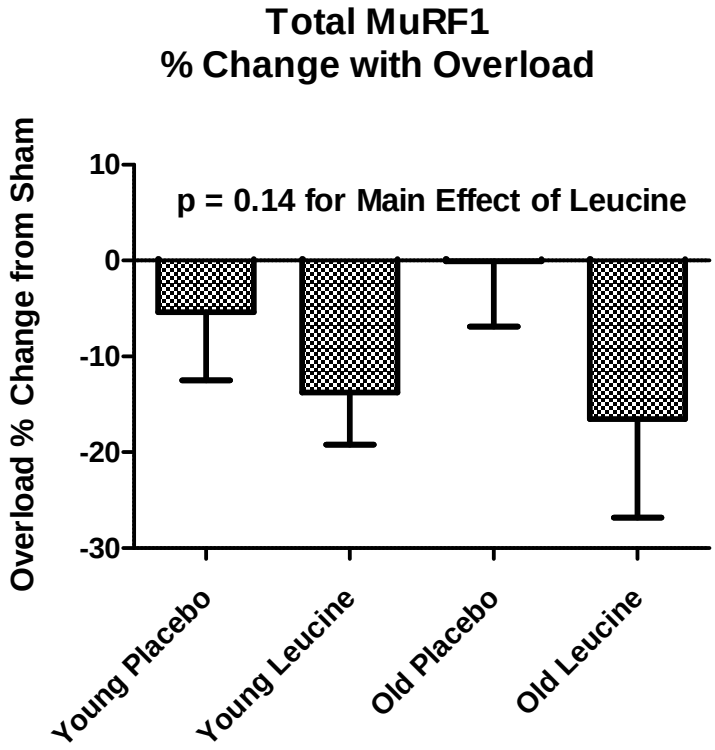

Figure 4.14. Mean + SEM percent change in total muscle RING finger 1 (MuRF1) contents of 7-day overloaded vs. sham-operated plantaris muscles in young adult (8 mo.) vs. old (33 mo.) rats fed normal chow (placebo) or chow with 5% dietary leucine supplementation.

Chapter V: Discussion

The purpose of this study was to determine if leucine supplementation prevented the increase in markers of protein degradation and rescued overload induced skeletal muscle hypertrophy in aged rats to levels comparable to their younger counterparts. We hypothesized that dietary leucine supplementation during 7 days of fast-twitch plantaris muscle overload would enhance plantaris muscle hypertrophy in aged rats to levels observed in young adult rats not receiving leucine. We also hypothesized that dietary leucine supplementation during the overload period would alter markers of protein degradation (enhance FoxO3 phosphorylation and reduce the levels of AMPK phosphorylation, Atrogin-1 protein content, and MuRF1 protein content) in the overloaded fast-twitch plantaris muscles of the aged rats to levels observed in young adult rats not receiving leucine.

As hypothesized, leucine supplementation did have a significant effect on hypertrophy in the fast-twitch plantaris muscle. Although the main fiber type analyzed in this study are the type II fibers found in the plantaris, the slow-twitch soleus muscle percent hypertrophy was not significantly smaller in the aged leucine group compared to young adult counterpart. The aged rats that were not fed leucine had significantly less hypertrophy in the soleus muscle as compared to their young adult counterparts. Following the one-week intervention, there was an observable difference in the markers of protein degradation, in particular phospho-AMPK and the phospho-/total AMPK ratio. There was a significant main effect of overload regardless of age or dietary condition. The aged placebo group showed significantly higher levels of phospho-AMPK at rest and with overload compared to their young adult counterparts. However, the leucine supplemented group showed significantly less phospho-AMPK levels compared to the

old rats not supplemented with leucine. The levels of phospho-AMPK observed in the aged leucine group were comparable to those seen the young adult rats. The phospho/total AMPK ratio was significantly less in the supplemented aged rats compared to aged rats consuming normal rat chow. Leucine and overload also had a significant effect on phospho-FoxO3 levels in the young adult and aged groups. There was a main effect of age in total Atrogin-1 and MuRF-1 protein content regardless of dietary or loading condition. Overload in all groups had a significant main effect on decreasing total MuRF-1 protein content.

It has been well documented that there is a decrease in the amount of achievable muscular hypertrophy with advancing age. Research in this area has shown that there are many factors at play in determining the amount of muscle mass in aged muscle: total number of fibers (Lexell, 1995), total number of type II fibers (Grimby et al., 1982, Lexell, 1995), re-innervation of type I fibers (Macaluso & De Vito, 2003), alterations in protein synthesis and degradation at rest and following resistance exercise (Tamaki et al., 2000). Although interventions in the elderly involving resistance training programs can increase muscle hypertrophy and strength, their increases are drastically reduced compared to their young counterparts (Kosek et al., 2006).

A recent study (Ispoglou et al., 2011) showed when 4 grams per day of leucine is added to the diet in conjunction with a 12-week period of resistance training, young males can increase strength nearly 10% more in the 5 rep max than when leucine is not supplemented. In this study we observed a novel finding: that an overload stimulus in combination with a dietary intervention of increased leucine content increases muscle hypertrophy significantly greater than the overload stimulus alone in aged rats.

Thomson and Gordon (Thomson & Gordon, 2005) have shown that in an intervention involving unilateral 1-week overload, aged rats had significantly less increase in fast-twitch muscle growth compared to their young counterparts (9.7% compared and 30.0%, respectively). Due to the fact that type II muscle fibers are primarily responsible for the significant increases in muscular hypertrophy and strength, this lack of overload induced hypertrophy can be detrimental for individuals wanting to attenuate or reverse the age-related effects of sarcopenia. In our study the aged animals showed a 6.5% increase in muscle mass with overload, however the aged rats supplemented with leucine showed a 14.3% increase in muscle with overload (Figure 4.2). The percent increase observed in the aged supplemented group is comparable to the young adult group not taking leucine, 15.6%.

It is interesting to note that leucine supplementation also aided in the aged rats' ability to hypertrophy the slow-twitch soleus muscle. Without that addition of leucine to the diet, the aged rats had an average percent hypertrophy of 11.0% with overload (Figure 4.4). This observed increase in muscle wet weight is nearly half of the observed in the young adult placebo group, 22.4%. With the addition of leucine to the diet in the aged rats, the percent increase from overload was 19.4%. These findings are in contrast to the Thomson and Gordon investigation (Thomson & Gordon, 2005), that did not observe a significant decrease in the ability to hypertrophy slow-twitch muscle with increasing age. One potential reason for why we observed a change in the slow-twitch soleus but Thomson and Gordon did not, is the advanced age of the rats in our study. The eldest rats in their study were three months younger than our eldest. The 33-month old rats in our study may have reached a time point in their life that slow-twitch muscle fibers were affected by pathways similar to those affecting the fast-twitch fibers earlier in life. Fisher 344 x Brown Norway F1 Hybrid rats have been predominantly studied for their age

related atrophy model and fast-twitch fiber adaptation capacity. To gain a better understanding, future studies may wish to investigate slow-twitch adaption later in the aging process (>33 months).

As we hypothesized, phospho-AMPK was decreased with the addition of leucine to the diet. AMPK has been named the "energy sensor" of the cell. During periods of reduced cellular energy, through decreases in ATP/AMP ratio, AMPK activation is increased (Miranda et al., 2007). It has been documented that during the aging process, AMPK activity is elevated in aged rats (Thomson & Gordon, 2005) at rest or following overload and humans (Drummond, Dreyer et al., 2008) at rest or following an acute bout of resistance training . Thomson and Gordon (Thomson & Gordon 2005) have shown that hyperphosphorylation of AMPK is present in aged rat skeletal muscle, more specifically fast-twitch muscle. Our study also showed that with overloaded aged skeletal muscle, AMPK hyperphosphorylation does occur. The old rats fed the standard rat chow showed a nearly 2-fold increase in AMPK phosphorylation status compared to their young counterparts. However, with the introduction of leucine into the diet, the supplemented rats reduced their phospho-AMPK levels by almost half in comparison to the aged placebo group. The old leucine group showed levels of AMPK phosphorylation similar to the young leucine group. From this intervention it is reasonable to assume that leucine supplementation impacted AMPK phosphorylation through improving the cellular energy stores.

Leucine is one of three amino acids known as branched-chain amino acids. Branched-chain amino acids differ from other amino acids in their structure and area of metabolism in the body. Due to the fact that the liver does not have the enzyme branched-chain amino acids aminotransferase, the branched chain amino acids pass through and are metabolized in skeletal muscle (Sweat et al., 2004). A study by Du et al. (Du et al., 2009) showed that treating C2C12

myotubes with leucine increased their levels of ATP. Du et al. also compared leucine's affect on ATP production to substances known to increase cellular energy: glucose and pyruvate. These three compounds stimulated ATP production and showed similar increases in ATP/AMP ratios. By improving ATP stores and increasing the ATP/AMP ratio, leucine supplementation resulted in a reduction of AMPK activation.

In comparison to this study, the Du et al. study did not take place in overloaded skeletal muscle. Overloading skeletal muscle and stimulating protein synthesis for rebuilding muscle tissue can have a very taxing effect on the cellular energy stores. One may be led to believe that although leucine is able to increase ATP stores in myotubes, this same effect may not hold up under the robust overload process demonstrated in our study. However, the phosphorylation status of AMPK in the overloaded rat skeletal muscle of the leucine group was shown to be similar in comparison to the sham-operated young leucine group. Even during a period of robust muscle hypertrophy and cellular stress, phospho-AMPK levels of the aged leucine group were similar to those of the young adult placebo group (Figure 4.5).

Thomson and Gordon (Thomson & Gordon 2009) have previously shown that there is a decrease in the total AMPK protein content present in aged muscle and our findings are in line with their previous investigations. Because AMPK hyperphosphorylation has been shown to be present in overload aged rat muscle, the decrease in total AMPK may be a safety mechanism for limiting the amount of atrophy with age. If the age related increase in AMPK phosphorylation was not accompanied by decrease in total AMPK protein, muscle wasting would possibly be severely increased and the ability to hypertrophy aged muscle would be even more diminished.

Analysis of FoxO3 phosphorylation at $Ser^{318/321}$ did show a significant difference between the old leucine versus old placebo group. FoxO3 is phosphorylated by Akt, which in turn sequesters FoxO3 in the cytosol, blocking nuclear translocation and transcriptional activity of FoxO3. Increased AMPK activity inhibits Akt activation (King, Song & Jope, 2006) and phosphorylation of FoxO3, allowing translocation into the cell nucleus. Leucine has been shown to direct Akt signaling (Drummond & Rasmussen, 2008) and decrease AMPK activity (Du et al., 2009), both of which down regulate FoxO3 phosphorylation. In turn, down regulated AMPK activity would be negatively correlated with leucine supplementation while phospho-FoxO3 would be positively correlated with the addition of leucine.

To our knowledge, this is the first study to analyze the effect of leucine and overload on FoxO3. With the multiple pathways affecting the phosphorylation status of FoxO3, one may be led to believe that with ability of leucine to affect AMPK and Akt activity, there would be a subsequent increase in phospho-FoxO3 (decrease in activity). This effect was seen in the aged supplemented rats and their young adult counterparts. Phospho-FoxO3 in the young leucine group was more than 2-fold higher than the young placebo group. This effect did not carry over as greatly into the aged supplemented group, but was still present. The observed increase in phospho-FoxO3 suggest that there could be a multiple pathway interactions between Akt and leucine supplementation on FoxO3 activity. These differences point in the direction that there is an underlying affecter in the aged rats that diminishes the phosphorylation of FoxO3. This may be in part due to an age related decrease in Akt response. Drummond, Dreyer et al. (Drummond, Dreyer et al., 2008) have found that with an increase in age, there is a subsequent decrease in the phospho-Akt response from resistance training.

There was a significant main effect of age on both Atrogin-1 and MuRF1 total protein content. Also, overload had a significant main effect on total MuRF1. Leucine did appear to decrease the percent change in Atrogin-1 levels in the aged groups (Figure 4.12), however this effect did not reach statistical significance ($p \leq 0.07$) (Figure 4.14). There was also an interaction with leucine in decreasing the amount of MuRF1 percent change with overload that did not reach significance ($p \leq 0.14$). The effect of leucine on these atrogenes is still not completely understood and its combined effect with overload is even less clear. The outcome observed depends on the type of overload or immobilization model and type of diet being analyzed (Sugawara et al., 2008; Zanchi et al., 2009). Our findings are in line with those of Sugawara et al. (Sugawara et al., 2008), that leucine feeding does not attenuate Atrogin-1 and MuRF1 gene expression. Sugawara et al. supplemented leucine in a protein deficient diet and did not use an overload model. One possible explanation for why we saw a similar outcome compared to Suguwara et al. may be that leucine does not have as strong of an effect when moving to these downstream pathways. The significant main effect of age may be a possible safety mechanism to prevent further increases in atrophy during the aging process.

Overloading the skeletal muscle did have a significant main effect on MuRF1 protein content as compared to the sham but this effect was not compounded by the addition of leucine. A recent study by Zanchi et al. (Zanchi et al., 2009) showed that chronic resistance training decreases MuRF1 and Atrogin-1 gene expression in rats. However, there are a few main differences between our study and the one by Zanchi et al. Instead of using a 1-week overload through tenotomy, these rats were given a resistance training protocol that consisted of squat-like exercises performed for two sessions a week for 12 weeks. The time period in this study may have been long enough to show a greater manifestation of atrogene expression as compared to

our one-week model. Mascher et al. (Mascher et al., 2007) have shown that following a second session of resistance exercise, Atrogin-1 gene expression is decreased to a greater extent than after the first exercise session. However, Marino et al. (Marino et al., 2008) have demonstrated that MuRF1 gene expression peaks after 3 days of surgical ablation and returns to basal (control) levels by day 7. Further investigations may be necessary to determine the effect of different overload models on atrogene expression.

Although differences between the placebo and leucine groups were not significant in regards to atrogene expression it may be through a combination of an increase in protein synthesis pathways (data not shown) that changes were noticed in the amount of hypertrophy in the aged rats.

During the investigation, food intake was not affected by leucine supplementation. The aged rats did consume less food (Table 4.2) during the overload period, which may have been due to decreased recovery compared to the young adult rats.

There were some limitations in this study that could be accounted for in future studies. First is the duration period of overload. In future investigations looking at this protocol on MuRF1 and Atrogin-1 protein levels in the first few rather than later on after a possible leveling off period may be of interest. A shorter duration may show that leucine has an effect on these atrogenes. Second, each animal served as an overload intervention and control. This does help in the comparison of the analyses of markers of degradation; however it may not have been a true control. Due to the fact that an entire muscle group, the gastrocnemius, is tenotomized, it may cause the animal to favor one side more than the other, and diminishing the controllability

of the model. In the future, if more animals can be tested so as to control for this limitation, it could give a better representation of the amount of overload induced hypertrophy.

In summary, we showed that supplementing leucine during a period of overload, increases the amount of hypertrophy in aged, fast-twitch and slow-twitch rat skeletal muscle. This investigation showed that leucine supplementation decreases phospho-AMPK activity measured in aged, fast-twitch skeletal muscle. We also demonstrated that leucine supplementation increases phospho-FoxO3 in overloaded muscle regardless of age. The next step in this research is to transition the study into aged humans. Due to the nature of the type of overload protocol used in this study, a similar study performed in humans would need to be extended for a significantly longer period of time to notice beneficial effects of leucine in conjunction with a resistance training program.

Practical Application

For individuals reaching the latter decades of life who want to combat the detrimental effects of sarcopenia, incorporating a resistance training program with the inclusion of a leucine supplementation may help increase their ability to hypertrophy skeletal muscle. This may in turn help increase an individual's independence instead of relying more heavily on others for their activities of daily living. Those looking to maximize the effects of their resistance training programs may benefit from leucine supplementation, regardless of age.

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APPENDIX A: ECU ANIMAL USE PROTOCOL APPROVAL DOCUMENT

East Carolina University

Animal Care and Use Committee
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