ABSTRACT

Effects of Hind Limb Unweighting on the Expression of Physiological Markers of Muscle Atrophy and Myofibrillar Protein Content in the Soleus and Extensor Digitorum Longus of Sprague-Dawley Rats

Anthony J. Vacanti

Committee Chairperson: Darryn S. Willoughby, Ph.D.

The purpose of this study was to determine the effects of hind limb unweighting on the expression of physiological markers of muscle atrophy and myofibrillar protein content in the soleus (SOL) and extensor digitorum longus (EDL) of Sprague-Dawley rats. After the 2-week unweighting period, the rats (4 HU; 4 months old, 4 CON; 5 months old) were euthanized, EDL and SOL excised and sent to the Exercise and Biochemical Nutrition Laboratory at Baylor University. The samples were analyzed for myofibrillar protein content, MHC isoform mRNA expression, calpain1, 2 activities, and ubiquitin proteolytic enzymes E2, E3, Atrogin-1, RF-1. In HU a reduction in myofibrillar content was witnessed (49% SOL, 81% EDL). A significant main effect for group was observed ($p = 0.047$) indicating HU produced a significant reduction in myofibrillar protein content and changes in MHC mRNA expression. Calpain 1, 2 mRNA expression in HU EDL increased with significant interactions ($p = 0.003$) for calpain 1 mRNA. E3 mRNA witnessed a significant interaction ($p = 0.037$), and E2, atrogin-1, and RF-1, displayed no significant interactions ($p > 0.05$).

The Effects of Rat Hind Limb Un-Weighting on Molecular Mechanisms Associated with Skeletal Muscle Atrophy

by

Anthony J. Vacanti, BS BA

A Thesis
Approved by the Department of Health, Human Performance and Recreation

Richard B. Kreider, Ph.D

Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of

Master of Science in Education

Approved by the Thesis Committee

Darryn S. Willoughby, Ph.D, Chairperson

Richard B. Kreider, Ph.D

Terrill F. Saxon, Ph.D

Accepted by the Graduate School
December 2005

J. Larry Lyon, Ph.D., Dean

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGMENTS

Sincere thanks to Dr. Darryn Willoughby for his hard work and unrelenting guidance, patience, and time! It is deeply appreciated and admired. I would like to thank my family for all the support they gave over the last two years. They helped me keep a positive attitude even when trouble presented itself. I would also like to thank my committee members for their patience and goodwill. Finally, my fellow students Ashli, Chris, and Dan for their friendship and hard work that enabled me dedicate myself to completing my goal.

CHAPTER ONE

Introduction

Skeletal muscle atrophy is a change that occurs in the muscle of adult animals as a result of the conditions of disuse (e.g. immobilization, denervation, muscle unloading), aging, starvation, and a number of disease states (i.e., cachexia). Regardless of the inciting events, skeletal muscle atrophy is characterized by a decrease in protein content, fiber diameter, force production, and fatigue resistance (Jackman et al., 2004). Consequently, atrophy of skeletal muscles lead to longer hospitalization recovery times following injury or surgery, as well as affect the ability of diseased populations (i.e., cancer, sepsis, AIDS) to recover efficiently and restore quality of life. Conversely, resistance training or combination of interventions such as prescriptive medication and nutritional supplementation has shown to reduce muscle atrophy and aid in the hypetrophic process of skeletal muscle regeneration, thus aiding rehabilitation and reduced muscle wasting (Jones et al., 2004). These methods, though successful, do not fully counter the dehabilitating effects of atrophy. The further development of pharmacological and rehabilitative approaches to reduce muscle atrophy and/or stimulate hypertrophy is highly desirable as it could reduce mortality rate, improve recovery times, quality of life, and reduce health care costs (Jones et al., 2004). The developments of such interventions are contingent for the inclusive understanding of atrophies underlying mechanisms.

The fundamental physiological event leading to atrophy inherently seems to be the loss of muscle tension. A substantial amount of the physiology of muscle atrophy has been characterized, but little is known about the mechanisms or the molecular signaling events underlying these processes. Decreased protein synthesis and increased protein degradation both have been shown to contribute to muscle protein loss due to disuse and recent work has delineated elements of pre-translational control, as well as synthetic and proteolytic processes underlying muscle atrophy. It is also becoming apparent that interactions among the proteolytic pathways (ubiquitin-proteasome, lysosomal, and calpain) are involved in muscle proteolysis during atrophy. Factors such as myostatin cytokine activity and reactive oxygen species can induce muscle protein loss under specific disease conditions. Also, it is now apparent several signaling pathways such as calcineurin/NFAT and Akt/mTOR, as wells as numerous transcription factors particularly NF-κB and Foxo- (which transcribes atrogin-1/MAFbx and MuRF1) play a key role in signal transduction with disuse atrophy. Transcriptional profiles of atrophying muscle show both up- and downregulation of various genes over time, thus providing further evidence that there are multiple concurrent processes involved in muscle atrophy. The intention of this study is to determine the effects of hind limb unweighting on the expression of physiological markers of muscle atrophy and myofibrillar protein content in the soleus (SOL) and extensor digitorum longus (EDL) of Sprague-Dawley rats. After a 2-week hindlimb unweighting period, the rats (4 HU; 4 months old, 4 CON; 5 months old) will be euthanized; the EDL and SOL excised and sent to the Exercise and Biochemical Nutrition Laboratory at Baylor University. The samples will then be analyzed for myofibrillar protein content, MHC isoform mRNA expression, calpain1, 2

activities, and ubiquitin proteolytic enzymes E2, E3, Atrogin-1, RF-1. These results will hopefully help elucidate some of the mechanisms and processes involved, adding to the overall understanding of the condition and current literature pool.

Purpose(s) of the Study

1. To examine the expression of several physiological markers of muscle atrophy such as muscle wet weight and myofibrillar protein content in response to hind limb unweighting (HU) in the soleus (SOL) and extensor digitorum longus (EDL) of Sprague-Dawley rats.
2. Identify and evaluate transcriptional activity of myosin heavy chain (MHC) isoforms in HU and control (CON) EDL and SOL muscles.
3. Identify and evaluate gene expression of enzyme systems impacting protein degradation (calpain-1, calpain-2; plus enzymes associated with polyubiquitin processes) in HU and CON EDL and SOL muscles.

Hypotheses

Ho_1 There will be a significant difference ($p<0.05$) in the physiological markers of muscle atrophy in both the EDL and SOL muscles in the HU group compared to CON.

Ho_2 There will be a significant reduction in MHC isoform mRNA expression in both the EDL and SOL muscles of the HU group compared to CON.

Ho_3 There will be a significant increase in the gene expression of the proteolytic proteins calpain-1, calapain-2, E2, E3, atrogin-1/MAFbx and MuRF1 in both EDL and SOL muscles of the HU group compared to CON.

Delimitations

The delimitations of this study are as follows:

1. Eight male Sprague-Dawley rats (~400 g) housed and monitored at Texas A&M University.
2. Rats group age; HU 4 months, and CON 5 months.
3. Rats were randomly assigned to a CON or HU group after arrival from the breeder.
4. Rats were subjected to a habituation period of 1 week surfaces while the forelimbs maintained contact with the cage floor. This allowed the animal free range of movement about the cage.
5. CON animals were maintained in a normal cage environment while HU rats were unweighted for 2 weeks (2 weeks HU in Rat population is relative to 1.05 Human years).
6. After the 2 wk unweighting period, the rats were euthanized, and the EDL and SOL muscles excised.
7. All analysis reported herein will be conducted by the Exercise and Biochemical Nutrition Laboratory (EBNL) in the Department of Health, Human Performance, and Recreation at Baylor University, in Waco, TX.

Limitations

1. Altered sleeping patterns between and within groups.
2. Dominance and territorial nature of the population in regards to eating pattern and availability.
3. Stress unrelated to physical stress of being HU.

4. Abnormal energy expenditure of rats with HU.
5. Texas A&M University handling, preparation, and monitoring of rats
6. Smaller than intended sample size.

Assumptions

1. All HU rats were subjected to identical guidelines allowing the animal free range of movement about the cage.
2. All CON animals were maintained in a normal cage environment
3. All rats were housed in a temperature-controlled (23 ± 2°C) room with a 12:12-h light dark cycle
4. All rats were apparently healthy with no contraindications to any of the prescribed testing procedures.
5. All rats had similar dietary habits.

Definitions

- Hindlimb Unweighting - suspension of the hindquarters in rodent or various animal models to induce atrophic conditions
- Soleus - weight bearing postural muscle located in the hindlimb; plantar flexion (Slow twitch).
- Extensor Digitorum Longus – non-weight bearing extensor located in the hindlimb, muscle used for plantar flexion and extension of the toes (Fast twitch)
- Myosin - A family of motor ATPases that interact with F actin filaments**.**
- Actin - A filamentous proteins (42 kD) involved in muscle contraction in both smooth and striated muscle and also serves as an important structural molecule for the cytoskeleton of many eukaryotic cells.
  It is the main constituent of the thin filaments of muscle fibers. The filaments (known also as filamentous or f-actin) can be dissociated into their globular subunits; each subunit is composed of a single polypeptide 375 amino acids long. This is known as globular or g-actin. In conjunction with myosin, actin is responsible for the contraction and relaxation of muscle.

- Myosin Heavy Chain - The heavy chains of the muscle protein myosin. Each molecule of myosin is composed of two heavy chains and two pairs of light chains. The heavy chains have a molecular weight of about 230 kD and each heavy chain is associated with a dissimilar pair of light chains.

- Calpain-1 - Calpain small subunit 1. Cysteine proteinase found in many tissues. Hydrolyzes a variety of endogenous proteins including neuropeptides, cytoskeletal proteins, proteins from smooth muscle, cardiac muscle, liver, platelets and erythrocytes. Two subclasses having high and low calcium sensitivity are known. Removes Z-discs and M-lines from myofibrils. Activates phosphorylase kinase and cyclic nucleotide-independent protein kinase.

- Calpain-2 - Calpain 2, large [catalytic] subunit precursor. Cysteine proteinase found in many tissues. Hydrolyzes a variety of endogenous proteins including neuropeptides, cytoskeletal proteins, proteins from smooth muscle, cardiac muscle, liver, platelets and erythrocytes. Two subclasses having high and low calcium sensitivity are known. Removes Z-discs and M-lines from myofibrils. Activates phosphorylase kinase and cyclic nucleotide-independent protein kinase.

- Polyubiquitin - A highly conserved 76-amino acid peptide universally found in eukaryotic cells that functions as a marker for intracellular PROTEIN TRANSPORT and degradation. Ubiquitin becomes activated through a series of complicated steps and forms an isopeptide bond to lysine residues of specific proteins within the cell. These “ubiquitinated” proteins can be recognized and degraded by proteosomes or be transported to specific compartments within the cell.

- Ubiquitin enzymes - E1 enzymes known as Ub-activating enzymes. These enzymes modify Ub so that it is in a reactive state (making it likely that the C-terminal glycine on Ub will react with the lysine side-chains on the substrate protein).

- E2 - enzymes known as Ub-conjugating enzymes. These enzymes actually catalyze the attachment of Ub to the substrate protein.

- E3 - enzymes known as Ub-ligases. E3’s usually function in concert with E2 enzymes, but they are thought to play a role in recognizing the substrate protein.

- Atrogin-1/MAFbx - atrophy-related ubiquitin ligase that causes skeletal muscle atrophy induced by Foxo transcription factors.

- MuRF1 (muscle RING finger 1) - an ubiquitin ligase that mediates muscle atrophy.

CHAPTER TWO

A Review of Literature

Muscle Atrophy Paradigms

All living organisms possess the inherent capacity to alter the structural and functional properties of their organ system. These changes are largely the manifestation of altered protein expression in which the amount or type of protein are altered to meet the imposed functional demands (Baldwin et al., 2002). These adaptations, in turn, alter the size and/or metabolic properties of the muscle fibers, resulting in physiological capabilities that match the new functional demand (Allen et al., 1999). This adaptive plasticity of protein expression involves a complex process centered on the general theme of altered gene expression.

It is important to have a global understanding of the plasticity of the organ system in which this condition and/or disease state occurs. Michael and colleagues (2000) recently defined the relationship of skeletal muscle plasticity during atrophy and functional status. Based on three muscle research paradigms, the relationship of muscle atrophy to function is portrayed as a bi-directional interaction wherein form and function influence each other by way of superficially physical and acutely internal interwoven and dependent cellular and molecular mechanisms. The first paradigm is referred to as *Adaptation,* which reflects the plastic nature of muscle when placed under certain conditions, ranging from disuse (which will be our main focus) to high-resistance exercise. The second paradigm is *Injury/loss* which describes damage to muscle tissue from ischemia, medication, cachexia, or reloading or reperfusion trauma. Also, within

this category of injury/loss, the loss of muscle tissue due to aging is incorporated. The final paradigm, *Integrity*, relates to the muscle's tendency to protect itself and maintain structural adjacencies and cellular/molecular proportion (Michael, 2000). These paradigms help simplify the complexity of the associated condition/disease and develop a basis for the cellular and molecular environment witnessed. It is important to remember that atrophy occurs from a wide range of stimuli, and each stimulus initiates the molecular atrophic response in a specific fashion. Though commonality exists between mechanisms, it is imperative each atrophic state is viewed separately and mechanisms are reviewed independently to fully delineate all the mechanisms involved.

To understand the marked degradation associated with atrophy, focus needs to be placed on the most basic unit involved in muscle form and function, the regulation of contractile proteins known as myosin heavy chain (MHC). This protein, which is both an important structural and regulatory protein comprising the contractile apparatus, can be expressed as different isoforms, thereby having an impact on the functional diversity of the muscle. The regulation of the MHC gene family is under the control of a complex set of processes including, but not limited to, activity, hormonal, neural stimuli, and metabolic factors. Thus, this protein will serve as a cellular "marker" for studies of plasticity in response to various mechanisms (Baldwin et al., 2002).

Muscle Isoform Plasticity Concepts

Muscle plasticity is the ability of a given muscle cell to alter quantity (amount) of protein or type of protein (phenotype or isoforms) comprising its different subcellular components in response to any stimulus that disrupts its normal homeostasis (Baldwin et al., 2002). For example in the case of atrophy, a given muscle fiber may respond to

chronic disuse, disease, age, etc., decreasing the mechanical stress and neural stimuli, thus inversely causing a cascade of mechanisms which ultimately lead to a reduction in cross-sectional area such that all of the subcellular components change (i.e. phenotype), thereby altering MHC isoforms that it expresses in the myofilaments. In this situation, as the muscle becomes both smaller and weaker because of a decrease in contractile protein, its intrinsic contractile properties also become transformed due to alterations in MHC phenotype that is expressed. Thus, the muscle's plasticity may involve a change in the amount of protein, the type of protein isoforms expressed, or a combination of both (Baldwin et al., 2002).

MHC Protein Isoforms

A significant factor dictating a muscle fiber's capacity for adaptation is related to its genetic capability of expressing different isoforms (molecular species). Protein isoforms are molecules with slight variations in amino acid sequence and/or composition, thereby altering either the structural, functional, or enzymatic properties of that protein. Isoforms species have been identified for a large number of muscle proteins and vary quite drastically depending on the mechanisms that they are involved (Baldwin et al., 2002). Thus a general classification scheme has been adopted and is similar between species. Adult mammalian skeletal muscle fibers have been shown to express at least four different types of MHC: slow type I MHC, fast types IIa, IIx, and IIb. Each of these isoforms displays different functional, cellular, and molecular mechanisms. Type I MHC is typically categorized as a slow isoform with large oxidative capacity. Type I is also exhibits low adenosine triphosphate activity. Common muscles associated with type I MHC fiber type are locomotive muscles, which endure, and function under chronic

aerobic conditions, i.e. soleus. At the other end of the functional spectrum, isoforms typically recruited for acute, high-intensity-explosive power, (muscles involved in power lifting or sprinting); express the IIx and IIb MHC. These isoforms are typically categorized as anaerobic, meaning the isoforms have the ability to readily resynthesize adenosine triphosphate for immediate utilization. Type IIa are considered an intermediate fast isoform typically used during continuous physical activity, with varied intensities. IIa are characterized as a blend between types I, IIx, and IIb, and relatively efficient under both aerobic and anaerobic conditions. Thus, there seems to be definite functional, mechanistic, and energetic correlations to the pattern of MHC expression (Baldwin et al., 2002). Understanding the conditions and potential mechanisms associated with these isoforms assist in determining there functional, cellular, and molecular demands.

Protein Turnover

As stipulated, muscle fiber capacity for adaptation is related to its genetic ability to manipulate and express different isoforms, thereby altering either the structural, functional, or enzymatic properties. To achieve the demand placed on the muscle tissue system, there is a constant flux of intracellular and extracellular proteins continually "turning over"; i.e., being hydrolyzed into their constituent amino acids and replaced by new synthesis (Lecker et al., 1999). Furthermore, under conditions/disease states associated with atrophy it has been shown that protein synthesis is reduced while protein degradation is increased. Consequently, the process of protein turnover provides a mechanism in which both type and amount of protein comprising cellular systems can be adapted in accordance with the environmental conditions imposed (Baldwin et al., 2002).

Until recently, research has been limited in this area, but growing evidence supporting protein degradation's essential role in muscle atrophy and skeletal muscle functional subunits has sparked interest.

Atrophic Effects on Myosin Heavy Chain Gene Expression

Each isoforms has slightly different biochemical properties that impact the shortening properties of the fiber (Barany et al., 1967). Different MHC isoforms are expressed in various muscle fiber types depending on the functional characteristics of the muscle fiber. For example, the weight bearing, slow twitch hindlimb soleus muscle expresses primarily type I MHC isoforms, while the nonweight-bearing, fast twitch extensor digitorum longus muscle expresses a predominance of IIb MHC isoforms (Haddad et al., 1997). Despite the specific fiber types, there is a high degree of MHC phenotype plasticity that occurs in response to variations in contractile activity, neural input, hormone status, disease, and disuse (Bishop et al., 1997; Diffee et al., 1993; Haddad et al., 1997; Swoop et al., 1994). Atrophy induced by unloading and other various stimuli are frequently correlated with a reduction in the expression of type I MHC and a shift in the MHC isoforms (Caiozzo et al., 1994; Haddad et al., 1998). The plasticity of the type I MHC phenotype is theorized to be regulated by transcriptional processes (Haddad et al., 1998; Knotts et al., 1995). However the specific transcriptional mechanisms by which exogenous signals, such as unloading, influence the expression of the type I MHC gene are unknown. Several elements within the promoter sequence of the type I MHC gene and corresponding transcription factors along with the behavior of several of the potential regulatory mechanisms have been the subject of numerous studies (Giger et. al. 2000).

Pre-Translational Control Mechanisms

The reduction in MHC isoforms as discussed previously purports a decrease in transcriptional activity of specific MHC genes as a major contributing factor. Haddad et. al. (2003), in a two-component study examined this theory as well as the role pretranslational and translational control has on protein accretion. Other basic molecular events were also characterized in this study and will also be discussed.

The initial study was designed to characterize the cellular process linked to marked muscle atrophy. Two hypothesizes were examined. First, that atrophy induced by spinal cord transection (SI- a procedure which renders the muscle virtually electrically silent while maintaining a functionally and anatomically intact neuromuscular connectivity), occurs rapidly due to the inability of the muscle to maintain sufficient ribosomal RNA and sarcomeric protein mRNA levels necessary for translating and maintaining sufficient amounts of muscle protein to counteract the ongoing protein degradation processes. Second, contrasting previous models of unloading-induced atrophy (Baldwin et al., 2001; Roy et al., 1996; Talmadge et al., 2000; Thomason et al., 1990), Haddad et. al. (2003), believed protein translational occurs throughout the atrophy response, until the muscle has reached a new steady state of reduced muscle mass.

To test these hypotheses, levels of myofibril protein pools and the total myosin heavy chain (MHC) and actin protein, as well as corresponding relative and total mRNA expressions (combined accounting for ~40% of total muscle protein) were assessed in two rat populations, SI and control. The SI and control groups were studied at 0, 2, 4, 8, and 15 days for slow soleus muscle atrophy. Approximately 50% atrophy was witnessed in the SI group during the first 8 days. Throughout the SI duration, muscle protein

concentration was maintained at the control level, whereas myofibril protein concentration steadily declined between 4 and 15 days of SI, and this was associated with a 50% decrease in MHC normalized to total protein. Actin relative to the total protein was maintained at the control level, while there was a marked reduction occurred in the total RNA and DNA content in total MHC and actin mRNA expressed relative to 18S ribosomal RNA. These findings suggest that two key factors may contribute to the muscle atrophy of SI (and potentially other unweighted models); a reduction in ribosomal RNA that is consistent with the reduction in protein translational capacity, and insufficient mRNA substrate for translating key sarcomeric proteins comprising the myofibril fraction, such as MCH and actin. This data reaffirms the involvement of pretranslational and translational processes in muscle atrophy.

In the second component of the study, SI again served as the model of inactivity. As reported in the first portion of the study, mRNA levels of both actin and type I MHC were markedly reduced in response to SI. Consistent with this response, during the first 7 days of SI, there were 60-70% reductions in pre-mRNA levels of type I MHC and actin in the soleus muscle. The significant reduction in transcriptional activity was determined not to be caused by a general reduction of transcriptional activity of the SI muscle, but because significant increases in the pre-mRNA levels of the fast-type IIb MHC gene were observed, which is consistent with the redirection of gene expression to a faster phenotype.

In accordance with pre-mRNA reduction of type I MHC and actin, the SI model was also shown to induce marked reductions in the transcriptional regulation of the type I MHC promoter. The findings reflecting the endogenous regulation of both slow type I

MHC and actin pre-mRNA in view of marked reductions in actin, total MHC, and especially type I MHC mRNA reported in the preceding component, demonstrate that decreased gene transcription likely plays a role in reducing the amount of mRNA of key sarcomeric proteins. The smaller mRNA pools for actin and type I MHC in the slow soleus muscle also potentially limit the ability of the muscle to synthesize these key proteins, especially taking into consideration that the ribosomal RNA pool is also reduced under these conditions. Therefore, it can be concluded from the work of Haddad et. al. (2003) that decreased transcriptional and translational regulation of key skeletal muscle genes play a significant role in regulating the atrophy response to muscle inactivity as induced by SI.

Proteolytic Enzymes and Activity

At least three proteolytic processes are known to be involved in muscle protein degradation. These include the cytosolic calcium-dependent calpain system, the lysosomal protease (i.e. cathepsins), and the ATP-dependent ubiquitin-proteasome system. What is now being understood is that these systems work as partners during muscle proteolysis rather than one system being used exclusively during atrophy (Jackman et al. 2004).

Role of Calpains in Atrophy

Controversial results surround the role of the calcium-dependent calpain system. While isolated proteasomes are able to degrade soluble actin and myosin they are not able to degrade intact myofibrils (Jagoe & Goldberg, 2001). This suggests that myofibrillar proteins must be released from the sarcomere before they can be degraded by the proteasome. It has been suggested under specific conditions of atrophy

(disuse/unweighted) that calcium-dependent calpains mediate this disassembly (Huang & Forsberg, 1998). Previous studies have shown calpain activation during disuse, but results have been mixed (Tischler et al., 1990; Taillandier et al., 1996; Ikemoto et al., 2001, Tidball & Spencer, 2002).

One of the first studies to test directly the role of calcium-mediated protein degradation during skeletal muscle atrophy was from the Tischler lab as noted (1990). Protein degradation rates were monitored in whole soleus caused by 3 days of unloading, using inhibitors of either calcium-dependent or lysosomal proteolysis. With respect to calcium dependency, unloading-induced protein loss was significantly attenuated when the muscles were directly injected with mersalyl during unloading, and protein degradation rate was attenuated when 3-day-unloaded muscles were removed and incubated in a bath with inhibitor of calcium-activated proteases. These changes were not seen when lysosomal inhibitors were tested. The study used both in vivo and in vitro approaches, and the in vivo experiment was administered throughout the period of unloading. The results of the study were crucial for understanding the action of calpains, because it seems calpains are involved in myofibrillar disassembly, perhaps the earliest event in muscle-specific protein degradation.

In more recent studies Taillandier et. al. (1996) have reported that calpain 2 (m-calpain) is upregulated 1.8-fold after 9 days of hindlimb unloading in rats, while Ikemoto et al. (2001) and Tidball & Spencer (2002) have shown no change with disuse. Consistent with the previous studies, which exhibited no change, Stevenson et al. (2003) performed a global gene expression analysis of rat soleus at 1, 4, 7, and 14 days of hindlimb unloading and found no significant change in calpain 2. Calpain 3, a muscle

specific isoform specifically bound to titin in the z-disk (Sorimachi et al. 2000), was significantly down-regulated by 1.5 and 1.6-fold by days 7 and 14, respectively. Another unexpected result from the Stevenson study was the upregulation of calpastatin, a specific inhibitor of calpain 1 and calpain 2, which was upregulated 2.8-fold by day 1 and reached 4-fold upregulation by day 14. Moreover, the calpain activator, diazepam-binding inhibitor (Dbi) was down-regulated by 2.2-fold at day 7. These specific protein levels or activation state of calpain 3, calpastatin (which may participate in attenuation of atrophy during unloading), or Dbi during disuse atrophy are relatively unexplored and will serve as an additional key to understanding calpains regulation of atrophy.

Activation of Lysosomal Proteases

Lysosomal enzymes, the cathepsins, can also target cellular proteins. A survey of he literature shows marked increases in various isoforms of cathepsins (C, D, and L) mRNAs in disuse atrophy (Ikemoto et al., 2001; Taillandier et al., 1996; Stevenson et al., 2003). However, when atrophied muscle resulting from disuse are treated with agents that block lysosomal acidification (Tischler et al., 1990) or with agents that directly inhibit cathepsins (Furuno et al., 1986; Ikemoto et al., 2001; Stevenson et al., 2003), myofibrillar protein degradation rates were not significantly affected and total protein degradation rates are only slightly reduces. These findings are consistent with the current notion that cathepsins do not degrade cytosolic proteins like myofibrils but rather, their role is to degrade membrane proteins, including receptors, ligands, channels, and transporters (Mayer, 2000). Therefore, it is expected from these results that inhibition would not have much of an effect on total or myofibrillar protein degradation during muscle atrophy (Jackman et al., 2004). Conversely, the membrane-associated proteins

that are likely degraded by lysosomal pathways during atrophy, while not contributing to the bulk of total muscle protein, may be key proteins contributing to the atrophied muscle phenotype. Thus the patterns of activation expressed by the cathepsins may indicate their role in protein degradation occurs concurrently with other mechanism later in the atrophy process. The activation of cathepsins B, C, and L have been shown with atrophy (Bey et al., 2003; Ikemoto et al., 2001; Taillandier et al., 1996) but the time course of this activation with respect to other proteolytic genes has not been demonstrated. A related area of research on proteolysis during muscle atrophy also has suggested the interaction of lysosomal and ubiquitin-proteasomal mechanisms. Just as the calpain system seems to operate in conjunction with the ubiquitin-proteasome system in degrading myofibrillar protein, it seems that the lysosomal and ubiquitin-proteasomal systems also work together to degrade specific protein substrates (Hicke et al., 1999, 2003).

IGF-I Pathway and Influence of the Ubiquitin-Proteasome system

Indeed, many of the underlying processes of atrophy are characterized by the activation of distinct pathways, in particular the ATP-dependent ubiquitin-proteasome proteolysis pathway. This system has been noted in recent literature to be one of the primary components contributing to all cause atrophy. The complete understanding of this system is integral to our understanding of all the proteolytic systems, which contribute to protein degradation.

Ubiquitin is a short peptide that can be conjugated to specific protein substrates. A chain reaction might then ensue in which a second ubiquitin peptide is ligated to the first, and a third to the second. In this way, a chain of polyubiquitin is built into a

substrate, and this ubiquitin chain targets the substrate to a structure called the proteasome, where the substrate is proteolyzed into small peptides (Jagoe et al. 2002).

The ubiquitin-proteasome system has been purported to be a major contributing factor of protein breakdown induced by disuse atrophy (Jagoe et al., 2001; Stevenson et al., 2003). Stevenson et. al. (2003), noted that proteins are marked for degradation by ubiquitin through a combined action of ubiquitin activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin protein ligases (E3). The E3 ubiquitin ligases are the components that confer substrate specificity. Several hundred distinct E3 have already been identified and it is likely that each modulates the ubiquitination of a distinct set of substrates (Glass et al., 2003). Thus the regulation of ubiquitination appears to be a coordinate signaling pathway, analogous to phosphorylation, in which key pathways might be activated by the enhanced proteolysis of a key inhibitor protein, or in which pathways might be inactivated via the degradation of an activating enzyme.

Recently in genetic screens aimed at identifying markers of muscle atrophy, Forkhead box O (Foxo), and two particular genes in particular, atrogin-1/MAFbx and MuRF1 were found to be dramatically upregulated prior to the onset of muscle loss in multiple experimental models (Bodine et al., 2001; Gomez et al., 2001; Stevenson et al., 2003). Both of these genes are purported to encode the proteins known as E3 ubiquitin ligases, which are responsible for substrate specificity of ubiquitin conjugation. Furthermore, the observation has been made that genetic deletion of either atrogin-1/MAFbx or MuRF1 results in a partial protection against muscle wasting and demonstrate that dissection of molecular regulations of these particular proteins is of

great significance with regard to resulting effects of muscle atrophy (Bodine et al., 2001; McKinnell et al., 2004).

As noted by McKinnell (2004), the presence of distinct ubiquitin proteolytic processes raises questions regarding whether muscle atrophy as the converse of muscle hypertrophy occurs via entirely independent processes or if these two mechanisms are in some way coregulated. Significant advances in the understanding of molecular control of muscle has arose from recent work of several groups that focus on the role of insulin growth factor (IGF) signaling cascades that converge to control the balance between hypertrophy and atrophy. To fully understand this cascade and its ultimate regulatory affects on atrophy a general understanding of IGFs signaling role during hypertrophy is imperative. These two potentially coregulated processes have much commonality and ultimately may be more closely related than previously expected.

IGF-1 is a secreted growth factor that regulates numerous biochemical pathways after binding to its membrane bound receptor, the IGF-1 receptor (IGFR). Upon binding to the IGFR, which is a tyrosine kinase, IGF-1 becomes phosphorylated and recruits the insulin receptor substrate 1 (IRS1), leading to the activation of the lipid kinase phosphatidylinositol 3-kinase (Pl3K). Pl3K catalyses the transfer of a phosphate group to the membrane bound phosphatidylinositol 4,5 bisphosphate (PIP_2). The phosphorylation of PIP_2 generates phosphatidylinositol 3,4,5-triphosphate (PIP_3), which recruits two additional kinases, Akt1 and phophoinositide-dependent protein kinase-1 (PDK-1) (Cantley, 2002). Akt is bound, phosphorylated, and activated by PDK-1 (Nicholson & Anderson, 2002). Once Akt is activated, it initiates a cascade of phosphorylation events targeting mammalian target of rapamycin (mTOR) (Nave et al., 1999) which in turn

phosphorylates the p70S6K and glycogen synthase kinase (GSK-3β) (Cross et al. 1995). Phosphorylated IRS1 also stimulates the Ras-Raf-MEK-ERK pathway, a mitogen-activated protein kinase (MAPK) pathway. Activation of this pathway may actually prevent hypertrophy because Ras-Raf-MEK-ERK inactivation, rather than activation, appears to characterize skeletal muscle hypertrophy (Rommel et al., 1999). Phosphorylation of mTOR represses, through an adaptor protein known as Raptor, eukaryotic initiation factor 4E (eIF-4E)-binding protein 1 (4EBP1) [4EBP1, also known as phosphorylated heat- and acid-stable protein (PHAS-1)], and inhibitor of (eIF-4E) (Hara et al 1998). Thus, mTOR-mediated inhibition of 4EBP1 results in activation of eIF-4E and subsequent increased protein synthesis. Therefore, mTOR phosphorylation activates eIF-4E and p70S6K, two positive regulators of protein synthesis. Similarly, eukaryotic initiation factor eIF-2B is activated through Akt-mediated phosphorylation and inactivation of GSK-3β. In summary, activation of the IGF-1 is imperative for the (partial) activation and regulation of protein synthesis, what role it may have on atrophy is still to be determined. Whether IGF-1 response is mediated through translation control or eventually interrupted during one of the numerous signaling intermediates is unknown. As noted earlier the possibility of an entwined hypertrophic and atrophic cascade is being theorized.

To support the correlation between hypertrophy and atrophy, the molecular cascades involving Akt1, p70S6k, and other translational components, were examined in atrophied hindlimb rat muscles. Atrophied hindlimb muscles, when reloaded after a period of disuse, undergo a hypertrophic recovery of their original muscle weight over the course of weeks (Thomason et al., 1987). In a recent study by Bodine et al. (2001)

rats were subjected 14 day of hindlimb suspension (HS) followed by period of reloading. Depending on the particular hindlimb muscle examined (MHC type I or II), a 25-55% loss of muscle weight was witnessed. After release from HS, the atrophied muscles were reloaded and hypertrophied, achieving a 15-20% recovery in muscle weight after 7 days of reloading. The activation of Akt1 and its targets were correlated with the trophic state of the muscle during both atrophy through disuse and hypertrophy on recovery. That is, Akt protein and phosphorylation levels decreased markedly during the atrophy induced HS, as did the activation of p70S6k, where as the PHAS-1/4EBP1 inhibitory complex with eIF4E increased. These changes reverted during recovery of the muscle when it was removed from HS.

Another recent study examined the degradation of fast-type contractile myosin heavy chain protein and expression of key enzymes, particularly ubiquitin-proteases, in skeletal muscle of young rats subjected to microgravity environment and HS (Ikemoto, et al. 2001). Conditions produced similar results between groups. Both groups witnessed a significant increase in the degradation and ubiquitination of fast-type MHC. The degradation was contributed but not limited to increased levels of the 20S proteasome components, E2 conjugating enzymes, polyubiquitin mRNA, and cathepsins L mRNA. The main concern of the study was to determine if microgravity or HS could induce conjugation of ubiquitin to muscle protein. Both groups did witness conjugation of ubiquitin but the mechanisms were not elucidated. Another interesting observation noted within this study suggested the MHC myofibrils do not function, as effective substrates for the poly-ubiquitin pathway, suggesting structural alteration, such as cleavage by other proteases, may be required before ubiquitination and degradation by 26S proteasome.

Thus supporting the held belief- multiple mechanisms influence the final functional and molecular condition of muscle.

Other recent studies involving the Akt pathway have also displayed similar results correlating well with its possible atrophic signaling mechanism. Sandri et al. (2004) and Stitt et al (2004) both focused on the possibility that an alternative target of IGF-1/Pl3K/Akt signaling cascade is the Forkhead box O (Foxo) family of transcription factors, which may be a possible mediator of atrophy.

In two different models of muscle cultures, a clear atrophy was induced together with a decrease in IGF-1/Pl3k/Akt pathway activity. Concurrent with the decrease was a rapid and robust induction of ubiquitin ligases, most strongly atrogin-1/MAFbx, suggesting the mechanism of atrophy was increased protein catabolism via ubiquitin-ligase mediated proteolysis. Conversely, addition of IGF-1 or constitutive activation of Pl3k/Akt pathway was able to prevent muscle loss in these models, apparently by suppressing atrogin-1/MAFbx induction.

An active role of both MuRF1 and MAFbx in mediating muscle atrophy was best displayed in a study by Bodine et al. (2001); this experiment used mice in which both alleles for either MAFbx or MuRF1 were deleted(-). MAFbx(-) and MuRF1(-) were both subjected to denervation of the gastrocnemius. Atrophy was attenuated in both populations. MAFbx(-) gastrocnemius atrophy was attenuated by 56% at 14 days, this was correlated with an decrease in E3 ligase. MuRF1(-) gastrocnemius atrophy was attenuated by 36% at 14 days. The reduction in atrophy exhibited by these models signifies a potentially strong correlation between the role of the ubiquitin-ligase and atrophy.

Although it is clear conditions associated with muscle atrophy are linked to activation of the ubiquitin proteasome system, the mechanisms leading to system activation in muscle are poorly understood. One reason for these difficulties is that a "rate-limiting" step in muscle proteolysis has not been identified (Price, 2003). Potentially important steps helping decipher the mechanisms involved have and continue to be discovered. The macro-complementary nature of the atrophic process though diverse, slowly is being elucidated. The research involved and discussed provides a framework for future research.

CHAPTER THREE

Methodology

Animals and Animal Care

Eight male Sprague-Dawley rats weighing ~400 g were obtained and (4 HU; 4 months old, 4 CON; 5 months old) (Harlan, Indianapolis, IN), housed in a temperature-controlled (23 ± 2°C) room with a 12:12-h light-dark cycle, and water and rat chow were provided *ad labitum* throughout the duration of the study. Upon arrival at Texas A&M Univeristy from the breeder, rats were subjected to 1-week habituation period. During this time, it was concluded that all rats were apparently healthy with no contraindications to any of the prescribed testing procedures and had similar dietary habits.

Hind Limb Un-Weighting Protocol

After the habituation period, rats were randomly assigned to a control (CON) or hindlimb un-weighting (HU) group. The hind limbs of the HU animals were elevated to an approximate spinal angle of 40-45° from horizontal. Briefly, the animals were injected with pentobarbital sodium (Nembutal, Abbott Laboratories, 30 mg/kg ip) to induce anesthesia. While anesthetized, the animals' tails were washed and dried, and a length of breathable non-elastic adhesive tape (Curity Porous tape, Kendall) with a hook attached to the end was placed on the proximal two-thirds of the tail, which allowed the end of the tail to remain unattached. The ends of the adhesive tape were further bonded to the tail with an additional adhesive and allowed to dry for 20 min before suspension. The hook attached to the adhesive tape was connected by a small chain to a swivel apparatus fixed at the top of the cage. Inspection of the animals was performed daily.

Adjustments to the length of the chain were made as necessary to prevent the rat hind limbs from touching any supportive surfaces while the forelimbs maintained contact with the cage floor. This allowed the animal free range of movement about the cage. All HU rats were subjected to identical guidelines allowing the animal free range of movement about the cage. The CON animals were maintained in a normal cage environment for 2 weeks while HU rats were un-weighted for 2 weeks. After the 2-week un-weighting period, the rats were anesthetized with pentobarbital (35 mg/kg ip), euthanized by decapitation, and the extensor digitorum longus (EDL) and soleus (SOL) muscles were excised and sent to the Exercise and Biochemical Nutrition Laboratory at Baylor University. All procedures performed in this study were approved by the Texas A&M University Institutional Animal Care and Use Committee and conform to the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* [DHHS Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, Bethesda, MD 20892].

Total RNA Isolation

Total cellular RNA was extracted from the homogenate of biopsy samples with a monophasic solution of phenol and guanidine isothiocyanate (Willoughby et al., 2000, 2002, 2003, 2004) contained within the TRI-reagent (Sigma Chemical Co., St. Louis, MO). The RNA concentration was determined by optical density (OD) at 260 nm (by using an OD_{260} equivalent to 40 mg/ml), and the final concentration adjusted to 1 mg/ml (Willoughby et al., 2000, 2002, 2003, 2004). Aliquots (5 ml) of total RNA samples were separated with 1% agarose gel electrophoresis, ethidium bromide stained, and monitored under an ultraviolet light to verify RNA integrity and absence of RNA degradation. In

line with our previous work (Willoughby et al., 2000, 2002, 2003, 2004), this procedure yielded un-degraded RNA, free of DNA and proteins as indicated by prominent 28s and 18s ribosomal RNA bands, as well as an OD_{260}/OD_{280} ratio of approximately 2.0 (data not shown). The RNA samples were stored at -70°C until later analyses.

Reverse Transcription and cDNA Synthesis

Two hundred μg of total skeletal muscle RNA were reverse transcribed to synthesize cDNA (Willoughby et al., 2000, 2002, 2003, 2004). A reverse transcription (RT) reaction mixture [2 mg of cellular RNA, 10x reverse transcription buffer (20 mM Tris-HCL, pH 8.3;50 mM KCl;2.5 mM $MgCL_2$; 100 mg of bovine serum albumin/ml), a dNTP mixture containing 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.8 mM MgCl2, 1.0 u/ml of rRNasin (ribonuclease inhibitor), 0.5 mg/ml of $oligo(dT)_{15}$ primer, and 25 u/mg of AMV reverse transcriptase enzyme (Promega, Madison, WI)] were incubated at 42°C for 60 min, heated to 95°C for 10 min, and then quick-chilled on ice. Starting template concentration were standardized by adjusting the RT reactions for all samples to 200 ng prior to PCR amplification (Willoughby et al., 2000, 2002, 2003, 2004).

Oligonucleotide Primers for Real Time-PCR

Antisense and sense oligonucleotide primer pairs were constructed using commercially available Primer Express software (Bio-Rad, Hercules, CA) from known mRNA sequences published in the GenBank nucleotide database (www.ncbi.nlm.nih.gov). Each target mRNA primer pair was optimized so that they amplified genes product between 150-300 base pairs in length.

Relative Control Standard Oligonucleotide Primers for Real Time-PCR

Due to its consideration as being a constitutively expressed "housekeeping gene," β-actin was used as an external reference standard for detecting the relative change in the quantity of mRNA using PCR. Previous research shows β-actin to not be differentially expressed in response to resistance exercise (Mahoney et al., 2004). For β-actin mRNA, we have previously shown these primers to amplify a PCR fragment of 152 bp (unpublished observations).

Real Time-PCR Amplification

For real time quantitative PCR, 200 ng of cDNA were added to each of the 100 ml PCR and specifically, each PCR reaction contained the following mixtures: [10x PCR buffer, 0.2 mM dNTP mixture, 1.0 mM of a cocktail containing both the sense and antisense RNA oligonucleotide primers for GAPDH and the target mRNAs (Ransom Hill Biosciences, Ramona, CA), 2 mM $MgCL_2$, 1.0 u/ml of Taq DNA polymerase (Sigma, St. Louis, MO), and nuclease-free dH_2O]. Each PCR reaction was amplified in real time with the IQ-ICycler real time PCR system (Bio Rad, Hercules, CA). The amplification profile involved 40 cycles with a denaturation step at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and extension at 72°C for 60 sec (Willoughby et al., 2000, 2002, 2003, 2004). To help control for differences in amplification efficiency during thermocycling, all PCR reactions were prepared from the same stock solution. The specificity of the PCR was demonstrated with an absolute negative control using a separate PCR reaction containing no cDNA. To assess reliability between amplifications, two separate PCR amplifications were performed for each sample to control for systemic differences between samples that could affect amplification efficiencies.

mRNA Quantitation

Real time quantitative PCR was run for 40 cycles and fluorescence measured after each cycle. The fluorescence resulted from the incorporation of the fluorophore SYBR green into the double-stranded DNA produced during the PCR reaction. A single gene product was confirmed using DNA melting curve analysis. The quantity of mRNA for each gene product was expressed relative to the expression of β-actin. ΔC_T values were used to compare basal gene expression between the HMB and placebo group, while $\Delta\Delta C_T$ was used to compare changes in gene expression with the treatments. The expression of each gene was then expressed using $2^{-\Delta\Delta C_T}$.

Gel Electrophoresis and Verification of Single Gene Products

After melting curve analysis, the DNA within each amplified PCR reaction was purified of contaminants such as primer dimers and amplification primers using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI). Aliquots (20 ml) of the purified PCR reaction mixtures were electrophoresed in 1.5% agarose gels in 1X Tris-Acetate-EDTA (TAE) buffer (Willoughby et al., 2000, 2004) to verify positive amplification of mRNA. The oligonucleotides within the gels were stained with ethidium bromide (present in the TAE buffer at 1 mg/ml) and illuminated with a UV transilluminator.

Myofibrillar Protein Quantitation

Total protein remaining from the total RNA isolation procedure was isolated with isopropanol, ethanol, and 0.3 M guanidine hydrochloride (Willoughby et al., 2000, 2002, 2003, 2004). Myofibrillar protein was further isolated with 1.0% SDS. Based on our previous work, myofibrillar protein content was then determined spectrophotometrically

based on the Bradford method at a wavelength of 595 nm and using bovine serum albumin as the standard. All assays were performed in duplicate and the average concentrations reported.

Statistical Analysis

A 2-way ANOVA [treatment groups (CON, HU) x muscle types (EDL, SOL)] was used to analyze the dependent variables. Significant differences in mean values for main effects or interactions were determined using a Newman-Keuls post hoc test. Effect size and significance test were also determined. A probability level of $p<0.05$ was set as the alpha level for all statistical procedures.

CHAPTER FOUR

Results

Myofibrillar Protein

The effects of CON and HU on myofibrillar protein content in SOL and EDL muscle were determined and results showed group means of 3.22 ± 1.16 mg/g and 2.16 mg ± .93 mg/g for CON SOL and HU SOL, respectively, and 5.08 ± 2.49 and 2.78 ± .88 mg/g, respectively, for CON EDL and HU EDL. These data represent reductions in myofibrillar content as a result of HU of 49% and 81%, respectively, for SOL and EDL. A significant main effect for group was observed ($p = 0.047$) in Figure 1 indicating that HU produced a significant reduction in myofibrillar protein content. These results impart supports our original hypotheses, representing a significant concomitant decrease in myofibrillar protein (physiological marker of atrophy) within HU EDL and SOL as compared to the CON.

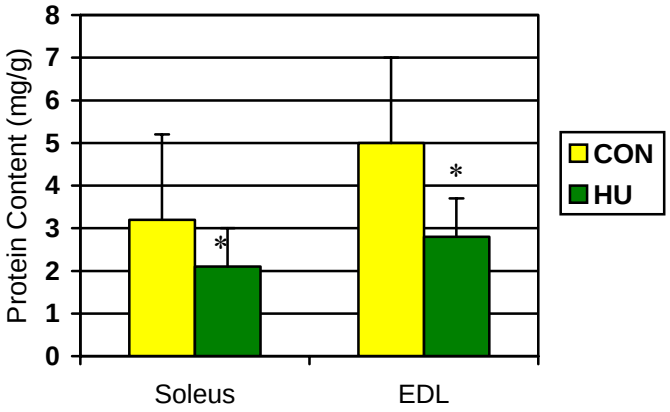

Figure 1. Myofibrillar protein (mg/g) concentrations for CON and HU groups in rat SOL and EDL muscle. Data are means ± SD, *N*=4. A significant main effect for groups was observed * ($p = 0.047$) and showed HU to produce greater reductions in myofibrillar protein than CON.

MHC Isoform mRNA Expression

Compared to the CON, more dramatic changes were observed within the HU group for MHC isoform mRNA expression as observed in Figure 2. In the HU SOL there appeared to be a transition toward a faster phenotype. There were significant main effects for muscle, and while the increases in MHC I were not significant ($p > 0.05$) (Fig.2A), there were significant muscle increases observed in MHC IIb ($p = 0.047$) (Fig.2C) and MHC IIx ($p = 0.05$) (Fig.2D) for HU SOL. Also, while not significant ($p = 0.068$), a trend was shown for MHC IIx expression differentially increases in SOL and differentially decreases in the EDL.

The HU EDL, however, revealed a transition toward a faster phenotype, which was demonstrated by decreases in MHC I and MHC IIx that likely can be explained by the large increases in MHC IIa. A significant interaction existed ($p = 0.029$) for MHC IIx mRNA indicating a difference between CON and HU and SOL and EDL. As for MHC IIa ($p = 0.05$) (Fig 2B) and IIb ($p = 0.047$), significant main effects for muscle were noted, but no interactions for either isoform were observed. In SOL, MHC IIa mRNA expression decreased with HU, whereas in MHC IIa mRNA was increased in EDL. Though our hypotheses were not formally supported in these results for MHC isoform mRNA expression, concomitant reductions and inverse increases of MHC isoform mRNA expressions of HU in varying muscle help give a better understanding of isoform transitions and adaptations during induced muscle atrophy.

Markers of Muscle Proteolysis: Calcium-Activated Protease System

In view of the significant myofibrillar protein loss with HU, it was of interest to determine if the myofibrillar disruption was due to possible up-regulation in calpain 1 and 2 since this proteolytic system is known to disrupt intact myofibrillar proteins.

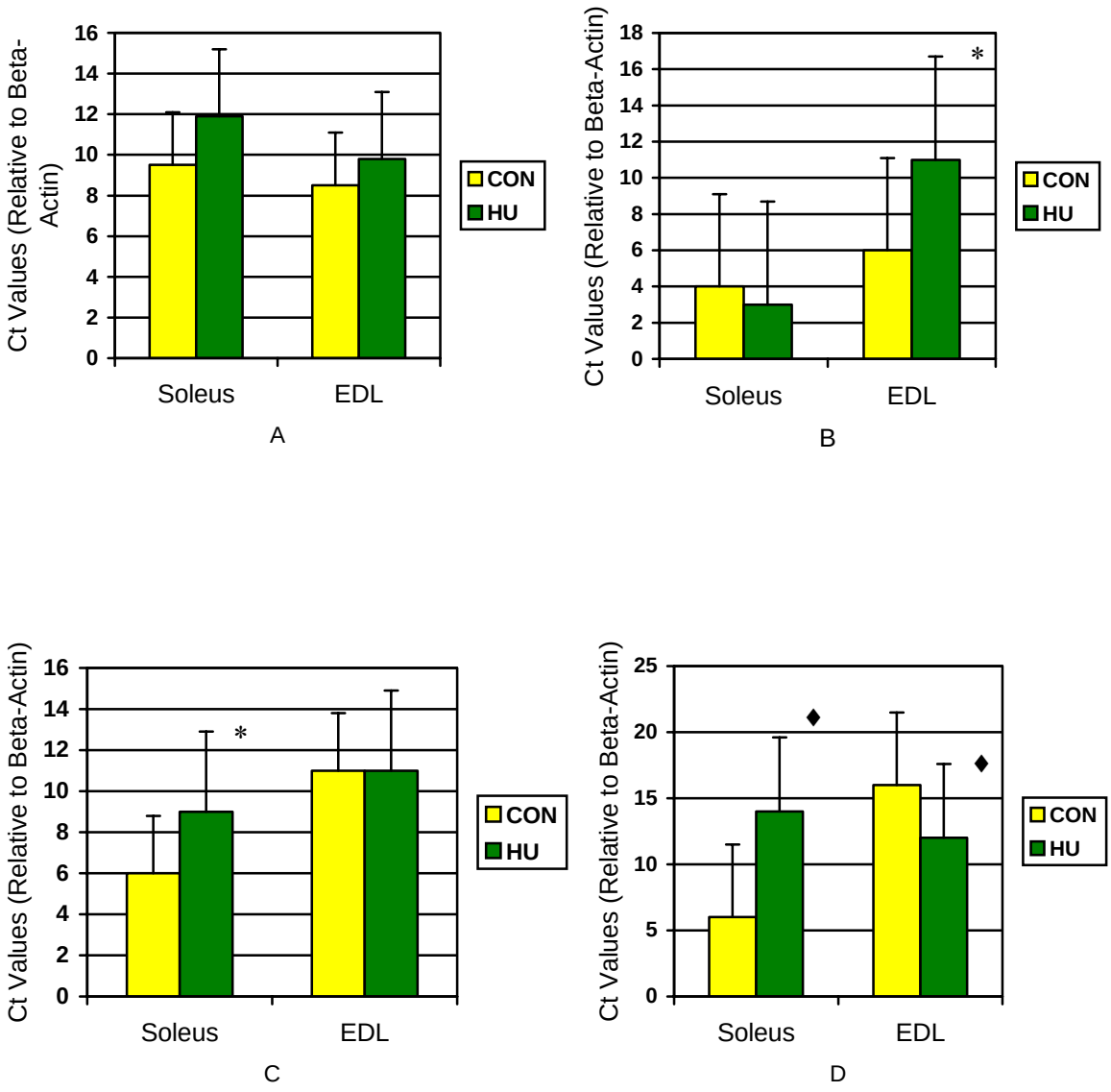

Figure 2. MHC mRNA isoform concentrations for CON and HU groups in rat SOL and EDL muscle. MHC mRNA isoforms were determined by RT-PCR. Data are means ± SD, *N*=4. A significant interaction existed for MHC IIx mRNA indicating a difference between groups and muscle ♦ ($p < 0.05$) (D). MHC IIa (B) and IIb (C) demonstrated a significant main effect for muscle * ($p < 0.05$), but no interactions for either isoform were observed.

The results demonstrated a pattern of increased calpain 1 and 2 mRNA expression in the HU EDL; however, a significant interaction was shown ($p = 0.003$) for calpain 1 mRNA only (Fig. 3A), indicating a difference between CON and HU and SOL and EDL. EDL. Specifically, calpain 1 mRNA expression was decreased in SOL but significantly increased in the EDL

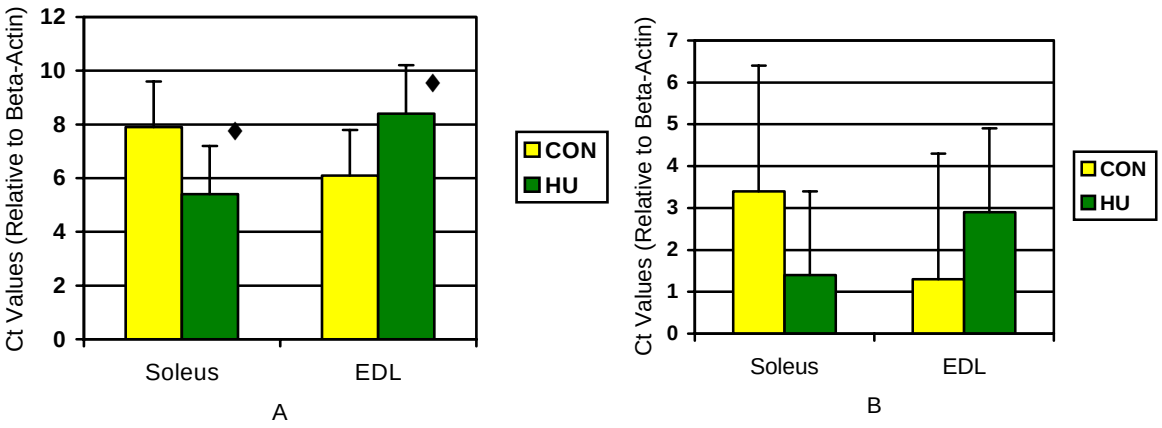

Figure 3. Calpain 1 (A) and 2 (B) enzyme concentrations for CON and HU groups in rat SOL and EDL muscle. Data are means ± SD, *N*=4. Results demonstrated a pattern of increased calpain 1 and 2 mRNA expression in the HU EDL; however, a significant interaction existed for calpain 1 mRNA indicating a difference between groups and muscle ♦ ($p < 0.05$).

Markers of Muscle Proteolysis: Ubiquitin System

In view of the decrease in myofibrillar protein content and up-regulation in calpain 1 and 2 mRNA expression, it was of interest to ascertain whether there was altered gene expression of various enzymes that regulate the ubiquitin proteolytic pathway because the ubiquitin pathway has been proposed to primarily degrade myofibrillar proteins disrupted by the calpain system. Results showed no significant interaction ($p > 0.05$) in E2 mRNA expression (Fig. 4A) indicating no difference between CON and HU and SOL and EDL. However, for E3 mRNA there was a significant

interaction ($p = 0.037$) indicating increased expression in HU EDL (Fig 4B). For atrogin-1 (Fig, 4C) and RF-1 (Fig.4D), there were no significant interactions ($p > 0.05$), suggesting no difference in the expression of these two genes in the CON and HU groups and also in SOL and EDL. Collectively these result do not formally support the hypotheses, however there seems to be strong correlative support for many of the proteolytic relations, which leads us to believe with a larger sample size significant trends may be found. In the HU EDL these results suggest that increases in the calcium activated protease system by calpain 1 and 2 (particularly calpain 2) is correlated to increases in the activity (up-regulation in gene expression of E2, E3, RF-1 and Atrogin-1) of the ubiquitin proteolytic pathway. Overall increases in the calpain system and increases in the ubiquitin system likely initiate proteolysis and subsequent decreases in myofibrillar protein. This decrease in myofibrillar protein (and overall proteolysis) may play a role in preferentially up-regulating MHC 2A gene expression. In soleus however, with the exception of E3, HU does not seem to activate the calpain and ubiquitin system as in the EDL. These results bring attention to the difference in how SOL and EDL (slow and fast) muscle adapt and respond molecularly under atrophic conditions.

Effect Size

To quantify the size and significance of the differences witness between two groups, and therefore a more powerful measure of the significance of the differences, we quantified the effect size for Group (CON, SOL), Muscle (HU, SOL) and overall Interaction Effect. The results for Group Effect demonstrated a significant interaction for both calpain 1 ($p <. 002$), and 2 ($p <. 003$) lending support to our previous finding between groups for calpain activity. Of particular interest were the results for RF-1 ($p < .000$). A significant interaction was noted. This lends one to believe that because of a limited

sample size this interaction may have been underestimated. Several other group effects were noted, even though not significant, MHC IIa ($p < .054$) and IIx ($p < .061$).

Muscle Effect results also noted a significant interaction for calpain 2 ($p < .004$), and though not significant, an effect of ($p < .057$) for calpain 1 supporting previous findings between muscle. And similar to the results for group effect a significant interaction for RF-1 ($p < .003$) was found for muscle. E3 ubiquitin ligase also displayed a significant interaction between muscle groups ($p < .019$) 4

As for Interaction Effect, MHC I ($p < .009$) and Atrogin-1 ($p <. 001$) were the only variables found to have significant interactions. However, due to the low sample size it was not overly surprising that more interactions were not found.

Correlations

With regards to the data presented herein, collectively, there were several significant correlations between both group and muscle. In CON SOL, MHC I was significantly correlated to E3 ligase ($r = 0.956$, $p = 0.044$), MHC IIb significantly correlated to E2 ($r = -0.991$, $p = 0.009$) and E3 ($r = -0.986$, $p = 0.014$), and MHC IIx significantly correlated to atrogin-1 ($r = -0.994$, $p = 0.006$). In CON EDL, MHC IIa was significantly correlated to RF1 ($r = -0.982$, $p = 0.018$) and calpain 2 ($r = -0.995$, $p = 0.005$). In HU SOL, E2 was significantly correlated to atrogin-1 ($r = 0.996$, $p = 0.004$), whereas in HU EDL, MHC I was significantly correlated with RF1 ($r = 0.983$, $p = 0.017$), MHC IIa significantly correlated to E3 ($r = 0.961$, $p = 0.039$) and calpain 2 ($r = 0.985$, $p = 0.015$), and calpain 2 significantly correlated to E3 ($r = 0.955$, $p = 0.045$) and atrogin-1 ($r = 0.957$, $p = 0.043$)

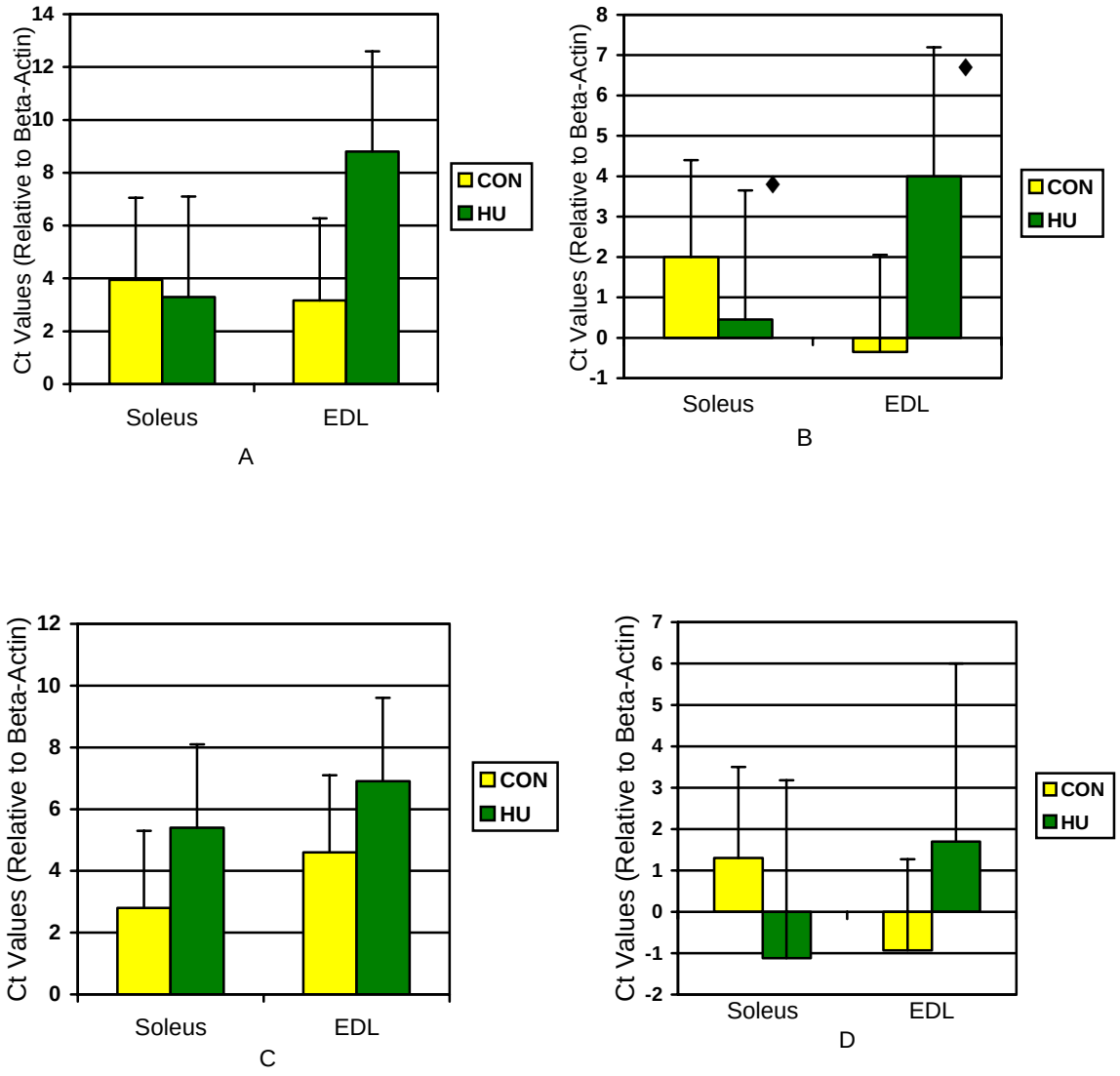

Figure 4. E2 (A), E3 (B), Atrogin-1(C) and Ring Finger-1 (D) concentrations for CON and HU groups in rat SOL and EDL muscle. Data are means ± SD, *N*=4. Results showed no significant interaction ($p > 0.05$) in E2 mRNA expression indicating no difference between groups and muscle. However, a significant interaction for muscle ♦ in E3 mRNA was observed. There were no significant interactions ($p > 0.05$) for Atrogin-1 and RF-1, suggesting no difference in the expression of the genes in either of the two groups and muscle types.

CHAPTER FIVE

Discussion

Myofibrillar Protein

Reductions in myofibrillar content are associated with molecular factors dictating a muscle fiber's capacity for adaptation related to various forms of physiological stress. The myofibrillar protein content typically varies between fast- and slow-twitch muscle fibers (Baldwin et al., 2002). This is evident in the difference exhibited between the different muscles and groups within our study. A significant dichotomy seemed to exist between the SOL and EDL in each group. Similar to current research in disuse models, a significant reduction in myofibrillar content was witnessed in the HU group. A significant main effect for groups was observed indicating that the HU produced a significant reduction in myofibrillar protein content. Also of particular interest were the percent reductions in myofibrillar protein content with in the HU group. The SOL underwent a 49% decrease while the EDL decreased 81%. The difference in myofibrillar protein reductions between muscle types can be theoretically explained on a multitude of levels; changes in the steady-state levels of genes encoding mRNA induced by a given stimulus commonly are reflections of variations in the rate of transcription of the gene encoding the mRNA (Haddad et al., 2003). Also, increases in the rate and/or magnitude of muscle proteolytic activity can play a role in affecting the accretion of myofibrillar protein (Huang et al., 1998; Solomon et al., 1996; Taillandier et al., 1996). Unless specific information is available concerning involvement of transcriptional processes in the regulation of a given gene, change in mRNA content for that gene are typically

regulated by pretranslational (including transcriptional) processes. Control mechanisms that alter the rate of protein synthesis and degradation at constant mRNA levels are typically associated with translational regulation (Bodine et al., 2001). Thus, from the results presented it may be assumed that both of these regulatory processes may be occurring; however, but further analysis and a larger sample size is required before more conclusions can be made.

MHC Isoform mRNA

As mentioned, marked reductions in both total mRNA concentration and content have been mechanisms suggested to regulate the capacity for protein translation. The rapid atrophy phase in our HU model of muscle inactivity demonstrates the existence of well-coordinated sets of molecular events that can lead to a smaller and weaker muscle. Also our findings make it apparent that the MHC proteins, in particular the slower isoforms were preferentially atrophied. Numerous studies on rats have also confirmed that mechanical unloading of the hindlimbs induces slow-to-fast transition (Pette et al., 1997; Thomason et al., 1990). Besides alterations in contractile properties, these transitions are best exhibited by changes in the pattern of MHC isoforms. Collectively with our findings, as well as complementing previous research (Caiozzo et al., 1998; Cros et al., 1999; Fauteck et al., 1995), our collective findings demonstrate a clear pattern supporting an increase in MHC IIa, IIx, and IIb mRNA expression with HU.

The present study demonstrates typical slow-to-fast transition in MHC isoform expression in response to unloading. We conclude these findings may be due to the fact that two processes may be occurring in parallel: 1) decreased gene expression and 2) increased muscle proteolysis. These issues regarding isoform transition, gene expression,

and atrophy are supported with reference to the trends and significance of the MHC IIa, IIx, and IIb mRNA in the HU and CON groups.

Because of the two groups and differing phenotype of the muscles assessed, the results from this study are particularly interesting despite the small population size (n = 4). Especially, in regards to the MHC isoform mRNA that are up-regulated in the HU muscle, and the apparent increase in proteolytic activity.

The most noteworthy finding between groups was a dramatic change within the HU for MHC isoform mRNA expression. Surprisingly, the HU SOL demonstrated a transition toward a slower phenotype with increases in MHC I; however, also witnessed were concomitant increase in IIb and IIx activity. These results though slightly divergent from recent studies (using similar methods of inactivity) found similar results. Haddad and colleagues (2003), using a spinal cord isolation model, suggested there was a net decrease in total MHC mRNA expression and that the loss was selective to slower MHC isoforms (I and IIa). However, other factors operating at the transcriptional and pretranslational levels most likely impacted the enhanced expression of the type IIx MCH isoform, which has typically been noted as the major MHC isoform in muscle after extended periods of unweighting. Coinciding with the recent literature, the present findings in the HU EDL demonstrated a transition toward a faster phenotype, which was noted by decreases in MHC I and MHC IIx, and more particularly with increases in MHC IIa. Even with significant increases MHC I expression within the HU SOL, what remains evident in the present findings is that reductions in myofibrillar protein are not due to general reduction of transcriptional activity of MHC isoforms in HU muscle. The present study also demonstrated a significant increase in mRNA levels of the fast–type isoforms

in both the SOL and EDL, which is consistent with the redirection of gene expression to a faster phenotype.

A significant interaction was also noted for MHC I mRNA, signifying a difference between and CON and HU and SOL and EDL. This interaction helps validate the previous-stated inferences, in that even with concomitant increases witnessed in HU MHC I, differences in expression between the atrophying and normalized muscle were present. Also noted in the results was that MHC IIa and IIb exhibited a significant main effect only between muscles and not between groups. As for MHC IIx, expression was shown to differentially increase in the soleus and differentially decrease in the EDL.

Integrated Systems of Muscle Proteolysis: Calcium-Activated Protease (Calpain) System

As reviewed earlier and demonstrated in our results, the protein degradation processes play a significant role in the turnover of muscle protein, and any alteration in the functional level of these pathways can significantly affect the balance of skeletal muscle myofibrillar protein (Haddad et al., 2003). Evidence supporting the role the ubiquitin proteasome pathway plays in myofibrillar protein degradation is increasing. This evidence also suggests that individual myofibril proteins (e.g., myosin and actin) are not readily degraded by the ubiquitin proteasome (Huang et al., 1998; Solomon et al., 1996; Taillandier et al., 1996). Thus, the approach of the present study was modeled after a similar study by Haddad and colleagues (2003), which proposed several proteolytic systems operating in synergy with the ubiquitin proteasome axis to bring about degradation of the complex structure of the muscles, such as the myofibrils.
As identified in recent research, the bulk of myofibrillar proteolysis resulting from muscle disuse involves the ubiquitin-proteasome pathway, yet it is also known that the

ubiquitin-proteasome system cannot degrade intact myofibrils (Attaix 1998, Solomon et al., 1998, Taillandier et al., 1996). Thus our research focused on several theories, which suggests that myofibril proteins must be released from the sarcomere before they can be degraded by the proteasomes. Calpains seem to be a likely candidate, which actively help facilitate this role by mediating the disassembly. Previous studies have shown calpain activation during disuse, but results have been mixed and controversial (Tischler et al. 1990, Taillandier et al. 1996, Ikemoto et al. 2001, Tidball & Spencer, 2002). Taking into consideration the significant myofibrillar protein loss with HU, it was of interest to determine if the myofibrillar disruption was due to possible up-regulation in calpain 1 and 2 since this proteolytic system is know to disrupt intact myofibrillar proteins.

The results demonstrated a clear pattern of increased calpain 1 and 2 mRNA expression in the HU EDL. A significant interaction was also noted for calpain 1 mRNA indicating a difference between CON and HU and SOL and EDL. Also of specific interest was the decreased expression of both calpain 1 and 2 in the soleus with marked increases in the EDL. These findings potentially suggest a more significant pattern of proteolytic activity within fast muscle with disuse, suggesting a possible difference in molecular patterns of remodeling between muscle and fiber types.

With decreases in myofibrillar protein content and an up-regulation in calpain 1 and 2 mRNA expression, it was of interest to ascertain whether there was altered gene expression of various enzymes that regulate the ubiquitin proteolytic pathway because the ubiquitin pathway has been proposed to primarily degrade disrupted myofibrillar proteins. Our finding noted a concomitant relationship with E3, calpain 1, and calpain 2

enzyme systems, which demonstrated significant interactions between the CON and HU groups and muscle types, as well as decreased activity in SOL and increased activity in EDL.

Integrated Systems of Muscle Proteolysis: Ubiquitin Proteolytic Pathway

Because muscle atrophy seems to be a direct result of myofibrillar protein loss, several well-defined ubiquitin protein ligases, specifically E2 and E3, were analyzed in the present study. Two other genes responsible for encoding ubiquitin protein ligase (E3) were also identified. One of the genes, RF1, a RING finger protein that interacts with titin, a large myofibrillar protein that has numerous functions was assessed, along with a similar protein Atrogin-1 (belonging to the F-box family of proteins, which forms a subunit of E3 ligase) (Price et al., 2003). Due to the complexity of the pool of muscle proteins involved, it is likely that multiple ubiquitin conjugation enzymes are involved in the accelerated atrophic process associated with HU. Understanding the difficulty of discerning all of the potential proteolytic contributors and their tedious nature, our study limited itself to the purported major contributors.

As for E2, atrogin-1, and RF-1, no significant differences were observed between CON and HU groups or muscle types. This data is somewhat surprising in the fact that increases in E3 typically correlate with upregulation of the atrogin-1. This typical correlation is intriguing in that E3 enzyme contains a specific sequence to suggest that it may be preferentially located in the nucleus (Bodine et al., 2001). Though atrogin-1 showed no significance, our relatively small sample size raises the question if a larger sample had been used might a significant difference exist? Regardless, E3 was significantly upregulated leading to the assumption that other concomitant mechanisms of

the polyubiquitin ligase system play a role in the HU atrophy process. Because of the upregulation seen with E3, it is possible that E3 and other ligases within this family target the degradation of transcription factors, inversely negatively influencing the transcription of key sarcomeric genes. Other potential theories surrounding housekeeping genes have also been questioned. Further analyses thus are needed to delineate the true nature of this multifaceted process.

Integration Between Calpain and Ubiquitin Systems of Muscle Proteolysis

Collectively, in EDL these results suggest that increases in the calcium activated protease system by calpain 1 and 2 (particularly calpain 2) is correlated to increases in the activity (up-regulation in gene expression of E2, E3, RF1 and AT1) of the ubiquitin proteolytic pathway. Overall increases in the calpain system and increases in the ubiquitin system likely initiate proteolysis and subsequent decreases in myofibrillar protein. This decrease in myofibrillar protein (and overall proteolysis) may play a role in preferentially up-regulating MHC IIa gene expression. In soleus, however, with the exception of E3, HU does not seem to activate the calpain and ubiquitin system as in the EDL.

Effect Size, Error, and Limitation

Effect size quantifies the size of the difference between two groups, and therefore may be said to be a true measure of the significance of the difference. For Group Effect significance interactions were found calpain 1 and 2, RF-1, and though not significant 2A and 2X. As for Muscle Effect significant interactions were found for E3, RF-1, and Calpain 2, and though not significant Calpain1. For Interaction Effect significance was

found for MHC 1 and At-1. The results for Effect Size help validate and add strength our findings for these specific dependent variables between groups and muscle.

Collectively our findings suggest, increases in the calcium activated protease system by calpain 1 and 2(particularly calpain 2) in the EDL correlating to increases in the activity (up-regulation in gene expression of E2, E3, RF1 and AT1) of the ubiquitin proteolytic pathway. Overall increases in the calpain system and increases in the ubiquitin system likely initiate proteolysis and subsequent decreases in myofibrillar protein. This decrease in myofibrillar protein (and overall proteolysis) may play a role in preferentially up-regulating MHC 2A gene expression. In soleus, however, with the exception of E3 HU does not seem to activate the calpain and ubiquitin system as in the EDL.

In regards to the limitations of this study, most noteworthy the smaller than desired sample size, and handling, preparation, and monitoring procedures of Texas A&M University, we feel relatively confident in the relationships and significance witnessed. In regards to the possibility of committing a type II error, which is typically associated with a smaller sample size (i.e. declare that there is no significant effect) we feel we have addressed sufficiently in our statistical model any relevant question in regards to the power of the results. Therefore concluding our findings as relevant and worthy of adding strength to the current literature surrounding muscle atrophy.

Conclusion

The appearance of atypical MHC isoform mRNA expression is difficult to explain, but as stated earlier may be due to the fact that transcriptional and translational factors affecting transitions in MHC isoform expression and also factors associated with

the calpain and ubiquitin systems of proteolysis are likely occurring simultaneously. What is obvious is that HU has an immediate affect on myofibrillar protein content and normally ordered expression of MHC isoforms. In the present investigation, the HU muscle contained less overall myofibrillar protein, and moreover, the results on discrepancies between MHC isoforms at the transcript and protein level suggests that unloading possibly disrupts the regulatory processes of transcription and translation.

As for enzymatic activity correlating with theses processes, the present study demonstrated that both the calcium activated proteolytic calpain system and ATP-ubiquitin-dependent proteolytic pathway appear to be involved in the atrophy of HU muscle. The possible connection between these proteolytic pathways though, still needs further elucidation. Although the ubiquitin proteolytic process might degrade the major contractile proteins, the ubiquinated enzymes, specifically atrogin-1 and RF-1 remain to be completely understood. Thus, further experiments must be designed to demonstrate whether or not the increased mRNA levels for some of these potential ubiquitin-activating enzymes result in elevated proteolytic activity.

Finally, it is important to realize that there were several restrictions to the present study, the potentially most influential being the small sample size (n = 4). Nonetheless, the present results are promising and further adds to the depth of molecular research surrounding disuse muscle atrophy.

BIBLIOGRAPHY

Allen, D. L., R. R. Roy, et al. (1999). "Myonuclear domains in muscle adaptation and disease." *Muscle Nerve* 22(10): 1350-60.

Attaix D., Aurousseau E., Combaret L., Kee A., Larbaud D., Ralliere C., Souweine B., Taillandier D., Tilignac T (1998). "Ubiquitin-proteasome-dependent proteolysis in skeletal muscle." *Reprod Nutr Dev* 38(2):153-65.

Baldwin, K. M. and F. Haddad (2001). "Effects of different activity and inactivity paradigms on myosin heavy chain gene expression in striated muscle." *J Appl Physiol* 90(1): 345-57.

Baldwin, K. M. and F. Haddad (2002). "Skeletal muscle plasticity: cellular and molecular responses to altered physical activity paradigms." *Am J Phys Med Rehabil* 81(11 Suppl): S40-51.

Bey, L., N. Akunuri, et al. (2003). "Patterns of global gene expression in rat skeletal muscle during unloading and low-intensity ambulatory activity." *Physiol Genomics* 13(2): 157-67.

Bishop, D. L. and R. L. Milton (1997). "The effects of denervation location on fiber type mix in self-reinnervated mouse soleus muscles." *Exp Neurol* 147(1): 151-8.

Bodine, S. C., E. Latres, et al. (2001). "Identification of ubiquitin ligases required for skeletal muscle atrophy." *Science* 294(5547): 1704-8.

Bodine, S. C., T. N. Stitt, et al. (2001). "Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo." *Nat Cell Biol* 3(11): 1014-9.

Caiozzo, V. J., M. J. Baker, et al. (1994). "Effect of spaceflight on skeletal muscle: mechanical properties and myosin isoform content of a slow muscle." *J Appl Physiol* 76(4): 1764-73.

Caiozzo V.J., Baker M.J., Baldwin K.M., (1998). "Novel transitions in MHC isoforms: separate and combined effects of thyroid hormone and mechanical unloading." *J Appl Physiol* 85(6):2237-48.

Cantley, L. C. (2002). "The phosphoinositide 3-kinase pathway." *Science* 296(5573): 1655-7.

Cros N., Muller J., Bouju S., Pietu G., Jacquet C., Leger J. J., Marini J. F., Dechesne C. A. (1999). "Upregulation of M-creatine kinase and glyceraldehyde3-phosphate dehydrogenase: two markers of muscle disuse." *Am J Physiol* 276(2 Pt 2): R308-16.

Cross, D. A., D. R. Alessi, et al. (1995). "Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B." *Nature* 378(6559): 785-9.

Diffee, G. M., S. McCue, et al. (1993). "Interaction of various mechanical activity models in regulation of myosin heavy chain isoform expression." *J Appl Physiol* 74(5): 2517-22.

Fauteck, S. P., Kandarian S. C. (1995). "Sensitive detection of myosin heavy chain composition in skeletal muscle under different loading conditions." *Am J Physiol* 268(2 Pt 1):C419-24

Furuno, K. and A. L. Goldberg (1986). "The activation of protein degradation in muscle by Ca2+ or muscle injury does not involve a lysosomal mechanism." *Biochem J* 237(3): 859-64.

Giger, J. M., F. Haddad, et al. (2000). "In vivo regulation of the beta-myosin heavy chain gene in soleus muscle of suspended and weight-bearing rats." *Am J Physiol Cell Physiol* 278(6): C1153-61.

Glass, D. J. (2003). "Molecular mechanisms modulating muscle mass." *Trends Mol Med* 9(8): 344-50.

Gomes, M. D., S. H. Lecker, et al. (2001). "Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy." *Proc Natl Acad Sci U S A* 98(25): 14440-5.

Haddad, F., C. Arnold, et al. (1997). "Interaction of thyroid state and denervation on skeletal myosin heavy chain expression." *Muscle Nerve* 20(12): 1487-96.

Haddad, F., A. X. Qin, et al. (1998). "Effects of isometric training on skeletal myosin heavy chain expression." *J Appl Physiol* 84(6): 2036-41.

Haddad, F., R. R. Roy, et al. (2003). "Atrophy responses to muscle inactivity. II. Molecular markers of protein deficits." *J Appl Physiol* 95(2): 791-802.

Hara, K., K. Yonezawa, et al. (1998). "Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism." *J Biol Chem* 273(23): 14484-94.

Hicke, L. (1999). "Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels." *Trends Cell Biol* 9(3): 107-12.

Hicke, L. and R. Dunn (2003). "Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins." *Annu Rev Cell Dev Biol* 19: 141-72.

Huang, J. and N. E. Forsberg (1998). "Role of calpain in skeletal-muscle protein degradation." *Proc Natl Acad Sci U S A* 95(21): 12100-5.

Ikemoto, M., T. Nikawa, et al. (2001). "Space shuttle flight (STS-90) enhances degradation of rat myosin heavy chain in association with activation of ubiquitin-proteasome pathway." *Faseb J* 15(7): 1279-81.

Jackman, R. W. and S. C. Kandarian (2004). "The molecular basis of skeletal muscle atrophy." *Am J Physiol Cell Physiol* 287(4): C834-43.

Jagoe, R. T. and A. L. Goldberg (2001). "What do we really know about the ubiquitin-proteasome pathway in muscle atrophy?" *Curr Opin Clin Nutr Metab Care* 4(3): 183-90.

Jagoe, R. T., S. H. Lecker, et al. (2002). "Patterns of gene expression in atrophying skeletal muscles: response to food deprivation." *Faseb J* 16(13): 1697-712.

Jagoe, R. T., C. P. Redfern, et al. (2002). "Skeletal muscle mRNA levels for cathepsin B, but not components of the ubiquitin-proteasome pathway, are increased in patients with lung cancer referred for thoracotomy." *Clin Sci (Lond)* 102(3): 353-61.

Jones, S. W., R. J. Hill, et al. (2004). "Disuse atrophy and exercise rehabilitation in humans profoundly affects the expression of genes associated with the regulation of skeletal muscle mass." *Faseb J* 18(9): 1025-7.

Knotts, S., H. Rindt, et al. (1995). "Position independent expression and developmental regulation is directed by the beta myosin heavy chain gene's 5' upstream region in transgenic mice." *Nucleic Acids Res* 23(16): 3301-9.

Lecker, S. H., V. Solomon, et al. (1999). "Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states." *J Nutr* 129(1S Suppl): 227S-237S.

Lecker, S. H., V. Solomon, et al. (1999). "Ubiquitin conjugation by the N-end rule pathway and mRNAs for its components increase in muscles of diabetic rats." *J Clin Invest* 104(10): 1411-20.

Mahoney, D. J., K. Carey, et al. (2004). "Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise." *Physiol Genomics* 18(2): 226-31.

Mayer, R.J. (2004). "The meteoric rise of regulated intracellular proteolysis." *Nat Rev Mol Cell Biol.* 1(2): 145-8.

McKinnell, I. W. and M. A. Rudnicki (2004). "Molecular mechanisms of muscle atrophy." *Cell* 119(7): 907-10.

Michael, K. (2000). "Relationship of skeletal muscle atrophy to functional status: a systematic research review." *Biol Res Nurs* 2(2): 117-31.

Nave, B. T., M. Ouwens, et al. (1999). "Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation." *Biochem J* 344 Pt 2: 427-31.

Nicholson, K. M. and N. G. Anderson (2002). "The protein kinase B/Akt signalling pathway in human malignancy." *Cell Signal* 14(5): 381-95.

Pette, D (1997). "Functional and biochemical adaptations to low-frequency stimulation: possible applications to microgravity." *Int J Sports Med* 18(4):S302-4.

Price, M. G., D. R. Caprette, et al. (1994). "Different temporal patterns of expression result in the same type, amount, and distribution of filamin (ABP) in cardiac and skeletal myofibrils." *Cell Motil Cytoskeleton* 27(3): 248-61.

Rommel, C., B. A. Clarke, et al. (1999). "Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt." *Science* 286(5445): 1738-41.

Roy, R. R., K. M. Baldwin, et al. (1996). "Response of the neuromuscular unit to spaceflight: what has been learned from the rat model." *Exerc Sport Sci Rev* 24: 399-425.

Sandri, M., C. Sandri, et al. (2004). "Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy." *Cell* 117(3): 399-412.

Solomon V., Goldberg A. L. (1996). "Importance of the ATP-ubiquitin-proteasome pathway in the degradation of soluble and myofibrillar proteins in rabbit muscle extracts." *J Biol Chem* 271(43): 26690-7

Sorimachi, H. (2000). "[Structure and function of calpain and its homologues]." *Seikagaku* 72(11): 1297-315.

Stevenson, E. J., P. G. Giresi, et al. (2003). "Global analysis of gene expression patterns during disuse atrophy in rat skeletal muscle." *J Physiol* 551(Pt 1): 33-48.

Stitt, T. N., D. Drujan, et al. (2004). "The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors." *Mol Cell* 14(3): 395-403.

Swoap, S. J., F. Haddad, et al. (1994). "Interaction of thyroid hormone and functional overload on skeletal muscle isomyosin expression." *J Appl Physiol* 77(2): 621-9.

Taillandier, D., E. Aurousseau, et al. (1996). "Coordinate activation of lysosomal, Ca 2+-activated and ATP-ubiquitin-dependent proteinases in the unweighted rat soleus muscle." *Biochem J* 316 (Pt 1): 65-72.

Talmadge, R. J. (2000). "Myosin heavy chain isoform expression following reduced neuromuscular activity: potential regulatory mechanisms." *Muscle Nerve* **23**(5): 661-79.

Thomason, D. B. and F. W. Booth (1990). "Atrophy of the soleus muscle by hindlimb unweighting." *J Appl Physiol* 68(1): 1-12.

Thomason, D. B., R. E. Herrick, et al. (1987). "Activity influences on soleus muscle myosin during rodent hindlimb suspension." *J Appl Physiol* 63(1): 138-44.

Thomason, D. B., R. E. Herrick, et al. (1987). "Time course of soleus muscle myosin expression during hindlimb suspension and recovery." *J Appl Physiol* 63(1): 130-7.

Thompson W. J., Condon K, Astrow S.H. (1990). "The origin and selective innervation of early muscle fiber types in the rat." *J Neurobiol* 21(1):212-22.

Tidball, J. G. and M. J. Spencer (2002). "Expression of a calpastatin transgene slows muscle wasting and obviates changes in myosin isoform expression during murine muscle disuse." *J Physiol* 545(Pt 3): 819-28.

Tischler, M. E., S. Rosenberg, et al. (1990). "Different mechanisms of increased proteolysis in atrophy induced by denervation or unweighting of rat soleus muscle." *Metabolism* 39(7): 756-63.

Willoughby, D. S. (2004). "Effects of heavy resistance training on myostatin mRNA and protein expression." *Med Sci Sports Exerc* 36(4): 574-82.

Willoughby, D. S. and M. J. Nelson (2002). "Myosin heavy-chain mRNA expression after a single session of heavy-resistance exercise." *Med Sci Sports Exerc* 34(8): 1262-9.

Willoughby, D. S., J. W. Priest, et al. (2000). "Myosin heavy chain isoform and ubiquitin protease mRNA expression after passive leg cycling in persons with spinal cord injury." *Arch Phys Med Rehabil* **81**(2): 157-63.

Willoughby, D. S., M. Taylor, et al. (2003). "Glucocorticoid receptor and ubiquitin expression after repeated eccentric exercise." *Med Sci Sports Exerc* **35**(12): 2023-31.