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Heat Shock Protein 70 Regulates Tumor Necrosis Factor-Alpha and Myogenin in Skeletal Muscle Following Chemical-Induced Injury

Cory W. Baumann

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This dissertation, HEAT SHOCK PROTEIN 70 REGULATES TUMOR NECROSIS FACTOR-ALPHA AND MYOGENIN IN SKELETAL MUSCLE FOLLOWING CHEMICAL-INDUCED INJURY, by CORY W. BAUMANN, was prepared under the direction of the candidate's Dissertation Advisory Committee. It is accepted by the committee members in partial fulfillment of the requirements for the degree, Doctor of Philosophy, in the College of Education, Georgia State University.

The Dissertation Advisory Committee and the student's Department Chairperson, as representatives of the faculty, certify that this dissertation has met all standards of excellence and scholarship as determined by the faculty.

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HEAT SHOCK PROTEIN 70 REGULATES TUMOR NECROSIS FACTOR-ALPHA AND

MYOGENIN IN SKELETAL MUSCLE FOLLOWING CHEMICAL-INDUCED INJURY

by

CORY W. BAUMANN

Under the Guidance of Jeff S. Otis, Ph.D.

ABSTRACT

Skeletal muscle injury results in functional deficits that can take several weeks to fully recover. Ultimate recovery of function is dependent on the muscle's ability to regenerate, a highly coordinated process that involves transient muscle inflammation and the replacement of damaged myofibers. Instrumental in the inflammatory response, is the pro-inflammatory cytokine TNF-α. Expression of TNF-α is thought to be regulated, in part, by the stress sensing 70 kDa heat shock protein (Hsp70). However, it remains unclear how Hsp70 alters TNF-α following injury, and if so, how these changes affect skeletal muscle repair. Therefore, we up-regulated Hsp70 expression using 17-allylamino-17-demethoxygeldanamycin (17-AAG) prior to and following BaCl2-induced injury, and assessed TNF-α and myogenin content. Regenerating

fiber cross-sectional area (CSA) and in vivo isometric torque were also analyzed in the weeks

following the injury. Treatment of 17-AAG resulted in a ~5 fold increase in Hsp70 of the

uninjured muscle, but did not affect any other biochemical, morphological or functional variables

compared to controls. In the days following the injury, TNF-α and myogenin were elevated and

directly correlated. At these earlier time points (≤7 days), treatment of 17-AAG increased TNF-

α above that of the injured controls and resulted in a sustained increase in myogenin. However,

no differences were observed in regenerating fiber CSA or in vivo torque production between the

groups. Together, these data suggest that Hsp70 induction increases TNF-α and myogenin

content following BaCl2-induced injury, but does not appear to alter skeletal muscle

regeneration or attenuate functional deficits in otherwise healthy young mice.

INDEX WORDS: Heat shock protein, Injury, Myogenin, Skeletal Muscle, TNF-α

HEAT SHOCK PROTEIN 70 REGULATES TUMOR NECROSIS FACTOR-ALPHA AND

MYOGENIN IN SKELETAL MUSCLE FOLLOWING CHEMICAL-INDUCED INJURY

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Cory W. Baumann

A Dissertation

Presented in Partial Fulfillment of Requirements for the

Degree of

Doctor of Philosophy

in

Kinesiology (Exercise Physiology)

in

the Department of Kinesiology and Health

in

the College of Education

Georgia State University

Atlanta, GA
2015

ACKNOWLEDGMENTS

I would like to thank all those that have contributed to my academic growth including my dissertation research committee and previous mentors. I thank Drs. Jeffrey Rupp, Brett Wong and Christopher Ingalls for their invaluable insight. I also thank my dissertation committee chair, Dr. Jeffrey Otis. Without his prompt and critical review, and encouragement I would have not been able to complete my dissertation in the time I did. I would also like to express my sincere gratitude to Dr. Thomas Wetter, my undergraduate mentor, colleague and friend for his trust and belief in my abilities.

I thank all my friends back home and in Georgia. I thank my fellow colleagues on campus for their presence, whether it was assisting in an experiment or going for an afternoon run. I express my sincere graduate to my close friends Ian and Rachel McCann, and Michael Gerber for welcoming me into their homes and treating me like family.

Lastly, I want to convey my deepest appreciation to my family for all their encouragement throughout my academic career. Their love and commitment to my success was and will continue to be my motivation. In particular, I want to thank my parents for their countless phone calls and check-ups. Even though some of the conversations were brief, hearing their voices always made me smile.

Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

17AAG	17-allylamino-17-demethoxy-geldanamycin
$BaCl_2$	Barium chloride
BW	Body weight
Ca^{2+}	Calcium
CK	Creatine kinase
CSA	Cross sectional area
DOMS	Delayed onset muscle soreness
DHPR	Dihydropyridine receptor
E-C	Excitation-contraction
EDL	Extensor digitorum longus
HSF1	Heat shock factor 1
Hsp	Heat shock protein
HSP	Heat shock protein family
IκB	Inhibitor of kappa B
IL	Interleukin
IL-1β	Interleukin-1 beta
Ip	Intraperitoneal
IKK	IκB kinase
JP	Junctophilin
mTORc1	Mammalian target of rapamycin complex 1
MAPK	Mitogen-activated protein kinases
MPC	Muscle progenitor cell
MHC	Myosin heavy chain
MHCe	Myosin heavy chain embryonic
MHCn	Myosin heavy chain neonatal
NFAT	Nuclear factor of activated T cell
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SR	Sarcoplasmic reticulum
SC	Satellite cell
TA	Tibialis anterior
T-tubules	Transverse tubules
TNFα	Tumor necrosis factor alpha
WT	Wild-type
YY1	Yin Yiang 1

1 SKELETAL MUSCLE INJURY AND RECOVERY

Introduction

Skeletal muscle is a remarkable tissue; it allows for force production to perform tasks required to sustain life, such as breathing and locomotion. When injured, the ability of the skeletal muscle to perform these tasks is compromised. Skeletal muscle injury often results in the physical disruption of muscle structures involved in the production or transmission of force (126). Furthermore, chronic injury or genetic diseases can blunt its regenerative capacity and lead to muscle wasting and further dysfunction. Therefore, it is vital to study the mechanisms of strength loss associated with injury and degeneration, as well as the regenerative processes that follow.

In response to injury (i.e., contraction- or trauma-induced), a well-orchestrated and coordinated series of cellular and molecular events are observed (54, 60). The injury results in necrosis of damaged tissue, which induces a rapid and sequential inflammatory response. Morphological analysis reveals interstitial edema with invading leukocytes (e.g., neutrophils, monocytes, macrophages) (21). These immune cells phagocytize necrotic tissue and release several cytokines. Initially, pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) are released that increase satellite cell (SC) activation and muscle progenitor cell (MPC) proliferation. When proliferation is blocked through irradiation, the muscle's ability to functionally return to its pre-injured state is significantly delayed (101). MPC differentiate and fuse to form small basophilic centro-nucleated myotubes (the hallmark of skeletal muscle regeneration). Fusion of myoblasts and

further growth of the newly regenerated centro-nucleated myofibers result in the restoration of the cellular architecture within the following weeks of injury (Figure 1) (48, 106).
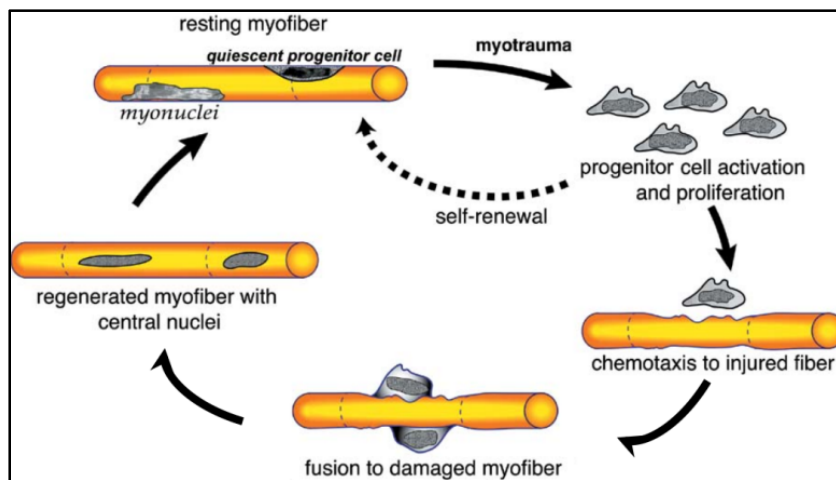
Figure 1. Depiction of regeneration mediated through SC activity.

In the event that any of these steps are blunted or inhibited, the ultimate recovery of the muscle will be prolonged. The transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), often referred to as the central mediator of the human immune response (97) is a key regulator in skeletal muscle degeneration and regeneration. On one hand, NF-κB orchestrates the inflammatory response by releasing the aforementioned pro-inflammatory cytokines that recruit leukocytes, which release reactive oxygen species (ROS) and proteases that assist in phagocytosis (21). In parallel, these cytokines also activate SCs and cause MPC proliferation (96). However, ROS (8) and TNFα (34, 67) are also known to inhibit MPC differentiation, thereafter blunting and delaying regeneration. Furthermore, NF-κB-derived ROS and pro-inflammatory cytokines can activate NF-κB (97), thus creating a positive feedback loop that prolongs NF-κB-derived signaling.

Many proteins are involved in the regulation of NF-κB (97). For example, heat shock protein 70 (Hsp70) is generally known for its role as a molecular chaperone and stress sensor that refolds denatured proteins (50, 70), but it is also an inhibitor of NF-κB (35, 82, 95, 105). Several recent studies have demonstrated that up-regulation of Hsp70 reduces histological damage (63, 64, 80, 84) and attenuates strength deficits following injury (63, 80). Though the precise mechanisms for this enhancement are unknown, increasing evidence suggest NF-κB is a central player (35, 82, 95, 105). Therefore, this review will outline skeletal muscle injury and recovery, the roles of Hsp70 in muscular regeneration and how regeneration is orchestrated between Hsp70 and NF-κB.

Models of skeletal muscle injury

Numerous injury models have been used to examine skeletal muscle regenerative mechanisms. Injury models are broadly characterized into two categories: contraction- or trauma-induced. Contraction-induced injury is the result of strenuous exercise or eccentric contractions. In comparison to concentric or isometric muscle actions, eccentric contractions are unique in the fact that they generate higher forces (10, 29) and for a given muscle force, require the requirement of fewer muscle fibers (65, 87). Thus, these high forces distributed across a relatively small population of fibers place a great deal of stress and strain on the muscle, making them susceptible to damage. Contraction-induced injury is more common than traumatic injuries (137).

In contrast, trauma-induced models occur independently of muscle contraction and include exposure to toxins, extreme temperatures, crushing, laceration, blunt impact or ischemia. The most reproducible traumatic injuries utilize chemical agents such as snake venoms (e.g.,

notexin, cardiotoxin) or barium chloride ($BaCl_2$) (28, 30, 96). A notexin is a myotoxic phospholipase A_2 derived from the venom of the Australian tiger snake, while a cardiotoxin is a set of small proteins found in cobra venom that causes skeletal muscle contracture and interferes with neuromuscular transmission. Alternatively, $BaCl_2$ induces necrosis by causing muscle depolarization by stimulating exocytosis and blocking the efflux of calcium (Ca^{2+}).

Regardless, the healing phases for an injured muscle including degeneration, inflammation, regeneration and remodeling are similar between injury types (54, 60), even though the initial mechanism of damage may differ. Furthermore, the current therapeutic approaches for treating muscle injuries are dependent not on the type of injury but rather the clinical severity (60). In the event the muscle does not heal properly, it will lead to an incomplete functional recovery, a tendency for recurrent injuries and/or scar tissue formation (54).

Characteristics of skeletal muscle injury

Many different markers are used to characterize injury, including alterations to fiber morphology, changes in intracellular proteins levels and localization, loss of intracellular muscle proteins to the surrounding environment and reductions to muscle function (126). By measuring changes in these markers it is possible to describe the magnitude and duration of the injury. Evidence of injury can be classified as direct, biochemical/histochemical or functional (126).

Direct evidence of muscle damage observed from muscle biopsies

Using light microscopy, focal disruptions to the striated banding patterns and ultra-structural features are evident immediately after injury, and worsen between 30 hours and 3 days post contraction-induced injury (41). Similarly, Z-line streaming is also observed at these later time

points using electron microscopy (41). Z-line streaming is one of the principle ultra-structural abnormalities seen after eccentric exercise (39) and may represent a weak link in the contractile chain of the myofibril (40). Other abnormalities include disrupted A band (41) and I band (77) filaments, transverse (T)-tubules and triads (116), sarcoplasmic reticulum (SR) (94), and extracellular matrix and capillaries (113), the presence of autophagic vacuoles (41) and central nuclei (44), swollen or missing mitochondria (41) and alterations to the cytoskeletal structure (39).

Biochemical and histochemical evidence of muscle damage

Muscle damage is also evident using biochemical analyses of bodily fluids and histochemical analysis of tissue. After injury it is common to assess blood levels or activity of circulating neutrophils and macrophages, cytokines and/or muscle proteins. Some of more common muscle proteins analyzed after injury include: creatine kinase (CK), myoglobin, lactate dehydrogenase, aspartate aminotransferase and Beta-glucuronidase (126).

Functional evidence of muscle damage

Functional evidence of muscle damage includes changes in range of motion and stiffness of muscle and joints, swelling, soreness, low frequency fatigue and strength loss and recovery. Following contraction-induced injury, voluntary range of motion (i.e., the distance and direction through which a joint can move) decreases, while circumference measurements increase due to swelling (93). Pain typically develops 2 to 4 days after exercise and is abated at 5 to 7 days post-injury (26). This pain, first described by Hough (53), is referred to as delayed onset muscle soreness (DOMS). DOMS is one of the hallmark manifestations of exercise-induced damage and is often assessed qualitatively in human studies as a marker of injury.

Other markers of injury include low frequency fatigue, and strength loss and recovery. Low-frequency fatigue is characterized by a relative loss of force at low frequencies of stimulation that slowly recovers over the course of hours/days due to reductions in Ca^{2+} release (62). The final marker of damage is loss and recovery of muscular strength. Warren et al. (137) considers loss of strength to be one of the most valid and reliable indirect markers of exercise-induced muscle damage. Strength deficits following eccentric exercise outlast changes in metabolite levels and therefore are not associated with fatigue (38). Strength loss and recovery is dependent on the mode, intensity and duration of the exercise. For example, downhill running typically results in immediate reductions in strength ranging from 10 to 30% (9, 25) that often recovers in 1 week. On the other hand, electrically stimulated maximal eccentric contractions in animal models can exceed strength deficits of 50% and take several weeks to fully recover (10, 74, 101).

Traumatic muscle injuries including crush, contusion, laceration or freezing occur relatively infrequently but when they do occur, can have dramatic and prolonged effects on the muscle's functional capacity. For example, Warren et al. (139) reported freeze injury reduced maximal isometric strength ~70% while Rogers, Baumann and Otis (unpublished data) demonstrated strength deficits of 80% after $BaCl_2$-induced injury.

Strength deficits associated with skeletal muscle injury

The contributors of strength loss will be dependent on the injury model. Over the past 2 decades a great deal of effort has been directed into understanding the mechanisms of strength loss after contraction-induced injury and thus will be highlighted in detail in the following sections.

Because the initial damaging events differ between contraction and trauma induced-injury models, trauma-induced injury will discussed after contraction-induced injury.

Damage to force generating structures after contraction-induced injury

Based on the principle that muscle force production is directly related to a muscle's functional area (i.e., physiological cross sectional area: CSA), immediate strength deficits were initially thought to be due solely to damage of force bearing proteins (3). However, further research has suggested physical damage only accounts for a portion of the strength loss immediately and up to 3 days post-injury (74, 134-136).

The first line of evidence to suggest that physical damage is not the sole cause of early strength deficits is the disconnect between histological damage and strength loss both temporally and quantitatively. This disconnect was demonstrated by Warren et al. (134) who found that the performance of 15 eccentric contractions *in vitro* in mouse extensor digitorum longus (EDL) muscle resulted in specific force deficits of over 60%. However, signs of damage per cross section were only visible in ~3% of the fibers immediately after the injury. Further, Lowe et al. (74) reported for mouse muscles exhibiting strength loss of 50%, no more than 5% of the fibers in cross-section showed histopathology at any time in the first 2 weeks following injury. In contrary, it is also possible to have robust muscle damage without a marked reduction in force production (52, 91, 92, 114). Street and Ramsey (114) suggest the ability of the muscle to generate near maximal force despite being damaged may be attributed to lateral transmission of force propagated by the still-intact sarcolemma. From these results, it is clear that a great deal of variability exists when comparing direct histological damage and strength loss, making it difficult to draw accurate conclusions.

The second line of evidence is achieved through direct activation of the myofilaments

through the application of maximally activating Ca^{2+} in skinned or intact fibers. Warren et al. (134) found peak voltage-induced isometric force was reduced 69% after EDL muscle performed 15 eccentric contractions *in vitro*, while maximal Ca^{2+} activated force produced by single fibers from these injured EDL muscles was only down by 34%. These results indicate that damage to force bearing proteins could account for ~50% of the immediate force deficits after injury (134). To the contrary, Balnave and Allen (6) reported ~80% of the strength loss was accounted by damage to force bearing structures within the fibers after eccentric exercise. Warren et al. (135, 136) suggested that these discrepancies may be due to the muscle fibers being stretched beyond non-physiological lengths (i.e. >130%), which may have predisposed the force bearing structures to overt damage. Additionally, measures of fiber maximal Ca^{2+} activated force may overestimate how much damage the force-bearing structures contributed to strength loss. This overestimation is due, in part, to the removal of lateral transmission of force to neighboring fibers in these single fiber preparations (55).

Damage to the force bearing structures within the fibers can account for a widely varying proportion of the strength loss. However, several studies have documented strength deficits are greatest immediately and up to 3 days post-injury while a very small amount of histological damage is present at the light microscope level 2 to 4 days after injury. Further, strength deficits are attenuated after exposure to maximally activating Ca^{2+} which strongly suggests other mechanisms may also be responsible for reductions in strength immediately after injury (74, 134).

Failure to activate intact force generating structures after contraction-induced injury
This category of mechanisms includes those resulting in failure of the excitation-contraction (E-C) coupling process, a sequence of events linking the release of acetylcholine at the

neuromuscular junction to the release of Ca^{2+} from the SR (37). E-C coupling failure, or E-C uncoupling, in response to exercise was first suggested by Davies and White (33) in the late 1970s and early 1980s. However, the relative contribution of E-C uncoupling to strength loss was not investigated until Warren and colleagues (138). To determine how much E-C uncoupling contributes to strength loss, pharmacological agents (e.g., caffeine, 4-chloro-m-cresol) are used that act to promote ryanodine receptor (RyR) opening and increase free cytosolic Ca^{2+} levels. Therefore, force elicited during caffeine exposure would not be dependent on acetylcholine release, action potential conduction along the plasmalemma or T-tubules, or the communication of the depolarization induced conformation change in the dihydropyridine receptor (DHPR). Thus, any reduction in caffeine elicited force would imply damage to the force bearing elements, decreased Ca^{2+} sensitivity and/or intrinsic SR dysfunction.

By isolating mice soleus muscle after 20 eccentric contractions, Warren et al. (138) demonstrated that caffeine elicited force of the injured muscles was not different from that of control muscles despite the fact that maximal isometric torque was down by 43% in the injured muscle vs. 4% for the controls. These data suggest that E-C uncoupling could account for the majority of the strength deficits early after injury. To further support this concept, Balnave and Allen (6) showed depressed levels of free cytosolic Ca^{2+} levels during tetanic stimulation in a single fiber preparation following eccentric contractions. Furthermore, by adding caffeine to the bathing medium in fibers that lost moderate amounts of strength (28%), free Ca^{2+} concentrations increased to normal levels. Ingalls et al. (57) reported similar results by demonstrating force from injured EDL muscle was ~50% down immediately and up to 3 days after injury. Free cytosolic Ca^{2+} was also reduced by 25-45% compared to uninjured muscles during electrical stimulation. By comparing forces elicited by pharmacological agents (i.e., caffeine and 4-chloro-

m-cresol) to forces stimulated electrically, Ingalls et al. (57) further estimated that E-C

uncoupling could account for 57-75% of the isometric strength loss in the first 5 days after

injury, and concluded that the remaining strength deficits at these early time points would

therefore be attributed to physical damage to the force producing structures and/or myofibrillar

Ca^{2+} sensitivity.

The specific mechanisms involved in E-C uncoupling are currently being investigated,

but likely involve several proteins and protein-protein interactions. Warren et al. (140) found

EMG amplitude in injured and uninjured muscles (i.e., muscles that were exercised

concentrically) was depressed (~9%) to a similar extent after exercise and recovered by 24 hours

even though strength was still significantly down in the eccentrically injured fibers.

Furthermore, Ingalls et al. (58) found *in vitro* strength deficits were similar when the EDL was

exposed to normal or high concentrations of potassium (which act to depolarize all membranes).

These studies suggest normal plasma and T-tubular membrane function after injury

demonstrating the ability to generate action potentials after eccentric exercise did not impact

strength deficits. DHPR and RyR number and sensitivity appear to be normal immediately after

injury on the basis of receptor binding experiments (e.g., scatchard analysis) (59). Further,

Ingalls et al. (57) found RyR release rates were not significantly impaired after injury when

measured using confocal laser scanning microscopic or fluorometric techniques. However when

measured with a Ca^{2+} minielectrode, a significant reduction (6%) in RyR release rates was

detected. Taken together these studies suggest eccentric contraction-induced skeletal muscle

injury disrupts the communication between the DHPR and RyR, and also the intrinsic ability of

the RyR to release Ca^{2+}.

The physical uncoupling between DHPR and RyR seen after injury would reduce voltage-gated SR Ca^{2+} release and subsequently reduce the number of actin-myosin cross-bridges. In order to better elucidate this physical uncoupling, Corona et al. (29) analyzed junctophilin 1 and 2 (JP1 & 2) content, proteins responsible for maintaining the apposition of the T-tubule and SR membranes, before and after a bout of eccentric or concentric contractions. Through the first 3 days, significant reductions were observed in JP1 (50%) and JP2 (35%) content after the eccentric exercise while no changes were seen after the concentric exercise. Regression analysis of JP1 and JP2 content indicated that loss of JPs after eccentric injury was significantly associated with early reduction in strength. In addition to disruptions in JP1 and JP2 content, early strength deficits may also stem from reductions in RyR function mediated by ancillary binding proteins such as FKBP12.

FKBP12 is a 12 kDa protein that binds to the subunits of the RyR homotetramer (61) and stabilizes the closed state of the channel preventing channel opening to subconductance states (i.e., SR Ca^{2+} leak) (18). Similar to JPs, FKBP12 content was disrupted after eccentric exercise and not concentric exercise. Baumann et al. (10) observed a 43% decrease in FKBP12 content in the pelleted fraction of mouse tibialis anterior (TA) muscle immediately after exercise which correlated with strength deficits after injury. The fact that both JPs (29) and FKBP12 (10), two proteins known to be involved in SR Ca^{2+} release, are reduced after injury and associated with early strength deficits lends further support to the notion that E-C uncoupling is a major factor contributing to loss of function after contraction-induced skeletal muscle injury.

Loss of force generating structures after contraction-induced injury

The initial damage to the force bearing structures is preceded by a marked increase in protein degradation rates between 1 and 5 days post-injury. The marked increase in proteolysis is likely

due to several mechanisms including activation of calpains (11, 12), the ubiquitin-proteasome pathway (4, 123) and the inflammatory response (74). Lowe et al. (74) suggests that these degradation pathways, specifically the inflammatory response, play a key role in removal of damage proteins before regeneration ensues.

In order to determine if there was a relationship between myofibrillar content and peak isometric force, Ingalls and colleagues mice (56) measured EDL force and myosin heavy chain (MHC) and actin following injury. Through the first 3 days, no alterations were seen in total or myofibrillar protein content nor the amount of MHC or actin. By day 5, total and myofibrillar protein content was significantly reduced (56) and remained down until day 14 (56, 74). In parallel MHC and actin were ~19% less than control muscle at day 5 and 21-22% less at day 14. Both total and myofibrillar protein content and MHC and actin returned to normal by day 28. Peak tetanic force was reduced 37-49% for up to 3 days after the injury and slowly returned in the following weeks (14 days, ~24%; 28 days, ~11%). Because protein content was not altered within the first 3 days after injury, while force deficits were peaked, decreases in the EDL force producing capacity could not be accounted for by a loss of force generating proteins (i.e., MHC and actin). However, by day 5, reductions in MHC and actin content could account for 58% of the force reduction and nearly all the decrements at days 14 and 28.

Together, these data suggest that because peak EDL force recovers at the same time MHC and actin content are declining, degradation of myofibrillar proteins is likely restricted to the initial sites of damage and therefore little to no unnecessary proteolysis occurs. In addition, because E-C uncoupling has been shown to explain some of the strength deficits after eccentric injury (10, 29, 57, 135, 136, 138), recovery of the E-C coupling machinery may also explain the paradoxical force recovery while contractile proteins content was declining. This concept has

been supported by recent research that reported E-C coupling proteins are significantly reduced early after injury and return to normal around the same time when muscular strength begins to recover (10, 29).

Destruction and loss of muscle fibers after trauma-induced injury

Two popular traumatic injury models include the use of intramuscular injections of either a snake venom (e.g., cardiotoxin - a snake venom that disrupts membranes and induces tissue necrosis) or $BaCl_2$ (which causes muscle depolarization and myofiber death by stimulating exocytosis while blocking Ca^{2+} efflux). In response to these toxins, 75–90% of the muscle is destroyed when employed in a mouse model (106). During the acute post-injury period ($\leq$6 h), myofibers become hyalinized and vacuolated and their nuclei are pyknotic or lysed (Figure 2) (45, 106). Therefore, toxin- and chemical-induced injury models drastically reduce muscular function through the destruction and loss of the muscle fibers, similar to decline in MHC and actin content after eccentric-contraction induced injury, but on a much larger scale. In this way, traumatic injuries are ideal for characterizing regeneration rather than the mechanisms behind the loss of function.
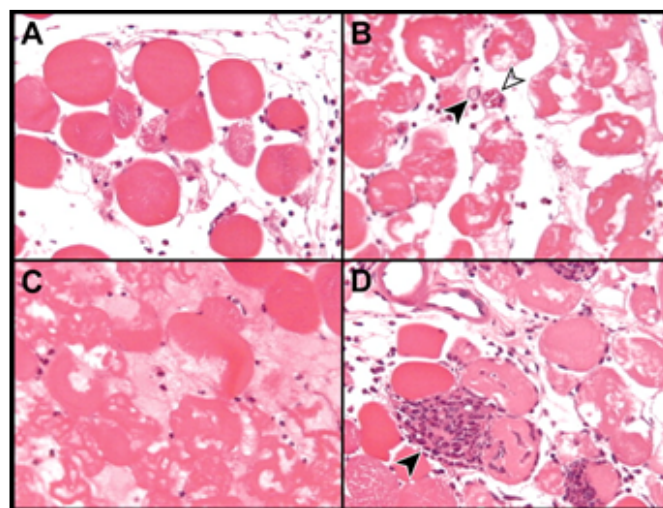
Figure 2. Morphological analysis following cardiotoxin-induced injury.

Time periods 6-12 h post-injury (A-D) (45).

Recovery and regeneration after skeletal muscle injury

Inflammatory response

Following injury, the muscle fibers and infiltrating immune cells release several pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα (76). These cytokines increase the expression of adhesion molecules within the endothelium and allow for margination of neutrophils. Further, these adhesion molecules release additional cytokines (IL-6 and IL-8) that, in turn, recruit additional neutrophils (14). Accumulation of neutrophils occur in skeletal muscle within 2 hours, reach peak levels by 1 day post-injury and slowly decline back to control levels by day 7 (99, 100). Infiltrating neutrophils degrade proteins by releasing proteases and remove cellular debris through phagocytosis. In addition, neutrophils produce a host of cytotoxic substances, including reactive oxygen species (ROS), such as superoxide anions, hypochloride, and hydrogen peroxide (13, 16). Furthermore, the cytokines IL-1β, IL-6, and TNFα may stimulate pathways that contribute to the activation of the enzyme NADPH oxidase, a key enzyme that initiates the ''respiratory burst'' and subsequent release of ROS (21, 31). These ROS are thought to delay recovery and/or cause secondary damage and loss of force after the initial injury (124).

Macrophages, another population of immune cells, generally appear after the arrival of neutrophils. Macrophages are divided into two subpopulations, ED1+ and ED2+ (21, 81, 112). The ED1+ macrophages are found in the damaged sites 1 day after neutrophil invasion and are activated by pro-inflammatory cytokines (TNFα and IL-1β) (51). Similar to neutrophils,

activated ED1+ macrophages act as phagocytes and function to remove cellular debris (112), and release pro-inflammatory cytokines such as prostaglandins and IL-1β (104). One to 5 day after contraction-induced injury in mouse muscle, Lowe et al. (74) demonstrated protein degradation rates were elevated and correlated with the infiltration of phagocytic macrophages, suggesting that the inflammatory response plays a key role in removal of damaged proteins before regeneration ensues.

After the clearance of ED1+ macrophages near the later stages of inflammation (e.g., ≥2 days), ED2+ macrophages generally appear (21) and regulate tissue repair and regeneration. ED2+ macrophages locate to the extracellular matrix and do not enter damaged tissue (112), suggesting their primary role in tissue repair is through cell signaling and growth regulatory cytokine production such as fibroblast and insulin-like growth factors. The release of these growth factors facilitate MPC differentiation and orchestrate intracellular signaling pathways associated with repair (21, 24).

Satellite cells (SCs)

Skeletal muscle exhibits a great capacity for regeneration after various injuries and diseases, largely due to a population of skeletal muscle stem cells, termed SCs. Under noninjured conditions, SCs are quiescent and located between the sarcolemma and the basal lamina of the myofiber (Figure 3) (79, 106). In this state, SCs display low transcriptional activity and dense heterochromatin. In response to cellular and extracellular signals (e.g., cytokines, mechanical stress) associated with muscle damage, SCs are activated and exit their quiescent state, proliferate, differentiate and migrate to the site of injury to repair or replace damaged muscle fibers by fusing together, fusing to existing muscle fibers, or both (48, 117). Repaired and newly synthesized fibers can be identified by their centrally located nuclei (Figures 1 and 4).
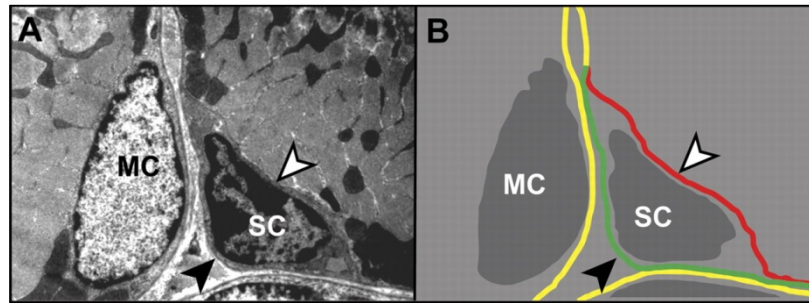
Figure 3. Location of SC in adult skeletal muscle. (A) Electron micrograph of adult skeletal muscle demonstrating the myocyte nucleus and the SC nucleus. (B) Schematic of (A) emphasizing that the SC lies between the basal lamina (black arrowhead or green line) and the sarcolemma (white arrowhead or red line) (106).

Satellite cells (SCs) are activated and begin to proliferate during the early phases of inflammation. As the inflammatory response decreases, differentiation of SCs proceeds and ultimately the healthy myofiber population is reconstituted. Otis et al. (96) demonstrated this process in the TA muscle of mice after $BaCl_2$-induced injury. One day after injury, the TA muscles showed edema and loss of myofiber content. By 5 days of regeneration, the inflammatory response was reduced, the muscles were less edematous, macrophages had invaded the myofibers and the SCs were in a proliferative state. Furthermore, numerous small basophilic, central-nucleated myofibers (i.e., newly regeneration myofibers) were visible. By 10 days post-injury, regenerating myofibers increased in size and continued to enlarge through day 28 when regeneration was essentially complete (96). A similar coordinated response occurs after freeze and eccentric contraction-induced skeletal muscle injury (139).

Rathbone et al. (101) demonstrated the critical importance of SCs on recovery by exposing mice anterior crural muscle to irradiation and documenting recovery of torque up to 35

days after 150 eccentric contractions. Interestingly, irradiated and nonirradiated muscular

strength recovered at the same rate through 7 days demonstrating recovery of strength during the

first week after contraction-induced injury does not require SC proliferation. By 28 days into

recovery, nonirradiated muscular torque recovered while torque was depressed 21-25% in the

irradiated muscle through day 35. Rathbone et al. (101) concluded that the SC dependent portion

of strength recovery occurs 1-2 weeks post-injury and most likely resulted from a restoration of

contractile protein and increased protein synthesis that requires SC to proliferate and presumably

to mature and fuse with injured myofibers.
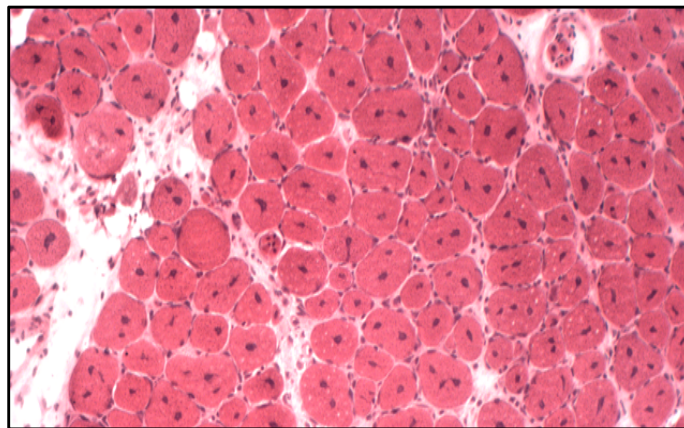
Figure 4. Centrally located nuclei – depicted by dark dots within the fibers.

Mammalian target of rapamycin complex 1 (mTORc1)

The mammalian target of rapamycin complex 1 (mTORc1) plays a vital role in controlling

protein synthesis rates (133). mTORc1 has been implicated in muscle hypertrophy (15), and in

recovery from freeze-(83) and toxin-(42) induced injury models. mTORc1, a serine/threonine

kinase, coordinates muscle regeneration and growth by phosphorylating p70s6k and the

translation repressor 4EBP1, two proteins known to affect mRNA-binding steps during

translation (49, 133). Baumann et al. (unpublished data) recently demonstrated that both mTORc1 and p70s6k activity significantly increased 3 days after eccentric injury. Furthermore, by inhibiting mTORc1 and p70s6k with daily treatment of rapamycin, long-term strength deficits were exacerbated by ~20% and delayed the recovery of peak isometric torque. These results provide further support that the mTORc1 pathway is activated after damage and instrumental to the recovery of muscle function following injury.

Heat shock proteins (Hsps)

Heat shock proteins are vital to regeneration in injured, atrophied, and aged skeletal muscle. Heat shock proteins are a family of proteins that serve diverse roles that include chaperoning, assisting in the removal of damaged proteins, protein folding and transport, regulating cell signaling pathways, and protecting against cellular stress. Because of these roles, Hsps are thought to restore protein homeostasis and contribute to cell survival (50, 66, 70).

Skeletal muscle Hsps

The term 'heat shock protein' was first coined in 1974 (127), but their discovery was over a decade earlier by Ritossa (102) in drosophila melanogaster larvae. Heat shock proteins occur in all organisms and appear in both prokaryotic and eukaryotic cells, while displaying a high level of conservation. Several different forms of Hsps exist and therefore are categorized into families on the basis of their molecular weight. For this reason, the Cell Stress Society International has developed nomenclature to describe the protein family and the specific protein, with HSP referring to the protein family and Hsp to the specific protein.

Heat shock proteins range in molecular mass from 8 to 110 kDa and can be found in the cytosol, mitochondria, endoplasmic reticulum and nucleus. In skeletal muscle, the most

abundant Hsps are small Hsps, HSP70, Hsp60 and HSP90. Small Hsps range in molecular mass from 8 to 27 kDa and include ubiquitin, Hsp20, Hsp25, Hsp27 and alpha B-crystallin (70). These small Hsps facilitate the targeting and removal of denatured proteins (22), stabilize microfilaments and cytokine signal transduction (88), and facilitate protein refolding and muscle development (59). Hsp60 is a mitochondrial Hsp responsible for folding and the assembly of proteins as they enter the mitochondria. Hsp60 also stabilize mitochondrial proteins under stress (78). Like other Hsps, HSP90 folds proteins, but are also thought to regulate the activity of steroid hormone receptors (32). HSP70 consists of four major Hsps: Hsp72, Hsp73, Hsp75 and Hsp78. Hsp75 and Hsp78 are termed GRP75 and GRP78, and are activated by glucose deprivation, calcium influx or agents that perturb glycolysis (72). Hsp73, also termed heat shock cognate (Hsc73), is synthesized in most cells and is constitutively expressed in the cytoplasm under unstressed conditions. Upon stress, Hsp73 translocates to the nucleus where it facilitates reintegration of denatured or unfolded pre-ribosomes (140). Unlike the other Hsps, Hsp70 (also known as Hsp72) is highly inducible and is synthesized in response to multiple stressors (70). In the unstressed state, Hsp70 is barely detectable. But with significant cellular stress, Hsp70 content rapidly increases and can constitute up to 20% of the total protein content (36). For this reason, Hsp70 and its function in skeletal muscle will be the focus of this review.

Structure and Regulation of Hsp70

Hsp70 is a highly conserved protein composed of three domains, the N-terminal ATPase domain, a peptide-binding site and C-terminus (Figure 5). Hsp70 shares a high correspondence of its amino acid residues with several different species (131). Human Hsp70 is 73% identical to the drosophila protein and 50% identical to the E coli dnaK product (69).
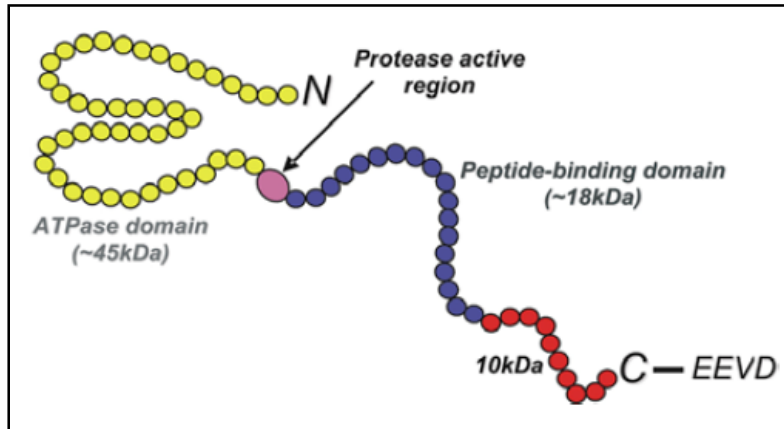
Figure 5. Schematic illustration of the molecular structure of Hsp70 (70).

Hsp70 content is regulated by heat shock factor 1 (HSF1), a transcription factor that is bound to Hsp70 in the unstressed state. In the bound state, Hsp70 and HSF1 are both inactive. Upon stress (e.g., elevated temperature, oxidative stress, contraction related stress, changes in energy metabolism, induction of stress related messengers and/or disturbances in oxygen perfusion and transport) cellular proteins unfold allowing hydrophobic regions of the peptide to be exposed. These hydrophobic regions attract Hsp70 causing it to disassociate with HSF1. Further, the presence of denatured proteins stimulates the ATPase activity on Hsp70 resulting in the hydrolysis of ATP to ADP. ATP hydrolysis increases Hsp70's affinity for its substrate (i.e., denatured or unfolded proteins) which, in turn, stabilizes the Hsp70/ADP-substrate complex. Upon proper folding, Hsp70 exchanges ADP for ATP that reduces Hsp70's affinity for its substrate, resulting in its release from the complex (70, 86).

When Hsp70 is bound to ADP in complex with its substrate, free HSF1 monomers become phosphorylated by protein kinase C or other serine/threonine kinases. They then form a DNA binding homo-trimetric structure that is free to translocate into the nucleus. In the nucleus,

the active HSF1 trimers bind to short nucleotide sequences on DNA promoter sites of the Hsp70

gene, called heat shock elements. This binding results in increased transcription of Hsp70 and

subsequently Hsp70 protein content. Increased levels Hsp70 provide aid and protection to the

cell decreasing the number of denatured proteins. At this point, the free Hsp70s rebind HSF1

preventing further expression of Hsp70 (Figure 6) (70, 86).
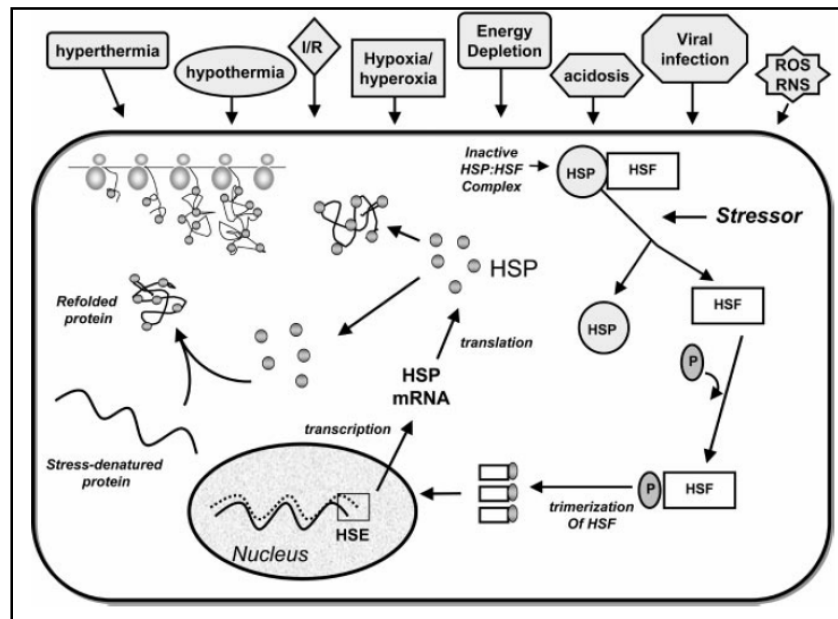
Figure 6. Summary of the pathway involved in Hsp70 synthesis (66).

General functions of Hsp70 in skeletal muscle

Intracellular molecular chaperone

Newly synthesized peptides probably do not fold instantly; but rather they are assisted at the

ribosome by molecular chaperones (i.e., heat shock proteins). Molecular chaperones, such as

Hsp70, are proteins and protein assemblies that help other proteins fold into their proper native

conformation. As mentioned above, Hsp70 binds to the hydrophobic regions of denatured

proteins in attempts to repair the defective protein by giving it another chance to refold. Furthermore, by binding and thus covering the hydrophobic regions of these proteins, Hsp70 temporarily prevents denatured proteins from aggregating (1).

Stress sensor

Hsp70 also plays a pivotal role during cellular stress that may occur due to elevated temperature, oxidative stress, contraction related stress, changes in energy metabolism, induction of stress related messengers and/or disturbances in oxygen perfusion and transport are all stressors that can unfold cellular proteins (Figure 6). In response, Hsp70 content will increase to repair the unfolded proteins. In parallel, Hsp70 prevents aggregation and degradation of unfolded proteins by the proteasomes.

Proteasomes are large protein complexes located in the cytosol and are responsible for degrading aberrant proteins. Proteins destined for destruction are tagged with a small protein called ubiquitin. If Hsp70 does not bind the unfolded protein before ubiquitin or Hsp70 is unable to repair it, the protein will be marked for degradation and transported to the proteasome. The ubiquitin-proteasome can also facilitate degradation of newly synthesized proteins that are misfolded or contain abnormal amino acids (1).

Inhibitor of apoptosis

Hsp70 inhibits apoptosis by acting on several steps both upstream and downstream of caspase activation. The intrinsic pathway is characterized by the release of mitochondrial pro-apoptotic factors into the cytosol, while the extrinsic pathway is triggered by external signaling factors that induce the apoptotic process. Siu et al. (109) investigated apoptotic adaptations from exercise training in skeletal muscle and found that exercise decreased Bax mRNA levels (pro-apoptotic signaling) and increased Bcl-2 (anti-apoptotic signaling). Furthermore, Siu et al. (109) reported

that Hsp70 content was negatively correlated to Bax mRNA and caspase-3 activity and positively correlated to Bcl-2 mRNA and protein content. The inhibitory potential of Hsp70 over apoptosis occurs through many signaling mechanisms (e.g. JNK, NF-κB and Akt), which are directly or indirectly blocked by Hsp70, or through increased Bcl-2 protein content. These mechanisms are thought to be responsible for Hsp70's anti-apoptotic function in cells under stressful conditions (103).

Inhibitor of nuclear factor-κB (NF-κB)

Nuclear factor-κB (NF-κB) is a dimeric transcription factor that regulates a broad range of biological processes, including innate and adaptive immunity, inflammation, stress responses, B cell development and apoptosis. NF-κB signaling is regulated by binding with inhibitor of kappa B (IκB) proteins (43). In the presence of known inducers of NF-κB (e.g., ROS, IL-1β, TNFα) (23, 43), IκB is phosphorylated by IκB kinase (IKK) resulting in the release of IκB. Free NF-κB translocates to the nucleus and increases gene expression of numerous chemokines and cytokines, many of which are the same proteins that initially activated it (Figure 7) (43). Increased Hsp70 content and heat shock treatment are known to inhibit NF-κB activity (35, 82, 95, 107), likely mediated through increased levels of IκBα (35, 95) that retain NF-κB proteins in cytosol preventing gene expression of inflammatory cytokines. It therefore has been suggested that Hsp70 may exert an anti-inflammatory effect in skeletal muscle.
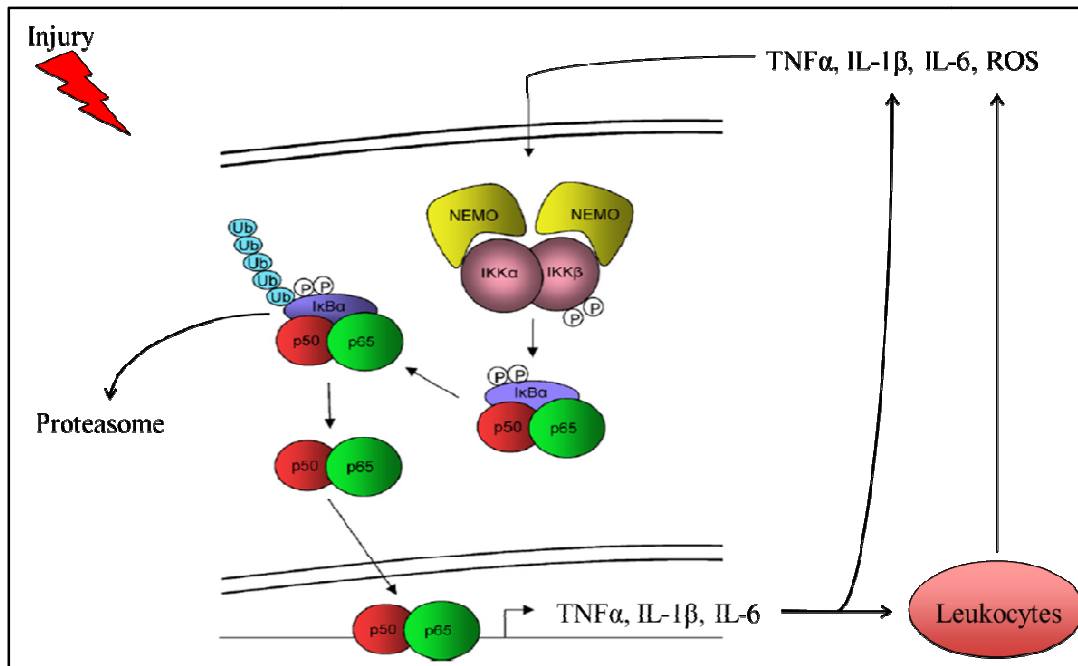
Figure 7. Depiction of the NF-κB pathway.

Changes in Hsp70 content

Eccentric contraction-induced alterations to Hsp70

Eccentric exercise is known to be a strong inducer of Hsp70 (7, 56). Expression of Hsp70

increased 4.27 fold in mouse TA muscle 48 h after a bout of 50 eccentric contractions (7).

Further, Hsp70 was elevated 6 h post-injury, peaked between 1 and 5 days, and gradually

declined up to 14 days following injury (56). The changes in Hsp70 were accompanied, but not

paralleled, by reductions in intramuscular MHC and actin, suggesting a relationship between

myofibrillar proteolysis and Hsp70 induction (56). In both studies (7, 56), the bout of eccentric

contractions resulted in large strength deficits that remained reduced for several days.

Eccentric exercise has also been shown to up-regulate Hsp70 in human muscle (98, 120-

122, 130, 141). Following a single bout of a 30 min of bench-stepping at 60 steps per minute,

Hsp70 expression in the vastus lateralis was increased at 3 (8.9 fold) and 24 (1.8 fold) hours

post-exercise, after which it declined to baseline levels 7 days into recovery (130). Willoughby et al. (141) reported similar finding in the vastus lateralis, demonstrating Hsp70 mRNA and protein content increased 97% and 128%, respectively, 48 h after eccentric exercise of the knee extensors (11). Further, Hsp70 content was found to be 227-1065% higher in the biceps brachii 48 h after resistance exercise (2 sets of 25 repetitions) with the elbow flexor muscles (7, 8). Paulsen et al. (98) recently demonstrated that increased Hsp70 content is also location specific. For example, Hsp70 content in the cytosolic fraction tended to be lower in the injured muscle 30 min after exercise but increased thereafter and was 203% higher 24 h after exercise. In contrast, Hsp70 content in the myofibrillar fraction was elevated by ~2 fold at 0.5 h and ~10 fold at 96 h post-injury.

Pharmallogically-induced alterations to Hsp70

Altering Hsp content with pharmaceuticals was initially developed for cancer therapies (111), but has extended to skeletal muscle injury models over the past decade (27, 63). Two drugs that have received recent attention are radicicol (27, 84) and 17-allylamino-17-demethoxy-geldanamycin (17AAG) (63). Both drugs are Hsp90 inhibitors and prevent association with HSF1. Then, HSF1 is free to translocate to the nucleus and subsequently increase the expression of Hsp40, Hsp70 and Hsp90 (111).

Radicicol is a natural product with antibiotic, antifungal, and antimalarial properties. When radiciol is administered intramuscularly (0.05 mg/20 g) into the hindlimb of mice there is a marked increase in Hsp70 1 day after administration (27, 84). The increase in Hsp70 was found to be similar to that of mice genetically overexpressing Hsp70 from birth (27). Alternatively, 17AAG, a derivative of the antibiotic geldanamycin, can also increase Hsp70 in

mice (63, 73). Intraperitoneal (ip) injections of 17AAG (40 mg/kg) significantly increased EDL

Hsp70 for up to 10 days after treatment, with content increased 218% at day 3 (63).

Genetically-induced alterations in Hsp70

McArdle et al. (20, 80) found a 10-20 fold increase in Hsp70 in the EDL and TA at rest of

transgenic mice. However, overexpression of Hsp70 in the skeletal muscles reduced body mass

by ~10% and muscle mass by ~20% (80). To prevent the large increase in Hsp70 content in

transgenic mice, which may not be physiologically attainable, and any other alterations that may

occur from genetic manipulation from birth, Senf et al. (105) and Dodd et al. (35) transduced rat

soleus muscle with an Hsp70 expression plasmid to specifically overexpress Hsp70. This

transduction caused an approximately 2.5 to 5-fold increase in Hsp70 protein expression over

endogenous levels (35, 105).

Heat-induced alterations in Hsp70

Heat shock proteins (Hsps) can be increased by heat shock treatment. Heat shock treatment can

be obtained through the use of warm-hot water (46, 128) or air temperature (64, 108). By

supporting rats in a hot water bath at 43°C (core 41 and 41.5°C) for 20 min (46, 128), Hsp70

expression increased, peaking at 48 h in the soleus muscle (128). Similarly, placing rats in a

heated chamber (ambient temperature, 42 ± 1.0°C) for 60 min resulted in similar elevations in

Hsp70 expression (64, 108). Immediately after heat shock treatment, Hsp70 mRNA expression

significantly increased and stayed elevated for 3 days (108).

Hsp70 reduces characteristics of skeletal muscle injury

Histological damage

Elevated levels of Hsp70 attenuate histological damage and reduce phagocytic cells infiltration

in injured muscles (63, 64, 80, 84, 108, 128). Leukocyte infiltration after heat shock treatment

was significantly lower immediately, 2 and 7 days after rats exercised downhill for 60 min compared to control rats. Furthermore, injured muscles that received heat shock treatment had fewer degenerated fibers 2, 3, and 7 days after exercise (108). Similar findings have recently been reported by Touchberry et al. (128) who demonstrated that heat shock treatment before a downhill running protocol reduced the number of infiltrating mononuclear cells and the number of swollen fibers 2 days post-injury. Heat shock treatment before or after cardiotoxin-induced injury reduced the inflammatory response and resulted in more newly formed myotubes compared to controls 7 days into recovery (64). Additionally, by day 14 the diameter of the regenerating fibers was considerably larger in the heat-treated muscle.

Alternatively, transgenic mice overexpressing Hsp70 display similar protection against injury. Three days after a bout of 450 eccentric contractions *in situ*, transgenic mice had a higher percentage of intact EDL fibers compared to wild-type mice that did the same injury protocol (80). The same group later reported that using the drug 17AAG reduced the number of centrally positioned nuclei 28 days after 450 eccentric contraction in treated vs. untreated mice (22.5 vs. 39.3%), indicating the muscle fibers regenerated quicker after 17AAG treatment (63). Further, 10 days after freeze injury, mice muscle overexpressing Hsp70 displayed less inflammation and larger fiber CSA compared to wild-type mice (84).

Blood creatine kinase (CK) levels

Blood levels of CK are used to detect skeletal muscle inflammation (i.e., myositis) or damage. Heat shock treatment prior to injury reduced CK release in cell culture (75) and human skeletal muscle (110). However, these studies did not document how heat shock treatment affected levels of Hsp70 and therefore, it is difficult to determine if the reduction in serum CK was due to an increase in Hsp70. To confirm this, Touchberry et al. (128) heat shocked rat soleus muscle in

a water bath before a downhill running protocol and analyzed serum CK 2 and 48 hours after the exercise bout. Heat shock treatment increased Hsp70 and significantly lowered levels of serum CK 2 hours after exercise compared to control treated rats. By 48 hours CK levels returned to baseline levels in both groups (64). Furthermore, heat shock treatment in rats prevented elevations in CK levels after cardiotoxin-induced injury (64).

Strength deficits

Hsp70 is significantly increased after exercise (see above) and, therefore, may play a vital role in the recovery of strength following injury. By injuring the quadriceps of male subjects through a single bout of 300 maximal voluntary eccentric contractions, Paulsen et al. (98) demonstrated that the number of Hsp70 positive fibers increased during the first 8 hours after injury and remained elevated for at least 7 days. During the same period, maximal concentric torque at 60°/s was reduced 47% immediately after exercise and remained down for the next 7 days. By comparing peak levels of Hsp70 in the days after exercise and strength deficits immediately after the bout of 300 eccentric contractions, Paulsen et al. (98) concluded that with greater strength deficits more Hsp70 was synthesized, suggesting Hsp70 is involved in the remodeling/adaptation process in skeletal muscle.

In support of this, McArdle et al. (80) reported transgenic mice overexpressing Hsp70 recovered significantly faster than their wild-type (WT) counter parts. Three hours following a bout of 450 isolated eccentric contractions, WT and Hsp70 transgenic mice exhibited similar force deficits of 50–70%. Between 3 h and 3 days, peak isometric torque of the WT mice showed a further decrease of ~20%, while no secondary loss of strength was evident in the Hsp70 mice. By day 14, peak isometric torque in the Hsp70 mice was not significantly different than pre-injury torque. In contrast, the WT mice still exhibited reductions of ~50% in peak

isometric torque. Strength deficits were eliminated 28 days into recovery in the adult WT (i.e.,

10-12 months), and adult and old (i.e., 26-28 months) Hsp70 mice, but peak isometric torque

remained 44% down in the old WT mice. McArdle et al. (80) concluded that the rapid recovery

of EDL strength of old mice overexpressing Hsp70 was remarkable considering old WT mice

sustained large force deficits even after 28 days of recovery.

In attempt to mimic the protective effects of overexpressing Hsp70 in the transgenic

mice, McArdle's group (63) used an Hsp90 inhibitor to increase Hsp70 content in old WT mice.

Treatment of 17AAG significantly increased Hsp70 muscle content for 10 days, with content

peaking after day 1. To determine if 17AAG could attenuate strength deficits after eccentric

injury (i.e., 450 eccentric contractions), mice were injected 3 days before exercise and then after,

every 7 days for up to 4 weeks. Treatment of 17AAG was associated with significant

improvements in recovery of force 28 days post-injury compared to the untreated mice. In fact,

peak isometric force generated by the EDL muscle of 17AAG-treated mice returned to pre-

injury values while untreated EDL force remained ~50% down (63). Their results (63, 80)

indicate that pharmallogically increasing Hsp70 content shortly before eccentric exercise can

expedite the recovery process in a similar fashion to that of Hsp70 overexpression using

transgenic mice. Though these studies reported that Hsp70 enhanced the functional return of

strength following eccentric injury, they did not elucidate the mechanistic properties for Hsp70's

beneficial effects. However, in both studies, histological damage and secondary injury were

attenuated and prevented, respectively. Because of these observations, the authors suggested

Hsp70 may reduce the activity of proteins and/or signaling pathways associated with oxidative

stress and inflammation (63, 80).

Hsp70 facilitates recovery after injury

Protein folding and chaperone activity

Hsp70 are chaperone proteins that refold denatured proteins and fold newly synthesized proteins and therefore are vital components involved in cellular repair. Hsp70 is known to translocate to myofibrillar proteins after eccentric injury, suggesting it may be a stabilizer of the myofibrillar structure, refolding denatured contractile proteins before they irreversibly aggregate and denatured and are degraded (98). Increased Hsp70 content in the myofibrillar fraction after exercise could indicate that Hsp70 plays a role in the assembly and incorporation of new proteins in the myofibrillar framework (98). McArdle et al. (80) suggested that the concentration of Hsp70 may be the rate limiting factor of protein synthesis in skeletal muscle.

Protein synthesis: Intracellular signaling and SC activity

One of the first observations that demonstrated heat shock treatment impacted protein synthesis and content *in vivo* was by Kojima et al. (64) in 2007. They demonstrated that heat shock treatment before or shortly after a cardiotoxin injection resulted in significantly higher levels of TA muscle protein content 28 days into recovery than rats that did not receive heat shock treatment. The heat-treated rats also exhibited fewer infiltrating mononucleated cells and had larger regenerating muscle fibers 2 weeks after the injury. By day 28, the heat-treated rats had almost fully recovered while the untreated rats still presented small regenerating fibers (64). Touchberry et al. (128) recently confirmed the results of Kojima et al. (64) by analyzing total soleus muscle protein and MHC neonatal (MHCn) content 2 h and 2 days after heat-treated rats performed a downhill running protocol. Two hours following exercise there were no differences in total protein content between heat-treated and untreated rats; however, 2 days after injury total protein content was higher in the heat-treated group compared to the untreated group.

Furthermore, at both time points, MHCn content was significantly higher in the heat-treated group (128). Touchberry et al. (128) speculated that heat shock treatment prior to muscle damage increased protein synthesis, and/or prevented the loss of intracellular proteins following damage. Preventing the loss of intracellular proteins may be mediated by stabilization of the sarcomeres by Hsp70 binding cytoskeletal/myofibrillar proteins (98) and ultimately stopping the global diffusion of proteins out of damaged myofibrils (128). Alternatively, increased total protein and MHCn content may be orchestrated through intracellular signaling pathways and/or SC activity.

To assess changes in intracellular signaling, Touchberry et al. (128) analyzed Akt/p70s6k signaling and mitogen-activated protein kinases (MAPK) (i.e., ERK1/2/JNK), two signaling pathways involved in protein synthesis. ERK1/2 activity 2 h after injury was significantly lower in the heat-treated rats, but returned to control levels by day 2. No differences were observed between groups for Akt, p70sk6 and JNK at either time points (128). Because heat shock treatment did not increase the activity of the Akt/p70s6k or MAPK pathways (128), an alternative mechanism is that heat stress and thus evaluated levels of Hsp70 increased SC activity (27, 64, 85, 95, 128). In support of this, 3 days after heat shock treatment, the relative population of SCs at rest significantly increased in rat soleus muscle compared to control muscle (95). Similar findings have been observed after injury (27, 64) and in atrophied muscle (85). Radicicol-treated mice had more satellite cells from 1 (31%) to 10 (63%) days after crotoxin injury compared to vehicle-treated mice (27). Similarly, 3 days after cardiotoxin injury, SC activity in heat-treated rats was found to be significantly higher than untreated rats (64). Miyabara et al. (85) demonstrated that after 7 days of immobilization the number of SCs in mouse EDL and soleus muscle was reduced, and by overexpressing Hsp70 this reduction was

prevented. Furthermore, Hsp70 muscle displayed centrally positioned nuclei and regenerating

fibers earlier in recovery compared to controls. Miyabara et al. (84) demonstrated that 10 days

after cyrolesion, Hsp70 transgenic mice had fewer MHCn-positive cells than did wild-type mice,

while Kayani et al. (63) reported that increased Hsp70 content reduced the number of centrally

positioned nuclei 28 days after 450 eccentric contraction, indicative of complete recovery of the

regenerating fibers. Taken together, these results suggest that elevated levels of Hsp70 prior to

injury may expedite the recovery process through increasing SC activity.

Inflammation and secondary damage

Infiltration of phagocytes following injury is accompanied by an increased production of free

radicals and lipid and protein oxidation. Free radicals, specifically ROS, are primarily produced

by activated neutrophils shortly after injury. Neutrophils orchestrate repair by removing cellular

debris, but can also damage the fiber if excess ROS and proteases are released (13, 21, 124).

This secondary damage or injury can delay the muscle's regenerative capabilities and increase

strength deficits in the days following the initial exercise bout. Pizza et al. (99) suggested that

ROS may exacerbate pre-existing muscle injury by damaging previously uninjured muscle.

Several reports have demonstrated that increased Hsp70 levels reduce the number of phagocytic

cells infiltrating (108, 128) the injured muscle fibers and the amount of fiber swelling (128) after

injury. Elevated levels of Hsp70 may also protect the fibers from an unnecessarily strong

inflammatory process (i.e., excess ROS and protease production) that can cause secondary

damage (80, 98, 108, 129) and delay recovery.

Proposed mechanisms by which Hsp70 enhances recovery

Hsp70 content inhibits NF-κB signaling

It is currently unknown how Hsp70 content enhances recovery after injury, however previous research would suggest there is a link between Hsp70 and the NF-κB pathway. As mentioned previously, the activity of NF-κB is primarily regulated by its interaction with inhibitory IκB proteins. Inactive NF-κB is sequestered in the cytoplasm by the IκBα protein (19, 43). Upon receiving a multitude of extracellular signals, IκB proteins are phosphorylated, ubiquitinated, and targeted to the proteasome for degradation (43, 143). NF-κB is dissociated from the IκB inhibitory proteins and then translocated to the nucleus leading to transcriptional activity. NF-κB is a major pleiotropic transcription factor modulating inflammation (43, 107). Proteins of particular interest that are transcribed by NF-κB include IL-1β, IL-6, and TNFα. Importantly, these proteins are known to orchestrate neutrophil and macrophage infiltration, SC activity and regulate NF-κB signaling (Figure 7).

Although, Hsp70 has been shown to inhibit NF-κB signaling, the specific details and models remain to be explored. Ohno et al. (95) demonstrated that heat shock treatment resulted in increased IκBα and decreased phospho-NF-κB content. Furthermore, several studies have shown that deceased NF-κB activity is caused by the upstream increase in Hsp70 (82, 107, 142). Heat shock treatment in BEAS-2B cells, a human bronchial epithelium cells, increased expression of IκBα mRNA and subsequently inhibited NF-κB (142). Meldrum et al. (82) reported liposomal transfer or thermal induction of Hsp70 prevented NF-κB activation and translocation to the nucleus in renal tubular cells. Furthermore, in a macrophage cell line, overexpression of Hsp70 inhibited the nuclear translocation of p65, the transcriptionally active

component of the NF-κB complex, and prevented the degradation of IκBα (107). More recently, it was reported that overexpression of Hsp70 in rats increased IκBα levels and prevented an increase in NF-κB activity that is associated with immobilization (35). Taken together, these findings suggest that Hsp70 may inhibit NF-κB activity through increasing the levels IκBα that are available to bind and retain NF-κB in the cytosol.

NF-κB gene products are also inhibited by Hsp70. For example, Meldrum et al. (82) reported that liposomal transfer or thermal induction of Hsp70 prevented TNFα gene transcription. Similarly, Shi et al. (107) demonstrated Hsp70 overexpression could inhibit TNFα, as well as other pro-inflammatory cytokines like IL-1. In rat soleus muscle, heat shock treatment reduced TNFα content after 3 days (95). Importantly, this decrease in TNFα was observed following an increase in Hsp70 and a decrease of phospho-NF-κB content. Because up-regulation of Hsp70 prevents IL-1 and TNFα gene transcription (82, 107) and NF-κB activation synthesizes these pro-inflammatory cytokines (43), inhibition of IL-1and TNFα is likely mediated through reduced NF-κB activity (95).

Role of NF-κB in inflammation

NF-κB signaling leads to the production of cytokines (e.g. IL-1β, IL-6, and TNFα) that orchestrate the inflammatory response. Inflammation increases neutrophil and macrophage accumulation in the cell. Neutrophils produce a host of cytotoxic substances, including ROS, such as superoxide anions, hypochloride, and hydrogen peroxide (13, 16). Furthermore, the cytokines IL-1β, IL-6, and TNFα may all stimulate pathways that contribute to activation of NADPH oxidase, a key enzyme that initiates the ''respiratory burst'' and subsequent release of ROS (21, 31). Though neutrophil activation leads to beneficial proteolysis and removal of debris, they can also release high concentrations of cytolytic and cytotoxic molecules (e.g.

proteases, ROS) that can damage healthy muscle (125). Whether a positive or negative response will prevail depends on many variables such as, the site of ROS production, persistence of ROS flow and the cell's antioxidant status. Prolonged, severe oxidative stress may imbalance intracellular antioxidant homeostasis and overall muscle health (8). High levels of ROS cause functional oxidative damage to proteins, lipids, nucleic acids and cell components, induce a significant rise in intracellular Ca^{2+} concentration and promote signaling cascades for apoptosis or autophagy via NF-κB or FoxO pathways (8). For these reasons, high ROS levels are often contributing factors in muscle degeneration, atrophy, sarcopenia, wasting, and chronic muscle diseases. Recent evidence has shown that administration of an antibody that blocks the "respiratory burst" and degranulation of neutrophils prior to lengthening contractions also produces large reductions in microscopically discernable muscle damage (17).

With injury, excessive inflammation and ROS production is thought to cause secondary damage and loss of force (124). Together with antioxidants, Hsp70 may mediate this process by protecting the cell against oxidative stress (71, 98) and reducing levels of pro-inflammatory cytokines (58) and ROS through inhibition of NF-κB signaling (35, 82, 105, 107). In parallel, leukocytes infiltration was abated in muscle overexpressing Hsp70 or muscle that received heat shock treatment prior to injury (108, 128). Hsp70 may also facilitate recovery through increased ROS scavenger/antioxidant activity within the fiber (84, 108). Specifically, superoxide dismutase activity was increased 3 days after heat-treated rats preformed eccentric exercise (108). Further, glutathione peroxidase activity was found to significantly higher 10 days after freeze-injury in mice overexpressing Hsp70 compared with muscles of wild-type mice (84).

Differentiation of SCs into myotubes is a fundamental prerequisite for muscle regeneration. NF-κB is associated with a negative regulation of skeletal muscle differentiation (47, 68, 132). NF-κB is thought to reduce myogenesis by inhibiting the synthesis of MyoD, a muscle-specific helix-loop-helix transcription factor central to muscle development and repair (47). In addition, NF-κB is able to suppress myofibrillar gene expression through the regulation of the myogenic transcriptional repressor Yin Yiang 1 (YY1) (132). In skeletal muscle, YY1 is considered to play a negative role in myogenesis by directly repressing the synthesis of late-stage differentiation genes, including skeletal actin, muscle CK and MHCII. In support of these findings, treatment of primary myoblasts with the NF-κB inhibitor curcumin stimulated myoblast fusion and enhanced myogenesis and repair, characterized by increased expression of MHCe after freeze-induced injury in mice (118). Similarly, 10 days after cardiotoxin-induced injury, mouse muscle depleted of NF-κB signaling (IKK knockdown mice) showed improved morphology, reduced intracellular space, and larger size of new myofibers compared to wild-type mice. Furthermore, fiber size and strength were preserved as late as 28 days after denervation, while wild type mice were not protected against atrophy (89).

Additionally, TNFα, one of inflammatory cytokines synthesized by NF-κB activation, can suppress SC differentiation (34, 67) through the inhibition of the myogenic regulatory factors MyoD and myogenin (115). TNFα activates NK-κB creating a positive feedback loop resulting in increased pro-inflammatory cytokine production and further inhibition of myogenesis, but also an increased production of ROS. Prolonged elevations in ROS mediated through neutrophils may cause further injury through oxidative damage and delay SC differentiation (8, 124). Ardite

et al. (2) showed that ROS depleted intracellular glutathione which caused further intracellular accumulation of ROS. The elevated ROS levels favor NF-κB activation and reduce the expression of MyoD impairing myogenesis.

Working hypothesis

Skeletal muscle is a force producing tissue that is required for locomotion, metabolism, generating heat and the maintenance of posture. Upon injury, the muscle's functional capacity is diminished and its return is dependent on the ability of the muscle to undergo remodeling. This remodeling occurs through a coordinated inflammatory response that includes SC activation, proliferation and differentiation followed by muscle growth (48, 124). Numerous transcription factors, such as NF-κB, regulate these inflammatory and myogenic processes (5). For example, NF-κB is activated by pro-inflammatory cytokines (e.g. TNFα) and ROS that are produced and released by the injured muscle or invading leukocytes (97). TNFα acts as a mitogen in muscle proliferation (96), but also as a negative regulator of differentiation (67). Furthermore, TNFα increases ROS production, which in excess inhibits myogenesis (8, 119). Following injury, sustained NF-κB signaling is thought to blunt skeletal muscle regeneration (8). For this reason, many therapeutic trials have used NF-κB inhibitors to ameliorate muscle recovery following injury or disease (90).

The drug 17AAG, a Hsp90 inhibitor (111), increases Hsp70 content *in vitro* (63) and *in vivo* (63, 73). Recently, treatment of 17AAG has been reported to improve recovery following contraction-induced injury in old muscles (63) and attenuate atrophy following unloading (73). Hsp70 is known for its role as a molecular chaperone (50, 70), but the beneficial effects observed in these recent studies are likely mediated by other means. By increasing Hsp70 through heat

37

shock treatment, genetic manipulation or the use of pharmaceuticals, several investigators have documented reductions in NF-κB activity (35, 82, 95, 105, 107, 142) and TNFα content (82, 95, 107), and enhanced SC (27, 64, 85, 95, 128) and ROS scavenger/antioxidant activity (84, 108). However, how Hsp70 content stimulates skeletal muscle regeneration has not yet been explored. Therefore, the purpose of this study will be to: (1) determine the effects of 17AAG on histological alterations and functional deficits following muscle injury and, (2) quantify the effects of 17AAG on NF-κB activity, NF-κB-dependent signaling components and ROS/antioxidant levels (Figure 8).
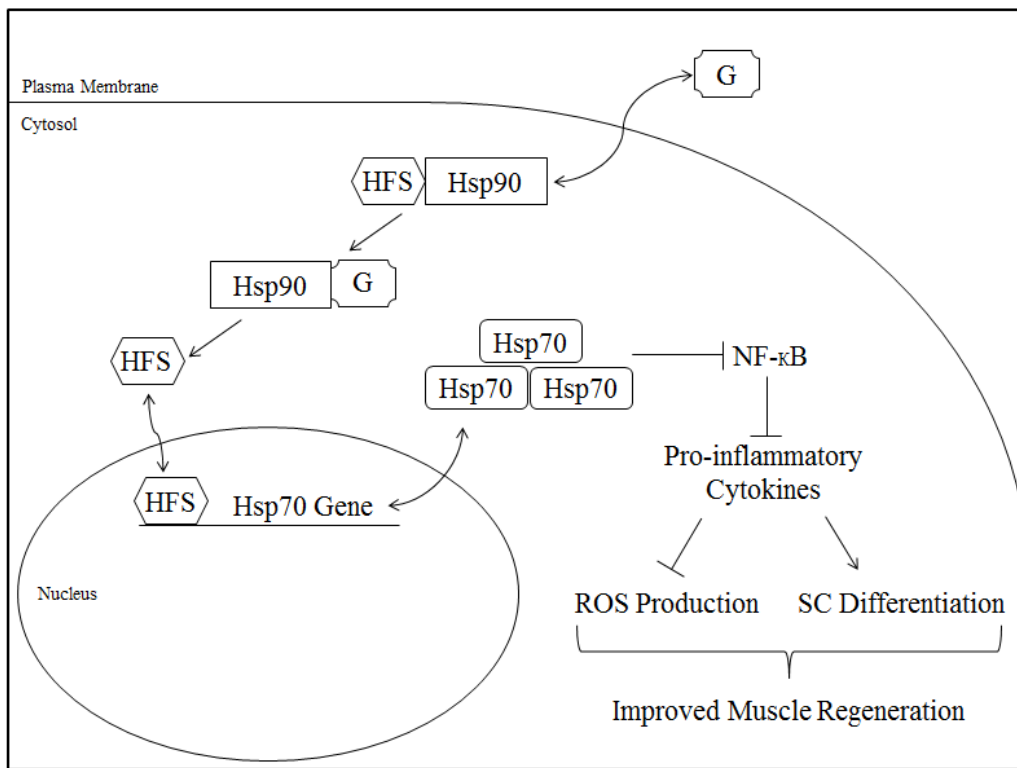
Figure 8. Proposed mechanisms by which 17AAG (G) improves muscle regeneration.

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2 HEAT SHOCK PROTEIN 70 REGULATES TUMOR NECROSIS FACTOR ALPHA AND MYOGENIN IN SKELETAL MUSCLE FOLLOWING CHEMICAL-INDUCED INJURY

Introduction

Skeletal muscle is required to sustain life and is involved in daily tasks such as locomotion, movement and the maintenance of posture. These tasks are achieved through the muscle's capacity to produce force, which is dependent upon the number of sarcomeres acting in parallel and the force generated per sarcomere. Following chemical or toxin-induced injury, ~70-90% of the afflicted myofibers are lost or lysed (47, 57), drastically reducing the muscle's ability to produce force. Reductions in force have been reported to be over 90% in mouse skeletal muscle and take several weeks to fully recovery (9). The return of strength, and thus function, is dependent on the muscle's ability to regenerate, a highly coordinated process that involves inflammation, degeneration of damaged myofibers and the formation of new myofibers (i.e., myogenesis) (47, 52, 53).

The inflammatory response begins shortly after the injury, coincident with the influx of phagocytic neutrophils and macrophages. These inflammatory cells, mainly macrophages, facilitate muscle regeneration through phagocytosis of cellular debris and the release of soluble factors, including chemoattractants, growth factors and cytokines (52, 53). Of particular interest is TNF-α, a cytokine that peaks 1-3 days post-injury (10, 56, 58) during both the inflammatory and subsequent myogenic phases of recovery, suggesting TNF-α has a binary role in skeletal

muscle regeneration (9, 10, 53, 56). Classically portrayed as a pro-inflammatory mediator, TNF-α activates NF-κB inducing TNF-α transcription, further promoting NF-κB activity and thus inflammation (22). However, TNF-α also acts as a mitogen increasing satellite cell activation and proliferation (33, 43, 50). The impact of TNF-α on myoblast differentiation appears to be more complex, with some reporting TNF-α stimulates p38 MAPK activating key differentiation mediators including myocyte enhancer factor (MEF)-2, myogenin and the Cdk inhibitor p21, and suppression of cyclin D1 (9, 10), while others demonstrate TNF-α inhibits differentiation by destabilizing MyoD mRNA and degrading the MyoD protein (24, 30, 31, 50). These contradictory findings likely reflect the concentration of and persistence at which TNF-α remains in the microenvironment (10). Despite considerable efforts to understand the mechanisms that control skeletal muscle regeneration, the complete profile of intrinsic and extrinsic cues that mediate TNF-α expression remain unknown.

Heat shock protein 70 (Hsp70, so-called Hsp72) also responds to skeletal muscle trauma and increases several fold shortly following skeletal muscle injury (16, 25, 51). Induction of Hsp70 mediates skeletal muscle mass, regeneration and function, and therapies targeting Hsp70 have proven beneficial in animal models of skeletal muscle atrophy, aging and injury (44). These beneficial effects are, in part, due to its role as a molecular chaperone, refolding denatured proteins and controlling protein quality (38). In addition, Hsp70 also regulates the activity of various transcription and signaling factors, including NF-κB and TNF-α (4, 5, 37, 39, 48). Some evidence suggests that Hsp70 initiates the inflammatory response (1, 2, 21, 46) by activating neutrophils (23, 40), macrophages and peripheral blood monocytes (3, 4, 28) increasing TNF-α expression (4, 5). Conversely, Hsp70 has also been reported to increase levels of IκBα (15, 39), an NF-κB inhibitor, thereby decreasing NF-κB activity (15, 37, 39, 48) and TNF-α expression

(37, 39, 48). These divergent findings are likely due to variability in experimental designs, including the type of cell line or tissue examined, the methods used to manipulate Hsp70 (e.g., heat shock treatment, genetic manipulation, pharmaceuticals) and when skeletal muscle is targeted, the type of stress or insult imposed on it (e.g., hindlimb unloading, injury, aging). Nonetheless, because TNF-α and Hsp70 expression increase shortly following insult and knowing skeletal muscle regeneration is dependent on inflammation and myogenesis, it is difficult to reconcile why Hsp70 would inhibit TNF-α expression, particularly following skeletal muscle injury.

Therefore, we sought to determine if the pharmaceutical 17-allylamino-17-demethoxy-geldanamycin (17-AAG), a known stimulator of Hsp70, would alter TNF-α content following skeletal muscle injury induced by an intramuscular injection $BaCl_2$. An intramuscular injection of $BaCl_2$ produces damage similar to that of snake toxins in which degeneration and inflammation develop more quickly than other types of injury (32), thereby amplifying TNF-α signaling. Next, we evaluated the relationship between TNF-α and myoblast differentiation by analyzing myogenin content. Finally, to determine if these molecular markers of regeneration have any measureable impact on muscle structure and function, we assessed regenerating fiber cross-sectional area (CSA) and *in vivo* torque deficits following the $BaCl_2$-induced injury. We hypothesized treatment of 17-AAG would increase TNF-α and myogenin content above that of the injured control muscle in the days following the injury, thereby enhancing both skeletal muscle regeneration and function.

Methods

Animals

Male C57BL6 mice ~2-4 months old were used in this study. Mice were housed in groups of 5 animals per cage, supplied with food and water ad libitum, and maintained in a room at 20–22°C with a 12-h photoperiod. The mice were euthanized with an overdose of isoflurane followed by cervical dislocation. All procedures were approved by the Georgia State University Institutional Animal Care and Use Committee and met the guidelines set by the American Physiological Society.

Experimental design

Mice were randomly assigned to one of two groups: vehicle-treated or drug-treated. The vehicle-treated (i.e, control) group received intraperitoneal (i.p.) injections of dimethyl sulfoxide (DMSO; Sigma, St Louis, MO), while the drug-treated group received i.p. injections of 17-AAG (LC Laboratories, Woburn, MA) at a dose of 40 mg/kg body weight in 100 µl of DMSO. This dose of 17-AAG and route of delivery has been shown to result in 99% bioavailability of the drug in skeletal muscle (19). Treatment of DMSO or 17-AAG began 3 days prior to a $BaCl_2$-induced injury to the left tibialis anterior (TA) muscle. Following this initial dose, mice continued to receive treatment every 7 days for up to 3 weeks as previously described (26). Thus, injections occurred 3 days prior, and 4, 11 and 18 days post-injury.

One in a half, 3.5, 7, 14 or 21 days following the injury, the right and left TA muscles were excised to determine if treatment of 17-AAG altered markers of regeneration.   Following dissection, TA muscles were weighed and immediately frozen in liquid nitrogen and stored at -80°C for Western blots analysis of Hsp70, TNF-α, myogenin and GAPDH. A subset of TA

muscles from days 7, 14 and 21 were mounted in a medium (O.C.T., Merck, UK), frozen rapidly in 2-methylbutane cooled in liquid nitrogen, and sectioned for histological analysis to determine the CSA of the regenerating fibers. For both groups, the contralateral (right) TA muscles served as an uninjured baseline, but also to determine if 17-AAG affected the muscle in the unstressed state. In the event that uninjured muscles did not differ across time, they were combined into their respective groups and listed as "uninjured control" or "uninjured 17-AAG".

To assess if 17-AAG attenuated strength deficits following the $BaCl_2$-induced injury, *in vivo* isometric torque produced by the left anterior crural muscles (TA, extensor digitorum longus and extensor hallucis muscles) was measured immediately prior to the injury (day 0), and 7, 14 or 21 days into recovery. Due to significant swelling and edema, torque was not assessed 1.5 and 3.5 days post-injury. See Figure 9 for the timeline of the experimental design.
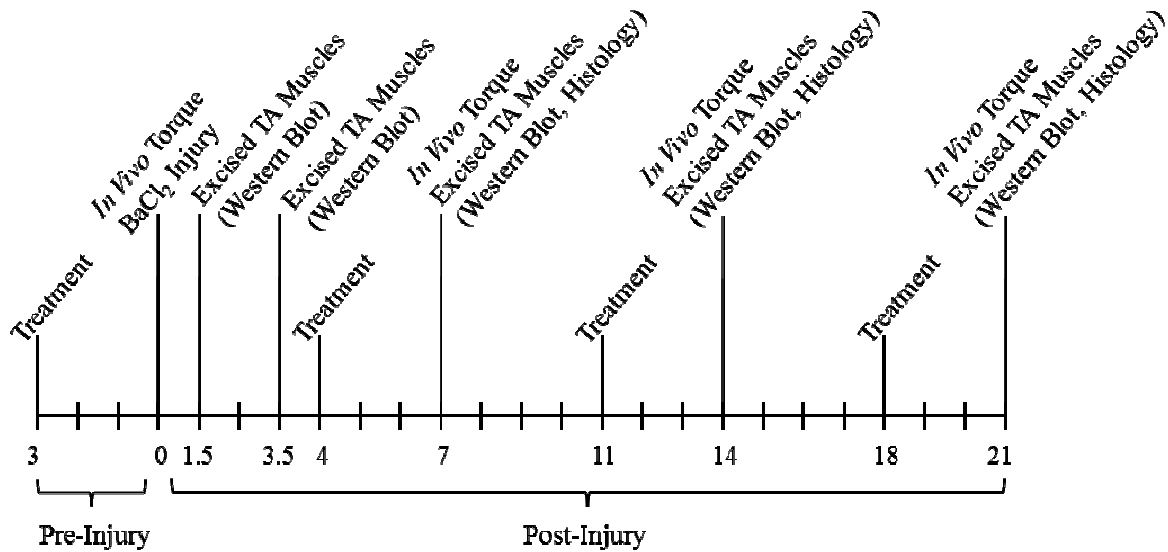
Figure 9. *Timeline (in days) of the experimental design. Treatment was either an intraperitoneal injection (i.p.) of 40 mg/kg of 17-AAG suspended in DMSO or a vehicle, DMSO. Tibialis anterior (TA) muscles were removed from anesthetized mice and stored for Western blot analysis or histology. On day 0 (immediately pre-injury) and 7, 14 or 21 days post-injury in vivo torque was assessed.*

Experimental methodology

Barium chloride (BaCl$_2$)-induced skeletal muscle injury

Immediately after recording peak isometric torque, 50 μl of a 1.2% BaCl$_2$ solution (diluted in sterile saline) was injected into the left TA muscles of the anesthetized mice using a 28 guage needle as previously described (43). Briefly, the needle was inserted at the origin of the TA, extended past the mid-belly of the muscle to a region just superior to the distal tendon. Next, the diluted BaCl$_2$ solution was continuously injected into the TA muscle as the syringe was slowly removed.

Protein extraction, protein quantification and Western blotting

A portion of TA muscles (10-15 mg/muscle) were homogenized in ice-cold RIPA lysis and extraction buffer (Thermo Scientific, Rockford, IL) supplemented with a protease inhibitor cocktail (Thermo Scientific, Rockford, IL). Muscle homogenates were centrifuged at 10,000 g for 15 min at 4°C and protein content of the soluble portion (i.e., supernatant) was quantified using the bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL). A portion of the muscle homogenate was then diluted in a loading buffer and boiled for 4 min. Equal amounts of protein (30 μg) were loaded onto a 12% SDS polyacrylamide gel and separated according to molecular weight (100 V for 80 min). The protein was then transferred to a nitrocellulose membrane using a trans-blot turbo transfer system at a constant 1.3 A, up to 25 V for 7 min (Bio-Rad Laboratories, Hercules, CA) and blocked for 1 h at room temperature in 5% nonfat dried milk (w/v) in tris-buffered saline with 0.1% Tween-20 (TBS-T) on an orbital shaker. Following the block, the membranes were probed with Hsp70 (HSPA1A, 1:6000; R&D Systems, Minneapolis, MN), TNF-α (52B83, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), myogenin (F5D, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and GAPDH (GA1R,

1:7500; Thermo Scientific, Rockford, IL) primary antibodies for 2 h at room temperature on an orbital shaker. Following incubation in the primary antibodies, membranes were washed with TBS-T (3 × 15 min) and then probed with the appropriate secondary antibody (goat anti-rabbit IgG, 1:15000; Sigma-Aldrich, St. Louis, MO or donkey anti-mouse IgG, 1:15000; Thermo Scientific, Rockford, IL) for 1 h at room temperature with shaking and washed as previously stated. Membranes were treated with an enhanced chemilumescent solution (Thermo Scientific, Rockford, IL) prior to detection using a BioRad ChemiDoc imaging station (Bio-Rad Laboratories, Hercules, CA) and analyzed by densitometry using QuantityOne software (Bio-Rad Laboratories, Hercules, CA).

Histology

Transverse, serial sections at 10 μm were cut through the mid-belly of the TA muscles using a cryostat (Leica CM1850, Leica, Germany). Sections were then processed for hematoxylin and eosin staining, dehydrated, mounted, and visualized at 10X with a VanGuard light microscope (VEE GEE Scientific, Kirkland, WA) as previously described (41-43). Regenerating fibers were identified by the presence of centrally positioned nuclei. Cross-sectional areas (CSA) of approximately 200 regenerated fibers per muscle were calculated using ImageJ software (National Institutes of Health, Bethesda, MD).

In vivo analysis of muscular torque

Contractile function (i.e., torque–frequency relationship) of the left anterior crural muscles was measured *in vivo* immediately before (day 0), and 7, 14 or 21 days after injury induction as previously described (7, 8, 35). Briefly, mice were anesthetized with isoflurane (1.5% isoflurane and 400 mL O_2 per min) and placed on a temperature controlled platform to maintain core body temperature between 35 and 37°C. The left knee was clamped and the left foot was secured to

an aluminum "shoe" that is attached to the shaft of an Aurora Scientific 300B servomotor (Aurora Scientific, ON, Canada). Sterilized platinum needle electrodes were inserted through the skin for stimulation of the left common peroneal nerve. Stimulation voltage and needle electrode placement were optimized with 5-15 isometric contractions (200 ms train of 0.1 ms pulses at 300 Hz). Following optimization, contractile function of the anterior crural muscles was assessed by measuring isometric torque as a function of stimulation frequency (20-300 Hz), with peak isometric torque recorded at 300 Hz.

Statistical Analysis

An independent t-test or factorial ANOVA (treatment by time) was used for comparisons before or across the study, respectively. A Bonferroni's post hoc test was performed in the event of a significant ANOVA. An α-level of ≤ 0.05 was used for all analyses. Values are presented in mean±SEM. All statistical testing was performed using SigmaPlot version 11.0 (Systat Software, San Jose, CA).

Results

Mouse body weights (BW) and tibialis anterior (TA) wet weights

Mouse body weights did not differ between control and 17-AAG groups before or after the injury. However, as a result of aging, body weights were 8-12% greater at day 21 relative to the pre-injury, and 1 and 3 day post-injury body weights ($p<0.01$) (Table 1).

Uninjured TA muscle wet weights were not different between groups or across time. Injured TA muscle wet weights also did not differ between control and 17-AAG groups. Among the groups, the injury caused significant edema and increased muscle wet weights 20% 1.5 day post-injection ($p<0.001$). By day 3.5, edema was still present, but large amounts of the muscle

fascicles were disorganized which reduced muscle weights comparable to that of the uninjured muscle. Seven days post-injury, swelling was reduced and muscle wet weights declined 34% from day 3.5 (p<0.001). By days 14 and 21, muscle wet weights were greater than that of the uninjured muscle (p<0.01) (Table 1).

Table 1. *Mouse body weights (BW) and tibialis anterior (TA) weights.*

	Control						17-AAG					
	0d	1d	3d	7d	14d	21d	0d	1d	3d	7d	14d	21d
n	27	12	10	10	10	7	27	11	14	10	10	7
BW (g)	24.3 ± 0.3^a	23.4 ± 0.5^a	24.4 ± 0.7^a	$24.6\pm0.3^{a,b}$	$24.5\pm0.6^{a,b}$	$26.8\pm0.4^{b,c}$	24.6 ± 0.3^a	23.6 ± 0.4^a	23.7 ± 0.4^a	$24.8\pm0.6^{a,b}$	$25.2\pm0.5^{a,b}$	$26.0\pm0.7^{b,c}$
TA (mg)	42.4 ± 0.6^a	50.6 ± 1.4^b	44.6 ± 1.7^a	35.1 ± 1.2^c	$48.2\pm1.9^{b,d}$	58.8 ± 2.9^e	43.3 ± 0.7^a	51.9 ± 2.0^b	40.3 ± 1.3^a	33.1 ± 1.6^c	$46.8\pm1.2^{b,d}$	55.1 ± 0.4^e

Values are means ±SEM. Mouse body weights (BW) and tibialis anterior (TA) weights across the study. Time points 1.5, 3.5, 7, 14 and 21 reflect days post-injury whereas 0 indicates uninjured or pre-injury data. No differences were detected in mouse BW or TA weights between the control and 17-AAG groups at any time point. Sample sizes (n) also reflect the number of mice used for torque assessments before (day 0) and 7, 14 or 21 days following the $BaCl_2$-induced injury. Values with same letter are not significantly different from each other.

Soluble protein and GAPDH Content

Treatment with the vehicle or 17-AAG did not alter soluble protein content of the uninjured TA muscles. Further, soluble protein content of the injured TA muscles did not differ between groups in the days following the injury. However, the $BaCl_2$ injection reduced soluble protein content 1.5 (40%) and 3.5 (22%) days post-injury (p<0.001). By days 7, 14 and 21 soluble protein content was similar to the uninjured TA muscles (Figure 10 *A*).

No differences were observed in GAPDH content of the uninjured control and 17-AAG groups across the study. The $BaCl_2$ injection resulted in large reductions in GAPDH content 1.5 (96%) and 3.5 (82%) days post-injury (p<0.001), which were similar between groups. By day 7, there as a trend for GAPDH content to be reduced when compared to pre-injury levels, (22%, p=0.07). In the following weeks, GAPDH content was similar to that of the uninjured muscle

(Figure 10 **B**). Because GAPDH, a common normalizing protein, was significantly altered

following the injury, Hsp70, TNF-α and myogenin were expressed as absolute values.
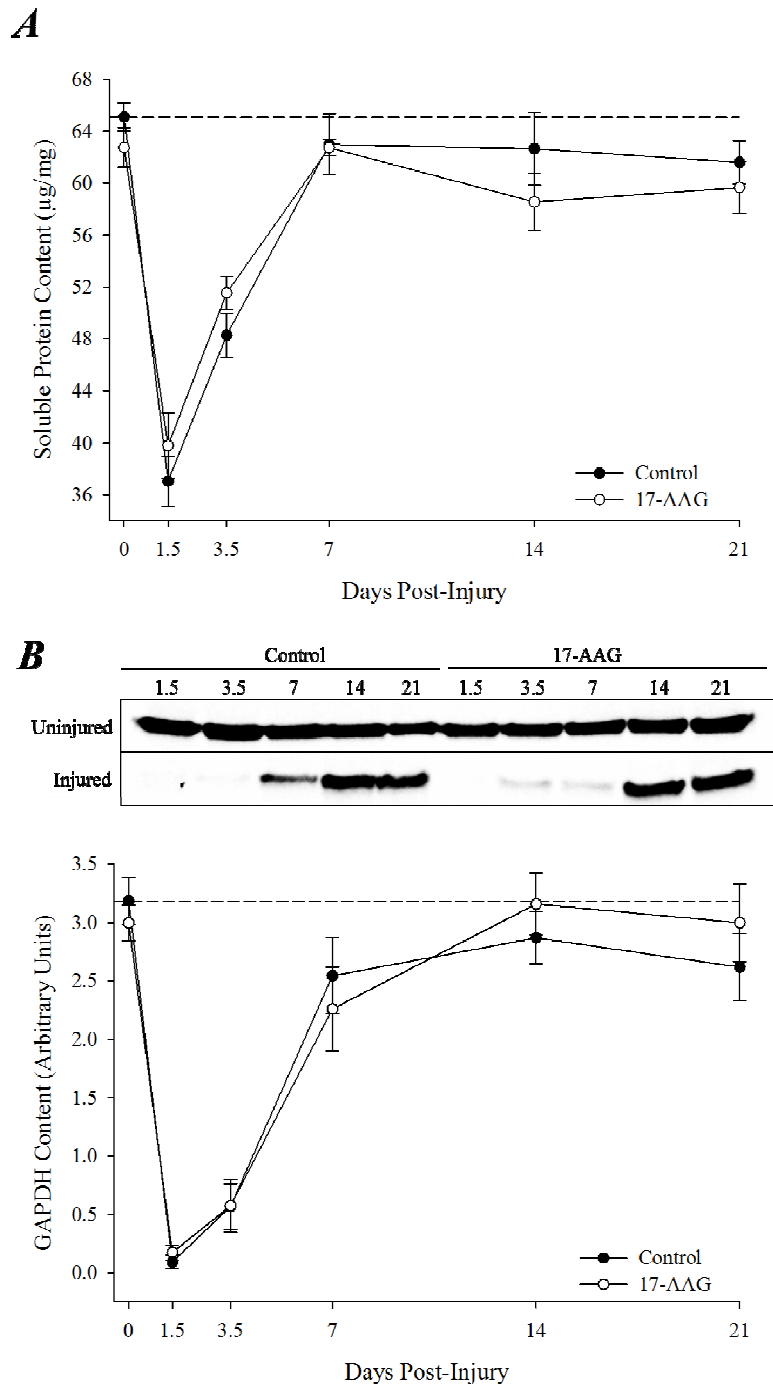
Figure 10. *(A) Soluble protein and (**B**) GAPDH content of tibialis anterior (TA) muscle before and after the BaCl$_2$-induced skeletal muscle injury. Soluble protein content was determined using a bicinchoninic acid (BCA) assay while GAPDH was determined using Western blot*

analysis. Muscles were assessed at day 0 (uninjured) and 1.5, 3.5, 7, 14 or 21 days post-injury. No differences were observed in soluble protein or GAPDH content between control and 17-AAG groups at any time point. The dashed line indicates content of uninjured control muscle. Uninjured: n=19-21/group, Injured: n=4-6/group. Values are mean±SEM.

Hsp70 Content

Treatment with 17-AAG increased Hsp70 content ~500% in the uninjured muscle (p<0.001). Following the injury, Hsp70 content in both groups was below that of the uninjured control muscle, but these levels were similar between the groups. However, due to the significant increase in Hsp70 content in the uninjured 17-AAG group, the drop in Hsp70 at 1.5 days (90%, p<0.001) was more pronounced when compared to the vehicle treated group (41%, p=0.11). At 3.5 days post-injury, Hsp70 content remained similar between the groups, but was still depressed by 85% (p<0.001) in the 17-AAG treated muscle while Hsp70 content in the control group was back to pre-injury levels. By day 7, Hsp70 content in the control muscle increased 150% above pre-injury content (p=0.17), which slowly returned to baseline by day 21. Hsp70 content in the muscle treated with 17-AAG was back to its pre-injury level by day 7 and remained there or slightly above through day 21. Furthermore, at these time points (i.e., 7, 14 and 21 days), Hsp70 content was greater in the 17-AAG groups when compared to the injured controls (p<0.001) (Figure 11 *A*).

TNF-α Content

No differences were detected in TNF-α content in the uninjured control or 17-AAG groups over the duration of the study. The injury resulted in large increases in TNF-α content at days 1.5 and 3.5 (p<0.001), which returned to pre-injury levels by day 7 and remained down through day 21. Specifically, levels of TNF-α in the injured control muscle were elevated 864 and 404%, and 893 and 603% in the 17-AAG groups at 1.5 and 3.5 days post-injury, respectively. By day 7, TNF-α

content in the control group was elevated 68% while TNF-α content remained 242% above its

pre-injury levels in the 17-AAG group. By days 14 and 21, TNF-α content was near or slightly

above baseline levels. Although no treatment by time interaction was observed (p=0.35), a

significant treatment effect was found (p=0.045). That is, TNF-α content was higher following

the injury in the muscle treated with 17-AAG when compared to the vehicle treated muscle

(Figure 11 *B*).

Myogenin Content

No differences were observed in myogenin content of the uninjured control and 17-AAG groups

across the study. As with TNF-α content, the injury resulted in a large increase in myogenin

content at days 1.5 (~545%) and 3.5 (~415%) (p<0.001), which did not differ between the

control and 17-AAG groups. By day 7, myogenin content in the control group returned to pre-

injury levels while myogenin content in the 17-AAG group remained elevated by 360%

(p<0.001) and was significantly greater than control content (p<0.001). By days 14 and 21,

myogenin content did not differ from baseline or between groups (Figure 11 *C*). The results of a

linear regression revealed that there was a positive relationship between TNF-α and myogenin

content across the study (R^2=0.71, p<0.001) (Figure 11 *D*).
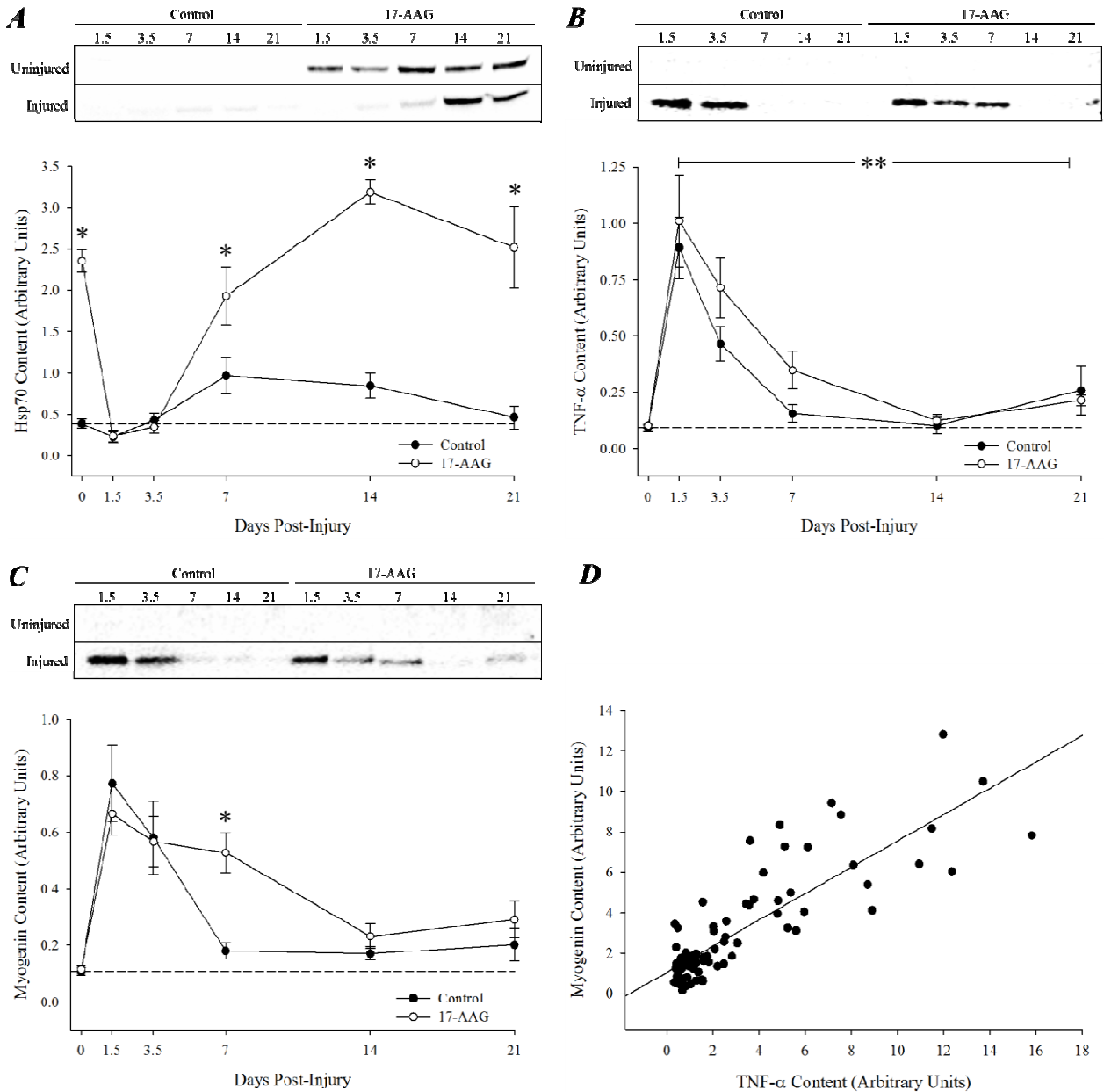
Figure 11. *(A) Hsp70, (B) TNF-α and (C) myogenin content of the of tibialis anterior (TA)*
muscle before and after BaCl₂-induced skeletal muscle injury. Protein content was determined
using Western blot analysis. Muscles were assessed at day 0 (uninjured) and 1.5, 3.5, 7, 14 or
21 days post-injury. The dashed line indicates protein content of uninjured control muscle. (D)
Regression analysis of TNF-α and myogenin content before and after the BaCl₂-induced skeletal
*muscle injury (R^2=0.71, p<0.001). *Significantly different from control muscle at that given*
*time point (p≤0.05). **Significantly different from control muscle following the injury (p≤0.05).*
Uninjured: n=17-19/group, Injured: n=4-6/group. Values are mean±SEM.

Histology

No differences in CSA were detected between groups or across time in the uninjured muscles.

As the inflammatory response abated, small regenerating fibers were observed that matured in

the weeks following the injury. No differences in regenerating fiber CSA were detected between

the control and 17-AAG groups. Regenerating fiber CSA was 47% smaller than the uninjured

fibers 7 days post-injury (p<0.001). By day 14, the regenerating fibers increased in diameter, but

were still decreased by 25% compared to the uninjured fibers (p<0.001). Twenty-one days after

injury induction, the CSA of the regenerating fibers was similar to the uninjured fibers (Figure

12 *A* and *B*).
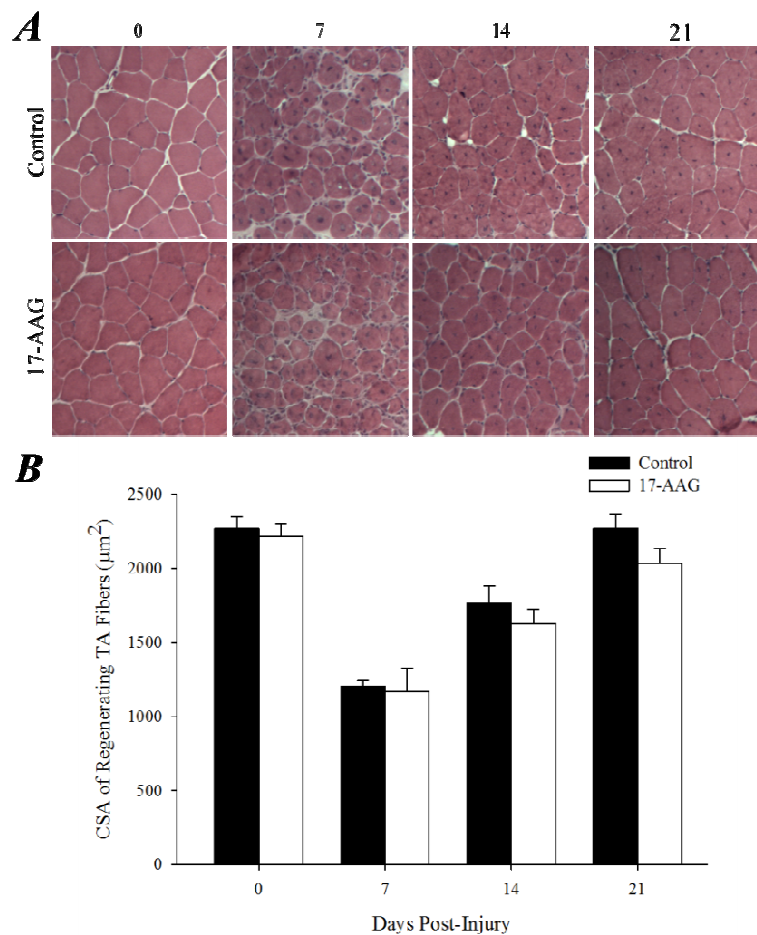
Figure 12. *(A) Histological and (B) graphical depiction of the cross-sectional area (CSA) of the TA muscle fibers. Uninjured data (day 0) was obtained from the right TA muscles while regenerating fibers, those with centralized nuclei, were assessed from the injured left TA muscles 7, 14 or 21 days post-injury. No differences were detected between control and 17-AAG groups at any time point. Uninjured: n=10-11/group, Injured: n=3-4/group. Values are mean±SEM.*

In vivo peak isometric torque

The initial treatment of 17-AAG had no effect on absolute or normalized peak isometric torque before the $BaCl_2$-induced injury. In parallel, recovery of absolute and normalized peak isometric torque was also similar between the control and 17-AAG groups. In general, absolute peak isometric torque was depressed 76% and 27% at days 7 and 14 ($p<0.001$), but not different than pre-injury torque by day 21. Normalized peak isometric torque followed a similar progression through day 14; however, remained 13% down 21 days post-injury ($p<0.001$) (Figure 13 *A* and *B*), likely due to the increase in body weight observed at this time point.
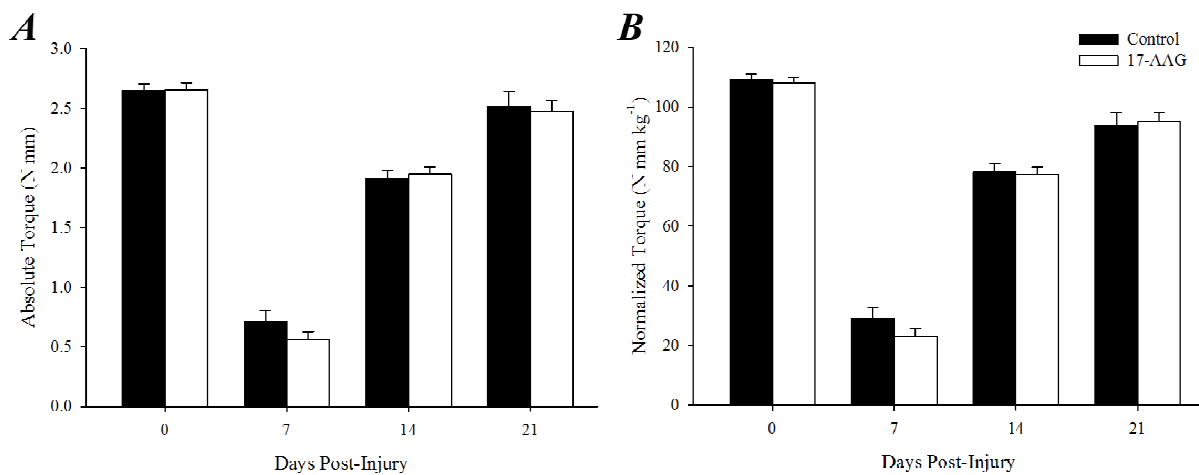
Figure 13. *(A) Absolute and (B) normalized in vivo torque produced by mouse anterior crural muscle before (day 0) and 7, 14 or 21 days post-injury. No differences were observed between the control and 17-AAG groups before or after the $BaCl_2$ injection. Sample sizes (n) are listed in Table 1. Values are mean±SEM.*

Discussion

Recovery from injury is dependent on the muscle's ability to regenerate, a highly coordinated process involving inflammation and myogenesis (52, 53), both of which are controlled by intrinsic and extrinsic signaling proteins. Of particular interest is TNF-α, a cytokine central to both the inflammatory and myogenic processes, and therefore a key regulator of skeletal muscle regeneration (53). By up-regulating Hsp70 content, a stress sensor involved in inflammation, we were able to further increase TNF-α content in injured muscle. Together, induction of Hsp70 and thus TNF-α resulted in a sustained increase in myogenin content, a marker of myoblast differentiation. However, these changes did not enhance regenerating fiber CSA or *in vivo* maximal isometric torque production. These findings demonstrate that Hsp70 regulates TNF-α content, which in turn mediates myogenic signaling, but not regeneration or functional recovery following $BaCl_2$-induced skeletal muscle injury in healthy young mice.

Several pharmaceutical agents have been used to increase Hsp70 content in skeletal muscle, one of which is the Hsp90 inhibitor 17-AAG (26, 34). Others that have used similar dosing strategies of 17-AAG have reported four-fold increases in Hsp70 content (26) which is comparable to the five-fold increase we observed. Furthermore, they reported 4 weeks of 17-AAG treatment in uninjured muscle did not result in any significant changes in other heat shock proteins (e.g., Hsp73 and Hsp25), muscle mass, CSA, isometric force production, or twitch characteristics (26). To that end, we also report treatment of 17-AAG does not affect muscle mass, CSA or maximal isometric torque, in addition to mouse body weight, soluble protein content or GAPDH, TNF-α or myogenin content in uninjured muscle. These observations, in particular that TNF-α and myogenin are not altered by 17-AAG treatment in the unstressed state,

suggest that the changes due to 17-AAG treatment in the damaged muscle were dependent on the injury.

Chemical- and toxin-induced injuries (e.g. $BaCl_2$, cardiotoxin, notexin) result in myofiber disruption (12, 43, 57), mass necrosis (12, 43, 57) and loss of function (9). Following the intramuscular injection of $BaCl_2$, Hsp70 content in the control group was reduced below baseline levels and slowly increased until day 7. This increase is delayed when compared to other types of injury. For example, Hsp70 content increases in the hours to days after eccentric contraction-induced skeletal muscle injury (25, 51). However, our laboratory has noted significant differences between these injury models. Specifically, in the days following a bout of eccentric contractions soluble protein and GAPDH content remain unchanged (7) while only 5-10% of the myofibers show signs of histological degeneration (14, 35), which is in stark contrast to the dramatic histological perturbations and reductions in soluble protein and GAPDH content that pursue a single intramuscular injection of $BaCl_2$. Torque deficits are also more pronounced following $BaCl_2$-induced injury when compared to that of eccentric exercise. We have shown that a single bout of eccentric contractions can reduce maximal isometric torque up to ~55% which is, in part, mediated through disruptions to key excitation-contraction coupling proteins (7, 13), whereas $BaCl_2$ reduces torque by ~80% due to the frank loss of nearly the entire muscle. Therefore, the finding that Hsp70 content did not significantly increase in the injured control muscle is a reflection of both the nature and severity of the $BaCl_2$-induced injury. We propose that in the days following the injury, Hsp70 content, including any newly synthesized Hsp70, was either degraded or lost from the myofiber. Although the latter has not been well documented following trauma-induced skeletal muscle injury, it seems entirely plausible Hsp70 could translocate into circulation like other muscle proteins (e.g., creatine kinase, lactate

dehydrogenase) via disruptions in the T-tubule and plasma membranes. Moreover, loss of

skeletal muscle proteins is a common consequence of eccentric exercise (6, 35, 49) and given

that $BaCl_2$ results in greater damage, it is likely to occur following this type of injury, and to a

greater extent.

Release of Hsp70 into the extracellular environment has been reported following physical

exercise (20, 55) and injury to heart (17, 18), liver (27) and skin (28) tissue. Extracellular Hsp70

(eHsp70) is thought to modulate the innate immune response by facilitating pro-inflammatory

processes through the recruitment of immune cells (1, 2, 21, 46). Previous studies have

demonstrated eHsp70 can interact with various surface receptors expressed on specific immune

cells, such as neutrophils (23, 40), macrophages and peripheral blood monocytes (3, 4, 28)

thereby activating them. Until recently, little evidence was available to support this notion in

skeletal muscle following injury. Using an Hsp70 knockout mouse model, Senf et al. (46)

reported that acute inflammation was delayed in cardiotoxin injured muscle, which was followed

at later time points by sustained inflammation and muscle fiber necrosis, fibrosis and reduced

CSA of regenerating myofibers. By reintroducing Hsp70 into the muscle prior to the injury via

Hsp70 intracellular expression plasmids, they were able to completely restore early immune cell

infiltration and prevent any changes that would have followed due to the ablation of Hsp70.

Interestingly, they also found that TNF-α expression was significantly lower in the Hsp70

knockout muscle 1 day post-injury when compared to injured controls, and as with the

inflammatory response, TNF-α expression was restored when recombinant Hsp70 was injected

into the muscle at the time of the injury. In support of this, antigen presenting cells have been

shown to significantly increase the release of TNF-α as early as 2-4 hours after exposure to

exogenous eHsp70 (4, 5). TNF-α appears to be critical to skeletal muscle regeneration, as we

and others report increased expression of TNF-α (10, 56, 58) and its receptors (58) 1-3 days into recovery, while TNF-α neutralization (56) and TNF-α receptor ablation (9, 56) significantly exacerbated long-term strength deficits following trauma-induced injury. Using 17-AAG to induce Hsp70 expression, we found that these treated mice had significantly higher levels of TNF-α when compared to the vehicle-treated controls. Furthermore, this effect was only observed in injured muscle, most evident 1.5 to 7 days following the BaCl$_2$ injection. Knowing this type of injury results in severe damage and that eHsp70 can initiate the inflammatory response. We propose that TNF-α was higher in the 17-AAG groups due to the release of a larger amount of intracellular Hsp70 in the days following the injury, subsequently increasing eHsp70 content and thereby enhancing immune cell recruitment and the release of TNF-α from antigen presenting cells.

In skeletal muscle, TNF-α can be released by infiltrating immune cells or expressed by the myofiber following injury (11, 29, 58). TNF-α activates NF-κB signaling which further increases TNF-α expression and promotes the inflammatory response (22). Thus, TNF-α is a critical modulator of acute inflammation which is necessary for normal regeneration after injury because immune cells phagocytize damaged cellular debris and secrete growth factors that promote myogenesis. In addition to its role as an inflammatory mediator, TNF-α can also stimulate myogenesis through p38 MAPK (9, 10, 59). Data from Chen et al. (9) demonstrated multiple p38 MAPK-mediated steps in myoblast differentiation are TNF-α signaling dependent, with physiological levels of TNF-α stimulating differentiation through MEF-2 phosphorylation, induction of myogenin and the Cdk inhibitor p21, and suppression of cyclin D1. In the present study, myogenin content was ~4-6 fold above baseline levels for up to 3.5 day post-injury in both the control and 17-AAG groups. Intriguingly, myogenin content remained significantly

evaluated until day 7 in the 17-AAG group, even though both groups were injured to the same degree as evident by regeneration fiber CSA and isometric torque production. We propose that as a result of injury, eHsp70 increased TNF-α expression thereby activating p38 MAPK, subsequently increasing myogenin expression. In support of this, we found that TNF-α and myogenin content were directly related. In that, TNF-α and myogenin were barely detectable in the uninjured muscle, but increased in parallel in the days following the injury. Furthermore, TNF-α expression has been shown to positively correlated with the regenerative activity of injured muscle (29), while ablation of the TNF-α receptors is known to suppress myogenin expression following cardiotoxin-induced injury (9).

Despite the fact that 17-AAG increased Hsp70, TNF-α and myogenin content, treatment did not result in any observable changes in recovery as assessed by regenerating fiber CSA and maximal isometric torque production. In contrast, others have shown Hsp70 induction decreases muscle damage, improves morphological recovery (36) and attenuates strength deficits (26, 36) following eccentric contraction-induced injury. For example, using the same drug, dosage and treatment, Kayani et al. (26) reported improved recovery of maximal tetanic force following a bout of eccentric contraction in aged mice. It is possible that Hsp70 induction prior to eccentric injury is involved in refolding denatured proteins such as myosin heavy chain and actin, that otherwise would have been degraded in the absence of Hsp70, a concept that would not be valid after $BaCl_2$-induced injury due the unpreventable necrosis and degradation that follows. Additionally, if the muscle's ability to express Hsp70 and/or induce its expression is diminished following injury, as seen with aging (36, 54), Hsp70 content may be rate limiting. Up-regulation of Hsp70 not only mediates this issue after eccentric contraction-induced injury in aged rodents (26, 36), but also has been shown to be advantageous in models of age- (36) and disuse- (15, 34,

45) skeletal muscle atrophy. As with Hsp70, TNF- α and myogenin are also not likely to be rate limiting in injured but otherwise healthy young muscle and therefore, as we show, increasing their expression does not translate into improved skeletal muscle regeneration or recovery.

We report that up-regulation of Hsp70 by the pharmaceutical 17-AAG increased TNF-α content above that normally observed following $BaCl_2$-induced injury. Furthermore, changes in TNF-α were found to be directly correlated to that of myogenin, indicating TNF-α expression is associated with myoblast differentiation. The elevated levels of Hsp70 and thus TNF-α were able to sustain the increase in myogenin content observed in the days following the injury. However, the increased content of these proteins was not followed by any significant changes in regenerating fiber CSA or *in vivo* maximal isometric torque production. In conclusion, Hsp70 induction in healthy young mice prior to $BaCl_2$-induced skeletal muscle injury increases TNF-α and myogenin content but does not appear to alter skeletal muscle regeneration or attenuate functional deficits in the weeks following the insult.

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APPENDICES

Appendix A: IACUC Original Protocol and Amendments

Georgia State University
Institutional Animal Care and Use Committee
RESEARCH PROTOCOL FOR ANIMAL CARE AND USE

For Office Use Only
Date Received:
Protocol Number:
Protocol Title:
Principal Investigator:
Veterinary Review Date:
Revision Date:
Final Approval Date:
Protocol Number Replacing:
Biosafety Approval Needed: Yes ☐ No ☐ Approval Date_____ Approval Number_____
Radiation Safety Approval Needed: Yes ☐ No ☐ Approval Date_____ Approval Number_____

Signature of Attending Veterinarian _____

Signature of IACUC Chair _____

The Institutional Animal Care and use Committee (IACUC) is by law responsible for ensuring that the use of animals at Georgia State University is performed according to the highest standards and in an ethical manner. This responsibility is shared with university faculty, staff, and students. The use of animals at the university is a privilege, not a right. Maintaining this privilege requires compliance with the following regulations, policies, and guidelines:
☐Animal Welfare Act Regulations
☐Public Health Service Policy on Humane Care and Use of Laboratory Animals
☐The Guide for the Care and Use of Laboratory Animals
☐U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training
The authority of the IACUC is derived from these laws and policies. The IACUC's role in institutional self-regulation ensures that animals are not subject to unnecessary pain and distress. Furthermore, by assuring compliance with these animal welfare laws and guidelines the IACUC also protects the investigator and the institution. The IACUC must review all aspects of the animal care and use program. The animal care and use program must include:

A properly constituted and functioning IACUC
Procedures for self-monitoring
An adequate veterinary care program
An occupational health and safety program
A training program for personnel
An environment, housing, and management program for animals
Appropriately maintained facilities for housing and support

Central to the IACUC's mandated functions are (1) reviewing and approving animal use protocols submitted by investigators and (2) semi-annual program reviews and facility inspections.
This form is intended to facilitate review of requests to use animals for specific research, instruction, or biological testing projects.

- This completed form must be reviewed and approved by the IACUC before the project or course is initiated and before animals can be procured.

- After 3 years a new complete AUP must be submitted, approved and assigned a new number.

- The number of animals used must be declared annually and their documentation is the responsibility of the Principal Investigator.

The Georgia State University IACUC Policies and Procedures Manual can be found on line at: GSU IACUC Policies and Procedures Manual This document contains information on Completing the Protocol Form, Protocol Review and Approval, Required Training, The Occupational Health Program, Additional Considerations Pertaining to Protocols, and more.

PLEASE COMPLETE A SEPARATE PROTOCOL FOR EACH DIFFERENT TYPE OF ANIMAL OR PROJECTS WITH DISTINCTLY DIFFERENT PURPOSES USING THE SAME TYPE OF ANIMAL (e.g. experimental protocol and breeding protocol).

SECTION 1. Basic Protocol Information

1.1   **PROTOCOL TITLE:** ➜**Alcoholic myopathy and skeletal muscle regeneration**
1.2   **DATE:** ➜
1.3   **PRINICPAL INVESTIGATOR:** ➜**Jeffrey S. Otis, PhD**
1.4   **DIVISION:** ➜**College of Education: Dept. of Kinesiology and Health**
1.5   **E-MAIL:** ➜**jotis@gsu.edu**

1.6   **General Animal Information**

    1.6.1   **Over a period of three (3) years** I would like to use a total of ➜ __**440**____ (number) animals.
    1.6.2   Common name: ➜Mouse
    1.6.3   Scientific name: ➜*Mus musculus*
    1.6.4   I would like to begin using these animals on (date): ➜October 1, 2013
    1.6.5   I will obtain animals from (name of supplier): ➜Harlan
    1.6.6   I will breed these animals:
        ☐ Yes (If yes, justify in Section 14 "Animal Housing & Husbandry" below).
            Where will breeding stock be obtained? (Name of supplier): ➜
        ☒ No

1.7   **List All Funding for the Proposed Animal Work:**

GRANT TITLE	GRANTING AGENCY	GRANT #
(1) Chronic alcohol-induced skeletal muscle myopathy: etiology and physiology	NIH/NIAAA	5K01AA17190
(2) Departmental start-up funds		

    1.7.1. **If Funding for the Proposed Animal Work is from the following sources, please submit the required documents listed below.**
        NIH funding - please attach the following:
        ☐Project/Performance Site Location(s)
        ☐Project Summary Abstract
        ☐Project Narrative
        ☐Research Plan Attachments:
        ☐Specific Aims

☐Research Strategy
☐Vertebrate animals (grants.nih.gov/grants/olaw/VASfactsheet_v12.pdf)

NSF funding - please attach the following:
☐Cover sheet
☐Project Summary
☐Results from prior NSF support
☐Explanation of Revisions
☐Project Description
☐Research Plan

1.8   **Three Year Renewal**
        If this is a 3 year renewal, please answer the following questions. If not, please skip to section 1.10.

        1.8.1   What is the number of the protocol this is replacing? ➜
        1.8.2   Do you presently have any live animals under this number? ➜
        1.8.3   If yes, will these animals be transferred to your new protocol once it is approved? ➜
        1.8.4   As this is a 3-year renewal, the IACUC requests that you provide a very brief description of the
                outcomes of the work conducted under the existing approval: ➜

1.9   **Location of work on project**

        1.9.1   Will any aspect of the study (course) or animal husbandry be conducted at **another institution**? If
                so, please name the institution: ➜**No**
        1.9.2   If yes, please provide the PHS Assurance number of the institution where this work will occur: ➜
        1.9.3   Has this proposal been approved by the IACUC of that institution? ➜
                If yes, please attach a copy of the approved protocol and a copy of the dated and signed approval
                letter.

1.10   **Veterinary Care and Consultation:** Principal Investigators may discuss the proposed project with the
        Attending Veterinarian before submission of the application to the IACUC. Procedures involving more
        than momentary or slight pain or distress (USDA Pain Category "D" or "E") must be discussed with the
        Attending Veterinarian in the planning of the research project. All protocols and amendments will be sent
        to the Veterinarian for review of animal care issues after submission.

1.11   <u>**Certification of Compliance: I will comply with the procedures described in the NIH Guide for the
        Care and Use of Laboratory Animals, with PHS policy, the Animal Welfare Act, and the GSU
        IACUC Policies and Procedures Manual. I acknowledge responsibility for the procedures described
        and assure that the faculty, staff and students who perform these procedures are qualified (or will be
        adequately trained) to conduct them in a humane manner. Failure to comply may result in sanctions
        by the IACUC including, but not limited to suspension of research activities.**</u>

        Signature of the Principal Investigator _____

        Date _____

SECTION 2. Lay Project Summary and Overview

2.1   **Objective(s) (What are you doing?)**
        Please provide a brief statement, in LAY TERMINOLOGY understandable by someone with a college
        education, with no acronyms or scientific jargon, outlining the objective of the procedures of this protocol.
        Begin with a broad statement concerning the overall problem (*e.g.*, "Pancreatic cancer kills ~50,000
        Americans each year. We are addressing this problem by …."). Include a statement of your experimental
        hypothesis or objectives. **Please do not submit your grant proposal abstract for this section.** Define all
        abbreviations the first time they are used and explain medical terms. You will be asked to provide a
        scientific summary of the project in a later section.

➔ The NIH has estimated that nearly 18 million Americans abuse alcohol. Chronic alcohol ingestion may produce a variety of complications that include skeletal muscle wasting (termed alcoholic myopathy). Our goal is to improve skeletal muscle function in chronic alcoholics (using well-established rodent models of chronic alcohol ingestion). We and others have shown that chronic alcohol ingestion is associated with oxidant stress. Importantly, we are able to reduce this alcohol-induced oxidant stress by providing alcohol-fed animals the antioxidant glutathione (as S-adenosylmethionine, SAMe). In parallel, we have shown that alcoholic muscle has a reduced ability to regenerate following injury – an effect we believe to be due, in part, to elevated oxidant stress.

We are addressing these problems via nutritional intervention and SAMe supplementation. As such, we hypothesize that alcoholic myopathy in both injured and uninjured skeletal muscle may be improved by glutathione restoration.

2.2 **Rationale and Significance (Why are you doing it?)**
Please provide a brief statement about how contributions from your proposed work might be relevant to human/animal wellbeing or the expansion of knowledge. This must be written in **LAY TERMINOLOGY**, understandable by someone with a college education, with no acronyms or scientific jargon. **Please do not submit your grant proposal abstract for this section.** Define all abbreviations the first time they are used and explain medical terms.
➔ About 45-70% of chronic alcoholics, defined as those that drink in excess of 100 g of ethanol (EtOH) for more than 10 years, show significant damage to skeletal muscle, including muscle wasting, debilitating fatigue, and weakness. Chronic alcohol abusers may be more likely to suffer skeletal muscle injuries due in part to muscle atrophy and weakness, nerve damage, increased incidence of risky behaviors, and general misbalance. However, no research exists on the capacity of alcoholic skeletal muscle to regenerate following injury.

While there are several causes for these myopathies, we have focused on the powerful antioxidant glutathione and the potential effects of elevated oxidant stress. Glutathione is the principle free radical scavenger (antioxidant) in skeletal muscle and is crucial for normal skeletal muscle function. Unfortunately, we and others have shown that the available pool of glutathione is drastically reduced in alcoholic muscle which may be responsible for the extent of alcohol-induced skeltal muscle wasting. Further, reduced glutathione levels may leave alcoholic muscle at a significant disadvantage to repair itself following muscle injury. <u>Accordingly, we hypothesize that restoring glutathione balance in alcoholic, injured muscle with antioxidant supplementation therapy will improve skeletal muscle health following injury.</u>

2.3 **Justification for the Use of Animals**
Please justify why animals must be used instead of using methodology that does not require vertebrate animal use. Provide a brief statement justifying your use of animals in the proposed project.
➔The skeletal muscle degenerative and regenerative response following injury requires complex multi-organ communication, including metabolic, hematological, immunological, and pathophysiological involvement. Therefore, these effects of skeletal muscle injury and the consequent regenerative process can only be modeled *in vivo*. Similarly, the catabolic effects of alcohol and the anti-oxidant effects of glutathione replacement therapy (SAMe) must be modeled *in vivo* to account for appropriate drug metabolism.

SECTION 3. Overall Animal Use Category Information

If the answer to any of the following questions is YES, please provide a <u>brief</u> explanation.
3.1 Will any technique be performed which will involve **prolonged physical restraint (> 30 minutes)** other than routine caging and handling? ➔No
If yes, describe the type, method and the length of time the animal is restrained. ➔
3.2 Will any substance such as **Complete Freund's Adjuvant** or other adjuvants be injected which could cause chronic inflammation and/or pain? ➔No
If yes, describe what will be used, volumes, and the schedule for the injections. ➔

3.3   Will it be necessary for live **animals to be removed** from the animal facility? ➔Yes

3.4   Will this experiment involve **stress, pain, or abnormal behavior** in live animals, which cannot be alleviated with drugs because their use would interfere with the research goal? ➔Yes

       3.4.1   Will any adverse effects or overt **signs of illness** be expected? ➔No

3.5   Will animals be subjected to **more than one major survival surgical procedure?** ➔No

Major surgery penetrates and exposes a body cavity, produces substantial impairment of physical or physiologic function, or involves extensive tissue dissection or transection. Please note that routine injections are not considered surgical procedures. Please refer to OLAW Position Statement 5) Multiple Surgical Procedures to determine whether it is appropriate to perform more than one major surgical procedure.

3.6   Will **food or fluid be restricted and/or regulated**? ➔No

3.7   Will field investigations be employed? ➔No

If yes, please provide all the relevant permits.

SECTION 4. USDA Animal Use Category Classification

USDA Classifications and Examples of Pain Categories:

Classification B: Animals being bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery, but not yet used for such purposes.

Examples:

- Breeding colonies - Includes parents and offspring.

- Animals held under proper captive conditions or wild animals that are being observed.

Classification C: Animals upon which teaching, research, experiments, or tests will be conducted involving no pain, distress, or use of pain-relieving drugs.

Examples:

- Procedures performed correctly by trained personnel such as the administration of electrolytes/fluids, administration of oral medication, blood collection from a common peripheral vein per standard veterinary practice or catheterization of same, standard radiography, parenteral injections of non-irritating substances.

- Euthanasia performed in accordance with the recommendations of the most recent AVMA Panel on Euthanasia, utilizing procedures that produce rapid unconsciousness and subsequent humane death.

- Manual restraint that is no longer than would be required for a simple exam; short period of chair restraint for an adapted nonhuman primate.

Classification D: Animals upon which experiments, teaching, research, surgery, or tests will be conducted involving accompanying pain or distress or leading to illness to the animals and for which appropriate anesthetic, analgesic, or tranquilizing drugs will be used.

Examples:

- Surgical procedures conducted by trained personnel in accordance with standard veterinary practice such as biopsies, gonadectomy, exposure of blood vessels, chronic catheter implantation, laparotomy or laparoscopy.

- Blood collection by more invasive routes such as intracardiac or periorbital collection from species without a true orbital sinus such as rats and guinea pigs.

- Administration of drugs, chemicals, toxins, or organisms that would be expected to produce pain or distress but which will be alleviated by analgesics.

Classification E: Animals upon which teaching, experiments, research, surgery, or tests will be conducted involving accompanying pain or distress or leading to illness to the animals and for which the use of appropriate anesthetic,

analgesic, or tranquilizing drugs will adversely affect the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests.

Examples:

- Procedures producing pain or distress unrelieved by analgesics such as toxicity studies, microbial virulence testing, radiation sickness, and research on stress, shock, or pain.

- Surgical and postsurgical sequella from invasion of body cavities, orthopedic procedures, dentistry or other hard or soft tissue damage that produces unrelieved pain or distress.

- Negative conditioning via electric shocks that would cause pain in humans.

- Chairing of nonhuman primates not conditioned to the procedure for the time period used.

4.1 **Selection of Pain Category**

Please classify the project according to the level of perceived pain / stress / distress experienced by the animal(s). Animals must be claimed under the highest class involved at any point prior to euthanasia or release. Highest Pain Category within this protocol: ➜ (enter B, C, D, or E)

Class E

4.2 **Justification of Pain Category "E"**

If category E is selected, please provide a scientific justification for withholding pain and/or distress relief. We have previously shown that COX2 inhibition (via non-steroidal anti-inflammatory drug delivery) can reduce skeletal muscle cell proliferation (*Otis et al., Exp Cell Res 2005, 310(2):417-425.*). Thus, we hesitate to use analgesics following $BaCl_2$-induced injury as it may impede skeletal muscle repair.

In our experience with this model (Dekeyser and Otis, *Reg Med Res* 2013, in press), mice ambulate normally upon awakening from anesthesia and do not appear to be in pain post-procedure or during the 28 day recovery process (i.e., no signs of failure to groom or roughed coat, decreased appetite, vocalization). Further, we have monitored body weight and show no significant difference between groups – either due to diet or due to injury.

4.3 **Monitoring of Animal Pain and Comfort Levels**

4.3.1 Who will be responsible for monitoring the animals for pain and/or distress during the experimental procedure(s)?

➜Jeffrey S. Otis, DAR staff including veterinarians

4.3.2 How will the comfort level of the animals be determined?

➜Pain or distress will be visually monitored. Potential signs of distress due to injury may include increased vocalization or favoring the injured hindlimb. However, we have not seen any of these signs in the animals. Animals will be monitored 2-3 hours following $BaCl_2$ injection and then 2 to 3 times per work week by the PI.

4.3.3 In the event that an animal needs to be euthanized or removed from the experiment, please list the criteria for the decision. Describe the humane endpoint criteria to be applied and the frequency of monitoring for these humane endpoints.

➜Should an animal require euthanasia because of significant stress, appropriate Georgia State University guidelines will be followed as identified in the GSU IACUC policies and procedures guideline located on the IACUC website (http://www.gsu.edu/research/iacuc.html).

Accordingly, euthanasia will be performed via CO_2 inhalation and confirmed by thoracotomy and diaphragm removal.

4.3.4 Who will determine this action?

➜Jeffrey S. Otis and/or GSU veterinary staff

4.3.5 Will animal models with tumors be utilized? ➜No

If yes, please review the following:

http://www.gsu.edu/images/vp_research/GSU_IACUC_Policy_on_Mouse_Tumor_Burden.doc

I will adhere to the standards specified in this policy.

☐ Yes
☐ No (If No, scientifically justify below):
➔

Section 5. Alternatives and Non-duplication

5.1 **Explanation for the Consideration of Alternatives for Category "D" or "E" Animal Use.**
Federal Regulations (The Public Health Service Policy and the <u>Animal Welfare Act) and University Policy</u> require assurance that this project does not unnecessarily duplicate research projects/courses performed at this or other institutions, and that the use of alternatives to live animal models and alternative procedures that may cause more than momentary or slight pain/distress (Class "D" and "E" procedures) to animals have been considered. The information in this section should include adequate information for the IACUC to assess that a reasonable and good faith effort was made to determine the availability of alternative models or methods.

The following is a guide for answering 5.1.1 and beyond.
Your literature search is done as part of the OLAW requirement to address the "3Rs" (Refinement, Reduction, and Replacement) issues. For example:

* Refinement of procedures to eliminate or minimize pain or distress, the use of remote telemetry to decrease the distress of restraint; the use of humane endpoints.

Other examples of refinement are: ways to enhance the well-being of animals and the use of analgesics to decrease pain or anesthetics to decrease distress. These should be addressed only in Section 10.

For the literature searches

Ask "Am I using the least painful technique(s)?"
 - **Search terms:** Your species + animal/experimental model/technique + scientific keywords

* Replacement of live animals with non-animal procedures or a less sentient species. Examples include the use of non-animal models such as in-vitro work, cell culture, tissues culture, computer models or simulations.

Ask "Do I need to use animals?":
 - **Search terms:** Your experimental model + simulation + in vitro +scientific keywords

Ask "Am I using the lowest species possible?":
 - **Search terms:** Your species + all applicable lower species (For example if using mice, specifically use "invertebrate", "fish" and "frog" in your search terms) + your animal/experimental model + scientific keywords

Note: **There are NO search terms that will direct the searcher to all examples of replacement or refinement. The onus is on the researcher to read and evaluate the literature.**

*Reduction in the number of animals used in the study. (**This only needs to be addressed in Section 9.2 of the protocol not here**). Examples include the use of shared control groups; preliminary screening in non-animal systems; innovative statistical packages.

The selection of databases depends on the work performed and the species used in the protocol. Samples of databases available through the Georgia State University Library www.library.gsu.edu/database web pages

· PubMed·   Web of Knowledge · Biological Abstracts·   Web of Science   PsychINFO   AltWeb   NLM Gateway   AVAR   Galileo   AGRICOLA

OVID (allows a search of multiple databases including Agricola, BIOSIS, CAB Abstracts, Medline, Zoological Record, etc.)

*Please visit the Animal Welfare Information Center (**AWIC**) Literature Searching and Databases page (http://awic.nal.usda.gov/nal_display/index.php?tax_level=1&info_center=3&tax_ subject=184) for a list of databases that can be used to search for alternatives. The AWIC site also recommends using the Literature Search Worksheet (http://www.nal.usda.gov/awic/alternatives/searches/altwksht.pdf) to assist in performing a successful alternatives database search. The worksheet helps to identify relevant searchable terms and concepts.*

5.1.1 **Literature search for alternatives to painful procedures**

If you chose category "D" or "E" above, please do literature searches using the broadest database for your area of study, and provide a brief summary of the results obtained to verify that you investigated the use of alternatives to painful or distressful procedures. If you have not selected either category D or E, skip to section 6.2.

➔ **Alcohol studies:**

The vast majority of research on the toxic effects of alcohol on skeletal muscle have been performed *in vivo* (for review, see: Preedy et al., *J Muscle Res Cell Motili.* 24(1): 55-63, 2003. While culturing muscle cells may uncover novel signaling mechanims, the complex metabolic response secondary to alcohol metabolism is best investigated using whole animals models.

Muscle injury studies:

Muscle repair can be modeled *in vitro* by looking at the ordered sequence of events during myogenesis (i.e., myoblast proliferation, differentiation, fusion, and myotube growth) (for review see: Abmayr and Pavlath, *Development*, 139(4): 641-656, 2012). However, effective muscle repair requires input and signaling from multiple cell types such as infiltrating immune cells – events that are best modeled *in vivo*.

5.1.1.1 Name of the databases used: ➔ Pubmed, ALTBIB

Date the databases search(es) were done: ➔ August 19, 2013

Did the search cover the entire date range of the databases with no restriction on dates?

☒ Yes

☐ No (If No, provide dates covered by the search(es) below)

Dates covered by the databases search(es): ➔

5.1.1.2 Search keyword(s) used: include number of hits for combinations of terms. **You must use 'alternative' and 'animal welfare' in the search combinations for invasive or painful procedures** (*See above for 3R criteria.*)

➔ alcoholic myopathy, skeletal muscle regeneration, skeletal muscle injury, animal testing alternatives, animal welfare, and alternative

(1) Alcoholic myopathy + animal welfare = 0

(2) Alcoholic myopathy + alternative = 0

(3) Skeletal muscle regeneration + animal welfare = 0

(4) Skeletal muscle regeneration + alternative = 10

(5) Skeletal muscle injury + animal welfare = 0

(6) Skeletal muscle injury + alternative = 5

5.1.1.3 Summarize the information found in the hits below. (Note: this should be a general summary. You do not need to go into the details of each hit but rather summarize hits by relevant groups)

Searches 4 and 6 from above returned similar studies. These studies describe experiments performed *in vivo* using mouse models, or in primary cells isolated from rodent tissues. The *in vivo* studies track regeneration over time (as we intend to do in the protocol), while the *in vitro* studies use a surrogate model of muscle regeneration. As we have stated in 5.1.1, using an in vitro model system does not accurately represent complications that arise from chronic alcohol ingestion.

5.1.2 Explain what alternatives exist in place of using animals for this protocol and provide a justification if these cannot be used.
➔ Alternatives do not include less sentient species and are limited to *in vitro* systems. These can not be used for reasons described in 5.1.1.

5.1.3 **Other Sources of Information on Alternatives to Painful Procedures**

5.1.3.1 Consultation with Experts: (Names, credentials, and dates): ➔ Mike Huerkamp, DVM 2005-2013. I sat on the Emory IACUC for 4 years and had frequent conversations with Mike (Director of DAR) and other IACUC members about animal welfare.
5.1.3.2 Scientific Meetings: Specify: ➔ NA

Note: A careful literature search is usually the best way to determine whether a proposed study is unnecessarily duplicative of previous work. However, it is ok to repeat a published experiment to make sure that it works in a different lab. This reason just needs to be stated as the justification.

5.2 **Consideration of non-duplication**

5.2.1 Please provide a written assurance that the proposed work is not unnecessarily duplicative.➔ All experiments described in this protocol are novel, and do not duplicate previously published work.

Section 6. Method(s) of Euthanasia

6.1 Describe in detail the method of euthanasia (if any) you will use. If the method involves the use of pharmaceuticals, please specify agent, dose, and route of administration. Please note that methods of euthanasia must be in accordance with the most current American Veterinary Medical Association Panel on Euthanasia. References must be cited to justify deviations from this document, or to justify use of cervical dislocation or decapitation without anesthesia.
➔Should an animal require euthanasia because of significant stress or has completed their experimental time period, appropriate GSU guidelines will be followed. Euthanasia will be performed via CO_2 inhalation followed by thoracotomy to confirm death.
6.2 How will death be confirmed? ➔ thoracotomy
6.3 How will remains be disposed? ➔ Animal carcasses will be disposed by putting them in the DAR designated freezers within the animal facility.
6.4 If you chose Carbon Dioxide inhalation method above, please review the GSU IACUC Carbon Dioxide Euthanasia Policy. ➔
I will adhere to the standards specified in this policy.
☒ Yes
☐ No (If No, scientifically justify below):
➔

SECTION 7. Justification for Animal Species Selection

7.1 **Animal characteristics**
7.1.1 Species Name: ➔Mouse
7.1.2 Strain (if any): ➔C57Bl/6
7.1.3 Age: ➔~8-10 wks
7.1.4 Sex: ➔Male

7.2   **This species has been selected because (check all that apply)**

☒   Previous work in the biomedical literature validates the use of this species as an animal model for this disease or biological process.

☒   This is the lowest sentient species that provides appropriate size, tissue or anatomy of the proposed work.

☒   There is a large body of existing data that would need to be repeated if another species was used instead.

☐   Available reagents or research tools necessary for this research are unique to this species.

☐   Characteristics of this species make it uniquely suited for the proposed research. Explain:

☐   Other (Explain): ➔

SECTION 8. Animal Use Narrative

8.1   **Please describe in narrative form all experimental or instructional procedures to be performed on the animals.** Please note that it is not necessary to provide the details already provided elsewhere in the protocol (e.g. procedure descriptions, volumes of blood collected, dosages, routes of administration, use of aseptic procedures, etc.). **However**, it is important that one is able to ascertain what procedure or set of procedures is conducted on each group of animals. Include the time frames and intervals between procedures and describe the procedures in the order they will be performed.

➔**Alcohol (EtOH) in drinking water**

Male, adult BALB/c mice will receive EtOH in drinking water for 14-18 weeks (depending on group) and be compared to mice without EtOH in the drinking water. EtOH will be   added to the animals' drinking water at an initial concentration of 5% and then gradually increased by 5% every 4-5 days until a final concentration of 20% (weight:volume) in the   drinking water is achieved. The animals are then continued on this regimen for 10-14 weeks and have ad lib access to solid rodent chow. Subgroups of control or alcohol-fed   animals will be supplemented with the glutathione precursor, S-adenosyl methionine   (SAMe, in drinking water).

Glutathione replacement therapy using S-adenosyl methionine (SAMe)

Injured or noninjured, control or EtOH-fed mice will be provided SAMe in the drinking water (0.4% w/v, Nutramax Labs). SAMe-supplemented animals in the injury groups will begin glutathione replacement therapy <u>one week prior to skeletal muscle injury</u>. Thus, animals may receive SAMe for 9 days to 3 weeks. Non-injured animals will be matched acordingly.

Drug is pulverized, dissolved in control water or water + 20% EtOH, passed through 100 um filters, and poured into water bottles. During the work week (Mon-Fri), drug-treated water will be replaced every two days. Volumes will be sufficient to drug-treat animals over the weekend (Fri-Mon). Static racks of drug-treated mice are noted with appropriate "SPECIAL WATER" signage. Discussions with our colleagues at Emory University (Viranuj Sueblingvong, David Guidot) suggest that the animals tolerate SAMe with ease and hydrate normally.

Barium chloride-induced muscle degeneration

Once mice have been on 20% alcohol water for 10 weeks, they will be anesthetized by isoflurane (2l O$_2$/min). Intramuscular injections of barium chloride (1.2% BaCl2 in sterile PBS, 50 µl final volume) will be injected into mouse tibialis anterior (TA) muscles. Uninjured, contralateral TA muscles serve as controls which greatly reduces are animal numbers. Briefly, the anterior shank of sedated animals will be shaved and the TA muscle will be identified via palpatation. Next, a 27 G needle is inserted from the origin of the TA muscle and continued to the midbelly. The syringe is slowly removed as the contents are dispersed into the muscle tissue.   We have signficant experience with this model and histological examination of the anterior leg muscles confirm TA injury with no injury to deeper skeletal muscles such as the extensor digitorum longus (EDL). TA muscles will be collected 2, 7, 14, or 28 days post-BaCl2 injection (n=20/time) and assessed for markers of degeneration and regeneration (inflammatory response, cross sectional area, fibrosis) or processed for protein and gene expression analyses (CNTF, IL-6, IGF, anti-oxidants such as superoxide dismutase 1-3 and catalase).

Mice undergoing BaCl$_2$ injections recover from anesthesia on heated pads and are monitored for 2-3 hours immediately post-injury and then daily (Monday-Friday) to identify

any signs of stress. Should an animal require euthanasia because of significant stress, appropriate GSU guidelines will be followed. Euthanasia will be performed via CO_2 inhalation followed by thoracotomy.

BrdU administration

Subgroups of mice (n=10/group) will receive 2 BrdU injections (0.3 ml of 100 ug/g separated by 8 hrs, delivered i.p.) <u>on the day of muscle injury</u> in order to label replicating cells. Thereafter, BrdU (0.8 mg/ml drinking water) will be added to the drinking water for up to 7 days after muscle injury. Lab personnel will be responsible for providing and changing the drinking water once the animals start on this special drinking water. Muscles are collected post-mortem for histochemical analyses of BrdU incorporation.

Voluntary Wheel Running

Subsets of animals will be housed individually in modified rat cages that contain standard running wheels (PetsMart) equipped with a CatEye bike monitor. Daily running distances will be recorded daily for up to 28 total days, as previously described (see: Waters et al., Voluntary running induces fiber type-specific angiogenesis in mouse skeletal muscle. Am J Physiol Cell Physiol 287: C1342-48, 2004.) In general, we hypothesize that chronic alcohol abuse +/- injury will reduce voluntary activity that may be partially improved following SAMe supplementation.

Animal usage

Our estimates of the number of mice required to complete the goals of this project are based on power analyses (significance accepted at $p \leq 0.05$, one sided), our extensive experience with these models, and our expected productivity. Mice are continually purchased, acclimatized to the diet, and euthanized prior to tissue collection. In our vast experience and in the absence of a power analysis, designating **20 mice per group** is a sufficient number to identify a statistical significance between treatments and to account for animal attrition (see Figure below that depicts 300 animals).
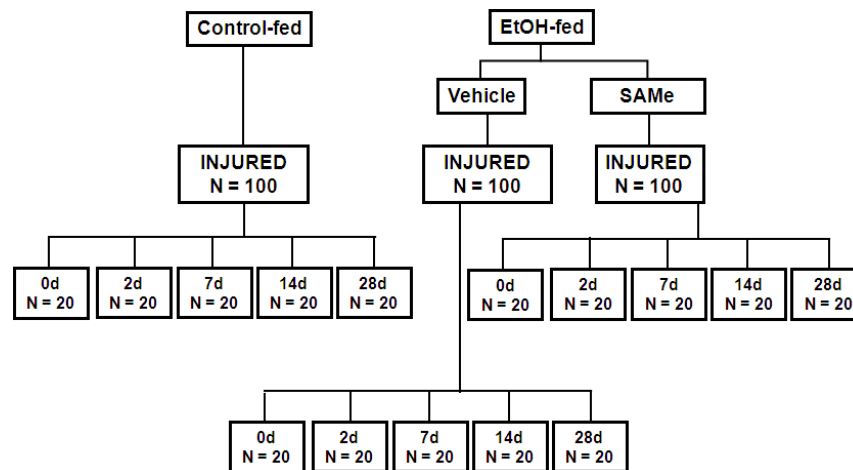
In addition, we are requesting **140 mice** to complete the Voluntary Wheel Running experiments. These animals will be grouped as:
(1) control-fed, uninjured (n=20),
(2) control-fed, injured (n=20),
(3) control-fed, injured + SAMe (n=20),
(4) alcohol-fed, uninjured (n=20),
(5) alcohol-fed, uninjured + SAMe (n=20),
(6) alcohol-fed, injured (n=20),
(7) alcohol-fed, injured + SAMe (n=20)

In all, we are requesting **440 mice** over the next 3 years.

SECTION 9. Justification for the number of animals that will be used

9.1   Group sizes are expected to represent the minimum number of animals that are needed to achieve the scientific or instructional objectives. Please underline indicate all the methods used to determine these numbers.

☒   Statistical tools, such as power analysis, were employed to determine appropriate group sizes to ensure statistically valid outcomes. (Please retain print outs from calculations.)

☒   Previous experience with this experimental paradigm indicates this is the minimum number of animals needed.

☐   Consultation with a biostatistician

☐   This is a pilot study used to determine feasibility before proceeding with larger, more tightly controlled experiments.

☐   This is an instructional activity. This is the minimum number of animals needed based on class size and optimal student to instructor ratios.

☐   Other (Explain): ➔

9.2   Using the specifics of your experimental plan demonstrate how the numbers of animals required to achieve your scientific objectives for this project were calculated. Include details of numbers of animals per group, control groups, treatment groups, pilot studies, and potential experimental failure. Information may be provided in the form of a table or flow chart. Justify the number of animals required for each procedure/experiment described in Section 10 using power analysis, if possible. For help with power analyses see: http://www.psycho.uni-duesseldorf.de/aap/projects/gpower/
Justify the number of animals requested.
➔ Power analyses were originally calculated by senior PI several years ago. We have been routinely using these group sizes as mouse tissue can not be used for multiple experiments (e.g., physiology, biochemistry, histology). As such, we have successfully used these animal numbers for the past 8 years and are confident that they are accurate and group sizes are sufficient to identify significant differences.

Section 10. Procedures

Please describe in detail all of the procedures you will be doing with animals below. Indicate whether infectious agents, chemical or physical restraint, radioactivity or adjuvants will be used. Describe possible/known side effects of each procedure.

10.1   **Prolonged physical restraint (> 30 minutes) that will be used under this protocol.**

    10.1.1   How is the animal acclimated to the restraint device? ➔ NA
    10.1.2   Describe the monitoring of the animal during the time of restraint. ➔ NA
    10.1.3   In the event of an animal welfare issue, what are the criteria for removal of the animal from the restraint device? ➔NA

10.2   **Non-surgical procedures that will be used under this protocol.**
    Non-surgical Procedures Description and Guidance
    Describe each non-surgical procedure that will be used and state the maximum number of times each procedure will be done to any animal.
    ➔ Please see 8.1 for complete research description. Each procedure (e.g,. muscle injury) will be   done one time per animal.

10.3   **Surgical procedures that will be used under this protocol.**
    Surgical Procedures Description and Guidance
    Please indicate if surgical procedures will be used under this protocol
    ☐ Yes
    ☒ No (If No, then skip to Question 11)

    10.3.1   Review the ASEPTIC TECHNIQUE FOR ANIMAL SURGERY.
            I will adhere to the standards specified in this policy.

☐ Yes
☐ No (If no, scientifically justify below*)
➔

10.3.2   Review the <u>PREPARATION OF SURGICAL INSTRUMENTS, DEVICES, AND SUPPLIES POLICY</u>.
I will adhere to the standards specified in this policy.
☐ Yes
☐ No (If no, scientifically justify below*)
➔

10.3.3   Describe surgical procedures that will be used. Indicate type of surgery, provide a general description and indicate if it will be **survival (indicate <u>major or minor</u>) or non-survival** surgery.
.➔

10.3.3.1 Describe pre- and post-op procedures and monitoring. ➔
10.3.3.2 Will analgesics will be given pre- or post-procedurally? ➔
10.3.3.3 If analgesics are not used, justify why this is the case. ➔
10.3.3.4 If sutures or wound clips will be used, indicate when they will be removed. ➔
10.3.3.5 If multiple major surgeries in a single animal are proposed, please scientifically justify the necessity. ➔
10.3.3.6. If multiple surgeries (major or minor) in a single animal are proposed, please indicate the minimum time interval between surgeries. ➔

10.3.4   Describe any non-pharmacological control of pain post procedure such as:
☐ quiet darkened recovery area; and/or
☐ increased ambient warmth; and/or
☐ soft resting surface, etc.; or
☐ other; please explain: ➔

SECTION 11. Substance Administration

11.1   **Substance Description**
If anesthetics, analgesics, or other substances are administered during the conduct of a procedure, please describe: *Please copy and paste the set of questions as needed to list each additional substance(s) to be administered*.

11.1.1   Name of Substance: ➔Ethanol
11.1.2   Is this a non-pharmaceutical grade chemical or substance? ➔ No
If yes, please justify and indicate how sterility and purity are achieved. ➔
11.1.3   Dose: ➔ up to 20% v/v diluted in drinking water
11.1.4   Route of Administration: ➔ oral
11.1.5   Frequency of administration: ➔ daily
11.1.6   If applicable, state how anesthetic depth will be assessed: ➔NA

11.1.1   Name of Substance: ➔ S-adenosyl methionine (SAMe)
11.1.2   Is this a non-pharmaceutical grade chemical or substance? ➔ yes
If yes, please justify and indicate how sterility and purity are achieved. ➔ S-adenosyl methionine is delivered as Denosyl provided by NutraMax Laboratories, Inc. This company specializes in animal nutrition. Our colleagues have used Denosyl and report no untoward side effects.
11.1.3   Dose: ➔ 0.4% w/v
11.1.4   Route of Administration: ➔ oral
11.1.5   Frequency of administration: ➔ daily, up to 28 days following injury
11.1.6   If applicable, state how anesthetic depth will be assessed: ➔NA

11.1.1   Name of Substance: ➔ Barium chloride
11.1.2   Is this a non-pharmaceutical grade chemical or substance? ➔ yes

If yes, please justify and indicate how sterility and purity are achieved. ➔ A pharmaceutical grade BaCl$_2$ solution is not available. Working BaCl$_2$ solution is prepared fresh and under the hood on the morning of the procedure. Working solution is diluted in sterile PBS and passed through a filter before being introduced into the animal.

11.1.3   Dose: ➔ 1.2% in sterile PBS delivered in 50ul
11.1.4   Route of Administration: ➔ i.m.
11.1.5   Frequency of administration: ➔ once on the day of muscle injury
11.1.6   If applicable, state how anesthetic depth will be assessed: ➔NA

11.1.1   Name of Substance: ➔ BrdU
11.1.2   Is this a non-pharmaceutical grade chemical or substance? ➔ no
         If yes, please justify and indicate how sterility and purity are achieved. ➔
11.1.3   Dose: ➔ i.p.: 100 ug/g, maxiumum volume 0.3 ml followed by oral: 0.8 mg/ml drinking water
11.1.4   Route of Administration: ➔ i.p. and oral
11.1.5   Frequency of administration: ➔ i.p.: twice on day of injury, separated by 8 hours, oral: daily for up to 7 days
11.1.6   If applicable, state how anesthetic depth will be assessed: ➔NA

11.1.1   Name of Substance: ➔ Isoflurane
11.1.2   Is this a non-pharmaceutical grade chemical or substance? ➔ no
         If yes, please justify and indicate how sterility and purity are achieved. ➔
11.1.3   Dose: ➔ 2l O$_2$/min flow rate
11.1.4   Route of Administration: ➔ inhalant
11.1.5   Frequency of administration: ➔ once on the day of muscle injury
11.1.6   If applicable, state how anesthetic depth will be assessed: ➔toe pinch, righting reflex

SECTION 12.   Personnel and Their Experience and Training

List the experience and/or training of the personnel and the procedures that each will conduct below. If personnel are not experienced, please list the name of the individual(s) who will be responsible for training on all procedures. Please note that DAR and/or the IACUC reserve the right to observe procedures being performed prior to protocol approval.

If training has not been completed, visit http://www.gsu.edu/research/iacuc.html and click on "How to Enroll in and Use the AALAS Learning Library". Complete the Required Education SECTION prior to submitting this protocol application.

All employees of Georgia State University who work with vertebrate animals must enroll in the Medical Monitoring Program for Vertebrate Animal Exposure whether or not they chose to participate. To enroll click on the following link: http://www.gsu.edu/images/vp_research/MMPVAE_Enrollment_Form.pdf

Principal Investigator:
Name: ➔ Jeffrey S. Otis, PhD
Phone number and location: ➔ TBD – start date at GSU is August 19, 2013
Procedures will do on animals: ➔ All
Experience: ➔ 15+ years working with animals models of myopathy. 8+ years working with chronic alcohol ingestion and glutathione supplementation models in rodents. 2+ years experience working with muscle injury and BrdU models.
GSU training completed: ➔ Yes
Is this person enrolled in the Medical Monitoring Program for Vertebrate Animal Exposure?
☒ yes
☐ no   If "no", see above.

Note: You can copy and paste the set of questions as needed to list additional personnel.

Postdoctoral personnel:
Name: ➔NA
Phone number and location: ➔
Procedures will do on animals: ➔
Experience (Indicate who will be responsible for training): ➔
GSU training completed: ➔
Is this person enrolled in the Medical Monitoring Program for Vertebrate Animal Exposure?
☐ yes
☐ no   If "no", see above

Graduate Students:
Name: ➔None yet, but they will be added to the protocol as any interested students join the lab
Phone number and location: ➔
Procedures will do on animals: ➔
Experience (Indicate who will be responsible for training): ➔
GSU training completed: ➔
Is this person enrolled in the Medical Monitoring Program for Vertebrate Animal Exposure?
☐ yes
☐ no   If "no", see above

Technicians:
Name: ➔NA
Phone number and location: ➔
Procedures will do on animals: ➔
Experience (Indicate who will be responsible for training): ➔
GSU training completed: ➔
Is this person enrolled in the Medical Monitoring Program for Vertebrate Animal Exposure?
☐ yes
☐ no   If "no", see above

Undergraduates:
Name: ➔NA
Phone number and location: ➔
Procedures will do on animals: ➔
Experience (Indicate who will be responsible for training): ➔
GSU training completed: ➔
Is this person enrolled in the Medical Monitoring Program for Vertebrate Animal Exposure?
☐ yes
☐ no   If "no", see above

SECTION 13. Hazard Use

All Hazardous Materials used must be identified.
For more information on the Biosafety program please click: GSU Biosafety Program.
For more information on the Radiation Safety program please click: GSU Radiation Safety.
For more information on Lab Safety and Hazard Communication please click: GSU Lab Safety and Hazard Communication.

13.1   Are you working with infectious agents and/or biologically-derived toxins?
☒ No
☐ Yes I have approval from the Biosafety Committee
    Date: ➔_____ Approval # ➔_____

13.1.1   Describe all special precautions recommended for personnel who handle animals, equipment, or do housekeeping. Describe how contaminated caging or tanks, bedding, and equipment should be handled.
    ➔

13.2   Does the project use any recombinant DNA (i.e. cloning/expression systems, viral vectors, etc.) material in animals?

☒   No

☐   Yes I have approval from the Biosafety Committee
        Date: ➔_____ Approval # ➔_____

    13.2.1   Describe all special precautions recommended for personnel who handle animals, equipment, or do housekeeping. Describe how contaminated bedding, cages or tanks or equipment should be handled.
            ➔

13.3   Will any carcinogenic or toxic compounds to be used in animals?

☐   No

☒   Yes

    BrdU usage has received IRB approval. Approval date is August 26, 2013.

    13.3.1   Describe all special precautions and training recommended for personnel who handle animals, equipment, or do housekeeping. Describe how contaminated caging or tanks, bedding, and equipment should be handled.
            ➔All personnel working with animals must successfully complete GSU training (e.g., working with mice, biosafety). Biological tissues will be disposed of in appropriate biohazard bags. Sharps will be disposed of in appropriate sharps containers in the lab. Cages and bedding will be returned to DAR facilities for processing – no special considerations are required for this protocol. Personnel working with BrdU will be trained by the PI. Appropriate signage will clearly indicate BrdU use (in storage form and in drinking bottles).

13.4   Will any radioactive compounds be administered to the animals?

☒   No

☐   Yes I have approval from the Radiation Safety Committee
        Date: ➔_____ Approval # ➔_____

    13.4.1   Describe all special precautions recommended for personnel who handle animals, equipment, or do housekeeping. Describe how contaminated caging, bedding, and equipment should be handled.
            ➔

13.5   Will Controlled Substances be used?

☒   No

☐   Yes

    13.5.1   Do you have a Controlled Substance Permit?
            ☐   No
            ☐   Yes

SECTION 14.   Animal Housing and Husbandry

14.1   Will you breed these animals? See OLAW Position Statement 2c) Rodent Housing and GSU IACUC Standard Operating Procedures on Social Housing of Social Species.

☒   No

☐   Yes Please address the following: describe method of mating (harem, monogamous, et cetera); describe age of weaning; describe genetic monitoring and methods to assure inbred / outbred status; justify breeding versus obtaining commercially. If only one sex of offspring will be used in experiments, please justify. A separate breeding protocol (Use breeding protocol form) may be submitted to generate animals for this and other experimental protocols.
        ➔

14.1.1 If breeding Genetically Modified Animals (GMAs), with their inherent potential for unanticipated phenotypes, please describe monitoring procedures for unexpected outcomes: ➔

14.1.2 Please state if there is a plan in place for preservation of critcal or irreplaceable animals such as transgenics: ➔

14.2 Do the animals require housing other than standard caging/bedding or tanks/water?
☐ No
☒ Yes (List housing required and explain below):
➔ Mice in the voluntary wheel running groups are housed individually in modified rat cages. This is required as measurements are taken from one mouse at a time.

14.3 Will animals be housed singly? ➔ Yes, see 14.2 for explanation.
Non-Human Primate Housing and Social Housing of Social Species criteria options can be found at GSU IACUC Standard Operating Procedure on Social Housing of Social Species and OLAW Position Statement 2a Nonhuman Primate Housing.

14.4 Do the animals require special care?
☒ No
☐ Yes (List special care and explain below)
➔

14.5 Do the animals require diet other than the standard diet for this species used at GSU?
☐ No
☒ Yes (List diet and explain below)
➔ A significant percentage of our animals will be on an alcohol diet. This diet will be replenished and tracked by the PI. Appropriate signage will clearly indicate to the DAR staff that a special diet is in use and that the PI should be contacted with any questions or issues.

14.6 Will food and/or fluid restriction and/or regulations be necessary?
☒ No
☐ Yes (List restrictions and explain below how they will be managed). ➔
Describe how the animal(s) will be monitored to ensure that food and fluid intake meets their nutritional needs. ➔ In our vast experience with these models, animals gain weight comparable to controls. Therefore, we do not routinely check body weight to ensure adequate nutrition. Regardless, water is replenished every 1-2 weeks.

14.7 Will animals remain outside the IACUC approved Animal Housing Area for more than 12 hours?
☒ No
☐ Yes (List building and room number and explain below)
➔

14.8 Will animals be transported outside of an IACUC approved Animal Housing Area (e.g. to your lab or off campus)?
☒ Yes
☒ I agree to adhere to the GSU IACUC Animal Transportation Policy.
☐ I request a deviation from the GSU IACUC Animal Transportation Policy. Please state the deviation and provide a justification for the deviation below:.
➔
☐ No

14.9 Will animals undergo experimental manipulations outside the IACUC approved Animal Housing Area?
☒ Yes (List building and room number and explain below)
➔ Room to be names, but it is next to Dr. Chris Ingalls' lab in the basement of the Sports Arena (G19)

☐ No

14.10 Can animals be provided environmental enrichment? See OLAW Position Statement 2b) Environmental Enrichment for more information. Enrichment options can be found at: Enrichment Options

☒ Yes (List enrichment requested below)
➔ group housing, nestlets, voluntary free wheels for singly housed mice
☐ No (Explain below) ➔

IACUC Original Protocol

GEORGIA STATE UNIVERSITY
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
Request for Amendment to an Approved Protocol

Information for the protocol you wish to amend:
Protocol Number: A13025 (0816)
Principal Investigator: Jeffrey S. Otis, PhD
Department: Kinesiology and Health
Protocol Title: Alcoholic myopathy and skeletal muscle regeneration
Animal Type and Quantity: Mouse
Proposal Period: 8/23/2016

1. I wish to amend this protocol by making the following changes:
➔ This amendment is requesting the addition of *in vivo* isometric torque measurements made in our mouse model.

2. Please provide scientific justification for the changes
➔ In the approved parent protocol and its amendment, we are interested in quantifying improvements in skeletal muscle regeneration following nutritional supplementation with S-adenosyl methionine (parent) and/or resveratrol (amendment). To do this, we have relied solely on histochemical analyses of regenerated fiber cross-sectional area and fibrosis post-injury. Here, we would like to add physiological measures of skeletal muscle performance (i.e., isometric torque). These measures complement our histochemical outcomes and give us a better idea of the true structure-function relationship within regenerating muscle.

Proposed experimental procedure in detail:
Isometric torque of the anterior crural muscles will be measured *in vivo* immediately before and at the end of 2, 7, 14, and 28 days of muscle recovery following barium chloride-induced tissue injury. Depending on group, isometric torque measures may occur in a single mouse from 2-5 times.

Immediately before testing, mice will be anesthetized with isoflurane (5% induction 1000 ml/min of 100% medical grade oxygen and 1-2% maintenance in 500 ml/min of 100% medical grade oxygen). Depth of anesthesia will be assessed by absence of toe pinch reflex and rate/depth of respiration. Body temperature will be maintained via heating pad. Hair will be removed using clippers and then the skin covering the biceps femoris and lateral gastrocnemius muscles will be aseptically prepared. Once complete, the mouse will be transferred and secured (tail, forelimbs, and contralateral hindlimb) to a temperature-controlled platform. The ankle and knee of the experimental hindlimb will be held in place using a clamp device as previously described (*J. Appl. Physiol.* 79:1260, 1995). Two sterilized platinum subdermal needle electrodes will be inserted into the skin proximal to the common peroneal nerve. Anterior crural muscle strength will be measured by stimulating the common peroneal nerve via the electrodes. Torque will then be measured at the following stimulation frequencies: 20, 40, 60, 80, 100, 125, 150, 200, 250, 300, 350, and 400 Hz. Mice will be identified via ear hole punch for subsequent torque measures. Depending on group, mice will be returned to their cages and monitored for recovery from anesthesia, or sacrificed as described in the parent protocol.

Importantly, the isometric torque test is well-tolerated by the mice and does not result in ill-effects on health (i.e., loss of body mass, poor appetite, failure to groom, loss of muscle function in the tested

limb). Finally, multiple anesthetic bouts are well-tolerated in mice.

3. Justification for non-duplication of work.
   Please provide a written assurance that the proposed work is not unnecessarily duplicative.
➔   We have search Pubmed (February 5, 2014) and have concluded that performing *in vivo* isometric torque analyses in our mouse model of alcoholic myopathy +/- injury +/- SAMe +/- resveratrol is completely novel and does not duplicate previous work.

4. Personnel: If new procedures are proposed, list the name(s), experience and/or training of the personnel and the procedures that each will conduct. List the names(s) of the individuals who will be responsible for training:
➔   I have submitted a personnel amendment application to include Cory Baumann and Russell Rogers. They are PhD students in the laboratory of Chris Ingall and have extensive knowledge, training, and expertise with the *in vivo* isometric torque model. Importantly, these techniques mirror the approved protocol of Dr. Ingalls (#A12045).

5. <u>Highest Pain Category for experiments described in this amendment:</u> ➔ (enter B, C, D, or E)
Class D.
Please note, however, that the approved parent protocol categorizes animals undergoing skeletal muscle injury via BaCl$_2$ injection as Class E.

USDA Classifications and Examples of Pain Categories:
Classification B: Animals being bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery, but not yet used for such purposes.
Classification C: Animals upon which teaching, research, experiments, or tests will be conducted involving no pain, distress, or use of pain-relieving drugs.
Classification D: Animals upon which experiments, teaching, research, surgery, or tests will be conducted involving accompanying pain or distress to the animals and for which appropriate anesthetic, analgesic, or tranquilizing drugs will be used.
Classification E: Animals upon which teaching, experiments, research, surgery, or tests will be conducted involving accompanying pain or distress to the animals and for which the use of appropriate anesthetic, analgesic, or tranquilizing drugs will adversely affect the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests.
Is this a change in Pain Category? ☐ **Yes** ☒ **No**

6. If the Pain Category Is "B" or "C" please go to question number 8.
7. Explanation for the Consideration of Alternatives for Category "D" or "E" Animal Use.
   Federal Regulations (The Public Health Service Policy and the Animal Welfare Act) and University Policy require assurance that this project does not unnecessarily duplicate research projects/courses performed at this or other institutions, and that the use of alternatives to live animal models and alternative procedures that may cause more than momentary or slight pain/distress (Class "D" and "E" procedures) to animals have been considered. The information in this section should include adequate information for the IACUC to assess that a reasonable and good faith effort was made to determine the availability of alternative models or methods.
   The following is a guide for answering 7.1 and beyond.
   Your literature search is done as part of the OLAW requirement to address the "3Rs"
   (Refinement, Reduction, and Replacement) issues. For example:
   * Refinement of procedures to eliminate or minimize pain or distress, the use of remote telemetry to decrease the distress of restraint; the use of humane endpoints.
   Other examples of refinement are: ways to enhance the well-being of animals and the use of analgesics to decrease pain or anesthetics to decrease distress.
   For the literature searches
   Ask "Am I using the least painful technique(s)?"
    - **Search terms:** Your species + animal/experimental model/technique + scientific keywords
   * Replacement of live animals with non-animal procedures or a less sentient species. Examples include the use of non-animal models such as in-vitro work, cell culture, tissues culture, computer models or simulations.
   Ask "Do I need to use animals?":

- **Search terms:** Your experimental model + simulation + in vitro +scientific keywords
Ask "Am I using the lowest species possible?":
- **Search terms:** Your species + all applicable lower species (For example if using mice, specifically use "invertebrate", "fish" and "frog" in your search terms) + your animal/experimental model + scientific keywords
Note: **There are NO search terms that will direct the searcher to all examples of replacement or refinement. The onus is on the researcher to read and evaluate the literature.**

*Reduction in the number of animals used in the study. (**This only needs to be addressed in Question 14 below**). Examples include the use of shared control groups; preliminary screening in non-animal systems; innovative statistical packages.
The selection of databases depends on the work performed and the species used in the protocol. Samples of databases available through the Georgia State University Library www.library.gsu.edu/database web pages
· PubMed· Web of Knowledge · Biological Abstracts· Web of Science PsychINFO AltWeb NLM Gateway AVAR Galileo AGRICOLA
OVID (allows a search of multiple databases including Agricola, BIOSIS, CAB Abstracts, Medline, Zoological Record, etc.)
*Please visit the Animal Welfare Information Center (**AWIC**) Literature Searching and Databases page (http://awic.nal.usda.gov/nal_display/index.php?tax_level=1&info_center=3&tax_ subject=184) for a list of databases that can be used to search for alternatives. The AWIC site also recommends using the Literature Search Worksheet (http://www.nal.usda.gov/awic/alternatives/searches/altwksht.pdf) to assist in performing a successful alternatives database search. The worksheet helps to identify relevant searchable terms and concepts.*

7.1 **Literature search for alternatives to painful procedures**
If you chose category "D" or "E" above, please do literature searches using the broadest database for your area of study, and provide a brief summary of the results obtained to verify that you investigated the use of alternatives to painful or distressful procedures. If you have not selected either category D or E, skip to section 6.2.
➔

7.1.1   Name of the databases used: ➔ Pubmed, ALTBIB
Date the databases search(es) were done: ➔ February 5, 2014
Did the search cover the entire date range of the databases with no restriction on dates?
☒ Yes
☐ No (If No, provide dates covered by the search(es) below)
Dates covered by the databases search(es): ➔

7.1.2   Search keyword(s) used: include number of hits for combinations of terms. **You must use 'alternative' and 'animal welfare' in the search combinations for invasive or painful procedures** (*See above for 3R criteria.*)
➔ alcoholic myopathy, skeletal muscle regeneration, skeletal muscle injury, animal testing alternatives, animal welfare, isometric torque, and alternative

(1) Alcoholic myopathy + animal welfare = 0

(2) Alcoholic myopathy + alternative = 0

(3) Skeletal muscle regeneration + animal welfare = 0

(4) Skeletal muscle regeneration + alternative = 10

(5) Skeletal muscle injury + animal welfare = 0

(6) Skeletal muscle injury + alternative = 5

(7) Skeletal muscle injury + isometric torque + animal welfare = 0

(8) Skeletal muscle injury + isometric torque + alternative = 1

71.3   Summarize the information found in the hits below. (Note: this should be a <u>general summary</u>. You <u>do not</u> need to go into the details of each hit but rather summarize hits by relevant groups)
➔ Search 8 returned 1 hit and was not an alternative to animal use, but described torque measurements in human subjects.

7.1.4   Explain what alternatives exist in place of using animals for this protocol and provide a justification if these cannot be used.
➔ Alternatives do not include less sentient species. Similarly, alternatives are limited to *in vitro* systems that do not adequately reflect the complex physiological response that occurs *in vivo*.

7.1.5   **Other Sources of Information on Alternatives to Painful Procedures**
7.1.5.1 Consultation with Experts: (Names, credentials, and dates): ➔
(1) Mike Huerkamp, DVM 2005-2013. I sat on the Emory IACUC for 4 years and had frequent conversations with Mike (Director of DAR) and other IACUC members about animal welfare.
(2) Chris Ingalls, PhD. I have discussed adding this procedure to my protocol with Dr. Ingalls and his graduate students, Cory Baumann and Russell Rogers.

7.1.5.2 Scientific Meetings: Specify: ➔ NA

Note: A careful literature search is usually the best way to determine whether a proposed study is unnecessarily duplicative of previous work. However, it is ok to repeat a published experiment to make sure that it works in a different lab. This reason just needs to be stated as the justification.

8. If additional animals are needed, please justify their use.
    Note: This Amendment Form may be used to request additional animals. If a different species is required you must submit a new protocol.
We are <u>NOT</u> requesting additional animals to assess the effects of our injury or treatments on isometric torque. All animals currently approved will be subject to isometric torque measures as described below (modified from approved protocol A12045, PI: Ingalls).

9. Are there any changes in the Animal housing/husbandry for this protocol?
    If so please describe below
    ➔ NA

Jeffrey S. Otis
_____**February 5, 2014**_____
Investigator's Signature   Date

IACUC Original Protocol

GEORGIA STATE UNIVERSITY
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
Request for Amendment to an Approved Protocol

Information for the protocol you wish to amend:
Protocol Number: A13025
Principal Investigator: Jeffrey S. Otis, PhD
Department: Kinesiology and Health
Protocol Title: Alcoholic myopathy and skeletal muscle regeneration
Animal Type and Quantity: Mouse
Proposal Period: end date of approved parent protocol is 8/23/2016

1. I wish to amend this protocol by making the following changes:

➔ This amendment requests: (1) a new drug, 17-allylamino-17-demethoxygeldanamycin (17AAG), to be delivered to mice i.p., and (2) an additional 150 mice.

2. Please provide scientific justification for the changes

Rationale

We are currently approved to injure tibialis anterior muscles of mice with intramuscular injections of 1.2% $BaCl_2$ to study the mechanisms of degeneration and regeneration. Recent work has suggested that early inflammatory events following injury (e.g, cytokine induction) may play a crucial role in the successful repair of damaged muscle fibers[1]. Accordingly, this amendment requests the use of 17AAG, an inhibitor of heat shock protein 90 (HSP90), to subsequently modulate the inflammatory cytokine, NFκB. Specifically, 17AAG binds HSP90, which then allows for heat shock transcription factor 1 (HSF1) to translocate into the muscle cell nucleus. HSF1 binds to HSF1 response elements and increases HSP70 expression[2,3] which has been shown to significantly reduce NFκB activity[5]. 17AAG treatment has been shown to enhance recovery following contraction-induced injury in old muscles[3] and attenuate atrophy following unloading[4], however, it is unclear if these findings extend to otherwise, healthy young mice. Further, these experiments will lay the foundation for our subsequent studies using the chronic alcohol model and the effects of HSP70 on regeneration.

Experimental Design and Methods

The overall goal of this proposal is to identify the role of HSP70 and NFκB on the regenerative response of injured skeletal muscle following injury. To accomplish this, mice will be randomly assigned to one of two groups (n=75/group): (1) Injured, vehicle-injected, or (2) Injured, allylamino-17-demethoxygeldanamycin (17AAG)-injected. Contralateral (uninjured) muscles will serve as controls. Mice in treatment group will receive 100-μl of 17AAG (40 mg/kg, i.p.) suspended in dimethyl sulfoxide 3 days prior to a $BaCl_2$-induced injury. 17AAG will be sterile filtered using .22μm filters prior to injection. This dose of 17AAG and route of delivery has been shown to result in 99% bioavailability of drug in skeletal muscle[6]. Depending on group, mice may receive up to 4 injections of 17AAG (initial injection, and once every 7 days for up to 3 weeks) as previously described[2]. Thus, injections will occur 4, 11 and 18 days following the $BaCl_2$ injury. The figure below displays our experimental timeline for 17AAG injection (treatment), muscle injury ($BaCl_2$ injection at time 0), and animal sacrifice and muscle excision.
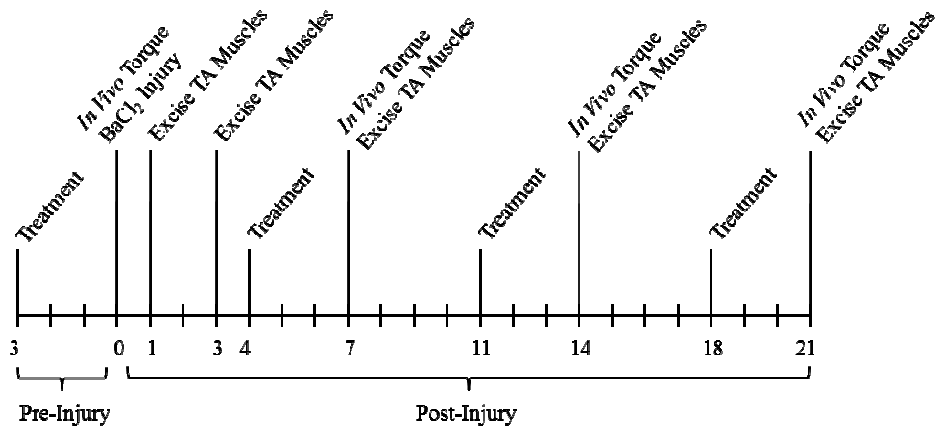
Timeline (in days) of the experimental design. Treatment will be either an intraperitoneal injection of 40 mg/kg of 17AAG suspended in DMSO or the vehicle, DMSO. Tibialis anterior (TA) muscles will be removed from anesthetized mice and stored for histological and biochemical analysis.

Again, uninjured, contralateral TA muscles from 17AAG-injected mice will be assessed to determine if the drug has any effect on the unstressed TA muscles. Likewise, vehicle-treated contralateral TA muscles from vehicle-injected mice will serve as the uninjured controls as we have previously described[1]. Using the contralateral muscle as the control greatly reduces the animal numbers required to calculate meaningful statistical differences.

Monitoring: We do not expect significant untoward effects of 17AAG administration. For example, Lomonosova has reported no change in body weight of rodents that received similar concentrations of the drug. Regardless, we will monitor our mice three times per week for any signs of distress (e.g., failure to groom). In addition, we will work closely with the DAR room staff to identify any (although unexpected) signs of distress and request euthanization of any ill-affected animal accordingly.

Approved procedures for the parent protocol (in brief)
Muscular function will be determined by assessing *in vivo* isometric torque produced by the anterior crural muscles (tibialis anterior; TA, extensor digitorum longus and extensor hallucis muscles) immediately before the $BaCl_2$ injury, and 7, 14 or 21 days into recovery. Mice will be sacrificed at 1, 3, 7, 14 or 21 days post-injury (n=15/time point) and the TA muscles will be excised. Within each time point, five mice will have their TA muscles mounted in a medium (O.C.T., Merck, UK), frozen rapidly in 2-methylbutane cooled in liquid nitrogen, and sectioned for histological analysis. The remaining 10 mice from each group will have their TA muscles rapidly frozen in liquid nitrogen and stored at −80°C until biochemical analysis.

References:
1. Otis JS, Niccoli S, Hawdon N, Sarvas JL, Frye MA, Chicco AJ, Lees SJ. Pro-inflammatory mediation of myoblast proliferation. PLoS One. 2014; 9(3):e92363.

3. Justification for non-duplication of work.
    Please provide a written assurance that the proposed work is not unnecessarily duplicative.
➔ 17AAG has been proven effective in an animal model of advanced age. However, the biochemistry and physiology of muscle regeneration is comparatively different in an otherwise healthy, younger animal. No research exists on the impact of HSP70 modulation on skeletal muscle physiology, inflammation, oxidant stress, nor NFkB-dependent signaling following chemical-induced injury. These experiments could provide the foundation for treatment paradigms that improve skeletal muscle repair and hasten the return of optimal muscle health following significant trauma.

4. Personnel: If new procedures are proposed, list the name(s), experience and/or training of the personnel and the procedures that each will conduct. List the names(s) of the individuals who will be responsible for training:

➔ Cory Baumann (PhD student) is currently approved on the parent protocol and will be responsible for 17AAG injections. Cory has approximately 5 years of reseach experience that requires animal handling, small animal surgery, and animal care. He has been adequately trained on these techniques by Chris Ingalls. In addition, Cory intends to contact Matt Davis to re-train on scruff techniques.

5. Highest Pain Category for experiments described in this amendment: ➔ (enter B, C, D, or E)
Class C.

Please note, however, that the approved parent protocol categorizes animals undergoing skeletal muscle injury via $BaCl_2$ injection as Class E. As the contralateral TA muscle is used as the control, all mice receive $BaCl_2$ injection on one limb.

USDA Classifications and Examples of Pain Categories:
Classification B: Animals being bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery, but not yet used for such purposes.
Classification C: Animals upon which teaching, research, experiments, or tests will be conducted involving no pain, distress, or use of pain-relieving drugs.
Classification D: Animals upon which experiments, teaching, research, surgery, or tests will be conducted involving accompanying pain or distress to the animals and for which appropriate anesthetic, analgesic, or tranquilizing drugs will be used.
Classification E: Animals upon which teaching, experiments, research, surgery, or tests will be conducted involving accompanying pain or distress to the animals and for which the use of appropriate anesthetic, analgesic, or tranquilizing drugs will adversely affect the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests.
Is this a change in Pain Category? ☐ **Yes** ☒ **No**

6. If the Pain Category Is "B" or "C" please go to question number 8.
7. Explanation for the Consideration of Alternatives for Category "D" or "E" Animal Use.
    Federal Regulations (The Public Health Service Policy and the Animal Welfare Act) and University Policy require assurance that this project does not unnecessarily duplicate research projects/courses performed at this or other institutions, and that the use of alternatives to live animal models and alternative procedures that may cause more than momentary or slight pain/distress (Class "D" and "E" procedures) to animals have been considered. The information in this section should include adequate information for the IACUC to assess that a reasonable and good faith effort was made to determine the availability of alternative models or methods.
    The following is a guide for answering 7.1 and beyond.
    Your literature search is done as part of the OLAW requirement to address the "3Rs"
    (Refinement, Reduction, and Replacement) issues. For example:
    * Refinement of procedures to eliminate or minimize pain or distress, the use of remote telemetry to decrease the distress of restraint; the use of humane endpoints.
    Other examples of refinement are: ways to enhance the well-being of animals and the use of analgesics to decrease pain or anesthetics to decrease distress.
    For the literature searches
    Ask "Am I using the least painful technique(s)?"
     - **Search terms:** Your species + animal/experimental model/technique + scientific keywords
    * Replacement of live animals with non-animal procedures or a less sentient species. Examples include the use of non-animal models such as in-vitro work, cell culture, tissues culture, computer models or simulations.
    Ask "Do I need to use animals?":
     - **Search terms:** Your experimental model + simulation + in vitro +scientific keywords
    Ask "Am I using the lowest species possible?":
     - **Search terms:** Your species + all applicable lower species (For example if using mice, specifically use "invertebrate", "fish" and "frog" in your search terms) + your animal/experimental model + scientific keywords
    Note: **There are NO search terms that will direct the searcher to all examples of replacement or refinement. The onus is on the researcher to read and evaluate the literature.**

*Reduction in the number of animals used in the study. (**This only needs to be addressed in Question 14 below**). Examples include the use of shared control groups; preliminary screening in non-animal systems; innovative statistical packages.

The selection of databases depends on the work performed and the species used in the protocol. Samples of databases available through the Georgia State University Library www.library.gsu.edu/database web pages
· PubMed· Web of Knowledge · Biological Abstracts· Web of Science   PsychINFO   AltWeb   NLM Gateway   AVAR   Galileo   AGRICOLA
OVID (allows a search of multiple databases including Agricola, BIOSIS, CAB Abstracts, Medline, Zoological Record, etc.)
Please visit the Animal Welfare Information Center (AWIC) Literature Searching and Databases page (http://awic.nal.usda.gov/nal_display/index.php?tax_level=1&info_center=3&tax_ subject=184) for a list of databases that can be used to search for alternatives. The AWIC site also recommends using the Literature Search Worksheet (http://www.nal.usda.gov/awic/alternatives/searches/altwksht.pdf) to assist in performing a successful alternatives database search. The worksheet helps to identify relevant searchable terms and concepts.

7.1   **Literature search for alternatives to painful procedures**
If you chose category "D" or "E" above, please do literature searches using the broadest database for your area of study, and provide a brief summary of the results obtained to verify that you investigated the use of alternatives to painful or distressful procedures. If you have not selected either category D or E, skip to section 6.2.
   ➔
   7.1.1   Name of the databases used: ➔
   Date the databases search(es) were done: ➔
   Did the search cover the entire date range of the databases with no restriction on dates?
   ☒ Yes
   ☐ No (If No, provide dates covered by the search(es) below)
   Dates covered by the databases search(es): ➔

   7.1.2   Search keyword(s) used: include number of hits for combinations of terms. **You must use 'alternative' and 'animal welfare' in the search combinations for invasive or painful procedures** (*See above for 3R criteria.*)

   71.3   Summarize the information found in the hits below. (Note: this should be a general summary. You do not need to go into the details of each hit but rather summarize hits by relevant groups)

   7.1.4   Explain what alternatives exist in place of using animals for this protocol and provide a justification if these cannot be used.

   7.1.5   **Other Sources of Information on Alternatives to Painful Procedures**
   7.1.5.1 Consultation with Experts: (Names, credentials, and dates): ➔
   7.1.5.2 Scientific Meetings: Specify: ➔

Note: A careful literature search is usually the best way to determine whether a proposed study is unnecessarily duplicative of previous work. However, it is ok to repeat a published experiment to make sure that it works in a different lab. This reason just needs to be stated as the justification.

8. If additional animals are needed, please justify their use.
   Note: This Amendment Form may be used to request additional animals. If a different species is required you must submit a new protocol.
Here, we request an additional 150 mice to be grouped as:

(1) Injured, vehicle-injected (n=75)
(2) Injured, allylamino-17-demethoxygeldanamycin (17AAG)-injected (n=75)

Within each group, 15 animals will be sacrificed at 5 different time points post-injury (i.e., 1, 3, 7, 14 or 21 days). Harvested tissues will then be subjected to various histological or biochemical analyses. Again, please see figure above for clarification.

9. Are there any changes in the Animal housing/husbandry for this protocol?
   If so please describe below
   ➔ No

Jeffrey S. Otis
_____**August 15, 2014**_____
Investigator's Signature   Date

Appendix B: Raw Data

In Vivo Data

Subject ID	Group	Day	BW-Pre	BW-Post	L-TA	R-TA	Absolute Torque-Pre	Absolute Torque-Post	Normalized Toque-Pre	Normalized Torque-Post	Torque Deficit
1	VEH	1		22.46	49.13	43.76					
2	VEH	1		23.00	52.64	38.20					
3	VEH	1		22.03	54.65	40.28					
4	VEH	1		24.70	61.35	40.51					
5	VEH	1		21.89	43.21	43.14					
6	VEH	1		22.27	46.61	33.02					
7	VEH	1		23.75	53.70	43.96					
8	VEH	1		20.99	47.46	33.21					
9	VEH	1		27.34	52.46	49.13					
10	VEH	1		26.13	46.73	40.36					
11	VEH	1		24.19	49.30	40.76					
12	VEH	1		22.11	49.85	41.18					
1	HSP	1		22.90	47.45	40.60					
2	HSP	1		22.87	46.40	42.00					
3	HSP	1		24.96	54.69	38.83					
4	HSP	1		23.47	59.83	39.98					
5	HSP	1		21.39	62.04	49.35					
6	HSP	1		23.14	47.75	38.07					
7	HSP	1		25.21	53.06	43.77					
8	HSP	1		22.09	51.64	42.47					
9	HSP	1		25.74	59.72	46.62					
10	HSP	1		25.19	43.00	49.18					
11	HSP	1		22.28	45.02	39.49					
1	VEH	3		21.31	52.39	48.56					
2	VEH	3		25.11	41.88	41.86					
3	VEH	3		27.25	45.21	47.63					
4	VEH	3		27.11	38.12	42.40					
5	VEH	3		25.90	47.98	47.22					
6	VEH	3		24.00	44.47	43.41					
7	VEH	3		24.00	46.22	44.83					
8	VEH	3		20.90	38.26	39.79					
9	VEH	3		24.02	38.93	34.22					
10	VEH	3		24.01	52.11	39.97					
1	HSP	3		23.86	45.74	45.12					

2	HSP	3		21.65	41.52	44.56					
3	HSP	3		23.32	37.69	45.37					
4	HSP	3		23.65	46.42	40.85					
5	HSP	3		25.31	41.52	33.02					
6	HSP	3		25.20	44.70	46.05					
7	HSP	3		22.19	23.56	37.68					
8	HSP	3		25.88	43.42	46.53					
9	HSP	3		24.99	39.73	46.83					
10	HSP	3		23.28	34.25	45.13					
11	HSP	3		21.11	33.61	39.32					
12	HSP	3		24.60	32.67	32.95					
13	HSP	3		21.43	39.03	34.56					
14	HSP	3		24.83	43.05	43.30					
1	VEH	7	25.17	25.43	38.21	36.61	2.47	1.25	98.17	49.27	49.81
2	VEH	7	21.54	22.21	30.47	39.34	1.91	0.55	88.68	24.95	71.87
3	VEH	7	26.01	25.10	31.41	40.28	2.80	0.59	107.78	23.52	78.18
4	VEH	7	23.80	23.94	34.65	43.20	2.70	0.96	113.41	40.13	64.61
5	VEH	7	25.03	25.31	40.20	40.28	2.86	0.48	114.21	19.10	83.28
6	VEH	7	24.51	24.88	30.56	46.07	2.71	0.54	110.36	21.70	80.34
7	VEH	7	24.64	24.14	38.04	45.63	2.52	0.64	102.17	26.48	74.08
8	VEH	7	24.04	24.65	36.81	41.34	2.41	1.09	100.36	44.17	55.98
9	VEH	7	24.43	25.61	35.02	41.14	2.99	0.35	122.33	13.51	88.96
10	VEH	7	24.20	24.57	30.86	44.53	2.74	0.64	113.36	26.22	76.87
1	HSP	7	25.51	24.29	36.79	43.36	2.48	0.75	97.15	30.84	68.26
2	HSP	7	23.90	23.33	35.37	38.20	2.29	0.93	95.79	40.03	58.21
3	HSP	7	23.36				2.43		103.84		
4	HSP	7	24.28	22.50	35.41	40.61		0.59		26.37	
5	HSP	7	24.64	25.43	34.75	39.04	2.30	0.70	93.21	27.61	70.38
6	HSP	7	26.93	28.08	32.11	50.18	2.75	0.30	102.26	10.57	89.66
7	HSP	7	25.60	25.64	39.74	49.01		0.49		18.94	
8	HSP	7	21.60	21.89	24.06	41.01		0.43		19.62	
9	HSP	7	24.53	25.51	37.34	44.81	3.06	0.34	124.73	13.18	89.43
10	HSP	7	25.39	25.04	28.87	53.09		0.46		18.19	
11	HSP	7	25.11	26.55	26.51	43.56	2.79	0.63	111.09	23.73	78.64
1	VEH	14	25.72	25.60	46.21	42.70	2.72	1.88	105.84	73.38	30.67
2	VEH	14	21.80	22.55	42.89	42.88	2.41	1.73	110.45	76.51	30.74
3	VEH	14	23.81	24.37	45.24	41.81	2.70	2.01	113.30	82.30	27.36
4	VEH	14	25.02	26.02	48.40	46.22	2.76	1.97	110.43	75.70	31.45
5	VEH	14	21.79	23.53	48.87	42.81	2.65	1.78	121.73	75.65	37.86
6	VEH	14	22.08	22.78	38.39	39.98	2.10	1.51	95.30	66.32	30.41
7	VEH	14	22.94	23.76	45.60	42.59	2.24	1.98	97.83	83.26	14.90

8	VEH	14	26.96	27.30	56.02	48.06	2.71	1.81	100.64	66.28	34.14
9	VEH	14	21.60	22.26	53.45	46.95	2.67	2.03	123.51	91.02	26.31
10	VEH	14	24.93	26.35	57.41	46.38	2.98	2.39	119.73	90.60	24.33
1	HSP	14	25.56	25.08	47.42	46.52	2.84	2.09	111.10	83.50	24.84
2	HSP	14	23.00	24.38	46.56	38.26	2.09	1.80	90.93	73.83	18.81
3	HSP	14	24.73	23.52	45.92	32.57	2.66	2.04	107.38	86.58	19.37
4	HSP	14	22.11	23.08	44.14	43.82	2.74	2.06	123.92	89.38	27.87
5	HSP	14	23.84	24.15	44.91	45.18	2.51	1.61	105.37	66.46	36.93
6	HSP	14	24.66	26.65	47.86	46.12	2.59	1.89	104.88	70.78	32.51
7	HSP	14	26.15	28.78	55.70	44.65	3.03	2.24	116.02	77.87	32.88
8	HSP	14	25.27	26.74	49.05	44.63	2.86	2.02	113.17	75.57	33.22
9	HSP	14	25.68	25.63	42.78	44.34		1.80		70.29	
10	HSP	14	22.96	24.46	44.09	34.75	2.66	1.93	115.68	78.98	31.72
1	VEH	21	21.26	26.76	65.28	40.23	2.48	2.13	116.74	79.60	31.81
2	VEH	21	27.39	28.50	73.94	53.74	3.11	2.98	113.67	104.69	7.90
3	VEH	21	27.47	27.22	57.30	45.40	2.95	2.14	107.46	78.55	26.90
4	VEH	21	25.11	26.78	56.55	48.77		2.86		106.89	
5	VEH	21	26.52	27.45	69.59	42.20	2.67	2.45	100.78	89.25	11.44
6	VEH	21	23.29	25.69	51.97	31.60		2.57		100.18	
7	VEH	21	23.68	25.52	52.21	37.57	2.93	2.49	123.91	97.56	21.26
1	HSP	21	26.95	27.87	55.85	50.91	2.69	2.44	99.80	87.41	12.42
2	HSP	21	24.25	25.13	56.17	43.12	2.52	2.61	104.06	103.83	0.22
3	HSP	21	23.58	23.50	54.23	48.66	2.39	2.07	101.53	88.00	13.32
4	HSP	21	26.61	27.71	56.01	52.11	3.28	2.47	123.24	89.05	27.74
5	HSP	21	25.67	26.39	54.08	54.78	2.65	2.81	103.38	106.36	-2.88
6	HSP	21	27.53	27.11	63.77	45.76	2.97	2.66	107.89	98.06	9.11
7	HSP	21	21.86				2.42		110.55		
8	HSP	21	21.94	24.05	54.40	40.04	2.74	2.24	124.82	93.34	25.22

Histology Data

Subject ID	Group	Day	TA	CSA
2	VEH	7	Left	1262
3	VEH	7	Left	1108
6	VEH	7	Left	1204
7	VEH	7	Left	1251
2	VEH	7	Right	2020
3	VEH	7	Right	2229
6	VEH	7	Right	2607
7	VEH	7	Right	1975
2	HSP	7	Left	1640
4	HSP	7	Left	1004
6	HSP	7	Left	1025
11	HSP	7	Left	1016
2	HSP	7	Right	2112
4	HSP	7	Right	2033
6	HSP	7	Right	2566
1	VEH	14	Left	1996
2	VEH	14	Left	1429
3	VEH	14	Left	1851
4	VEH	14	Left	1774
1	VEH	14	Right	2322
2	VEH	14	Right	2059
3	VEH	14	Right	2333
4	VEH	14	Right	2382
1	HSP	14	Left	1890
2	HSP	14	Left	1389
3	HSP	14	Left	1619
4	HSP	14	Left	1591
1	HSP	14	Right	2392
2	HSP	14	Right	2224
3	HSP	14	Right	2284
4	HSP	14	Right	1662
1	VEH	21	Left	2170
2	VEH	21	Left	2472
3	VEH	21	Left	2159
1	VEH	21	Right	2759
3	VEH	21	Right	1869
4	VEH	21	Right	2386

3	HSP	21	Left	1824
4	HSP	21	Left	2128
5	HSP	21	Left	2143
3	HSP	21	Right	1949
4	HSP	21	Right	2619
5	HSP	21	Right	2296

Protein Assay Data

Subject ID	Group	Day	TA	ug/mg of Muscle Wet Weight
1	VEH	1	Left	60.64
2	VEH	1	Left	45.78
3	VEH	1	Left	33.18
4	VEH	1	Left	36.47
5	VEH	1	Left	34.96
6	VEH	1	Left	33.28
7	VEH	1	Left	38.58
1	VEH	1	Right	64.29
2	VEH	1	Right	61.01
3	VEH	1	Right	57.33
4	VEH	1	Right	70.59
1	HSP	1	Left	67.24
2	HSP	1	Left	44.41
3	HSP	1	Left	32.17
4	HSP	1	Left	36.36
5	HSP	1	Left	40.37
6	HSP	1	Left	45.65
1	HSP	1	Right	69.50
2	HSP	1	Right	59.72
3	HSP	1	Right	54.30
4	HSP	1	Right	66.14
1	VEH	3	Left	66.27
2	VEH	3	Left	50.21
3	VEH	3	Left	53.30
4	VEH	3	Left	43.43
5	VEH	3	Left	45.99
6	VEH	3	Left	48.55
1	VEH	3	Right	69.54
2	VEH	3	Right	59.84
3	VEH	3	Right	65.31
4	VEH	3	Right	70.62
1	HSP	3	Left	63.33
2	HSP	3	Left	52.23
3	HSP	3	Left	55.25
4	HSP	3	Left	47.83
5	HSP	3	Left	52.48
6	HSP	3	Left	49.97

1	HSP	3	Right	67.07
2	HSP	3	Right	83.60
3	HSP	3	Right	62.81
4	HSP	3	Right	62.44
1	VEH	7	Left	62.22
2	VEH	7	Left	62.99
3	VEH	7	Left	71.22
4	VEH	7	Left	56.47
5	VEH	7	Left	61.99
1	VEH	7	Right	60.39
2	VEH	7	Right	63.63
3	VEH	7	Right	68.65
4	VEH	7	Right	69.60
1	HSP	7	Left	61.69
2	HSP	7	Left	64.83
3	HSP	7	Left	63.41
4	HSP	7	Left	62.12
5	HSP	7	Left	63.73
6	HSP	7	Left	60.59
1	HSP	7	Right	74.57
2	HSP	7	Right	62.72
3	HSP	7	Right	56.46
4	HSP	7	Right	65.12
5	HSP	7	Right	63.06
1	VEH	14	Left	65.72
2	VEH	14	Left	60.77
3	VEH	14	Left	71.26
4	VEH	14	Left	50.73
5	VEH	14	Left	63.49
6	VEH	14	Left	63.96
1	VEH	14	Right	74.33
2	VEH	14	Right	61.19
3	VEH	14	Right	63.88
4	VEH	14	Right	66.22
5	VEH	14	Right	70.52
1	HSP	14	Left	50.60
2	HSP	14	Left	60.79
3	HSP	14	Left	63.09
4	HSP	14	Left	61.04
5	HSP	14	Left	57.26
1	HSP	14	Right	64.22

2	HSP	14	Right	60.60
3	HSP	14	Right	62.62
4	HSP	14	Right	65.22
1	VEH	21	Left	63.29
2	VEH	21	Left	61.43
3	VEH	21	Left	55.23
4	VEH	21	Left	64.36
5	VEH	21	Left	63.68
1	VEH	21	Right	62.76
2	VEH	21	Right	62.00
3	VEH	21	Right	59.83
4	VEH	21	Right	64.17
5	VEH	21	Right	69.86
1	HSP	21	Left	55.12
2	HSP	21	Left	55.94
3	HSP	21	Left	67.86
4	HSP	21	Left	56.47
5	HSP	21	Left	60.46
6	HSP	21	Left	62.14
1	HSP	21	Right	50.81
2	HSP	21	Right	61.57
3	HSP	21	Right	52.81
4	HSP	21	Right	61.08
5	HSP	21	Right	63.97
6	HSP	21	Right	63.60

Western Blot Data

Subject ID	Group	Day	TA	Hsp70	GAPDH	TNF-alpha	Myogenin
3	VEH	1	Left	0.11	0.04		
8	VEH	1	Left	0.55	0.02	0.51	0.73
5	VEH	1	Left	0.23	0.05	0.59	0.40
9	VEH	1	Left	0.09	0.01	0.81	0.63
4	VEH	1	Left			0.87	0.54
7	VEH	1	Left	0.17	0.38	1.20	1.28
10	VEH	1	Left	0.23	0.06	1.37	1.05
3	VEH	1	Right	0.37	3.20		
5	VEH	1	Right	0.16	5.17	0.09	0.14
4	VEH	1	Right	0.51	3.16	0.10	0.17
8	VEH	1	Right	0.36	3.92	0.11	0.16
3	HSP	1	Left	0.23	0.07		
8	HSP	1	Left	0.31	0.41	0.48	0.40
4	HSP	1	Left	0.53	0.29	0.61	0.72
10	HSP	1	Left	0.13	0.08	1.15	0.82
5	HSP	1	Left	0.17	0.01	1.24	0.60
7	HSP	1	Left	0.06	0.19	1.58	0.78
3	HSP	1	Right	1.82	4.10		
5	HSP	1	Right	1.48	3.53	0.06	0.14
4	HSP	1	Right	2.39	1.95	0.06	0.12
8	HSP	1	Right	1.98	2.09	0.11	0.12
3	VEH	3	Left	0.49	0.06		
10	VEH	3	Left	0.63	1.08	0.26	0.36
7	VEH	3	Left	0.32	0.77	0.34	0.44
8	VEH	3	Left	0.51	0.35	0.49	0.83
9	VEH	3	Left	0.12	0.05	0.52	0.32
4	VEH	3	Left	0.54	1.10	0.71	0.94
3	VEH	3	Right	0.44	3.40		
10	VEH	3	Right	0.72	3.86	0.03	0.06
8	VEH	3	Right	0.38	3.09	0.10	0.13
4	VEH	3	Right	0.57	4.16	0.10	0.04
3	HSP	3	Left	0.39	0.11		
4	HSP	3	Left	0.25	0.23	0.36	0.44
7	HSP	3	Left	0.36	1.51	0.48	0.46
8	HSP	3	Left	0.68	0.75	0.75	0.88
5	HSP	3	Left	0.27	0.76	0.89	0.41
10	HSP	3	Left	0.15	0.08	1.09	0.64

3	HSP	3	Right	2.01	2.90		
4	HSP	3	Right	2.04	2.61	0.06	0.06
5	HSP	3	Right	1.61	2.67	0.13	0.06
8	HSP	3	Right	1.76	2.50	0.15	0.06
9	VEH	7	Left	0.45	2.25	0.08	0.20
4	VEH	7	Left	0.84	3.45	0.11	0.18
1	VEH	7	Left	1.05	3.19	0.13	0.20
10	VEH	7	Left	1.77	1.90	0.15	0.07
5	VEH	7	Left	0.73	1.92	0.31	0.25
4	VEH	7	Right	0.31	3.28	0.04	0.12
10	VEH	7	Right	1.10	2.63	0.04	0.05
5	VEH	7	Right	0.22	1.52	0.07	0.02
1	VEH	7	Right	0.53	2.71	0.16	0.16
5	HSP	7	Left	2.89	2.94	0.05	0.32
9	HSP	7	Left	2.00	1.49	0.36	0.76
7	HSP	7	Left	0.78	1.54	0.38	0.46
8	HSP	7	Left	1.72	3.24	0.42	0.60
10	HSP	7	Left	2.27	2.08	0.54	0.50
10	HSP	7	Right	2.68	2.55	0.04	0.08
9	HSP	7	Right	3.00	2.78	0.10	0.15
5	HSP	7	Right	2.48	3.77	0.13	0.15
8	HSP	7	Right	2.47	3.45	0.18	0.18
6	VEH	14	Left	1.23	3.06	0.04	0.23
10	VEH	14	Left	1.02	2.99	0.04	0.15
9	VEH	14	Left	0.32	2.14	0.05	0.10
5	VEH	14	Left	0.84	2.66	0.17	0.17
8	VEH	14	Left	0.84	3.49	0.21	0.22
10	VEH	14	Right	0.31	3.53	0.05	0.05
6	VEH	14	Right	0.26	2.87	0.06	0.07
5	VEH	14	Right	0.34	3.10	0.07	0.12
8	VEH	14	Right	0.36	2.02	0.07	0.07
6	HSP	14	Left	2.75	3.07	0.04	0.34
8	HSP	14	Left	3.61	3.29	0.10	0.18
5	HSP	14	Left	3.21	3.92	0.12	0.13
10	HSP	14	Left	3.37	3.24	0.16	0.18
7	HSP	14	Left	3.00	2.28	0.20	0.33
6	HSP	14	Right	2.76	3.12	0.05	0.05
5	HSP	14	Right	2.36	3.06	0.06	0.18
10	HSP	14	Right	3.53	3.15	0.08	0.17
8	HSP	14	Right	3.69	2.47	0.08	0.04
2	VEH	21	Left	0.54	3.02	0.04	0.06

3	VEH	21	Left	0.17	2.57	0.18	0.15
4	VEH	21	Left	0.82	3.05	0.25	0.28
5	VEH	21	Left	0.33	1.83	0.56	0.31
2	VEH	21	Right	0.27	2.10	0.06	0.16
1	VEH	21	Right	0.04	4.32	0.14	0.11
5	VEH	21	Right	0.12	2.47	0.28	0.18
2	HSP	21	Left	3.92	2.22	0.16	0.45
3	HSP	21	Left	2.50	2.71	0.20	0.31
5	HSP	21	Left	1.89	3.38	0.25	0.14
6	HSP	21	Left	1.76	3.69	0.25	0.25
1	HSP	21	Right	2.09	4.58	0.09	0.08
2	HSP	21	Right	2.48	2.53	0.12	0.19
5	HSP	21	Right	2.17	3.14	0.22	0.13