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Suppression of Calcineurin Signaling and PGC-1α Expression During the Chronic Skeletal Muscle Atrophy Associated with Diabetes Mellitus: Implications for Muscle Function

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Tiffany K. Roberts-Wilson

B.S., The University of Georgia, 2004

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Abstract

Suppression of Calcineurin Signaling and PGC-1α Expression During the Chronic Skeletal Muscle Atrophy Associated with Diabetes Mellitus: Implications for Muscle Funtion

By Tiffany K. Roberts-Wilson

Skeletal muscle atrophy frequently indicates a poor prognosis for patients with systemic pathologies, including diabetes mellitus (DM). These patients demonstrate reduced muscle size as well as decreased strength and endurance, indicating that a link exists between muscle size and functional capacity in these conditions. PGC-1α is a transcriptional coactivator that controls energy homeostasis through regulation of glucose and oxidative metabolism. Both PGC-1α expression and oxidative capacity are decreased in skeletal muscle of diabetic patients and animals undergoing atrophy, suggesting that PGC-1α participates in the regulation of muscle mass. This dissertation focuses on elucidating the mechanisms that regulate PGC-1α expression *in vivo* in a model of DM as well as the potential physiological effects of PGC-1α downregulation.

Our work reveals that suppressed calcineurin (Cn) signaling contributes to decreased PGC-1α expression in chronic DM rat skeletal muscle and this may result in a muscle fiber-type switch from an oxidative phenotype to a more glycolytic phenotype. Specifically, we demonstrate that expression of Cn, a calcium-dependent phosphatase, was decreased in the skeletal muscle of rats with streptozotocin-induced diabetes (STZ-DM) for 21 days. PGC-1α expression is regulated by two Cn substrates, MEF2 and NFATc, both of which showed significantly reduced activity in the same muscles. MEF2 and NFATc activity as well as PGC-1α expression were also decreased in muscles of $CnA\alpha^{-/-}$ and $CnA\beta^{-/-}$ mice without diabetes indicating that decreased Cn signaling, rather than changes in other signaling pathways, were responsible for decreased PGC-1α expression. These findings demonstrate that Cn activity is a major determinant of PGC-1α expression in skeletal muscle during diabetes and possibly other conditions associated with loss of muscle mass. We also found that STZ-induced atrophy is associated with fiber type switching from MHCI and the oxidative phenotype towards MHCII and the glycolytic phenotype along the MHC gene expression continuum. Furthermore, there was a preferential decrease in the cross-sectional area of MHCII fibers in the skeletal muscles of STZ-DM animals. These results indicate that the chronic muscle atrophy associated with DM predominantly affects MHCII fibers and that switching from MHCI to MHCII may serve as a mechanism to sustain atrophy. A more thorough understanding of the signaling pathways that regulate protein degradation in different fiber types will be important for the development of therapies to treat the chronic atrophy associated with DM and other systemic diseases.

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I started out life on a three-square-mile island in the Bahamas where the first several years of my educational career were spent in a single room school-house. It has been a long, exciting, and difficult journey from those humble beginnings to the successful completion of this doctorate; a journey that I could never have made without the help and support of many, many people. I have not enough paper, words, or memory to thank all of them here, but there are several who have been most instrumental that I want to mention.

Firstly, I want to acknowledge my family. They have made great sacrifices to allow me the opportunities that I have had. I will never forget their dedication and support and I am truly grateful to have their love. In this I am now privileged to include my husband, Scott. Without his unfailing and unconditional love and support over the past seven years I would not have had the fortitude to complete this work. Scott has held me up when I would have fallen and pushed me to new heights of achievement that I would never have thought to reach for on my own. It may be cliché, but he is truly the wind beneath my wings.

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the work load threatened to overwhelm me. Lastly, all of my “horsey” friends have been essential to my sanity. Horses and horseback-riding and all the friends that I have made through horses have been my group therapy, keeping me grounded through it all.

Certainly, I could not have done any of this work or earned this degree without my advisor, Russ Price. Russ not only served as my supervisor but also encouraged and challenged me throughout my graduate school career. He guided me through my research, never accepting less than my best efforts. Russ and my committee members always had the highest expectations of me and, though at times I perceived them to be unreasonable, they have made me not only a better scientist, but a better professional and a better person as well.

I render my deepest and sincerest thanks and gratitude to all of the people mentioned here as well as to all those who have touched my life, inspired me to always do my best, told me that I can do anything… and believed it.

TABLE OF CONTENTS

FIGURES

TABLES

ABBREVIATIONS

Atrogin-1…….AT-1 and/or MAFbx

Calcium……Ca^{2+}

Calcium-dependent Kinase…….…..CaMK

Calcineurin……..Cn

Calcineurin A (catalytic subunit)……...CnA

Calcineurin B (regulatory subunit)……CnB

cAMP Response Element Binding Portein……CREB

Control…….CTL

Cross Sectional Area……...CSA

Diabetes Mellitus…….DM

Dihydropyridine Receptor α1 subunit……DHPRα1s

Gastrocnemius……...……GAST

Glycogen Synthase…….GS

Glycogen Synthase Kinase 3 β……..……GSK3β

Histone Deacetylase……...HDAC

Modulatory Calcineurin Inhibitory Protein 1.4…….MCIP1.4

Muscle-specific Ring Finger 1……MuRF-1

Myocyte Enhancement Factor 2…….MEF2

Myogenic Regulatory Factor 4……MRF4

Myosin Heavy Chain……..MHC

Nuclear Factor of Activated T-cells……...NFAT

Peroxisome Proliferator Activated Receptor γ – coactivator 1 α…..PGC-1α

Protein Kinase A…….PKA

Force…….…….…….P_0

Soleus…….SOL

Streptozotocin…….STZ

Transducer of Regulated CREB……..TORC

Ubiquitin…….Ub

CHAPTER 1

INTRODUCTION

Many common pathologic conditions, including diabetes mellitus (DM), are frequently accompanied by skeletal muscle atrophy, which is often the primary prognostic indicator of mortality in these patients. DM can result from insulin deficiency or resistance and is characterized by a variety of metabolic disturbances including reduced cellular glucose uptake, decreased fatty acid oxidation, and increased amino acid catabolism (Felig, Wahren et al. 1977; DeFronzo, Jacot et al. 1981; Sun, Liu et al. 2008). Skeletal muscle is the primary site of insulin action and the principal reservoir for amino acids making it a most important contributor to whole body metabolism. A loss in skeletal muscle mass and function is intimately linked to the diabetic condition.

DIABETES: ATROPHY AND FIBER-TYPE SWITCHING

Insulin modulates many of the molecular signaling pathways that regulate protein homeostasis in muscle, including the PI3K/Akt, MEK/ERK, and NFκB pathways. Defective insulin signaling in skeletal muscle results in impaired whole body glucose homeostasis, as well as elevated rates of protein degradation, reduced protein synthesis and, ultimately, pronounced muscle atrophy. Numerous studies have suggested that DM-induced muscle weakness results not only from changes in muscle mass and structure, but also from the functional alterations that occur in the muscle.

Whole muscles, such as the gastrocnemius or soleus, are often thought of as fast-twitch or slow-twich. In reality, these muscles are a heterogeneous mix of fiber-types that is highly plastic and adaptable. Slow-twitch fibers are defined as expressing MHC (myosin heavy chain) I and have highly oxidative metabolism. Fast-twitch fibers express MHCII and are primarily glycolytic. The ratio of slow:fast fibers can change in response

to contractile activity, neural inputs and functional demands. There is evidence that fiber-type switching can also occur in systemic models of atrophy, including DM.

DM patients have demonstrated reduced exercise tolerance, as well as impaired oxygen uptake and reduced oxidative capacity. These observations suggest that there should be a reduction in the number of MHCI, oxidative fibers in DM. However, while there is evidence that there are a reduced number of oxidative fibers in DM, these have never been identified as to MHC type (Armstrong, Gollnick et al. 1975). Nonetheless, even in the diabetic state, oxidative fibers retain their ability to undergo hypertrophy under increased loading whereas glycolytic fibers do not (Bassel-Duby and Olson 2006). These data together suggest that DM may induce fiber-type switching from MHCI to MHCII, thus decreasing functional capacity as well as perpetuating the atrophy process since glycolytic fibers appear to be more susceptible to wasting during DM. However, there have been no complete studies performed at the single-fiber level. Our studies are the first to thoroughly examine both phenotypic changes of individual fibers in muscle of diabetic rats in conjunction with the molecular signaling pathways that control oxidative capacity and fiber-type transitions.

THE ROLE OF CN/NFAT/MEF2 SIGNALING

Muscle remodeling has been well studied in the context of exercise physiology. Several lines of evidence indicate that the Cn (calcineurin) – NFAT (nuclear factor of activated T-cells) pathway is the best candidate for an activity-dependent mechanism for maintaining as well as inducing the slow-oxidative fiber gene program in adult skeletal muscle. Transgenic mice expressing Cn specifically in skeletal muscle, under an MCK (muscle creatine kinase) promoter, demonstrate an increased proportion of MHCI and

MHCIIa oxidative fibers compared to MHCIIb and MHCIIx glycolytic fibers in several muscle groups (Chin, Olson et al. 1998). Their muscles also have increased expression of myoglobin and of the enzymes responsible for mitochondrial oxidative phosphorylation and lipid metabolism (Chin, Olson et al. 1998). These data suggest that Cn is a key regulator not only of MHC isoform expression but also the corresponding metabolic gene program.

Cn has also been implicated in muscle hypertrophy. Inhibitors of Cn activity blocked the hypertrophy that occurs in mice with overloading and in C_2C_{12} mouse muscle cells with IGF-1 treatment (Chin, Olson et al. 1998; Dunn, Burns et al. 1999). However, transgenic mice overexpressing Cn do not demonstrate hypertrophy (Naya, Mercer et al. 2000). Nonetheless, Cn is activated by the same calcium oscillations that induce muscle contractile activity, which blunts muscle atrophy even in the face of catabolic signals. These observations suggest that while Cn alone may not be responsible for inducing hypertrophy, it is likely involved in muscle adaptation in response to various stimuli. Thus, suppression of the Cn pathway in systemic conditions like DM may contribute to muscle wasting and sustaining atrophy long-term. Our studies examine the activity of the Cn signaling pathway including NFAT and MEF2 transcriptional activity in a chronic model of atrophy.

THE ROLE OF PGC-1α

PGC-1α is likely a major effector protein of the Cn mediated regulation of muscle mass and fiber-type composition. PGC-1α is a transcriptional co-activator that regulates energy homeostasis and is potent regulator of the MHCI fiber phenotype in skeletal muscle. Transgenic expression of PGC-1α in muscles that are usually primarily MHCII

results in fiber type switching to a more oxidative, MHCI type phenotype, mimicking the effects of transgenic overexpression of Cn (Lin, Wu et al. 2002). Conversely, skeletal muscle-specific PGC-1α knockout mice exhibit a shift from oxidative, MHCI fibers to more glycolytic, MHCII fibers accompanied by exercise intolerance (Handschin, Chin et al. 2007). Additionally, Sandri et. al. recently demonstrated that fasted mice transgenically overexpressing PGC-1α experienced less atrophy than controls (Sandri, Lin et al. 2006). Examination of the protective mechanism indicated that the enhanced level of PGC-1α suppressed FoxO3-mediated induction of the muscle-specific E3 ubiquitin ligases, MAFbx/AT-1 and MuRF-1, which have been highly correlated with the atrophy phenotype (Sandri, Lin et al. 2006).

PGC-1α expression is typically downregulated in numerous models of atrophy, including DM. Our studies are unique because they are the first to validate the upstream signaling pathways that regulate PGC-1α in vivo in a model of chronic muscle atrophy. Furthermore, we examined the consequences of DM on some functional properties of selected muscles that have been linked to PGC-1α. Together, our results suggest that Cn signaling through PGC-1α may protect oxidative fibers from wasting. Therefore, the Cn pathway and PGC-1α may be pivotal regulators of both muscle size and fiber composition in systemic models of atrophy, like DM.

CHAPTER 2

BACKGROUND AND SIGNIFICANCE

Skeletal muscle is the largest organ in the body, comprising 40-60% of total mass. It plays an obvious role in mobility and is fundamentally involved in respiration, speech, and vision. The importance of muscle mass and strength as they relate to the activities of daily living are unquestionable. Muscle also plays a key, and yet underappreciated, role in metabolism. For example, skeletal muscle is the major site of insulin action, which affects not only glucose metabolism, but also lipid and protein metabolism as well (DeFronzo, Jacot et al. 1981; Sun, Liu et al. 2008). The major biological action of insulin is to regulate blood glucose. This is accomplished primarily by stimulating glucose uptake in skeletal muscle while simultaneously inhibiting gluconeogenesis in liver and kidney (Sun, Liu et al. 2008). Defects in the insulin signaling pathway in skeletal muscle have been implicated as a major contributing factor to insulin resistance and, ultimately, diabetes (Taylor, Accili et al. 1994). As such, alterations in muscle metabolism can contribute to pathologies associated with many common diseases.

Skeletal muscle also plays a central role in whole-body protein metabolism by serving as the principal reservoir for amino acids. Maintenance of the protein content of certain tissues and organs, like the brain and heart, is essential for survival. These organs rely on a steady supply of amino acids from the blood to serve as precursors and maintain protein homeostasis (Felig, Owen et al. 1969; Cahill 1970; Biolo, Zhang et al. 1995). In the absence of amino acid absorption from the gut (the fasting state), skeletal muscle serves as the principal reservoir for maintaining blood levels of amino acids that are absorbed by other tissues (Biolo, Zhang et al. 1995). Importantly, these amino acids also serve as precursors for hepatic gluconeogenesis allowing for maintenance of plasma glucose levels in the fasting state (Felig 1973).

Under normal conditions, skeletal muscle protein turnover is highly dynamic. In the fed state, protein synthesis incorporates dietary amino acids into muscle protein to balance or exceed the loss of muscle protein via degradation. Conversely, muscle protein degradation exceeds protein synthesis during the fasting state. Through this balance between protein synthesis and protein degradation, muscle mass is maintained (Goll, Neti et al. 2008). In some pathological conditions, this balance can be perturbed, resulting in a net depletion of muscle mass. This process is known as atrophy and, if allowed to persist, it is incompatible with life. Extensive studies have shown that death by starvation occurs when muscle protein breakdown becomes inadequate to maintain the necessary supply for gluconeogenic precursors due to loss of overall mass (Keys, Brozek et al. 1950; Winick 1979). Furthermore, many common chronic diseases like cardiac failure, cancer, and diabetes, are frequently associated with skeletal muscle atrophy (Wolfe 2006). While the mechanisms by which atrophy occurs in these conditions are poorly understood, atrophy is the primary prognostic indicator of mortality in these patients. Therefore, an improved understanding of the cellular mechanisms regulating skeletal muscle protein breakdown is imperative for developing therapies for some of the most increasingly prevalent clinical conditions.

SKELETAL MUSCLE STRUCTURE

The bulk of skeletal muscle protein comprises the contractile apparatus, which is constructed primarily of the myofibrillar proteins, actin and myosin (Fig. 2.1) (Clark, McElhinny et al. 2002). Polymers of actin bound to nebulin form the thin filaments. Tropomyosin protein dimers coil around the actin core and troponin is also bound to the thin filaments. Polymers of myosin form the thick filaments, which are thought to be

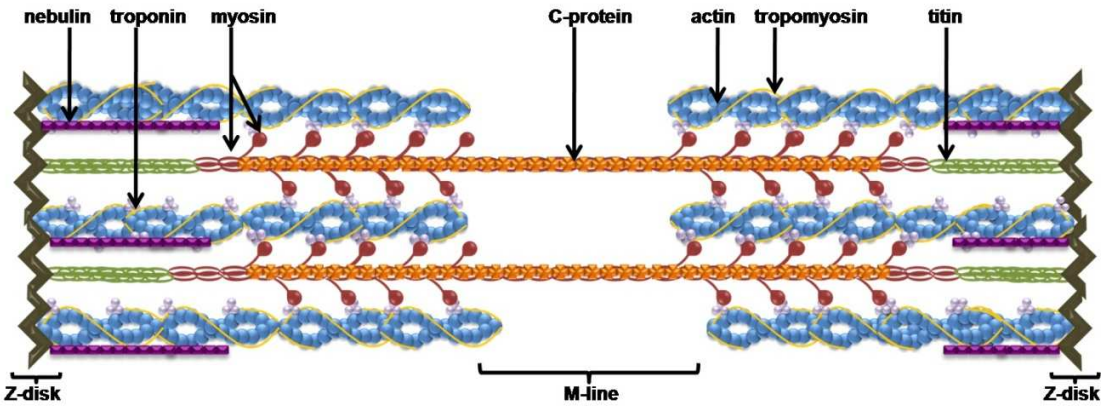

Figure 2.1. **Schematic of human skeletal muscle sarcomere structure and protein components.**

stabilized by binding of C-protein. These myofilaments are bundled into myofibrils, which are organized as sarcomeres. Sarcomeres are defined as the segment between the Z-discs, which are the attachment sites for the thin filaments. The middle of the sarcomere, known as the M-line, is the junction of the thick filaments. The thick filaments are connected from the M-line to the Z-disc by titin. Sarcomeres are the smallest contractile unit of the muscle and repeat every 10µM along the myofibril. Myofibrils form the basic structural component of muscle cells or myofibers. The myofibers are single, multinucleated cells, enclosed in a membrane called the sarcolemma, that span the length of the muscle. The myofiber nuclei are usually localized around the exterior of the fiber just beneath the sarcolemma. Several myofibers are bundled and several of these bundles together comprise a fascicle. Fascicles are surrounded by connective tissue and several of these comprise a single, functional muscle (Clark, McElhinny et al. 2002). When muscle is injured, however, satellite cells, which are normally quiescent and reside between the sarcolemma and the basement membrane, differentiate and fuse to the pre-existing fibers to regenerate the injured tissue (Mauro 1961). The result is that recently injured, regenerating myofibers have centralized nuclei.

The most basic function of muscle is contraction, which takes place at the level of the single myofiber. Each myofiber is innervated by a single nerve but that nerve can innervate several myofibers to form a motor unit. The strength of a muscle contraction is dependent on the number of motor units that are recruited to produce the action. Release of the stimulatory neurotransmitter acetylcholine from the nerve end results in depolarization of the sarcolemma and t-tubules, stimulating the release of intracellular calcium stores from the sarcoplasmic reticulum (Sandow 1965). The released calcium

then binds to troponin C, which resides on the thin filaments. Troponin C allosterically modulates tropomyosin, which obstructs myosin binding sites on the thin filament. A conformational change in troponin caused by calcium binding changes the position of tropomyosin and allows myosin to bind to actin. Myosin hydolyzes ATP and undergoes a conformational change into a high-energy state. This allows the head group of myosin to bind to actin, thus forming a cross-bridge between the thick and thin filaments. The energy stored by myosin is released and ADP and inorganic phosphate dissociate from myosin. The resulting relaxation of the myosin molecule entails rotation of the globular head, which induces longitudinal sliding of the filaments, resulting in muscle shortening and force generation (Sandow 1965; Cooke 2004).

SKELETAL MUSCLE FIBER TYPES

Myofibers can express different isoforms of myosin that control not only the contractile properties, but the metabolic properties of the fibers as well (Brooke and Kaiser 1970; Schiaffino, Sandri et al. 2007). Eight genes encoding MHC (myosin heavy chain) isoforms have been identified in humans. Two are located on chromosome 14. One of these encodes the isoform which is expressed in the atrial myocardium (Bredman, Wessels et al. 1991). The other is expressed in the ventricular myocardium and in slow-twitch skeletal muscle fibers (Lompre, Nadal-Ginard et al. 1984; Saez, Gianola et al. 1987). Slow-twitch muscles are responsible for posture and anti-gravity activities or sustained endurance type locomotor activities. Slow-twitch muscle fibers, which express MHCI have a significant number of mitochondria and, as a result, rely primarily on oxidative metabolism. These muscles are often called the “red” muscles, as they are redder in color than their fast-twitch counter-parts due to increased expression of

myoglobin and increased vasculature. As a result, slow-twitch muscles also carry a higher oxygen load and are able to sustain aerobic activity. The slow contraction of these fibers is due to reduced ATPase activity associated with MHCI, which makes ATPase staining a good marker for differentiating slow-twitch fibers from fast-twitch fibers (Brooke and Kaiser 1970).

The remaining MHC genes are located on chromosome 17. Three encode the embryonic, neonatal, and extraocular isoforms while the remaining three encode isoforms that are expressed in fast-twitch skeletal muscle fibers (Karsch-Mizrachi, Travis et al. 1989; Karsch-Mizrachi, Feghali et al. 1990; Weiss, McDonough et al. 1999; Weiss, Schiaffino et al. 1999; Smerdu and Erzen 2001). Fast-twitch fibers are primarily utilized for movements involving strength and speed because they exert quick contractions, but they also fatigue quickly. These fibers express one of three MHCII isforms; MHCIIa, MHCIIb, or MHCIIx. Fibers expressing MHCIIa exhibit an intermediate phenotype. They are metabolically oxidative like slow-twitch fibers, but contract at speeds similar to those of other fast-twitch, glycolytic myofibers. Additionally, in humans, MHCIIx gene transcripts are expressed in fibers that were identified by marker staining to correspond to rodent MHCIIb fibers (Smerdu, Karsch-Mizrachi et al. 1994). Humans also have a gene for MHCIIb in the cluster on chromosome 17, but its expression in human muscle has not been confirmed (Horton, Brandon et al. 2001).

The metabolic and functional properties of myofibers are actually quite plastic and can switch phenotype, depending upon which MHC isoform is expressed, to adapt to functional requirements (Bassel-Duby and Olson 2006; Schiaffino, Sandri et al. 2007). The various isoforms of MHC are expressed from different genes and each one is

associated with a specific transcriptional program of many other genes involved in prescribing the metabolic and functional properties of the myofiber (Bassel-Duby and Olson 2006). The MHC genes form a continuum of expression: MHCI ↔ MHCIIa ↔ MHCIIx/b, meaning that for a MHCI fiber to switch to MHCIIx/b it must transition through the MHCIIa phenotype along the continuum (Schiaffino, Sandri et al. 2007).

The fiber type composition of muscles is heterogeneous and can be affected by neuronal imput. The speed and pattern of motor-unit firing and the associated calcium oscillations translate the functional requirements into the actual myofiber phenotype that is expressed (Schiaffino, Sandri et al. 2007). In fact, fiber-type switching can be induced in adult muscle by electrical stimulation studies (Pette and Vrbova 1999). Phasic, high frequency electrical stimulation, resembling the firing pattern of fast motoneurons, induces a slow-to-fast switch (Schiaffino and Bormioli 1973). Conversely, tonic, low frequency electrical stimulation, resembling the firing pattern of slow motoneurons, induces a transition in the other direction from fast-to-slow (Kirschbaum, Kucher et al. 1990). Thus, fiber-type plasticity and switching is often a direct result of changes in neural input as defined by changing functional requirements on the muscle.

Changing functional requirements can also affect changes in muscle size. Increased load often results in hypertrophy or growth of muscle. Conversely, disuse or denervation can lead to atrophy or muscle wasting. Disuse or denervation generally results in selective wasting of the specific muscle group that is experiencing reduced functional demands. However, general muscle atrophy across all muscles is a frequent consequence of many systemic disease states including diabetes, cancer, sepsis, and AIDS when the balance between protein synthesis and protein degradation is perturbed.

In fact, loss of lean body mass is the primary predictor of mortality for each of these conditions (Wolfe 2006).

PROTEIN DEGRADATION

Loss of muscle mass occurs at the single myofiber level and atrophy is a reduction in the size of each myofiber as opposed to a decrease in the number of myofibers. Individual myofibers shrink as a result of increased protein turnover. Skeletal muscle, in addition to being very plastic metabolically, is also very dynamic, turning over 1.5 kg of total wet weight per day in a delicate balance between protein synthesis and protein degradation. At that rate of turnover, it is clear that even small changes in either synthesis or degradation can have dramatic effects on the size of the muscle. Atrophy has typically been studied in the context of elevated rates of protein degradation and the cellular mechanisms regulating protein breakdown are beginning to be understood.

THE LYSOSOMAL SYSTEM

Skeletal muscle has three classes of proteins based on their solubility; 1) stroma proteins, 2) sarcoplasmic proteins, and 3) myofibrillar proteins. Skeletal muscle also has four proteolytic systems that work coordinately to degrade these various classes of proteins. The stroma proteins constitute 10-15% of total protein in muscle. These proteins are insoluble in an aqueous solvent at neutral pH. This group includes collagen, extracellular matrix proteins, and some membrane proteins (Goll, Neti et al. 2008). Stroma proteins are degraded by the lysosomal system. Proteases in this system, known as the cathepsins, are located inside membrane-bound vesicles or lysosomes (Bechet, Tassa et al. 2005). These proteases function optimally at acidic pH and, for this reason, are not active in the cytoplasm. Therefore, any role for cathepsins in muscle protein turnover must occur inside lysosomes. Besides the fact that there are very few lysosomes

present in skeletal muscle cells, myofibrils and myofilaments are too large to be engulfed by lysosomes. However, the lysosomal system is ideal for degradation of stroma proteins that have been taken up by pinocytosis or receptor-mediated endocytosis and then transported by a series of vesicles to the lysosome (Bechet, Tassa et al. 2005; Goll, Neti et al. 2008).

THE UBIQUITIN PROTEASOME SYSTEM

Sarcoplasmic proteins constitute 30-35% of total protein in muscle (Goll, Neti et al. 2008). These proteins are cytoplasmic and, therefore, are very easily targeted for degradation by the ubiquitin proteasome system (Fig. 2.2). The proteasome is a large proteolytic complex that has two parts; a 20S core particle and a 19S regulatory complex that binds to the 20S particle to form the 26S proteasome. The 20S core particle is a barrel-like complex of approximately 700-kDa and containing 28 different subunits grouped into two classes; α and β. The 20S particle is arranged in four rings of seven subunits each. The α subunits form the two outer rings and the β subunits form the inner two rings. Proteolytic activity of the proteasome resides exclusively with the β subunits and all of the catalytic sites are located inside the central cavity of the barrel-like structure.

Access to the central cavity is restricted by the 19S regulatory particle, which is involved in substrate recognition and unfolding. Protein molecules cannot enter the catalytic chamber without first being unfolded. The 19S regulatory particle is composed of lid and base components. The lid contains eight polypeptides that bind polyubiquitin chains and remove them from the susbstrate so that they can be recycled. The base contains six homologous ATPases that unfold the polypeptides entering the chamber in

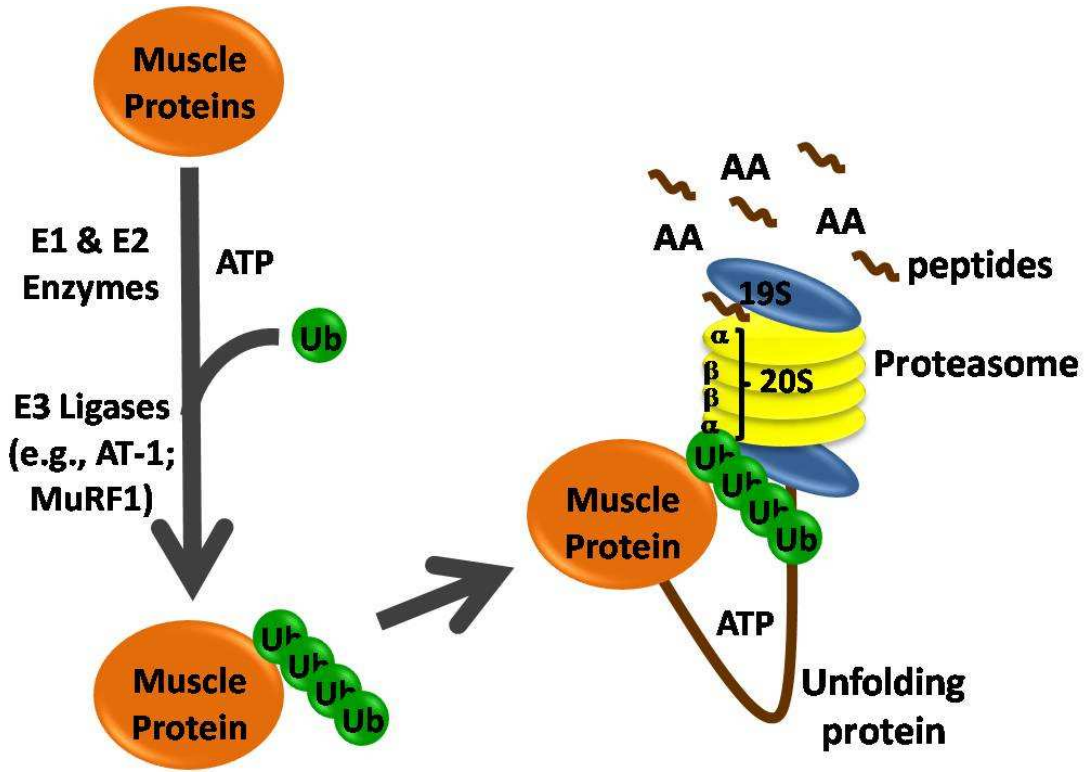

Figure 2.2. **The ubiquitin proteasome system.** A series of enzymatic steps conjugates a tail of four ubiquitin monomers onto a protein, thus targeting it to be degraded by the 26S proteasome.

an energy-dependent process. In fact, proteasomal degradation is an energy intensive process. It has been estimated that as many as 300-400 molecules of ATP may be required for degradation of a protein via this system (Goll, Neti et al. 2008).

For a protein to be degraded by the proteasome, a chain of four ubiquitin molecules must be conjugated to the target polypeptide in a process that also requires ATP hydrolysis (Goll, Neti et al. 2008). Ubiquitin is an 8.5-kDa protein that is highly conserved between bacteria and mammals. Ubiquitin conjugation proceeds in a series of enzymatic steps. The first enzyme is known as the E1 ubiquitin activating enzyme. The E1 activates a ubiquitin monomer in an ATP-dependent reaction and forms an intermediate high-energy E1- ubiquitin complex. The activated ubiquitin is transferred to one of a small family of E2 ubiquitin conjugating enzymes. The E2s then interacts with one of a very large family of E3 ubiquitin ligating enzymes. The E3s represent the specificity component of the ubiquitin proteasome system and over 500 E3s have been identified in the human genome. The E3-ubiquitin conjugate recognizes a substrate protein and transfers the ubiquitin to the substrate. The same process, with the same enzymes, is repeated for each additional ubiquitin monomer that is added to the tail of the elongating ubiquitin chain. The poly-ubiquitinated substrate is recognized by the proteasome and degraded (Fig. 2.2) (Goll, Neti et al. 2008).

E3 Ubiquitin Ligases

E3 ligases fall into two broad categories. They contain either HECT (homologous to E6-AP carboxy terminus) domains or RING finger domains, so named for a core of cysteine and histidine amino acids arrayed in a C3HC4 “RING” (Cao, Kim et al. 2005). HECT E3 ligases are single proteins that bind directly to activated ubiquitin; however,

this is the smaller of the two groups. The vast majority of E3 ligases fall into the RING finger class. RING finger E3s catalyze ubiquitin conjugation by a less direct mechanism, by creating a scaffolding complex for transfer of activated ubiquitin on the E2 to a lysine residue on the protein target. RING finger proteins are typically one subunit of a larger complex that together demonstrates ubiquitin ligase activity. The prototype of such an E3 is the SCF (Skp1-Cullin-Fbox) complex (Cardozo and Pagano 2004). The F-box contributes specificity to the complex by binding to target proteins prior to binding to the Skp protein. Skp1 is the bridging protein linking the F-box to Cullin, which is the main scaffolding protein linking Skp to the RING finger domain of Rbx1, a fourth subunit. The E2-ubiqutin complex then binds to the SCF complex via Rbx1. However, RING fingers have also been found in many single subunit E3s such as E3α and MuRF-1(Cao, Kim et al. 2005).

E3α

The first E3 ligase to be implicated in muscle atrophy was E3α, also called UBR1(Solomon and Goldberg 1996). E3α is a large, 200-kDa, single subunit ubiquitin ligase that contains a modified RING-H2 domain and acts in concert with the ubiquitin carrier protein, $E2_{14k}$. It is thought to recognize substrate proteins that begin with unblocked hydrophobic or basic amino acids, commonly referred to as the N-end rule (Solomon, Baracos et al. 1998; Solomon, Lecker et al. 1998). The connection between E3α and muscle atrophy began with the finding that mRNA for $E2_{14k}$ and E3α are increased in atrophying muscle (Wing and Banville 1994; Lecker, Solomon et al. 1999). Furthermore, inhibitors of E3α reduced rates of ubiquitin conjugation back to control levels in cellular extracts from atrophying muscle (Solomon, Lecker et al. 1998; Lecker,

Solomon et al. 1999). These findings implicated E3α in the accelerated ubiquitin conjugation that occurs during the atrophy process.

More recently, an E3α homologue, named E3αII, was identified. This protein shares 60% overall sequence homology with E3α and was found to be dramatically upregulated in atrophying muscles of tumor-bearing rodents (Kwon, Xia et al. 2001; Kwak, Zhou et al. 2004). Transfection of either E3α or E3αII into muscle cells in culture markedly increases the rate of N-end rule ubiquitination (Kwak, Zhou et al. 2004). However, there is at least one other protein with homology to E3α in mammalian muscle tissue and this redundancy makes it difficult to draw definitive conclusions regarding the role of the N-end rule pathway in muscle wasting (Kwon, Reiss et al. 1998; Kwon, Xia et al. 2001).

MuRF-1 and MAFbx/AT-1

In 2004, two studies found that transcriptional expression of two E3 ligases were significantly upregulated specifically in skeletal muscle undergoing atrophy. The first study using tissue from several rat models of atrophy, including fasting and diabetes, identified the ligases MuRF-1 (muscle specific ring finger) and MAFbx (muscle atrophy F-box) (Bodine, Latres et al. 2001). The second study used tissue from a denervated mouse model of atrophy and identified a ligase, which they named AT-1 (atrogin-1) (Gomes, Lecker et al. 2001). Subsequent sequence analysis showed that AT-1 was 96% homologous to MAFbx and, therefore, the two studies separately identified the same gene in different species and several different atrophy models. Throughout this dissertation, MAFbx/AT-1 will be referred to as AT-1.

AT-1 contains an F-box domain suggesting that the 42-kDa protein could form the specificity subunit of a larger SCF complex (Gomes, Lecker et al. 2001). There are no clear motifs present in the AT-1 sequence to suggest which proteins it may recognize in muscle. Nonetheless, it does contain both a PDZ-binding domain, implicated in protein-protein interactions, and a nuclear localization sequence (Doyle, Lee et al. 1996; Sandri, Sandri et al. 2004). Early studies of AT-1 indicated that expression was restricted to striated and cardiac muscle with induction at least 12hr before significant muscle weight loss occurred (Gomes, Lecker et al. 2001). AT-1 knockout mice demonstrate greatly reduced atrophy after 14d of denervation; however, the unchallenged knockout animals have no obvious phenotype (Bodine, Latres et al. 2001). These data strongly suggest a role for AT-1 in the early phases of atrophy. Possibly, AT-1 recognizes key nuclear regulatory proteins or transcription factors, targeting them for degradation, which may lead to a decrease in protein synthesis.

MuRF-1, the second muscle-specific E3 that was identified, is a 40-kDa protein with a RING-finger at the amino-terminal region and two coiled-coil domains in the central region (Spencer, Eliazer et al. 2000; Bodine, Latres et al. 2001; Centner, Yano et al. 2001). MuRF-1 is also upregulated in various models of atrophy (fasting, diabetic, uremic, etc.) and mice lacking MuRF-1 are protected against atrophy, although to a lesser extent that those lacking AT-1 (Bodine, Latres et al. 2001). Like the AT-1 knockout mice, the MuRF-1 null mice have no overt phenotype unless challenged by an atrophy-inducing stimulus. The targets and specific role of MuRF-1 in skeletal muscle remains unclear but troponin I and myosin heavy chain protein have been suggested to be targets (Centner, Yano et al. 2001).

TRIM32

TRIM32 has also been implicated in protein degradation in skeletal muscle. TRIM32 belongs to the tripartite motif (TRIM) family, which is defined by three linked motifs including a RING finger, a B-box, and a coiled-coil domain (Kudryashova, Kudryashov et al. 2005). TRIM32 demonstrates E3 ubiquitin ligase activity attributable to its RING finger (Kudryashova, Kudryashov et al. 2005). However, unlike the other E3s discussed above, TRIM32 expression is not restricted to skeletal muscle. Rather, TRIM32 has been implicated in diverse pathologic conditions. TRIM32 has been suggested to be an oncogene, but that link remains unclear (Albor, El-Hizawi et al. 2006; Kano, Miyajima et al. 2008). A mutation in the B-box domain of TRIM32 is one of 12 gene mutations that have been linked to Bardet-Biedl Syndrome, which is a multi-systemic disorder characterized by obesity, diabetes, cardiac and renal abnormalities, cognitive impairment, gastro-urinary tract malformation, and retinopathy (Chiang, Beck et al. 2006). A mutation near the C-terminal of TRIM32 has been associated with limb-girdle muscular dystrophy and sarcotubular myopathy, both of which are hereditary disorders (Kudryashova, Kudryashov et al. 2005; Borg, Stucka et al. 2009; Locke, Tinsley et al. 2009).

TRIM32 knockout mice display a phenotype similar to human muscular dystrophies (Kudryashova, Wu et al. 2009). However, closer examination of these mice indicated that the phenotype maybe due to a neurogenic defect. Loss of TRIM32 resulted in atrophy of motor axons (Kudryashova, Wu et al. 2009). As it is known that larger axons innervate MHCII fibers and smaller axons innervate MHCI fibers, Kudryashova et. al. examined the MHC fiber type composition of these mice. Consistent with the reduction in motor axon diameters, they have an increased proportion of MHCI fibers

compared to control (Kudryashova, Wu et al. 2009). Therefore, the mechanism of TRIM32 on muscle may be primarily through its role in the nervous system and not directly through its E3 ubiquitin ligase activity in muscle cells.

THE CALPAINS

The proteasomal system is ideal for degradation of sarcoplasmic proteins which are easily accessible to the ubiquitinating enzymes. However, the myofibrillar proteins, which comprise 55-60% of total protein in muscle, cannot be directly degraded by the proteasome (Goll, Neti et al. 2008). Both the myofibril and the thick and thin filaments are too large to enter the central chamber of the proteasome where the catalytic residues reside. In fact, studies have shown that although the proteasome can degrade myosin and actin, it does not directly act on intact myofibrils, suggesting that the rate-limiting step in degradation of myofibrillar proteins is their release from the myofibril (Solomon and Goldberg 1996).

Even during atrophy and wasting, it is essential to survival that muscle function be maintained to sustain mobility, respiration, vision, etc. To maintain the structural and functional integrity of the muscle, individual myofilaments must remain intact. Therefore, skeletal muscle atrophy requires that myofibrillar proteins be removed from the myofibril before being degraded to amino acids; otherwise, individual myofilaments would become destabilized and lose the ability to contract and create tension. It has long been proposed that myofilaments can be released from the surface of a myofibril, leaving a myofibril with a diameter that is smaller by one layer of myofilaments. This is consistent with the observations that atrophy results in smaller myofibers, with decreased diameters and cross-sectional areas, as opposed to a reduction in the number of

myofibers. The calpains are thought to be responsible for the release of myofilaments from the surface of myofibrils, because the calpains cleave many of the proteins that are involved in keeping the myofilaments attached (Smith and Dodd 2007; Goll, Neti et al. 2008).

The calpains are a family of 14 different Ca^{2+}-dependent proteinases, two of which are expressed in skeletal muscle. Upon activation, the calpains can rapidly cleave titin and nebulin at the point where these two polypeptides enter the Z-disk (Goll, Thompson et al. 2003). The calpains also cleave desmin and filamen, which encircle the myofibril at the Z-disk and tether it to the sarcolemma (Goll, Dayton et al. 1991). These cleavages release α-actinin, the principal Z-disk protein, resulting in the release of thin filaments from the surface of the myofibril (Goll, Dayton et al. 1991). The calpains also degrade M-line proteins (Goll, Thompson et al. 1992). This cleavage, together with titin cleavage, severs the attachments of the thick filament to the myofibril (Goll, Neti et al. 2008). In the presence of ATP to dissociate the myosin cross-bridge to the thin filaments, the thick filaments are released from the myofibril. Calpain cleavage of troponin and tropomyosin would facilitate disassembly of the thin filaments into actin monomers. Further, calpain cleavage of the C-protein would facilitate disassembly of the thick filament to myosin monomers (Goll, Neti et al. 2008). However, the calpains do not cleave proteins into amino acids, but rather make selective cuts that produce large peptide fragments, which are susceptible to degradation by either the proteasome via the ubiquitination pathway or the lysosomal system via autophagy.

THE CASPASES

Caspases are proteolytic enzymes that are activated during apoptosis and by other cell stressors. They can selectively cleave a variety of cellular proteins. A relevant substrate of the effector caspase-3 in muscle is actin. Caspase-3 cleaves actin at several locations, thus producing multiple fragments including a characteristic 14-kDa fragment (Du, Wang et al. 2004). The caspase system becomes upregulated in numerous models of atrophy and the 14-kDa actin fragment is now a well accepted marker of atrophy in both experimental animals and patients (Du, Wang et al. 2004). It is likely that subsequent to myofibril release due to calpain activity, actin is further processed by caspase-3 prior to degradation to amino acids by the proteasome.

To summarize the current school of thought, protein degradation in skeletal muscle involves four systems. The Ca^{2+}-dependent calpains release myofibrils from myofilaments and, possibly, cleave thick and thin filaments into myosin and actin monomers. Caspase-3 then further cleaves actin into smaller fragments. Polypeptide fragments produced by either the calpains or caspase-3 are now subject to further degradation to amino acids through either autophagy and the lysosomal system or the ubiquitin proteasome system. There are several lines of evidence in support of this mechanism. First, the ubiquitin ligase E3α operates based on the N-end rule, recognizing substrates that have unblocked hydrophobic or basic amino acids at the N-terminal (Lecker, Solomon et al. 1999). However, as the vast majority of cellular proteins possess an N-terminal methionine and are acetylated, specific targets of E3α or how they may be generated are unclear. A likely mechanism would involve upstream proteolytic systems clipping the muscle proteins to yield free amino-terminal residues that can be recognized by E3α. Both the calpains and caspases are candidates for this role. As a second example,

the muscle specific E3 ubiquitin ligase MuRF-1 has been shown to bind to titin at the Z-disk and M-line regions (Goll, Neti et al. 2008). This localization positions MuRF-1 for immediate ubiquitination and proteasomal degradation of titin polypeptides that are released by the calpains. It is estimated that 80-90% of all muscle proteins are ultimately degraded via the proteasome pathway into amino acids, which are necessary to sustain total protein content in other essential organs like the brain and heart (Wolfe 2006; Goll, Neti et al. 2008).

MOLECULAR MECHANISMS REGULATING PROTEIN HOMEOSTASIS

A number of diverse molecular signals regulate muscle protein turnover. It has been suggested that a common program of transcriptional events, including increased expression of components of the ubiquitin proteasome system, occurs in response to various catabolic stimuli (Price 2003; Lecker, Jagoe et al. 2004). Hormones such as insulin, IGF-1 (insulin-like growth factor) 1, and glucocorticiods influence protein homeostasis. Insulin and IGF1 play well documented roles in hypertrophy and protein synthesis.

PI3K/AKT

Binding of either insulin or IGF-1 to their respective receptors initiates phosphorylation of the IRS (insulin receptor substrate) proteins (Fig. 2.3). IRS1 activates PI3K (phosphatidylinositol-3-kinase), resulting in the production of PIP3 (phosphatidylinositol-3, 4, 5-triphosphate) from PIP (phosphatidylinositol-4, 5-bisphosphate). PIP3 provides a docking site at the plasma membrane for the serine/threonine kinase, Akt. Phosphorylation of Akt by PDK1 at the membrane activates the enzyme which phosphorylates downstream target proteins that mediate cell proliferation, survival,

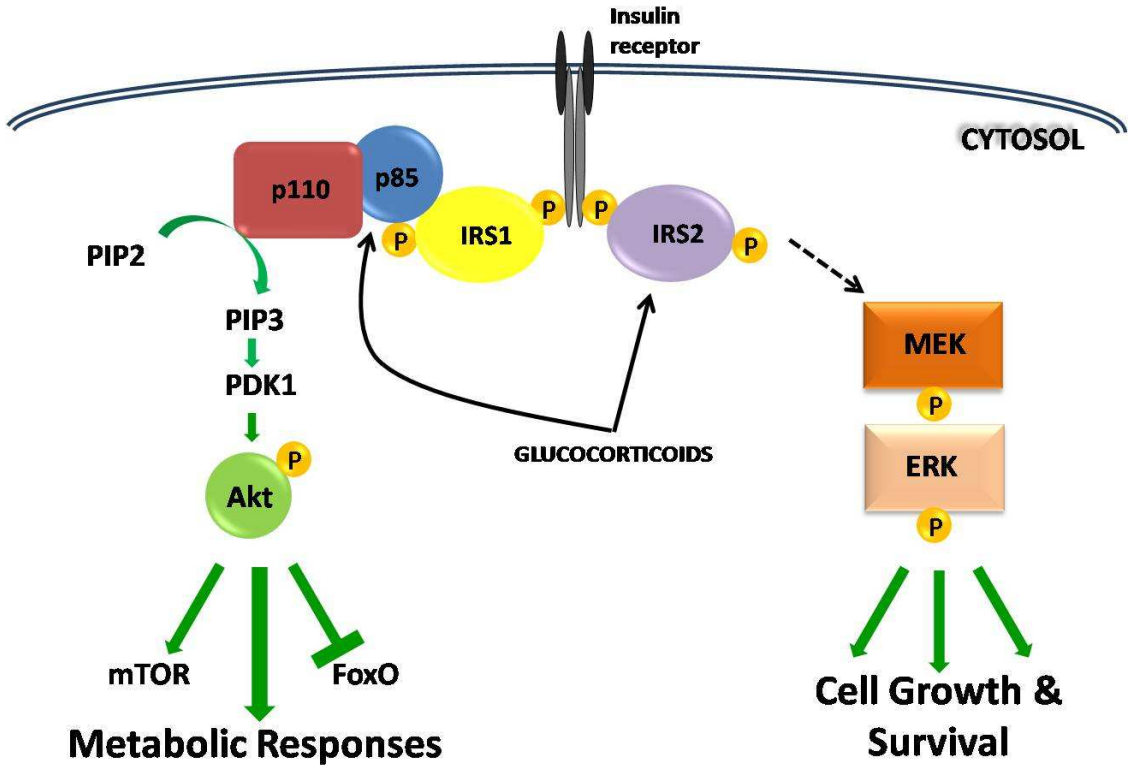

Figure 2.3. **Insuling signaling activates both the PI3K/Akt and MEK/ERK pathways.** Glucocorticoids increase the amount of PI3K p85 regulatory subunit, which causes inhibition of PI3K complex function through competition for IRS1 binding. Glucocorticoids also increase IRS2 expression, possible through a cross-talk mechanism with the PI3K/Akt pathway.

protein synthesis, and the activity of transcription factors. Phospho-Akt activates mTORC1 (mTOR-raptor complex), which increases translation initiation and ribosome biosynthesis in response to growth factors and nutrients (Guertin, Stevens et al. 2006). In muscle, mTOR and its target, S6 kinase, are activated by the PI3K/Akt pathway and are crucial for muscle growth (Rommel, Bodine et al. 2001; Ohanna, Sobering et al. 2005). Muscle hypertrophy in response to IGF-1 can be abolished by treatment with the mTOR specific inhibitor, rapamycin, in C_2C_{12} murine muscle cells (Rommel, Bodine et al. 2001). Hypertrophy of rat muscle caused by *in vivo* transfection of constitutively active Akt is also blocked by the oral administration of rapamycin, indicating that mTOR is crucial for muscle growth (Bodine, Stitt et al. 2001). Many of mTOR's actions on muscle appear to be mediated by the mTOR substrate S6K (S6 kinase). S6K knockout mice have decreased muscle mass and muscle fiber size compared to wildtype (Ohanna, Sobering et al. 2005). Furthermore, primary myoblasts from S6K knockout mice demonstrate decreased rates of protein synthesis (Mieulet, Roceri et al. 2007).

The PI3K/Akt pathway also regulates the FOXO transcription factors. There are four members of the FOXO family, three of which (1, 3a, and 4a) are substrates of Akt. FOXO6 is consitutively nuclear. Active Akt inactivates the FOXO transcription factors through phosphorylation, which results in sequestration of the FOXOs in the cytoplasm (Takaishi, Konishi et al. 1999; De Ruiter, Burgering et al. 2001). The FOXOs are closely linked to cell growth and numerous target genes have been identified including the cell cycle inhibitors, p15, p19, p21, and p27 (Nakae, Kitamura et al. 2003; Motta, Divecha et al. 2004; Katayama, Nakamura et al. 2008), the apoptotic mediators, Bim and Bid (Stahl, Dijkers et al. 2002; Luo, Puig et al. 2007), the autophagy mediators, Bnip3 and LC3

(Mammucari, Schiaffino et al. 2008), and the muscle specific E3 ligases AT-1 and MuRF-1 (Sandri, Sandri et al. 2004; Stitt, Drujan et al. 2004). Mice transgenically overexpressing constitutively active FOXO1 have smaller muscle mass compared to wild-type and reduced levels of mTOR, raptor, and phosphorylated S6K, which are all important for protein synthesis (Southgate, Neill et al. 2007). Active FOXOs also upregulate AT-1 and MuRF-1 *in vitro*, and *in vivo*, accelerating muscle atrophy (Sandri, Sandri et al. 2004; Stitt, Drujan et al. 2004).

Glucocorticoids and insulin deficiency reduce the activity of the PI3K/Akt pathway. Insulin deficiency directly decreases signaling through the pathway via decreased ligand binding to the insulin receptor. Glucocorticoids suppress PI3K signaling via upregulation of the p85 regulatory subunit of PI3K, without a corresponding increase in the p110 catalytic subunit (Fig. 2.3) (Giorgino, Pedrini et al. 1997). The result is competition for binding between free p85 and the PI3K holocomplex to phosphorylated IRS-1 (Giorgino, Pedrini et al. 1997; Zheng, Ohkawa et al. 2010). However, the effect of glucocorticoids on the PI3K pathway seems to be multifaceted as glucocorticoid treatment also causes a decrease in IRS-1 protein, thus potentially blocking PI3K induction (Zheng, Ohkawa et al. 2010). When the PI3K/Akt pathway is suppressed, activity of the FOXO transcription factors is increased, which suppresses protein synthesis via downregulation of mTOR and accelerates protein degradation via upregulation of the E3 ligases MuRF-1 and AT-1 as well as proteins involved in autophagy.

MEK/ERK

In muscle, chronically activated glucocorticoids activate the MEK/ERK pathway, which is one of four subfamilies of MAPK (mitogen-activated protein kinase) pathways (Fig.2. 3). ERKs (extracellular signal regulated kinases) are activated in response to extracellular signals. These signals, transduced via integrins, G-protein coupled receptors, or tyrosine kinase receptors, induce recruitment of the serine/threonine kinase Raf to the membrane by Ras. Active Raf then phosphorylates and activates MEK, which in turn activates downstream ERKs. There are two isoforms of ERK in mammals, ERK1 and ERK2. Both are ubiquitously expressed and thought to have distinctive roles based on the data showing that ERK2 can compensate for ERK1 but not vice versa (Pages, Guerin et al. 1999). However, what distinct roles the two might have remain unclear. The insulin receptor is a tyrosine kinase receptor that activates the MEK/ERK pathway signal transduction through IRS2. Recently, our lab demonstrated that MEK/ERK activation by glucocorticoids is likely through a cross-talk mechanism in coordination with the PI3K/Akt pathway in which downregulation of IRS1, which suppresses PI3K/Akt signaling, resulted in upregulation of IRS2 and MEK/ERK signaling (Zheng, Ohkawa et al. 2010). In this model, glucocorticoids likely bypass the activation of Raf and Ras.

The MEK/ERK pathway contributes to the atrophy process. Glucocorticoids activate the MEK/ERK pathway resulting in phosphorylation and activation of the transcription factor Sp1, which subsequently upregulates ubiqutin transcription (Marinovic, Zheng et al. 2002; Zheng, Ohkawa et al. 2010). First characterized as a factor that binds to GC boxes in the promoter regions of mammalian “housekeeping” genes, Sp1 has since been recognized as a complex protein with multiple regulatory domains

that can be modulated by posttranslational modifications and cofactors (Mitchell and Tjian 1989; Lee, Suh et al. 2005).

NFκB

The NF (nuclear factor)-κB dependent pathway is activated by glucocorticoids. NFκB is a family of dimeric transcription factors composed of p50/p105, p52,p100, c-Rel, Rel A, and Rel B subunits, all of which share similarities in the Rel homology domain at the amino-terminus (Chen and Ghosh 1999). The Rel homology domain includes sequences required for DNA binding, dimerization, nuclear localization and inhibitor factor binding. These subunits form homodimers or heterodimers that bind to specific DNA sequences known as κB sites. The binding affinity of every dimer is variable depending on the combination of subunits and each κB site (Chen and Ghosh 1999). Inactive NFκB is normally associated with an endogenous inhibitor, IκB, in the cytosol. In response to extracellular stimuli, IκB is phosphorylated by IKK (IκB kinase), which causes the dissociation of the NFκB-IκB complex and the release of active NFκB (Rayet and Gelinas 1999). NFκB is upregulated in muscle in response to glucocorticoids, exercise, immobilization, and cytokines, including TNF-α (Du, Mitch et al. 2000; Li, Tupper et al. 2003; Ji, Gomez-Cabrera et al. 2004). Interestingly, NFκB activation by exercise can be blocked by as much as 76% by inhibitors of ERK, which suggests a possible link to that pathway and may explain how NFκB is often activated in concert with the ubiquitin proteasome system in conditions of atrophy (Ji, Gomez-Cabrera et al. 2004; Ji, Zhang et al. 2004). NFκB increases transcription of MuRF-1 and the α2 subunit of the 20S proteasome particle (Cai, Frantz et al. 2004).

CALCINEURIN/NFAT/MEF2 SIGNALING

Cn (calcineurin) is involved in the regulation of muscle size in many conditions. It has been extensively studied in the field of exercise physiology and has been shown to induce muscle hypertrophy when activated by exercise (Dunn, Burns et al. 1999; Olson and Williams 2000). The response is due to its function as a sensor of neural activity, specifically the calcium oscillations that accompany contractile activity. Exercise and contractile activity have long been known to blunt the atrophy that occurs with systemic conditions. The exact mechanisms regulating muscle size in the face of catabolic signals are unknown, but the Cn signaling pathway is an excellent candidate.

Calcineurin

Cn, so named for its ability to bind calcium and high expression in the nervous system, is a Ca^{2+}/calmodulin dependent serine/threonine protein phosphatase (Klee, Crouch et al. 1979; Klee, Krinks et al. 1983). It exists as a heterodimer of an A catalytic subunit and a B regulatory subunit and multiple isoforms of each subunit exist. Skeletal muscle expresses the CnB1 isoform of the regulatory subunit and the CnAα and CnAβ isoforms of the catalytic subunit. The binding between CnA and CnB is very tight, can only be dissociated under denaturing conditions, and is not thought to be a regulated process (Perrino, Fong et al. 1992). Although CnB is considered to be the regulatory subunit since it is essential for high enzymatic activity of calcineurin, the precise role of the subunit remains unclear. The CnA subunit has two regulatory domains. A calmodulin binding domain confers the calcium sensitivity of the enzyme. In contrast, an autoinhibitory domain inhibits enzyme activity in the absence of $Ca^{2+/}$calmodulin by binding to the catalytic site.

Several endogenous inhibitors of Cn have been identified. The first was AKAP79, which was shown to bind and inhibit Cn by scaffolding it away from substrates (Coghlan, Perrino et al. 1995). CAIN binds directly to Cn and inhibits its activity through an as yet unknown non-competitive mechanism (Lai, Burnett et al. 1998; Sun, Youn et al. 1998). CHP demonstrates a high degree of homology to CnB and likely inhibits Cn through competition for CnA binding (Lin and Barber 1996; Lin, Sikkink et al. 1999). Lastly, the MCIP proteins are Cn inhibitors that are expressed at high levels in skeletal muscle (Fuentes, Genesca et al. 2000; Rothermel, Vega et al. 2000). Interestingly, MCIP1 expression is regulated by Cn and is postulated to be a negative feed-back pathway (Yang, Rothermel et al. 2000). The physiological roles of endogenous Cn inhibitors remain unknown; however, they have frequently been used as experimental tools to inhibit Cn activity through overexpression or, in the case of MCIP1, as a read-out of Cn activity (Fenyvesi, Racz et al. 2004).

The first described function of Cn was in T-cell receptor signaling and the generation of immune responses. NFAT (nuclear factor of activated T-cells) is a modulator of T-cell activation and its activation via nuclear translocation was blocked by the pharmacological agents FK506 and Cyclosporine A (CsA) (Flanagan, Corthesy et al. 1991). A parallel study discovered that FK506 and CsA interacted with intracellular receptors called immunophilins and inhibited Cn activity (Liu, Farmer et al. 1991). Subsequent studies linked the two pathways by demonstrating that Cn is a key regulator of T-cell activation and that NFAT is a target of Cn (Clipstone and Crabtree 1992; Jain, McCaffrey et al. 1993; McCaffrey, Perrino et al. 1993). The role of Cn in T-cell signaling is supported by the clinical use of CsA and FK506 as immunosuppressants.

NFAT

The mammalian NFAT family of transcription factors is comprised of five members. The mechanisms regulating NFAT5 activity are poorly understood and may or may not involve Cn (Lopez-Rodriguez, Aramburu et al. 1999; Miyakawa, Woo et al. 1999). However, the other four isoforms of NFAT are regulated directly by Cn and are therefore known as NFATc1-c4 (Mancini and Toker 2009). All four of the NFATc isoforms are expressed in skeletal muscle but NFATc1 is the predominant species. Cn is constitutively bound to NFATc at a conserved docking site within the NFAT homology domain (Mancini and Toker 2009). Active Cn dephosphorylates NFATc resulting in an unmasking of a nuclear localization sequence. NFATc then translocates to the nucleus where it binds to target gene promoters and induces transcription of a selected set of genes (Zhu and McKeon 1999; Mancini and Toker 2009). Glycogen synthase kinase-3β and casein kinase 1/2 are the major kinases that regulate NFATc1 nuclear export in skeletal muscle (Beals, Sheridan et al. 1997; Zhu, Shibasaki et al. 1998; Neal and Clipstone 2001; Okamura, Garcia-Rodriguez et al. 2004).

In vivo reporter studies have shown that NFATc1 is localized primarily in the cytoplasm in the TA (tibialis anterior), a predominantly MHCII muscle, but it is localized primarily in the nucleus in the soleus, a predominantly MHCI muscle (Thomson, Porter et al. 2007). Nuclear translocation of NFATc1 is rapidly induced in the TA by low-frequency electrical stimulation (Tothova, Blaauw et al. 2006). Furthermore, NFAT transcriptional activity is higher in oxidative than in glycolytic muscles. Constitutively active NFATc1 induces MHCI expression in slow muscles, but not in fast muscles even though it can bind the MHCI promoter in the latter muscles (McCullagh, Calabria et al.

2004). One reason for this discrepancy may be that NFAT is thought to require interaction with other transcription factors for transcriptional activity. Specifically, the transcription factor, MEF2 (myocyte enhancer factor 2) is thought to work synergistically with NFATc1 to regulate the MHCI gene program (Wu, Naya et al. 2000). In fact, some known MEF2 gene targets include MHCI, myoglobin, and troponin 1(Wu, Naya et al. 2000; Wu, Rothermel et al. 2001).

MEF2

MEF2 proteins are a subclass of the MADS (MCM1, agamous, deficiens, SRF) family of transcription factors (Shore and Sharrocks 1995). The N-terminal of MEF2 proteins contains a highly conserved MADS-box and a MEF2 domain, which together mediate dimerization and DNA-binding (Black and Olson 1998; McKinsey, Zhang et al. 2002). The C-termini are highly divergent among family members as they are subject to complex patterns of alternative splicing, which complements their function as transcriptional activation domains (Martin, Miano et al. 1994). There are four isoforms in vertebrates, MEF2A-D, which function as homo- or heterodimers and have distinct, but often overlapping functions (Edmondson, Lyons et al. 1994). MEF2 is scaffolded away from its target genes by binding to the class II HDACs (histone deacetylases) (Lu, McKinsey et al. 2000). Contractile activity activates CaMKII, which phosphorylates the HDACs resulting in their export from the nucleus and cytoplasmic sequestration via 14-3-3 binding (Zhang, McKinsey et al. 2002; McKinsey and Olson 2005). This releases MEF2 to interact with NFATc and bind target genes. Low frequency electrical stimulation, characteristic of slow fibers, has been shown to induce HDAC translocation to the cytoplasm as well as increase MEF2 transcriptional activity (Wu, Naya et al.

2000). There is evidence, however, that Cn may dephosphorylate MEF2 and this may increase the efficiency of binding to target promoters (Wu, Rothermel et al. 2001).

The Cn/NFAT/MEF2 signaling pathway has been implicated in maintaining the type I fiber phenotype and regulates many of the genes for prototypical type I proteins such as MHCI, troponin I, and myoglobin (Chin, Olson et al. 1998; Bigard, Sanchez et al. 2000; McCullagh, Calabria et al. 2004). However, another facet of the type I phenotype is increased oxidative capacity and mitochondrial content. The signaling pathways regulating these genes remain elusive. Recently, PGC (Peroxisome proliferator activated receptor γ coactivator) 1α has been implicated in mediating this component of the type I fiber phenotype.

PGC-1α

PGC-1α is a transcriptional coactivator that participates in the regulation of skeletal muscle metabolism, particularly energy homeostasis and glucose metabolism (Puigserver and Spiegelman 2003). Transcriptional coactivators mediate the functional connection between transcription factors and the general transcription apparatus, including the polymerase. The coactivators themselves usually exist and function as multiprotein complexes, which contain proteins to mediate docking on transcription factors and others that mediate functions necessary for transcription such as histone modification and chromatin remodeling. Transcription factors themselves have been the targets of much study when it comes to transcriptional control. However, more recently we are discovering that transcriptional coactivators add another level to the complexity of transcriptional regulation. Indeed, PGC-1α demonstrates a remarkable degree of regulation in different tissues and physiological states.

PGC-1α is part of a very small family of transcriptional coactivators that includes PGC-1β and PGC-1 related coactivator. The N-terminus of all of these proteins contains a transcriptional activation domain and includes the major nuclear hormone receptor-interacting motif. The C-terminal contains an RNA-binding motif and a serine-arginine rich domain. The presence of a transcriptional activation domain and RNA-processing motifs in the same molecule is an unusual feature of the PGC-1 family that is highly conserved across species. This feature makes the PGC-1 family proteins excellent candidates for studying integration of pre-mRNA splicing and transcription, which have been suggested to be linked *in vivo* (Misteli 2000). PGC-1α also contains a number of "docking domains" for transcription factors such as PPARγ and MEF2C.

PGC-1α was first identified in BAT (brown adipose tissue), the primary site of adaptive thermogenesis, or changes in heat dissipation in response to environmental temperatures. BAT is rich in mitochondria, which contain a specific uncoupling protein (UCP) -1 that is necessary for cold-induced thermogenesis. UCP-1 gene expression is highly cold-inducible through the activation of the sympathetic nervous system and is mediated by β-adrenoreceptors and cAMP. Several activated nuclear hormone receptors also play an important role in the differentiation of BAT and UCP-1 expression, including the PPARs (peroxisome proliferator activated receptors). Activation of PPARγ by synthetic ligands promotes differentiation of BAT precursor cells and PPARγ knockout animals do not have BAT, indicating that PPARγ is required for formation of this tissue (Foellmi-Adams, Wyse et al. 1996; Tai, Jennermann et al. 1996; Barak, Nelson et al. 1999; Rosen, Sarraf et al. 1999). PGC-1α was identified as a PPARγ-interacting

protein expressed preferentially in BAT compared to white adipose tissue, which is mainly involved in energy storage (Puigserver, Wu et al. 1998). When mice are exposed to cold, PGC-1α is strongly induced (Puigserver, Wu et al. 1998). Furthermore, when a BAT cell line is treated with β-adrenergic receptor agonist, PGC-1α mRNA is induced (Puigserver, Wu et al. 1998). In the thermogenic response, β-adrenergic receptors and protein kinase A mediate the induction of PGC-1α, which subsequently binds to PPARγ. These proteins form a complex with other transcriptional coactivators at the UCP1 enhancer and activate transcription.

Skeletal muscle is the other major tissue involved in adaptive thermogenesis and it expresses high levels of PGC-1α. Skeletal muscle has a high level of oxidative metabolism due to a high number of mitochondria. Indeed, mitochondrial biogenesis is an important component of adaptive thermogenesis. Exposure to cold temperatures induces mitochondrial biogenesis and increases uncoupling of respiration. The NRF (nuclear regulatory factor) transcription factors -1 and -2 transactivate several mitochondrial genes that are encoded in the nucleus including mtTFA (mitochondrial transcription factor A), which translocates to the mitochondria and activates mitochondrial DNA replication and transcription. PGC-1α dramatically induces gene expression of NRF-1, NRF-2, and mtTFA (Wu, Puigserver et al. 1999). Furthermore, PGC-1α interacts with NRF-1 to augment its transcriptional activity (Wu, Puigserver et al. 1999). A dominant negative allele of NRF-1 completely blocks the ability of PGC-1α to induce mitochondrial proliferation (Wu, Puigserver et al. 1999). Thus, PGC-1α and its associated functions provide a plausible molecular basis for the connection between environmental and

hormonal stimuli and mitochondrial biogenesis and respiration under conditions of changing energy or thermogenic requirements.

Adaptive thermogenesis and the increased energy expenditure ultimately requires increased fuel metabolism. PGC-1α has been shown to stimulate genes required for increased fatty acid oxidation in adipocytes and heart (Lehman, Barger et al. 2000; Vega, Huss et al. 2000). The effects of PGC-1α on glucose uptake and metabolism are of particular interest in diabetes because there are numerous studies indicating that the rates of mitochondrial oxidation can affect glucose uptake (Randle, Priestman et al. 1994). Indeed, PGC-1α expression is upregulated in the liver in response to fasting, insulin deficiency, and insulin resistance (Herzig, Long et al. 2001; Yoon, Puigserver et al. 2001). Exogenous overexpression of PGC-1α at comparable levels in primary hepatocytes stimulated expression of the key enzymes required for hepatic gluconeogenesis: PEPCK (phosphoenol pyruvate carboxykinase), fructose-1, 6-bisphosphatase, and glucose-6-phosphatase (Yoon, Puigserver et al. 2001). PGC-1α also induces gene expression for the insulin-sensitive glucose transporter (GLUT4) and increases glucose uptake (Michael, Wu et al. 2001). This effect on GLUT4 gene expression is partially mediated through PGC-1α in conjunction with MEF2C.

Molecular Mechanisms Regulating PGC-1α

Post-translational Regulation

P38 MAPK

The role of PGC-1α in energy metabolism is multi-faceted, therefore, it is not surprising that its transcription is complexly regulated. The first pathway that was shown

to regulate PGC-1α expression was p38-MAPK. Cytokines are elevated in cachexia, a chronic state of negative energy balance, which led researchers to examine how cytokines stimulate metabolic rates. They found that cytokines, such as IL (interleukin) -1α, IL-1β, and TNFα enhance the activity of PGC-1α through direct phosphorylation by p38-MAPK (Puigserver, Rhee et al. 2001). Three residues in PGC-1α (T262, S265, and T298) are directly phosphorylated by p38-MAPK leading to increased stability and half-life (Puigserver, Rhee et al. 2001). Interestingly, these residues fall within a region of the PGC-1α protein that plays a regulatory role in binding to transcription factors, specifically NRF-1 (Puigserver, Adelmant et al. 1999). Docking of transcription factors at this site causes a conformational change that accelerates recruitment of other transcriptional effector proteins to the complex. Whether the p38-MAPK mediated phosphorylation affects transcription factor docking or recruitment of other coactivators remains undetermined.

AMPK

In both BAT and skeletal muscle, PGC-1α is regulated by AMPK (AMP-activated kinase). AMPK directly phosphorylates PGC-1α on T177 and S538 (Jager, Handschin et al. 2007). T177 is within the domain where PPARγ docks to PGC-1α and S538 lies within the MEF2C docking domain, which suggests that these AMPK phosphorylation sites are involved in transcription factor docking (Jager, Handschin et al. 2007). Interestingly, PGC-1α has been shown to mediate its own transcription through a feed-back loop (Handschin, Rhee et al. 2003). AMPK phosphorylation increases PGC-1α dependent activation of its own promoter.

Transcriptional Regulation of PGC-1α

CREB

PGC-1α is induced by cold temperatures in BAT via β-adrenergic stimulation of a cAMP-mediated pathway. In the liver, PGC-1α induction and subsequent regulation of gluconeogenesis is also regulated by a cAMP-mediated pathway. cAMP induces phosphorylation of Ser133 in the cAMP response element binding protein (CREB) via PKA (protein kinase A). Activated CREB forms a complex with other proteins (e.g., p300, CREB Binding Protein or CBP) which then bind to a cAMP response element located in the promoter region of the PGC-1α gene, thus inducing PGC-1α transcription (Fig. 2.4) (Gonzalez and Montminy 1989; Herzig, Long et al. 2001).

Calcineurin/NFAT/MEF2

Lastly, PGC-1α expression can be regulated by calcium-dependent pathways, including both Cn, and the calcium dependent protein kinase, CaMK (Fig. 2.4). Activated CaMK catalyzes a variety of protein phosphorylation events. It activates CREB, which subsequently induces PGC-1α. CaMK also mediates the release of MEF2 from an inhibitory complex, which includes the histone deacetylases HDAC1/2 and HDAC4/5. After phosphorylation by CaMK, the HDACs are exported from the nucleus. As a consequence, MEF2 becomes transcriptionally active.

MEF2 and NFAT can also be regulated by Cn via direct dephosphorylation. Active MEF2 binds directly to the PGC-1α promoter to transduce PGC-1α expression (Czubryt, McAnally et al. 2003). While NFAT alone seems to have little effect on induction of PGC-1α transcription, co-transfection experiments in cultured C_2C_{12} mouse

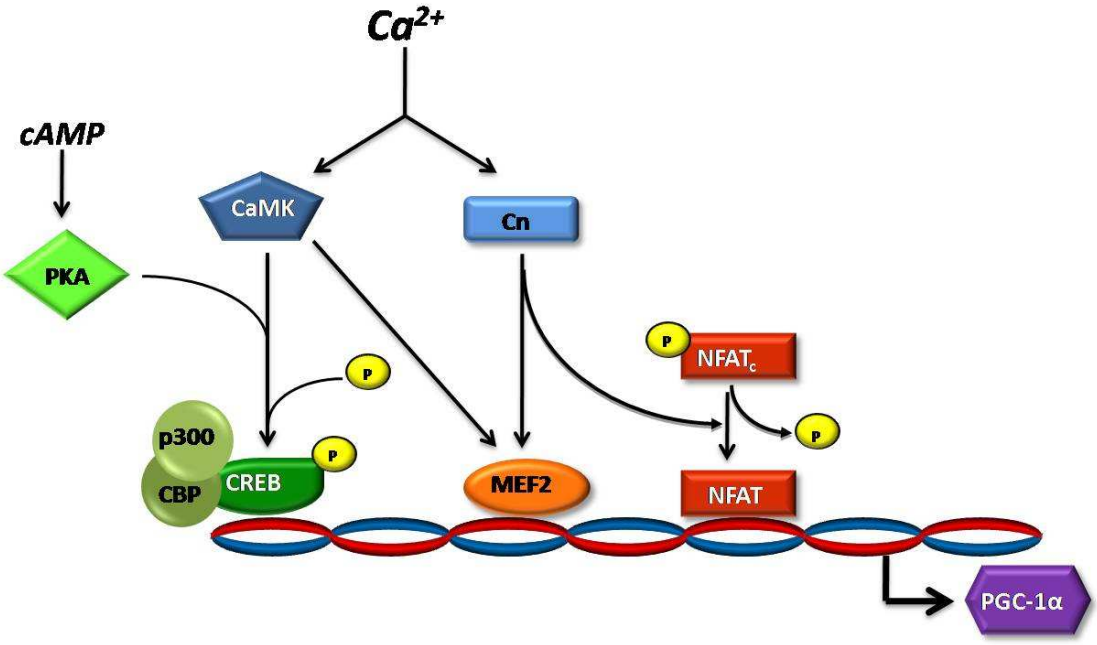

Figure 2.4. **Transcriptional regulation of PGC-1α.** cAMP induces PKA phosphorylation and activation of CREB. CREB can induce PGC-1α transcription through a CRE in its promoter. Two parallel calcium-dependent pathways involving calcium-calmodulin kinase (CaMK) and calcineurin (Cn) can regulate MEF2 whereas Cn is the primary pathway that regulates NFAT. The MEF2 and NFAT transcription factors have been suggested to act synergistically to promote PGC-1α transcription.

myotubes suggest that NFAT and MEF2 work synergistically to regulate PGC-1α (Handschin, Rhee et al. 2003). PGC-1α contains a MEF2 docking site and co-activates MEF2 transcriptional activity, resulting in a positive feed-back loop maintaining PGC-1α expression (Handschin, Rhee et al. 2003). NFAT is likely also recruited to the complex formed between PGC-1α and MEF2 and participates in the positive feed-back loop.

CHAPTER 3

MATERIALS AND METHODS

MATERIALS

Streptozotocin (STZ) was purchased from Sigma-Aldrich (St. Louis, MO). Commercially available antibodies were used: anti-actin (Sigma-Aldrich), anti-Akt and anti-phospho(Ser473)Akt (Cell Signaling: Danvers, MA), anti-calcineurin pan A (Chemicon: Billerica, MA), anti-CREB and anti-phospho(Ser133)CREB (Cell Signaling), anti-glycogen synthase and anti-phospho(Ser641)glycogen synthase (Cell Signaling), anti-GSK3β and anti-phospho(Ser9)GSK3β (Cell Signaling), anti-laminin (Developmental Studies Hybridoma Bank, Iowa City, IA), anti-MEF2 (Santa Cruz: Santa Cruz, CA), anti-MHCI (Sigma), anti-MHCII (Sigma), anti-MHCIIa (Developmental Studies Hybridoma Bank), anti-NFATc1 (Abcam: Cambridge, MA), anti-PGC-1 (Calbiochem: San Diego, CA), HRP-conjugated donkey-anti-rabbit IgG (Amersham: Piscataway, NJ)m, and HRP-conjugated goat-anti-mouse (Jackson Immunoresearch Laboratories: West Grove, PA). Oligo-nucleotide primers were purchased from Invitrogen (Carlsbad, CA) for real-time RT-PCR. Primer sequences and references are listed in Table 3.1.

ANIMALS

STZ-DM RATS

All studies were approved by the Emory University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing ~150 g were anesthetized using isoflourane and given a single tail-vein injection of STZ (60mg/kg body weight) prepared fresh in 0.1M sodium citrate buffer, pH4.0; control (CTL) rats were injected with the vehicle alone. Chronically diabetic animals were fed the standard diet *ad libitum* for

Table 3.1. **Oligonucleotide primers used for real time RT-PCR.**

TRANSCRIPT	FWD PRIMER	REV PRIMER	REFERENCE
DHPRα1s	5' TTTCCCACAGGCCGTGCTGCTGCTCTTCA 3'	5' CATGTAGAAGCTGATGAA 3'	FWD: Sheridan, D et. al. (2003) REV: Savignac, M et. al. (2004)
MCIP1.4	5' ACGGTGATGTCTTCAGCGAAA 3'	5' ATTCGGACACGCTTGAAGCTC 3'	Frank, D et. al. (2007)
MRF4	5' CTACATTGAGCGTCTACAGGACC 3'	5' CTGAAGACTGCTGGAGGCTG 3'	Patapoutian, A et. al. (1995)
PGC-1α	5' ACTGAGCTACCCTTGGGATG 3'	5' TAAGGATTTCGGTGGTGACA 3'	Rogers, J et. al. (2005)
UbC	5' GTTAACACCAAGAAGGTC 3'	5' GGAATGCAAGAACTTTATTC 3'	Marinovic, A et. al. (2000)

21days. At the time of sacrifice, animals were anesthetized by an intraperitoneal injection of ketamine/xylazine, muscles were dissected, and arterial blood was collected for glucose measurements.

STZ-DM TRANSGENIC MICE:

Transgenic mice expressing a NFAT-luciferase (NFAT-luc) reporter gene in all tissues were created by Dr. J. Molkentin (Cincinnati Children's Hospital, Cincinnati, OH) (Bueno, Wilkins et al. 2002; Wilkins, Dai et al. 2004). Mice weighing between 25 and 30 g were given an interperitoneal injection of STZ (55mg/kg body weight) prepared fresh in 0.1M sodium citrate buffer, pH. 4.0, once daily for four days; CTL mice were injected with vehicle alone. Blood glucose levels were monitored daily by tail-snip for one week following the last injection of STZ. Mice with blood glucose levels more than 200 mg/dL were considered diabetic. Diabetic mice were fed standard diet and water *ad libitum* for 21 days and blood glucose levels were monitored weekly by tail-snip. At the time of sacrifice the mice were anesthetized using isoflourane, muscles were dissected, and arterial blood was collected for glucose measurements.

CALCINEURIN KNOCK-OUT MICE

Mice lacking the gene for the α isoform of the CnA catalytic subunit were created by Dr. J. Seidman (Howard Hughes Medical Institute, Harvard Medical School, Boston, MA) (Zhang, Zimmer et al. 1996). Mice lacking the gene for the β isoform of the CnA catalytic subunit were created by Dr. J. Molkentin (Cincinnati Children's Hospital, Cincinnati, OH) (Bueno, Wilkins et al. 2002; Wilkins, Dai et al. 2004).

MEASUREMENT OF MUSCLE PROTEIN DEGRADATION

RATE OF TYROSINE RELEASE

The rate of total protein degradation was measured in isolated, mixed-fiber epitrochlearis muscles as described (Price, Bailey et al. 1996). Briefly, freshly isolated muscles were incubated for 2h at 37^{o}C in Krebs Ringer Bicarbonate buffer containing cyclohexamide to inhibit protein synthesis. Free tyrosine released into the media by proteolysis was measured fluorometrically (Waalkes and Udenfriend 1957). The rate of tyrosine release was calculated as the nmol tyrosine released/ mg wet muscle weight/ hour.

ACTIN DEGRADATION

Gastrocnemius muscles for measurement of the actin fragment were immediately snap frozen upon dissection and stored at -80^{o}C. Muscle samples were later homogenized in 20µl/mg of a hypotonic buffer consisting of 50mM Tris (pH7.5), 1mM EDTA, 1mM EGTA, 0.5mM DTT, 0.1% NP-40, and complete mini protease inhibitor cocktail tablets (Roche: Indianapolis, IN). The homogenates were centrifuged at 2000xg for 10min at 4^{o}C and the pellet was resuspended in Laemmli sample buffer (without dye). The pellet was then homogenized in a ground glass homogenizer and recentrifuged at 2000 x g for 10 min. The supernatant was used for the subsequent analysis. The protein concentration of each sample was measured using the Bio-Rad DC protein assay (Hercules, CA) and equal amounts (50 µg) were separated by SDS-polyacrylamide gel electrophoresis (SDS PAGE); equal protein loading and transfer was confirmed by Ponceau S staining. The blot was developed using antibodies generated to the C-terminal 14 amino acids of actin, an HRP-conjugated goat-anti-rabbit secondary antibody (see Materials) and standard chemiluminescence methods.

WESTERN BLOT ANALYSIS

Muscles to be used for Western blot analysis were snap frozen in liquid nitrogen upon dissection and stored at -80^{o}C. Gastrocnemius muscle samples were later homogenized in 20µl/mg of lysis buffer. Two different lysis buffers were used depending on the stability of the protein of interest. A hypotonic buffer consisting of 50mM Tris (pH7.5), 1mM EDTA, 1mM EGTA, 0.5mM DTT, 0.1% NP-40, and complete mini protease inhibitor cocktail tablets (Roche: Indianapolis, IN) was used for Cn, NFAT, MEF2, and PGC-1α. The samples were subjected to three freeze/thaw cycles using liquid nitrogen and a 37^{o}C water bath. The homogenates were centrifuged and the supernatant was used for analysis. A buffer consisting of 50mM Hepes (pH 7.4), 137mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mM Na pyrophosphate, 10mM Na fluoride, 2mM EDTA, 10% glycerol, 1% NP-40, 2mM Na_3VO_4, 2mM PMSF, 10µg/mL aprotinin, 10µg/mL leupeptin, and 10mM benzamidine, was used for analysis of AKT, CREB, GSK-3β, and glycogen synthase. Supernatant protein concentrations were measured using a BioRad DC Protein Assay Kit (BioRad: Hercules, CA). Protein samples (50µg) were separated by SDS-PAGE, transferred to nitrocellulose membranes and blocked in Tris-buffered saline (pH7.5) containing 5% non-fat milk and 0.1% Tween-20 (Sigma: St. Louis, MO). Blots were incubated with primary antibodies (see Materials; dilutions and washes according to manufacturer's instructions) followed by a goat-anti-rabbit (1:5000) HRP conjugated secondary antibody (see Materials) and detected using chemiluminescence technology. Equal protein loading and transfer were confirmed by Ponceau S Red staining of the membranes.

Films of the developed immunoblots were scanned using a desktop scanner and quantitative densitometric analysis was performed using ImageJ software (National Institutes of Health.

REAL-TIME RT-PCR

RNA was isolated from previously snap frozen gastrocnemius muscles using Trizol Reagent (Invitrogen: Carlsbad, CA) according to the manufacturer's instructions. RNA (5-10μg) was treated with DNase and reverse transcribed using M-MLV reverse transcriptase and random hexamer primers. Real-Time PCR was performed using a BioRad iCycler with target-specific primers (see Materials; Table 1) and iQ SYBR Green (BioRad: Hercules, CA); the 18S rRNA was used as a normalization control. The data were analyzed for fold change (ΔΔCt) using the iCycler software.

IMMUNOHISTOCHEMISTRY

Gastrocnemius and soleus muscles to be used for immunohistochemistry were immediately mounted in Tissue Tek® O.C.T. compound (Sakura Finetek: Torrance, CA), frozen in liquid nitrogen cooled 2-methylbutane, and stored at -80^{o}C for cross-sectioning. Serial transverse cross-sections (12 μm) were preared from soleus and gastrocnemius muscles of five control and five STZ rats. For each cross-section, an origin was randomly selected from which 100 contiguous fibers were counted. Results were compared using different origins in several cross-sections.

Serial cross-sections were stained using antibodies to detect MHCII (pan) (1:400), MHCIIa (1:5), MHCI (1:800), and laminin (1:25) as previously described (Snow, McLoon et al. 2005; Snow, Sanchez et al. 2005) followed by an HRP-conjugated goat-anti-mouse secondary (see Materials) and detected with 3, 3'-diaminobenzidine (DAB)

stain. Laminin immunostaining defined the plasmalemma allowing for clear definition of fiber edges in serial cross-sections.

After identification of fiber type, the first ten MHCI fibers and the first ten MHCII fibers adjacent to the origin were selected for determination of CSA. The perimeter of each fiber of interest was traced using a contour mapping software program that creates a CSA map (Neurolucida version 8, Bioscience MicroBrightField, Inc.) that is processed using a program that calculates CSA (Neurolicida Neuroexplorer program). Hybrid MHCI/II fibers were not included. In the rare cases when the contiguous 100 fibers did not include ten each of the MHCI and MHCII fiber types, additional contiguous fibers were added to reach n=10.

ENDOGENOUS NFAT ACTIVITY ASSAY

Luciferase activity in the gastrocnemius muscles of control and STZ NFAT-luc mice was measured using a commercial kit (Promega: Madison, WI). Dissected muscles were immediately snap frozen in liquid nitrogen and stored at -80^{o}C. Later, tissue samples were homogenized in 20µl/mg passive lysis buffer and particulate matter removed by centrifugation. Total protein content of the supernatant was measured using a BioRad DC Protein Assay Kit (Hercules, CA) and all lysates were diluted to a concentration of 1µg/µL with passive lysis buffer. Luciferase assay reagent (50µL) was added to 10µl of supernatant and luminescence was measured for 10 seconds using a luminometer (Turner Biosystems, Sunnyvale, CA).

SINGLE FIBER ANALYSIS

SINGLE FIBER PREPARATION

Dissected gastrocnemius and soleus muscles to be used for single fiber preparations were immediately placed in a permeablizing solution of 50% glycerol and pCa 8.0 (Alley and Thompson 1997) and stored at -20^{o}C. The muscles were later rinsed and moved to a relaxing solution of pCa 8.0, which was kept cold (4^{o}C) for further dissection. Under a dissecting scope, single fibers were carefully isolated from the whole muscle using a fine probe.

SINGLE FIBER FORCE ASSAY

Both ends of the single permeabilized fiber were mounted in aluminum foil clips, which were then attached to the separate poles of a force transducer and fixed servo-arm (Permeabilized Fiber System, Aurora Scientific, Inc.). Digital images of each fiber at 40X magnification were recorded (Nikon high pixel density monochrome camera with NIS Elements version 2.31 software) pre-contraction and post-contraction. The images were used to measure average pre- and post-contraction sarcomere length, average fiber diameter (D), and sarcomere length (SL) at 3 places along the length of the mounted fiber to ensure fiber integrity.

Specific Force is defined as P_0/CSA where P_0 (mN) is the maximum steady state force generation as determined by the increase in steady state isometric force at saturating concentrations of Ca^{2+} (pCa 4.0 or 3.8) over baseline at pCa 8.0 and CSA (mm^2) is derived from fiber diameter (π $[D/2]^2$) (Donaldson and Kerrick 1975; Donaldson, Hermansen et al. 1978). A fiber was excluded if P_0 declined more than 10% of the original value or post-contraction sarcomere length was not within 0.2 μm of initial value, indicating that the integrity of the contractile apparatus was compromised.

SINGLE FIBER MHC-TYPE DETERMINATION

Fibers removed from mounting clips were dissolved in Laemmli sample buffer and stored at -80^{0}C as previously describe (Alley and Thompson 1997). Total solubilized proteins were separated by SDS-PAGE and MHC types were detected by either silver staining or by Western blot. Silver staining of the SDS-PAGE gel allows for determination of MHC type based on the differing sizes of the different isoforms (Talmadge and Roy 1993) . Alteratively, for Western blot analysis, the proteins that were separated by SDS-PAGE were transferred to nitrocellulose membranes and blocked in Tris-buffered saline (pH7.5) containing 5% non-fat milk and 0.1% Tween-20 (Sigma: St. Louis, MO). Blots were incubated with primary antibodies (see Materials; 1:10^5) followed by an HRP-conjugated goat-anti-mouse secondary antibody (see Materials: 1:10^4) HRP and detected using chemiluminescence technology (Sugawara, Fujimura et al. 1999). These methods identify fiber type by the presence of a single MHC isoform (pure MHC type) or multiple MHC isoforms (hybrid MHC type); hybrids were identified by the specific combination of MHC isoforms. Determination of MHC fiber type was blinded to force data and if fiber type could not be determined, the corresponding force and size data were discarded.

STATISTICAL ANALYSIS.

The analysis used for each data set is given in the Results section. All analyses were performed using GraphPad Prism version 4.00 and InStat version 3.0 (GraphPad Software, San Diego, CA).

CHAPTER 4

CALCINEURIN SIGNALING AND PGC-1α EXPRESSION ARE SUPPRESSED DURING MUSCLE ATROPHY DUE TO DIABETES

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R. N. Reddy maintained the CnA-/- mice, which were funded by J.L. Gooch.
J. L. Bailey assisted with 21d STZ-DM rats.
B. Zheng contributed Fig. 4.6 A and C.
R. Ordas contributed Fig. 4.3 A.

INTRODUCTION

Diabetes mellitus (DM) is an epidemic public health issue that threatens the health and quality of life of people globally. DM can result from insulin deficiency or resistance and is characterized by a variety of metabolic disturbances including reduced cellular glucose uptake, decreased fatty acid oxidation, and dysfunctional mitochondria (Sun, Liu et al. 2008). Skeletal muscle is a major site of insulin action and loss of insulin signaling induces muscle atrophy, which leads to reduced functional capacity and muscle weakness (Sun, Liu et al. 2008).

PGC-1α is a transcriptional coactivator that participates in the regulation of skeletal muscle metabolism, particularly energy homeostasis. It controls glucose transport and is necessary for mitochondrial biogenesis and the maintenance of the oxidative phenotype of muscle fibers (Michael, Wu et al. 2001). Skeletal muscles of mice transgenically overexpressing PGC-1α have an increased proportion of oxidative fibers (Lin, Wu et al. 2002). Recently, PGC-1α has been implicated in the regulation of muscle mass and protein turnover. Glycolytic fibers express a lower level of PGC-1α and they exhibit a greater degree of atrophy than oxidative fibers in disease or systemic models of muscle atrophy (Tiao, Lieberman et al. 1997; Sandri, Lin et al. 2006; Sacheck, Hyatt et al. 2007). In general, PGC-1α expression is lower in skeletal muscles undergoing atrophy, including those of Type II DM patients (Mootha, Lindgren et al. 2003; Patti, Butte et al. 2003; Arany 2008). The mechanism of the atrophy-related reduction of PGC-1α in skeletal muscle is unknown.

Given the multi-faceted role of PGC-1α in energy metabolism, it is not surprising that the control of its transcription is complex. Several signaling pathways (e.g., MAP

kinase, cAMP/PKA) induce PGC-1α transcription by phosphorylating Ser133 in the cAMP response element binding protein (CREB). Activated CREB forms a complex with other proteins (e.g., p300, CREB Binding Protein or CBP) which then bind to a cAMP response element located in the promoter region of the PGC-1α gene (Gonzalez and Montminy 1989; Herzig, Long et al. 2001). Thus, a reduction in CREB activity could be responsible for the reduction in PGC-1α during muscle atrophy. Another pathway known to regulate PGC-1α involves the calcium-dependent phosphatase calcineurin (Cn). Two downstream substrates of calcineurin, NFAT and MEF2 have been proposed to work in concert to increase the transcription of prototypical type I oxidative muscle fiber genes, including PGC-1α (Chin, Olson et al. 1998). Importantly, transgenic expression of Cn in skeletal muscle recapitulated the effects of PGC-1α overexpression by inducing a switching from glycolytic to oxidative fibers (Naya, Mercer et al. 2000; Olson and Williams 2000; Lin, Wu et al. 2002). Thus, a second possible mechanism that could lead to down-regulation of PGC-1α expression is a reduction in Cn signaling.

In the present study, we found that chronic DM induced by a low-dose of streptozotocin increased skeletal muscle protein degradation and decreased PGC-1α expression. This led us to examine the possible mechanisms that could be responsible for reducing PGC-1α transcription. Our results demonstrate that insulin deficiency leads to suppression of Cn signaling and a resulting decrease in PGC-1α transcription.

RESULTS

RATS TREATED WITH STREPTOZOTOCIN EXPERIENCE SKELETAL MUSCLE ATROPHY DUE TO INCREASED PROTEIN DEGRADATION.

Streptozotocin (STZ) was administered to rats to induce Type I DM (i.e., insulin deficiency and hyperglycemia) and cachexia. After 21 days, STZ-rats were hyperglycemic with significantly smaller body and wet muscle weights than controls (Table 4.1). To confirm that increased muscle protein turnover contributed to the lower muscle mass, total protein degradation was measured in isolated epitrochlearis muscles. The epitrochlearis is a small, flat, mixed-fiber type muscle located in the fore-limb. The size of the muscle allows for extended incubation times after dissection without induction of necrosis. Additionally, protein degradation rates measured in the epitrochlearis are comparable to those of the perfused hind-limb(Clark and Mitch 1983). Therefore, the rate measured in the epitrochlearis is representative of whole body protein breakdown. The proteolytic rate was increased in epitrochlearis muscles of STZ-treated rats (Fig. 4.1A). A second independent measure of protein degradation, expression of rat ubiquitin (*UbC*) mRNA, was also increased in gastrocnemius muscle from STZ-treated rats versus controls (Fig. 4.1B).

PGC-1α EXPRESSION IS DECREASED IN MUSCLE FROM STZ-TREATED RATS.

Expression of the PGC-1α transcriptional co-activator has been reported to be downregulated in skeletal muscle of humans with insulin resistance or Type II DM (Patti, Butte et al. 2003). To determine whether PGC-1α is similarly regulated in skeletal muscle of rats with chronic diabetes mellitus, we evaluated PGC-1α protein and found it was decreased in the gastrocnemius muscle of STZ-treated rats (Fig. 4.2A). The

Table 4.1 **Overall body weight and muscle weights are reduced in 21day STZ-treated rats.**

	CTL	**STZ**
Glucose (mg/dL)	153 ± 14.7	427 ± 29.8*
Initial Body Wt. (g)	157 ± 5.3	154 ± 5.9
Final Body Wt. (g)	346 ± 11.7	254 ± 8.4*
Gastrocnemius. Wt. (g)	2.2 ± 0.08	1.3 ± .011*
Epitrochlearis. Wt. (mg)	57.1 ± 3.4	39.1 ± 4.7*

21d DM rats presented with hyperglycemia and significantly smaller body and wet skeletal muscle (gastrocnemius and epitrochlearis) weights compared to controls. Mean ± SEM, n=8, *P<0.05.

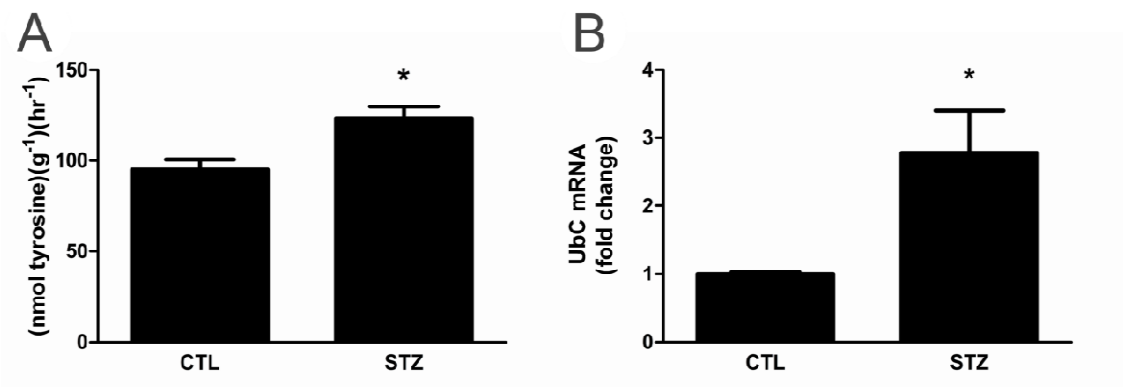


Figure 4.1. **The rate of protein degradation and ubiquitin expression are increased in 21day STZ-treated rat muscle. (A)** Protein degradation was measured in isolated mixed-fiber epitrochlearis skeletal muscles. Muscles from control and 21d STZ-treated rats were incubated in Krebs-Ringer Bicarbonate Buffer with cycloheximide to inhibit protein synthesis. Free tyrosine released into the media was measured fluorometrically after being converted to a nitrosonapthol derivative. **(B)** Ubiquitin C (rUbC) mRNA in the gastrocnemius muscles was measured by real-time RT-PCR. Data were normalized to 18S RNA and expressed as mean fold increase over control. Data are expressed as the mean ± SEM; n=7/group, $P<0.05$

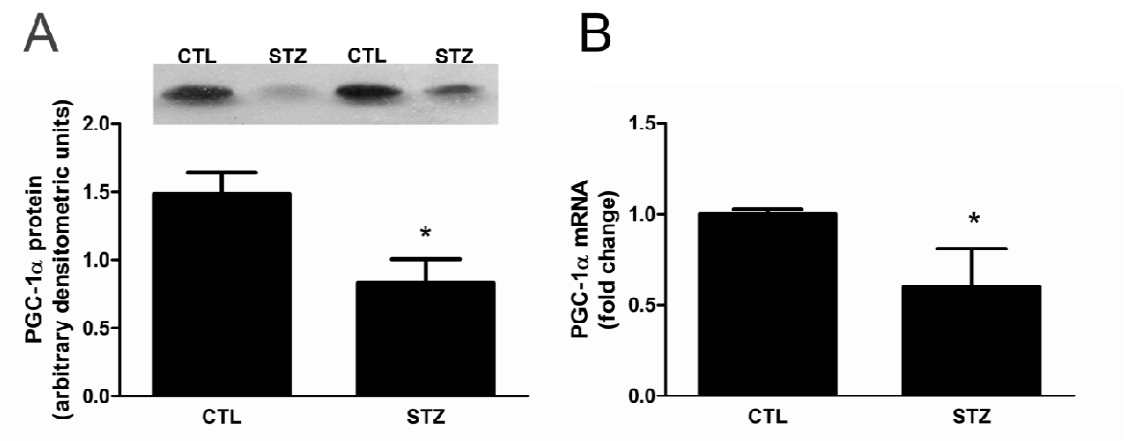


Figure 4.2. **PGC-1α expression is decreased in 21day STZ-treated rat muscle. (A)** PGC-1α protein was evaluated by Western blot analysis. Equal protein loading and transfer was confirmed by Ponceau S staining. n=9/group, **P*<0.05. **(B)** PGC-1α mRNA in gastrocnemius muscle was measured by real time RT-PCR, normalized to 18S RNA, and expressed as mean fold change relative to control ± SEM; n=7/group, **P*<0.05.

decrement in PGC-1α protein appears to result from a pre-translational mechanism because PGC-1α mRNA was also significantly decreased in STZ-treated rat muscle (Fig. 4.2B).

DECREASED PGC-1α TRANSCRIPTION IS NOT DUE TO DECREASED CREB ACTIVITY.

One of the major signaling pathways that regulates PGC-1α transcription involves the cAMP regulatory element binding protein (CREB) via a CREB binding element in the cofactor's promoter (Herzig, Long et al. 2001). CREB is activated by phosphorylation of Ser 133 by various Ca^{2+}-sensing and stress pathways including CaMKII, PKA, and ERK, thus making it relevant in the context of reduced insulin-signaling (Mayr and Montminy 2001). To determine if the reduction in PGC-1α transcription could be attributed to attenuated CREB activity, we evaluated the amounts of CREB phospho-Ser133 relative to total CREB protein. In contrast to the reduction of PGC-1α, the amount of activated CREB was markedly increased ~8 fold in STZ-treated rat muscle compared to controls (Fig. 4.3A). This finding suggests that CREB function may be abnormal in the muscle of STZ-treated rats and led us to evaluate the expression of a known CREB target. The dihydropyridine receptor (DHPR) α1s subunit is expressed exclusively in skeletal muscle, and its transcription is regulated by CREB (Zheng, Wang et al. 2002). The level of DHPRα1s mRNA was decreased in muscles from STZ-treated rats (Fig. 4.3B).

CALCINEURIN SIGNALING IS DOWN-REGULATED IN MUSCLE FROM STZ-TREATED RAT.

The calcium-dependent calcineurin (Cn) signaling pathway has also been implicated in regulating PGC-1α transcription (Schiaffino and Serrano 2002), prompting

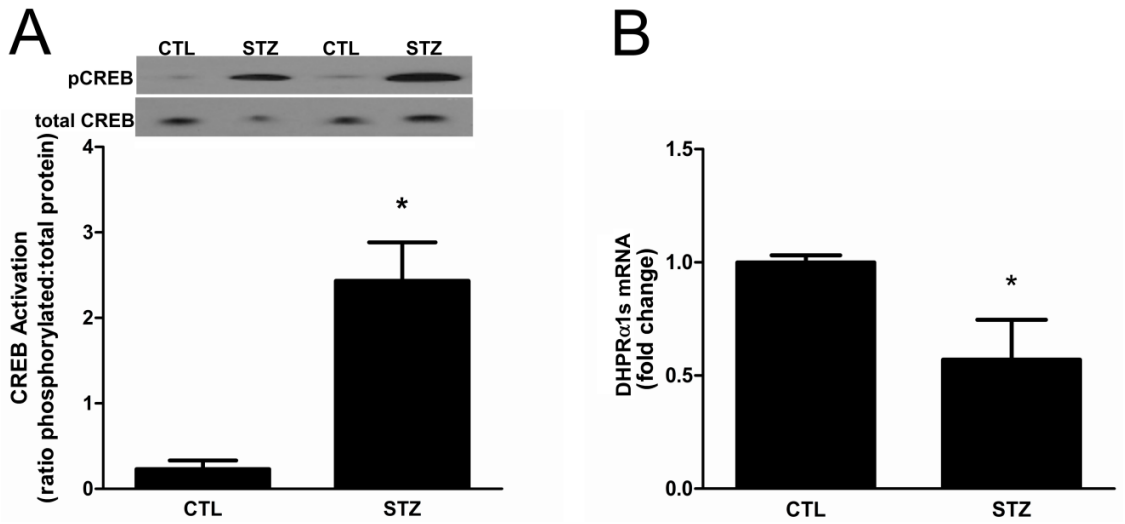


Figure 4.3. **CREB signaling is abnormal in 21day STZ-treated rat muscle. (A)** The phosphorylation (i.e., activation) status of CREB in gastrocnemius muscles of control and 21d STZ-treated rats was examined by Western blot analysis using antibodies that detect phospho-Ser133 and total CREB. Data are expressed as the mean ratio of phosphorylated protein to total protein ± SEM. Equal protein loading and transfer were confirmed by Ponceau S staining. n=6/group, **P*<0.05. **(B)** To evaluate CREB function, the amounts of DHPRα1s, a gene target of CREB, were measured in gastrocnemius muscles by real time RT-PCR. Data are expressed as the mean ± SEM; n=8/group, **P*<0.05.

us to investigate its role in regulating PGC-1α in our 21d STZ-treated rats. The amount of Cn catalytic A subunit (CnA) was decreased in muscles from STZ-treated rats (Fig. 4.4). One of the best characterized substrates of Cn is the NFAT transcription factor family. To determine if NFAT activity was reduced, we measured the amount of mRNA encoding the modulatory calcineurin interacting protein MCIP1.4. MCIP1.4 is an NFAT responsive gene that has been used previously as a surrogate reporter for Cn activity in skeletal muscle (Yang, Rothermel et al. 2000; Fenyvesi, Racz et al. 2004). MCIP1.4 expression was decreased in gastrocnemius of STZ-rats (Fig. 4.5A). To determine whether the reduction in NFAT activity was a result of a change in NFAT protein, the amount of NFATc1 protein was measured since it is the major subclass of NFAT in muscle (Olson and Williams 2000). NFATc1 protein expression was not significantly changed in STZ-rat muscles (Fig. 4.5B). As a way of confirming that STZ-induced, chronic diabetes mellitus attenuated NFAT activity in muscle, we compared luciferase activity in the gastrocnemius muscle of control and STZ-treated mice expressing a NFAT responsive luciferase transgene in all tissues. Luciferase activity was decreased in STZ-treated mice (Fig. 4.5C).

The activities of NFAT proteins can be regulated by either Cn or glycogen synthase kinase -3β (GSK-3β) which phosphorylates and inactivates the transcription factors (Beals, Sheridan et al. 1997). In response to insulin, GSK-3β is inactivated by AKT-mediated phosphorylation (Cross, Alessi et al. 1994). To exclude the possibility that DM activated GSK-3β, we examined the phosphorylation status of GSK-3β Ser-9. There was no difference in the level of phosphorylated enzyme or enzyme expression in STZ-treated and control rat muscles (Fig. 4.6A). To confirm that GSK-3β activity was

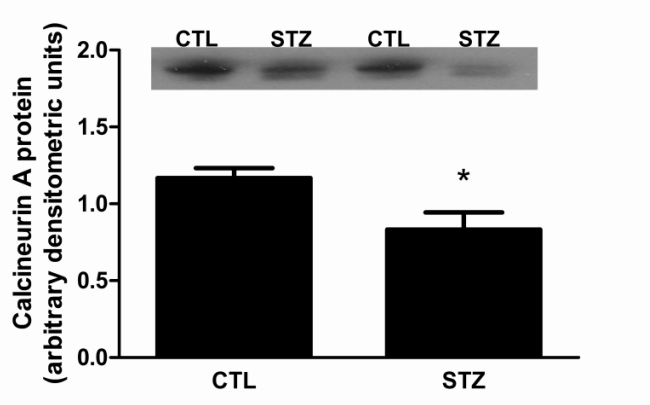


Figure 4.4. **Cn catalytic A subunit protein is decreased in 21day STZ-treated rat muscle.** The levels of Cn A subunit (CnA) protein in gastrocnemius muscles of control and 21d STZ-treated rats were measured by Western blot analysis using a pan antibody that recognized all CnA isoforms. Equal protein loading and transfer were confirmed by Ponceau S staining. Data are expressed as the mean ± SEM; n=12/group, *P<0.05.

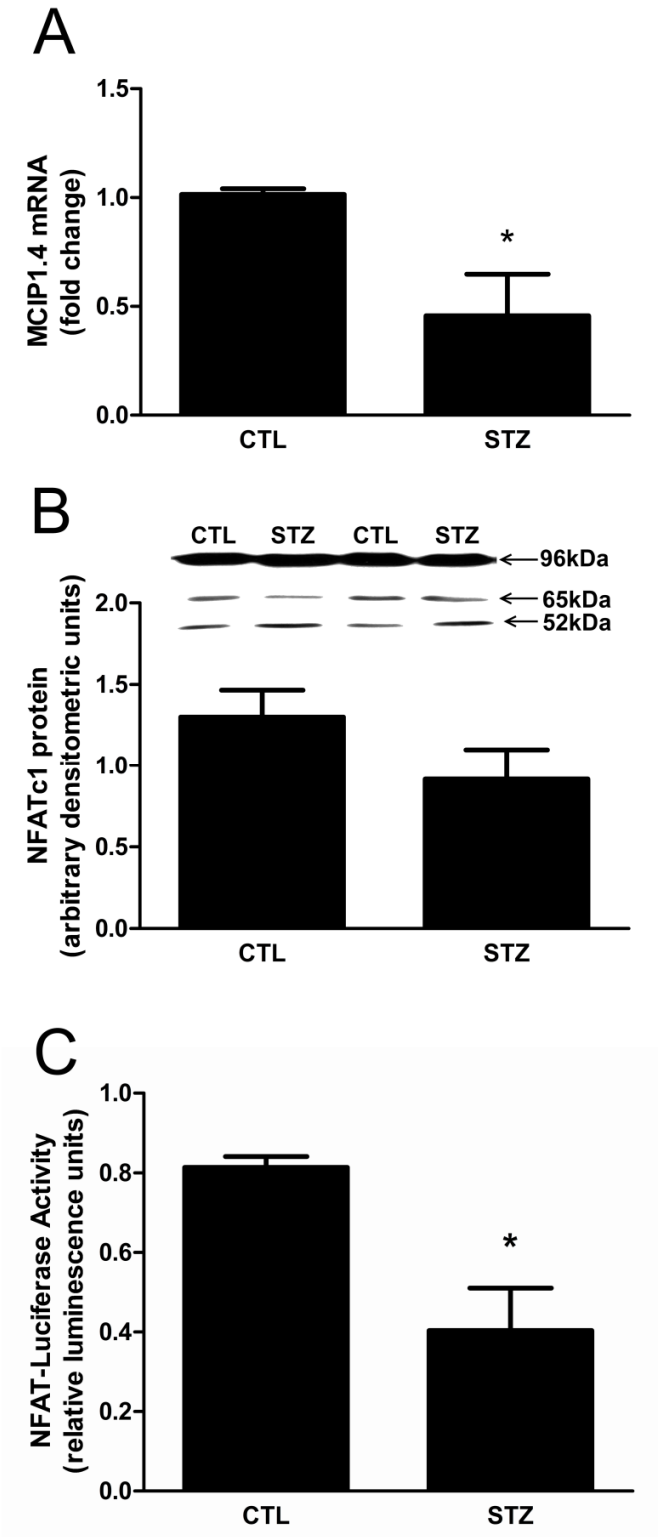
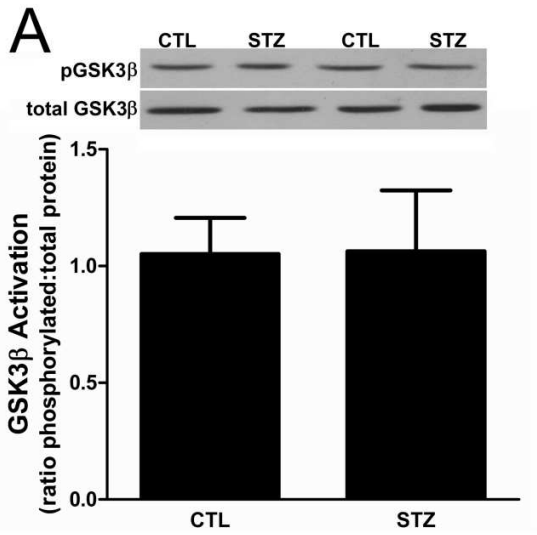

A
MCIP1.4 mRNA (fold change)
1.5
1.0
0.5
0.0
*
CTL
STZ
B
CTL
STZ
CTL
STZ
96kDa
65kDa
52kDa
NFATc1 protein (arbitrary densitometric units)
2.0
1.5
1.0
0.5
0.0
CTL
STZ
C
NFAT-Luciferase Activity (relative luminescence units)
1.0
0.8
0.6
0.4
0.2
0.0
*
CTL
STZ

Figure 4.5. **NFAT activity is decreased in 21 day STZ-treated rat muscle.** Changes in Cn activity are reflected in the activity of the transcription factor NFAT. **(A)** The amount of mRNA encoding the NFAT target MCIP1.4 in gastrocnemius muscles was measured using real time RT-PCR. Values were normalized to 18S RNA and expressed as mean fold change relative to control ± SEM; n=8/group, **P*<0.05. **(B)** The levels of NFATc1 proteins were evaluated by Western blot analysis. n=10/group, P=0.14. **(C)** Transgenic mice expressing a luciferase reporter gene under the control of a NFAT-responsive promoter were injected with STZ (an intraperitoneal injection of STZ (55 mg/kg) daily for four days). After 21d muscle NFAT activity was evaluated by measuring luciferase activity in lysates prepared from gastrocnemius muscles. Data are expressed as mean luminesence units ± SEM; n=3/group, **P*<0.05.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CTL
STZ
CTL
STZ
pGSK3β
total GSK3β
GSK3β Activation
(ratio phosphorylated:total protein)
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1.0
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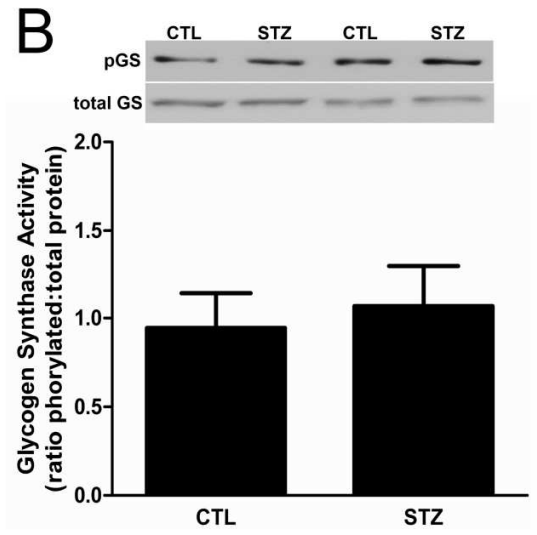
0.0
CTL
STZ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CTL
STZ
CTL
STZ
pGS
total GS
Glycogen Synthase Activity
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1.5
1.0
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0.0
CTL
STZ

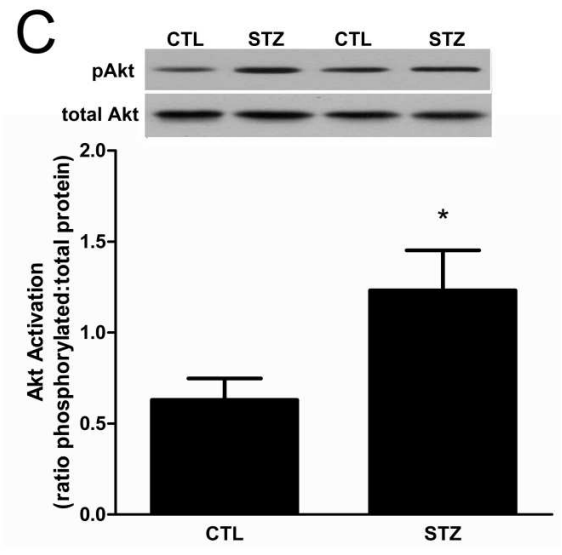
C
CTL
STZ
CTL
STZ
pAkt
total Akt
Akt Activation
(ratio phosphorylated:total protein)
2.0
1.5
1.0
0.5
0.0
*
CTL
STZ

Figure 4.6. **GSK-3β signaling is unchanged in 21day STZ-treated rat muscle. (A)** The phosphorylation (i.e., inactivation) of GSK-3β on Ser-9 in gastrocnemius muscles was examined by Western blot analysis using antibodies that detect phospho-S9 and total GSK-3β. Data are expressed as the mean ratio of phosphorylated protein to total protein ± SEM. n= 8/group; *P*=0.31 **(B)** The phosphorylation of glycogen synthase on Ser-641 also was examined as a downstream target of GSK-3β activity by Western blot analysis using antibodies that detect total and phospho-Ser-641 glycogen synthase. Data are expressed as the mean ratio of phosphorylated protein to total protein ± SEM. n= 8/group; *P*=0.69 **(C)** Western blot analysis of Akt phosphorylation was performed using antibodies that detect total and phospho-Ser-473 Akt. Data are expressed as the mean ratio of phosphorylated protein to total protein ± SEM: $P<0.05$. For each experiment equal protein loading and transfer were confirmed by Ponceau S staining. n=7/group.

unchanged, we measured the levels of glycogen synthase (GS) phosphorylation at Ser-641, the canonical target of GSK-3β. There was no difference in either the phosphorylation status or total protein expression between STZ-treated versus control rat muscles (Fig. 4.6B). We also analyzed the phosphorylation status of Ser-473 in Akt and found that it was increased slightly but significantly in STZ-treated rat muscle (Fig. 4.6C). Together, these results indicate that GSK-3β signaling was unchanged in STZ-treated animals.

MEF2 is another transcription factor that can regulate PGC-1α expression and Cn has been shown to affect its activity. To evaluate MEF2 activity, the amount of myogenic regulatory factor 4 (MRF4) mRNA was measured because MEF2 is its primary regulator in differentiated muscle cells (Rhodes and Konieczny 1989; Black, Martin et al. 1995). In STZ-treated rat muscle, MRF4 mRNA was decreased relative to control rat muscle (Fig. 4.7A). This response was not due to a change in the amount of MEF2 protein (Fig. 4.7B).

LOSS OF CALCINEURIN SIGNALING RESULTS IN DECREASED PGC-1α TRANSCRIPTION IN SKELETAL MUSCLE.

To directly examine the relationship between Cn signaling and PGC-1α expression, the level of PGC-1α mRNA was measured in gastrocnemius muscles of CnAα$^{-/-}$ and CnAβ$^{-/-}$ mice. PGC-1α expression was drastically reduced in both CnA$^{-/-}$ strains. Moreover, NFAT activity, as measured by MCIP1.4 expression, was significantly decreased when either CnA subunit was absent (Fig. 4.8). MRF4 expression, a measure of MEF2 activity, was also significantly decreased in CnAβ$^{-/-}$ mice and tended to be lower in CnAα$^{-/-}$ mice (Fig. 4.8).

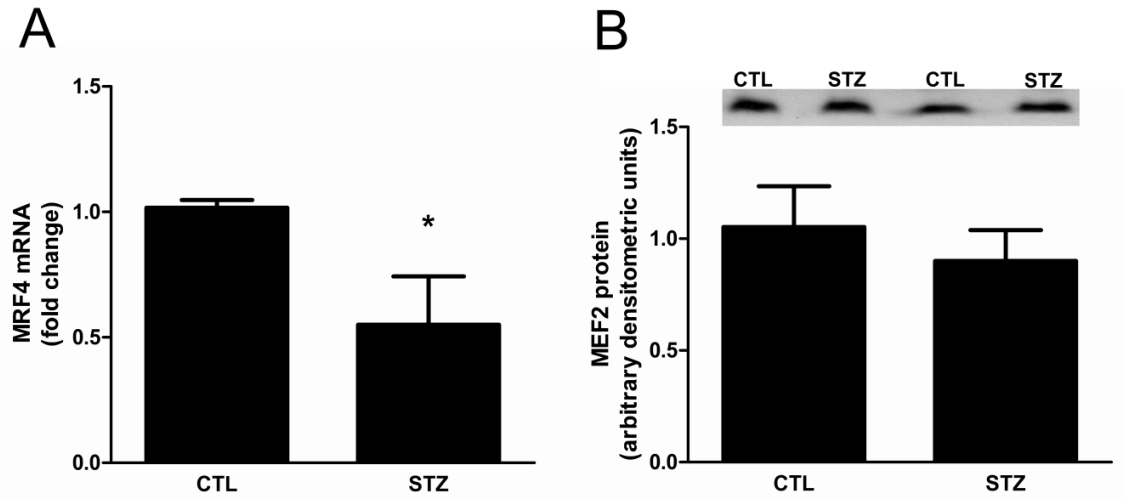

Figure 4.7. **MEF2 activity is decreased in 21day STZ-treated rat muscle. (A)** The amount of mRNA encoding the MEF2 target MRF4 in gastrocnemius muscles was measured using real time RT-PCR, normalized to 18S RNA and expressed as mean fold change relative to control ± SEM. n=6/group, **P*<0.05. **(B)** MEF2A protein in gastrocnemius muscles of control and 21d STZ-treated rats was evaluated by Western blot analysis. Equal protein loading and transfer were confirmed by Ponceau S staining. n=8/group, *P*=0.74

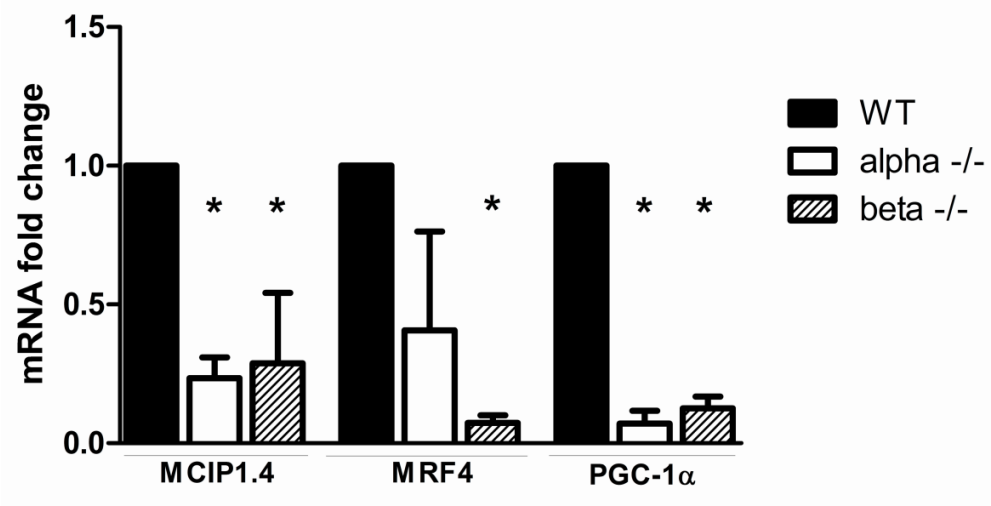

Figure 4.8. **MEF2 and NFAT signaling and PGC-1α mRNA are decreased in muscles of CnAα$^{-/-}$ and CnAβ$^{-/-}$ mice.** MRF4, MCIP1.4 and PGC-1α mRNAs in the gastrocnemius muscle of CnAα$^{-/-}$ and CnAβ$^{-/-}$ mice were measured by real-time RT-PCR, normalized to 18S RNA, and expressed as mean fold change relative to control ± SEM; n≥3/group, *P<0.05.

DISCUSSION

PGC-1α is a coactivator protein that participates in the transcriptional regulation of a variety of genes involved in energy metabolism, including the glucose transporter 4 (GLUT4) and mitochondrial biogenesis (Michael, Wu et al. 2001; Lin, Tarr et al. 2003; Rodgers, Lerin et al. 2005; Finck and Kelly 2006; Uldry, Yang et al. 2006). Hyperglycemia in Type II DM patients has been attributed, in part, to decreased expression of PGC-1α (Patti, Butte et al. 2003; Soyal, Krempler et al. 2006). Other complications of Type I and Type II DM in experimental animals include skeletal muscle atrophy and PGC-1α has been linked to the regulation of skeletal muscle protein turnover (i.e., muscle mass) and fiber type (Price, Bailey et al. 1996; Sandri, Lin et al. 2006; Wang, Hu et al. 2006). Furthermore, atrophy occurs preferentially in muscles comprised predominantly of type II glycolytic fibers during DM and other systemic disease states are also associated with loss of muscle oxidative capacity (Sandri, Lin et al. 2006; Phielix and Mensink 2008). This preferential atrophy could be linked to the reduction in PGC-1α expression since the cofactor helps to maintain the type I oxidative fiber phenotype in muscle (Lin, Wu et al. 2002).

In the present studies, the levels of PGC-1α protein and mRNA in skeletal muscle were decreased in a model of chronic (21d) STZ-induced DM. To elucidate the mechanism regulating PGC-1α expression in this system, we first tested whether CREB-mediated signaling was altered because CREB is a potent regulator of PGC-1α expression (Puigserver and Spiegelman 2003). Paradoxically, the activation state of CREB was increased in skeletal muscle of 21d STZ-treated rats. This led us to examine a relevant downstream target of CREB, DHPRα1s. DHPRα1s forms the L-type calcium

channel in the T-tubules and is a voltage sensor of excitation-contraction (EC) coupling (Pietri-Rouxel, Gentil et al.). A recent report demonstrated that knock-down of DHPRα1s results in the induction of muscle atrophy (Pietri-Rouxel, Gentil et al.). In our study, DHPRα1s mRNA was decreased in muscles from STZ-treated rats, a finding that is suggests that CREB-mediated transcription is abnormal. Several proteins are known to interact with CREB to mediate its activity, including p300 or CBP (Gonzalez and Montminy 1989). One or more of these interacting proteins could be altered in skeletal muscle of STZ-treated rats, which would result in dysfunctional CREB signaling and decreased expression of downstream targets like DHPRa1s and PGC-1a.

The Cn/NFAT/MEF2 signaling pathway has been linked to the maintenance of overall muscle mass. Cn activity, which is induced by exercise or other stimuli that raise intracellular calcium, activates two relevant downstream substrates, MEF2 and NFAT (Bassel-Duby and Olson 2006; Schiaffino, Sandri et al. 2007). These transcription factors regulate the expression of genes involved in maintenance of overall muscle mass and the oxidative fiber phenotype, including PGC-1α (Fig.9) (Bassel-Duby and Olson 2006).

Since, NFAT can be inactivated by phosphorylation by the kinase GSK-3β, we examined Akt/GSK-3β signaling and found no evidence that the pathway was activated in STZ-treated rats. Although phosphorylation of Akt on Ser-473 was slightly elevated, the increase did not seem to be physiologically relevant with regard to GSK-3β signaling or suppression of protein degradation. Tong et. al. reported similar paradoxical findings involving increased atrophy-related responses despite augmented levels of phosphorylated Akt (Tong, Yan et al. 2009).

In muscles of STZ-treated rats, extensive evidence was found to indicate that the decrease in CnA protein was the primary cause of the reduction in overall pathway signaling. The role of Cn in the regulation of PGC-1α gene expression was confirmed when PGC-1α mRNA was found to be lower in the muscles of CnAα$^{-/-}$ and CnAβ$^{-/-}$ mice. Together, these results suggest three possible interpretations. First, CnAα and CnAβ may have overlapping actions with regard to activating NFAT and MEF2. Second, CnAα and CnAβ may exhibit selectivity for either MEF2 or NFAT and inactivation of either transcription factor is sufficient to reduce PGC-1α expression regardless of the activation status of the other protein. Lastly, MEF2 and NFAT could act cooperatively to regulate PGC-1α and inactivation of either protein is sufficient to affect cofactor expression. Further studies will be necessary to delineate the intricacies of this mechanism in the context of diabetes. The reduction in PGC-1α expression may contribute to the chronic muscle atrophy induced by DM. This cofactor has been proposed to protect against atrophy through several different mechanisms. First, PGC-1α can antagonize FoxO-dependent transcription of AT-1 (Sandri, Lin et al. 2006). Decreased PGC-1α protein could perpetuate atrophy through the loss of FoxO inhibition and subsequent upregulation of AT-1. In light of the fact that AT-1 mRNA was not increased in 21 day STZ-treated rat muscle, it would seem other mechanisms are responsible for sustaining muscle wasting (data not shown). Second, the loss of PGC-1α could affect oxidative metabolism as PGC-1α is necessary to maintain the oxidative muscle fiber phenotype and helps to protect muscle cells against increased oxidative stress (Lin, Wu et al. 2002; St-Pierre, Drori et al. 2006). In other studies, we found that chronic DM reduced the proportion of oxidative MHC Type I fibers relative to glycolytic

MHC II fibers in soleus muscles (TKR et al., manuscript submitted). Therefore, the loss of PGC-1α may contribute to the progression of muscle atrophy by increasing the number of glycolytic fibers, which are more prone to undergo atrophy induced by systemic pathologic conditions (Tiao, Lieberman et al. 1997; Acharyya, Ladner et al. 2004). This could account for the perpetuation of atrophy over long periods of time.

In summary, our studies have shown that DM suppresses the Cn/NFAT/MEF2 signaling pathway and that this may perpetuate muscle wasting by downregulating PGC-1α expression. Recent reports indicate that increasing muscle contractile activity through resistance exercise may help to attenuate the muscle atrophy process (Fluckey, Dupont-Versteegden et al. 2002; Alkner and Tesch 2004). Our findings suggest that contractile activity may attenuate chronic wasting by increasing the activation state of Cn or by activating alternative calcium signaling pathways that bypass Cn, such as calmodulin-dependent kinase (CaMK) (Fig. 2.4). Lastly, our results provide a possible explanation for the myopathy that frequently develops in transplant recipients who receive Cn inhibitors as part of their immunosuppressive regimen and suggests that these drugs may make the post-surgical rehabilitation of these patients more difficult (Rennie and Wilkes 2005). A better understanding of the role of Cn in muscle atrophy will be important for improving rehabilitation of patients suffering from systemic disease or receiving immunosuppressive therapies.

CHAPTER 5

SINGLE SKELETAL MUSCLE FIBER TYPE SPECIFICITY AND SWITCHING IN ATROPHY ASSOCIATED WITH STREPTOZOTOCIN-INDUCED INSULIN-DEFICIENCY

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This chapter is in preparation for submission for publication.

H. Li contributed Fig. 5.3 and Fig. 5.4.
E. F. Eaton contributed Fig. 5.2

INTRODUCTION

Skeletal muscle atrophy is a common co-morbidity of many systemic diseases and is often an indicator of a poor prognosis for patients. The hallmark feature of atrophy is abnormally elevated rates of protein degradation (Stitt, Drujan et al. 2004; Nader 2005). Streptozotocin (STZ) treatment recapitulates diabetes mellitus (DM) in rats as the animals develop insulinopenia resulting in sustained hyperglycemia. STZ also induces skeletal muscle atrophy. Furthermore, recent studies have indicated that fast, glycolytic fibers may be more susceptible to atrophy in this chronic DM model than slow, oxidative fibers (Sandri, Lin et al. 2006). Preferential wasting of primarily fast or primarily slow muscles has been observed consistently in the past several decades. These investigations were based on whole muscle size, measured protein breakdown in whole muscle, and cross-sectional staining for markers of oxidative metabolism such as succinate dehydrogenase (Mendell and Engel 1971; Dahlmann, Rutschmann et al. 1986). Howevever, preferential wasting has never been studied on a single fiber level as defined by myosin heavy chain (MHC) isoforms. Single fiber analysis may provide critical insight into how metabolism affects the signaling programs controlling atrophy in systemic conditions like diabetes because the metabolic profiles of the fibers are coordinated based on MHC isoform expression.

Mammalian skeletal muscles are a variable mix of fiber phenotypes, distinguishable by their molecular myosin heavy chain (MHC) isoform(s), mechanical performance (fast vs. slow), metabolic properties (oxidative vs. glycolytic) (Hilber, Galler et al. 1999; He, Bottinelli et al. 2000), and contractile activation properties (Donaldson, Hermansen et al. 1978). The skeletal muscle fiber type categories derive primarily from the content and

properties of the proteins that they contain and the most definitive method of identifying single fiber type is by MHC isoform type (Schiaffino, Sandri et al. 2007). Separate genes encode four MHC isoforms in rats: MHCI, MHCIIa, MHCIIx/d and MHCIIb. Single rat muscle fibers display MHC isoform types along a gene continuum of MHCI ↔ MHCI/IIa ↔ MHCIIa ↔ MHCIIa/IIx/d ↔ MHCIIx/d/IIb ↔ MHCIIb. Rat fibers can transform as to MHC isoform/functional type from "slow" (MHCI) to "fast" (MHCII) or the reverse along this continuum (Schiaffino and Reggiani 1994; Staron, Kraemer et al. 1999). In a single fiber, only one MHC gene is active at a given time and it coordinates the expression of a program of genes that determine its contractile and metabolic phenotype (Pette and Vrbova 1999; McCullagh, Calabria et al. 2004; Schiaffino, Sandri et al. 2007).

The changes in mRNA expression of MHC and coordinated phenotypic genes are orderly and follow the MHC gene continuum, but a fiber's mRNA expression may not reflect its MHC isoform(s) and coordinated proteins at a given point in time. The rate limiting step in fiber MHC isoform type transformation is fiber protein turnover, which may take 2-3 weeks (Goll, Neti et al. 2008). Changes in mRNA levels for different contractile protein isoforms precede fiber protein turnover by variable time intervals which makes the time pattern of changes in mRNA levels non-predictive of fiber protein isoform content (Pette and Vrbova 1999). As a result, a change in fiber MHC isoform is the critical indicator of actual transformation of fiber type rather than changes in mRNA expression.

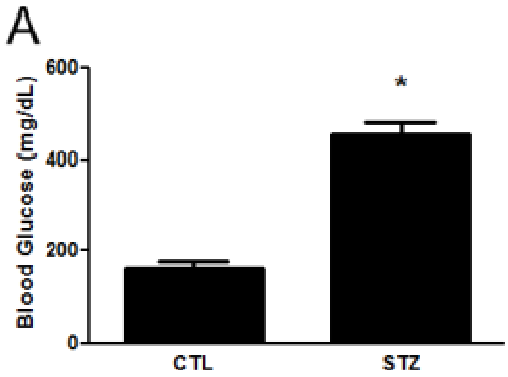
In this study, animals were not sacrificed until 21 days post-STZ injection in order to allow time for fiber MHC type switching to manifest as a change MHC isoform. Since

fast, glycolytic and slow, oxidative muscles are of predominant MHCII and MHCI fiber type, respectively, there is reason to believe that the genetic responses associated with MHC type may play a role in STZ-induced atrophy. The purpose of this study was to determine if MHCII fibers atrophy to a greater extent than MHCI fibers, irrespective of muscle of origin, during STZ-induced skeletal muscle atrophy and if this is accompanied by switching of myosin heavy chain (MHC) isoforms at the single fiber level. Our findings demonstrate fiber type switching from MHCI to MHCII in muscles that are predominantly type I (soleus) accompanied by a selective decrease in the size (cross-sectional area) of MHCII fibers regardless of muscle of origin. MHC proteins are long-lived and 21d is the earliest time point at which changes in MHC fiber-type would be detectable. The data presented here are likely indicative of the very beginning of the fiber-type transition. These data suggest that in this chronic DM model muscle fibers switch to a phenotype that is more susceptible to atrophy, thus potentially perpetuating skeletal muscle wasting.

RESULTS

STZ REDUCED BODY WEIGHT BY ELEVATING PROTEIN BREAKDOWN IN SKELETAL MUSCLE.

Initially, all of the 6 wk-old rats had normal plasma glucose levels and similar body weights. By the time of sacrifice, 21 days post-injection, the STZ treated rats were severely hyperglycemic and significantly smaller than CTL (Fig. 5.1A and B). Elevated abundance of the actin fragment in the gastrocnemius of STZ rats confirmed that the noted difference in size was partially due to increased protein degradation (Fig. 5.1C). The 14kDa actin fragment detected is generated by cleavage of full length actin by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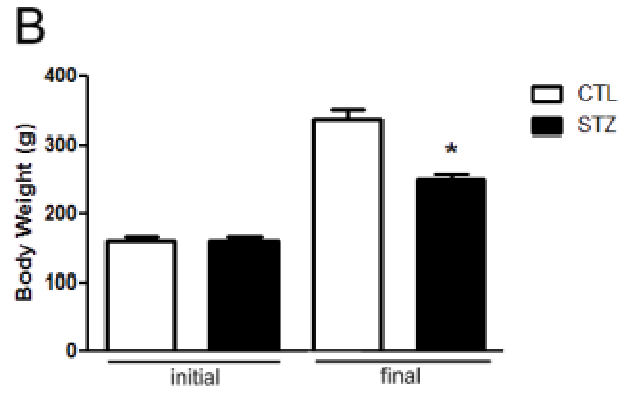
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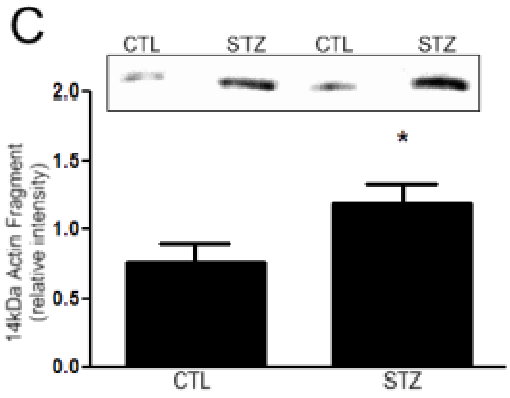
C
CTL
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2.0
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STZ

Figure 5.1. **STZ-treated rats have elevated blood glucose, decreased body mass, increased protein degradation.** (A.) Blood glucose levels confirmed that STZ-treated rats were severely hyperglycemic (392 ± 25mg/dL) at the time of sacrifice compared to CTL (134 ± 18mg/dL). (B.) Initially all animals were approximately 160g at the time of injection. At the time of sacrifice, STZ-treated rats had decreased body mass (251 ± 6g) compared to CTZ (336 ± 13g). (C.) Protein levels of the 14kDa actin fragment, which is generated by caspase-3 cleavage of full length actin, are elevated in STZ-treated animals ~1.5X compared to CTL. *P<0.05. All data are Mean ± SEM, n=7.

caspase-3 and is frequently used as a marker of protein breakdown in skeletal mucle (Du, Wang et al. 2004).

STZ DID NOT AFFECT SPECIFIC FORCE OR STRUCTURAL INTEGRITY OF FIBERS.
No significant difference in single fiber specific force (P_0/CSA) was observed in STZ-treated rats vs. CTL for any fiber type (Fig. 5.2A). Stable specific force values indicate that the integrity of the contractile apparatus was maintained in fibers of STZ-treated animals, which was confirmed by pictures taken of mounted fibers post- contraction (Fig. 5.2B). The sarcomere length of the mounted fibers was decreased as a result of contraction, but STZ-treated fibers did not differ from CTL (Fig. 5.2B).

STZ CAUSED MUSCLE FIBER TYPE SWITCHING FROM MHCI TO MHCII.
STZ SOL muscles demonstrated a trend of fiber type switching from MHCI to MHCIIx/d+b fibers compared to CTL (Fig. 5.3A). There was approximately a 9% reduction in the number of MHCI fibers, ~7% increase in the number of MHCIIa fibers, and ~2% increase in the number of MHCIIx/d+b fibers (Fig. 5.3A). These data are consistent with a fiber-type switch along the gene continuum. A chi square trend analysis was used to analyze histogram data of all the fiber types from a pool of 500 fibers from five rats per treatment group. GAST muscles from CTL and STZ-treated rats were similar with respect to MHC fiber type proportions (Fig. 5.3B).

STZ CAUSED A PREFERENTIAL REDUCTION OF CSA OF MHCII FIBERS.
In SOL muscle MHCI is the predominant fiber type and those fibers do not demonstrate a significant decrease in CSA in this model (Fig. 5.4A and B). Only the MHCII fibers in the SOL are significantly smaller (Fig. 5.4B). In the GAST muscle, which is predominantly MHCII, both MHCI and MHCII fibers demonstrate a significant

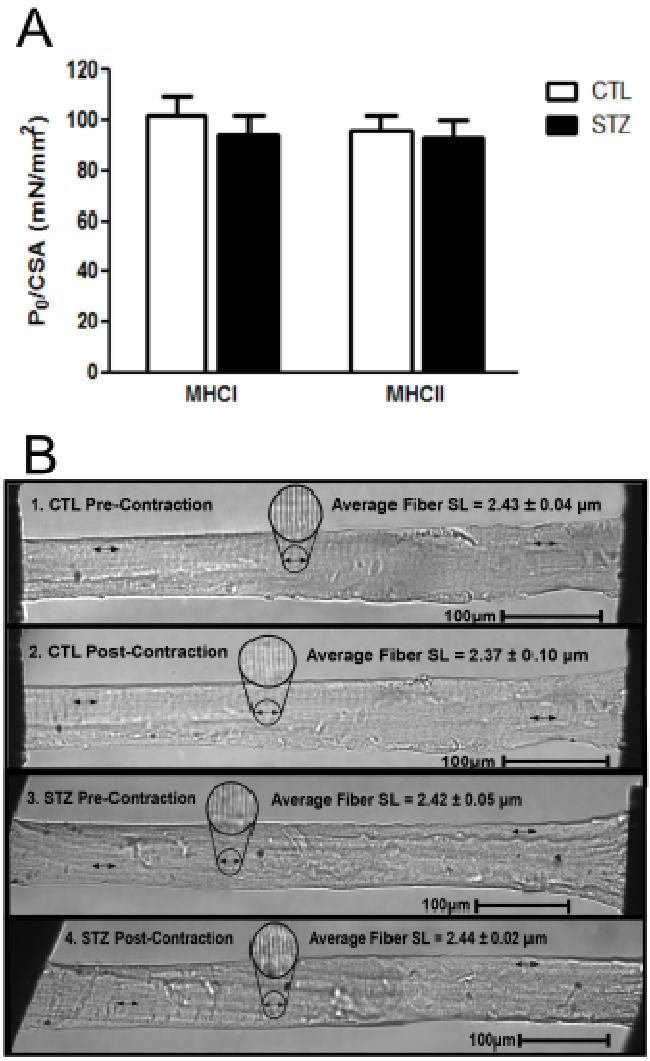


Figure 5.2. **Fibers from STZ-treated rats maintained structural integrity.** (A.) No significant difference was observed in single fiber specific force (P_0/CSA) by treatment group for MHCI and MHCII fibers. Date is mean ± SEM, n= 97 fibers. (B.) Pre-contraction image indicates sarcomere length (SL) at three places along the fiber. Inset is a magnification to show z-lines. Post-contraction image indicates the same measurements of SL with a magnification inset. The SL decreased slightly as a result of contraction, but the longitudinal and transverse integrity of the z-lines is preserved.

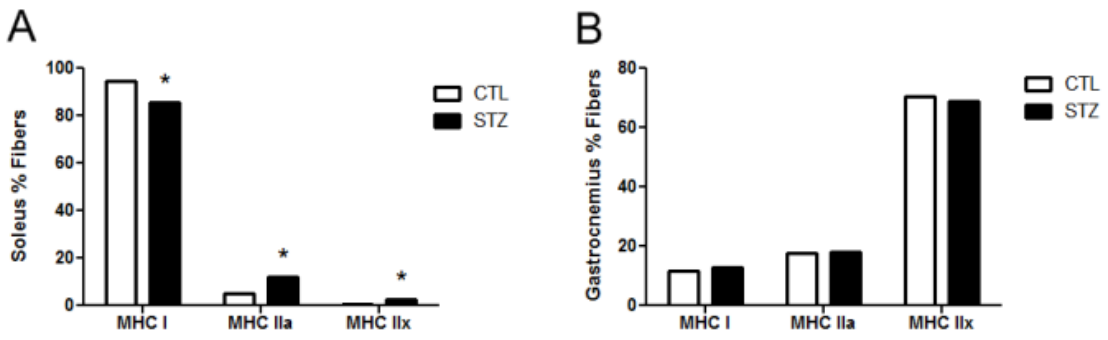
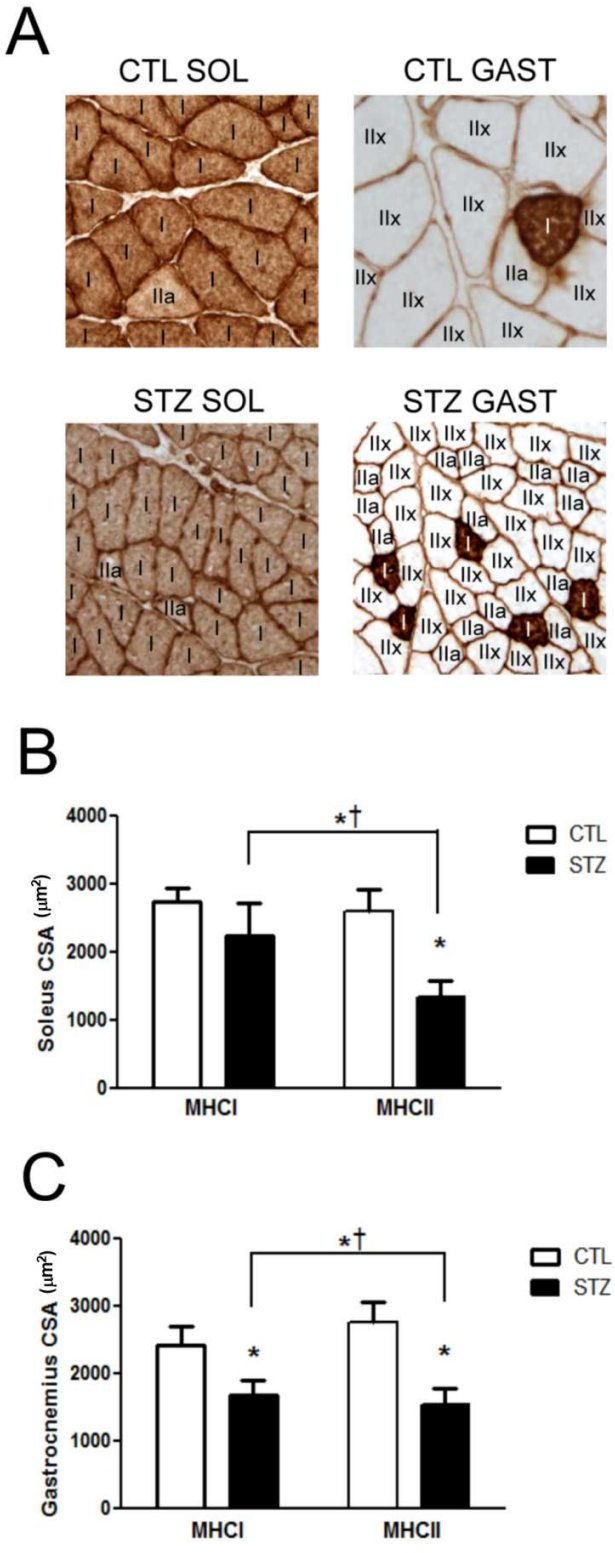


Figure 5.3. **Fiber type switching from MHCI to MHCII in soleus muscle of STZ-treated rats.** (A.) Chi square analysis for linear trend was used to compare MHC fiber type proportions in SOL muscles from CTL and STZ-treated rats. STZ-SOL contained significantly more MHCII fibers compared to CTL-SOL (*P<0.05). (B.) In contrast, GAST muscles from STZ-treated rats contained similar proportions of fiber types compared to CTL-GAST. Data are expressed as percentages, n=500 fibers from each of the four muscle types.


A
CTL SOL
CTL GAST
STZ SOL
STZ GAST
I
IIa
IIx
B
Soleus CSA (μm²)
4000
3000
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*†
*
CTL
STZ
MHCI
MHCII
C
Gastrocnemius CSA (μm²)
4000
3000
2000
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*
CTL
STZ
MHCI
MHCII

Figure 5.4. **MHCII fibers are more susceptible to STZ-induced atrophy than MHCI fibers in both the soleus and gastrocnemius.** (A.) SOL and GAST from CTL and STZ-treated rats were immunostained for MHCI. Decreased CSA was apparent in STZ-treated rats. Cross-sections are shown at 20X magnification. (B.) Soleus muscle sections were analyzed for CSA based on MHC type. MHCII fibers decreased significantly. *P<0.05. (C.) Similar analyses were performed using GAST muscle, which also contained significantly smaller MHCII fibers as well as MHCI fibers. However, the decrease was significantly greater for MHCII than MHCI fibers (†P<0.001), indicating that MHCII fibers are more susceptible to wasting. *P<0.05. Data are mean ± SEM , n=10 fibers per mouse from four mice.

decrease in CSA (Fig. 5.4B and C). However, the MHCII atrophy significantly more (-757μm^2) than the MHCI (-1228μm^2) (Fig. 5.4C). These data indicate that MHCII fibers are more susceptible to the muscle wasting that accompanies STZ-induced insulin deficiency than MHCI fibers.

DISCUSSION

The results of this study indicate that the skeletal muscle wasting that accompanies systemic diseases like DM is selective at the single fiber level and that fiber type switching may perpetuate chronic atrophy. Numerous studies have suggested preferential atrophy of glycolytic myofibers in systemic disease models based on changes in whole muscle size or rates of protein degradation (Sandri, Lin et al. 2006). Others have found decreased CSA selectively in glycolytic myofibers based on staining with markers for mitochondrial content (Armstrong, Gollnick et al. 1975). This study demonstrates for the first time that atrophy in a model of STZ-induced insulin deficiency occurs predominantly in fast, glycolytic myofibers as designated by MHC isoform II irrespective of the muscle of origin.

Metabolism has been implicated as having a major role in the atrophy that occurs with systemic diseases like diabetes. In fact, in the skeletal muscle of diabetic patients there is a perturbation not only of glycolytic metabolism (decreased glucose uptake) but also of oxidative metabolism (accumulation of lipids) (Pan, Lillioja et al. 1997). Different muscle fiber types are characterized by glycolytic or oxidative metabolism, expressing specific gene programs associated with their unique metabolic properties. These metabolic gene programs are coordinated by the MHC gene that is being expressed in the fiber. Therefore, studying the mechanisms of atrophy in single fibers may provide insights into how the specific metabolic properties affect the signaling pathways responsible for protein degradation in various conditions, without the possible complications of a mixed fiber type population. Futhermore, single fiber analysis would

help to delineate the roles of intrinsic muscle signaling pathways versus those of other cell populations within the muscle (immune cells, vasculature, etc.).

Decreased CSA of myofibers in STZ-treated rats is a consequence of increased protein degradation, manifested as an increase in the level of a 14-kDa actin fragment in STZ GAST muscles. The rate of proteolysis, measured as tyrosine release, and ubiquitin expression are also upregulated in these muscles (Chapter 4, Fig. 4.1) indicating an overall increase in protein degradation. Specific force (P_0/CSA) was unchanged in muscle from STZ-treated rats compared to controls demonstrating that STZ-induced atrophy does not affect the force generating function of the contractile apparatus (Donaldson, Hermansen et al. 1978). Therefore, the decrease in gross force (P_0) generation in STZ-treated animals is wholly a result of decreased CSA. These results suggest that prevention of increased myofibrillar protein degradation in MHCII fibers is critical to the success of any therapy for atrophy associated with insulin deficiency.

Fiber type switching from MHCI, which are more resistant to wasting in this model, to MHCII may perpetuate chronic atrophy. Previous studies reported changes in oxidative capacity of fibers, based on mitochondrial stains, during STZ-induced atrophy in rats (Armstrong, Gollnick et al. 1975; Armstrong and Ianuzzo 1977) but oxidative capacity of a population of fibers can change in the absence of a change in MHC gene expression (He, Bottinelli et al. 2000; Schiaffino, Sandri et al. 2007). This is the first study to demonstrate MHCI to MHCII fiber type switching in STZ-induced atrophy.

Previous studies that have identified MHC isoform expression patterns in animal models have used fiber counts from entire microscopic fields of muscle cross-sections to

compare fiber numbers or relative proportions of fiber types. However, this methodology is impractical for clinical studies, which must rely on muscle biopsies where the number of fibers is severely limited. In this study we validate a methodology using 100 contiguous fibers in a cross-section for fiber-typing. In control animals, the proportions of MHCI and MHCII fibers was similar to those reported previously indicating that a count of 100 contiguous fibers is sufficiently representative of the fiber type composition of the whole muscle (Song, Ryder et al. 1999; Buhl, Jessen et al. 2001). Futhermore, this analysis demonstrated a significant trend in changing gene expression in the direction of MHCI to MHCII in the SOL muscle of STZ-treated rats and is the first demonstration of MHC fiber type switching in a model of chronic atrophy associated with insulin deficiency.

The results of this study suggest that skeletal muscle atrophy occurs preferentially in MHCII fibers in this model of DM and may be similar to other systemic diseases. Furthermore, overall muscle wasting in patients experiencing chronic atrophy may be perpetuated by fiber-type switching from MHCI to MHCII. Further understanding of the differences in cellular signaling mechanisms regulating proteolysis in MHCI and MHCII myofibers is necessary to development of successful therapies for atrophy associated with chronic insulin deficiency and other systemic diseases.

CHAPTER 6

DISCUSSION AND CONCLUSIONS

Diabetes mellitus (DM) is a common disease that significantly affects quality of life and shortens life expectancy. The established pathology of DM is impaired cellular glucose uptake, resulting in persistent hyperglycemia. Skeletal muscle is closely linked with the development of DM as it is the major system for glucose uptake and utilization. However, the pathophysiological mechanisms of DM also cause tremendous changes in skeletal muscle in terms of mass, functional capacity, and metabolism. Skeletal muscle atrophy results in smaller individual fibers and DM patients exhibit reduced strength, endurance, and muscle functional capacity (Sun, Liu et al. 2008). Studies also indicate that fiber-type switching occurs with atrophy and that oxidative fibers are more resistant to DM-induced atrophy than glycolytic fibers (Armstrong, Gollnick et al. 1975; Vondra, Rath et al. 1977; Hegarty and Rosholt 1981; Lithell, Lindgarde et al. 1981; Krotkiewski, Bylund-Fallenius et al. 1983; He, Watkins et al. 2001). These studies suggest a close relationship between muscle fiber size and composition, although the signaling mechanisms allowing for cross-talk between the two are poorly understood, the transcriptional co-activator PGC-1α appears to play a pivotal role in regulating the oxidative metabolism of muscle fibers and has been implicated in maintaining muscle size. A recent study by Sandri et. al. demonstrated that muscle specific transgenic overexpression of PGC-1α blocked denervation-induced atrophy by antagonizing Foxo-dependent upregulation of AT-1 and MuRF-1 (Sandri, Lin et al. 2006). Furthermore, PGC-1α is thought to be an integral signaling component related to glucose uptake and utilization both in the liver and in skeletal muscle. Adenoviral overexpression of PGC-1α in primary hepatocytes stimulated expression of key gluconeogenic enzyme genes, including PEPCK, fructose 1,6-bisphosphatase, and glucose-6-phosphatse (Yoon,

Puigserver et al. 2001). In both C_2C_{12} (mouse) and L6 (rat) cultured myotubes, adenoviral overexpression of PGC-1α increased GLUT4 gene expression and stimulated both basal and insulin-stimulated glucose transport (Michael, Wu et al. 2001). These two roles of PGC-1α make it especially relevant in the context of DM, where hypoinsulinopenia and hyperglycemia contribute to structural and functional changes in muscle. In fact, PGC-1α has previously been shown to be downregulated in several models of muscle atrophy including DM (Sandri, Lin et al. 2006). However, our studies are the first to evaluate both the signaling mechanisms regulating PGC-1α and the potential physiological effects of its downregulation in muscle *in vivo* during chronic atrophy.

CELLULAR MECHANISMS REGULATING PGC-1α IN STZ-DM

CREB SIGNALING IS ABNORMAL IN STZ-DM

PGC-1α expression can be regulated by the transcription factor CREB. In canonical signaling models, CREB is activated by phosphorylation by PKA at Ser133 in response to elevated cAMP. In our STZ-induced DM rats, CREB phosphorylation in skeletal muscle is increased significantly (Chapter 4, Fig. 4.3), whereas PGC-1α expression is decreased, suggesting that CREB is not the major transcription factor regulating PGC-1α in skeletal muscle during STZ-DM (Chapter 4). Therefore, we examined another known gene target of CREB, DHPR (DiHydroPyridine Receptor)-α1s, and found it to be transcriptionally downregulated as well (Chapter 4, Fig. 4.3). These data indicate that CREB signaling is abnormal and its transcriptional activity misregulated in this model. Besides phosphorylation by PKA, CREB has several other levels of regulation.

Ca^{2+} Regulation of CREB

Several lines of evidence purport that CREB is regulated by Ca^{2+} signals in addition to cAMP (Sheng, McFadden et al. 1990; Dash, Karl et al. 1991; Sheng, Thompson et al. 1991). *In vitro* studies indicated that CaMKIV can phosphorylate CREB at Ser133; however, this mechanism seems to be less effective *in vivo* (Dash, Karl et al. 1991; Sheng, Thompson et al. 1991; Matthews, Guthrie et al. 1994; Sun, Enslen et al. 1994). More recent studies indicated that Ca^{2+} signals can destabilize the CREB:CBP (CREB binding protein) complex through secondary phosphorylation events, suggesting that the effect of Ca^{2+} signaling on CREB activity is through a mechanism other than Ser133 phosphorylation (Kornhauser, Cowan et al. 2002). Indeed, evidence exists to implicate the bZIP (basic leucine zipper) domain in mediating CREB association with a Ca^{2+} regulated cofactor (Sheng, Thompson et al. 1991). As the TORC (transducer of regulated CREB) coactivator proteins are known to interact with the bZIP domain, they are likely candidates for this role.

TORC proteins regulate CREB in response to Ca^{2+}-signaling

There are three TORC family members (TORC1-3) that were identified in a screen for modulators of CREB activity and all share a N-terminal coiled-coil domain that associates with the CREB bZIP domain (Conkright, Canettieri et al. 2003). Overexpression of TORC1 and and TORC2 in HEK-293 cells induced several cAMP-responsive genes and co-expression of a dominant negative CREB polypeptide blocked the induction, indicating that TORC is a direct modulator of CREB activity (Conkright, Canettieri et al. 2003). Furthermore, TORC potentiates CREB activity independently of Ser133 phosphorylation by PKA, suggesting that it represents a second level of CREB regulation (Conkright, Canettieri et al. 2003).

A 2004 study by Screaton et. al. elaborated on the role of Ca^{2+}-driven TORC-dependent activation of CREB (Screaton, Conkright et al. 2004). In this study, TORC2 was found to be sequestered in the cytoplasm through a phosphorylation-dependent interaction with the scaffolding protein 14-3-3. Phosphorylation at Ser171 by SIK (salt inducible kinase) 2 prompted nuclear export of TORC2 by masking the nuclear localization sequence and was the major phosphorylation site involved in the interaction with 14-3-3 (Fig. 6.1). Cyclosporine A, a Cn inhibitor, interfered with dephosphorylation of TORC2 at Ser171and blocked CREB target gene activation (Screaton, Conkright et al. 2004). These data suggest that the activation of CREB by Ca^{2+} signaling is likely mediated by the Cn pathway, through dephosphorylation of TORC allowing for its nuclear translocation and co-activation of CREB (Fig. 6.1).

Studies in primary mouse myotubes have shown that the TORCs are potent inducers of PGC-1α expression (Wu, Huang et al. 2006). Adenoviral overexpression of all three TORCs in myotubes increased PGC-1α expression, PGC-1α driven mitochondrial biogenesis, and oxidative capacity (Wu, Huang et al. 2006). Therefore, the TORCs are likely candidates as central mediators of both cAMP and Ca^{2+} regulation of PGC-1α and oxidative capacity in muscle.

Future Directions for Cn-dependent TORC regulation of CREB

Our lab has recently initiated studies to determine whether the incongruity between PGC-1α transcription and CREB phosphorylation (Fig. 4.3) is due to downregulation of TORC. Preliminary experiments indicate that TORC1 protein levels are decreased in muscle from STZ-rats as well as L6 rat muscle cells treated with glucocorticoids. It will be important, given that active TORC localizes to the nucleus, to

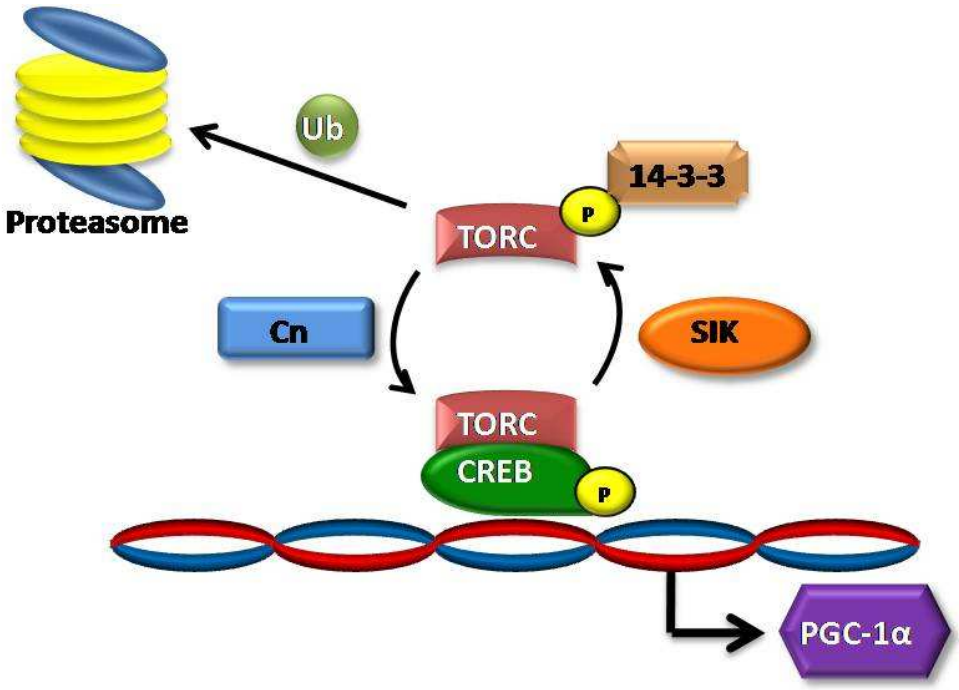

Figure 6.1. **Phosphorylation-dependent regulation of TORC.** Phosphorylated TORC is sequestered in the cytoplasm through its interaction with 14-3-3. Upon dephosphorylation by Cn, TORC can translocate to the nucleus and coactivate CREB dependent transcription of PGC-1α.

separate nuclear and cytoplasmic fractions of TORC to give a clearer picture of activation status. Furthermore, cytoplasmically sequestered TORC is targeted for proteasomal degradation. Proteasome inhibitors can be used in L6 rat muscle cells treated with glucocorticoids to determine whether there is increased TORC protein turnover. If TORC protein is being degrade, blocking the proteasome should result in a buildup of polyubiquitinated TORC protein that can be easily detected by western blot. Concurrently, a series of luciferase reporter studies in L6 cells will be used to determine whether overexpression of TORC can rescue PGC-1α expression in the face of glucocorticoid treatment and whether knock-down of TORC inhibits CREB-dependent regulation of PGC-1α transcription. Furthermore, a CHIP (chromatin immuno-precipitation) assay in the same cells will determine whether TORC and CREB are present at the same CRE-site in the PGC-1α promoter, thus indicating a direct mechanism. These experiments would determine if STZ-DM results in downregulation of TORC activity, thereby reducing its ability to co-activate CREB to drive PGC-1α transcription.

As TORC is regulated by Cn signaling, and Cn activity is lower in STZ-DM rat muscle (Fig. 4.4), a decrease in TORC function could provide a plausible explanation for the reduction in CREB transcriptional activity despite increased CREB phosphorylation. Decreased Cn signaling could contribute to increased phosphorylation and cytoplasmic sequestration of TORC. It would be interesting to examine TORC protein levels and the ratio of cytoplasmic: nuclear TORC in the muscle of CnA-/- mice or in rats treated with cyclosporine A as compared to control. Furthermore, luciferase reporter assays in L6 cells co-expressing activated Cn could reveal whether Cn can induce CREB-mediated

expression of PGC-1α. These experiments would determine if decreased Cn signaling inhibits TORC activity, effectively blocking CREB-mediated PGC-1α transcription in STZ-DM.

Potential role for Cn and TORC regulation of CREB in DM and transplant patients

In our rat model of STZ-induced DM, both Cn signaling and CREB activity (independent of its phosphorylation state) are decreased (Fig. 4.3 and 4.4). These data support the hypothesis that decreased Cn activity results in cytoplasmic sequestration of TORC and subsequent decreased CREB activity in the skeletal muscle of these rats. Interestingly, in the course of our studies we attempted to recapitulate the effect of Cn suppression observed in STZ-DM using cyclosporine A treatment, but the animals became hyperglycemic, thus complicating the interpretation of results. Further review of the literature revealed that the use of cyclosporine A and other Cn inhibitors as immunosuppressive therapies is frequently associated with the development of pancreatic β cell failure and new-onset DM in organ transplant patients (Filler, Neuschulz et al. 2000; Al-Uzri, Stablein et al. 2001). The effect of Cn on TORC may provide an explanation for this phenomenon.

Elevations in blood glucose, such as occur after meals, stimulate insulin release from pancreatic β cells. A number of hormones like GLP (glucagon-like peptide) 1, are induced by elevations in blood glucose levels to modulate β cell gene expression, thus preventing inappropriate insulin release when glucose levels are low. CREB is thought to mediate the effects of GLP-1 and other glucose signaling hormones on the pancreatic β cells (Hui, Nourparvar et al. 2003). In fact, transgenic mice expressing a dominant negative CREB polypeptide in β cells develop DM due to reduced IRS2 expression, a

direct target of CREB (Jhala, Canettieri et al. 2003). These findings, taken together, lead to the hypothesis that cyclosporine A treatment and the subsequent suppression of Cn signaling in patients may result in decreased nuclear TORC and decreased CREB activity. This, in turn, would lead to a loss of glucose-regulated insulin release from β cells, development of hyperglycemia, and ultimately new-onset DM.

Future Directions for cyclosporine A and TORC

Hyperglycemia in transplant patients is a frequent side-effect of cyclosporine A treatment is often considered "the lesser of two evils" when confronted with the alternative of organ rejection. Nonetheless, these patients also develop new-onset DM and muscle atrophy post-surgically, which can both contribute to future organ damage. Previously unknown perturbations in metabolism and energy homeostasis caused by Cn suppression may be major contributors to both new-onset DM and muscle atrophy in these patients. A more complete understanding of the physiological effects of Cn suppression is critical to improve post-surgical management of transplant patients. To this end, it will be important to understand Cn regulation of TORC-mediated CREB activity in pancreatic β cells. Real-time RT-PCR or gene arrays of mRNA from the pancreas of cyclosporine A treated animals would be useful in determining whether cyclosporine A inhibits transcriptional expression of CREB-regulated genes that are known to be involved in the insulin response. If so, further analysis could determine whether misregulation of these genes is caused by defects in CREB phosphorylation at S133 or by downregulation of TORC. If the deficiency is attributable to TORC, it may be possible to overexpress or activate Cn to rescue the phenotype. Lastly, future experiments should

determine how suppression of Cn, TORC, and CREB transcriptional activity affect glucose metabolism in skeletal muscle as well, as it is the major site of insulin action.

CN SIGNALING IS SUPRESSED IN STZ-DM

In addition to its proposed role in regulating TORC, Cn signaling through the transcription factors NFAT and MEF2 has also been implicated in regulation of skeletal muscle differentiation, hypertrophy, and fiber-type specification (Olson and Williams 2000). Cn is activated by sustained low-amplitude calcium waves like those that are produced by endurance exercise-type contractile activity. Activated Cn drives the expression of a subset of genes which control the MHCI, oxidative muscle fiber phenotype. As such, Cn has long been thought of as a sensor of neural signaling associated with contractile activity and a master regulator of muscle adaptation (Bassel-Duby and Olson 2006). Our results are consistent with this theory; they also suggest that the primary effector of muscle adaptation in response to Cn signaling may be PGC-1α (Chapter 4). Cn signaling through NFAT and MEF2 maintains MHCI muscle fibers through transcription of "slow"-type myofibrilliar proteins like troponin I (Chin, Olson et al. 1998; Schiaffino, Sandri et al. 2007). However, the MHCI fiber phenotype also has a metabolic component. Muscle-specific Cn transgenic mice had an increased proportion of MHCI fibers that demonstrated a corresponding oxidative phenotype, yet it was unclear how Cn regulated the metabolic gene program (Naya, Mercer et al. 2000). The answer to this puzzle became clearer when it was learned that transgenic mice expressing PGC-1α specifically in muscle had the same phenotype as the Cn transgenic mice (Lin, Wu et al. 2002). PGC-1α, through its role as a transcriptional co-activator, drives genes that are involved in mitochondrial biogenesis and fatty acid oxidation, which are hallmarks of

MHCI, oxidative muscle fibers. Considered altogether, these data suggest that PGC-1α mediates the effects of Cn signaling on the metabolic gene program associated with type I muscle fibers. In fact, PGC-1α likely represents a convergence of several signaling pathways and coordinates the diverse genetic programs that regulate fiber type plasticity and adaptation.

Future Directions for Cn regulation of PGC-1α

Previously, *in vitro* co-transfection studies indicated that NFAT does not transduce PGC-1α transcription alone but rather acts synergistically with MEF2 (Handschin, Rhee et al. 2003). MEF2 alone was able to transduce PGC-1α, but the effect was enhanced when cells were transfected to co-express NFAT (Handschin, Rhee et al. 2003). PGC-α was also shown to co-activate MEF2, thus forming a positive feed-back loop driving its own expression (Handschin, Rhee et al. 2003). All of these studies were performed in C_2C_{12} mouse muscle cells and it remains to be proved that all of these transcriptional regulators interact *in vivo*. Using adenoviral vectors to overexpress tagged forms of these various proteins in the hindlimb muscles of animals as well as PGC-1α-luciferase constructs, it may be possible to characterize the individual roles of MEF2 and NFAT in mediating the effects of Cn on PGC-1α expression.

PHYSIOLOGICAL IMPACT OF SUPPRESSED PGC-1α EXPRESSION IN STZ-DM

MUSCLE FIBERS TRANSITION FROM MHCI TO MHCII IN STZ-DM

Given that PGC-1α and Cn signaling drive the MHCI gene program and transgenic overexpression of either is sufficient to increase the proportion of MHCI fibers in skeletal muscle, it follows that in STZ-rats, where we found decreased Cn signaling and PGC-1α(Chapter 4), there would be fewer MHCI fibers. In fact, we observed a

decreased proportion of MHCI fibers, and a corresponding increase in the proportion of MHCII fibers, in the soleus muscle of STZ-rats (Chapter 5, Fig. 5.3). The soleus is primarily composed of MHCI fibers. A similar fiber-type switching phenomenon was not observed in the gastrocnemius muscle, which is primarily composed of MHCII fibers, possibly because there are so few MHCI fibers to begin with. Importantly, this is consistent with the observation that diabetic patients have reduced oxidative capacity in skeletal muscle as well as decreased resistance to fatigue (Lithell, Lindgarde et al. 1981; He, Watkins et al. 2001).

MHCII FIBERS ARE MORE SUSCEPTIBLE TO ATROPHY IN STZ-DM

Our studies also demonstrate that MHCII fibers are more likely to undergo atrophy than MHCI fibers in 21d STZ-rats (Chapter 5, Fig. 5.4). These findings are consistent with observations by others that oxidative fibers are more resistant than glycolytic fibers to atrophy associated with systemic diseases(Armstrong, Gollnick et al. 1975; Sun, Liu et al. 2008). Clearly, this phenomenon of fiber-type switching from MHCI to MHCII and preferential wasting of MHCII fibers has implications for the strength, functional capacity, and endurance of DM patients. However, the physiological implications of fiber-type switching may extend to the actual mechanisms of perpetuating chronic atrophy. One can imagine a model in which the MHCII, glycolytic fibers would waste first upon initiation of the atrophy process and result in an immediate observable decrease in muscle size. However, subsequent fiber-type switching would reduce the number of atrophy-resistant MHCI, oxidative fibers and provide a new population of MHCII, glycolytic fibers. These new MHCII fibers would then waste, thus maintaining atrophy long-term (Figure 6.2). This prolonged muscle atrophy provides a constant

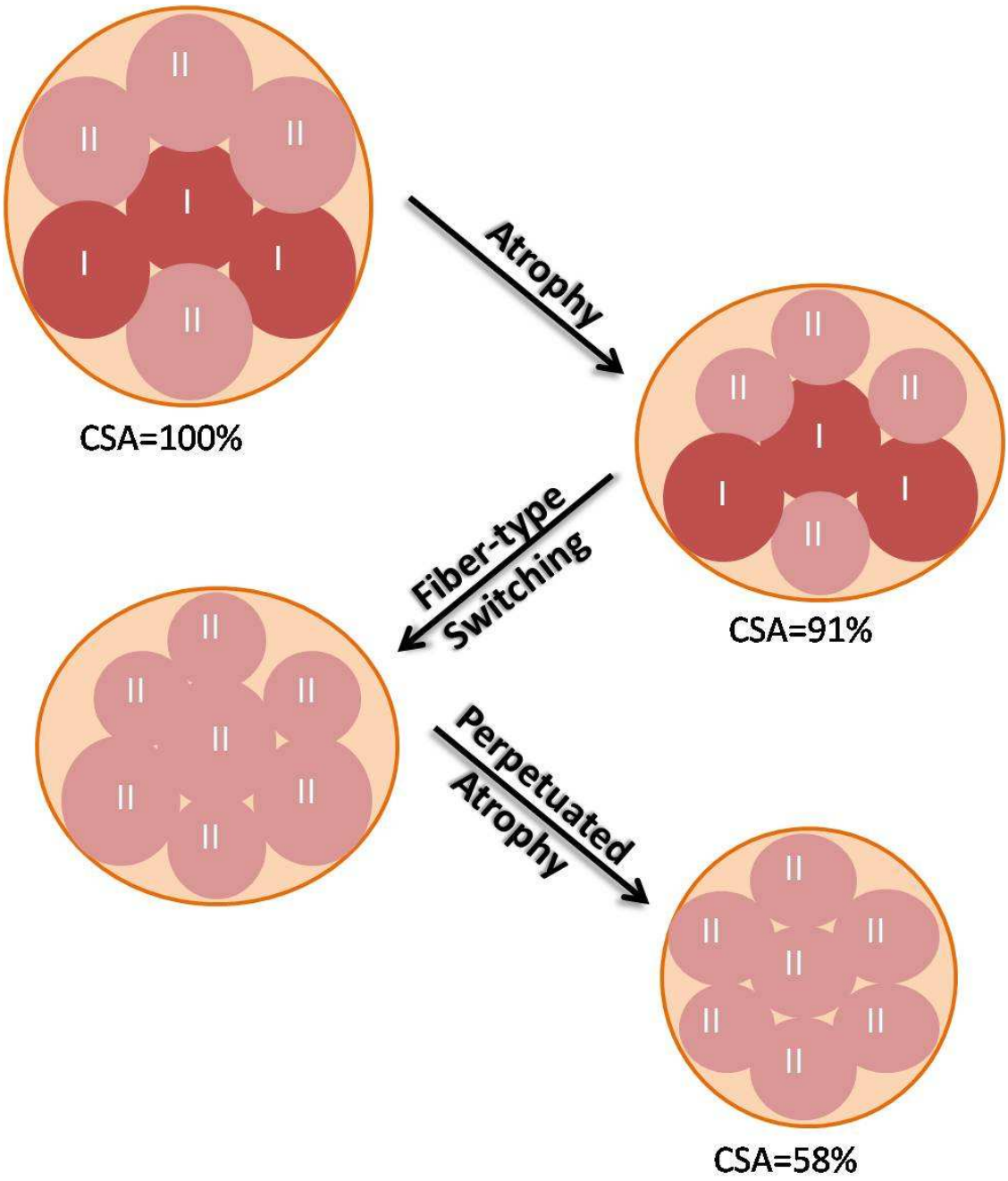
II
I
Atrophy
CSA=100%
CSA=91%
Fiber-type
Switching
Perpetuated
Atrophy
CSA=58%

Figure 6.2 **Hypothetical model showing the role of fiber-type switching in sustaining skeletal muscle atrophy.** In systemic disease, initial atrophy signals induce atrophy primarily in the MHCII fibers. To compensate for this loss, MHCI fibers switch to MHCII. Changes in PGC-1α expression likely contribute to this switching process. The new population of MHCII fibers then wastes further, perpetuating muscle atrophy.

source of amino acids and other energy precursors to more vital organs in conditions of systemic disease.

THE LINK BETWEEN FIBER-TYPE AND ATROPHY

Atrophy in systemic diseases does not occur acutely. While the signals that initiate the process may be acutely modulated, the atrophy continues over a long period, sometimes years, in the progression of diseases. Most of the animal atrophy models that have been studied to date are acute models, demonstrating severe atrophy in short periods of time. However, many myofibrillar proteins, like the MHCs, have much longer half-lives and fiber-type switching cannot be observed in these models. Moreover, many of the "atrogene" transcriptional programs that have been identified in acute models, such as the ubiquitin ligases AT-1 and MuRF-1, are likely to be involved in the initiation of the atrophy process but recent studies utilizing chronic atrophy models have demonstrated that these effectors return to basal levels within 7-14days after the stimulus (Sacheck, Hyatt et al. 2007). In sharp contrast, PGC-1α expression in these models decreased over the first several days and remained suppressed throughout the course of the study (Sacheck, Hyatt et al. 2007). In our model, PGC-1α expression was suppressed at 21d post-stimulus, but AT-1 mRNA was unchanged and MuRF-1 mRNA was significantly suppressed (Chapter 4 and data not shown). Interestingly, ubiquitin gene expression was substantially increased, indicating a continued need for new ubiquitin protein to maintain atrophy (Chapter 4, Fig. 4.1). These data indicate that some prototypical "atrogenes" may be initiators of atrophy but they are not required to sustain the process (Attaix and Baracos 2010). We propose that suppression of PGC-1α and other pathways involved in

muscle fiber metabolism and energy homeostasis may be necessary for maintaining the atrophy process.

The connection between skeletal muscle atrophy in disease pathology and energy homeostasis is often overlooked but the relationship may prove to be the key to understanding how atrophy occurs in these conditions. Skeletal muscle serves as the main amino acid reservoir for other organs and increased muscle protein breakdown is likely to be an adaptive response under conditions of perturbed energy metabolism such as DM, sepsis, cancer, etc. Therefore, the signaling pathways that coordinate muscle adaptation to energy requirements are excellent candidates for mediating sustained atrophy after the initial stimulus and "atrogene" programs have subsided.

Future Directions for PGC-1α and fiber-type in skeletal muscle atrophy

PGC-1α is a nexus of energy homeostasis and muscle plasticity and, therefore, is likely to play a major role in chronic muscle atrophy (Puigserver and Spiegelman 2003). We have shown that PGC-1α expression is decreased in skeletal muscle from STZ-DM rats and that this decrease is accompanied by fiber-type switching. Our lab is now developing adenoviral vectors to overexpress proteins, like PGC-1α and Cn, *in vivo* in the hind-limbs of STZ-DM rats. This experimental system will allow us to determine whether overexpression of PGC-1α can rescue the fiber-type switching phenotype as well as whether overexpression of constitutively active Cn can return PGC-1α expression to basal levels and block the fiber-type switching process. Lastly, both Cn signaling and PGC-1α are induced by exercise. It would be interesting to investigate whether exercise could rescue both Cn signaling and PGC-1α expression in STZ-DM rats, thus blocking the fiber-type switch and attenuating atrophy.

Our findings also raise the question of whether atrophy induces fiber-type switching, does the switch precede atrophy, or do the two events occur simultaneously? The observation that expression of AT-1 and MuRF-1 is induced early in atrophy and returns to basal while PGC-1α remains suppressed would suggest that the atrophy signals initiate the switch. PGC-1α regulation by FOXO, which also regulates AT-1 and MuRF-1, also supports this hypothesis. However, transgenic overexpression of PGC-1α can block atrophy through antagonism of FOXO-dependent transcription of AT-1 and MuRF-1 (Nakae, Kitamura et al. 2003; Sandri, Lin et al. 2006). These data suggest that the opposite is true, that fiber-type switching precedes atrophy. A first step towards answering this question would be time-point assays to determine the expression profiles of acute atrophy markers (AT-1, MuRF-1, ubiquitin) and fiber-type specific proteins (MHCs, troponins, PGC-1α). The results of these time-course studies would guide adenoviral expression studies in which PGC-1α would be overexpressed in the muscle before and after STZ-induction of DM. Animals that were infected before STZ administration should maintain PGC-1α expression levels even after induction of DM. If these animals atrophied to the same extent as the ones that were infected after, it would support the hypothesis that the atrophy signals initiate the fiber-type switch. However, if the animals that were infected prior to STZ administration atrophy less, it would indicate that either the fiber-type switch precedes the initiation of atrophy or is a parallel, but completely separate, event. While these experiments would not provide any definitive evidence, they would help to determine which scenario is most likely and to direct future experiments.

CONCLUSIONS

Skeletal muscle atrophy is a primary prognostic indicator of mortality for patients with DM, organ failure, or even advanced age. Atrophy in these contexts is not sudden, but rather progresses slowly rendering structural, functional, and physiological changes in skeletal muscle. The structural effects of atrophy, such as decreased size, strength, mobility, etc., have long been of interest. However, the physiological ramifications are often overlooked, especially in the context of a larger disease state which often presents more urgent complications.

Atrophy is an adaptive response. Studies in mechanical models, such as denervation or unloading, often take this concept into account and discuss atrophy as an adaptive response to decreased functional requirements. Skeletal muscle is a highly metabolic tissue and requires vast amounts of energy to maintain. If it is not being fully utilized, such as when functional demands are reduced, then it is logical, energetically, to decrease the size of the muscle tissue. Similarly, in systemic conditions like DM, energy homeostasis is already perturbed. Therefore, muscle atrophy is likely an attempt to buffer the energy imbalance by both decreasing the energy demand, through fiber-type switching from oxidative to glycolytic, and maintaining the amino acid content of other more critical organs, by degradation of muscle protein. When the skeletal muscle has atrophied to the point that it can no longer buffer these effects, the diaphragm no longer has the strength to support breathing, other organs begin to fail as they no longer have a supply of amino acids for protein synthesis, and the patient succumbs to their condition.

In order to improve the prognoses for these patients, it is critical that we develop a better understanding of the relationship between energy homeostasis and muscle atrophy. The Cn signaling pathway mediates muscle adaptation in response to functional demands

and PGC-1α is a key regulator of metabolism in multiple tissues. Furthermore, the work presented here has demonstrated that PGC-1α is the primary effector of muscle metabolic adaptation in response to Cn signaling. Therefore, this pathway is an excellent starting point for further investigation into muscle atrophy in the context of systemic disease. The role of Cn signaling in this process is doubly important because of the implications for immunosuppressive therapies in organ transplant patients. Overall these studies have broad-reaching clinical implications for developing therapies for long-term systemic atrophy.

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