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The Effects of ApoE Knock Out on Skeletal Muscle Regeneration: A Look into Markers of Protein Synthesis and Satellite Cell Function

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The Effects of ApoE Knock Out on Skeletal Muscle Regeneration: A Look into Markers of Protein Synthesis and Satellite Cell Function

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ABSTRACT

ApoE helps regulate serum cholesterol levels by adding in transport of cholesterol into the cells, as well as, to the liver. ApoE knockout mice (ApoE KO) present a model that demonstrates the effects of hypercholesterolemia. Damage to muscle stimulates a complex regenerative response. The effects of high cholesterol on this regenerative response are not known. **PURPOSE**: To determine if skeletal muscle regeneration is altered in apoE KO mice by measuring protein synthesis regulator IGF-1, Akt, mTOR, and p70S6K, cell cycle regulator cyclin D1, and myogenic regulatory factors myoD and myogenin. **METHODS**: Female C57/BL6 (WT) and apoE KO were assigned to wither an uninjured or injured group. To induce skeletal muscle damage the tibialis anterior (TA) was injected with either bupivacaine or phosphate buffered saline (PBS). TA muscle was extracted 3 days post-injection. Quantitative PCR was conducted to determine gene expression for IGF-1, myoD, myogenin, and Cyclin D1. Western blot analysis was performed to quantitate the markers of protein synthesis Akt, mTOR, and p70S6K. **RESULTS**: IGF-1 gene expression increased 5-fold ($p < 0.05$) and 3.5-fold ($p < 0.05$) during skeletal muscle regeneration in WT and aopE-KO mice, respectively. Cyclin D1 increased 1.75-fold ($p < 0.05$) in WT mice 3 days post-bupivacaine injection. However, cyclin D1 gene expression increased 12-fold ($p < 0.05$)3 days post-bupivacaine injection in apoE-KO mice. An attenuation of myoD was observed in apoE KO mice, as there was only an 1.5-fold increase occurred in the apoE KO group compared to a 3.5-fold in the WT controls. An increase in the phosphorylated forms of markers of protein synthesis was observed after bupivacaine injection; a 4.44-fold, 2.5-fold, and a 1.0-foldincrease was observed in Akt, mTOR, and p70 respectively. **CONCLUSION**: A deficiency in the apoE gene has no effect on markers of protein synthesis expression but does however have an effect on cyclin D1 and myoD expression.

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CHAPTER 1

INTRODUCTION

Atherosclerosis is a disease characterized by thickening of arterial walls due to plaque accumulation, this plaque is a mix of fat, cholesterol, calcium, and other substances found in the blood(1). Atherosclerosis can lead to many other life threating pathologies such as coronary artery disease (CAD), cardiovascular disease (CV), peripheral artery disease (PAD), and chronic kidney disease; it is also the leading cause of myocardial infarction (MI) in the United States(1).

Cholesterol is a fatty, wax-like substance produced naturally in the human body and can be found in some foods. Cholesterol is used to make hormones, support and repair cell membranes, make vitamin D, and to process ingested foods (1). Lipoproteins package and carry cholesterol, triacylglycerol (TAG), and fatty acids throughout the body. The interior of the lipoprotein is made of cholesterol, TAG, and fatty acids, while the exterior is made of protein (1,2,3). The binding of lipoproteins to receptors located in the cell membrane facilitate the release of the cholesterol into the cell (1,2,3). The protein of greatest importance is a class of proteins called Apolipoproteins. These proteins serve as the primary peptide in lipoproteins. The two classes of most concern are low density lipoprotein (LDL) and high density lipoprotein (HDL). HDL, often called “good cholesterol”, has the task of reverse cholesterol transport where cholesterol is transported back to liver where it is removed from the body. LDL-C, or “bad cholesterol”, transports cholesterol to the cells of the body. Having high LDL increases your risk for atherosclerosis, as well as, angina and myocardial infarction (1,4,5). Apolipoprotein E has a drastic effect on LDL levels as it causes an increase in circulating cholesterol. This excess stimulates an upregulation of LDL, which has negative effects on the total cholesterol level that can lead to CAD, CV, PAD, kidney disease and MI (4).

Protein synthesis is a crucial component in the regenerative response of skeletal muscle. Protein synthesis involves the copying of DNA to an mRNA transcript (transcription) and then taking that mRNA transcript and translating it into a protein (translation). Without protein synthesis the newly differentiated myofibers would lack the ability to contract properly, as well as maintain viability. There are multiple known markers of protein synthesis; these markers have been shown to up regulate following muscle damage and precede regeneration (6, 7, 8, 9). These markers include IGF-1, Akt, mTOR, and p70s6k. In the canonical pathway each of these markers phosphorylates the next and the end result is the upregulation of protein synthesis. The relationship of these markers to one another and to the regulation of protein synthesis has been extensively studied (6, 7, 8, 9).

Skeletal muscle regeneration is dependent on a group of myogenic cells called satellite cells; these cells are found in the basil lamina of skeletal muscle cells. The proliferation and differentiation of these cells cause new nuclei to be added to the fibers allowing for the fiber to increase in size, as skeletal muscle is postmitotic. It has been documented that satellite cell number and activity reaches its peak within three days of muscle damage, this has been confirmed using immunochemical analysis (10, 11, 12, 13, 14, 15). Cyclin D1 is a cell cycle regulator, responsible for the progression of somatic cells through G_1 phase of the cell cycle to S phase of the cell cycle. Cyclin D1 is up regulated during muscle regeneration; this upregulation indicates that cells have become mitotically active (16). Another regulator of satellite cell function are the myogenic regulatory factors (MRFs), these include myoD and myogenin. MyoD has a dual role in the function of satellite cells; it regulates satellite cell proliferation and regulates myogenin (10). Myogenin is responsible for the differentiation of satellite cells only;

as when myogenin was inhibited and MyoD was left to function normally proliferation occurred while a decrement in differentiation was observed (10).

The effects of hypercholesterolemia on arterial endothelial cells are well documented; however very little research has investigated the effects on skeletal muscle. Multiple studies have shown that high cholesterol impairs angiogenesis after arterial injury (17, 18, 19, 20, 21). The effect of hypercholesterolemia on the repair of arterial smooth muscle raises a question on the effects it could possibly have on skeletal muscle. Literature on high cholesterol's effects on skeletal muscle is limited. One study has shown that after ischemia induced muscle damage skeletal muscle healing is delayed (22). The mechanism attributed to this delay was a chronic inflammatory response that persisted longer in the hypercholeterolemic group than in the control. The inflammatory response was the only mechanism that was investigated in this study leaving questions on the regenerative response of skeletal muscle in hypercholesterolemic hosts. The purpose of this investigation is to examine the effects of hypercholesterolemia on skeletal muscle regeneration by quantitating markers of protein synthesis and satellite cell function in mice that have high cholesterol after a damaging event.

CHAPTER 2

REVIEW OF LIERATURE

This literature review is divided by topic into 4 main sections: 1) hypercholesterolemia's effects on muscle, 2) models of muscle damage and the response of muscle tissue, 3) muscular protein synthesis and its role in muscle regeneration, and 4) satellite cell function as it relates to the regeneration of damaged muscle tissue. This review will include general knowledge on cholesterol and how it has been shown to effect muscle regeneration in previous research, as well

as, how the regenerative response occurs in muscle under normal conditions. The two components of muscle regeneration that will be examined in this study, protein synthesis and satellite cell response. Previous literature on markers of satellite cell function; myoD, myogenin, and cyclin D1 and the role these play in skeletal muscle regeneration will be reviewed. Markers of protein synthesis including IGF-1, AKT, mTOR, p70S6K, will be reviewed in their role in the regenerative response.

I. Hypercholesterolemia and muscle

Hypercholesterolemia and smooth muscle

Hypercholesterolemia, or high cholesterol, is a disease that is characterized by a total serum cholesterol of 240 mg/dL, an LDL level of greater than 70 mg/dL, or an HDL level of greater than 170 mg/dL (1). It is a primary risk factor for many pathologies including heart attack, angina, and cardiovascular disease. Apolipoproteins, a ligand for lipoprotein binding in cellular membrane, helps to regulate circulating levels of cholesterol (23). A subtype, apoE, is responsible for facilitating the binding of LDL's to the cellular membrane (24). Without apoE LDL begins to accumulate in the blood and one begins to develop high cholesterol. Mice genetically altered to lack expression of the gene responsible for apoE production has given a consistent and proven model of hypercholesterolemia (25, 26). ApoE deficient mice have presented total cholesterol levels 5 times higher than normal litter mates (26). These mice also present lower HDL and higher LDL levels compared to normal litter mates (26, 27, 28). Because of the proven effectiveness investigators have consistently used this model in experiments involving high cholesterols effects on various interventions. Several studies have investigated the effects of high cholesterol on arterial smooth muscle (18, 20, 21, 25). These studies suggest that high cholesterol inhibits angiogenesis after arterial injury. These studies

suggest the primary mechanism for this delayed repair lies in the function of the regenerated endothelial cells (EC). The proliferative response to damage seems to be normal in a hypercholesterolemic environment; however, the newly formed EC show dysfunction in the ability to vasodilate and vasoconstrict (18, 21). Contrarily; Rosenbaum, Miyazaki, and Graham (2011) suggest a different mechanism for the delayed healing. In this study a decrease in the early EC proliferation and differentiation was observed suggesting that healing was delayed by a lack of viable cells to repair the injury. Regardless of the mechanism of dysfunction these studies suggest, a normal regenerative response was seen after seven days and the function of the regenerated tissue was below that of normal tissue. Although the mechanism behind the delayed angiogenesis in mice with high cholesterol is unclear, it is fairly definitive that high cholesterol causes a dysfunction in regeneration of arterial smooth muscle.

Hypercholesterolemia and skeletal muscle

While the effects of high cholesterol on angiogenesis are well documented the effects on skeletal muscle have not been extensively studied. To our knowledge only one study has investigated the effects hypercholesterolemia on skeletal muscle regeneration has been completed (22). In this study muscle was damaged by suspending the hind limbs of both WT and hypercholesterolemic (apoE deficient) mice causing ischemia due to lack of blood flow. A delay in muscle regeneration was observed in the apoE deficient mice but not in the WT controls. Results suggest the apoE deficient mice have a prolonged inflammatory response not seen in the WT controls. No significant difference was observed in a marker for satellite cell proliferation (myoD); however, a significant difference was observed in the marker for satellite cell differentiation (myogenin). A dual mechanism for the delayed regenerative response involving dysfunction in both satellite cell function and inflammatory response is suggested.

The suggested dysfunction of satellite cells in this experiment agrees with the results of Rosenbaum et al. (2011). The differentiation of satellite cells seems to be significantly impaired by hypercholesterolemia.

II. Models of muscle damage and the response of muscle tissue

Scientists use various models of muscle damage in research involving markers of regeneration. These studies are used to investigate disease related atrophy, age related atrophy, and many other disease related dysfunctions in muscle regeneration. Several models of muscle damage exist; however, some models are more popular because of the lack of limitations and confounding factors. One of the more diverse models used to investigate the regenerative response is the model of eccentric exercise. This model is unique in that it can be used in research involving muscle regeneration after exercise induced injury in a diseased or biologically deficient population, response to exercise in the elderly, and the hypertrophic response of muscle in a healthy population. A classic study on eccentric exercise induced muscle damage showed downhill running to be a more effective way to cause muscle damage compared to level or uphill running (29). Results showed a larger increase and a higher total level of plasma enzymes and macrophages associated with necrosis of muscle tissue common with muscle damage in the downhill running group compared to the uphill or level running group. An unintended result was the observation of mitotic "figures" resembling satellite cells near the damaged muscle fibers. Although these figures were not identified definitively the assumption can be drawn that eccentric exercise in the form of downhill running up regulates satellite cells in response to injury. Although this model is effective in causing muscle damage it does require a good deal of time and supplies. This model requires a specialized treadmill as well as a technician trained to use the equipment as the animals or human subjects must be monitored while performing the

action. This is also an exercise model causing it to be used primarily in hypertrophy studies or in research investigating the response of a particular population to exercise. Because of these factors other models of muscle damage are frequently used. Two models commonly used in research investigating atrophy are hind limb immobilization and suspension. In both models protein synthesis is decreased during the period inactivity, this is paired with increased protein degradation and a decrease in myonuclear domain(30). The increase in protein degradation and decrease in protein synthesis are indicative of muscle cell dysfunction caused by the immobilization of the limb, while the decrease in myonuclear number suggest muscle cell death as a result of this dysfunction. Upon remobilization of the limb increases in satellite cell proliferation, myonuclear number, and protein synthesis were observed. The increase in satellite cell proliferation in combination with an increase in myonuclear number suggests that in response to tissue damage satellite cells are mobilized and differentiate to aid in the regeneration of viable muscle tissue. Concurrently the increase of protein synthesis above baseline values suggests not only a return to normal function of the cell but also a role of protein synthesis in the regenerative response. The model chosen for this study was a bupivacaine (Marcaine) injection into the muscle, as it is the least time intensive models that can cause muscle damage. A classic study by Benoit and Belt (1970) investigated the effects of the long lasting anesthetic bupivacaine. Previously research had definitely proven that local anesthetics like lidocaine causes damage to skeletal muscle that is followed by regeneration of new muscle fibers. This study investigated the effects of the long lasting anesthetic, bupivacaine, on muscle fibers and the regenerative response to the damage it caused. After a single injection of bupivacaine the damaged muscle fibers were seen throughout the muscle, up to one half of the muscle thickness in depth. Fourteen hours post injection macrophages were observed invading the tissue that had

been injected, the first evidence that muscle damage had occurred. At three days post injection a few singly nucleated myoblasts were seen in combination with a significantly reduced number of macrophages and an increased amount of multinucleated tubes. All this in combination suggests after damage induced by bupivacaine injection a regenerative response involving satellite cell proliferation and differentiation occurs. Sequential injections of bupivacaine caused further damage to the muscle identical to that of a single injection followed by an identical regenerative response (11). The regenerative response was not delayed or prolonged by sequential injections of bupivacaine; the only differences were the amount of damage incurred and the total of regenerative markers observed.

III. Protein synthesis

Protein synthesis is an event that occurs in all the cells of the body. This process involves taking a portion of DNA code and copying it onto an mRNA transcript (transcription), then taking this copy and assembling the protein that the code is specific to (translation). This is an essential process in all cells as it provides the proteins needed to make the structural components of cells, as well as, creates the enzymes that catalyze the reactions of the cells. A cascade of reactions involving many intermediates is required to up regulate transcription and translation. These intermediates and reactions will be covered in this section of the literature review.

IGF-1

IGF-1 is a hormone produced in the body by the liver and also by organ that frequently use the hormone, this happens in an autocrine/paracrine fashion. When muscle receives an overload stimulus an upregulation of IGF-1 is observed (31, 32). This upregulation is attributed to an increase in production by the damaged myofibers using the aforementioned autocrine/paracrine relationship (31). Although these results do not provide insight into the

mechanism of action for IGF-1 it does provide us with evidence that when muscle damage occurs IGF-1 concentrations increase.

An increase in IGF-1 gene expression and IGF-1 concentration has been shown to precede hypertrophy in response skeletal muscle overload in rats (32, 33, 34). It has also been shown that a local overexpression of IGF-1 promotes an increase in contractile protein mRNAs that precedes a regenerative and hypertrophic response (35). mRNA is a component of the transcription stage of protein synthesis, the increase in mRNA suggests a possible increase in protein synthesis. This data leads us to postulate that an increase in IGF-1 causes an increase in protein synthesis. A study using mice with chronic renal failure showed that a decrease in IGF-1 caused by the disease lead to a decrease in skeletal muscle protein synthesis (9). The data from these two studies suggest that changing IGF-1 concentrations has an effect on protein synthesis in skeletal muscle.

Although IGF-1 has been shown to affect protein synthesis through an increase in mRNA, it does not act directly on DNA. A cascade of reactions occurs to cause this upregulation in protein synthesis. A study by Glass (2005) gave a detailed investigation of the canonical pathway involving IGF-1, AKT, mTOR, and p70s6k with each intermediate being activated, at least in part, by its predecessor in the pathway. A study by Rommel et al. (2001) suggests that IGF-1 induced hypertrophy requires an activation and phosphorylation of AKT. This was shown when a decrease in protein synthesis and hypertrophy occurred in mice where AKT was inhibited through gene manipulation. The same results were seen when other downstream targets of IGF-1 such as mTOR and p70s6k were blocked. Another study duplicated these results showing that an increase in IGF-1 alone is not sufficient to cause skeletal muscle regeneration or hypertrophy (36). The data from previous studies suggests that to

accurately assess the response of a host to damaging stimuli via protein synthesis, more than IGF-1 concentration must be quantified.

Another point of interest found in the literature is the role of IGF-1 in satellite cell function. Mice genetically altered to overexpress IGF-1 were shown to have significantly higher concentration of satellite cells after injury then wild type controls (13), suggesting that upregulation of IGF-1 causes satellite cells to proliferate. Support for this hypothesis can be found in the review by Phillippou et al. (2007). Another study suggests that IGF-1 is released by proliferating satellite cells and that the observed increase in IGF-1 expression is caused by the increase in proliferation not the reverse (37). An alternative hypothesis is that the IGF-1 mediated increase in protein synthesis causes quiescent satellite cells to proliferate (32). Although the role of IGF-1 in satellite cell function has not been extensively studied, the aim of this study was not to investigate these effects. The fact that IGF-1 is postulated to affect satellite cell function simply adds to the importance of quantifying its expression in this experiment.

AKT

The next step in the conical protein synthesis pathway is phosphorylation of AKT (protein kinase B). Phosphorylation of AKT is dependent upon a reaction with phosphoinositide-dependent-kinase-1 (PDK1). An increase in activation of AKT has been observed to precede skeletal muscle hypertrophy, a response similar to that observed in regenerative models (7, 38, 39, 40). It has also been shown that an increase in AKT phosphorylation, dependent of an increase in IGF-1 concentration causes a statistically significant increase in skeletal muscle hypertrophy (7, 38, 40). It should be noted that in Bodine et al. (2001) there were no damaging stimuli and consequently no increase in satellite cell proliferation, suggesting that an increase in AKT phosphorylation alone is sufficient to induce

skeletal muscle hypertrophy. Mice genetically altered to over express AKT were observed having skeletal muscle mass two- to three-fold larger than WT control animals (40). These mice were not introduced to any damaging stimuli and were fed identical diets, further supporting the postulation that AKT alone is sufficient to induce skeletal muscle hypertrophy assuming the proper function of its downstream targets. Although AKT can cause an increase of protein synthesis that can induce skeletal muscle hypertrophy, it is similar to IGF-1 in that it does not have this effect without activation of its downstream targets. Without the activation of mTOR by phosphorylated AKT muscle hypertrophy does not occur, this suggested in multiple studies where mTOR was inhibited by rapamycin after a damaging stimulus was induced (7, 39). An increase in phosphorylated AKT was observed with no increase in mRNA concentration or skeletal muscle hypertrophy. The data from these studies further support the importance of the downstream cascade of this pathway.

mTOR

As previously noted mTOR is biochemically regulated by phosphorylated AKT; however, in the absence of phosphorylated AKT skeletal muscle contraction can cause mTOR phosphorylation (41). It has also been observed that regardless of concentration of phosphorylated AKT, if IGF-1 concentration is elevated mTOR phosphorylation occurs (8). This suggests that although AKT is part of the conical pathway, increased protein synthesis is most dependent on an increase in IGF-1 expression and the subsequent phosphorylation of mTOR.

The primary role of mTOR is to phosphorylate p70s6k, p70s6k and its role will be discussed in the next section. Multiple studies suggest that a lack of mTOR phosphorylation leads to a decrease in mRNA synthesis, protein synthesis, and subsequent skeletal muscle

hypertrophy (7, 12, 39). It has also been shown that a complete inhibition of mTOR causes no negative effect on muscle (13), suggesting that mTOR's role lies strictly in the regeneration and hypertrophy responses in skeletal muscle and play no role in cell maintenance.

p70s6k

P70s6k is the primary downstream target of mTOR. Studies suggest that phosphorylation of mTOR is the only mechanism that causes an increase in p70s6k in skeletal muscle cells (35). P70s6k is a protein kinase that phosphorylates S6, a ribosomal protein that causes an upregulation of mRNA that code for translation of DNA codons. This increase in translation rate is the primary mechanism of protein synthesis (8, 42). It has been shown in multiple studies that an increase in p70s6k phosphorylation precedes an increase in translational rate and subsequent protein synthesis (8, 42, 43). Inhibition of p70s6k through genetic alteration of mice supports the previous data, as there was no phosphorylation of S6 seen in these mice (42). There was also no upregulation of mRNA synthesis or protein synthesis in this model, suggesting that an upregulation of protein synthesis is dependent on phosphorylation of p70s6k.

IV. Satellite cell function

Myosatellite cells or satellite cells are myogenic cells that line the basal lamina of all human muscle tissue. As mature muscle fibers are postmitotic, meaning they cannot forgo cell division, satellite cells are essential for myogenesis to occur. Three stages in satellite cell function exist, activation which occurs as a result of damaging stimuli, proliferation in which these cells become myoblasts, and finally differentiation in which these cells become a more complex

myofiber. Known genetic markers of the proliferation and differentiation can be quantified using a variety of laboratory tests.

MyoD/ Myogenin

MyoD and myogenin are two of the genetic markers used to quantitate satellite cell function and both have a crucial role in the regenerative response of skeletal muscle. Although concentration of satellite cells can be quantified by using immunochemical analysis (14, 15, 44) these results are only useful in quantitating the number of satellite cells not the mechanism behind the activation.

The role of myoD and myogenin in proper satellite cell proliferation was first postulated in a classic study by Fuchtbauer and Westphal (1991). This study suggested a dual mechanism by which an increase in myoD and myogenin caused satellite cell proliferation and differentiation, that fact has since been disputed. A more recent study suggests that the role of myoD is to initiate the proliferation of active satellite cells, as well as, to up regulate the production of myogenin (15), while myogenin has the role of specializing these cells (differentiating). Data suggest that in absence of myoD not only did activated satellite cells not proliferate; there was also a deficiency in myogenin not seen in wild type control animals. There was also a decrement in the differentiation of the satellite cells, this was attributed to the lack of myogenin upregulation because when myoD was left to function normally and myogenin was inhibited satellite cells proliferated but did not differentiate. A deficiency in myoD expression through genetic manipulation showed a dysfunction in the total regenerative response, regardless of myogenin concentration (14). This data supports the notion that the myogenic response to skeletal muscle damage is dependent on an increase in myoD concentration and the subsequent proliferation of satellite cells, as well as, the myoD dependent increase in myogenin.

A study by Lowe and Alway (1998) suggest that myoD and myogenin concentrations are increased as a response to overload. This study also suggests that the release and upregulation of these proteins originates from mature myofibers that were exposed to the damaging stimuli. From the results of this study it can be postulated that in a normally functioning host any damaging stimuli will cause an upregulation of these proteins and a dysfunction of this upregulation will effect skeletal muscle regeneration.

Cyclin D1

Cyclin D1 is a protein involved in regulation of the cell cycle. As cells enter G_1 phase (the first growth phase of the cell cycle) an increase in cyclin D1 is observed (46, 47). During this phase the cell is increasing in size and preparing for DNA synthesis. Cyclin D1 has a dual role during this phase of the cell cycle. The first is phosphorylating downstream targets that result in the upregulation of DNA polymerase, a key step in somatic cell mitosis. Another primary function of cyclin D1 is to bind to and phosphorylate cyclin- dependent kinases 4 and 6 to form CDK4 and CDK6, these complexes are responsible for the transition from G_1 to S phase of the cell cycle. It is postulated that the increase in the CDK4 and CDK6 complexes are responsible for communicating the correct time for the transition out of G_1 (48).

Cyclin D1 is relevant to satellite cell function because an increase in cyclin D1 concentration is seen following a damaging stimulus. This was suggested when cyclin D1 was measured following heavy resistance exercise regimen (49). It is postulated that the activation of cyclin D1 has a role in satellite cell function as an increase in cyclin D1 correlated with an increase in satellite cell proliferation and differentiation (49), suggesting that a similar increase in the CDK complexes causes satellite cells to progress through the steps of maturation into myofibers. It should be noted that no significant increase was observed in cyclin D1

concentration at any time point of detraining suggesting that cyclin D1 is only up regulated in response to damage, not in response to atrophy (49).

In summary, multiple factors play a role in both damage induced protein synthesis as well as satellite cell function in response to a damaging stimulus. There are multiple intermediates that play key roles in the downstream cascade of reactions involved in the upregulation of protein synthesis. Of these, it seems that IGF-1 and mTOR are of the utmost importance, as AKT phosphorylation is dependent on IGF-1 expression. Also, the absence of AKT, IGF-1 can cause the downstream phosphorylation of mTOR usually enacted by AKT. It is also important to note that p70s6k cannot be left out of the pathway as it is the intranuclear target of mTOR and has direct effect on mRNA synthesis. Similarly, many different proteins control the proper function of satellite cells. MyoD regulates proliferation of satellite cells, while myogenin controls the specialization (differentiation) of the proliferated cells. The cell cycle regulator cyclin D1 assists in this process with a mechanism similar to that seen in somatic cells, where it is responsible for the maturation and progression from proliferation to differentiation.

The data in total suggests that all of these markers up regulate in response to damaging stimuli, inferring that in a normally functioning cell these markers should be in seen at levels higher than baseline. This information gives proficient evidence that quantifying these markers and then comparing to baseline, as well as, normally functioning control hosts given the same experimental intervention should provide insight into the effects hypercholesterolemia on skeletal muscle regeneration.

CHAPTER 3

SPECIFIC AIMS

Hypercholesterolemia, commonly called high cholesterol, is a condition in which total serum cholesterol is greater than 240 mg/dL, LDL cholesterol above 70 mg/dL. Of most concern is LDL cholesterol level and its control as LDL's role in the body is to transport cholesterol into the cell. Over 38% of Americans have high LDL cholesterol (1). High cholesterol is a primary risk factor for cardiovascular disease (CVD), angina, atherosclerosis, and myocardial infarction.

Skeletal muscle regeneration involves the coordinated response of protein synthesis upregulation and satellite cell activation. Protein synthesis is up regulated in response to a damaging stimulus (30). One way to quantitate the function of skeletal muscle tissue is to measure the concentration of protein synthesis markers. These markers include IGF-1, Akt, mTOR, and p70s6k. Each of these markers up regulate during muscle regeneration (34, 7). Satellite cells react to a damaging stimulus by proliferating and differentiating. The cell cycle regulator cyclin D1 and myogenic regulatory factors (MRFs) control these processes. It has been shown that cyclin D1 and MRFs are up regulated during muscle regeneration in normal conditions (47, 44).

The effects of hypercholesterolemia on smooth muscle like that found in the arteries and veins have been extensively studied. A dysfunction in the repair of veins and arteries has been shown to occur in mice genetically altered to be hypercholesterolemic (apoE KO) (7, 9, 10, 14). In these studies the repair of the damaged smooth muscle occurred; however, the newly developed cells lacked normal function (7, 10). A study by Rosenbaum, Miyazaki, and Graham (2011) showed a dysfunction in proliferation and differentiation of EC cells (vascular satellite cells) 3 days post injury did not occur in mice with high cholesterol, while the wild type (WT)

controls maintained this response. This suggests that angiogenesis was delayed due to a lack of viable cells to repair the injury.

The existing literature gives insight into the effects of high cholesterol on cardiac smooth muscle regeneration following injury; however, investigation into high cholesterol and the effect it has on skeletal muscle regeneration is limited. Ischemia-reperfusion, a model of muscle damage, was used in aopE-KO mice. This study demonstrated that skeletal muscle regeneration was delayed in apoE KO mice and this was attributed to an increased and prolonged inflammatory response and decreased myogenin levels. Although this study examined the effects of hypercholesterolemia on skeletal muscle regeneration, it left significant investigation to be completed. Based on previous literature high cholesterol has some effect on satellite cell function, as well as, protein synthesis**.** This has serious real world implications as many patients suffering from hypercholesterolemia are also co-morbid with obesity, and common practice to combat obesity is resistance training. If having high cholesterol negatively effects muscle regeneration then these patients may be contraindicated for exercise. **Our central hypothesis is that abnormal muscle regeneration that involves a dysfunction in protein synthesis and/or satellite cell function will occur in apoE KO mice.**

Aim 1. To determine if hypercholesterolemia has an inhibitory effect on markers of protein synthesis during skeletal muscle regeneration. Experiments will examine the process of protein synthesis in a hypercholesterolemic host. ApoE KO mice will be used as these mice are proven models of hypercholesterolemia. Experiments will examine upstream and downstream markers of protein synthesis including IGF-1, Akt and p-Akt, mTOR and p-mTOR, p70s6k and p-p70s6k. This will allow investigators to determine high cholesterol effects protein

synthesis and where this effect occurs. These markers will be measured from tissue taken 3-days post bupivicaine injection. **We hypothesize that IGF-1 will be lower in apoE KO mice compared to WT mice during skeletal muscle regeneration. We also hypothesize that each downstream marker (Akt, mTOR, p70s6k, and their phosphorylated forms) will be lower. in apoE KO mice compared to WT mice during skeletal muscle regeneration. Aim 2. To determine if hypercholesterolemia has an inhibitory effect on markers of satellite cell function during skeletal muscle regeneration.** Experiments will examine three markers of satellite cell function. These markers include cell; cylin D1, myoD, and myogenin; as all of these are known to be up regulated when satellite cells function normally in a regenerative response. These markers will be measured in tissue taken 3-days post bupivicaine injection. **We hypothesize that cyclin D1 concentrations will be lower in apoE KO mice compared to WT mice during skeletal muscle regeneration. We hypothesis that myoD concentrations will be lower in apoE mice compared to WT mice during skeletal muscle regeneration. We also hypothesize that myogenin concentration will be lower in apoE KO mice compared to WT mice during skeletal muscle regeneration.**

CHAPTER 4

METHODS

Animals and housing

70-80 week old apoE KO mice were donated by Rigel Pharmaceuticals; 12 week old WT mice were purchased from Jackson Laboratories. Animals were housed in the University of Arkansas Central Laboratory Animal Facility. Animals were kept on a 12:12-h light-dark cycle, and given access to normal rodent chow and water for the duration of the study. The mice were randomly assigned to one of two groups: 1) uninjured (control; n= 12) or 2) injured (n = 6). All

procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC).

Bupivacaine Injection

Mice were anesthetized with a subcutaneous injection of a cocktail containing ketamine hydrochloride (45 mg/kg body weight), xylazine (3 mg/kg body weight), and acepromazine (1 mg/kg body weight). Muscle damage was induced by injecting 0.03ml of 0.75% bupivacaine (Marcaine) in the left and right tibialis anterior (TA). A 25-gauge, 5/8 (0.5 X 16 mm) needle was inserted along the longitudinal axis of the muscle, and the bupivacaine was injected slowly as the needle was withdrawn. Bupivacaine was delivered in an isotonic solution of NaCl. The control group was injected with 0.03ml of phosphate buffered saline (PBS).

Muscle and Tibia Extraction

Three days post-bupivacaine injection, the TA and tibias were extracted. Mice were anesthetized with a subcutaneous injection of a cocktail containing ketamine hydrochloride (90 mg/kg body weight), xylazine (3 mg/kg body weight), and acepromazine (1 mg/kg body weight). The left TA was snap frozen in liquid nitrogen and stored at -80°C for protein and gene expression analysis. After the TA was dissected out it was weighed, then the tibia was removed and measured with a plastic caliper.

Western blotting

Tissue will be homogenized in Muller Buffer and protein concentration was measured using the Qubit 2.0® (Invitrogen). Muscle homogenate (40ug) will be fractionated in a 7-12% SDS-polyacrylamide gels. Gels will be transferred overnight to polyvinylidene difluoride (PVDF) membranes. Membranes will be Ponceau S stained before blotting to verify equal loading of the gels. Membranes will be blocked in non-fat milk, in Tris-buffered saline with

0.1% Tween-20 (TBST), for 2 hours. Primary antibodies for Akt (CST-4691), pAkt (Ser 473; CST-4060). mTOR (CST-2983), p-MTOR (Ser 2448;CST-5536), p70s6k (CST-2708) and p-p70s6k (Thr 389; CST-9234) will be diluted 1:2000 to 1:10,000 in 5% non-fat milk, in TBST, and incubated at 4°C overnight. Anti-rabbit secondary antibodies (Santa Cruz) will be diluted 1: 10,000 in non-fat milk, in TBST, and then incubated at room temperature for 1 hour. Enhanced Chemiluminescence (ECL) will be performed using Fluorochem M imager (Protein Simple, Santa Clara, California) or the Li-Cor C-Digit ™ Blot Scanner to visualize antibody-antigen interaction. Blotting images will be quantified by densitometry using Alphaview™ software. The Ponceau-stained membranes will be digitally scanned, and the 45-kDa actin bands will be quantified by densitometry and used as a protein loading correction factor for each lane.

RNA Isolation, cDNA synthesis, and quantitative RT-PCR

RNA will be extracted using a Trizol reagent (Life Technologies, Grand Island, NY, USA). Total RNA will be isolated, DNase treated and concentration and purity will be determined by fluorometry using the Qubit 2.0 (Life Technologies). cDNA will be reverse transcribed from 1 µg of total RNA using the Superscript Vilo cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA). Real-time PCR will be performed, and results were analyzed by using the ABI StepOne Real-Time PCR System (Step One; Applied Biosystems, Foster City, CA, USA). cDNA will be amplified in a 25 µL reaction containing appropriate primer pairs and TaqMan Universal Mastermix (Applied Biosystems). Samples will be incubated at 95°C for 4 min, followed by 40 cycles of denaturation, annealing and extension at 95°C, 55°C and 72°C respectively. TaqMan fluorescence will be measured at the end of the extension step each cycle. Fluorescence labeled probes for MyoD (FAM dye), myogenin (FAM dye), cyclin D1 (FAM dye) and IGF-1 (FAM dye), dye will be purchased from Applied

Biosystems and quantified with TaqMan Universal mastermix. Cycle threshold (Ct) will be determined, and the ΔCt value calculated as the difference between the Ct value and the 18s Ct value. Final quantification of gene expression was calculated using the ΔΔCT method Ct = [ΔCt(calibrator) – ΔCt(sample)]. Relative quantification will then be calculated as $2^{-\Delta\Delta Ct}$.

Data analysis. Results are reported as mean SE. Data were analyzed using 2-way ANOVA test. To assess main effects and interactions and if significant main effects or interactions are found, group differences will be analyzed by Bonferonni analysis. Statistics will be performed with Sigma Stat with the significance level set at $p < 0.05$.

CHAPTER 5

RESULTS

Muscle and Bone Data

Immediately following extraction the TA was weighed and the tibia bone was measured. There was a significant decrease in the wet weight in both the injured B6 and apoE-KO groups when compared to the uninjured group, while no significant difference was seen in the length of the tibia in any group (Table 1). The ratio of muscle weight to bone length was significantly lower in both injured groups. TA mass to tibia length decreased 20.8% (2.4 ± 0.1 vs. 1.9 ±0.2 mg/mm) ($p < 0.05$) and 15.4% (2.98 ± 0.19 vs. 2.52 ± 0.12 mg/mm) ($p < 0.05$) 3 days post injection in B6 and apoE injured groups respectively.

Table 1
Muscle Mass, Bone Length, and Mass to Length Raatio *Note.* There was a significant difference between tibialis anterior length in both injured groups of mice while no difference was observed in tibia length in either group. A significant difference was observed in muscle mass/tibia/length in both te B6 and apoE KO groups respectively (20.8% and 15.4% respectively).

	Wild Type		**apoE Knockout**	
	uninjured	injured	uninjured	injured
Tibialis anterior (mg)	38.3 ± 1.4	31.9 ± 3.0*	50.63 ± 3.22	43.70 ± 2.16*
Tibia length (mm)	15.8 ± 0.6	16.8 ± 0.1	17.30 ± 0.05	17.36 ± 0.12
Muscle mass/Tibia length (mg/mm)	2.4 ± 0.1	1.9 ± 0.2*	2.98 ± 0.19	2.52 ± 0.12*
Body weight (g)	24.65 ± 1.59	24.13 ± 1.05*	24.37 ± 1.56	25.87 ± 1.56*

Gene Expression of Myogenic and Growth-related Factors

An increase in myoD expression of 2.4 fold was seen in injured B6 compared to uninjured while no change was observed following injury in apoe-KO mice (Figure 1). Although mean increase was seen in both groups following injury, only the increase observed in the B6 control group was significant.

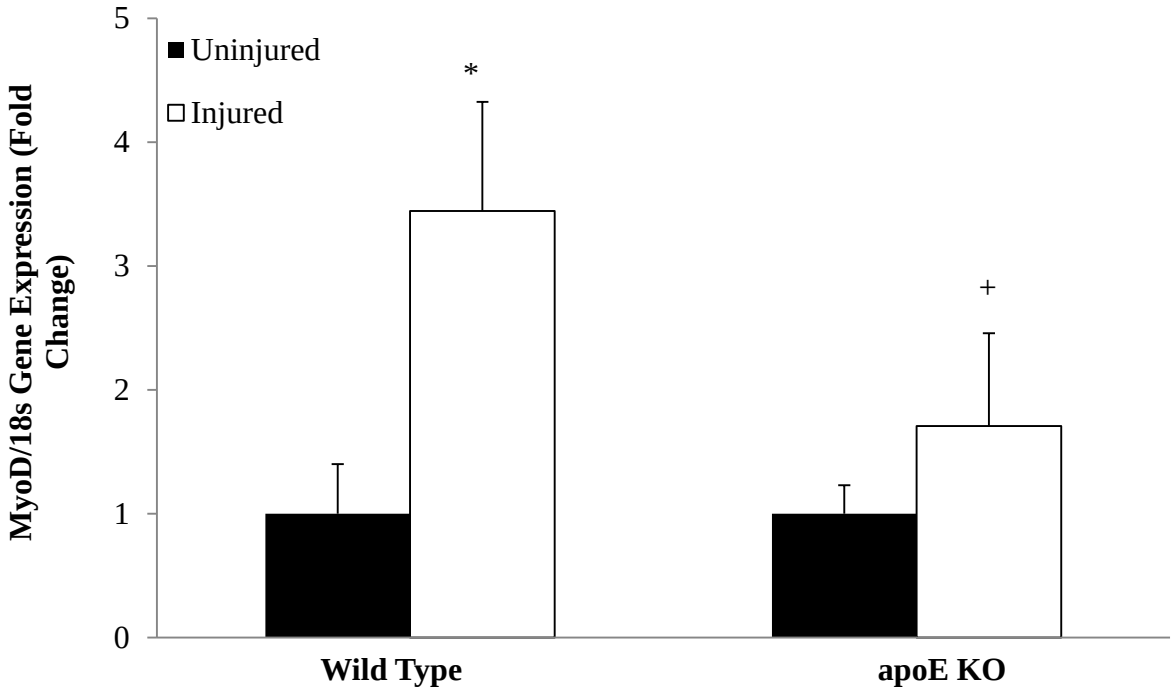


Figure 1.Comparison of fold change in MyoD expression in both WT and apoE KO mice for both injured and uninjured groups. A significant increase ($p<0.05$) was seen in the WT group of injured mice; however, there was a blunted response in the apoE KO injured group.
*Shows significance within group; + Shows significance between groups

Myogenin expression increased in both groups compared to their uninjured counterpart. A 2.1 and 3.3 fold increase was observed in the B6 and apoE-KO groups, respectively (Figure 2). Interestingly, the increase in the apoE-KO group trended towards being significantly greater when compared to the increase observed in the B6 group.

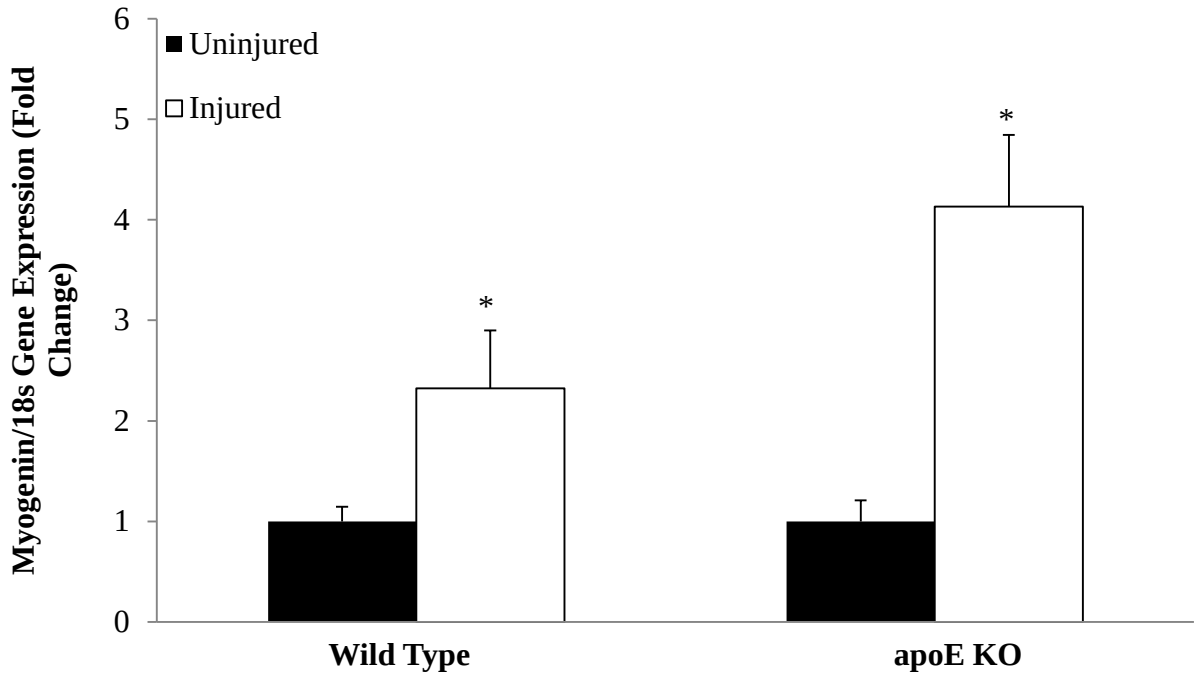


Figure 2. Comparison of fold change in myogenin expression in both WT and apoE KO mice for both injured and uninjured groups. A significant increase ($p<0.05$) in myogenin expression was seen in both groups of injured mice. There was no significant difference between the two injured groups, although there was a trend towards a significant increase in the apoE KO mice.
*Shows significance within group

Similarly, cyclin D1 expression was elevated in both the B6 and apoE-KO injured groups when compared to their uninjured counterparts. A 13 fold increase was observed in the apoE-KO group while no significant increase was observed in the B6 injured group.

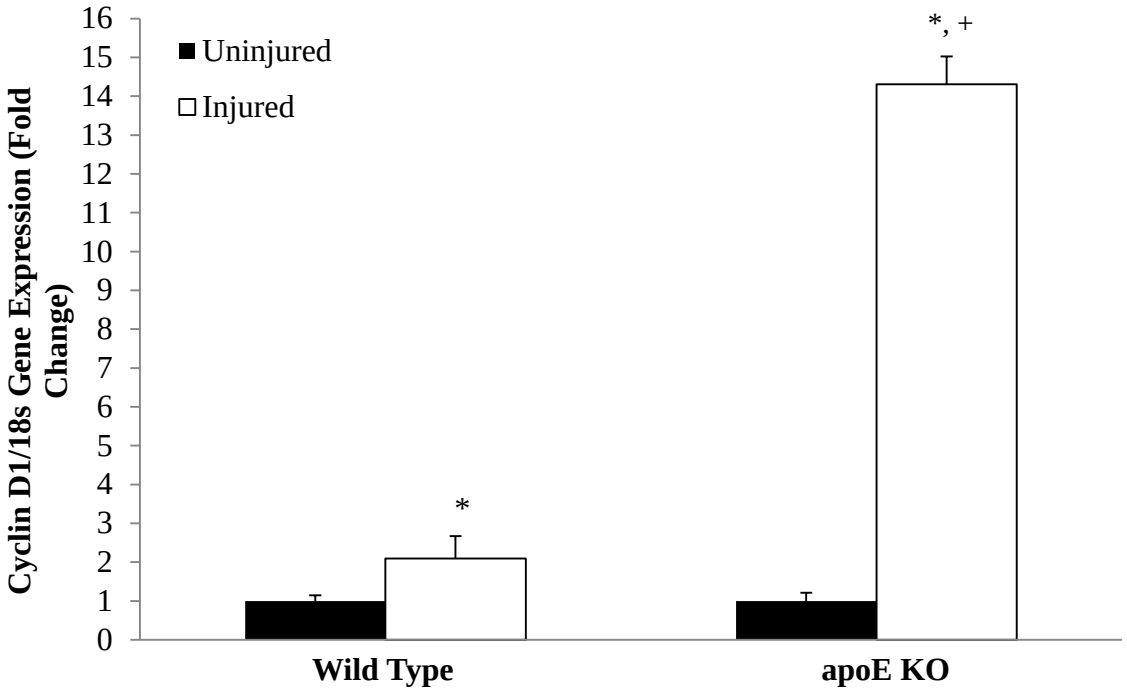


Figure 3. Comparison of fold change in cyclin D1 expression in both WT and apoE KO mice for both injured and uninjured groups. A significant increase ($p<0.05$) was seen in both groups of injured mice; however, a significantly larger increase in expression was observed in the apoE mice.
*Shows significance within group; + Shows significance between groups

IGF-1 expression was observed to increase in response to bupivacaine injection induced muscle damage. Although the apoE-KO group had a slightly lower increase (3.4 fold increases) compared to the B6 group (5 fold increase) there was no significant difference between the two groups.

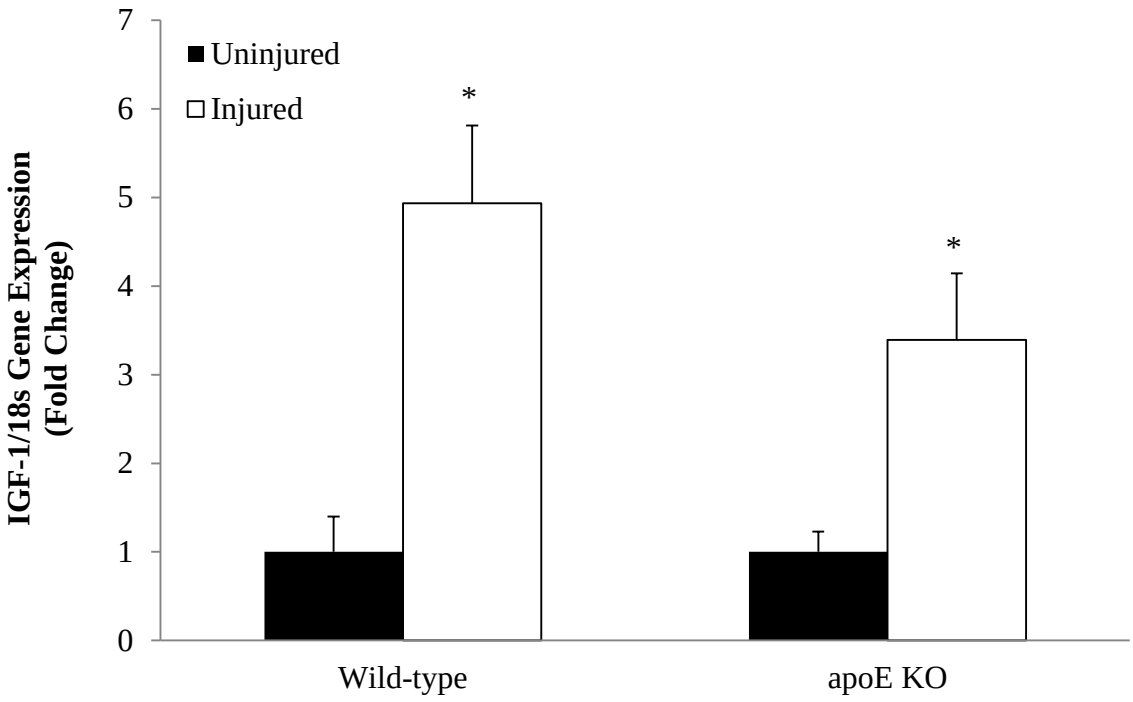
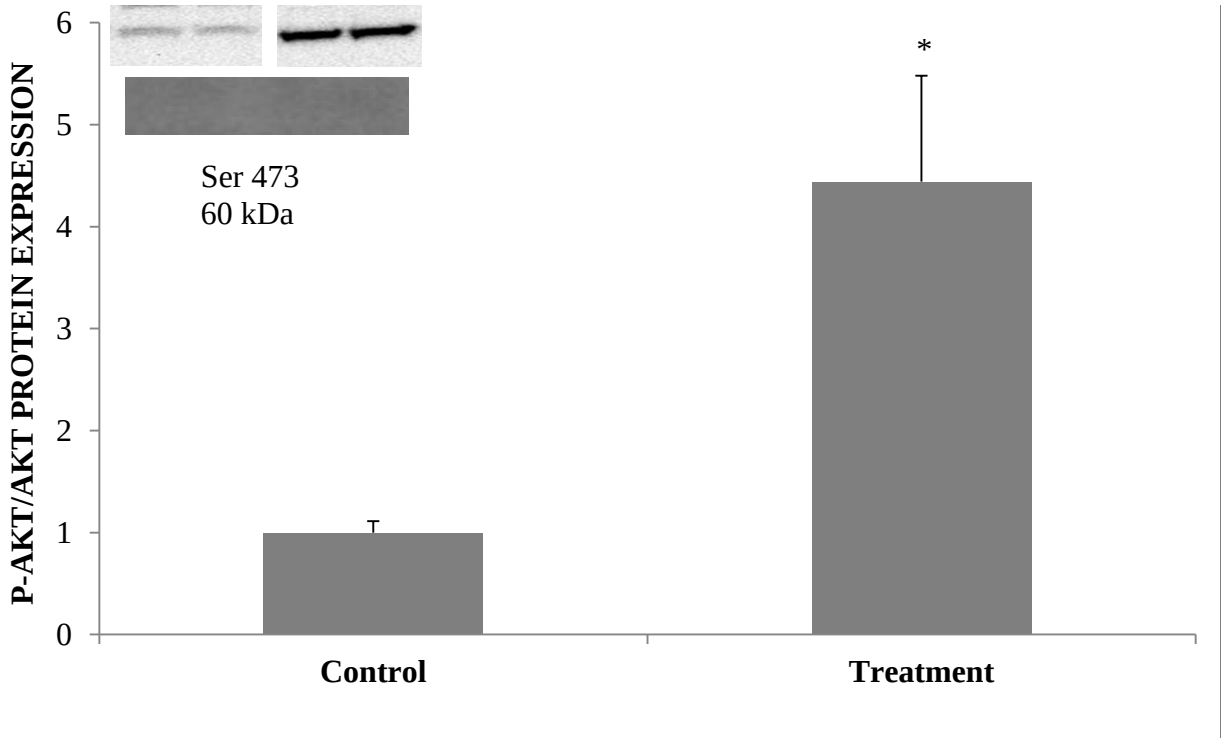


Figure 4. Comparison of fold change in IGF-1expression in both WT and apoE KO mice for both injured and uninjured groups. A significant increase ($p<0.05$) was seen in both groups of injured mice. There was no difference between the two injured groups.
*Shows significance within group

Expression of Markers of Protein Synthesis

Markers of protein synthesis downstream of IGF-1including Akt, mTOR, and p70S6K were measured as to allow for quantification of the response to muscular damage. It was observed that in the apoE model the phosphorylated version of all of these markers was significantly higher in injured mice compared to non-injured mice (Figure 5-7). This was reported as a ratio of the content of the phosphorylated version to the total content of the marker. A 4.44-fold, 2.5-fold, and 1.0-fold increase was seen in the ratio of phosphorylated Akt, mTOR, and p-70S6K to the total Akt, mTOR, and p-70S6K respectively.



Figured 5. Comparison of fold change in p-Akt compared to total Akt in apoE KO mice for both injured and uninjured groups. A significant increase ($p<0.05$) was seen in the injured (treatment) mice.

*Shows significance within group

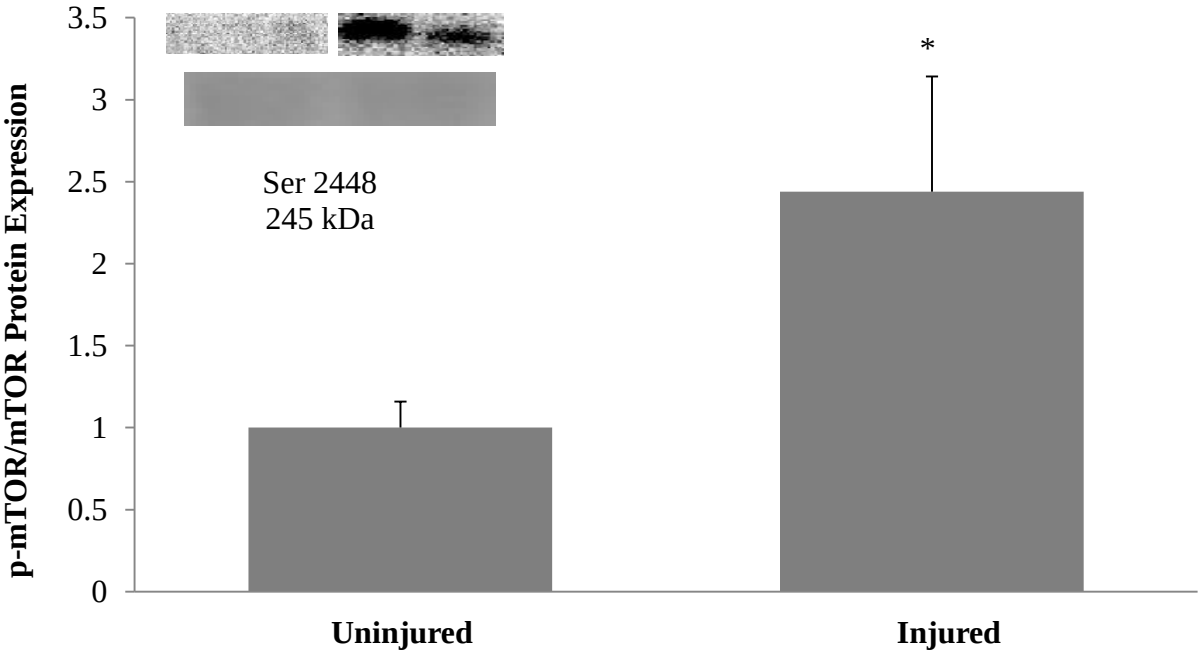


Figure 6. Comparison of fold change in p-mTOR compared to total mTOR in apoE KO mice for both injured and uninjured groups. A significant increase ($p<0.05$) was seen in the injured (treatment) mice.
*Shows significance within group

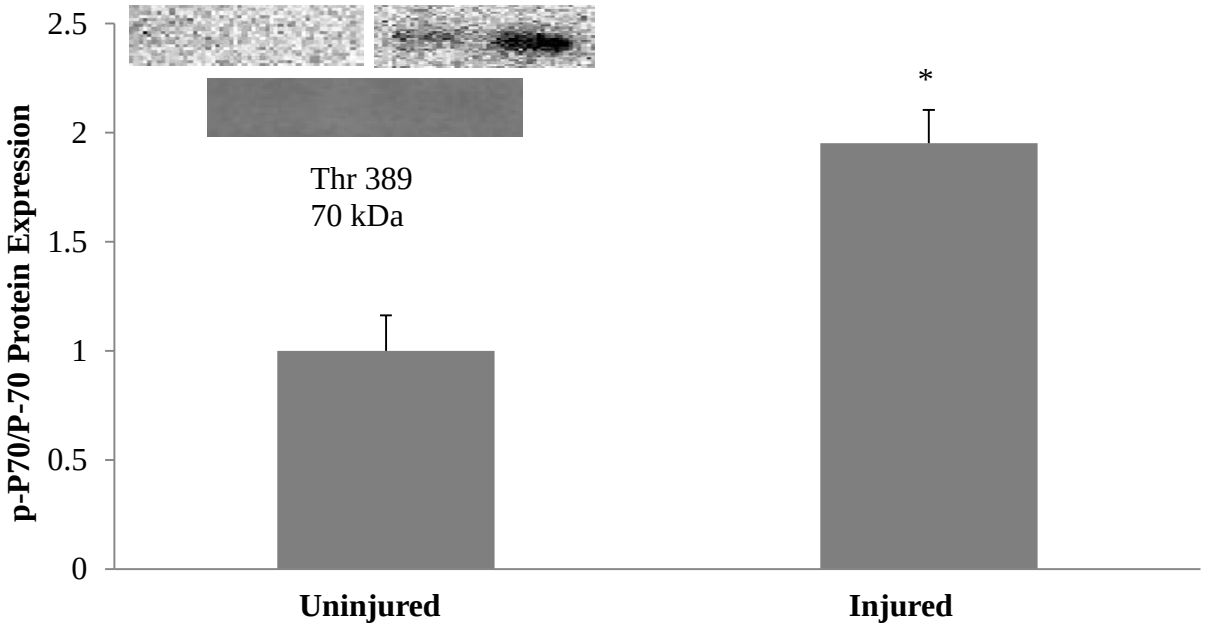


Figure 7. Graph showing fold change in p-p70 compared to total p-70 in apoE KO mice for both injured and uninjured groups. A significant increase ($p<0.05$) was seen in the injured (treatment) mice.
*Shows significance within group

CHAPTER 6

DISCUSSION

The currents study brought insight into the differences in the regenerative response in the apoE KO model as it relates to satellite cell function and protein synthesis. The effect the model has on markers of protein synthesis has not been previously demonstrated. Although the effects of apoE KO has on cyclin D1 has been previously demonstrated other markers of satellite cell function have not been quantified.

This study was intended to give insight into the effects hypercholesterolemia may have on skeletal muscle regeneration as only limited data is available on the topic at this time. It has been shown previously that in wild type mice IGF-1, Akt, and mTOR are up regulated three days after skeletal muscle damage, specifically with muscle damage resulting from bupivacaine injection (50). The up regulation of markers of protein synthesis that occurs as a result of bupivacaine injection has also been shown to occur in other models of muscle damage (31, 32, 38, 39, 41, 42), supporting the supposition that a lack of this up regulation in apoE KO mice would indicate that hypercholesterolemia negatively affects skeletal muscle regeneration.

We hypothesized that this up regulation of the IGF-1-Akt-mTOR pathway would be attenuated by hypercholesterolemia, and would present as low or non-existent levels of these proteins. Contrary to our hypothesis there was no difference in the phosphorylation of any markers of protein synthesis in the injured apoE KO mice compared to the uninjured counterpart. Although it has been noted that phosphorylation of Akt is not dependent on IGF-1 up regulation (39), and phosphorylation of mTOR can occur dependent of Akt in the presence of skeletal muscle contraction, in the current study phosphorylated forms of these markers are up regulated

in injured mice ruling out any of these mechanisms for the up regulation of the final product of the Akt- mTOR pathway, p70S6K. The lack of attenuation in these markers suggests that protein synthesis is unaffected by high cholesterol. This would mean that amino acid chains are formed correctly and in normal concentrations in apoE KO mice, allowing for these chains to be associated into newly forming proteins. This is a crucial mechanism in skeletal muscle regeneration as the structure of the muscle cells is damaged and requires these newly formed proteins to reestablish the structure of these cells. Although no data was obtained on the response to bupivacaine injection in wild type mice, the current data can be held in esteem as previous studies show a normal up regulation in protein synthesis in response bupivacaine induced muscle damage (50). Protein turnover is of significance when investigating the response to skeletal muscle damage, this is the ratio of protein synthesis to protein degradation. Although there was no difference in protein synthesis protein turnover may be affected leading to the decrease in muscle mass to tibia length observed in the apoE model.

Attenuation in myoD expression and an accentuation cyclin D1 was observed in injured apoE KO mice while no change was observed in myogenin expression compared to the uninjured apoE KO mice. These results partially support our hypothesis on markers of satellite cell function, in that some but not all markers of satellite cell function were attenuated in the apoE KO mice.An accentuation of cyclin D1 with attenuation of myoD suggests that while a dysfunction in satellite cell proliferation may occur other cells are progressing normally through the cell cycle. White et al. (2009) showed a similar attenuation of myoD and accentuation of cyclin D1 in mice lacking inflammatory cytokine interleukin-6 (IL-6), suggesting that the lack of a proper inflammatory response to muscle damage can cause dysfunctions in satellite cell proliferation. Somewhat similarly Kang et al. (2008) demonstrated attenuation of myogenin with

a concurrent prolonged inflammatory response resulting in lower than normal satellite cell differentiation in the apoE KO model.

The investigators postulated that the change in the inflammatory response caused unknown changes in satellite cell function following muscle damage. Taken together these studies provide novel insight into a possible mechanism for dysfunctional muscle regeneration related to the inflammatory response following muscle damage in a high cholesterol environment.

While increases in cyclin D1 and myoD expression are essential for proper satellite cell function and subsequent muscle regeneration, the relationship between the two is delicate. Previous studies demonstrate that overexpression of cyclin D1 causes satellite cell differentiation arrest (52, 53). This is a point of interest in that a similar overexpression was seen in apoE KO mice; however, examination of skeletal muscle at a time point greater than 3 days was not completed thus disallowing investigation into the long term effects of overexpression of cyclin D1. Expression of myoD has been shown to gradually increase during the cell cycle, this increase in myoD leads to a translocation of cyclin dependent kinase 4 (CDK4) to the nucleus which is essential for cyclin D1 mediated cell cycle progression (54). This data suggests an interdependence of cyclin D1 and myoD, possibly providing insight into the dysfunction observed in the current study. Interestingly Havely et al. (1995) suggests that increases in myoD expression cause an increase in the CDK inhibitor p21, which consequently decreases the reactions between cyclin D1 and CDK4 and allows the cell to withdraw from the cell cycle. Latella et al. (2001) shows that terminally differentiated cells can be driven into the cell cycle with increases in cyclin D1, further supporting the essential nature that a decrease in cyclin D1 has on progression of satellite cells through the cell cycle. These data together not only

demonstrate the importance of normal increases in myoD and cyclin D1 but how the two work in concert to illicit a complete and sufficient regenerative response and how decreases in cyclin D1 must occur to allow for proper satellite cell function. From the current research it can be seen that a decrement in apolipoprotein E causes dysfunction in the regulation of both myoD and cyclin D1 that leads to improper satellite cell function.

Future research involving the apoE KO model and muscle regeneration should investigate the effects the early dysfunction of satellite cells has at a time point of 21-28 days post muscle damage, as this has been shown to be when muscle regeneration is in its final stages in normal conditions. Another important area that should be investigated is the effect of apoE KO on markers of inflammation as data from this study resemble those in White et al. (2009) where inflammation was genetically disrupted. Future investigation into other MRF's are warranted, as the entire family of proteins are interrelated and affect the results brought on by one another and any dysfunction seen as a result of a decrement of one may be attenuated or nullified by the other. And finally further investigation should incorporate markers of protein degradation as to allow for complete analysis of protein turnover.

The current study shows that the apoE KO model has no effect on markers of protein synthesis at the three day time point. It also shows that there is a dysfunction in markers of satellite cell cell cycle regulation as well as satellite cell proliferation. This is important as it guides future research into the mechanisms behind this dysfunction. On completion of this study it is need be noted that skeletal muscle regeneration may be affected by high cholesterol and so resistance training may need be avoided in some capacity. Also, an intervention may be needed for sufferers of hypercholesterolemia to avoid muscle wasting from lack of regenerative response following muscle damage resulting from ADL's or other injury.

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