HIGH-FAT DIET INDUCED OBESITY INCREASES SERUM MYOSTATIN, BUT DOES NOT ACCELERATE SKELETAL MUSCLE ATROPHY

by

Steven L. Roseno

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Director of Thesis: Jeffrey J. Brault

Major Department: Kinesiology

Myostatin is a potent negative regulator of muscle mass, i.e. high levels of myostatin induce loss of muscle. Surprisingly, severely obese humans and obese mice have elevated levels of serum myostatin, but the role of myostatin in controlling muscle mass during obesity is largely unknown. The aim of this study is to determine if obesity induced by a high-fat diet will decrease muscle mass or sensitize muscles to other factors that induce muscle atrophy. Thirty male C57BL/6 mice were divided into three groups; 12-wk control diet (CD), 9-wk control diet then 3-wk high-fat diet (3wHF), and 12-wk high-fat diet (12wHF). At 10 weeks with each diet, the left sciatic nerve was cut (surgical denervation, a model of nerve damage), and contralateral sham operated. At 12 weeks, EDL, soleus, tibialis anterior (TA), plantaris, and gastrocnemius muscles were excised and weighed; body composition was measured by MRI; and serum myostatin was measured by ELISA. The EDL and soleus muscles were incubated and rates of protein degradation were determined by the net amount of tyrosine released. The TA muscles were cross-sectioned and mean fiber area estimated. As expected, the 12wHF resulted in a profound increase in fat mass (785%), with less in 3wHF (541%) and CD (216%). The concentration of myostatin in serum was between 1.5 to 2 fold higher ($p \leq 0.05$) in the 12wHF group (705 pg/ml ± 79) than the 3wHF (420 pg/ml ± 36) and CD (316 pg/ml ± 18). In spite of

this, there were no significant differences in muscle mass of the innervated muscles between diets. The percent atrophy due to denervation ranged from 15 to 39%, depending on the muscle, but there were no significant differences among diet groups, with the exception of greater atrophy ($p \leq 0.05$) of the soleus muscle in the 3wHF group (26% versus 16% for CD and 15% for 12wHF). The rate of protein degradation was ~55 ηmol/g/hr for the innervated EDL muscles and ~75 ηmol/g/hr for the innervated soleus muscles. No significant differences existed between diet groups for either muscle. There were no significant differences between the diet groups for the rate of protein degradation of the denervated EDL muscles, but the rate of protein degradation for the denervated soleus muscle of the 3wkHF group was 30% faster than the CD and 12wkHF groups in accordance with the muscle mass findings. No significant differences in mean fiber area were found between diet groups among the innervated or denervated TA muscles. In summary, muscles of mice on a 12-week high-fat diet are exposed to higher myostatin but have the same mass and atrophy at the same rate as mice fed a control diet. This demonstrates that obesity may cause muscles to become resistant to the catabolic actions of myostatin.

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Steven L. Roseno

APPROVED BY:

DIRECTOR OF THESIS: _____
Jeffrey J. Brault, PhD

COMMITTEE MEMBER: _____
Joseph A. Houmard, PhD

COMMITTEE MEMBER: _____
Carol A. Witczak, PhD

COMMITTEE MEMBER: _____
Terry E. Jones, PhD

CHAIR OF THE DEPARTMENT OF KINESIOLOGY: _____
Stacy R. Altman, JD

DEAN OF THE GRADUATE SCHOOL: _____
Paul J. Gemperline, PhD

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CHAPTER 1: INTRODUCTION

Skeletal muscle is one the most metabolically active tissues in the human body, being the primary tissue responsible for both glucose and lipid oxidation[1,2]. Additionally, it provides the largest reservoir of amino acids for use during periods of fasting, sickness, and tissue trauma[3–6]. Perhaps because of these important metabolic roles of muscle, low levels of muscle mass have been identified as a comorbidity in numerous conditions such as AIDS, sepsis, heart failure, cancer, kidney failure, uncontrolled diabetes, sarcopenia, and severe tissue injury[7–10]. Therefore, identifying means to prevent or reverse muscle wasting is critical to human health.

The prevalence of obesity has steadily increased in the United States over the last 20 years[11]. Currently, 35.7% of the adult population is classified by their body mass index (BMI $\geq$ 30 kg/m^2) as being obese. Obesity is associated with metabolic inflexibility, defined as the inability to appropriately respond to alterations in substrate availability[12]. This phenomenon has been well documented in relation to glucose uptake and lipid oxidization[12–14]. However, a new characteristic of this inflexibility may be the inability to effectively mobilize amino acids[15]. For example, when muscles from a rodent hind limb were functionally overloaded to induce hypertrophy, the gain in muscle mass was significantly less in the obese rodents when compared to lean[16]. A possible reason for this blunted hypertrophy response could be elevated levels of the transforming growth factor myostatin documented in both human and animal models of obesity[17,18]. Myostatin is a potent negative regulator of muscle mass and is well known to reduce muscle size, increase rates of protein degradation, and decrease rates of protein synthesis[19–21].

The effect of obesity on muscle mass in general is largely unknown. One study indicates that muscles from mice fed a high-fat diet for 5 months have an elevated rate of protein degradation as well as significantly lower muscle weights[22]. However, considering that muscles

of obese rodents hypertrophy less than those of lean rodents, it might be predicted that muscles of the obese would atrophy to lesser extent than those of the lean when exposed to an atrophying stimulus such as denervation[16]. If this is true, understanding what mechanisms control muscle mass in the obese may provide a new area of study and the potential for the development of novel treatments to prevent muscle wasting in a wide range of muscle wasting syndromes.

The purpose of this study is to determine if obesity changes the susceptibility of skeletal muscle to atrophy. The *specific aim* is to determine if C57BL/6 mice fed a high-fat diet for 3 or 12-weeks will have an altered response to 14 days of sciatic nerve sectioning when compared to mice fed a diet of standard rodent chow.

We will test the following *hypotheses*:

1. The percent muscle mass lost from the denervated muscles will be less in the high-fat fed groups than in the standard chow fed group.
2. The percent fiber cross-sectional area lost from the denervated tibialis anterior muscle will be lower in the high-fat fed groups than in the standard chow fed group.
3. The rate of protein degradation of the denervated extensor digitorum longus and soleus muscles will be lower in the high-fat fed groups than in the standard chow fed group.

CHAPTER 2: REVIEW OF LITERATURE

The Importance of Skeletal Muscle

Skeletal muscle is one of the most vital tissues in the human body. In healthy adults, measurements taken by magnetic resonance imaging (MRI) have shown that muscle accounts for up to 40% of total body mass[23]. Muscle is also important for everyday movement, as it is the tissue that applies force to the skeletal system moving it about the environment[24]. In relation to this, it has been shown that total muscle mass has a positive correlation with quality of life in both the elderly and chronically ill[25,26]. Additionally, a negative correlation exists between overall muscle mass and the risk of falling and survival during chronic illness[26,27]. Therefore, maintaining muscle mass is critical for quality of life and prolonging mortality.

Skeletal muscle, in part because of its large size, is a major disposal site of metabolic substrates in the body. It alone accounts for ~20% of basal glucose uptake, which increases to ~86% during periods hyperinsulinemia[1]. It is also the primary tissue involved in lipid oxidation with nearly 100% of the adenosine triphosphate (ATP) produced at rest coming from free fatty acids[2]. Furthermore, it is the largest source of amino acids available to the human body during times of fasting[3]. For example, during the fasting state skeletal muscle is degraded in favor of providing amino acids to support protein synthesis for other tissues as well as precursors for gluconeogenesis[3–6]. When the amount of skeletal muscle decreases, the ability to perform these metabolic processes decreases as well. Since the capacity to uptake glucose, oxidize fatty acids, and mobilize amino acids decreases as whole body skeletal muscle decreases, these metabolic functions might explain the relationship between skeletal muscle mass and survival during chronic illness.

Structure of Skeletal Muscle

Individual skeletal muscle cells look like long fibers with alternating areas of light and dark bands called striations[24]. Because of their fiber-like appearance they are commonly referred to as muscle fibers. They have similar structures to other cell types. For example, most cells have a plasma membrane and cytoplasm, whereas muscle fibers have a sarcolemma and sarcoplasm[24]. Additionally, muscle fibers have transverse tubules (t-tubules) and a sarcoplasmic reticulum instead of a golgi apparatus and endoplasmic reticulum[24]. A major difference between muscle fibers and other cells is that muscle fibers are multinucleated, meaning more than one nucleus exists per fiber[24].

Muscle fibers can be broken down into different structural components. Surrounding the muscle fiber is the sarcolemma[24]. Inside the sarcolemma are the nuclei, sarcoplasm, mitochondria, sarcoplasmic reticulum, t-tubules, and myofibrils[24]. Myofibrils consist of sarcomeres, the functional component of muscle fibers[24]. Sarcomeres are comprised of two contractile filaments, actin and myosin[24]. They line up end-to-end creating repetitive areas of overlapping and non-overlapping filaments creating the alternating areas of light and dark bands, thus giving skeletal muscle a striated appearance[24]. Although all skeletal muscle has these basic elements, it is an extremely diverse tissue and is classified into different fiber types, each with its own characteristics and purpose.

Muscles are Comprised of Different Fiber Types

Skeletal muscle of small mammals can be classified into at least 4 fiber types; type I, type IIa, IIb, and type IIx. Type IIx fibers do not exist in skeletal muscle of humans or large mammals. Each fiber type is defined by their respective isoform of myosin heavy chain (MHC),

which can be broken down into type I, type IIa, type IIb, or type IIx[28–30]. The MHC protein consists of a tail and a globular head. The head binds to actin during contractions and contains the enzyme myosin ATPase. The rate at which ATP can be hydrolyzed depends on the activity level of this enzyme, which correlates with the type of MHC it resides on. Type I MHC has the slowest ATPase rate, while type IIa, type IIx, and type IIb are successively faster [31].

Fiber types can be further classified by their oxidative capacity[24]. This relates to how much a muscle fiber relies on beta-oxidation for ATP instead of glycolysis[24]. Fibers with greater oxidative capacity, i.e. more mitochondria, are able to sustain production of ATP and therefore can contract for longer periods of time[24]. In other words, highly oxidative muscle fibers are fatigue resistant[24]. Type I fibers are generally more oxidative in nature than type II fibers and therefore are more fatigue resistant[24]. However, type IIa fibers are unique in the fact that they are capable of being highly oxidative as well as glycolytic[24]. Type IIb and type IIx fibers have less oxidative capacity and rely more heavily on glycolysis[24]. Therefore these fibers are the least resistant to fatigue[24].

A muscle fiber's maximal shortening velocity (V_0) depends on the rate at which ATP can be hydrolyzed[32]. The lower the activity level of myosin ATPase, the slower a particular fiber can contract[32]. In mammalian skeletal muscle, type I fibers have the lowest V_0, where as type IIb fibers have the highest[32]. When considering rodent skeletal muscle, type IIx fibers contract slightly slower than type IIb fibers[33].

The specific function of a whole muscle determines the fiber distribution within it[24]. Muscles used to maintain posture, stabilize movement, or perform tasks requiring fine motor control will predominately be composed of type I fibers, whereas muscles used during powerful movements will have a high number of type II fibers[24]. The amount of heterogeneity, or variety

of muscle fiber types, per muscle varies from species to species. Humans have a relatively even distribution of fiber types regardless of the muscle's role[24]. However, some animals, such as rodents, have muscles or sections of muscle that are composed predominantly of a single fiber type[34]. The extensor digitorum longus (EDL), tibialis anterior (TA), and plantaris muscles of a rodent are exclusively type II[34]. The majority of the gastrocnemius is also type II with a small percentage type I fibers, with the superficial medial portion being predominantly type IIb and the deep lateral portion being predominantly type IIa[34]. The soleus is the only muscle that has a significant percentage, ~58%, of fiber area occupied by type I fibers[34]. This kind of homogeneity makes rodents an ideal model for examining the effects of diet-induced obesity on protein metabolism in different fiber types.

As previously discussed, muscles that maintain posture, stabilize movement, or execute delicate movements are primarily composed of type I fibers[24]. The physiological demands of these roles do not require a fiber that generates powerful, high-velocity contractions. Instead the fiber must be able to contract for long periods of time. Type I fibers are ideal for this role because their myosin ATPases only require a moderate network of t-tubules and sarcoplasmic reticula[24]. An additional characteristic of this fiber is that they have numerous, large mitochondria capable of supplying the ATP required for continuous muscle contractions[24]. To help facilitate delicate movements, several small motor units innervate type I fibers[24]. This allows for the precise activation of specific muscle fibers, which make fine movements possible[24].

Type IIa and type IIb fibers are recruited for powerful, high velocity movements like sprinting or jumping[24]. To accommodate this kind of movement these fibers have well developed t-tubules and sarcoplasmic reticula, which allow them to quickly transmit action potentials as

well as release and sequester high amounts of calcium ions[24]. The combination of high myosin ATPase activity and the ability to transport large amounts of calcium ions allow type II fibers to cycle through contractions much faster than type I fibers[24]. Additionally, not only do large motor units innervate type II fibers, but also one single motor unit innervates more muscle fibers[24]. This design allows for more fibers to be recruited at one time, increasing the amount of force the muscle can generate[24]. Skeletal muscles are composed of several different fiber types. Each fiber type serves a specific purpose and usually has characteristics specific to the fiber's function. Because of this specificity, different fiber types may respond to the same stimuli in different ways.

Muscle Fiber Size is Influenced by Multiple Stimuli

Skeletal muscle fibers respond to stimuli by adapting structural components to better accommodate continued stimulus; this trait is known as plasticity[24]. Plasticity is fiber-type specific and has been documented for different types of training and atrophy. For example, when type I fibers are exposed to resistance training, the amount of hypertrophy, as measured by percent increase of cross-sectional area, is typically less than type II fibers[35,36]. In contrast, endurance training increases the diameter of type I fibers while having little or no effect on the size of type II fibers[37]. Endurance training has also been shown to increase capillary density and oxidative enzyme activities in both type I and type II fibers[38]. If the stimulus is intense enough, a small portion of fibers may cross from one fiber type to another. Type IIb and IIx fibers of mice have been documented to transform into type IIa fibers when exposed to a significant amount of endurance training[39]. In extreme circumstances, such as low frequency electrical stimulation,

type II fibers have adopted oxidative characteristics that closely resemble those of type I fibers[39]. However, this shift in fiber types is less clear in human muscle.

Fiber-type specific atrophy is more complex. Not only do different fiber types respond to atrophy differently, but also different types of atrophy influence each fiber type in a different way. For example, in young rats, surgically denervating the soleus and plantaris muscles for ten days can reduce their masses by up to 33% and 31%, respectively[41]. When the muscles are exposed to a large dose of corticosteroids the plantaris will again lose up to 30% of its mass, but the soleus muscle will only lose around 10%[41]. If exposed to starvation, the plantaris once again will lose approximately 30% of its mass, while the soleus only loses around 8%[42]. This indicates that type I oxidative fibers appear to be sensitive to denervation (inactivity) induced atrophy, but not other atrophy causes. Since both fiber types are influenced by denervation to relatively the same degree, denervation is an excellent model to explore other factors that may induce fiber-type differences during atrophy.

A lesser-known area of fiber type-specific plasticity is how muscle fibers respond to obesity. A study investigating different fiber types in the rectus abdominus of lean and obese women found that obese women had a lower percentage of type I fibers and significantly more type IIb fibers[43]. It was also found that the amount of weight lost after gastric bypass surgery was directly correlated to the percentage of type I fibers of the patient[43]. A similar study examined fiber type differences between rodents who were prone to dietary obesity and those who were resistant[44]. It was found that obesity prone rodents had significantly less type I fibers in the medial head of their gastrocnemius both before and after a high-fat feeding period of four weeks than obesity resistant rodents[44]. These studies suggest that a lower percentage of type I fibers may increase an individual's susceptibility of becoming obese. Alternatively, it is possible that

the distribution fiber types may shift during obesity from type I to type II and that a reduction in weight may shift type II fibers to type I.

A limited number of studies using rodent models of obesity have attempted to examine the effects of obesity on skeletal muscle. The results have been inconclusive. After 28 days of a high-fat diet, the amount of MHC type I protein in the quadriceps muscle was 5.1 times higher than those on the control diet[45]. Western blots were done on elements of the electron transport chain and revealed that the protein content of complexes I-V were significantly higher in the high-fat group[45]. These findings suggest that a short term high-fat diet may remodel skeletal muscle in a way that increases its oxidative capacity. In contrast, a similar study showed that a 5-week high-fat diet failed to induce significant changes in fiber-type composition or muscle capillariztion in the medial gastrocnemius, soleus, and EDL[46]. Currently there are no studies investigating the influence of long term high-fat feedings, periods of 12 weeks or greater, on fiber-type specific changes in skeletal muscle.

Role of Skeletal Muscle in Disease

Cachexia is a form of skeletal muscle wasting caused by an underlying illness[47]. It is commonly associated with conditions such as AIDS, sepsis, heart failure, cancer, kidney failure, uncontrolled diabetes, and severe tissue injury[42]. The amino acids lost from skeletal muscle presumably are used by other tissues during these conditions[48]. For example, the increased immune response requires a greater amount of amino acids to help rebuild tissue and synthesize antibodies[48]. In order to meet the elevated need for amino acids, protein from skeletal muscle is degraded at an increased rate[48]. In a fed state, and when healthy, amino acids absorbed from the digestive system replenish the lost amino acids through a relative increase in protein synthesis[48].

Because of an increased degradation rate during cachexia, protein synthesis cannot keep up and skeletal muscle is lost[48].

Inflammation and the myokine Myostatin are thought to play key roles in cachexia. During chronic illness the body often enters a proinflammatory state in which cytokines, such as interleukin-6 and tumor necrosis factor-α, are produced[49]. Cytokines inhibit growth factors and stimulate skeletal muscle protein degradation at the same time[49]. Increased expression of myostatin is also common in patients suffering from muscle wasting[7,50,51]. Myostatin, which is produced by muscle, negatively regulates skeletal muscle growth by increasing the rete of protein degradation, decreasing the rate of protein synthesis, and inhibiting myoblast differentiation and proliferation[20,21]. The combination of increased inflammation and elevated myostatin results in an accelerated rate of muscle catabolism.

Current Interventions for Muscle Wasting

Maintaining skeletal muscle is vital during times of health, illness, and advancing age. Currently there are no proven pharmaceutical therapies available to treat muscle wasting once it begins. Early interventions of nutritional supplementation and appetite stimulation result in little to no improvements in quality of life or survival[52]. On the other hand, exercise training has shown potential for maintaining and increasing muscle mass, especially when the muscle lost is due to advancing age or disuse[53]. For patients with chronic illness it is often difficult to exercise due to decreased muscle mass and malnourishment brought on by disease[53]. However, if patients start exercising at the onset of the disease they have a better chance of maintaining muscle mass, with some populations being successful at increasing it[53].

Several pharmacological therapies are currently being investigated. Promising medications include Thalidomide and Celebrax, which target cytokines associated with the

proinflammatory state of disease[54]. Current results have been a mixture of positives and negatives. While these medications have been successful in clinical trials, increasing muscle mass as well as improving strength and quality of life, they are often accompanied by adverse side effects making long-term administration difficult[55,56].

A second class of medications, Anabolic-androgenic steroids, is capable of stimulating anabolism of skeletal muscle and inhibiting glucocorticoid receptors. When androgen binds to its receptor, muscle protein synthesis is increased[57]. In contrast, when glucocorticoids bind to their receptors they result in the reduction of the rate of skeletal muscle protein synthesis and an increase in protein degradation[57]. Additionally, androgens are necessary for the production of Insulin-like Growth Factor I, which is a major stimulator of muscle protein synthesis[57]. These medications have been successful at increasing muscle mass in the chronically ill, but adverse side effects make them a high-risk option[57]. An alternative to steroid medications called selective androgen receptor modulators are currently being developed[58]. These medications promote muscle growth in the same manner as steroids but, presumably, with less side effects[58].

A third area of treatment being developed involves inhibiting myostatin[59,60]. This approach has been effective in mice for treating muscular dystrophies[61], sarcopenia[62], and various forms of disuse atrophy[63]. Its potential for treating cachexia has not been extensively investigated. However, in rodents with lung cancer, the administration of a myostatin inhibitor resulted in the increase of muscle mass, cross-sectional area, maximal force production, and oxidative enzyme activity[60]. Although this approach appears to have potential, the few studies looking at myostatin inhibition in humans has not been promising[64], and further research is needed.

The majority of interventions being developed today focus on stimulating muscle protein synthesis[54]. The converse approach is to look at the pathways regulating muscle protein degradation. Most models of cachexia occur via activation of the major intracellular degradation pathway, the ubiquitin-proteosome[65]. Therefore, if a medication is developed that could target directly, or indirectly, the activation of the proteasome it would be expected to be beneficial for preserving skeletal muscle in all forms of muscle wasting.

Mechanisms Behind Protein Degradation

Muscle mass is regulated by a balance between protein synthesis and protein degradation[65]. If synthesis is down regulated or degradation is up regulated, atrophy occurs. The majority of muscle atrophy is caused by an increase in protein degradation[65,66]. Protein degradation in muscle occurs either by the ubiquitin/proteasome pathway or an autophagy/lysosomal pathway[65,66].

Protein degradation occurring via the ubiquitin proteasome pathway (UPP) involves the use of ubiquitin as a marker for specific proteins selected for degradation[65]. A series of enzymes, known as E3 ubiquitin ligases, are used for the activation and attachment of ubiquitin to specific proteins[67–71]. These enzymes facilitate protein degradation by physically conjugating the ubiquitin and protein together[65]. The main E3 ubiquitin ligases involved in the degradation of skeletal muscle proteins are Muscle Ring Finger-1 (MuRF1)[67,71] and Atrogin-1 (MAFbx)[68–70]. MuRF1 is responsible for the degradation of several contractile components including myosin-binding protein C, myosin light chain 1, myosin light chain 2, and MHC[67,71]. MAFbx is associated with breakdown of MyoD, a protein responsible for myoblast differentiation, and eukaryotic initiation factor 3 subunit 5[68–70]. After proteins are conjugated with a chain of

ubiquitin molecules, they are fed through the protein degrading machine known as the 26S proteasome[65].

Proteins are also degraded by proteolytic enzymes found in lysosomes[66]. Here, portions of the cytoplasm and organelles are engulfed by vacuoles called autophagosomes[66]. The autophagosomes then fuse with the lysosome where their content is degraded by lysosomal enzymes[66]. This pathway is apparently not as specific as UPP, and the exact mechanisms for how proteins are selected for degradation is not well defined[66]. It is unknown how protein degradation is regulated during obesity.

Mechanisms Driving Protein Synthesis

Protein synthesis is stimulated by the insulin growth factor-1 (IGF-1)/phophatidylinositaol-3 kinase (PI3K)/Akt pathway[72]. IGF-1 is a peptide growth factor that has autocine/paracrine action known to stimulate hypertrophy in skeletal muscle[72]. When IGF-1 binds to it's membrane receptor it initiates a signaling cascade of intracellular kinases resulting in the activation of a serine/threonine kinase called Akt[72].

Akt is the central protein involved in the regulation muscle of growth[72]. It has been reported in transgenic mice where Akt is constitutively active that skeletal muscle mass and cross sectional fiber area is higher than in wild type mice[73]. Akt's activation leads to the activation of regulatory-associated protein of mTOR (RAPTOR) and mammalian target of rapamycin (mTOR), collectively known as TORC1. TORC1 then activates p70S6K, which promotes protein synthesis[73]. Simultaneously, the activation of Akt inhibits protein degradation by phosphorylating the transcription factor FOXO[74]. As long as FOXO is phosphorylated it is unable to enter the nucleus and thus cannot promote the expression of MuRF1 and MAfbx[74]. Conversely, if Akt is inactivated FOXO will not be phosphorylated and can then translocate to

the nucleus where it promotes protein degradation by increasing the expression of MuRF1 and MAfbx[74].

Obesity is Associated with Metabolic Inflexibility

Obesity is a growing epidemic in the United States of America. As of 2010, 35.7% of Americans 20 years old or older were obese as defined by having a body mass index (BMI) of 30 kg/m^2 or greater[11]. The increasing prevalence of obesity is also present in America's youth[75]. In children, age percentiles are used to evaluate obesity[75]. Here, obesity is considered having a BMI greater than or equal to the 95th percentile[75]. In 2010, 16.9% of all children between the age of 2 and 19 years old met this criteria[11].

Obesity is associated with developing what is called metabolic inflexibility[12]. Metabolic flexibility refers to the ability to switch from one substrate to another when it comes to producing energy[12]. Obese individuals have a reduced ability to switch between substrates, even when the cellular environment favors one substrate over the other[13,15]. Insulin resistance is perhaps the most well documented metabolic inflexibility that occurs as a consequence of obesity[14]. When a cell becomes insulin resistant it requires increasingly higher concentrations of insulin to take up a given amount of glucose from the blood stream[14]. Additionally, these individuals have a remarkably reduced ability to oxidize lipids[12]. When muscle tissue from lean and obese subjects was analyzed for the capacity to oxidize lipids, obese participants oxidized ~52% less lipid on average than lean participants[12].

A reasonable conclusion is that since obese individuals are metabolically inflexible in regards to both glucose uptake and lipid oxidation, then perhaps deficits in the area of protein metabolism are present as well. Indeed, under conditions of hyperinsulinemia and hyperaminoacidemia, two stimuli which promote protein synthesis and inhibit protein

degradation, obese participants had a lowered inhibition of protein degradation and decreased stimulation of protein synthesis[15]. This suggests that obese individuals cannot regulate the balance between protein degradation and protein synthesis in the same manner as lean individuals.

This topic has been further studied in animal models[16,22,76]. Lean and obese rodents had their Achilles tendon severed at the point of their gastrocnemius in order to overload their soleus muscle[76]. Both groups responded to the overload by increasing muscle mass[76]. However, the increase in mass of the obese mice was ~53% less than that of the lean mice[76]. In a similar study, mice were fed a high-fat diet for 14 weeks[16]. At the conclusion of the diet the mice had an ablation procedure, severing the gastrocnemius and soleus, resulting in the functional overload of the plantaris muscle[16]. After 14 days of functional overload the plantaris of the high-fat fed group weighed 20% less than the control diet[16].

This reduced ability to hypertrophy associated with obesity could be attributed to elevated levels of myostatin[17,18]. In humans, elevated levels of myostatin have been observed in muscle tissue of those who are extremely obese (BMI $\geq$ 40 kg/m^2)[17]. In rodents, 12 weeks of a high-fat diet results in a significant increase in mRNA for muscle myostatin when compared to standard rodent chow fed controls[18]. However, whether these elevated levels of myostatin have any effect on protein metabolism is unknown and additional research is necessary before further conclusions can be made.

There are a limited number of studies investigating the effect of a long-term high fat feeding on protein degradation. One study looked at rodents fed a high-fat diet for a period lasting 5 months. They observed slight decreases in mass of the EDL or soleus muscles for the

high-fat mice[22]. Additionally, the basal rate of protein degradation of the high-fat group was significantly elevated[22].

At this time there are no studies investigating how skeletal muscle of the obese responds to an atrophy stimulus. Given that moderately obese individuals cannot mobilize proteins in response to a hypertrophying stimulus[16,76], it would be logical to expect that they would also have difficulty mobilizing proteins in response to atrophy. Further, if basal protein degradation is elevated, as occurs with very long-term obesity[22], muscle might increase their rate of protein catabolism. Therefore, the duration of the high-fat diet, or extent of obesity, may play a critical role in determining how skeletal muscle mass is regulated. If obesity is in fact associated with a altered ability to mobilize amino acids in response to atrophy, further investigation into the causes may provide a new target area for medication development in the treatment of muscle wasting disease.

CHAPTER 3: METHODOLOGY

Experimental Animals

Six week old, male C57BL/6 mice were purchased from Charles River Laboratories. This mouse strain was selected because of their well-known susceptibility to obesity induced by a high-fat diet[16,18,22]. In addition to weight gain, a high-fat diet results in blood glucose levels nearly twice the amount of mice fed a standard diet, indicating that the high-fat diet induces insulin resistance[18]. All mice were housed at the East Carolina University (ECU) Brody School of Medicine animal care facility. The mice were kept three to a cage whenever possible. They had ad libitum access to water and food and were kept on a 12:12 hour light:dark cycle. This project was approved by the ECU Animal Care and Use Committee (Animal Use Protocol #P073).

Study Design

A grand total of sixty mice were randomly separated into three diet conditions. A control diet, 12-week high-fat diet (12wkHF), and 3-week high-fat diet (3wkHF), each lasting a total of 12 weeks (Figure 3.1). Diet duration was set at 12 weeks as it is a common model used to induce insulin resistance in the C57BL/6 mouse[16,18]. The control diet lasted the entire 12 weeks and mice were fed standard rat chow (Prolab, RMH 3000) where 14% of calories came from fat, 26% from protein, and 60% from carbohydrate. The 12wkHF diet also lasted all 12 weeks, but mice were fed a diet (Opensource Diets, D12492) where 60% of the calories came from fat, 20% from protein, and 20% from carbohydrate. The 3wkHF diet group was fed standard rat chow for the first 9 weeks and then switched to the high-fat diet for the remaining 3. This diet was designed

with the intent to examine the influence of a short term high-fat diet independent of the mice becoming insulin resistant.

Twenty mice were assigned to each diet condition. At 10 weeks, ten were randomly selected from each group for surgical, unilateral sectioning of the sciatic nerve (see Surgical Procedures). This procedure was selected for its effectiveness at inducing rapid atrophy in muscles of the rodent hind limb[41]. After surgery, mice continued on their diets for an additional 2 weeks so that tissue and blood samples were collected exactly 12 weeks after each mouse started their respective diet. The other ten mice of each diet group had surgery 11 weeks and two days into the diet, thus samples were collected from these mice with 5 days of denervation. Muscle-specific data of the 5 day denervated mice will be reported elsewhere.

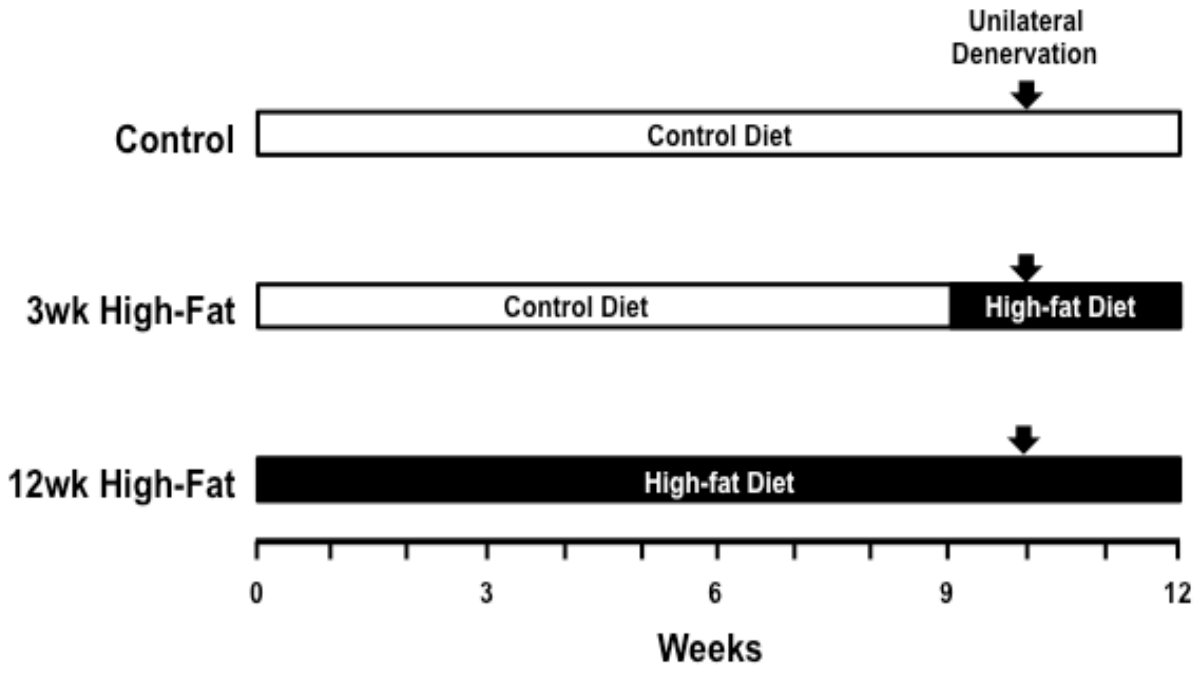


Figure 3.1. Six week old, male C57BL/6 mice were separated into three diet conditions (8-10 per group). Each group underwent a unilateral denervation at 10 weeks, indicated by an arrow.

Body Composition

Body weight was obtained using an electronic Ohaus scale measuring to 0.1 grams. Each mouse was weighed every week of the study to monitor weight gained. Body composition was measured every third week to determine fat mass and fat-free mass. It was measured by a magnetic resonance imager (EchoMRI 700 Body Composition Analyzer) capable of measuring to 0.01 grams. At the end of this study there were no significant differences in body weight, fat mass, and fat-free mass between mice in a respective diet group undergoing the 14-day denervation and those undergoing a 5-day denervation. In light of this, all weight and body composition measurements were pooled by diet condition.

Surgical Procedures

Mice were taken to a dedicated surgical room and anesthetized with isoflurane (Webster Veterinary). After removing the hair the on the lateral surface of the hind limb the bare skin was disinfected with iodine. An incision no larger than 5 mm was made at approximately mid-femur. Using blunt dissection, the quadriceps muscles were moved exposing the sciatic nerve. While the sciatic nerve was exposed, a 2 mm section was cut and removed to prevent nerve regrowth. The skin was then closed with surgical glue (3M Vetbond Tissue Adhesive). A sham surgery was performed on the contralateral limb. All steps for the sham surgery were identical to the denervation surgery with the exception of the sciatic nerve being cut and removed. This procedure is well characterized for inducing rapid atrophy in all muscle groups below the knee[41]. A subcutaneous dose of Buprenex (0.01mL/g) was given immediately post surgery as an analgesic.

Tissue Samples

Mice were euthanized using a lethal dose of ketamine followed by cervical dislocation and pneumothorax once all samples had been collected. The gastrocnemius, tibialis anterior (TA), plantaris, extensor digitorum longus (EDL), and soleus muscles were collected along with a sample of whole blood from the abdominal aorta. All muscles were weighed immediately using an analytical balance measuring to 0.01 milligrams. The gastrocnemius and plantaris were then put in cryogenic tubes, flash frozen with liquid nitrogen, and then stored at -80°C. The EDL and soleus were immediately washed and prepared for measuring rates of protein degradation (see below). The TA was used to measure mean fiber area (see below). Since the rapid freezing nature of liquid nitrogen can cause artifacts within the muscle, the TA was instead frozen with isopentene cooled by liquid nitrogen. It was then stored in a cryogenic tube at -80°C.

Whole blood was collected with a syringe from the abdominal aorta and then transferred to an eppendorf tube. The blood was left on ice until sufficient clotting had occurred. Once clotted, the blood was spun down at 5000 rpm for eight minutes to separate the red blood cells and from the plasma. The plasma was siphoned off and transferred to a clean eppendorf tube, which was immediately stored at -80°C. The red blood cells were discarded.

Rate of Protein Degradation

Rates of protein degradation were determined by measuring the amount of tyrosine released by muscles incubated at resting length[77]. Since tyrosine is neither synthesized nor degraded in skeletal muscle, the net accumulation of this amino acid in the medium reflects the net degradation of cell proteins[77].

In brief, each EDL and soleus muscle was washed in a 15 ml pyrex flask with

approximately 10 ml of Krebs henseleit buffer/ 5 mM glucose/ 0.15 mM pyruvate gased with 95% O_2 /5% CO_2. The muscles were then blotted dry, weighed and recorded to the nearest 0.1 miligrams, and secured with a custom plastic clip at approximately resting length. The clip was then placed, muscle side down, in the pre-incubation well for 30 minutes with 3 ml of Krebs henseleit buffer/ 5 mM glucose/ 0.15 mM pyruvate. During the pre-incubation, the well was covered and continuously gased with 95% O_2 /5% CO_2, and maintained at 37°C. Immediately before the 30 minutes expired, 3 ml of heated, bubbled Krebs henseleit buffer/ 5 mM glucose/ 0.15 mM pyruvate containing 0.5 mM cyclohexamide (a protein synthesis inhibitor) was added to the incubation well. Once the 30 minutes of pre-incubation expired, the muscles were removed, blotted, and placed in the gassed incubation well. After 2 hours, 2 mL of the final incubation media was removed and added to a cryogenic tube with 0.5 mL 1N perchloric acid to precipitate proteins. These samples were then stored at -80°C until a time when the tyrosine concentration could be measured. The muscles were also removed after 2 hours and blotted dry, and then frozen in liquid nitrogen and stored in cryogenic tubes at -80°C.

Tyrosine was measured by first derivitizing the samples with Waters AccQTag technology and then quantifying the derivitized amino acid using a Waters Aquity Ultra Performance Liquid Chromatograph. Rates of protein degradation are given as pmol tyrosine per mg muscle per two hours.

Mean Fiber Area

Serial cross sections of each TA were obtained using a cryostat-microtome (Microm HM 550). The frozen TA muscles were placed in the cutting chamber, which was set at -20°C. Once the temperature of the TA equilibrated with the temperature of the cutting chamber, it was cut in

two at the muscle belly using a razor blade cooled to -20°C. The uncut end was fixed to a cutting chuck with a dime-sized portion of O.C.T. compound (Tissue-Tek). After setting the cutting platform, a total of three 10 μm cross-sections were obtained per muscle. Four muscles were able to fit per slide.

After the desired number of cross-sections were obtained, the slides were left at room temperature for 30 minutes or until the cross-sections dried. A hydrophobic marker was used to make a barrier encircling all of the cross sections. A solution of 0.1% Triton-X100/ 1 X phosphate buffered saline (1 X PBS) was made and 200 μL of it pipetted onto each set of cross-sections. Triton-X100 is a detergent capable of denaturing some of the membrane proteins, making the cross-sections more permeable to a blocking solution. After 2 minutes of incubating the slides were washed one time in 1 X PBS for five minutes. After washing, the slides were removed and blotted dry. Once dried, 200 μl of a blocking solution consisting of 1 X PBS/ 0.5% Bovine Serum Albumin (BSA)/ 10% Rat Serum was pipetted onto each set of cross-sections. The blocking solution prevents nonspecific binding of the primary antibody. The slides were then incubated at room temperature for 20 minutes.

During the 20-minute incubation, a solution of 1 X PBS/ 0.5% BSA/ 2% Rat Serum was combined with a primary antibody for the protein laminin (Sigma-Aldrich Anti-Laminin antibody produced in rabbit, CAT.# L9393) in a working ratio of 1:500. After removal of the blocking solution, 200 μl of the diluted primary antibodies were pipetted onto each set of cross-sections. The slides were then incubated overnight at 4°C in a humidifier.

On day two, the slides were removed from 4°C and washed in 1 X PBS 3 times for 5 minutes each. During the wash, a solution of 1 X PBS/ 0.5% BSA/ 2% Rat Serum was combined with the secondary antibody (Invitorgen Alexa Fluor® 546 Goat Anti-Rabbit IgG (H+L), CAT.#

A11010) in a working ratio of 1:250. After the final wash, 200 μL of the diluted secondary antibody was pipetted onto each set of cross-sections. The slides were then incubated in complete darkness at room temperature for 1 hour. The slides then were washed in complete darkness 3 times in 1 X PBS for 5 minutes each.

After the final wash, the slides were removed and blotted dry with a kimwipe. Particular care was taken during this step to ensure that the cross-section did not get over blotted and then dry out. After blotting, a drop of mounting media containing DAPI (Invitrogen SlowFade® Gold Antifade Reagent with DAPI, CAT.# S36939), which stains for nuclear DNA, was placed directly on each set of cross-sections. A slide cover slip was then carefully placed on top of the slide and sealed using fingernail polish. At this point the cross-sections were ready for imagining.

Laminin is a protein located on the sarcolemma, the outer membrane of a muscle fiber. By labeling this protein with a fluorescent antibody it outlines each individual fiber in the cross-section. Images of this antibody were captured by a fluorescent capable microscope (Leica Microsystems) and Simple PCI 6 imaging software. The imagines were taken with a 10 X objective, which allowed for at least 300 fibers to be captured per image. Four to five images were taken per muscle. Mean fiber area was then calculated using the software program ImageJ[78], which was downloaded from the National Institutes of Health website. Mean fiber area was calculated for each muscle and reported as μm^2.

Blood Glucose, Serum Insulin, and Serum Myostatin

Prior to euthanization, a small incision was made at the tip of the tail and a small drop of blood collected on a test strip (OneTouch Ultra Blue) and read using a glucometer (OneTouch

Ultra 2). Blood glucose was reported in mg/dL. Serum insulin was measured using an enzyme-linked immunosorbent assay (ELISA) kit for rat / mouse insulin (Millipore, CAT.# EZRMI-13K). A concentration curve ranging from 0.2 ng/mL to 10 ng/mL was generated from the standards provided by the ELISA kit. The ELISA protocol was followed exactly as directed by the manufacturer. The plate was read using a microplate reader (Spectramax M4) at an absorbance of 450 nm and 590 nm. The difference of absorbance was recorded. The amount of insulin in ng/mL was calculated relative to the standard curve.

Serum myostatin was also measured using an ELISA kit for mouse myostatin (USCN Life Science Inc., CAT.# E9153Mu). A standard concentration curve ranging from 78 ρg/mL to 5000 ρg/mL was generated from the stock solution and standard diluent supplied with the ELIZA kit. Serum samples were prepared as a 10 fold dilution, combining 20 µL of serum with 180 µL of 1 X PBS. The ELISA protocol was followed exactly as directed by the manufacturer. The plate was read using a microplate reader (Spectramax M4) at an absorbance of 450 nm. The amount of myostatin in ρg/mL was calculated relative to the standard curve.

Calculations for Percent Atrophy and Percent Water

The amount of muscle mass lost was calculated by taking the innervated wet weight, subtracting the denervated wet weight, dividing the difference by the innervated wet weight, and multiplying by 100 to get a percent atrophy.

Low-grade inflammation is a common condition associated with a high-fat diet[22,79]. Inflammation can lead to edema, making it possible that any muscle mass lost may have been replaced by water. To account for this the EDL and soleus muscles were freeze-dried (LABCONCO) overnight. By taking the muscle's wet weight, subtracting its dry weight,

dividing the difference by the wet weight, and then multiplying by 100 a percentage of water was calculated. Water can account for as much as 80% of a muscle's mass[80]. If the percentage of water in the denervated muscles exceeded 80%, it is possible that their wet weights may have been inflated by excess water.

Statistical Analysis

All response variables in this study were quantitative. There was one explanatory variable, diet condition, which was a categorical variable with three levels making an analysis of variance the appropriate statistical analysis to determine if there were any differences in the sample means. Alpha was set at $p \leq 0.05$. A Tukey's honestly significant difference was used for post hoc analysis to determine which means were significantly different.

CHAPTER 4: RESULTS

Body Composition

Mice were divided into three groups, each fed a different diet lasting 12 weeks. The control diet (CD) group received standard rat chow where 14% of the total calories came from fat. Over 12 weeks, the control diet resulted in increases of total mass by 96% (Figure 4.1), fat mass by 216% (Figure 4.2), and fat-free mass by 24% (Figure 4.3), consistent with typical growth curves of young mice. The 3wkHF group was fed the control diet for 9 weeks and then a high-fat diet (60% kcal fat) for 3 weeks. Not surprisingly the body composition changes of the 3wkHF group mimicked that of the CD for the first 9 weeks of the diet. Once the group started the high-fat diet total mass and fat mass increased drastically. By the end of 12 weeks, the 3wkHF diet increased total mass by 72% (Figure 4.1), fat mass by 541% (Figure 4.2), and fat-free mass by 27% (Figure 4.3). The 12wkHF group, which was fed the 60% fat diet for the entire 12 weeks, gained the most total mass, fat mass, and fat free mass at 96%, 785%, and 33%, respectively. Both the 12wkHF and 3wkHF groups gained significantly more ($p \leq 0.05$) total mass (Figure 4.1) and fat mass (Figure 4.2) than the CD. Fat mass was 2.78 fold higher in the 12wkHF group and 2.18 fold higher in the 3wkHF group. Only the 12wkHF group gained significantly more fat-free mass (Figure 4.3).

Blood Glucose, Serum Insulin, and Serum Myostatin

Blood glucose and serum insulin were analyzed to estimate the presence of insulin resistance, a known complication of obesity[14]. While no significant differences were found among glucose measurements (Figure 4.4), serum insulin was higher than controls for the

3wkHF and 12wkHF groups, 287% and 910% respectively (Figure 4.5). However, because of the high variability of the 3wkHF group, a significant difference ($p \leq 0.05$) was only found for the 12wkHF group. Myostatin is a well-known negative regulator of muscle mass[21] and is increased during obesity[17,18]. Therefore, levels of serum myostatin were measured after each 12-week diet. Myostatin of the 3wkHF group was not different than controls, but the 12wkHF group was significantly higher ($p \leq 0.05$) at 123% (Figure 4.6).

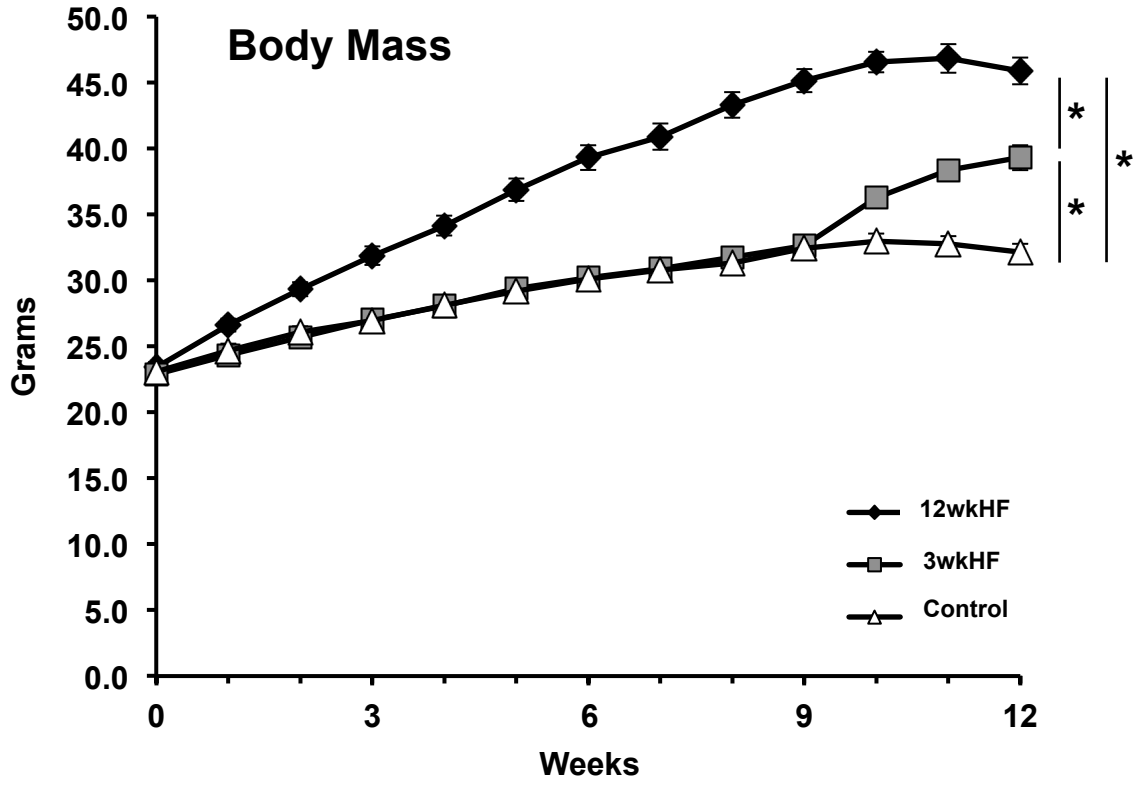


Figure 4.1. Changes in total body mass for the control group, 3-week high-fat group (3wkHF), and 12-week high-fat group (12wkHF) after each week of the study (means ± SE; 18-20 per group). *Significant difference at 12 weeks ($p \leq 0.05$).

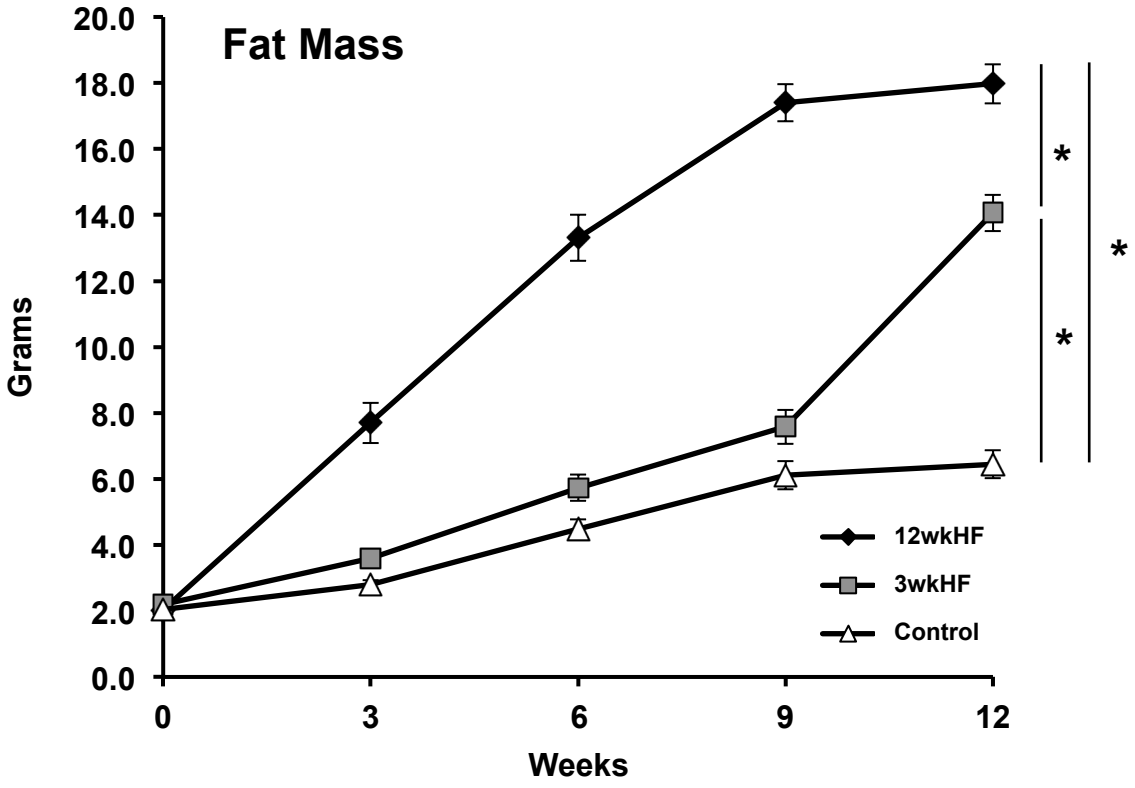


Figure 4.2. Changes in whole body fat mass for the control group, 3-week high-fat group (3wkHF), and 12-week high-fat group (12wkHF) after every third week of the study (means ± SE; 18-20 per group). *Significant difference at 12 weeks ($p \leq 0.05$).

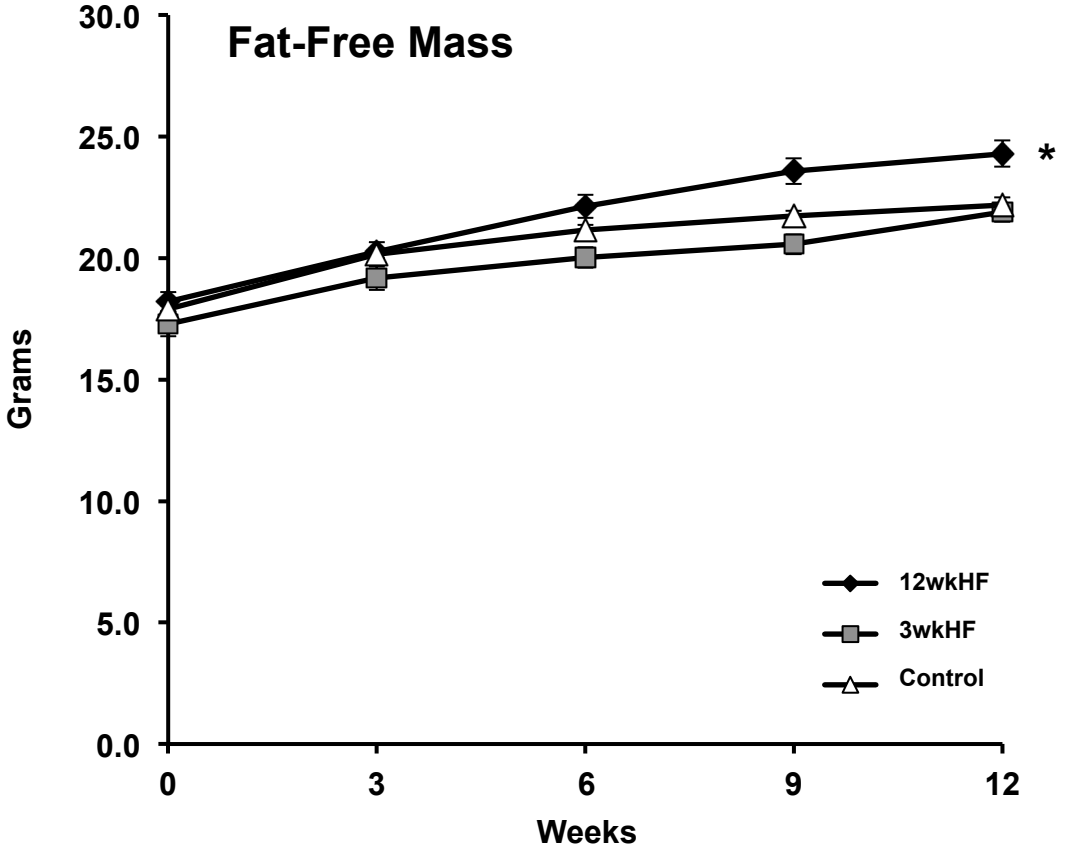


Figure 4.3. Changes in whole body fat-free mass for the control group, 3-week high-fat group (3wkHF), and 12-week high-fat group (12wkHF) after every third week of the study (means ± SE; 18-20 per group). *Significant difference at 12 weeks versus control and 3wkHF ($p \leq 0.05$).

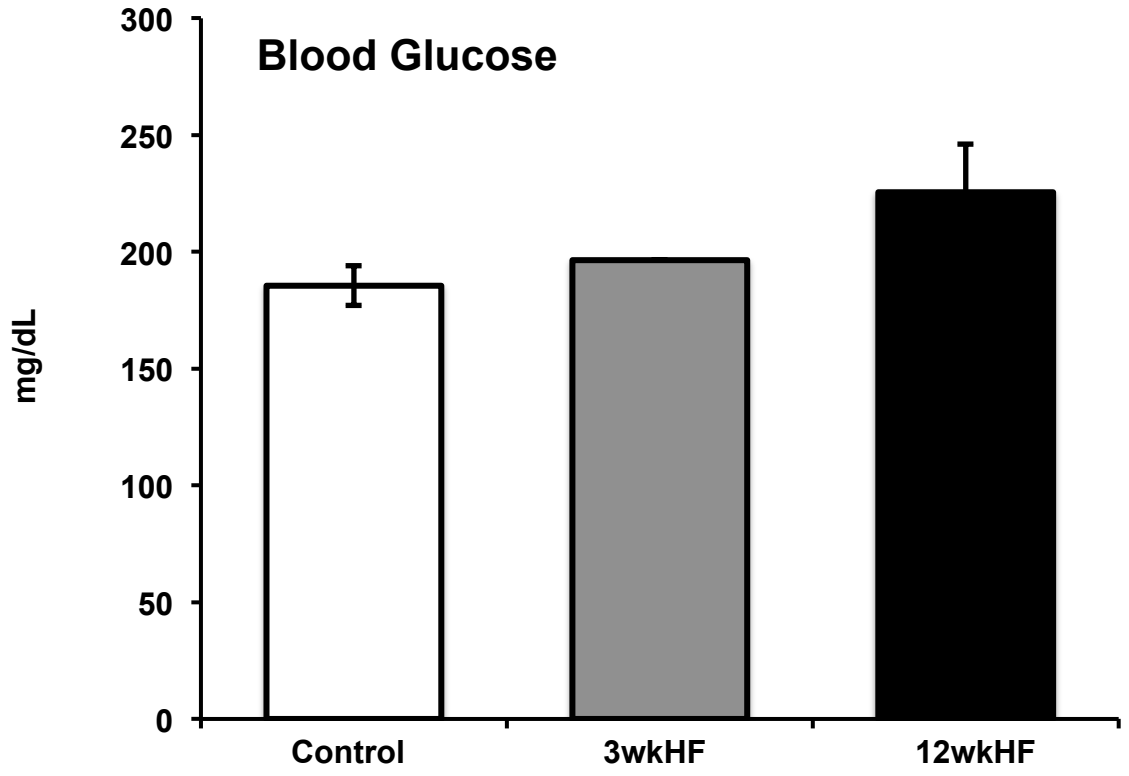


Figure 4.4. Blood glucose for the control group, 3-week high-fat group (3wkHF), and 12-week high-fat group (12wkHF) at weeks (means ± SE; 18-20 per group).

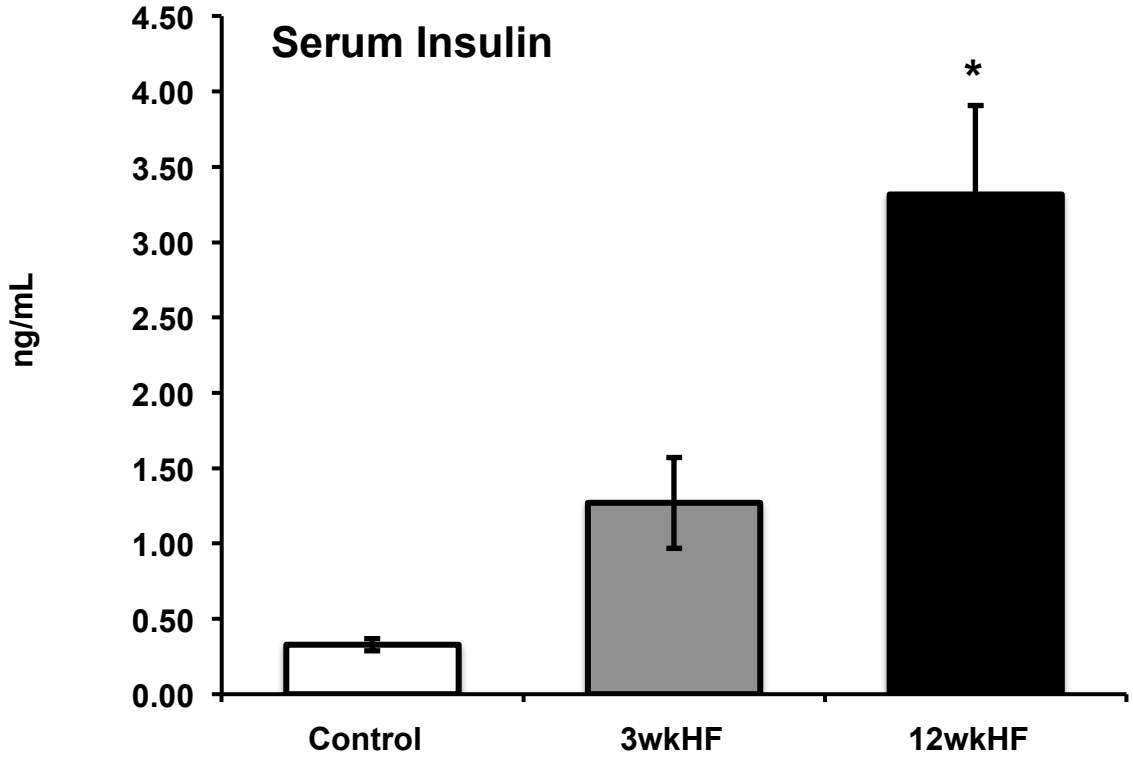


Figure 4.5. Serum insulin for the control group, 3-week high-fat group (3wkHF), and 12-week high-fat group (12wkHF) at 12 weeks (means ± SE; 18-20 per group). *Significant difference ($p \leq 0.05$) versus control and 3wkHF.

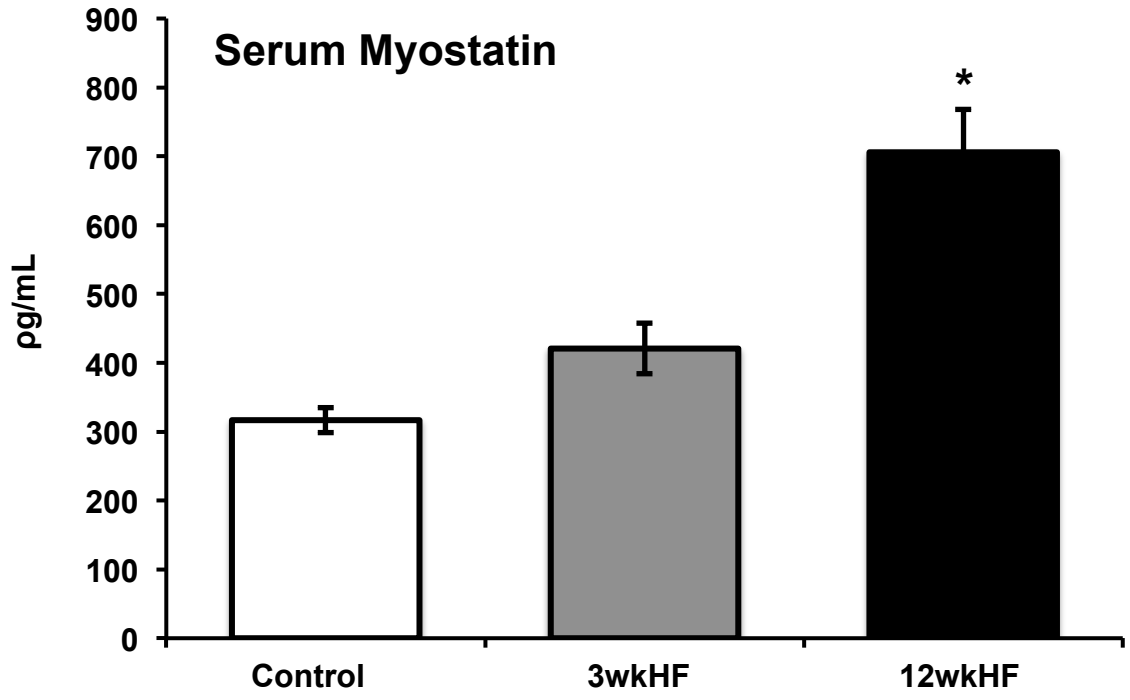


Figure 4.6. Serum myostatin for the control group, 3-week high-fat group (3wkHF), and 12-week high-fat group (12wkHF) at 12 weeks (means ± SE; 18-20 per group). *Significant difference ($p \leq 0.05$) versus control and 3wkHF.

Measures of Atrophy

To determine whether a high-fat diet increased the weight of the hind limb muscles, individual innervated muscles of the lower hind limbs, representing extremes of fiber-type in the mouse, were removed and weighed. Weights of the innervated gastrocnemius, tibialis anterior (TA), and extensor digitorum longus (EDL) muscles of the 12wkHF group were 3% to 7% higher than the control or 3wkHF groups (Figure 4.7). Significant increases ($p \leq 0.05$) were seen only in the plantaris and soleus muscles, which increase by 8% and 13%, respectively.

The amount of muscle lost due to the denervation was determined by taking the difference between the innervated and denervated muscle masses, dividing it by the innervated muscle mass, and multiplying by 100 to obtain a percent atrophy. The soleus muscle of the 3wkHF group was the only muscle to have a significantly higher ($p \leq 0.05$) percent atrophy, 26% compared to 16% and 15% in the CD and 12wkHF groups (Figure 4.8). To rule out the possibility of excess water masking the atrophy response, the soleus and EDL muscles were dehydrated via an overnight freeze-dry to determine the percent water (Table 4.1). Both muscles of the 3wkHF and 12wkHF groups either contained the same or a less water than the control group, suggesting that the atrophy is a true loss of protein.

To verify that the loss of muscle weight was due to atrophy of fibers (and not loss of the number of fibers), fiber size was measured by immunofluorescence microscopy. The tibialis anterior muscles were removed and 10 μm cross-sections were cut. The cross-sections were probed with an antilaminin antibody. The mean fiber areas of the denervated muscles were between 32% and 34% smaller than the innervated muscles as expected (Figure 4.9). There were no significant differences among the groups for either the innervated or denervated muscles, which is consistent with the findings for innervated muscle mass and percent atrophy.

To determine whether atrophy was due to accelerated protein degradation, the soleus and extensor digitorum longus muscles were incubated for 2 hours in an oxygenated krebs bath. The amount of tyrosine released was quantified to establish the rate at which skeletal muscle proteins were being degraded. The rate of protein degradation was elevated in the denervated muscles as expected. There were no differences among the diet groups for the extensor digitorum longus, but the rate of protein degradation of the soleus muscle for the 3wkHF group was significantly higher ($p \leq 0.05$) than both the control and 12wkHF groups by 30% (Figure 4.10). These findings are in agreement with the percent atrophy data.

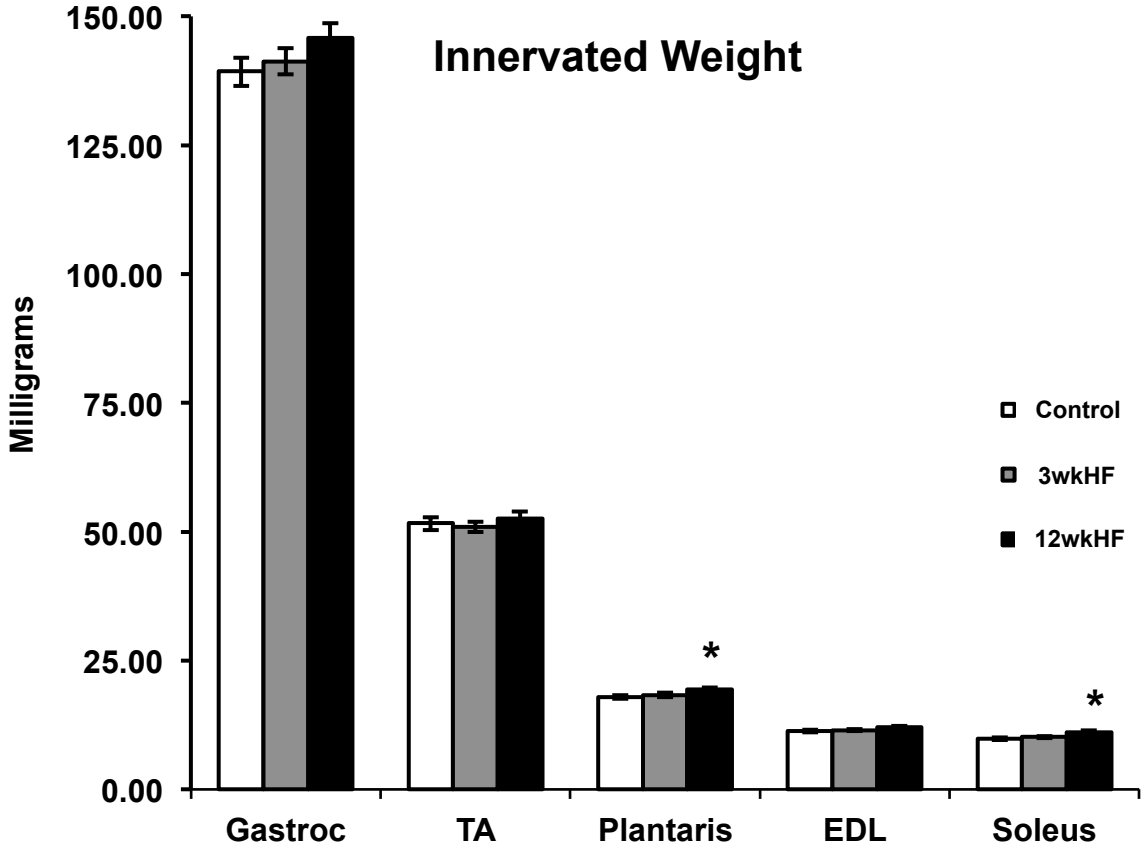


Figure 4.7. Muscle mass of the innervated gastrocnemius (Gastroc), tibialis anterior (TA), plantaris, extensor digitorum longus (EDL), and soleus for the control group, 3-week high-fat group (3wkHF), and 12-week high-fat group (12wkHF) (means ± SE; 18-20 per group). *Significant difference ($p \leq 0.05$) versus control and 3wkHF.

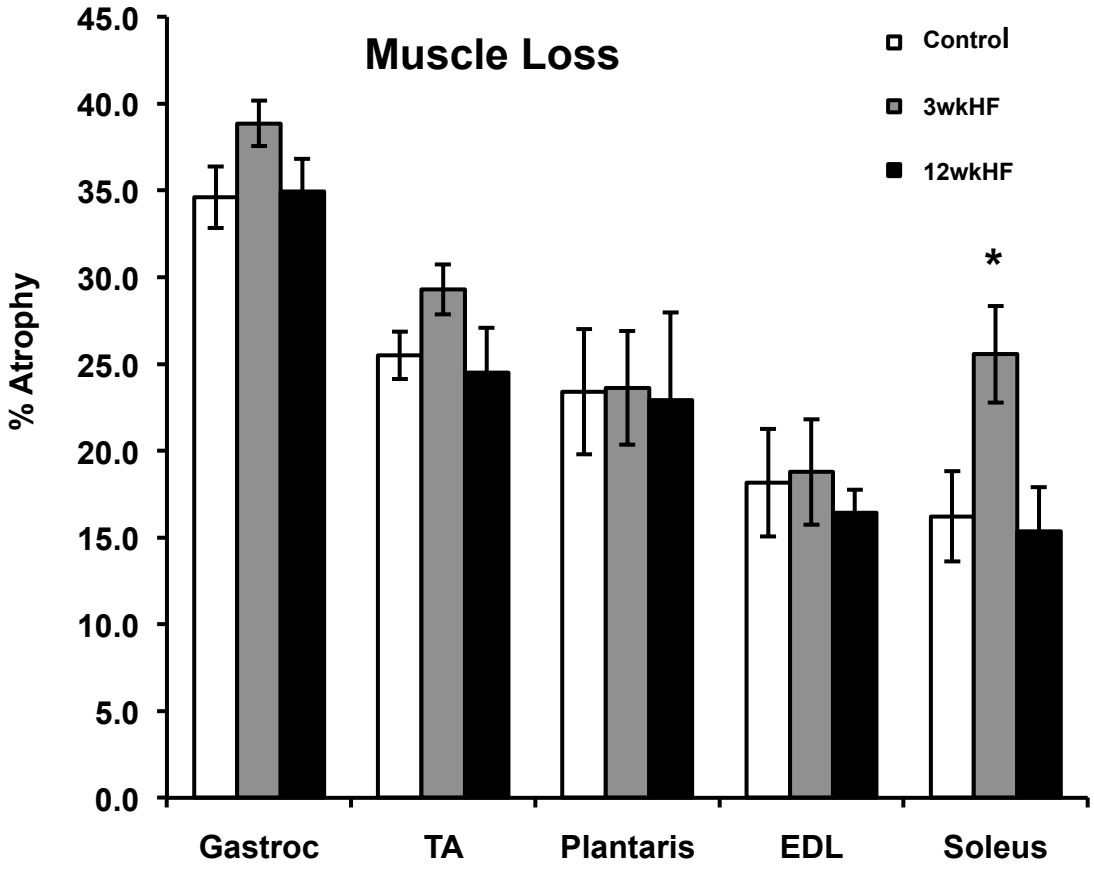


Figure 4.8. Percent loss of muscle mass, reported as % atrophy, of the gastrocnemius (Gastroc), tibialis anterior (TA), plantaris, extensor digitorum longus (EDL), and soleus for the control group, 3-week high-fat group (3wkHF), and 12-week high-fat group (12wkHF) (means ± SE; 8-10 per group). *Significant difference ($p \leq 0.05$) versus control and 12wkHF.

A.

Soleus	Innervated	Denervated
Control	76.43 ± 0.53	77.52 ± 0.65
3wkHF	74.61 ± 0.39	75.26 ± 0.71
12wkHF	74.10 ± 0.37	73.19 ± 1.68

B.

EDL	Innervated	Denervated
Control	74.13 ± 0.25	76.32 ± 0.38
3wkHF	73.97 ± 0.42	74.95 ± 0.45
12wkHF	73.86 ± 0.45	74.91 ± 0.91

Table 4.1. Percent water of the Soleus (Table A) and Extensor Digitorum Longus (EDL) (Table B) muscles.

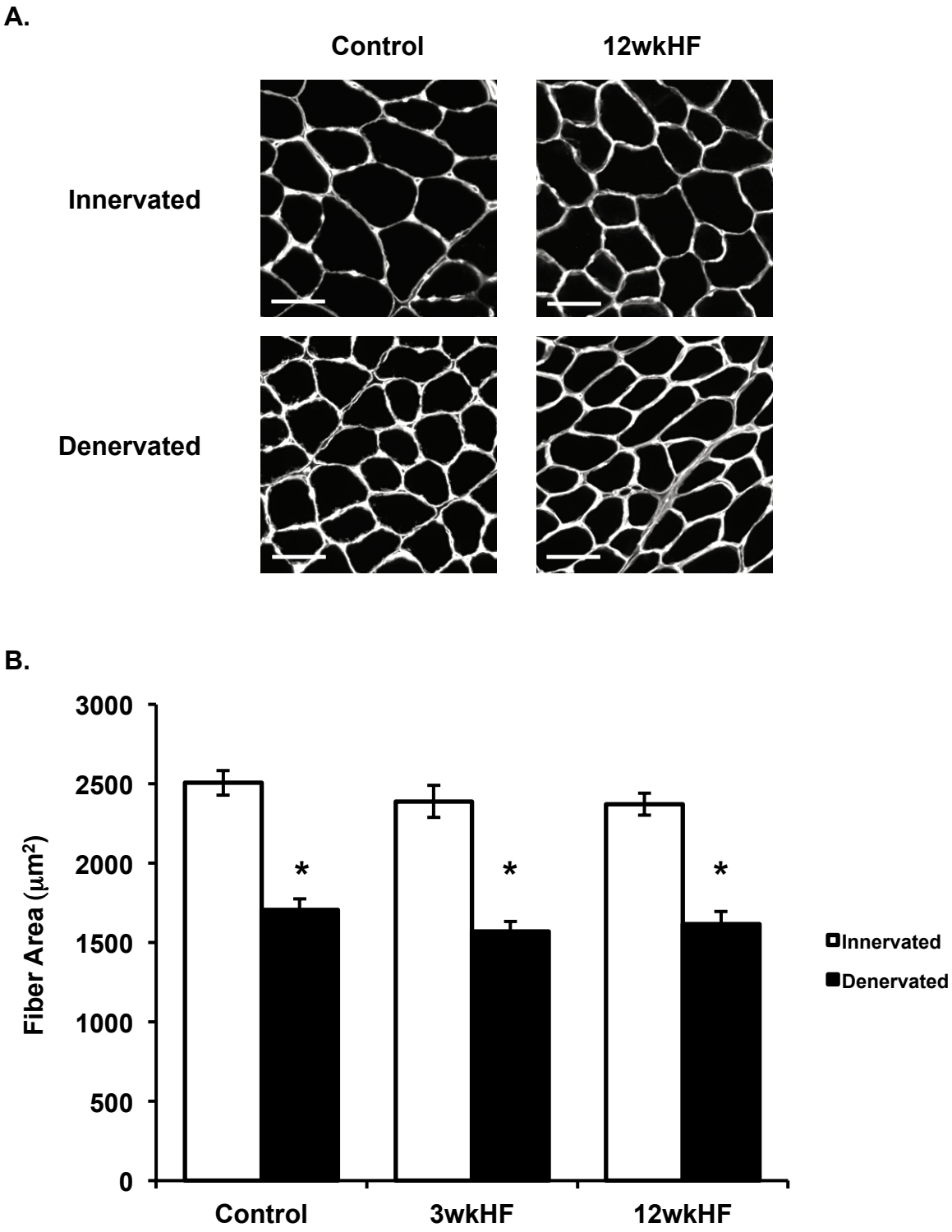
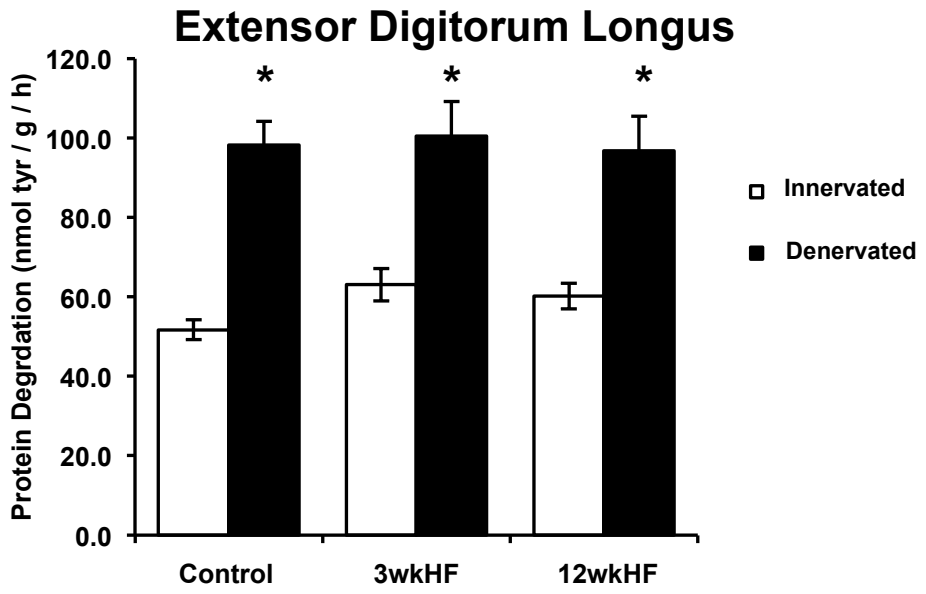


Figure 4.9. Cross-sections of innervated and denervated tibialis anteriors from the control and 12wkHF groups (Panel A) (Scale Bar = 50 μm). Fiber area of the tibialis anterior (Panel B) for the control group, 3-week high-fat group (3wkHF), and 12-week high-fat group (12wkHF) (means ± SE; 8-10 per group). *Significant difference ($p \leq 0.05$) versus innervated.

A.



B.

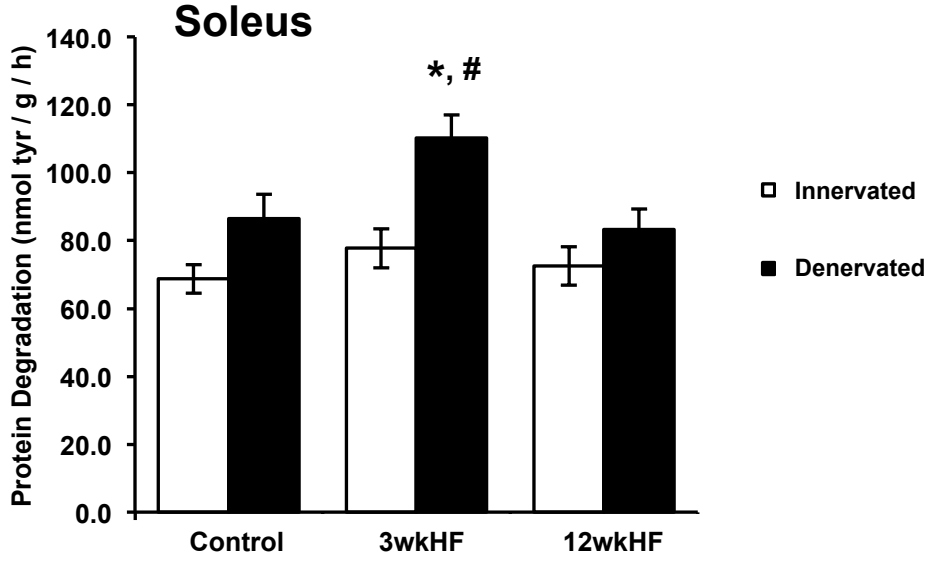


Figure 4.10. Rate of protein degradation for the extensor digitorum longus (Panel A) and soleus (Panel B) of the control group, 3-week high-fat group (3wkHF), and 12-week high-fat group (12wkHF) (means ± SE; 8-10 per group). *Significant difference ($p \leq 0.05$) versus innervated. #Significant difference ($p \leq 0.05$) versus control and 12wkHF, denervated.

CHAPTER 5: DISCUSSION

Summary of Findings

The major goal of our study was to determine if obesity changes the susceptibility of skeletal muscle to atrophy. Therefore, our diets were designed to induce short-term (3wkHF) or long-term (12wkHF) obesity. At 12 weeks with each diet, the 3wkHF group and 12wkHF group weighed significantly more than the control group (Figure 4.1). Differences in weight gain were largely attributed to increases in whole body fat mass, but fat-free mass was also significantly higher in the 12wkHF group (Figures 4.2 and 4.3). Although fat-free mass includes all non-fat tissue, increases in bone mass and other minerals is thought to have been minimal[81], meaning that increases in fat-free mass should mainly be a result of increased muscle mass. Increased fat mass is a common result of a high-fat diet in C57BL/6 mice[16,18,22,45,81,82]. For example, Sitnick et al[16] measured epididymal and retroperitoneal fat pads at the end of 14 weeks and saw increases of 117% and 106% above control values, respectively.

Current literature has not provided a definitive answer on whether increased muscle mass is to be expected with a high-fat diet. Sitnick et al[16] reported minor increases of 5% and 7% in the gastrocnemius and soleus, neither reaching significance, while Zhou et al[22] showed significant decreases of 11% and 12% in the soleus and extensor digitorum longus. Both studies used a diet lower in fat (~40% kcal fat) than ours (60% kcal fat), but a crucial difference between our diet and the one used by Zhou et al[22] was diet duration. A 12-week high-fat diet is sufficient to induce insulin resistance in a C57BL/6 mouse[18], as demonstrated by our data (Figure 4.5). Zhou et al[22] fed their mice a high-fat diet for 5 months. It is possible that these animals developed type II diabetes by the time of data collection. Unregulated type II diabetes is a condition well characterized by increased rates of protein degradation and decreased muscle

mass[8]. While increased mass overall is entirely consistent with other literature, increases in both fat and fat-free mass likely depend on factors such as diet composition and duration.

Myostatin is a major negative regulator of skeletal muscle mass. In our study, serum myostatin of the 12wkHF, but not 3wkHF, was over 2 fold higher than the control group (Figure 4.6). This finding is consistent with other studies in current literature. Lyons et al[18] demonstrated in mice that a 12-week high-fat diet increased mRNA for myostatin in muscle 2.5 fold. Further, Hittel et al[17] showed myostatin protein levels are increased in skeletal muscle and plasma from extremely obese humans. Considering that our findings agree with both human and rodent studies, our experimental design provides an excellent opportunity for studying how muscle mass is regulated during obesity.

Mass of the innervated muscles tended to be higher in the 12wkHF group, but no differences were found for the amount of muscle lost or the rate of protein degradation due to denervation among any of the diet groups (Figures 4.7 – 4.10). The only exception was the higher percent atrophy and rate of protein degradation for the denervated soleus muscle of the 3wkHF group. This goes against our hypotheses that the atrophy response will be reduced in the high-fat groups. As previously discussed, increased muscle mass is consistent with findings from Sitnick et al[16], but not Zhou et al[22]. Sitnick et al did not measure rates protein degradation, but Zhou et al showed increases of 18% and 25% in basal protein degradation for the soleus and extensor digitorum longus, respectively[22]. Again, these differences may be a result of the prolonged diet condition. Currently, no studies have examined how muscles of the obese react when atrophy is induced. Sitnick et al[16] did induce hypertrophy by functionally overloading the plantaris muscle and found obese mice hypertrophied 16% less than controls. They also found that a high-fat diet inhibited the pathway responsible for increasing protein synthesis[16]. In light

of this, it would seem that obese muscle would respond to atrophy by losing more muscle mass. However, our hypotheses were based on a second possibility, that obese individuals may not be as efficient at controlling amino acid metabolism in general. If this were the case, the muscle of the obese would atrophy less. Our data, though disproving our hypotheses, shows that muscle exposed to long-term (12-week) obesity is no more susceptible to atrophy induced by denervation than lean muscle. These findings are surprising considering that increased expression of myostatin alone has been shown to induce atrophy[20]. These mice were exposed to over 2 fold more myostatin than their lean counterparts yet they did not atrophy more (Figure 4.8); in fact, they tended to increase muscle mass (Figure 4.7).

While no differences were found for measures of atrophy between the control and 12wkHF groups, the soleus muscle of the 3wkHF group had a rate of protein degradation 30% higher and lost 10% more mass (Figure 4.10 and 4.8). Under our model, circulating factors such as insulin, free fatty acids, cytokines, and myostatin are presumably the same for all muscles. Therefore, why these differences exist between muscles is unclear. However, it could be related to the fiber type distribution of the soleus muscle in comparison to other muscles of a rodent hind limb. The soleus is highly oxidative and contains the highest percentage of type I fibers at about 58%[34]. This is a point of interest because Type I fibers have a tremendous resistance to most types of atrophy. When exposed to starvation[42] or excess corticosteroids[41], conditions known to induce atrophy, type I fibers only lose about 8% of their mass whereas type II fibers lose over 30% of their mass. Conversely, when type I fibers are exposed to denervation[41] the amount of mass lost increases to around 30%. Another point to consider is that a short-term high-fat diet causes oxidative adaptations in skeletal muscle. de Wilde et al[45] showed that after a 4-week high-fat diet, rodent skeletal muscle increased protein for type I myosin heavy chain and complexes I-

V of the electron transport chain. It is possible that this shift towards a more oxidative profile may have increased the soleus's susceptibility to denervation. To test this theory one could conduct a study using a similar short-term (3-week) high-fat diet and induce atrophy using excess corticosteroids, for example. If the fiber-type differences are no longer apparent, it suggests that an increased oxidative profile may in fact be making the soleus more sensitive to denervation.

Mice fed a typical long-term high-fat diet, 12 to 14 weeks, have over twice the concentration of circulating myostatin as controls, yet they do not appear to increase basal protein degradation or atrophy more in response to denervation. This suggests that these mice have become resistant to the effects of myostatin and may not be able to regulate muscle mass in the same manner as controls. What is causing this resistance is not known, but it could involve the metabolic inflexibility commonly associated with obesity[12,15]. Metabolic inflexibility refers to an individual's inability to switch between different substrates, even when the cellular environment favors a specific substrate[12]. The most notable form of metabolic inflexibly is insulin resistance, which impairs glucose and lipid metabolism[14]. It is reasonable to suspect that an additional component of metabolic inflexibility might be the inability to mobilize amino acids and it could be due in part to this myostatin resistance.

Clinical Relevance

Muscle mass and obesity has become a topic of interest over that last decade. This may be due in part to the congruent emergence of the obesity paradox and sarcopenic obesity. The obesity paradox refers to how obese individuals are at higher risk of developing chronic diseases than lean individuals, but they tend to live longer once the incident of disease occurs[83]. This is

particularly true for individuals who are classified by their body mass index (BMI) as overweight (BMI of 25 – 29.9 kg/m^2) or grade 1 obese (BMI of 30 – 34.9 kg/m^2)[84]. If an individual crosses into obesity grades 2 (BMI of 35 – 39.9 kg/m^2) or 3 (BMI ≥ 40 kg/m^2), mortality increases[84]. Mortality increases even higher if muscle mass is decreased, a condition known as sarcopenic obesity[85].

The reason the obesity paradox exists is unclear, but two theories have been suggested. First, disease states are often characterized by an increased state of catabolism causing energy requirements to drastically increase[48]. The increased adiposity of an obese individual allows them to survive longer while leaner individuals become malnourished and succumb to the disease sooner[86]. Secondly, it may be due to the genetic disposition of the leaner individual becoming sick in the first place. Obesity induced disease may be associated with better outcomes in general, where as a lean individual who develops disease due to genetic factors may be more susceptible from the beginning[86].

Since the genetic background of the mice in our study is identical between groups, our data supports the first theory. During a highly catabolic state it is typical for skeletal muscle to be degraded to mobilize amino acids for synthesizing proteins for antibodies or rebuilding damaged tissue[48]. In a healthy individual after the amino acid demand returns to normal, skeletal muscle mass is replenished by a dietary source of amino acids[48]. When an individual becomes sick the demand for protein is so high that dietary sources, including amino acid supplementation, is not enough to maintain muscle mass[48]. Eventually muscle mass is degraded to a point where it can no longer support vital metabolic processes and the person dies[48].

Our data might shed some light on the obesity paradox as it suggests that obese skeletal muscle is not as readily degraded by elevated levels of myostatin as lean skeletal muscle,

allowing obese individuals to survive longer with disease. Considering that myostatin is abundant in many of the disease states associated with muscle wasting[7,51,87,88], this resilience may be associated with the apparent myostatin resistance seen in our mice. If the mechanisms behind myostatin resistance could be established, perhaps a new target area for treating muscle wasting in all populations could be developed.

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APPENDIX: ANIMAL CARE AND USE COMMITTEE APPROVAL LETTER

East Carolina University

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

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

January 13, 2012

Jeffrey Brault, Ph.D.
Department of Kinesiology
Brody 3W-40A
ECU Brody School of Medicine

Dear Dr. Brault:

Your Animal Use Protocol entitled, "Muscle Atrophy in Diet-Induced Obesity" (AUP #P073) was reviewed by this institution's Animal Care and Use Committee on 1/13/12. The following action was taken by the Committee:

"Approved as submitted"

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

Sincerely yours,

Scott E. Gordon, Ph.D.
Chairman, Animal Care and Use Committee

SEG/jd

enclosure