Abstract

5'-AMP-Activated Protein Kinase and Eukaryotic Elongation Factor 2 Response to

Resistance Exercise in Young Versus Old Men and Women

By: Bradley M Harper
May, 2009
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It has been shown that skeletal muscle 5'-AMP-activated protein kinase (AMPK)

phosphorylation and/or activity is more greatly elevated in response to resistance exercise

or loading in aged rats, and that eukaryotic elongation factor 2 (eEF2) is more

phosphorylated with increased AMPK phosphorylation and/or activity, thereby

potentially decreasing the hypertrophic response of translation elongation with age.

Increased AMPK phosphorylation has also been shown to occur in old compared to

young humans following an acute resistance exercise bout. Thus, we hypothesized that

AMPK phosphorylation, AMPK activity, and eEF2 phosphorylation would be greater in

the vastus lateralis muscles of old compared to young men and women within the two

hours after an acute bout of resisted leg extensions. Subjects (N=6/age group) performed

an acute bout of leg extension resistance exercise consisting of 3 working sets. Muscle

biopsies were obtained pre-exercise (Pre-Ex), immediately post-exercise (0P), one hour

post-exercise (1P), and two hours post-exercise (2P). Phosphorylation of AMPK, acetyl-

CoA carboxylase (ACC; a measure of AMPK activity), and eEF2 were analyzed by

western blot. There were no differences in AMPK phosphorylation between age groups

or over time, while ACC phosphorylation was significantly ($P \leq 0.05$) increased at 0P and 1P compared to Pre-Ex for both age groups. The percent change in phospho-ACC from Pre-Ex was significantly higher in old compared to young subjects at all post-exercise timepoints. eEF2 phosphorylation was significantly elevated at 0P, and significantly lower at 1P and 2P, regardless of age group. Furthermore, total eEF2 was significantly elevated at 1P and 2P regardless of age group. These findings suggest that AMPK activity may be increased more in old compared to young subjects immediately following an acute bout of resistance exercise. This could lead to lower downstream translational signaling in older humans following resistance exercise, although eEF2 may not be a translational signaling protein affected by this phenomenon.

5'-AMP-Activated Protein Kinase and Eukaryotic Elongation Factor 2 Response to

Resistance Exercise in Young Versus Old Men and Women

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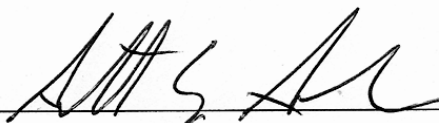
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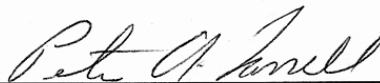
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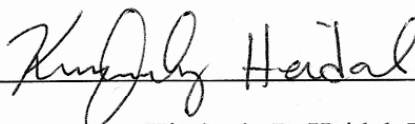APPROVED BY:

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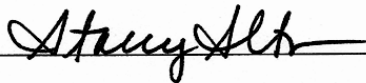Dr. Peter A. Farrell, PhD

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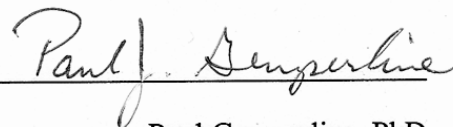COMMITTEE MEMBER: _____

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CHAPTER I: INTRODUCTION

Sarcopenia

According to the Center for Disease Control and Prevention (CDC), as of 2006, the average life expectancy in the United States was 77.7 years, showing a steady increase since 1900 (CDC, 2007). With an ever increasing life expectancy and increases in medical technology, the number of elderly individuals in society is on a continual rise. Approximately 39 million Americans have been reported to be $\geq$ 65 years old as of 2000 [reviewed by (Greenlund & Nair, 2003)]. Normal aging is accompanied by a decrease in muscle mass and strength (Rogers & Evans, 1993). Age-related decreases in muscle strength occur in both men and women (Hughes et al., 2001). The loss of muscle protein and mass with age is termed sarcopenia; one-third of this muscle protein loss occurs between ages 25 and 75 due to sarcopenia (Cohn et al., 1980).   Sarcopenia also leads to a decrease in muscle fiber cross-sectional area and isometric force (Korhonen et al., 2006), and has been shown to be significantly correlated with increased disability (Janssen, Heymsfield, & Ross, 2002). A lower skeletal muscle index in elderly individuals shows increased risk of disability (Janssen, Baumgartner, Ross, Rosenberg, & Roubenoff, 2004). As the elderly population loses muscle mass, they lose the ability to perform activities of daily living and eventually lose independence. For instance, per capita health care spending has greatly risen between 1966 and 2001 (Altman, Tompkins, Eilat, & Glavin, 2003) which can be partially attributable to disability from sarcopenia. As of 2001, it is estimated that by 2010, $183 billion will be spent for nursing home stays in the U.S. (Greenlund & Nair, 2003).

Resistance Training

Resistance training is one intervention thought to delay the progression of sarcopenia. Resistance exercise has been shown to increase strength and muscle fiber size (Brose, Parise, & Tarnopolsky, 2003; Kryger & Andersen, 2007; Rogers & Evans, 1993; Trappe et al., 2000); chronic strength training is proposed to help maintain muscle strength with increasing age (Aagaard, Magnusson, Larsson, Kjaer, & Krustrup, 2007; Bamman et al., 2003). However, a decrease in the ability of old skeletal muscle to hypertrophy compared to young skeletal muscle has been shown in rats (Degens & Alway, 2003), even when exercising under chronic overload conditions (Blough & Linderman, 2000). In humans, inhibition of gains in force production with age have also been shown despite resistance exercise (Korhonen et al., 2006). The age related decrease in myosin heavy chain (MHC) synthesis in humans (Balagopal, Rooyackers, Adey, Ades, & Nair, 1997) cannot be prevented by resistance exercise alone (Balagopal, Schimke, Ades, Adey, & Nair, 2001).

Protein Synthesis

Muscle protein synthesis (MPS) is the formation of proteins within the muscle, and leads to an increase in muscle mass and muscle fiber size if MPS is greater than protein breakdown. An age-related decrease in MPS exists at rest (Drummond, Miyazaki et al., 2008) and after resistance exercise in rats (Tamaki et al., 2000) and humans (Kumar et al., 2009; Sheffield-Moore et al., 2005). This decrease in MPS could strongly affect the increase in age-related atrophy. With decreased MPS, an imbalance of net protein balance may occur between MPS levels and muscle protein breakdown. Protein

synthesis consists of three stages: translation initiation, translation elongation, or translation termination. Of these, the most highly regulated are translation initiation and translation elongation.

mRNA Translation into Protein

A ribosomal initiation complex is first formed by an initiation t-RNA, an mRNA strand, a ribosome, and various eukaryotic initiation factors [as reviewed (Kimball, Farrell, & Jefferson, 2002; Nader, Hornberger, & Esser, 2002)]. During the first stage of translational initiation, eukaryotic initiation factor 4F (eIF4F) is formed by the combination of various initiation factors. Next, eIF4F binds mRNA thereby forming a ternary complex that proceeds to bind a 40s ribosomal subunit to create a preinitiation complex. This 40S complex scans the mRNA strand in the 5' to 3' direction until it reaches the start codon. Once the start codon is found, an 80S complex is formed, ready for translation elongation.

During translation elongation, amino acids are added to a polypeptide chain to form a protein. tRNAs with complimentary anticodon sequences bind to the aminoacyl (A) site of the 80S complex, next to the initiator tRNA (Frank, Gao, Sengupta, Gao, & Taylor, 2007). Polypeptide bonds are formed between amino acids as tRNAs are moved forward into the peptidyl site allowing new tRNAs to move into the A site (Nierhaus et al., 1998). Eukaryotic elongation factor 2 (eEF2) guides the movement of tRNAs into the exit (E) site for removal from the peptide [as reviewed (Browne & Proud, 2002)]. Inhibition of eEF2 occurs through phosphorylation by eukaryotic elongation factor 2 kinase (eEF2k), which inhibits translation elongation. One amino acid is added at a time

until a stop codon in the mRNA is reached. Translation termination then causes the release of a polypeptide chain from tRNA and the separation of ribosomal subunits (Bolster, Crozier, Kimball, & Jefferson, 2002).

Upstream Signaling Pathways

Both translation initiation and elongation are highly regulated by upstream signaling pathways. The Akt/mammalian target of rapamyacin (mTOR) signaling pathway is increased following a bout of resistance exercise (Drummond, Dreyer et al., 2008) and leads to an increase in skeletal muscle hypertrophy (Bodine et al., 2001) through increased protein synthesis (Phillips, Tipton, Aarsland, Wolf, & Wolfe, 1997). mTOR is activated partly via phosphorylation by Akt in respect to mechanical loading (Inoki, Li, Zhu, Wu, & Guan, 2002). Phosphorylation of mTOR can be inhibited by the tuberous sclerosis complex (TSC2) (Inoki, Li, Zhu, Wu, & Guan, 2002).

Downstream, mTOR affects initiation by phosphorylated deactivation of 4E-binding protein 1 (4E-BP1), which otherwise binds eIF4E and prevents the formation of the initiation complex eIF4F. mTOR can also phosphorylate and activate 70-kDa ribosomal protein S6kinase (p70^{s6k}) (Inoki, Li, Zhu, Wu, & Guan, 2002; Kimball, Farrell, & Jefferson, 2002), which stimulates translation by recruitment of the 5' tract of pyrimidine (5'TOP) mRNAs to the ribosome. p70^{s6k} elicits an effect on translation elongation by inhibiting eukaryotic elongation factor 2 kinase (eEF2k) from phosphorylating and deactivating eEF2 (Horman et al., 2002; Ryazanov, Shestakova, & Natapov, 1988) thereby leading to increased translation elongation.

Translational Signaling Response to Resistance Exercise

Downstream signals of mTOR are similarly activated in response to resistance exercise (see Upstream Signaling Pathways above). $p70^{s6k}$ activity has been shown to increase in response to resistance exercise in rats. Increases in $p70^{s6k}$ activity allows for increased eEF2 activity by inhibiting eEF2k (Horman et al., 2002).

AMPK

Upstream of mTOR lies 5'-AMP-activated protein kinase (AMPK). AMPK acts as a sensor of energy stores within the body (Winder & Hardie, 1999) and is activated by an increase in the AMP/ATP ratio (Carling, 2005; Miranda, Tovar, Palacios, & Torres, 2007). Essentially, AMPK acts to increase the production of energy stores within the body through processes such as glycolysis and fatty acid oxidation while inhibiting energy expensive anabolic processes (Hardie, Hawley, & Scott, 2006).

Protein synthesis is one such energy-expensive process (Schmidt, 1999), and thus AMPK inhibits protein synthesis in order to conserve energy stores. AMPK can inhibit mTOR through phosphorylation of TSC2 (Inoki, Zhu, & Guan, 2003), and can also act more directly on translation elongation by activation of eEF2k (Browne & Proud, 2002). AMPK phosphorylation (one mechanism by which AMPK is activated) is negatively correlated with chronic overload-induced muscle hypertrophy and has also been shown to be more activated in old (O) versus young (Y) rats under overload conditions(Thomson et al., 2009; Thomson & Gordon, 2005). Moreover, AMPK is more highly activated in response to resisted contractions in old vs. young rats (Thomson et al., 2009) as well as in response to resistance exercise in old vs. young humans (Drummond, Dreyer et al.,

2008). The potential mechanism for this occurrence is an elevation of the AMP/ATP ratio is old versus young skeletal muscle before and after exercise (Bastien & Sanchez, 1984).

Specific AIM

Muscle fiber atrophy and a lack of hypertrophy have been shown to increase with age (Hortobagyi et al., 1995; Thompson, 1994). Decreases in translational signaling with progressive age both at rest and during exercise (Drummond, Dreyer et al., 2008; Tamaki et al., 2000) may explain this lack of muscle maintenance in elderly populations. AMPK phosphorylation and/or activity has been shown to be increased in young and old subjects following essential amino acid ingestion after an acute bout of resistance exercise (Drummond, Dreyer et al., 2008). Increased eEF2 phosphorylation has been observed with increased AMPK activity in rats (Thomson, Fick, & Gordon, 2008). However, no studies in humans have displayed this increase in eEF2 phosphorylation with increased AMPK activity. Thus the aim of this study was to determine whether AMPK and eEF2 phosphorylation were higher in skeletal muscle of old versus young humans after an acute resistance exercise bout. We hypothesized that AMPK phosphorylation, AMPK activity, and eEF2 phosphorylation would be greater in the vastus lateralis muscles of old compared to young men and women within the two hours after an acute bout of resisted leg extensions.

CHAPTER II: REVIEW OF LITERATURE

Sarcopenia

The elderly population consists of approximately 20% of the US population

((CDC), 2007). There is often clinically significant muscle wasting with age, a condition

known as "sarcopenia". A steady decline in muscle mass is typically observed after age

45 (Hughes et al., 2001; Tzankoff & Norris, 1977), as well as a decrease in strength with

progressing age (Balagopal, Rooyackers, Adey, Ades, & Nair, 1997). Sarcopenia, the

muscle protein loss during aging, results in as much as 14% muscle loss and 34% muscle

protein loss between 25-75 years of age (Cohn et al., 1980).

A greater prevalence of disability is precipitated by this muscle atrophy

(Baumgartner et al., 1998; Janssen, Heymsfield, & Ross, 2002); as much as 20% of the

total population ≥65 years is shown to be disabled as of 1999 (Manton & Gu, 2001). In

the United States, the age-related decrease in muscle mass accounts for 85.6% of men

and 26% of women who are disabled (Janssen, Shepard, Katzmarzyk, & Roubenoff,

2004). Elderly populations lose their ability to perform normal daily activities such as

carrying one's own groceries or standing from a chair without help. Moreover as many

as 13.4% of men and 16.3% of women age 60 and up need help getting out of bed while

20.3% of men and 30.9% of women within the same age range have difficulty walking 10

steps; these percentages only increase with age (Ostchega, Harris, Hirsch, Parsons, &

Kington, 2000). Decreases in strength with age, are also related to bone density

(Jacobson, Beaver, Grubb, Taft, & Talmage, 1984), only compounding the impact of

sarcopenia; therefore increased prevalence of disability will occur from osteopenia and other bone related diseases due to sarcopenia (Hunter, McCarthy, & Bamman, 2004).

In 2000, health care costs related to the loss in muscle mass with age were around 18.5 billion dollars (Janssen, Shepard, Katzmarzyk, & Roubenoff, 2004). Worse, gross expenditure is projected to increase by 23 percent by 2011 from levels observed in the year 2000 (Altman, Tompkins, Eilat, & Glavin, 2003). With such a prevalence of age-related disability, it is thus important to examine the mechanism(s) underlying muscle atrophy with age, as well as potential compensatory methods to increase muscle mass in sarcopenic individuals and control for national and individual expenses due to dysfunctional status.

Sarcopenia can occur due to a number of various factors, including inherent changes in muscle mass with age, a sedentary lifestyle, or disease status. The inherent loss in muscle mass with age is typically due to both a decrease in muscle fiber size and number, which results in losses in whole muscle protein content (Trappe et al., 2003) as well as decreases in whole-muscle mass as assessed by cross-sectional area. Atrophy on the single fiber level is mostly confined to fast-twitch (FT) fibers (Korhonen et al., 2006). Exercise interventions such as resistance training can increase whole muscle mass to a certain extent, but hypertrophy appears limited in elderly individuals and increases in muscle mass are attenuated (Thompson, 1994).

Resistance Training

Resistance training is one method used to delay the progression of sarcopenia and to increase muscle growth. Resistance training has been shown to cause an increase in

cross-sectional area of muscle fibers in elderly individuals (Bamman et al., 2003; Kryger & Andersen, 2007) and attenuate the decline of muscle mass with age (Thompson, 1994). However, these exercise-induced increases only slow the process of protein loss and do not produce a complete reversal (Balagopal, Schimke, Ades, Adey, & Nair, 2001). The loss of effect of resistance training is less pronounced in ST muscle fibers with age (Frontera, Meredith, O'Reilly, Knuttgen, & Evans, 1988; Trappe et al., 2000) and elderly women show less myofiber hypertrophy and strength gains compared to elderly males (Bamman et al., 2003; Brose, Parise, & Tarnopolsky, 2003). A decreased hypertrophic response is also seen in FT muscles of aged rats (Haddad & Adams, 2006; Thomson & Gordon, 2005). The cellular and molecular mechanisms underlying the attenuated ability for fiber growth in aged muscle remain to be completely elucidated.

Protein Synthesis

A change in the rate of muscle protein synthesis is one potential factor underlying the age-related decline in muscle fiber cross-sectional area and perhaps fiber number. There is a decrease in resting muscle protein synthesis rate with age (Drummond, Miyazaki et al., 2008) as well as decreases in fractional synthesis rate in old compared to young individuals after an acute bout of resistance exercise (Sheffield-Moore et al., 2005) which may be one factor underlying age-related atrophy. The protein synthesis response appears to be delayed in older individuals (Drummond, Dreyer et al., 2008). Hypertrophy occurs through an increase in muscle protein synthesis within a given muscle fiber. An elevation of protein synthesis occurs in response to resistance training in young adults (Dreyer et al., 2006; Phillips, Tipton, Aarsland, Wolf, & Wolfe, 1997).

However, with age, there is a reduced muscle protein synthesis response to resistance training in both humans (Drummond, Dreyer et al., 2008; Welle, Bhatt, & Thornton, 1996) and rats (Tamaki et al., 2000). This may contribute to an altered balance in protein synthesis and degradation in the elderly.

There are three components of protein synthesis. These factors include initiation, elongation, and termination. Rate limiting factors of protein synthesis and thus muscle cell growth and maintenance may lie within the intracellular regulation of both initiation and elongation.

Translation Initiation

During translation initiation, formation of a ribosomal initiation complex occurs as reviewed elsewhere (Kimball, Farrell, & Jefferson, 2002; Nader, Hornberger, & Esser, 2002). This ternary complex is formed by the initiator tRNA (Met-tRNAi), an mRNA strand, a ribosome, and various eukaryotic initiation factors. First the eukaryotic initiation factor 4F (eIF4F) protein complex is formed by other eukaryotic initiation factors: eIF4A, eIF4E, and eIF4G. In this process, eIF4E availability is the rate limiting step, and is regulated by 4E-binding protein 1 (4E-BP1), which binds to eIF4E and prevents eIF4E association with eIF4G thereby preventing eIF4F formation and the first step of initiation. Phosphorylation of 4E-BP1 prevents its binding to eIF4E, and this phosphorylation step is controlled by factors to be discussed later. Once formed, eIF4F binds mRNA. A 40S ribosomal subunit next binds this ternary complex to form a preinitiation complex. The initiation factor, eIF3 guides this binding, scanning in the 5' to 3' direction, the 40S complex scans for the start codon (usually AUG) along an mRNA

strand that will match the tRNA anticodon sequence. AUG is a group of three nucleotides that code for a single amino acid, methionine. Once the start codon is coded, a 60S subunit joins the 40S initiation complex to yield an 80S complex ready for polypeptide elongation.

Translation Elongation

Translation elongation is the most energy expensive process within the cell (Schmidt, 1999) utilizing 4 GTPs (ATP equivalents) for each amino acid added to the polypeptide chain. With elongation, new tRNAs containing complimentary anticodon sequences bind to the aminoacyl (A) site of the 80S complex beside the initiatior tRNA that is attached to the peptidyl (P) site of the ribosome (Frank, Gao, Sengupta, Gao, & Taylor, 2007). Polypeptide bonds are formed between the amino acids and subsequent tRNAs are moved to the P site (Nierhaus et al., 1998). Methionine attaches to amino acids in the A site releasing tRNA (Frank, Gao, Sengupta, Gao, & Taylor, 2007). Eukaryotic elongation factor 2 (eEF2) propels the movement of the ribosome along the mRNA to aid in the removal of uncharged tRNAs by facilitating their movement to the exit (E) site on the ribosome [as reviewed (Browne & Proud, 2002)]. This leaves behind polypeptide chains attached to the tRNA in the adjacent A site. The initial uncharged tRNA is moved to the E site, the second tRNA moves to the P site leaving the A site open to bind new tRNA with a complimentary anticodon sequence (Frank, Gao, Sengupta, Gao, & Taylor, 2007). The process continues adding one amino acid at a time. Eventually, a stop codon is reached within the assembly and a releasing factor binds. Translation termination occurs, releasing a polypeptide chain from the tRNA followed by

the separation of the ribosomal subunits (Bolster, Crozier, Kimball, & Jefferson, 2002).

Although only a few key regulatory factors are mentioned in this review, each step of

protein synthesis is tightly regulated.

Control of protein translation via mTOR

Upstream cellular signaling pathways highly regulate both initiation and

elongation factors controlling protein synthesis. One of the most important signaling

pathways, the Akt/mammalian target of rapamycin (mTOR) pathway, partially controls

the protein synthesis response to loading and ultimately hypertrophy as well (Bodine et

al., 2001). Signaling through this Akt/mTOR pathway has been shown to increase

(Drummond, Dreyer et al., 2008) after an acute resistance exercise bout which leads to an

increase in protein synthesis (Phillips, Tipton, Aarsland, Wolf, & Wolfe, 1997).

Pharmacological blockade of mTOR signaling inhibits resistance exercise-induced

protein synthesis in rats (Kubica, Bolster, Farrell, Kimball, & Jefferson, 2005) and

humans (Drummond, Dreyer et al., 2008) and inhibits chronic overload-induced muscle

hypertrophy (Bodine et al., 2001). With mechanical loading of the muscle cell, Akt is

phosphorylated to activate mTOR through mTOR phosphorylation at Ser^{2448} (Inoki, Li,

Zhu, Wu, & Guan, 2002; Reynolds, Bodine, & Lawrence, 2002). Alternatively, mTOR

can be deactivated by activation of the tuberous sclerosis complex (TSC) pathway which

inhibits mTOR phosphorylation at Ser^{2448} (Inoki, Li, Zhu, Wu, & Guan, 2002; Inoki, Zhu,

& Guan, 2003). This TSC2 pathway can also be negatively regulated by Akt, which

deactivates TSC2, thereby allowing an increase in mTOR activity.

Downstream, mTOR stimulates protein translation through various signaling proteins mediating both translational initiation and elongation. mTOR facilitates initiation by phosphorylating 4E-BP1, which allows for increased formation of the translational initiation complex eIF4F by hindering 4E-BP1 binding to (and deactivation of) eIF4E. mTOR also activates 70-kDa ribosomal protein S6kinase (p70^{s6k}) (Inoki, Li, Zhu, Wu, & Guan, 2002; Kimball, Farrell, & Jefferson, 2002), which can have a two-pronged effect on protein synthesis. First, p70^{s6k} phosphorylates ribosomal S6 protein (rpS6), thereby recruiting 5'-tract of pyrimidine (5'TOP) mRNAs to the ribosome. 5'TOP mRNAs encode ribosomal proteins that are important for translation (Levy, Avni, Hariharan, Perry, & Meyuhas, 1991; Terada et al., 1994) and thus the translational mechanism itself is improved. Second, p70^{s6k} has also been shown to inhibit eukaryotic elongation factor 2 kinase (eEF2k), an eEF2 inhibitor (Wang et al., 2001). eEF2 kinase phosphorylates eEF2 at Thr^{56} (Redpath & Proud, 1993), thereby inactivating eEF2 (Ryazanov, Shestakova, & Natapov, 1988). Inactivation of eEF2k, and thus activation of eEF2, can lead to increased translational elongation (Horman et al., 2002). Phosphorylation of eEF2k on the Ser ^{366} residue occurs by either p70^{s6k} or by MAPK signaling through p90^{RSK} eEF2k activation(Wang et al., 2001).

AMPK

5'-AMP-activated protein kinase (AMPK) is another signaling protein mediating protein translation and synthesis; however its actions inhibit the process. AMPK is an energy sensor within the cell (Winder & Hardie, 1999), and its actions act to maintain energy stores within each cell. AMPK activation occurs as the AMP/ATP ratio is

increased (Carling, 2005; Miranda, Tovar, Palacios, & Torres, 2007; Ponticos et al.,
1998). AMPK activation occurs during processes that either decrease production of ATP
or processes within the cell that increase utilization of ATP such as hypoxia or skeletal
muscle contraction (Hardie, Hawley, & Scott, 2006). For instance, AMPK activation
occurs in response to low amounts of glucose within the cell (Salt, Johnson, Ashcroft, &
Hardie, 1998). Even at rest, low glycogen and energy levels can activate AMPK
(Wojtaszewski, 2003). Once AMPK is activated due to low energy stores, it acts to
promote ATP production through glycolysis, increasing glucose uptake, fatty acid
oxidation, and mitochondrial biogenesis, while inhibiting other energy expensive
processes within the cell (Hardie, Hawley, & Scott, 2006). AMPK essentially aids in
preservation of cell life through energy maintenance.

With protein synthesis being a high energy process (Schmidt, 1999), AMPK acts
to keep an energy sensitive homeostasis within the cell. AMPK can inhibit mTOR
through TSC2 phosphorylation at Thr^{1227} and Ser^{1345} and thus cause TSC2 activation
(Inoki, Zhu, & Guan, 2003) to inhibit protein synthesis and thus prevent a further
decrease in energy stores within the muscle cell. In rats, injection with the AMPK
activator 5-aminoimidazole-4carboxamide-1-beta-D-ribofuranoside (AICAR) inhibits
protein synthesis as well as reduces the phosphorylation (and presumably activation) of
the upstream regulators Akt, mTOR, $p70^{s6k}$, and 4E-BP1 (Bolster, Crozier, Kimball, &
Jefferson, 2002). Additionally, AMPK can activate eEF2k by phosphorylation at Ser^{398}
(Browne & Proud, 2002) which would decrease translation elongation. Interestingly, a
negative correlation between AMPK phosphoylation status and overload-induced muscle

hypertrophy was shown by Thomson and Gordon (Thomson & Gordon, 2005), indicating that AMPK may play a role in negatively mediating loading-induced muscle growth.

AMPK activity has been shown to be elevated in aged rat muscle under both resting and overloaded conditions (Thomson, Fick, & Gordon, 2008; Thomson & Gordon, 2005). This corresponds with a decrease in translational signaling and growth (Thomson & Gordon, 2005). Although this activation was shown to be elevated in FT muscle fibers, AMPK activity was not elevated in ST muscle (Thomson & Gordon, 2005, 2006). Following a resistance exercise model, rats show higher AMPK activation in old compared to young subjects(Thomson et al., 2009). AMPK is also elevated in the skeletal muscle of older versus younger humans after acute resistance exercise (Drummond, Dreyer et al., 2008). This may be due to energy stores, as aged skeletal muscle has an elevated AMP/ATP ratio before and after exercise (Bastien & Sanchez, 1984; Marcinek, Schenkman, Ciesielski, Lee, & Conley, 2005). Interestingly, translation signaling is lower after acute resistance exercise in aged versus younger rats (Funai, Parkington, Carambula, & Fielding, 2006; Parkington, LeBrasseur, Siebert, & Fielding, 2004). Similarly, aging also results in decreased translation signaling in chronically overloaded muscle (Thomson & Gordon, 2006). Thus, it is postulated that elevated AMPK activity may be responsible for diminished translational signaling in response to resistance exercise in aged skeletal muscle.

eEF2

eEF2 is an essential component of translational elongation. After being bound to GTP, thereby becoming activated, eEF2 assists in the removal of tRNAs from the

polypeptide chain [as reviewed (Browne & Proud, 2002)]. eEF2 is regulated in an energy sensitive manner through an mTOR dependent pathway. Elongation is a high energy process and may be affected by situations of high energy demand or low energy supply. Regulation and inhibition occurs through phosphorylation by eEF2k as discussed previously in translation elongation.

eEF2 activity has been shown to be inhibited by AMPK activation with overload (Thomson, Fick, & Gordon, 2008). This inhibition is more pronounced in old compared to young rats (Thomson & Gordon, 2006). Similarly, with AMPK inhibition, eEF2 phosphorylation was not significantly increased after overload (Thomson & Gordon, 2006). In humans, no changes in eEF2 phosphorylation has been seen after essential amino acid ingestion, which is known to stimulate mTOR (Drummond, Dreyer et al., 2008) however, we postulate that changes may develop without a post-exercise supplement.

Specific Aim

Muscle fiber atrophy and lack of hypertrophy has been shown to be increased with age (Hortobagyi et al., 1995; Thompson, 1994). This may be partly due to a decrease in muscle protein synthesis rate and translational signaling with age at both rest and with overload (Drummond, Dreyer et al., 2008; Tamaki et al., 2000). Furthermore, evidence of higher skeletal muscle AMPK phosphorylation and/or activity with age has been shown in response to resistance exercise in rats (Thomson et al., 2009) and humans (Drummond, Dreyer et al., 2008). eEF2 has been shown to be more phosphorylated (and thus theoretically less active) with increased AMPK activity in rats (Thomson, Fick, &

Gordon, 2008). Although one study in humans revealed no difference in eEF2 phosphorylation after resistance exercise in old vs. young men despite higher AMPK phosphorylation in the older group, all subjects had ingested an essential amino acid supplement prior to exercise (Drummond, Dreyer et al., 2008). Essential amino acids, particularly, Leucine, stimulate mTOR activity [as reviewed (Drummond & Rasmussen, 2008)] and thus could have masked downstream signaling differences between young and old subjects. Thus the aim of this study was to determine whether higher AMPK and eEF2 phosphorylation occurs in skeletal muscle of old versus young humans after an acute resistance exercise bout. We hypothesized that AMPK phosphorylation, AMPK activity, and eEF2 phosphorylation would be greater in the vastus lateralis muscles of old compared to young men and women within the two hours after an acute bout of resisted leg extensions.

CHAPTER III: METHODS

Subjects

Participants in this study consisted of 6 young subjects (3 male, 3 female) and 6

old subjects (4 male, 2 female). Subject characteristics are shown in Table 3.1. All

participants were healthy, lean men and women with no subjects reported previous

cardiovascular disease, diabetes, or hypertension. Each subject was initially screened on

the telephone or through e-mail to determine age, height, weight, BMI, medical history

including medications, and exercise history. This study was approved by the East

Carolina University and Medical Institutional Review Board for the use of human

subjects.   Subjects were recruited through the posting of flyers, word of mouth, and e-

mail to East Carolina University (ECU) faculty and staff.

Table 3.1. Subject Characteristics.

	Young Adults (n=6)	Old Adults (n=6)
Age (years)	22.17 ± 0.79	66.33 ± 4.37
BMI (kg/m^2)	23.06 ± 0.97	28.29 ± 1.07*
Body Fat (%)	20.94 ± 1.77	30.46 ± 0.85*
Fat-Free Mass (kg)	57.82 ± 3.90	60.8 ± 2.39
Weight (kg)	73 ± 5.0	88 ± 4.1*

*=Significantly different between groups. Data are presented as mean $\pm$ SEM (standard error of the mean). Old adults were had significantly higher BMI, weight and body fat percentage. There were no significant differences between young and old adults for fat-free mass. BMI: body mass index. Body fat and fat free mass were obtained with the use of a four-site Durnin and Womersley skinfold method of measurement (Durnin &

Womersley, 1974), which measured skinfolds at the bicep, triceps, subscapular, and suprailiac sites. Body fat and fat free mass were then calculated (Siri, 1961). A complete description of measurements is provided in later in the later Methods section, *Initial Visit.*

Experimental Design

We hypothesized that AMPK phosphorylation, AMPK activity, and eEF2 phosphorylation would be greater in the vastus lateralis muscles of old compared to young men and women within the two hours after an acute bout of resisted leg extensions. The experimental sessions are summarized in Table 3.2. The first session was performed in the Fitness Instruction, Testing, and Training (FITT) building at East Carolina University. This initial visit consisted of an initial screening and baseline measurements, familiarization with resistance exercise equipment, and determination of a 10-repetition maximum (10RM) on the Cybex leg extension machine (Cybex International; Serial#: 485097W272216). One to two weeks following the initial visit, subjects came in to Brody School of Medicine after an overnight fast for the experimental session. The 1-2 week waiting period was used as a recovery period to minimize muscle soreness or effects of 10RM testing on protein synthesis and breakdown during the experimental session. The 10RM testing was used for the resistance exercise bout in the experimental session in an attempt to stimulate AMPK and the downstream eEF2 signal. A muscle biopsy was obtained before exercise for pre-exercise measurements, immediately post exercise (after completing the final resistance exercise set and completion of preparation for the biopsy), one hour after taking the immediately post-exercise biopsy, and two hours after taking the immediately post-exercise biopsy. Biopsy

time points are based upon the results of Drummond et al. (Drummond, Dreyer et al., 2008), showing that skeletal muscle AMPK phosphorylation is greater in old versus young men by 1 hour post-resistance exercise, and continues until at least 2 hours post resistance exercise.

Initial Visit (non-fasted)	Experimental Session (overnight fast)
1) Informed consent and medical history 2) Height and weight measurements 3) Skinfold measurements 4) 10-repetition maximum determination	1) Exercise Session -bilateral leg extension -3 warm-up sets (50,70,90% of 10RM -3 sets to failure (100% of 10RM 2) Biopsy – alternating legs -pre-exercise, immediately post, 0 min, 60 min, 120 min post

Table 3.2. 10-repetition max(10RM) was determined on the leg extension machine in the FITT building at East Carolina University. Warm-up sets consisted of 10 repetitions, 5-7 repetitions, and 3-5 repetitions for each set (50%, 70%, 90% of the estimated 10RM), respectively. The 0 min time point begins after the completion of the last set of resistance exercise. 60 min and 120 min post are obtained 1 and 2 hours after the 0 min post biopsy, respectively

Initial Visit

During the initial visit, subjects filled out an informed consent (Appendix A), medical history (Appendix B), and a dietary food log (Appendix C) for a normal day's diet to ensure that there was not an absence of carbohydrate, protein, or fat in the diet. A

low carbohydrate diet can cause decreased muscle water by dehydration; while dehydration may attenuate resistance exercise performance (Judelson et al., 2007). A high protein diet could increase availability of amino acids for protein synthesis. Height and weight were then measured. Next, 4-site skinfold measurements were taken at the bicep, tricep, suprailiac, and subclavical. Body fat density was estimated with the Durnin and Womersley skinfold assessment and body density formula ([495/(1.1714 - 0.063 * LOG[sum of skinfolds] – 0.000406 * [age]) – 450]) (Durnin & Womersley, 1974; Siri, 1961).

The 10-repetition maximum was determined on the Cybex Leg Extension machine. Subjects were first acquainted with the leg extension machine in order to help reduce the likelihood of injury or cardiovascular events such as a hypertensive response. Proper etiquette, timing, and breathing during resistance exercise were explained and/or demonstrated. Before commencement of the 10-RM testing, subjects were asked to predict the maximum amount of weight they could lift 10 times. Half of this predicted weight was used as a warm-up. After completion of the first set and each subsequent step, the weight was increased 5-20 pounds depending upon the performance of the individual. Each set consisted of 10 repetitions followed by a 1-2 minute rest interval. Testing was repeated no more than 4 times. Upon determining the 10RM, subjects were unable to complete more than 10 repetitions, thus giving a true determination of the 10RM.

Subjects were given an information sheet which provided guidelines to follow in the 7-14 days preceding the experimental session. During the extent of the study, subjects refrained from exercise and donating blood. For the two days preceding the experimental session, subjects were directed to refrain from drinking alcohol, moderate the intake of caffeine, and drink 64 ounces of water per day. Alcohol consumption has been shown to decrease protein synthesis (Lang, Kimball, Frost, & Vary, 2001) and dehydration may cause decreased performance (Shirreffs, 2005). Caffeine must be controlled for since its ergogenic effects include a reduction in RPE and possible increase in contractile force of muscle. For the day preceding the experimental session, subjects were told to refrain from eating at least 12 hours prior to reporting to the laboratory and exercising the day before the session. On the day of the experimental session, subjects were to drink 16 oz. of water before reporting to the lab and wear exercise clothing. Food diaries were also recorded three days before the experimental session for later use, but those data were not analyzed for this report.

Experimental Session

One to two weeks following the initial visit, subjects reported to the Brody School of Medicine, room 3S08 after an overnight fast. Muscle biopsies were taken before exercise, directly after exercise, at one hour post exercise and at two hours post exercise. The timeline of the experimental sessions are presented in Figure 3.1.

Timeline of Experimental Day

-1	0	1 Hr	2 Hrs
Pre-exercise biopsy	Exercise session	1 hour post biopsy	2 hour post biopsy
	Immediately post biopsy		
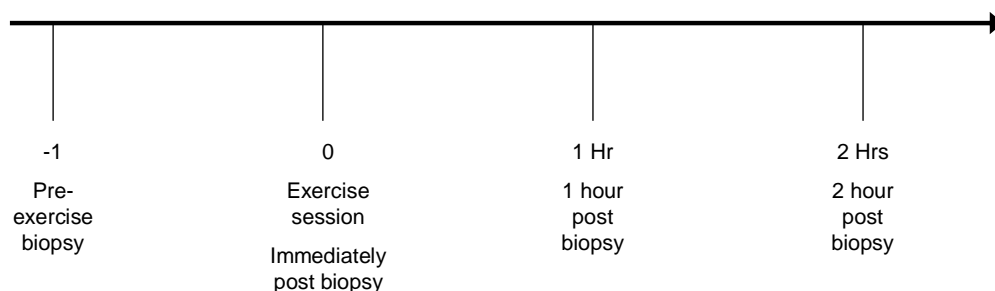
Figure 3.1. Timeline of events for the experimental day for each subject. -1 consists of the period before the exercise session. 0 is the exercise session. 1Hr is one hour after the resistance exercise session. 2 Hrs is two hours after the resistance exercise session.

Acute Resistance Exercise

The acute resistance exercise bout was performed on the Cybex leg extension machine (Cybex VR Leg Extension Model # 4850, Cybex International, Medway, MA) after the pre-exercise muscle biopsy was obtained. Proper etiquette was discussed again with subjects in order to prevent injury. The session began with a warm-up set starting at 50% of the predetermined 10RM for 10 repetitions. After a 1.5 minute rest interval, a second set of 5-7 repetitions was performed at 70% of the predetermined 10RM. The

final warm-up set began 1.5 minutes after the second set and consisted of 3-5 repetitions at 90% of the predetermined 10RM. After the standard 1.5 minute rest interval, the working sets began using the predetermined 10RM. Each working set was performed until failure, with the first and second sets being followed by the 1.5 minute rest interval. Upon reaching failure, the subjects were encouraged verbally to complete 1-3 forced repetitions with the assistance of the investigator directing the exercise session. If the repetitions performed reached 15 or more, the weight was increased by five to ten pounds for the subsequent set.

Muscle Biopsies

Skeletal muscle biopsy procedures were explained fully to the subjects before the procedure began. All procedures were performed under aseptic conditions. The vastus lateralis muscle was located, and the area was marked and shaved if necessary. The area was sterilized using iodine swabs and draped with a sterile field. Cold spray was used to numb the area and approximately 5 mL of local anesthetic (1% lidocaine) was injected into the biopsy site subcutaneously along the vastus lateralis. A small incision, approximately ¼ inch was made on the skin and through the fascia of the vastus lateralis muscle using a No.11 scalpel. Muscle biopsies were executed using a 5-mm Bergström needle using sterile procedure and suction was applied in order to maximize the amount of muscle extracted.

Immediately after each biopsy was taken, direct pressure and ice were applied to the site of the procedure. Once bleeding had ceased, a steri strip bandage was applied to

hold the incision site together, followed by a Band aide bandage, a Tegaderm patch, and a pressure wrap. This set-up allowed proper healing and aided in decreasing the likelihood of scars. The Tegaderm patch provided waterproof protection for the site.

Preparation of the first two biopsy sites occurred prior to exercise. The initial baseline biopsy was alternated between legs among subjects. Subsequent biopsies were obtained from legs in a bilaterally alternating order with the first and third biopsy being obtained from the same leg. After the Pre-exercise (Pre-Ex) biopsy, the subject performed the acute resistance exercise session (see *Acute Resistance Exercise* above). Directly after the resistance exercise bout, the subject moved to a bed and the immediately post biopsy was taken at time point 0P. After 60 minutes (1P), a third biopsy was taken approximately 5cm proximal from the initial biopsy site. A final biopsy was taken at 120 minutes post-exercise (2P) from the vastus lateralis approximately 5cm proximal from the second biopsy site. New incision sites were made for each biopsy in order to reduce the effect of the inflammatory response on the muscle being removed for analysis.

Once removed, muscle tissue was divided. One portion was immediately oriented for cross-sectional alignment and mounted in a mixture of tragcantham gum and OCT compound. After being embedded in the OCT mixture, samples were frozen in isopentane chilled in liquid nitrogen. The frozen oriented muscle section, to be used for histochemical analyses, was placed into a cryovial and temporarily stored in liquid nitrogen. The second portion of the muscle biopsy tissue was placed into a cryovial and

flash frozen in liquid nitrogen for later, western blot analyses. Samples were stored at -80°C until being analyzed.

Sample Analysis

Western Blotting Analysis for AMPK, P-AMPK, eEF2, P-eEF2, ACC, and P-ACC

The western blotting technique was used for signaling protein analyses. The primary antibodies are commercially available: anti-AMPK [Cell Signaling Technology (CST); Danvers, MA; Cat. # 2532], anti-phospho-AMPK (CST; Cat. # 2535), anti-eEF2 (CST; Cat. # 2332), anti-phospho-eEF2 (CST; Cat. # 2331), anti-acetyl CoA Carboxylase (streptavidin-HRP, GE Life Sciences, RPN1231), and anti-phospho-Acetyl CoA Carboxylase (Millipore Corporation; Temecula, CA; Cat. # 07-303; Lot#: LV1506382). A fraction of each frozen muscle biopsy 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 $Na_4P_2O_7 \cdot 10H_2O$, 100 mM ß-glycerophosphate, 25 mM NaF, 50 µg/ml leupeptin, 50 µg/ml pepstatin, and 33 µg/ml aprotinin. A ground glass homogenizer using a variable speed motor was used to perform all homogenizations. Homogenizations were performed in ice in order to prevent excessive heat build-up that may denature proteins.

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/ml and then boiled for 5 min. Proteins were separated by a 4-7.5% gradient sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE). Gel electrophoresis occurred for 1.5 hours at 4˚C on a PVDF membrane at 100V in a transfer buffer that contained: 25 mM Tris-base pH~8.3, 192 mM glycine, and 20% methanol. Ponceau S, stained the membranes after which they were dried and then scanned into a digital image. The digital image allowed 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 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, followed by incubation in the primary antibody diluted in1% bovine serum albumin in TBS-T overnight at 4˚C. Primary antibody dilutions consisted of anti-phospho-AMPK: 1/4000; anti-AMPK: 1/1000; anti-phospho-eEF2: 1/2000; anti-eEF2: 1/2000; anti-phospho-ACC: 1/1000; anti-ACC: 1/1000. Membranes were washed 4 x 5 minutes per wash in TBS-T, incubated in secondary antibody in blocking buffer for an hour while at room temperature followed by a 4 x 5 minutes wash period in TBS-T.

After the last wash period, detection of the HRP activity occurred using a chemiluminescence reagent (Amersham, Piscataway, NJ) and exposure to autoradiographic film (Classic Blue Sensitive; Midwest Scientific, St Louis, MO, USA). The integrated optical densities (IODs) were then quantified by densitometry and calculation of the concentration of the antigen present in each muscle as the IOD normalized to units of total muscle protein that was 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. Due to difficulty in obtaining data for phospho-AMPK, all analysis of AMPK consisted of 4 subjects per group instead of six subjects per group.

Immunohistochemistry Procedure

Fluorescent microscopy was attempted in order to measure phospho-AMPK (Cell Signalling; Catalog #:2535) after an acute resistance exercise bout in fast versus slow-twitch muscle fibers of young and old subjects.

Ten um serial cross-sections were cut from biopsies of the vastus lateralis muscle using a cryostatic microtome at -20°C. Cross-sections were individually placed on clean, uncoated microscope slides, aligned for cross-sectional analysis, and stored at -20°C until preparation for staining. Execution of immunohistochemical steps occurred at room temperature unless otherwise stated.

In preparation for staining, frozen slides were thawed and set to air-dry for 1 hour. Sections were fixated for five minutes in methanol followed by 1 minute in acetone. Sections were allowed to air dry and were then rehydrated for one minute using phosphorous buffered saline (PBS). Blocking was performed in PBS with 10% horse serum for 1.5 hours followed by two 5 minute rinses with PBS. Samples were then incubated in a 1:10 dilution of the primary antibody (phospho-AMPK) overnight at 4°C. During the second day of staining slides were rinsed twice for 5 minutes in PBS. Incubation of the biotinylated anti-rabbit IgG antibody (Vector Laboratories; Catalog#: BA-1000; Lot# U0702) in PBS then occurred in a 1:200 dilution for 45 minutes. This process was followed by the standard 2x5 minute washes using PBS. Lastly, the sections

were incubated for thirty minutes in a 1:400 dilution of Fluorescein Avidin DCS (Vector Laboratories; Catalog#: A-2001; Lot#: U0403) diluted in sodium bicarbonate (pH8.2) followed by 2x5 minute rinses in PBS. Slides were allowed to air-dry and were mounted with cover slips using Permount. The biotinylated anti-rabbit IgG and Fluorescein Avidin DCS were used to enhance the fluorescent signal obtained from the primary antibody.

Fiber Type Staining

Serial cross-sections were histochemically stained for fiber type to enable comparison of the phospho-AMPK signal intensities between fiber types. This staining was accomplished by the myosin ATPase method.

Slides were incubated for 5 min in acid preincubation solution (1.94g Sodium Acetate and 2.94g Sodium Barbital in 100mL distilled water) is prepared at room temperature at a pH of 4.54. Rinse three times with distilled water. Incubation then occurs for 45 min at 37°C in alkalai solution (2.25g Glycine, 2.40g Calcium chloride,1.76g Sodium chloride, 1.10g Sodium hydroxide in 300mL distilled water; pH 9.4). Rinse three times in distilled water. Incubate for three minutes in 1% calcium chloride (in distilled water), followed by three rinses with distilled water. Next a three minute incubation in 2% cobalt chloride (in distilled water) occurs, followed by the standard three rinses in distilled water. Sections are finally incubated in a 1% ammonium sulfide solution (in distilled water) for three minutes followed by three rinses in distilled water. Sections are allowed to air dry and mounted using Permount and a coverslip.

Imaging and Analysis

Slides were observed under the Leica DMI 4000 B microscope and images captured to computer. A FITC filter was used to observe fluorescein from the primary antibodies, while light microscopy was used to identify fiber types. Sections stained for signaling proteins were directly compared to serial cross-sections stained for fiber type. Fluorescent staining intensity was subjectively assessed.

Statistics

An analysis of Variance (ANOVA) with repeated measures was used to analyze differences between and within groups over time for all western blot data except the percent change data, for which a one-way ANOVA between groups was used. Fischer's LSD post-hoc test for measurement of post-hoc differences where necessary. Subject characteristic data was analyzed using independent samples t-tests. Significance was set at an alpha level of $p \leq 0.05$.

CHAPTER IV: RESULTS

Subject Strength and Work Volume

All subjects successfully completed the initial visit and the experimental session. Exercise performance was measured by the total and relative exercise volume of each group. Total volume was determined by the multiplying the total amount of weight lifted by the number of repetitions for each work set (not warm-up sets) in the experimental session. Relative volume normalized the total volume lifted by individual fat-free mass (kg) (Data shown in Table 4.1). Although the older subjects generally demonstrated reduced strength, no significant differences existed between groups for any measure

Table 4.1. Assessment of Ten-repetition Maximum Strength and Workout Volume Between Young and Old Adults

	Young Adults (n=6)	Old Adults (n=6)
Estimated 10 RM (kg)	97 ± 14	83 ± 9.6
Total volume (kg resistance x repetitions)	3510 ± 512	2925 ± 589
Relative volume (kg resistance/kg FFM)	59 ± 4.8	47 ± 7.7

No significant differences were found between groups on the measure of estimated 10 repetition maximal strength. Total volume (kg resistance) was calculated to assess the absolute amount of weight lifted during the working sets by each subject with the equation: Total volume = [(resistance $_{set\ 1}$(kg) x repetitions $_{set\ 1}$) +(resistance $_{set\ 2}$(kg) x repetitions $_{set\ 2}$) + (resistance $_{set\ 3}$(kg) x repetitions $_{set\ 3}$)]. Total volume was normalized to fat free mass in order to show the relative amount of weight lifted: relative volume = total volume (kg resistance) / fat free mass (kg). All data are presented as means $\pm$ SEMs.

Western Blotting Analysis

The aim of this study was to determine the response of AMPK, ACC, and eEF2 phosphorylation in the two hours following an acute resistance exercise bout in old and young men and women. Western Blotting analyses were performed to examine differences between groups and between time points.

AMPK

For the n of 4/group, no differences in AMPK phosphorylation at Thr^{172} (Figure 4.1), total AMPK (Figure 4.2), or the phospho/total AMPK ratio (Figure 4.3) existed between age groups, at any time point. A p-value of 0.14 existed for the main effect of age in phospho/total AMPK ratio (Figure 4.3). Furthermore, there was no effect of resistance exercise on AMPK phosphorylation. No significant effect on the percent change in phospho-AMPK at Thr^{172} (Figure 4.4) or the phospho/total AMPK ratio (Figure 4.5) compared to Pre-Ex values existed for either group.

Figure 4.1. Mean $\pm$ SEM phospho-AMPK at Thr[172] in young and old subjects and the representative western blots. Pre-Ex is the pre-exercise muscle biopsy, 0P is the immediately post-exercise muscle biopsy, 1P is the one hour post-exercise muscle biopsy, 2P is the two hour post-exercise biopsy. Phospho-AMPK was not significantly different between age groups or from Pre-Ex at any time point. Significance is set at $p \leq 0.05$.

Figure 4.2. Mean $\pm$ SEM total AMPK in young and old subjects and the representative western blots. Pre-Ex is the pre-exercise muscle biopsy, 0P is the immediately post-exercise muscle biopsy, 1P is the one hour post-exercise muscle biopsy, 2P is the two hour post-exercise biopsy. Total AMPK was not significantly different between age groups or from Pre-Ex at any time point. Significance is set at p ≤ 0.05.

Figure 4.3. Mean ± SEM phospho/total AMPK ratio in young and old subjects. Pre-Ex is the pre-exercise muscle biopsy, 0P is the immediately post-exercise muscle biopsy, 1P is the one hour post-exercise muscle biopsy, 2P is the two hour post-exercise biopsy. Phospho/Total AMPK was not significantly different between age groups (P=0.14 for main effect) or from Pre-Ex at any time point. Significance is set at p ≤ 0.05.
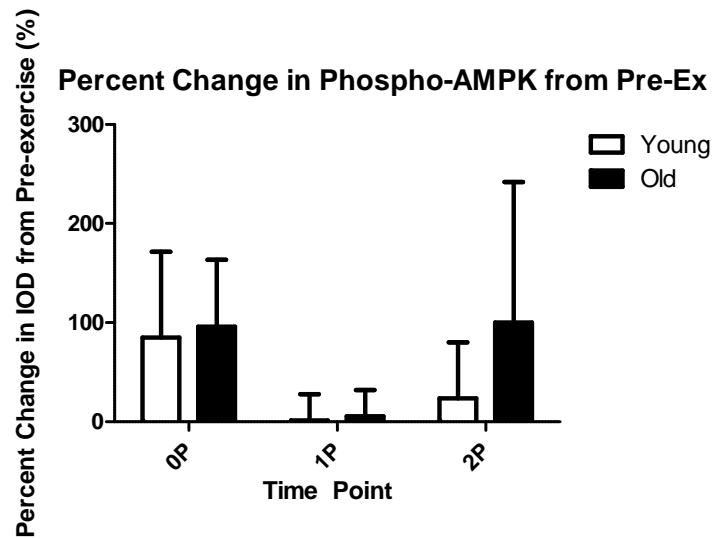
Figure 4.4. Mean $\pm$ SEM for percent change for phospho-AMPK at Thr[172] in young and old subjects at all time points from pre-ex. Pre-Ex is the pre-exercise muscle biopsy, 0P is the immediately post-exercise muscle biopsy, 1P is the one hour post-exercise muscle biopsy, 2P is the two hour post-exercise biopsy. No significant differences in percent change for phospho-AMPK exist between age groups or from pre-exercise at any time point. Significance is set at $p \leq 0.05$.
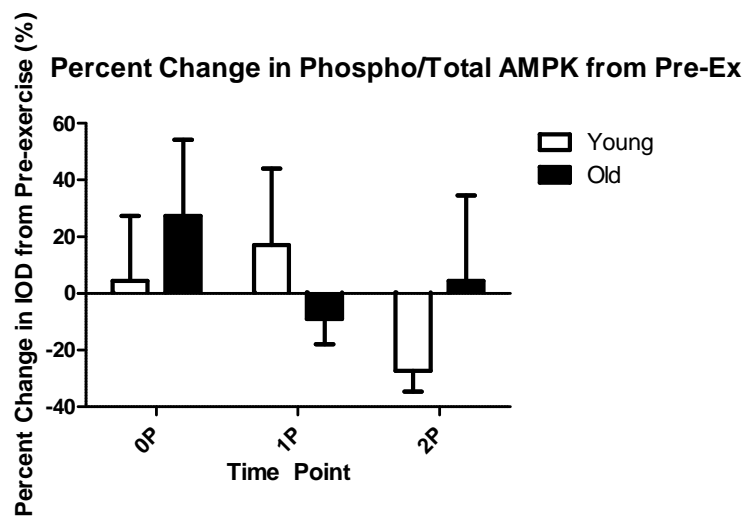
Figure 4.5. Mean ± SEM for percent change of phospho/total AMPK in young and old subjects from pre-exercise values. Pre-Ex is the pre-exercise muscle biopsy, 0P is the immediately post-exercise muscle biopsy, 1P is the one hour post-exercise muscle biopsy, 2P is the two hour post-exercise biopsy. No significant differences exist between age groups for phospho/total AMPK from Pre-Ex at any time point. Significance is set at $p \leq 0.05$.

ACC

Acetyl CoA carboxylase (ACC) showed significantly increased phosphorylation status at Ser^{79} immediately post exercise (0P) and at one hour poster exercise (1P) (Graph 4.6). However, no significant difference existed between age groups or time points for Total ACC (Graph 4.7). The phospho/total ACC ratio was significantly elevated immediately post resistance exercise (Graph 4.8). The percent change in phosphor-ACC at Ser^{79} (Graph 4.9) and the phospho/total ACC ratio (Graph 4.10) from Pre-Ex were significantly elevated in old compared to young subjects, however no differences existed between time points.
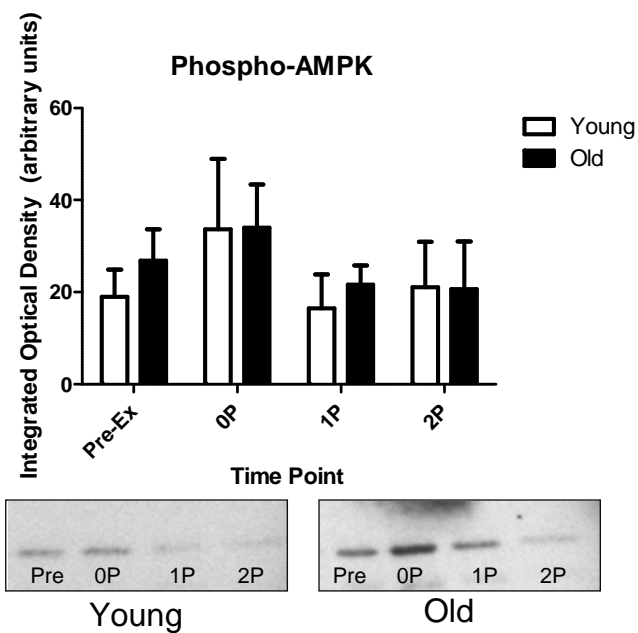
Figure 4.6. Mean $\pm$ SEM for phospho-ACC at Ser[79] in young and old subjects and the representative western blots. Pre-Ex is the pre-exercise muscle biopsy, 0P is the immediately post-exercise muscle biopsy, 1P is the one hour post-exercise muscle biopsy, 2P is the two hour post-exercise biopsy. * = significantly different than the Pre-Ex time point. No difference existed between age groups. However, phospho-ACC was significantly higher from Pre-Ex at 0P and 1P. Significance is set at $p \leq 0.05$.
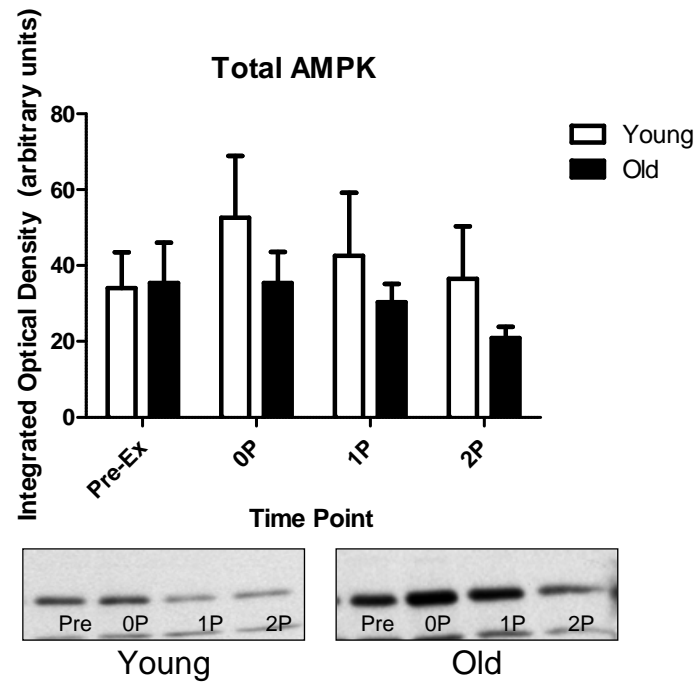
Figure 4.7. Mean ± SEM for total ACC in young and old subjects and the representative western blots. Pre-Ex is the pre-exercise muscle biopsy, 0P is the immediately post-exercise muscle biopsy, 1P is the one hour post-exercise muscle biopsy, 2P is the two hour post-exercise biopsy. No significant differences exist for total ACC between age groups or from Pre-Ex at any time point. Significance is set at $p \leq 0.05$.
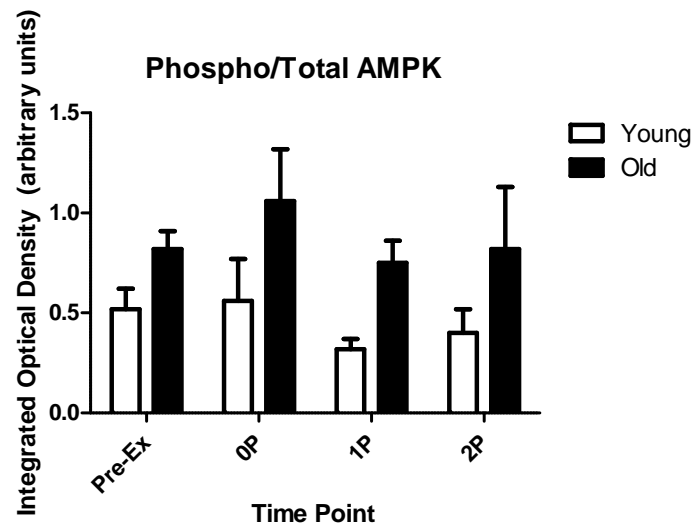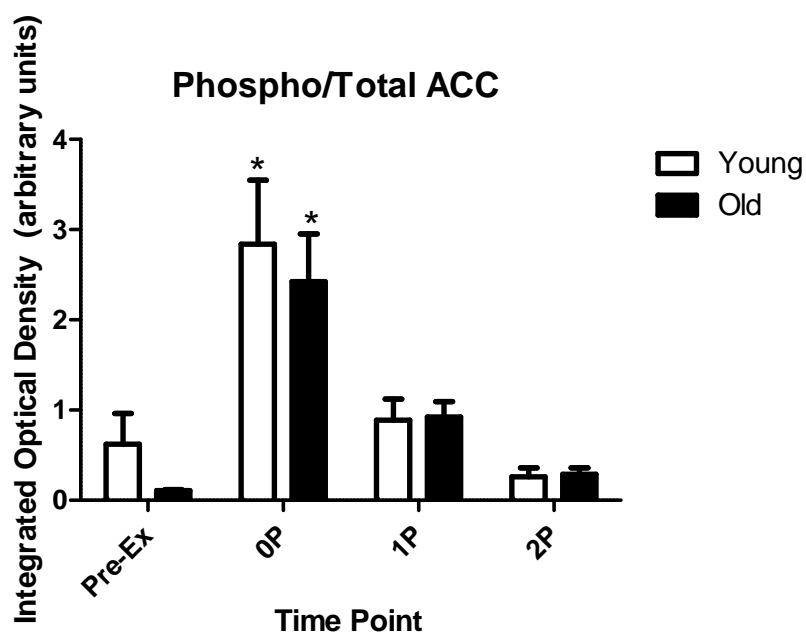
Figure 4.8. Mean ± SEM for the phospho/total ACC ratio in young and old. Pre-Ex is the pre-exercise muscle biopsy, 0P is the immediately post-exercise muscle biopsy, 1P is the one hour post-exercise muscle biopsy, 2P is the two hour post-exercise biopsy. * = significantly different than the Pre-Ex time point. The phospho/total ACC ratio was not different between age groups, however the phospho/total ACC ratio was significantly higher versus Pre-Ex in both Y and O subjects. Significance is set at $p \leq 0.05$.
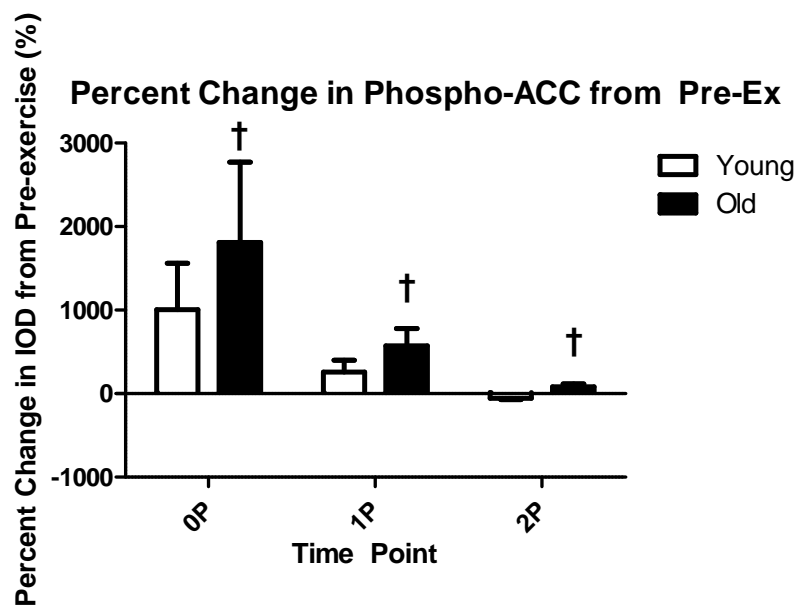
Figure 4.9. Mean $\pm$ SEM for the percent change in phospho-ACC at Ser[79] in young and old subjects from pre-ex. Pre-Ex is the pre-exercise muscle biopsy, 0P is the immediately post-exercise muscle biopsy, 1P is the one hour post-exercise muscle biopsy, 2P is the two hour post-exercise biopsy. † = significantly different between age groups. Old subjects had a significantly higher percent change in phospho-ACC versus Pre-Ex at all time points. Significance is set at $p \leq 0.05$.

eEF2

eEF2 phosphorylation at Thr[56] was not significantly different between age groups, however phospho-eEF2 was significantly higher 0P resistance exercise and significantly lower 1-2 hours post resistance exercise compared to Pre-Ex (Figure 4.11). Total eEF2 was significantly higher from Pre-Ex 1-2 hours post resistance exercise (Figure 4.12). Similar to phospho-eEF2, no significant differences existed between age groups in phospho/total eEF2 ratio, although the phospho/total eEF2 ratio was significantly higher than Pre-Ex at 0P and was significantly lower than Pre-Ex 1-2 hours post resistance exercise in both age groups (Figure 4.13). No significant difference existed between age group or time point comparing the percent change in phospho eEF2 at Thr[56] to Pre-Ex (Figure 4.14) or the percent change in phospho/total eEF2 to Pre-Ex (Figure 4.15).
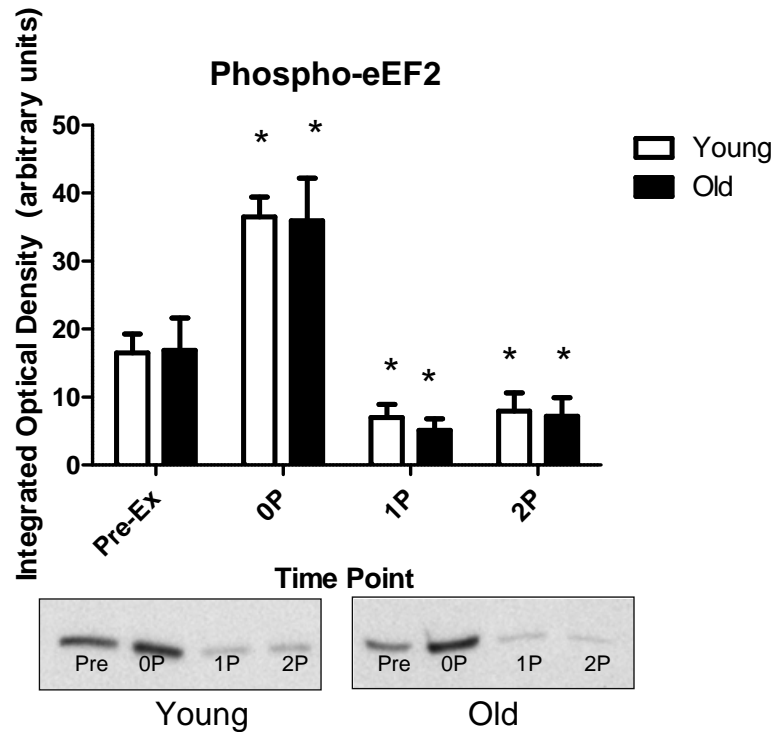
Figure 4.11. Mean $\pm$ SEM for phospho-eEF2 at Thr^{56} in young and old subjects and the representative western blots. Pre-Ex is the pre-exercise muscle biopsy, 0P is the immediately post-exercise muscle biopsy, 1P is the one hour post-exercise muscle biopsy, 2P is the two hour post-exercise biopsy. * = significantly different than the Pre-Ex time point. Phospho-eEF2 was significantly elevated at 0P and significantly lower at 1P and 2P compared to Pre-Ex. Significance is set at $p \leq 0.05$.
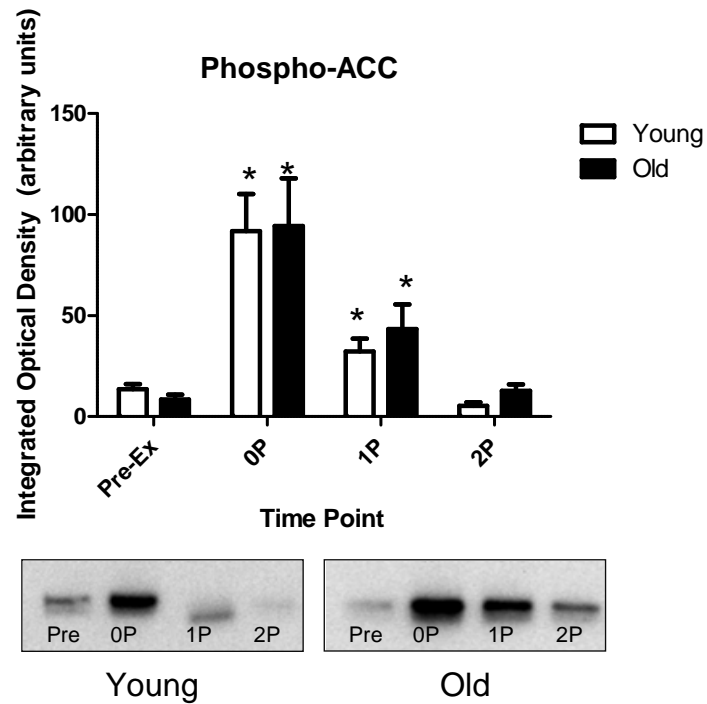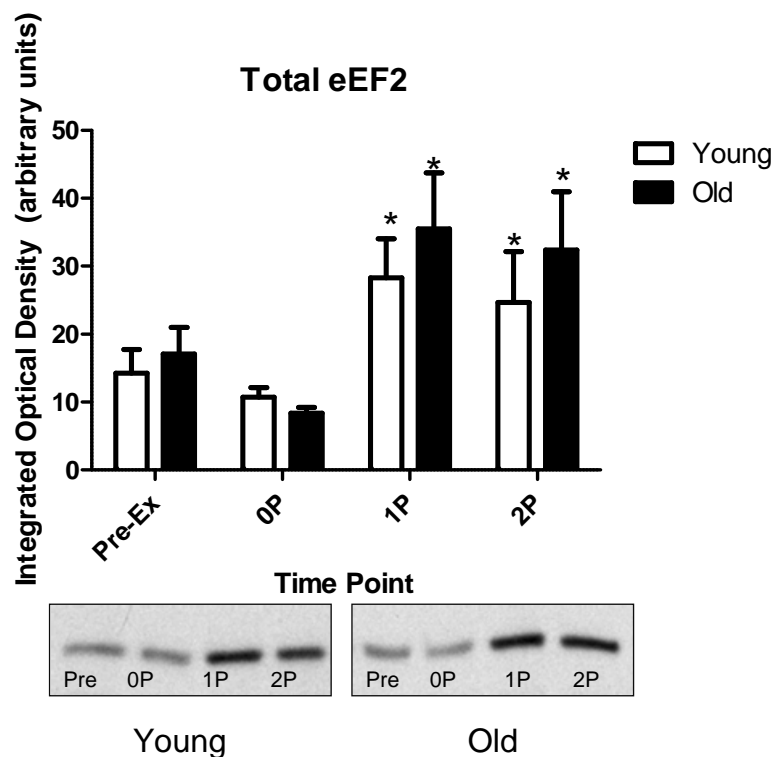
Figure 4.12. Mean $\pm$ SEM for total eEF2 in young and old subjects and the representative western blots. Pre-Ex is the pre-exercise muscle biopsy, 0P is the immediately post-exercise muscle biopsy, 1P is the one hour post-exercise muscle biopsy, 2P is the two hour post-exercise biopsy. * = significantly different than the Pre-Ex time point. Total eEF2 was significantly elevated at 1P and 2P compared to Pre-Ex. Significance is set at $p \leq 0.05$.

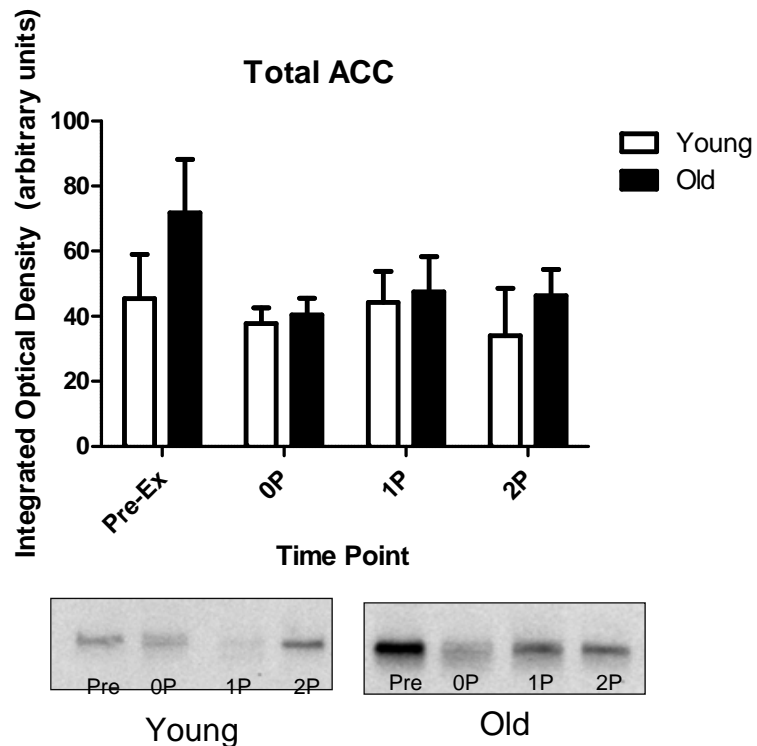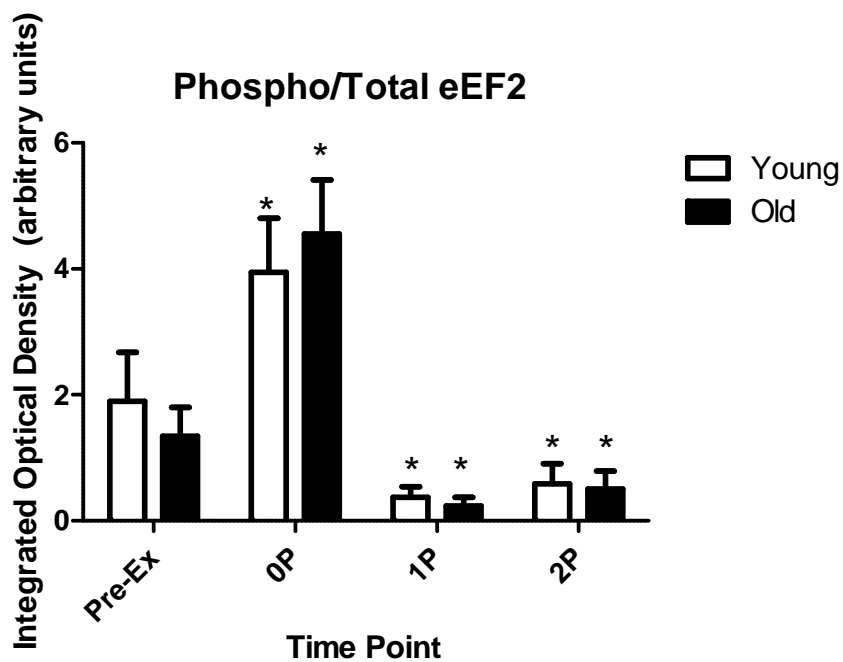Figure 4.13. Mean ± SEM for the phospho/total eEF2 ratio in young and old subjects and the representative western blots. Pre-Ex is the pre-exercise muscle biopsy, 0P is the immediately post-exercise muscle biopsy, 1P is the one hour post-exercise muscle biopsy, 2P is the two hour post-exercise biopsy. * = significantly different than the Pre-Ex time point. The phospho/total eEF2 ratio was significantly elevated at 0P and 1P compared to Pre-Ex. Significance is set at $p \leq 0.05$.
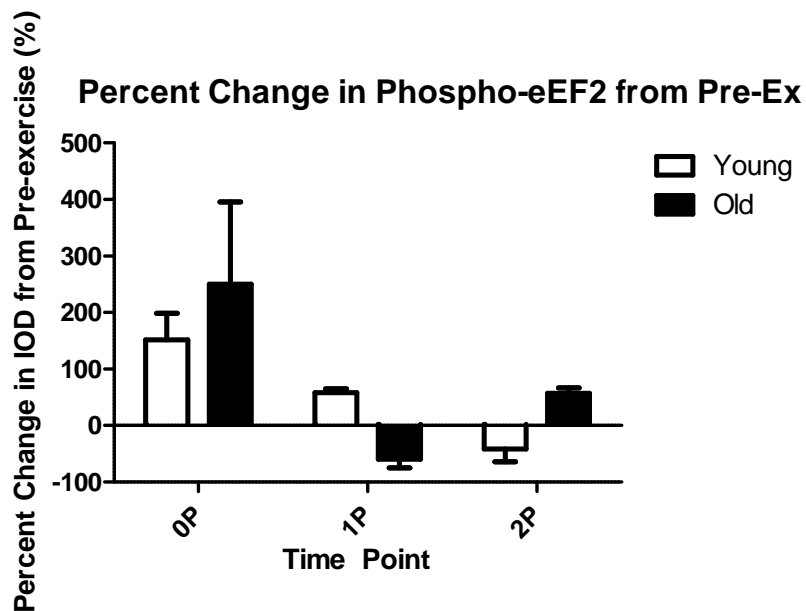
Figure 4.14. Mean $\pm$ SEM for the percent change in phospho-eEF2 at Thr[56] from pre-ex in young and old subjects. Pre-ex is the pre-exercise muscle biopsy, 0P is the immediately post-exercise muscle biopsy, 1P is the one hour post-exercise muscle biopsy, 2P is the two hour post-exercise biopsy. † = significantly different between age groups. No significant differences exist between age groups for the percent change in phospho-eEF2. Significance is set at $p \leq 0.05$.

Percent Change in Phospho/Total eEF2 from Pre-Ex
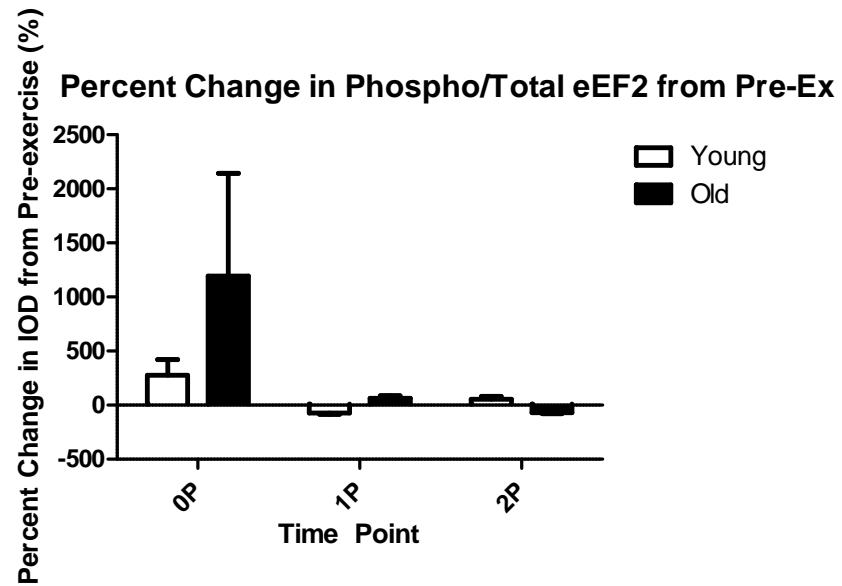
Figure 4.15. Mean ± SEM for percent change of phospho/total eEF2 from pre-ex in young and old subjects. Pre-ex is the pre-exercise muscle biopsy, 0P is the immediately post-exercise muscle biopsy, 1P is the one hour post-exercise muscle biopsy, 2P is the two hour post-exercise biopsy. † = significantly different between age groups. No significant differences exist between age groups for the percent change in Phospho/Total eEF2. Significance is set at $p \leq 0.05$.

CHAPTER V: DISCUSSION

The aim of this study was to determine whether AMPK and eEF2 phosphorylation was higher in skeletal muscle of old versus young humans after an acute resistance exercise bout. We hypothesized that AMPK phosphorylation, AMPK activity, and eEF2 phosphorylation would be greater in the vastus lateralis muscles of old compared to young men and women within the two hours after an acute bout of resisted leg extensions. Although AMPK phosphorylation was not significantly increased after resistance exercise in either group, AMPK activity, as measured by phosphorylation of the downstream target Acetyl CoA Carboxylase (ACC), was shown to be significantly increased immediately post (0P) and one hour post (1P) resistance exercise in both young and old subjects. While neither AMPK phosphorylation nor ACC phosphorylation were significantly different between age groups, the percent increase in phosphorylation of ACC was significantly higher in old versus young subjects 0P, 1P, and two hours following (2P) a resistance exercise bout. eEF2 phosphorylation was significantly higher at 0P and significantly lower at 1P and 2P as compared to before exercise; however there was no difference between age groups.

The current results for AMPK phosphorylation do not agree with previous research by Drummond et al. (Drummond, Dreyer et al., 2008). The previous study showed significantly elevated phosphorylation of AMPK in old compared to young men at 1 and 3 hours following a resistance exercise bout. Several differences between studies may have contributed to this difference.

First, Drummond et al. looked at only men, where as our study included both men and women. However, we observed no differences between genders. In fact women have been shown to have a similar muscle fractional synthesis rate as men, at least under basal conditions after normalizing to lean mass (Fujita, Rasmussen, Bell, Cadenas, & Volpi, 2007), and the menstrual cycle has been shown to have little effect on skeletal muscle protein synthesis [as reviewed (Burd, Tang, Moore, & Phillips, 2009)]. Older women, however, have been shown to have a decreased muscle protein synthesis response to resistance exercise compared to older men [as reviewed (Burd, Tang, Moore, & Phillips, 2009)]. Protein synthesis is an energy expensive process, therefore the AMP:ATP ratio may be decreased in old women because of decreased protein synthesis. Since the current study used both men and women, the protein synthesis response may have been slightly blunted in the older group following resistance exercise, thereby altering the phosphorylation of AMPK.

Drummond et al. also used 8 working sets of leg extension resistance exercise compared to 3 sets used in this study, and the increased workload may have elicited a higher AMPK response to resistance exercise. Subjects ingested an essential amino acid and carbohydrate supplement after the first hour of recovery post resistance exercise in the Drummond et al. study, however, the current study used fasted subjects. The use of non-fasted subjects in the Drummond et al. study may have played a role in affecting the AMPK response to exercise since AMPK is partially regulated by energy stores within the body (Winder & Hardie, 1999). Finally the AMPK data from our results consisted of only 4 subjects per group, and the difficulty in obtaining data from the remaining subjects

may have contributed to a low power in the statistical tests as there was a nonsignificant elevation in the older compared to younger subjects for the phospho/total AMPK ratio (P= 0.14).

ACC is directly phosphorylated by AMPK at Ser[79] (Park et al., 2002) and used as a marker of in vivo AMPK activity (Hawley et al., 1996). ACC and AMPK have shown a similar phosphorylation response to one week overload conditions in rats (Thomson & Gordon, 2005); however ACC phosphorylation does not always reflect AMPK phosphorylation because AMP can allosterically activate AMPK independent of phosphorylation (Winder & Hardie, 1999). For instance, Thomson et al. saw a larger increase in ACC phosphorylation compared to AMPK phosphorylation in response to a high frequency exercise stimulus and AICAR (Thomson et al., 2009). Dreyer et al. showed an increase in AMPK activity in response to resistance exercise in young men (Dreyer et al., 2008), however another study in the same research group failed to show an increase in AMPK phosphorylation in young men (Drummond & Rasmussen, 2008).

Dreyer et al. (Dreyer et al., 2006) showed elevated fractional synthesis rate immediately after resistance exercise followed by an increase in synthesis at one and two hours post resistance exercise, which showed an inverse relationship with eEF2 phosphorylation. The current results for eEF2 phosphorylation were similar to those seen by Dreyer et al. Although no differences occurred among age groups, total and phospho eEF2 were significantly higher from Pre-Ex values at 0P and significantly lower at 1P and 2P following an acute bout of resistance exercise. Drummond et al. showed a

significant decrease in eEF2 phosphorylation from baseline values three hours after a resistance exercise bout (Drummond, Dreyer et al., 2008).

Dreyer et al. showed that after a resistance exercise bout AMPK activity increased inversely with both fractional synthesis rate and eEF2 (Dreyer et al., 2006), however the change in AMPK activity appears to be smaller compared to eEF2. In the current study, AMPK and eEF2 phosphorylation respond similarly at all time points following a resistance exercise bout regardless of age.

The response seen by phosphorylation of eEF2 is important when looking at the protein synthesis response within the body after resistance exercise. This phenomenon shows a need for the body to decrease protein synthesis during resistance exercise and increase protein synthesis during the recovery, which accounts for an energy disturbance due to resistance exercise. The significant increases in total eEF2 at 1P and 2P resistance exercise, probably occurs because eEF2 is encoded in the 5'TOP mRNAs, which are specifically targeted for translation by p70^{s6k}. Similarly, Thomson and Gordon showed increased total eEF2 with chronic overload in young rats (Thomson & Gordon, 2006). This increase in total eEF2 allows for an increase in translational protein signaling. The combination of increased total eEF2 and decreased phospho eEF2 are two mechanisms that favor the upregulation of translation elongation.

There were no strength differences between young and old subjects. Values for 10-RM, total volume, and relative volume, were all insignificant. Drummond et al. (Drummond, Miyazaki et al., 2008) showed significantly greater absolute strength in young compared to old subjects, while total work was significantly higher in old

compared to young subjects. All values were insignificant when compared to lean body mass, similar to the current data.

The lack of age differences in the present study could be caused by a number of various factors. For example, the older subjects in the current study may have been more healthy and unrepresentative of the normal population. A lack of differences between strength measures could confirm this health effect or show a lack of effort in the young group. Secondly, work done in the fasted state, since fasted conditions are less than optimal for the normal protein synthesis response to resistance exercise conditions, may have dampened the protein synthesis response (Burd, Tang, Moore, & Phillips, 2009).

Fast twitch muscle fibers appear to be affected most by the aging process with significant decreases in cross sectional area of the fast-twitch fibers (Hortobagyi et al., 1995). No changes are typically observed in Slow-twitch (ST) muscle fiber area. Additionally, it has been shown that elderly muscle contains a lower percentage of FT fibers compared to muscle of young adults, [as reviewed (Andersen, 2003)] corresponding with the steady decline of FT fiber number with age, as ST fibers show little change (Kamel, 2003). In the current study homogenates were used, which may mask individual fiber type differences; especially because FT muscle fiber atrophy would lead to a decreased effect in old adults due to less representation in the homogenate. Immunohistochemistry was reviewed in order to account for fiber type differences within the muscle. Although quantification and analysis was incomplete for the present study, results for one subject seemed promising, showing increased AMPK phosphorylation in response to exercise as determined by subjective analysis (Figure 5.1).
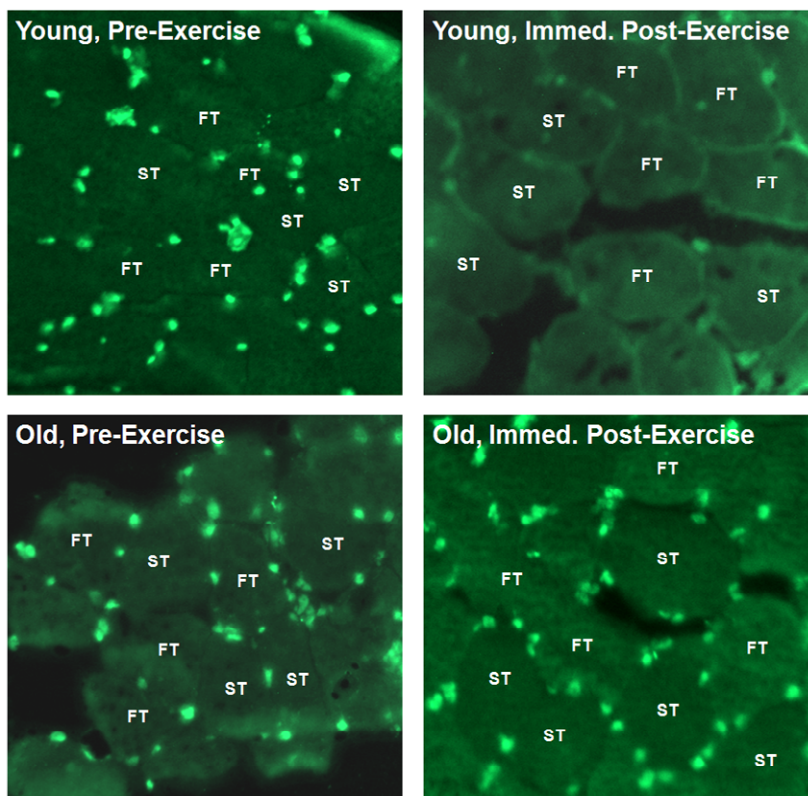
Figure 5.1. Fluorescent immunohistochemistry of AMPK phosphorylation at Thr^{172} Pre-exercise and 0 post resistance exercise. Fast-twitch (FT) muscle fibers showed a higher phosphorylation status as determined by objective measures than slow-twitch muscle fibers in old compared to young subjects. No differences occured pre-exercise.

Although no differences were seen between young and old subjects in AMPK phosphorylation and eEF2 phosphorylation, there is still potential for future investigations to determine differences between age groups. Studying effects of nutrition on AMPK further may be beneficial, as differences in AMPK phosphorylation have been seen between age groups after ingestion of an essential amino acid and carbohydrate supplement (Drummond, Dreyer et al., 2008). Furthermore, differentiation between fiber types may reveal differences in age groups as compared to the muscle homogenate used in western blotting.

In summary, AMPK phosphorylation status was unaffected by age group or by resistance exercise compared to pre-exercise values. AMPK activity, measured by ACC, after a bout of resistance exercise was increased in the first hour after a bout of resistance exercise regardless of age. Furthermore, the percent change of phospho-ACC and phospho/total ACC from Pre-Ex was significantly increased in old compared to young individuals. Increased AMPK inhibition of eEF2 activation within 2hrs post-exercise may not be responsible for the diminished hypertrophy response to resistance exercise with age. Additionally future studies are needed to determine if the AMPK signaling response may be different beyond the 2 hour time period observed. Older subjects may need to consist of more sarcopenic adults as well. Finally differences between fiber types following an acute bout of resistance exercise in young and old men and women may arise.

CHAPTER VI: REFERENCES

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APPENDIX A: ECU INSTITUTIONAL REVIEW BOARD APPROVAL DOCUMENT

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Title of research: Age-related Changes in Skeletal Muscle Signaling after Acute Heavy Resistance Exercise

Principal Investigator: Scott E. Gordon, Ph.D.

Sponsor:

Fund number for IRB fee collection (applies to all for-profit, private industry or pharmaceutical company sponsored project revisions requiring review by the convened UMCIRB committee):

Fund	Organization	Account	Program	Activity (optional)
		73059		

Version of the most currently approved protocol: 11/17/08
Version of the most currently approved consent document: 12/2/08

CHECK ALL INSTITUTIONS OR SITES WHERE THIS RESEARCH STUDY WILL BE CONDUCTED:

- [x] East Carolina University
- [ ] Pitt County Memorial Hospital, Inc
- [ ] Heritage Hospital
- [ ] Other
- [ ] Beaufort County Hospital
- [ ] Carteret General Hospital
- [ ] Boice-Willis Clinic

The following items are being submitted for review and approval:
- [ ] Protocol: version or date
- [ ] Consent: version or date
- [x] Additional material: Personal History Form   **version or date** 2/17/09

Complete the following:
1. Level of IRB review required by sponsor: [ ] full [x] expedited
2. Revision effects on risk analysis: [ ] increased [x] no change [ ] decreased
3. Provide an explanation if there has been a greater than 60 day delay in the submission of this revision to the UMCIRB.
4. Does this revision add any procedures, tests or medications? [ ] yes [x] no   If yes, describe the additional information:
5. Have participants been locally enrolled in this research study? [x] yes [ ] no
6. Will the revision require previously enrolled participants to sign a new consent document? [ ] yes [x] no

Briefly describe and provide a rationale for this revision

Questions concerning menstrual history and status were added to the medical history questionnaire to be able to account for menstrual status of our female subjects.

Signature	Scott E. Gordon	2/17/09
Principal Investigator Signature	Print	Date
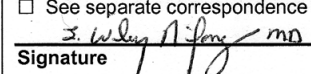
Box for Office Use Only

The above revision has been reviewed by:
- [ ] Full committee review on _____
- [x] Expedited review on 2/19/2009

The following action has been taken:
- [x] Approval for period of 2/19/09 to 12/9/09
- [x] Approval by expedited review according to category 45CFR46.110
- [ ] See separate correspondence for further required action.

S. Wiley N-fong MD		2/19/09
Signature	Print	Date

APPENDIX B: INFORMED CONSENT DOCUMENT

Principal Investigator: Scott E. Gordon, Ph.D.
Institution: Human Performance Laboratory
Address: 363 Ward Sports Medicine Building
Telephone #: (252) 737-2879

> This consent form may contain words that you do not understand. You should ask the study doctor or the study coordinator to explain any words or information in this consent form that you do not understand.

<u>INTRODUCTION</u>

You have been asked to participate in a research study being conducted by <u>Scott E. Gordon, Ph.D.</u> and his associates. This research is designed to determine the response of several molecules in muscle after one resistance exercise (strength training) session, and if this response changes with age. All of these molecules and cells are involved with muscle hypertrophy (growth).

We will study 10 younger (18-45 years) and 10 older inactive adults (45-85 years), at rest and during and after a resistance exercise session for the legs. Inactive is defined as not having participated in any regular form of exercise for the past six months (less than 30 minutes per day, one day per week). Studies will take place in the Human Performance Laboratory and Brody School of Medicine at East Carolina University.

<u>PLAN AND PROCEDURES</u>

Prior to any testing, I will report to the Human Performance Laboratory Fitness, Instruction, Testing, and Training (FITT) Building to read and sign this Informed Consent for research as well as fill out a medical history questionnaire and 3-day food record. I will be allowed to complete this process on the day of my first visit. On this day, I will undergo determination of my height and body weight, and percent body fat. I will also undergo strength testing and familiarization with the resistance exercise test. One to two weeks later, I will report to the Brody school of Medicine, Room 3S-08 in a fasted state for the resistance exercise testing session, four blood samples from a forearm vein, and 4 biopsies of the thigh muscles.

The following section is an outline of the experimental visits and the procedures to be accomplished on each visit. Note that more detailed descriptions of each procedure immediately follow this section. There will be 5 visits for a total of approximately 8 hours of total participation time spread over approximately 15-20 days:

First Visit (Human Performance Lab FITT Building) (1.5 hours):
1.) Thorough interview in person for informed consent, health history questionnaire, and 3-day food record.
2.) Determination of height, weight, and skinfold thickness (fat pinch) for percent body fat.
3.) Determination of the maximum weight I can lift 10 times (10 repetition maximum, also called a 10 RM) for the seated leg extension exercise.

Second Visit (Brody School of Medicine, Room 3S-08) (7-14 days after initial visit) (3.5 hours):
1.) I will report to the Brody SOM, Room 3S-08, in the morning after an overnight fast (not having eaten after midnight the night before).
2.) Four small blood samples will be obtained during this visit from a forearm vein (before, and after exercise).
3.) Four thigh muscle biopsies (tissue samples) will be performed during this session, two on each leg.
4.) A resistance exercise (strength training leg extension) bout of approximately 15 minutes focused on the thigh muscle group will be performed. The bout will consist of 6 sets of leg extension exercise.

Detailed Description of the Procedures to be Used During this Study:

- Body composition screening. My height and weight will be measured on my first visit. My body fat will be estimated by measuring skinfold thicknesses (fat pinch) with a skinfold caliper at four sites: biceps, triceps, back, and waist. I may feel a slight pinch or squeeze from the caliper at the skinfold sites, but no known risks are associated with this procedure.

- Strength testing. An exercise test to determine my strength levels and familiarize me with the resistance exercise protocol will be performed during my first visit (after the informed consent). As part of my familiarization and subject characterization, I will be assessed for maximal strength by 10-repetition maximum (10-RM) testing. This will entail determining the maximum amount of weight that I can lift in ten repetitions for the leg extension exercise. This procedure will consist of me initially lifting lighter weights and progressing to the heaviest weight that I can lift. An adequate amount of rest will be provided between repetition attempts. All exercises will be performed on Cybex weight machines. This session will also serve as a familiarization session to make me comfortable with the resistance exercise to be performed during the experimental session. I will be examined for proper exercise technique during this session and instructions or modifications will be provided if necessary. During this session, my tolerance for the heavy resistance exercise protocol will also be assessed.

- Leg resistance exercise workout. During my second visit to the laboratory, I will perform a 15-minute resistance exercise bout focused on the quadriceps (thigh) muscle group. The bout will consist of 6 sets of leg extension exercise. The first 3 sets will be warm-up sets performed at 50% (8 repetitions), 70% (6 repetitions), and 90% (4 repetitions) of my previously determined 10-RM weight. The fourth through sixth sets will be performed at 100% of the 10 RM weight and will be performed until I am no longer able to perform them on my own (approximately 10 repetitions). I will rest for 90 seconds between all sets

- Fasting blood draws. I will not have anything to eat 12 hours prior to my second visit to the lab so that blood can be drawn from my forearm vein by a needle. During the second visit to the lab, blood will be drawn before, and after the resistance exercise workout described above. Four total blood samples of 5 milliliters each will be obtained during this study. The total amount of blood obtained will be 20 milliliters, which is approximately 1/25 of a pint.

- Muscle Biopsies. I will undergo four muscle biopsies (tissue samples) to determine the levels of several molecules in muscle after one resistance exercise (strength training) bout. These biopsies will be obtained immediately before, immediately after, and 1 and 2 hours after the resistance exercise bout in visit # 2. For this procedure, I will have a small amount of anesthesia (3 cc of 1% Lidocaine) injected in a ½ inch area under the skin of my thigh. A small (1/4 inch) incision will then be made through the skin, fat and fibrous tissue that lies over the muscle. A biopsy needle (about ½ the width of a pencil) will then be inserted ½ to 1 inch into the muscle. A small piece of muscle (½ the size of an eraser on the end of a pencil) will then be clipped out with the biopsy needle. The needle will be withdrawn and the muscle sample immediately preserved by freezing. Dr. Robert Hickner, Ph.D. or Dr. Timothy Gavin, Ph.D. will perform the muscle biopsies. These investigators have performed a total of over 500 muscle biopsies.

POTENTIAL RISKS AND DISCOMFORTS

There are certain risks and discomforts that may be associated with this research,

including those listed below.

- The general performance of muscular exercise and physical effort can entail the potential hazards of injury from overexertion and/or accident. The possibility of cardiopulmonary (heart and lung) overexertion is slight. It will be minimized by screening, selection, and monitoring procedures which are designed to anticipate and exclude the rare individual for whom exercise might be harmful. It is

questionable whether it is possible to overexert the heart by voluntary physical effort unless there is some underlying disease. Nevertheless, there are a number of disorders, some of which can readily escape clinical detection, where strenuous exercise may be potentially hazardous or may cause disability. Some of these, such as aneurysms (blood vessel ruptures) in the brain, solitary pulmonary cysts (small sacs of fluid in the lung), or alveolar blebs (small lung lesions), are rare and not readily diagnosed in the absence of symptoms. For these disorders, a history of tolerance to prior physical effort must suffice. For other, more common conditions such as ischemic heart disease (low blood and oxygen flow to the heart), several risk factors can be identified through the preliminary medical history and physician screening process.

- The risks specifically associated with resistance exercise are very low, and this study will be planned to avoid injury to the musculoskeletal (muscle and bone) system. Possible risks include the possibility of strains or pulls of the involved muscles, delayed muscle soreness 24 to 48 hours after exercise, muscle spasms (cramping), and, in extremely rare instances, muscle tears. Such risks are very low. Dizziness and fainting may also occur infrequently. I understand that every effort will be made by the researchers to make this investigation safe for my participation through proper instruction of the techniques and proper warm-up prior to exercise and testing. Furthermore, risks will be reduced by close supervision by experienced personnel to ensure that I utilize proper form.

- The total amount of blood drawn (1/25 of a pint) is negligible. There is an extremely small risk of local hematoma (bruising) or infection associated with insertion of venipuncture needles. In obtaining blood samples from a vein with a needle, the risks to me are of local discomfort, syncope (faintness), and hematomas (bruising). Thrombosis (blood clot in the vein), embolism (a blood clot that has come loose and may lodge itself in an artery), and infections are potential risks but are of very rare occurrence. Risks will be reduced or eliminated by having investigators who are trained and proficient in phlebotomy (puncturing veins with needles) use aseptic techniques. Furthermore, I will be in a seated position while blood is being obtained. All blood samples will be drawn in the laboratory under aseptic conditions with biohazard protection for the investigators and myself.

- Robert Hickner, Ph.D. or Dr. Timothy Gavin, Ph.D. will perform all biopsies, and Dr. Walter Pofahl, M.D. will provide medical coverage for biopsies performed in this investigation. There is a small risk of hematoma (bruising) or infection around the biopsy site, as well as muscle cramping, mild muscle tenderness and occasional bruising. The risk will be minimized by using sterile procedures and applying pressure to the biopsy site for 10 minutes, or until bleeding has stopped if longer than 10 minutes. A steri-strip (bandage) will be applied over the incision and will remain in place for at least 4 days to close the incision during healing. A pressure wrap will be placed around the biopsied thigh and will remain in place

for 8 hours following the biopsy. There is an extremely remote risk of allergic reaction to the Lidocaine anesthesia. This risk will be minimized by using subject's who have had prior exposure to Lidocaine or Novocaine anesthesia. This precaution should eliminate this risk.

- The procedures and circumstances encompassed by this protocol provide for a high degree of safety. Every attempt will be made by the investigators to minimize any risks of this study to me. This includes familiarization, technique instruction and practice, supervision by experienced personnel, screening, and individualized testing and monitoring. The investigators will employ a close interaction with the physician in their clinical unit during this study. My safety will be enhanced in this study with individualized supervision during all laboratory visits. I will be asked to immediately alert a member of the research team if I have any injury or health problem. These factors should dramatically contribute to a reduction, if not an elimination, of any potential risks associated with this study.

- **To my knowledge, I am not allergic to "caine-type" anesthetics. For example, I have not had an allergic reaction to an injection at the dentist's office. To my knowledge, I do not possess any condition which would result in excessive bleeding and I do not have known heart disease, i.e., had a heart attack.**

- I am aware that there are unforeseen risks involved with this and all research studies.

POTENTIAL BENEFITS

There are potential benefits to subjects and to society. The results of this study will help to determine the response of several muscle growth-related molecules and cell types in muscle after one resistance exercise (strength training) session, and if this response changes with age. The benefits of this study far outweigh the risks.

I will gain information about my blood sugar (glucose) and insulin levels, which may be indicators of health status due to the importance of blood glucose regulation. I will obtain information about my percent body fat and body mass index (BMI, or weight/height squared), which is also an important indicator of risk for metabolic diseases such as diabetes or heart disease. I will also gain information on my muscle fiber type (slow-twitch or fast-twitch), which is important component of athletic ability characteristics.

I will be paid a total of $200.00 compensation upon completion of the entire study.

SUBJECT PRIVACY AND CONFIDENTIALITY OF RECORDS

Only the investigators associated with this study will have access to the data obtained. The identity of the subjects will be protected by numeric coding. The data will be stored in the office of the Principal Investigator, or in a locked storage room. No identifying information will be released.

TERMINATION OF PARTICIPATION

My participation in this research study may be terminated without my consent if the investigators believe that these procedures will pose unnecessary risk to myself. I may also be terminated from participation if I do not adhere to the study protocol.

COST AND COMPENSATION

I will be paid $50.00 for my time and inconvenience for each muscle biopsy for a maximum of $200 for completion of the entire study. There are no costs to me for participation in this study.

The policy of East Carolina University does not provide for compensation or medical treatment for subjects because of physical or other injury resulting from this research activity. However, every effort will be made to make the facilities of the School of Medicine available for treatment in the event of such physical injury.

VOLUNTARY PARTICIPATION

I understand that my participation in this study is voluntary. Refusal to participate will involve no penalty or loss of benefits to which I am otherwise entitled. Furthermore, I may stop participating at any time I choose without penalty, loss of benefits, or without jeopardizing my continuing medical care at this institution.

RESEARCH PARTICIPANT AUTHORIZATION TO USE AND DISCLOSE PROTECTED HEALTH INFORMATION

The purpose of the information to be gathered for this research study is to better understand the response of several molecules in muscle after one resistance exercise (strength training) session, and if this response changes with age. The individuals who will use or disclose your identifiable health information for research purposes include Dr. Scott Gordon, Dr. Timothy Gavin, Dr. Robert Hickner, Dr. Walter Pofahl, and Mr. Bradley Harper. Individuals who will receive your identifiable health information for research purposes also include Dr. Scott Gordon, Dr. Timothy Gavin, Dr. Robert Hickner, Dr. Walter Pofahl, and Mr. Bradley Harper. The type of information accessed for this research study includes 1) general medical history (including family health history, medications, nutrition, physical activity levels and body weight history), 2) body

composition information, blood levels of insulin, glucose, and other compounds related to muscle hypertrophy and metabolism, and 3) muscle fiber type percentage as well as growth-related molecules in your thigh muscle. The information will be used and disclosed in such a way as to protect your identity as much as possible; however, confidentiality cannot be absolutely guaranteed. Someone receiving information collected under this Authorization could potentially re-disclose it, and therefore it would no longer be protected under the HIPAA privacy rules (federal rules that govern the use and disclosure of your health information). There is not an expiration date for this Authorization.

You may not participate in this study if you do not sign this Authorization form. You may revoke (withdraw) this Authorization by submitting a request in writing to Dr. Scott Gordon. However, the research team will be able to use any and all of the information collected prior to your request to withdraw your Authorization.

To authorize the use and disclosure of your health information for this study in the way that has been described in this form, please sign below and date when you signed this form. A signed copy of this Authorization will be given to you for your records.

_____

Participant's Name (print)   Signature   Date

_____

Authorized Representative Name (print)-----Relationship   Signature   Date

_____

Person Obtaining Authorization   Signature   Date

If you have questions related to the sharing of information, please call Scott Gordon at 252-737-2879. You may also telephone the University and Medical Center Institutional Review Board at 252-744-2914. In addition, if you have concerns about confidentiality and privacy rights, you may phone the Privacy Officer at Pitt County Memorial Hospital at 252-847-6545 or at East Carolina University at 252-744-2030.

PERSONS TO CONTACT WITH QUESTIONS

The investigators will be available to answer any questions concerning this research, now or in the future. I may contact the primary investigators Scott E. Gordon, Ph.D. at 252-737-2879 (weekdays) or 252-321-7655 (nights and weekends). Also, if questions arise about my rights as a research subject, I may contact the Chairman of the University and Medical Center Institutional Review Board at phone number 252-744-2914 (weekdays).

CONSENT TO PARTICIPATE

I certify that I have read all of the above, asked questions and received answers concerning areas I did not understand, and have received satisfactory answers to these questions. I willingly give my consent for participation in this research study. (A copy of this consent form will be given to the person signing as the subject or as the subject's authorized representative.)

_____

Participant's Name (Print)

_____

Signature of Participant   Date   Time

WITNESS: I confirm that the contents of this consent document were orally presented, the participant or guardian indicates all questions have been answered to his or her satisfaction, and the participant or guardian has signed the document.

_____

Witness's Name (Print)

_____

Signature of Witness   Date

PERSON ADMINISTERING CONSENT: I have conducted the consent process and orally reviewed the contents of the consent document. I believe the participant understands the research.

_____

Person Obtaining Consent (Print)

_____

Signature of Person Obtaining Consent   Date

_____

Principal Investigator's Name (Print)

_____

Signature of Principal Investigator   Date

FUTURE TESTING OF BLOOD/MUSCLE SAMPLES

Upon termination of this study, the blood and muscle samples collected for this study will be stored for up to 7 years to research scientific questions specifically related to age-related changes in molecules regulating muscle mass in response to resistance exercise. I will continue to be the owner of the samples and retain the right to have the sample material destroyed at any time during this study by contacting the study principal investigator Scott Gordon, Ph.D. at 252-737-2879. During this study, the samples will be stored with number identifiers only; however, the number identifier will be linked to a specific name and will be kept on file in the possession of the principal investigator. The linked file will be stored password protected on the Principal Investigator's computer with CD backup. No other individuals will have access to these identifying materials unless the principal investigator is required by law to provide such identifying information. Data will not be publicly available and participants will not be identified or linked to the samples in publication. If a commercial product is developed from this research project, I will not profit financially from such a product. Furthermore, there are no plans for me to profit financially from such a product.

CONSENT TO PARTICIPATE IN FUTURE TESTING OF BLOOD/MUSCLE

SAMPLES

I certify that I have read all of the above, asked questions and received answers concerning areas I did not understand, and have received satisfactory answers to these questions. I willingly give my consent for participation in this research study. (A copy of this consent form will be given to the person signing as the subject or as the subject's authorized representative.)

_____

Participant's Name (Print)

_____

Signature of Participant   Date   Time

WITNESS: I confirm that the contents of this consent document were orally presented, the participant or guardian indicates all questions have been answered to his or her satisfaction, and the participant or guardian has signed the document.

_____

Witness's Name (Print)

_____

Signature of Witness   Date

PERSON ADMINISTERING CONSENT: I have conducted the consent process and orally reviewed the contents of the consent document. I believe the participant understands the research.

_____

Person Obtaining Consent (Print)

_____

Signature of Person Obtaining Consent   Date

_____

Principal Investigator's Name (Print)

_____

Signature of Principal Investigator   Date

PERSONAL HISTORY FORM   **(version 2-17-09)**

Technician _____   Contract _____   ID _____

PLEASE PRINT AND FILL OUT COMPLETELY

1.   **Name**: _____   Date: _____
        Phone#: (home) _____   (work) _____
        Address: _____
        City: _____   State _____   Zip _____
        e-mail address (if available):_____
2.   **Employer**: _____
        Occupation: _____

3.   Date of Birth: _____ Sex**:** _____ Age: _____ Race: _____

4. **General Medical History**   Circle one
Any medical complaints presently?   (if yes, explain) ....   yes   no
_____
_____
_____

Any major illnesses in the past? (if yes, explain) .....   (date) _____   yes no
_____
_____
_____

Any hospitalization or surgery? (if yes, explain) ......   (date) _____   yes   no
_____
_____
_____

Have you ever had an EKG (electrocardiogram) ? ......   (date) _____   yes   no

Are you diabetic? ....If yes, at what age did you develop diabetes: _____   yes   no

Are you currently taking any medications? ....   yes   no

Medication   Dosage   Reason   Times taken per day
_____
_____

5. **Family History**

	Age if alive	Age of death	Cause of death
Father	_____	_____	_____
Mother	_____	_____	_____

Do you have a family history of: (Blood relatives only: give age of occurrence if applicable)   Relationship

      Age of

      occurrence

			Relationship
--High blood pressure ...... yes	no		_____
--Heart attack.....................yes	no		_____
--By-pass surgery...............yes	no		_____
--Stroke.............................yes	no		_____
--Diabetes..........................yes	no		_____
--Gout................................yes	no		_____
--Obesity............................yes	no		_____

6. **Tobacco History** (check one)

_____	None		
_____	Quit months/years ago		
_____	Cigarette		
_____	Snuff		
_____	Chewing tobacco		
_____	Pipe		

Cigarette history
_____   1-10 daily
_____   11-20 "
_____   21-30 "
_____   31-40 "
_____   more than 40

Total years of tobacco use? _____

Snuff history
_____   < 0.5 cans daily
_____   0.5-2.5 cans "
_____   > 2.5 cans   "

Chewing history
_____   < 0.5 pouches daily
_____   0.5-2.5 pouches "
_____   > 2.5 pouches   "

7**. Weight History**
What do you consider a good weight for you? _____   Weight at age 21? _____
Weight since age 21? _____   Weight one year ago? _____
Weight now? _____

8. **Cardio-Respiratory History**

Any heart disease now?.. yes no

Any heart disease in the past?.. yes no

Heart murmur?.. yes no

Occasional chest pains?.. yes no

Chest pains on exertion?... yes no

Fainting?.. yes no

Daily coughing?... yes no

Cough that produces sputum?.. . yes no

High blood pressure?.. yes no

Shortness of breath --
     at rest.. yes no
     lying down... yes no
     sleeping at night.. yes no
     after 2 flights of stairs.. yes no

9. **Muscular History**

Any muscle injuries or illnesses now?.. yes no

Any muscle injuries in the past?... yes no

Muscle pain at rest?.. yes no

Muscle pain on exertion?.. yes no

10. **Bone-Joint History**

Any bone or joint (including spinal) injuries or illnesses now?........................ yes no

Any bone or joint (including spinal) injuries or illnesses in the past?............. yes no

Ever had painful joints?... yes no

Ever had swollen joints?.. yes no

Flat feet?... yes no

11. **<u>Menstrual History</u>** (Women only)

Are you post-menopausal (e.g., not had menstrual flow for at least one year)? ...   yes no

Have you had a hysterectomy? ....   yes no

If you have had a hysterectomy, were the ovaries removed? ……………….......   yes no

If pre-menopausal: On what date did your last period start (beginning of flow)?
_____

                Are your periods regular? ....   yes no
                Approximately how many days apart are your periods?
            _____

Are you on any hormonal supplements, such as a birth control pill or
estrogen replacement therapy? ....   yes no
              If so, what? _____

12. **<u>Nutritional Survey</u>**
How many times do you usually eat per day?

     _____

What time of day do you eat your largest meal?

     _____

How many times per week do you usually eat...
    ____ Hamburger   ____ Sausage   ____ Bacon
    ____ Beef   ____ Pork   ____ Cheese
    ____ Shellfish (shrimp, oysters, scallops, clams, etc.)
    ____ Fish   ____Poultry   ____ Fried Foods
    ____ Breads   ____ Cereals   ____ Vegetables
    ____ Eggs   ____ Desserts   ____ Ice Cream
    ____ Other

How many servings per week do you usually consume?
____ Whole milk   ____ Coffee
____ Low-fat milk (2% milk fat)   ____ Tea
____ Skim milk (non-fat)   ____ Soft drinks
____ Buttermilk   ____ Other

13. **Physical Activity Survey**

a. Compared to a year ago, how much regular physical activity do you currently get?
(Check One)

        ____ much less   ____ somewhat less   ____ about the same
        ____ somewhat more   ____ much more

b. For the last three months, have you been exercising on a regular basis?............ yes no

c. What type of exercise or physical activity do you currently do or have done regularly in the past?
   (For example: walking, swimming, weight lifting, gardening, etc.)

_____
_____
_____

d. On the average, how many days per week do you exercise? _____

e. How long do you exercise each time? For how many minutes? _____

f. How hard do you exercise on a scale from 1 to 5: with 1 being easy and 5 being very hard?

      ____ 1   ____ 2   ____ 3   ____ 4   ____ 5

g. Do you ever check your heart rate (pulse) to determine how hard you are exercising?
      ____ yes   ____ no

h. What aerobic activity or activities would you prefer in a regular exercise program for yourself?

____ Walking and/or running	____ Tennis	____ Bicycling
____ Racquetball	____ Swimming	____ Basketball
____ Aerobic dance	____ Stationary cycling	____ Soccer
____ Stair climbing	____ Rowing	_____Other

14. **Alcohol History**

Do you ever drink alcoholic beverages?   Yes ____   No ____
If yes, what is your approximate intake of beverages per week?
Beer _____   Wine _____   Mixed Drinks _____

15. **Sleeping Habits**

Do you ever experience insomnia (trouble sleeping)?   Yes ____   No ____
If yes, approximately how
often?_____
How many hours of sleep do you usually average per
night?_____

16. **Education**
Please indicate the highest level of education completed.
____ Grade School   ____ Junior High   ____ High School
____ College   ____ Graduate   ____ Postgraduate
Please indicate degree earned (i.e. B.A., M.S., Ph.D.)_____

17. **Motivation or reason for participating in the testing program?**
____ General health and fitness evaluation
____ Medical evaluation prior to starting and exercise program
____ Baseline for weight loss
____ Required by supervisors or employers
____ Other _____

18. **Family Physician**
Name: _____
Address:_____
Phone: _____
Should it be necessary, may we send a copy of your results to your physician?

19. **Insurance** I, _____understand that this evaluation is not
reimbursable under Medicare and the cost of the evaluation must be paid by me.

Signature: _____
Date: _____

APPENDIX D: DIETARY LOG FOR A TYPICAL DAY

Date _____   **Subject ID #** _____

Dietary Log for a Typical Day

Meal	Time of day	Serving Size	Food Item	Prepared by:

Please list all other vitamins, minerals, and supplements that you normally take in a day: _____

_____

Age-related Changes in Skeletal Muscle Signaling after Acute Heavy Resistance Exercise

Principal Investigator: Scott E. Gordon, Ph.D.
Telephone #: (252) 737-2879
Sub Investigator: Brad Harper
Telephone #: (252) 903-9088

EXPERIMENTAL SESSION INSTRUCTIONS

Human Performance Laboratory

For <u>two</u> full days prior to session (Start Date:_____):

1. **Do not drink alcohol.**

2. **If you consume caffeine, do so only in moderation.**

3. **Drink at least 64 oz. of water per day (i.e., eight 8-oz. glasses).**

On <u>day of</u> experimental session (Date:_____):

1. **Drink 16 oz. of water before reporting to the laboratory.**

2. ****Do not eat or drink anything <u>but</u> water for the 12 hours prior to reporting to the laboratory!!**

3. ****Do not exercise before the experimental session!!**

4. **Report to the Brody School of Medicine, room 3S08 at _____.**

5. **Wear exercise clothes, specifically shorts and athletic shoes, to the experimental session.**

For the duration of the experiments:
1. **Do not engage in exercise.**

2. **Do not donate blood or plasma.**

3. **If you begin taking new medications, please notify Brad Harper.**