CONTROL OF PROTEIN BALANCE DURING IMMOBILIZATION-INDUCED SKELETAL MUSCLE ATROPHY

A Thesis in
Physiology
by
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ABSTRACT

There is a general lack of understanding concerning the changes in protein balance underpinning skeletal muscle wasting. Such knowledge would substantially improve the prognosis and treatment of individuals subject to conditions causing loss of lean body mass. Therefore, the purpose of this thesis was to examine the mechanisms responsible for changes in protein metabolism underlying atrophy using a model of reduced skeletal muscle loading, hindlimb immobilization. Rats were unilaterally casted for up to five days. In the casted limb, gastrocnemius wet weight decreased after three days and thereafter remained constant. Further analysis revealed that this loss of muscle mass was the result of integrated atrophy and growth failure, and demonstrated that there was no defect in the rate of protein synthesis and essential regulators of translation at day five. This muscle atrophy was partially rescued \textit{in vivo} with a potent proteasome inhibitor and was associated with enhanced mRNA expression of factors that contribute to ubiquitin - proteasome dependent degradation, including the muscle-specific ubiquitin ligases MAFbx/Atrogin-1 and MuRF1. The precise means by which the expression of these ubiquitin ligases is controlled is unknown. For this reason, additional investigations using an \textit{in vitro} model system of skeletal muscle were performed to expand upon the understanding of the cellular signaling events regulating their expression. C2C12 myotubes were treated individually with several AMPK activators, resulting in a dose- and time-dependent modulation of mRNA content of MAFbx/Atrogin-1 and MuRF1 (characterized by an acute repression preceding a sustained induction). These treatments in conjunction with dexamethasone produced a pronounced synergistic effect on ligase mRNA expression at later time points. Stimulation of AMPK activity \textit{in vivo} via AICAR injection recapitulated the stimulation of MAFbx/Atrogin-1 and MuRF1 expression observed in culture. These data suggest AMPK may be a critical component of the intercalated network of signaling pathways governing skeletal muscle atrophy. In total, the work completed within this thesis has enhanced
the understanding of processes fundamental to protein balance (synthesis and degradation) during conditions of skeletal muscle atrophy and identified an additional signaling mechanism which may account for several of the phenotypes displayed by atrophic skeletal muscle.
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Chapter 1

Protein metabolism and the adaptation of skeletal muscle size

1.1 Introduction

One of the most distinctive properties of skeletal muscle is its ability to readily alter its size as a customary physiological response to a wide array of chronic environmental prompts. That this characteristic is so freely adaptable in muscle when compared with other tissue types underscores this flexibility as a key mechanism by which muscle contributes to the long-term homeostatic metabolic balance in an organism (e.g., as an important determinant of resting energy expenditure and as storage reservoir for gluconeogenic substrates). The etiology of the trophic state of muscle at any specific instance is necessarily multi-factorial and many of its phenotypic characteristics are caused by numerous contributing mechanisms, including regulation of distinct processes such as programmed cell death (apoptosis), the regenerative capacity of existing muscle fibers, and the de novo synthesis of new fibers (hyperplasia). While the interactions and causal influences of these processes are not completely understood, the majority of data indicate the predominant factor in determining adult muscle size change is an increase or decrease in the cross-sectional area of existing fibers (hypertrophy and atrophy, respectively). Therefore understanding the stimuli, responses, and effectors responsible for these changes in fiber area becomes crucial for deciphering the adaptive process of skeletal muscle size control.

While such knowledge is undoubtedly beneficial in appreciating control of muscle mass within a range of normal physiological parameters, such as those imposed by activities of daily living or repeated bouts of strenuous exercise, it is perhaps more important under conditions in
which programs for acclimatization fail or are executed to an extreme level. This is vividly demonstrated in conditions where skeletal muscle undergoes severe wasting. While atrophy is a normal and appropriate response to catabolic stimuli, if the muscle-associated changes manifest in loss of functional capacity, then the pronounced reduction in lean body mass has become a mechanism by which atrophy causes morbidity. Moreover, as skeletal muscle functions as part of an integrated system, its pathophysiological adaptation may be both a cause and a consequence of more widely debilitating or life-threatening conditions (e.g. in type II diabetes, where tissue insulin resistance contributes to a failure to properly control blood glucose concentrations). In such instances the mal-adaptation of muscle becomes a major contributing factor to clinically relevant conditions and ultimately, if left untreated, mortality.

Once the driving factors essential to both hypertrophy and atrophy are elucidated, appropriate behavioral and pharmacological strategies can be devised and implemented as both prophylactics and therapeutics to mitigate or circumvent the most injurious characteristics of these catabolic conditions.

1.2 Perturbations of protein balance drive the state of skeletal muscle trophy

A change in skeletal muscle mass elicited by some stress is, in essence, a gross demonstration of a perturbation in protein balance. A quiescent tissue is said to be “in balance” with respect to protein metabolism, in that rates of synthesis and degradation are matched so there is no net protein accretion or erosion and normal function is maintained. Stressors disrupt this homeostatic state and unbalance or shift the equilibrium of this equation. Chronic application or exposure would be predicted to result in either hypertrophy or atrophy of the muscle (depending on the stimulus) due to alterations in global rates of protein synthesis, protein degradation, or both. This concept has been supported experimentally by numerous models examining both the anabolic and catabolic responses of skeletal muscle to genetic,
pharmacological, and mechanical stimuli. As an example, a more detailed discussion of these processes in models of hypokinesia and hypodynamia can be found in sections 2.1 and 2.4. This trophic response in turn impacts the functional properties evidenced by the muscle. A classic example of such a connection is the established correlation between muscle cross-sectional area and maximum tetanic tension, where hypertrophy increases maximum tension and atrophy vice versa. Thus, in general terms, alterations in global protein balance influence both the form and function of skeletal muscle.

One remarkable aspect of physiology is the economy with which tissue adaptations can be achieved through modulation of one individual protein or subset of proteins. Consequently, responses evident at the organ level can be achieved via qualitative changes in specific protein synthesis and degradation at a molecular level within independent cells (in addition to the quantitative changes of global protein balance described above). Often these protein-specific alterations act at critical switch points in essential cellular processes. For instance, one of the most basic cellular behaviors, proliferation, is regulated in part by the ordered synthesis and degradation of D-type cyclins (which “sense” mitogenic stimulation and consequently induce the G₁ to S transition of the cell cycle) (161). As mature muscle fibers are terminally differentiated and post-mitotic, a more relevant example would be the shift in myosin heavy chain isoform composition observed in various models of muscle disuse (such as hindlimb suspension, immobilization, or denervation) (43, 167, 181), which contributes to the resultant changes in contractile function and fatigability (46, 190, 191).

It is important to note that a state of complete balance, while conceptually convenient, is unlikely to exist at any instant in vivo due to the constant interplay between the organism and its surroundings. The variability of protein metabolism within physiological limits affords the host the ability to respond properly to a fluctuating environment. Regardless, the elicitation of a hypertrophic or atrophic phenotype depends on the associated adaptations of skeletal muscle at the tissue, cellular, and molecular levels due to quantitative and qualitative changes in its
proteome. In turn, these changes in content are ultimately driven by asymmetry in rates of protein synthesis and degradation as the principal determinants of protein balance. The importance of macromolecular synthesis and degradation to virtually all biological processes is highlighted by their dependence on intricate machinery designed to incorporate multiple levels of regulation to achieve quality control and maximal efficiency in responding to perturbations of homeostasis.

1.3 The machinery of protein synthesis and degradation

1.3.1 Ribosomes: the site of protein synthesis

The genesis of new proteins involves the ordered assembly of ribosomal subunits into functional ribosomes, which act with assistance from accessory factors to decode and “translate” the nucleotide sequence of a bound messenger RNA into the corresponding polypeptide chain (143). These large (4.3 mDa) rRNA-protein complexes contain the catalytic sites where the synthesis of peptide bonds and polypeptide chain assembly occur, and therefore represent the authentic apparatus of protein synthesis (133, 143). This process of mRNA translation can be divided into four distinct phases: initiation, elongation, termination, and recycling (89). The majority of regulation occurs during initiation (where protein-protein and RNA-protein interactions dictate the alignment and assembly of the translational machinery) and elongation (where the successive rounds of peptide bond formation occur between the ultimate member of a growing polypeptide chain and the newly recruited aminoacyl charged tRNA). During each respective stage, eukaryotic initiation (eIF) and elongation (eEF) factors are the primary agents of translational control. These individual factors are frequently endpoints of numerous cellular signaling pathways (see section 1.4 below), and their interlocking sensitivities and functions during translation make the process of protein synthesis responsive to virtually all
extra- and intra-cellular stressors and cues. This regulatory network allows synthesis to proceed only when the cellular environment favors completion of this energy and resource expensive process with no threat to cell viability.

The field of translational control is expanding and under constant revision. As a result, an exhaustive review of all the contributing proteins is beyond the scope of this thesis. However, it is important to highlight and briefly discuss some of the more well-established steps and factors as they relate to the investigations completed herein.

1.3.1.1 Translation initiation

Regulation of translation initiation primarily occurs at two distinct events: the attachment of an initiator Met-tRNA$^i$, onto the small 40S ribosomal subunit (forming the 43S complex), and the loading of the 43S complex onto “capped” mRNA following dissolution of any secondary or tertiary structural elements at the 5' end (Fig. 1-1). Met-tRNA$^i$, binding falls under the purview of eIF2, a heterotrimer that in an active, GTP-bound state shuttles the methionylated initiator tRNA to the 40s subunit as part of an eIF2-GTP- Met-tRNA$^i$, ternary complex (89, 143, 163). Because eIF2 has a much greater affinity for and a slow release rate of GDP (163), its activation necessitates the involvement of an exchange factor responsible for catalyzing GDP-GTP recycling (89, 143). This is achieved by eIF2B (96), a multi-subunit enzyme that expresses guanine nucleotide exchange factor activity with respect to its substrate eIF2.

Loading of the 43S complex onto the mRNA is regulated by the activity of eIF4F, a multi-factor complex tasked with binding the 5’ end of mRNA and melting of any complex structure present (89, 143). eIF4F consists of eIF4E (65) and eIF4A (138) (whose functions are to directly bind the 5’-methylguanosine cap structure found in all non-organellar mRNAs and to unwind secondary structure via ATP-dependent RNA helicase activity, respectively) bound to the adaptor protein eIF4G. Control of this step in initiation is largely exerted through availability
of free eIF4E (112, 137). When bound to members of the translational repressor 4E-binding protein (4E-BP) family, eIF4E - while still free to associate with mRNA - cannot bind to eIF4G and participate in an active eIF4F complex (64). As the efficient translation of mRNAs, especially those with highly ordered 5’ UTRs, is facilitated by eIF4E (98) as part of an active eIF4F complex, the sequestration of eIF4E into inactive complexes with 4E-BPs impedes translation and consequently protein synthesis.

1.3.1.2 Translation elongation

Control of translation elongation is also exercised at two discrete steps, recruitment of tRNAs to and the traffic of the actively translating ribosome down the mRNA following each peptide bond formation. Recruitment of amino-acylated tRNAs to the A site during elongation is controlled by eEF1A (17, 89) in a manner loosely analogous to that of eIF2 for 43S complex formation during initiation. An eEF1A-GTP-aminoacyl-tRNA ternary complex enters the A site and, if cognate base-pairing is achieved, hydrolysis of GTP causes release of the aminoacyl tRNA (89). Following formation of a peptide bond and before the next amino acid addition, the ribosome must be translocated three nucleotides down the mRNA to allow for recruitment of the next associated aminoacyl tRNA. This movement of the ribosome is catalyzed by eEF2, which acts as a molecular “motor” for elongation, in a GTP-dependant manner (17, 89).

1.3.2 The proteolytic systems of skeletal muscle

Skeletal muscle possesses multiple systems capable of protein destruction (perhaps a reflection of its proclivity to change its protein content in response to varying stimuli, particularly catabolic ones). They are classified into four major categories based on their operative properties: calcium-dependent, caspase-mediated, lysosomal, and ubiquitin-proteasome
dependent (29). While each is typically presented as a distinctive entity with its own afferent and efferent components, notable interdependence and overlap in functionality (i.e. bulk or specific breakdown of proteins) between them has been observed. This is especially true for degradation of the primary constituents of the contractile apparatus, which appear to be protected from breakdown while interacting with each other (165). The need for a preceding step in the proteolysis of myofibrillar components has led to the proposal of a multi-step model, whereby calcium-dependent calpains initially cleave and release those proteins allowing for their breakdown by the ubiquitin-proteasome pathway (UPP) (29). Therefore, the coupled function of these pathways contributes to a coordinated proteolytic response that is more flexible and precise in its response to specific environmental demands or signals. Such a system poses obvious complications regarding interpretation of experimental results, and consequently there is considerable controversy as to the precise contribution of each system towards protein degradation in skeletal muscle, particularly regarding conditions of wasting. However, the majority of data suggest the UPP predominates in these states (125, 174), and hence forms the focus of the remaining discussion.

Proteolysis through the UPP encompasses two distinct processes - the identification and tagging of specific proteins for degradation and their successive destruction by the proteasome (59, 125). This pathway of degradation is distinguished by its high degree of specificity and regulation, characteristics which are achieved through both general (e.g. the ATP-dependence of multiple steps within the pathway) and specific (e.g. the role of E3 ubiquitin ligases, see section 1.3.2.1 below) mechanisms (59). Akin to the discipline of translational control described above, the UPP remains an avidly pursued area of research. Nevertheless, the cumulative effect of this investigation has been the identification of basic steps and enzymes forming the backbone of this pathway. An outline of portions of this elementary architecture is pertinent to the analysis located in subsequent chapters.
1.3.2.1 Protein ubiquitination

The first process, marking of the protein to be degraded, is itself a multi-step cascade involving the activation, transfer, and attachment of a “tag” to the protein substrate. As its name implies the UPP depends upon a co-factor, the small protein ubiquitin, as this universal indicator or tag (Fig. 1-2). In the proximal step of this cascade ubiquitin is activated by the E1 enzyme, resulting in the formation of an E1-ubquitin intermediate complex. This activated ubiquitin is then transferred to one of several E2 enzymes (also referred to as ubiquitin-carrying or ubiquitin-conjugating enzymes, UBCs), forming an E2-ubiquitin intermediate. These UBCs perform the actual labeling of the protein substrate. Naturally, this reaction necessitates the close spatial proximity of the substrate and the active site of the E2. This step in the tagging cascade is catalyzed by a diverse array of E3 ubiquitin ligase enzymes. E3s serve as the recognition factors of the UPP by binding a specific protein (or subset of proteins) that expresses a distinct degron, or degradation signal (59, 182). By binding to both the E2 and a specific protein substrate, E3s decrease the distance between the two, allowing for transfer of the ubiquitin moiety from the E2 to the substrate (59). This site of attachment usually corresponds to an internal lysine residue of the target, though exceptions have been noted (particulary mono-ubiquitination at the N-terminus of a protein). Thus, through the formation of multiple enzyme-ubiquitin intermediaries, ubiquitin is activated, transferred, and attached to a protein destined for destruction.

This ordered process of activation, transfer, and attachment occurs cyclically, resulting in the multi-ubiquitination of a substrate. Each successive round beyond the first results in the attachment of a free ubiquitin to the one previously anchored, causing the formation of a polymeric ubiquitin chain. The construction of a chain containing a minimum of four ubiquitins (7) appears to be necessary for efficient recognition by the 26S proteasome, the major functional component of the second stage of the UPP.
1.3.2.2 Proteasomes: final destination of poly-ubiquitinated proteins

26S proteasomes are large barrel-shaped proteases consisting of distinct subcomplexes - the 20S core particle and 19S regulatory particles that cap the core on either end (59) (Fig. 1-3). The 20S core is composed of stacked rings, two inner β-rings flanked by two outer α-rings. Each inner β-ring contains three active protease sites situated towards the inside of the barrel. The specific proteolytic activities of the core particle include chymotrypsin-like (cleaving after hydrophobic residues), trypsin-like (after basic residues), and peptidyl-glutamyl-peptide peptidase (after acidic residues) activities, which correspond to the β-5, β-2, and β-1 subunits, respectively (37). Access to this inner chamber is restricted by the α-rings through a gating-type mechanism, preventing degradation of protein through mass action or similar non-specific means. In addition to restricting access to the proteolytic barrel, the α-rings also serve as docking sites for the 19S regulatory particles. These 19S particles can be further divided into multi-subunit base and lid components, and are proposed to regulate substrate recognition, selection, editing and unfolding, and translocation into the 20S core (59). The exact contribution of each individual subunit to these processes is relatively unknown save for a select handful.

A ubiquitin chain of sufficient length can be recognized by the Rpn10/S5a/Mcb1 subunit of 19S regulatory particle, resulting in the binding of the tagged substrate to the 19S particle (59). Once bound the protein substrate is prepared for breakdown through several ATP-dependent processes involving its unfolding, deubiquitination (by several deubiquitinating enzymes, or DUBs), and translocation into the proteolytic barrel (59). Inside the lumen of the 20S core the substrate is degraded via the proteolytic activities of the β-subunits into short peptide stretches of 3 to 22 amino acids in length, which exit the proteasome by simple diffusion (95). Complete proteolysis of these remaining fragments is performed by cytosolic proteases and aminopeptidases. It is worth noting that proteasomal function in some cases is considerably more flexible and elaborate than the basic explanation offered here. Such
complexity is clearly illustrated in cases where breakdown of substrates by the proteasome is purposely incomplete, resulting in the activation of an enzyme or signaling protein from its latent form (e.g. the processing of the p105 protein to form an active subunit of NF-κB) (59).

1.4 The control of protein balance by intracellular signaling cascades

The connection between a wide variety of environmental signals and the control of cell mass necessitates a means of signal transduction that communicates extracellular cues to the aforementioned machinery controlling protein balance (discussed in the preceding section 1.3). This work has led to the characterization of several evolutionarily-conserved growth pathways that in most tissues, including skeletal muscle, control protein synthesis and/or degradation in response to these inputs (58). Specific signaling cascades - including the phosphatidylinositol-3-kinase/Akt (PI3K/Akt) and the mammalian target of rapamycin complex 1/ complex 2 (mTORC1/2) cascades - serve critical functions in controlling the adaptive response of skeletal muscle to environmental perturbations. The relationships between activation of each of these pathways in isolation, their crosstalk at multiple levels, and the consequent cellular phenotypes are exceptionally intricate and cannot be recounted in exact detail here. Regardless, the highlighting of key components and important points in relation to skeletal muscle is warranted for the research presented within this thesis.

1.4.1 Mitogein-induced Akt signaling in skeletal muscle

The PI3K/Akt pathway is initiated proximally by ligand-receptor binding leading to the eventual activation of the serine (Ser)/threonine (Thr) protein kinase Akt (also referred to as protein kinase B, PKB) by phosphoinositide-dependent kinase 1 (PDK1) (2) and mTORC2 (154) (Fig. 1-4). Activated Akt signals directly or indirectly to a number of important controllers of
protein synthesis, including glycogen synthase kinse-3 (GSK3) (31, 67, 149) and mTOR and its
downstream effectors ribosomal protein S6 kinase-1 (S6k1) and 4E-BP1 (13, 18, 41, 57, 131,
149, 158, 159, 172); the latter data allude to Akt as a connecting signaling kinase between
hormonal and growth factor inputs and the mTOR pathway through its phosphorylation and
control of tuberin (79, 80, 119, 142, 175) and/or the AMP-activated protein kinase (AMPK) (66).
Indeed, growth factor-induced stimulation or expression of constitutively active Akt in vitro and in vivo is sufficient to induce hypertrophy and increased protein synthesis by signaling to distal
regulators of the translational machinery in a cell-autonomous manner (13, 45, 67, 100, 135,
149, 186). Furthermore, the observation that genetic activation of Akt is sufficient to prevent
denervation atrophy (13, 135) suggests the kinase may also increase cell size by antagonizing
protein breakdown. This role was confirmed in vitro where activation of an inducible Akt
expression construct repressed proteolysis (45) and pharmacological inhibition of Akt activity
ablated the suppressive effects of insulin and IGF-I on protein degradation (45, 152).

1.4.2 mTOR, a master integrator of growth-related signals

mTOR (also known as FRAP, RAFT1, or RAPT1) serves as the foundation for a second
critical growth-associated kinase cascade (47, 72). In association with various adaptor proteins,
such as Raptor/mKOG1 (68, 93), mLST8/GβL (94), and Rictor/mAVO3 (82, 155), it forms two
distinct multimeric complexes, mTORC1 and mTORC2, that function in control of cell size and
number through a variety of processes including ribosomal biogenesis, cap-dependent
translation, and cytoskeletal organization (Fig. 1-4). In addition to nutrient-related growth
control, studies have also demonstrated the necessity of mTOR in conducting hormonal and
growth factor mediated signals to these processes as well (4, 21, 24, 25, 34, 38, 113, 135,
144). Thus mTOR is strategically positioned to serve as a hub for integration and propagation
of multiple afferent signals (including hormones, growth factors, amino acid availability, and
cellular energy status) to intermediaries thought to be essential for supporting the maintenance or induction of cellular growth (49) and proliferation (48).

The best characterized downstream targets of mTOR are the S6 kinases (S6k1 in particular) and 4E-BP1. S6k1 plays obligatory roles in promoting organism growth (127, 162) and hypertrophy of skeletal muscle \textit{in vitro} (134, 136), presumably by regulating both general rates of translation through indirect stimulation of eIF4A helicase activity via phosphorylation of eIF4B (75, 148) and specific translation of 5’-terminal oligopyrimidine (5’-TOP) mRNA (84, 91, 176) via phosphorylation of ribosomal protein S6 (rpS6) (85), though the latter observation has been questioned by more recent investigations (121, 141, 151). 4E-BP1 is the isoform of the 4E-BP family predominantly expressed in skeletal muscle and is a negative regulator of cell growth (49, 150), likely due to its inhibition of protein synthesis.

The control of proteolysis by mTOR is less clear-cut. Conflicting evidence from mammalian cell studies exists regarding the role of mTOR activity in growth factor and amino acid-induced inhibition of autophagy. While select investigations demonstrate the mTOR-dependant nature of one or both of these effects (11, 88), others suggest the kinase is dispensable for this suppression of protein breakdown (45, 128). The exact reasons for these discrepancies are unknown, though in some cases they may by attributable to cell-type differences in the stimulation of autophagy. For example, mTOR appears to control autophagy induced by insulin deprivation in primary rat hepatocytes (88), yet appears unnecessary for this response in partially transformed hepatoma cells (45). Additionally, there is no direct evidence that any of the downstream targets of mTOR control autophagic proteolysis (123). Studies in \textit{Saccharomyces cerevisiae} have shown that TOR antagonizes proximal steps in autophagy via control of the kinase Atg1. However, the role of homologs of Atg1 in the mTOR-dependent control of autophagy in rodent or human systems remains to be investigated (123). The potential role(s) of mTOR and its effectors in regulating other proteolytic pathways outside of conditions of nutrient starvation in skeletal muscle is unknown.
1.5 **Protein metabolism and skeletal muscle atrophy - more questions than answers**

The preceding sections have provided, in a broad sense, one prospective set of answers to the key questions that form the crux of understanding the physiological adaptation of a system to stress. In the context of adaptation of muscle size, those elements are: who (numerous signal transduction pathways, such as those involving Akt and mTOR) acts upon what (the basic apparatus and diverse array of supplementary factors contributing to the creation or destruction of proteins) where (within individual muscle cells, whose collective capacity to hypertrophy or atrophy determines skeletal muscle organ growth or wasting) when (in response to extra- and intra-cellular cues engaged by environmental input) why (to adapt fiber size in a manner that both preserves present function within homeostatic limits and enhances future resistance to further perturbations at the cellular, organ, and organism levels), and how (by altering quantitative and qualitative rates of global and specific protein synthesis and degradation).

Despite the apparent “tidiness” of such a holistic framework, in reality the specific details inherent to each of those steps are largely unresolved. In spite of the existence of a detailed foundation of knowledge concerning cellular events involved in protein synthesis and degradation (sections 1.3.1.1, 1.3.1.2, 1.3.2.1, and 1.3.2.2), by comparison little is known specifically in muscle regarding the alterations in cellular signaling pathways, the consequent changes in protein balance (especially concerning specific key proteins), and how these responses reflect or account for the integrated phenotype of a larger or smaller tissue mass. It could be argued that this information is exceptionally important for insults that result in excessive muscle wasting, such as alcoholism, starvation, cachexia, sepsis, diabetes, and prolonged reductions in weight-bearing due to restraint, bed rest, or mechanical ventilation. Such an understanding would represent a substantial advancement towards minimizing the deleterious consequences and improving the prognosis, management, and eventual recovery (if
possible) of individuals subject to these and other conditions. Therefore, the first portion of this thesis work was conducted in accordance with the following purpose and working hypothesis to investigate these issues.

1.5.1 Purpose

The purpose of these initial studies was to examine the potential mechanisms responsible for changes in protein metabolism underlying skeletal muscle atrophy during a reduction in mechanical loading imposed by hindlimb immobilization.

1.5.2 Working Hypothesis

The working hypothesis tested by this research was that this model of short-term immobilization would result in a decrease in skeletal muscle wet weight in muscles directly subjected to the reduction in loading. This atrophy would be attributable to changes in processes determining protein balance (protein synthesis and protein degradation) caused by concomitant modifications in the expression or activity of components of the synthetic and degradation machinery itself and/or signal transduction pathways controlling cellular size.
Fig. 1-1: The translation initiation pathway. The 40S ribosomal subunit is primed for initiating translation by binding of the ternary complex comprising eIF2, Met-tRNA$_{\text{Met}}$, and GTP (see left side of depicted scheme). In yeast, this is aided by the multifactor complex (MFC), an intermediate with roles in several steps of translation initiation. The resulting 43S preinitiation complex is recruited to the mRNA via interactions with the eIF4 factors bound at or near the cap structure of the mRNA (see top of schematic). The 43S complex then scans the 5'UTR to locate the initiator codon (centre-right of scheme). Following recognition of the AUG, involving base-pairing with the anticodon loop of Met-tRNA$_{\text{Met}}$, release of the bound factors accompanies two distinct GTP hydrolysis steps and joining of the 60S subunit to form an elongation competent 80S ribosome (see bottom of figure), poised to start the first peptide elongation cycle. GDP-bound eIF2 is recycled by eIF2B to allow further ternary complex formation (see bottom left of the diagram). For clarity, eukaryotic initiation factors are labelled with the unique portion of their respective names only, omitting the general ‘eIF’ prefix i.e., ‘4E’ instead of ‘eIF4E’). Every effort was made to design this schematic such that it accurately reflects the many interactions between factors. Due to geometric constraints, however, the figure fails to show the interaction between eIF1A, and eIF2 as well as eIF3. The ‘?’ near the looped-out 5'UTR in the center of the scheme indicates the speculative nature of this scanning intermediate. To simplify the bottom part of the diagram, the distinct requirements of GTP-bound eIF5B for 60S subunit joining and GTP hydrolysis after 80S ribosome formation for eIF5B release are not shown. The recently identified factor eIF4H is also not represented in the scheme.

Source: Adapted from Figure 1 in Preiss T and Hentze MW. Starting the protein synthesis machine: eukaryotic initiation translation. *Bioessays* 25: 1201-1211, 2003.
Fig. 1-2: The ubiquitin proteolytic pathway. 1: Activation of ubiquitin by the ubiquitin-activating enzyme E1, a ubiquitin-carrier protein, E2 (ubiquitin-conjugating enzyme, UBC), and ATP. The product of this reaction is a high-energy E2~ubiquitin thiol ester intermediate. 2: Binding of the protein substrate, via a defined recognition motif, to a specific ubiquitin-protein ligase, E3. 3: Multiple ($n$) cycles of conjugation of ubiquitin to the target substrate and synthesis of a polyubiquitin chain. E2 transfers the first activated ubiquitin moiety directly to the E3-bound substrate, and in following cycles, to the previously conjugated ubiquitin moiety. Direct transfer of activated ubiquitin from E2 to the E3-bound substrate occurs in substrates targeted by RING finger E3s. 3': As in 3, but the activated ubiquitin moiety is transferred from E2 to a high-energy thiol intermediate on E3, before its conjugation to the E3-bound substrate or to the previously conjugated ubiquitin moiety. This reaction is catalyzed by HECT domain E3s. 4: Degradation of the ubiquitin-tagged substrate by the 26S proteasome complex with release of short peptides. 5: Ubiquitin is recycled via the activity of deubiquitinating enzymes (DUBs).

Source: Adapted from Figure 1 in Glickman MH and Ciechanover A. The Ubiquitin-Proteasome Proteolytic Pathway: Destruction for the Sake of Construction. *Physiol Rev* 82: 373-428, 2002.
Fig. 1-3: Structure of the proteasome. The proteasome is a modular structure. One or two regulatory particles (RP) attach to the outer surface of the core particle (CP). The CP is made up of four heptameric rings: two outer identical \( \alpha \)-rings and two identical inner \( \beta \)-rings. Each ring is made of seven distinct homologous subunits. Certain \( \beta \)-subunits contain the protease active sites facing inward into the sequestered proteolytic chamber. Upon interferon-\( \gamma \) induction, three \( \beta \)-subunits can be replaced by \( \beta_i \) (LMP) homologs that alter the proteolytic specificities of the proteasome. The 19S RP is comprised of two eight-subunit subcomplexes, the lid, and the base. The base containing all six proteasomal ATPases attaches to the \( \alpha \)-ring of the CP. The lid can disassociate from the proteasome, resulting in a truncated base-CP complex. Rpn10 can interact with either the lid or the base and stabilizes the interaction between the two. Rpn10 is also found outside of the proteasome. Numerous associated proteins and auxiliary factors, such as chaperones and components of the ubiquitination machinery, can interact with the RP. Alternative regulatory complexes can also attach to the surface of the \( \alpha \)-ring.

Source: Adapted from Figure 5 in Glickman MH and Ciechanover A. The Ubiquitin-Proteasome Proteolytic Pathway: Destruction for the Sake of Construction. *Physiol Rev* 82: 373-428, 2002.
Fig. 1-4: A model of the mTOR and PI3K/Akt signaling pathways and their interconnections. Two mTOR-interacting proteins, raptor and rictor, define distinct branches of the mTOR pathway. The raptor–mTOR pathway regulates cell growth (accumulation of cell mass) through S6K1 and 4E-BP1 as well as unknown effectors. It responds to nutrients and growth factors in part through the upstream regulators TSC1/2 and rheb. The rapamycin-insensitive rictor–mTOR pathway regulates Akt/PKB, PKCa, and Rho/Rac to control cell survival, proliferation, metabolism and the cytoskeleton. The binding of growth factors to cell surface receptors activates PI3K to generate PtdIns(3,4,5)P3 and recruits the PDK1 kinase and Akt/PKB to the plasma membrane. Akt/PKB is activated by its phosphorylation on two different sites. The rictor–mTOR complex phosphorylates Akt/PKB on Ser473 in the hydrophobic motif which may facilitate the phosphorylation by PDK1 of the activation loop of Akt/PKB on Thr308. How the rictor–mTOR complex is regulated is unknown. Dashed lines indicate interactions that are likely not direct.

Source: Adapted from Figure 1 in Sarbassov Dos D, Ali SM, and Sabatini DM. Growing roles for the mTOR pathway. *Curr Opin Cell Biol* 17: 596-603, 2005.
Chapter 2

Hindlimb casting decreases muscle mass in part by proteasome-dependent proteolysis but independent of protein synthesis

Data and text within this chapter have been re-printed from the following published journal article (used with permission):


2.1 Introduction

Skeletal muscle demonstrates a remarkable degree of plasticity in response to alterations in mechanical loading. Removal of normal weight-bearing stress results in quantitative and qualitative adaptations in protein content that are phenotypically epitomized by decreased muscle wet weight, fiber diameter, force output, and fatigue resistance. As prefaced in section 1.4, changes in tissue mass are primarily determined at a cellular level by complex signaling pathways (58) that coordinate growth and proliferation (26). These circuits ultimately manage specific enzymatic machinery with intimate ties to fundamental biological events such as macromolecular synthesis or degradation (section 1.3) and gene expression. Indeed, the etiology of atrophy in various models of mechanical unloading or “disuse” is attributable to both
a repression in protein synthesis and/or an elevation in protein degradation (15, 19, 60, 61, 171, 179, 180).

The process of protein synthesis can be regulated by two general mechanisms: alteration of the ribosomal content (the base translational capacity of a cell, section 1.3.1) or of the translational activity of pre-existing ribosomes (the efficiency or rapidity with which they participate in cyclical rounds of translation). Acute repression of protein synthesis with casting immobilization is not a consequence of significant defects in total RNA concentration (15, 60), but instead can be attributed to a reduction in translational efficiency (60). These observations suggest a decrement in either signaling to or the function of one or more members of the cadre of eIFs and eEFs previously established as vital for controlling protein synthesis (sections 1.3.1.1 and 1.3.1.2). Accordingly, hindlimb suspension-induced atrophy of antigravity muscles is associated with decreased protein expression and phosphorylation of Akt (13). Furthermore, this reduction of skeletal muscle loading decreases the activation state of S6k1 (13) and increases the amount of 4E-BP1 bound to eIF4E (13). These observations are indicative of a direct responsiveness of the Akt and the mTOR pathways to the loading state of skeletal muscle, suggesting the pathology of disuse muscle wasting can be traced to cellular signaling defects that correlate with the allied muscular atrophy.

Similarly to many other wasting conditions, the ATP-dependent ubiquitin-proteasome system appears to predominate under conditions of muscle disuse (171). Because ubiquitin conjugation is thought to be the rate-limiting step of degradation through the UPP in these contexts, the upregulation of specific E2s and E3s and ubiquitin itself may be central to the atrophic response during catabolic states. This notion is supported by gene expression studies demonstrating that disparate atrophic stimuli drive the induction of a specific transcriptional program resulting in increased expression of components innate to the ubiquitin-proteasome system (106, 168). In particular, the muscle-specific E3 ligases Muscle Atrophy F-box (MAFbx)/Atrogin-1 (12, 62) and Muscle RING Finger 1 (MuRF1) (12) are increased by a
multitude of catabolic perturbations, such as treatment with interleukin (IL)-1 (12) or
dexamethasone (12) as well as cancer cachexia (62, 106), diabetes (62, 106), fasting (106),
and sepsis (193). Together these data suggest that execution of a specific transcriptional
response to catabolic cues contributes to the activation of the UPP and consequently enhances
proteolysis.

Collectively, the aforementioned studies establish a role for several critical events and
signaling pathways in control of skeletal muscle cell size under a variety of conditions of normal-
and dis-use. However, this body of knowledge represents an accrual of results obtained using a
wide breadth of atrophic models, time courses, and examined muscles. Therefore no complete
set of information exists for one well-defined model of disuse atrophy. Hence, the purpose of
these studies (as defined in section 1.5.1) was to pursue the first working hypothesis of this
thesis (section 1.5.2) by examining protein synthesis and translation control pathways in a
clinically and physiologically relevant model of reduced skeletal muscle loading, unilateral
hindlimb immobilization produced by casting. Furthermore, as atrophy could potentially be
ascribed to resistance to anabolic stimuli, the acute responsiveness of the above synthetic
pathways in immobilized muscle to exogenously administered IGF-I and insulin was determined.
Additionally, because glucocorticoids are necessary mediators of enhanced proteolysis in
multiple contexts (125), the role of circulating corticosterone in mediating the immobilization-
induced remodeling process was assessed with the glucocorticoid receptor antagonist RU-486.
Finally, the contribution of proteasomal function to skeletal muscle weight loss during disuse
atrophy was determined in vivo using the reversible proteasome inhibitor Velcade.
2.2 Materials and Methods

2.2.1 Animals

Pathogen-free male Sprague-Dawley rats (150-175 g; Charles River Breeding Laboratories, Cambridge, MA) were quarantined for 1 week while exposed to constant temperature and a 12:12 hour light-dark cycle. Standard rat chow (Harlan Teklad, Indianapolis, IN) and water were provided ad libitum. All experiments were performed in adherence with the National Institutes of Health Guide for Care and Use of Laboratory Animals and with the approval of The Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

2.2.2 Experimental protocols

Rats were anesthetized with an intraperitoneal (ip) injection of pentobarbital sodium (50 mg/kg) and subjected to unilateral hindlimb immobilization via a fiberglass cast. Briefly, the left hindlimb was shaved and wrapped in a protective layer of cast padding (Specialist brand; Johnson and Johnson, Raynham, MA). Multiple layers of fiberglass casting tape (3M VetCast Plus veterinary casting tape; 3M, St. Paul, MN) were then applied and allowed to harden. The foot was positioned in plantar-flexion to induce maximal atrophy of the gastrocnemius (60, 61, 195). Following casting, rats were resuscitated with 10 ml of 0.9% sterile saline administered subcutaneously. Results from pilot studies (data not shown) indicated that unilateral immobilization had no effect on various parameters of interest in skeletal muscle from the contralateral non-casted leg. Consequently the contralateral hindlimb served as the control in all subsequent experiments. Following casting, rats were housed individually and provided free access to standard rat chow and water.
Immobilization was imposed for a duration of 1, 3, or 5 days. Following an overnight fast, casts were removed under pentobarbital anesthesia and gastrocnemius from both the immobilized and control hindlimb was rapidly excised, weighed, and processed. A portion of each gastrocnemius for use in immunoblotting was homogenized immediately (Kinematica Polytron; Brinkmann, Westbury, NY). The homogenates were prepared in 4 volumes of ice-cold homogenization buffer (consisting of 20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM NaF, 100 mM KCl, 0.2 mM EDTA, 50 mM β-glycerophosphate, 1 mM DTT, 0.1 mM PMSF, 1 mM benzamidine, and 0.5 mM sodium vanadate). Studies that involved immunoblots for ubiquitin protein utilized buffer with 10 mM NEM substituted for 1 mM DTT. Homogenates were centrifuged at 10,000 g for 20 minutes at 4°C and supernatants aliquoted for storage. The remaining muscle was quickly frozen between aluminum clamps pre-cooled in liquid nitrogen. The order of collection with respect to immobilized and control limbs was alternated from one animal to the next to correct for the time elapsed during processing of the preceding leg.

2.2.3 Injections

To investigate whether immobilized skeletal muscle manifests a resistant state with respect to intracellular signaling pathways associated with protein synthesis, separate groups of rats were injected intravenously (iv) with either IGF-I (25 nmol/kg body weight), insulin (5 µU/kg body weight), or an equivalent volume (0.5 ml) of isotonic saline following cast removal. Twenty minutes after administration of growth factor or saline, muscles from immobilized and control limbs were processed as described above (section 2.2.2). In a separate experiment, unilateral hindlimb immobilization was imposed for 3 days (a time point that incurs a maximal atrophic response and transcriptional upregulation of components of the UPP, see sections 2.3.1 and 2.3.9). Rats were injected subcutaneously (sc) once daily with the cytosolic type II glucocorticoid receptor antagonist RU-486 (20 mg/kg body weight; Sigma-Aldrich, St. Louis, MO) or an
equivalent volume (0.5 ml) of vehicle (60% ethanol/40% saline) for the duration of the experiment. This dose of RU-486 has been used previously to attenuate glucocorticoid-induced changes in protein and mRNA content following thermal injury (103). In a separate experiment, rats were injected ip at the time of casting with Velcade (0.5 mg/kg body weight; Millenium Pharmaceuticals, Cambridge, MA) or an equivalent volume (0.5 ml) of vehicle (saline). This dose has been used previously to study proteasome function in in vivo cancer and alcoholic hepatitis models (6, 16, 105). Following 3 days of immobilization, muscles were processed as above.

### 2.2.4 Protein synthesis, RNA content, and translational efficiency

The rate of muscle protein synthesis was determined in vivo by the flooding-dose technique (27, 53, 101). Casts were removed and a catheter was placed in the carotid artery. At time zero, rats were administered either IGF-I or an equal volume of isotonic saline via percutaneous injection of the inferior vena cava. At the 10 minute time point all rats received a bolus injection of L-[³H]phenyalanine (150 mM, 30 μCi/ml, 1 ml/100g body weight) via the jugular vein. Blood samples (for determination of phenylalanine concentrations and specific radioactivity) were collected at 12, 16, and 20 minutes via the arterial catheter into heparinized syringes. Gastrocnemius was then excised and frozen as above. Total protein concentration of immobilized and control gastrocnemius was determined on powdered tissue via the Biuret method with a crystalline BSA standard. A fraction of powdered gastrocnemius was weighed, dried for 2 days in an oven (70°C), and reweighed to calculate the dry weight to wet weight ratio.

The specific radioactivity of deproteinized plasma was determined by HPLC (39). Specific radioactivity was calculated by dividing the radioactivity of the phenylalanine peak by the concentration of phenylalanine in the sample. A portion of powdered muscle was used to
determine the rate of incorporation of radioactive phenylalanine into protein as previously described (27, 101). Total RNA was determined by spectrophotometry using previously described methods (27, 101). Translational efficiency was calculated by dividing the rate of protein synthesis of mixed muscle proteins by the total RNA concentration and expressed as nmol phenylalanine incorporated into mixed protein per hour per mg RNA. The vast majority of cellular RNA in skeletal muscle is ribosomal; therefore measurements of total tissue RNA provide an accurate reflection of ribosomal content and are appropriate for the calculation of efficiency.

2.2.5 Immunoblotting and immunoprecipitations

The protein concentration of tissue supernatants was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA). An equal volume of 2X sample buffer was added to tissue supernatant. Samples were loaded according to total protein content on polyacrylamide gels for separation by SDS-PAGE. Proteins were transferred to PVDF membrane (Biotrace; PALL, Pensacola, FL), blocked in nonfat dry milk, and incubated overnight at 4°C with phosho-specific antibodies for the following proteins: Akt (Thr\textsuperscript{308}), mTOR (Ser\textsuperscript{2448} and Ser\textsuperscript{2481}), S6K1 (Thr\textsuperscript{389}), rpS6 (Ser\textsuperscript{235/236} and Ser\textsuperscript{240/244}), 4E-BP1 (Thr\textsuperscript{37/46}), eIF4G (Ser\textsuperscript{1108}), eIF2\textalpha (Ser\textsuperscript{51}), eEF2 (Thr\textsuperscript{56}), tuberin (Thr\textsuperscript{1462}) (all from Cell Signaling Technology, Beverly, MA), and eIF2B\textepsilon (Ser\textsuperscript{535}; Biosource International, Camarillo, CA). Excess primary antibody was removed by washing in TBST (1X TBS + 0.1% Tween 20) and membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Sigma-Aldrich) at room temperature. Blots were developed using enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ) in accordance with the manufacturer’s instructions and exposed to BioMax XAR X-ray film (Kodak, Rochester, NY) in a cassette equipped with a DuPont Lightning Plus intensifying screen. Developed film was scanned (ScanMaker IV;
Microtek USA, Carson, CA) and analyzed using ScionImage software (Version Beta 4.0.2; Scion Corporation, Frederick, MD).

Following development, antibody was removed from membranes by treatment with a solution containing 62.5 mM Tris, pH 6.8, 2% (weight/volume) SDS, and 100 mM β-mercaptoethanol in a 50°C water bath for 15 minutes. Blots were then blocked with nonfat dry milk and incubated overnight at 4°C with antibodies for: Akt, mTOR, rpS6, eIF2α, eEF2, ubiquitin (all from Cell Signaling Technology), S6k1, eIF2Bε, tuberin (all from Santa Cruz Biotechnology, Santa Cruz, CA), 4E-BP1, eIF4G (both from Bethyl Laboratories, Montgomery, TX), eEF1A (Upstate USA, Charlottesville, VA), or eIF2Bβ (provided by Drs. Jefferson and Kimball; Hershey, PA). Membranes were then processed as above. Signal densities for phosphorylated proteins were normalized to densities for their respective total proteins.

The association of eIF4E with eIF4G and 4E-BP1 was assessed by immunoprecipitation. Homogenates were incubated with an anti-eIF4E monoclonal antibody (provided by Drs. Jefferson and Kimball; Hershey, PA) at a final titer of 1:6 in a PBS solution containing 2% Triton X-100 on a rotating rack overnight at 4°C. Antibody-antigen complexes were collected by incubation with Biomag magnetic goat anti-mouse beads (PerSeptive Biosystems, Framingham, MA) suspended in buffer A (consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% β-mercaptoethanol, and 1% nonfat dry milk) for one hour. The beads were then captured in a magnetic rack and washed twice with the buffer A and once with buffer B (consisting of 50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 5 mM EDTA, 1% Triton X-100, 12 mM sodium deoxycholate, 0.1% (weight/volume) SDS, and .04% β-mercaptoethanol). Protein was eluted from the beads by boiling in 1X sample buffer and subjected to SDS-PAGE. Immunoprecipitated proteins including eIF4G and 4E-BP1 were detected by immunoblotting as described above.
2.2.6 RNA extraction, Northern blotting, and Ribonuclease Protection Assay

Total RNA was extracted from powdered gastrocnemius using TRI Reagent (Molecular Research Center, Cincinnati, OH). Total RNA (25 µg) was electrophoresed in a 1% agarose – 6% formaldehyde denaturing gel and transferred to Nytran Supercharge membranes (Schleicher and Schuell, Keene, NH). Blots were hybridized overnight at 42°C in ULTRAhyb (Ambion, Austin, TX) containing oligonucleotides for myostatin (5'-CAGCCCATCTTCTCTGGTGCTCTGGGAAGGTTACAGC-3'), MAFbx/Atrogin-1 (5'-CCCACCAGCACCAGCTTCTCTGGGACCAGCGTGC-3'), MuRF1 (5'-AGCGGAAACGACCTCCAGACATGGACACCGAGCCACCGC-3'), and polyubiquitin (5'-GGATCTTGGCTTTACGATGGTGACTGGGCTC-3') labeled by terminal transferase (Roche Diagnostics, Indianapolis, IN) tailing with [α-32P]dATP (Amersham Biosciences); a rat 18S oligonucleotide (5'-GTTATTGCTCAATCTCGGGTG-3') was identically labeled. A rat IGF-I probe was generated from cDNA (provided by Peter Rotwein; St. Louis, MO) using a Random Primed DNA Labeling kit (Roche). Membranes were washed twice in 2X SSC/0.1% SDS at 42°C for 5 minutes, once in 0.2X SSC/0.1% SDS at 42°C for 15 minutes, and once in 0.2X SSC/0.1% SDS at 48°C for 10 minutes to remove unbound probe. Blots were exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA), visualized, and analyzed using ImageQuant software (Version 5.2, Molecular Dynamics). Signal densities for mRNAs were normalized to densities for 18S mRNA. Cytokine mRNA was determined by RiboQuant Multi-probe RNase Protection Assay (RPA; BD PharMingen, San Diego, CA). Riboprobes were generated with the rat rCK – 1 template set by use of an in vitro transcription kit (BD PharMingen). Riboprobes were hybridized with 20 µg of total RNA as per the manufacturer’s protocol and protected RNAs were separated by electrophoresis on vertical 5% acrylamide gels. Gels were dried and exposed to PhosphorImager screens as outlined above. Data were analyzed using ImageQuant software and normalized to L32 mRNA.
2.2.7 Statistics

Experimental values are presented as means ± SE. Data were analyzed by unpaired Student’s $t$-test in two-group comparisons and ANOVA followed by Student-Neuman-Keuls test in multi-group comparisons to determine treatment effect when ANOVA indicated a difference among the means. Differences between groups were considered significant when $P < 0.05$.

2.3 Results

2.3.1 Unilateral immobilization induces skeletal muscle loss

Collectively, data from several separate studies indicated that five days of hindlimb immobilization decreased muscle wet weight 20 to 30% relative to time-matched contralateral control muscle. From the time-course data presented in Fig. 2-1, two distinct processes appear to underlie this wasted phenotype. No significant change was seen in wet weight following a single day of immobilization. By the third day, the gastrocnemius of the casted limb was significantly smaller than both the time-matched contralateral muscle (~19%, at day 3 compare b vs. c) and the one-day immobilized gastrocnemius (~8%, compare b at day 3 vs. a at day 1); this latter observation is demonstrative of skeletal muscle atrophy. By the fifth day the weight of the casted muscle was considerably reduced compared to control values (compare b at day 5 vs. d). However, unlike immobilized wet weight comparisons between day three and one, there was no difference in the size of the casted gastrocnemius between day five and day three (compare b at day 5 vs. b at day 3). This latter response is more indicative of growth failure in the muscle of the casted limb relative to the contralateral weight-bearing limb.
There was a concordant decrease in both total muscle protein concentration (179.4 ± 4.7 vs. 160.1 ± 3.9 mg protein/g wet weight, control vs. immobilized muscle, \( P < 0.05 \)) and protein content (306.7 ± 10.1 vs. 215.9 ± 6.5 mg protein/whole muscle, control vs. immobilized muscle, \( P < 0.05 \)) following five days of immobilization. Additionally, the calculated dry weight to wet weight ratios indicated a small but statistically significant decrease of the casted gastrocnemius (23.2 ± 0.2% vs. 22.6 ± 0.2%, control vs. immobilized muscle, \( P < 0.05 \)).

### 2.3.2 Activation of Akt

Full activity of Akt in response to stimulation by growth factors is dependent on phosphorylation at two residues, Thr 308 and Ser 473. Casted and control gastrocnemius from animals immobilized for five days displayed a comparable basal Akt phosphorylation that was enhanced equivalently in both muscles several-fold by IGF-I (Fig. 2-2). In the casted gastrocnemius total Akt protein decreased in proportion to the loss in protein content. However, the phosphorylation noted above was not a consequence of an alteration in the relative expression of total Akt when equivalent amounts of protein from immobilized and control samples were subject to SDS-PAGE. Hereafter this relative expression per equal amount of muscle protein will be referred to as the “total” amount.

Additionally, Akt plays an established role in PI3K-dependent phosphorylation of tuberin, the product of the tumor-suppressor gene \( TSC2 \) (section 1.4.1). Tuberin is a negative regulator of cell growth via its formation of a heterodimer with hamartin (the product of the \( TSC1 \) gene), consequently creating a functional complex capable of depressing activity of mTOR and its related effectors (79) (section 1.4.2). These growth suppressing properties can be precluded by Akt-directed phosphorylation of tuberin on Thr 1462 (119). Five days of immobilization was without effect on tuberin phosphorylation or content in any context (data not shown), arguing
against depression of the mTOR signaling pathway by tuberin under these experimental conditions.

### 2.3.3 Phosphorylation of S6k1 and rpS6

The laddering effect of S6k1 seen with IGF-I (Fig. 2-3A) is indicative of decreased electrophoretic mobility of the protein due to conformational changes in structure via phosphorylation. Thr 389 in the hydrophobic motif of the linker region is the principle rapamycin-sensitive, and therefore mTOR activity-dependent, residue whose phosphorylation is requisite for full kinase activity (140). Basal Thr 389 phosphorylation was virtually undetectable in immobilized and control muscle from saline-treated rats (Fig. 2-3B). IGF-I administration resulted in a marked multi-fold increase in phosphorylation, the magnitude of which was not different between casted and control muscle (Fig. 2-3B). Moreover, this phosphorylation was not a result of an increased protein expression, as total S6k1 remained unchanged under all conditions.

rpS6 is a component of the 40S ribosome and is postulated to play a role in mediating the effects of S6k1 on 5'-TOP translation (described previously in section 1.4.2). Furthermore, under many conditions phosphorylation of rpS6 correlates with increases in translational initiation and elongation rates (23, 42). Constitutive phosphorylation at two sets of sites in rpS6 (Ser 235/236 and Ser 240/244) was increased in five-day immobilized gastrocnemius (Figs. 2-4A and 2-4B, respectively). This immobilized-induced increase in basal phosphorylation was greater for Ser 235/236 (~three-fold) relative to Ser 240/244 (~50%). IGF-I treatment caused a disparate site-dependent increase in phosphorylation of rpS6. Growth factor stimulated phosphorylation of Ser 235/236 was equivalent between immobilized and control groups, whereas phosphorylation of Ser 240/244 was induced to a greater extent in muscle from the
casted limb (Fig. 2-4B). These changes occurred in the absence of altered total amounts of rpS6.

2.3.4 Phosphorylation of 4E-BP1, eIF4G, mTOR, and the distribution of eIF4E

When resolved by SDS-PAGE, 4E-BP1 separates into three distinct bands, \( \gamma \), \( \beta \), and \( \alpha \) (in order of hyper- to hypo-phosphorylation). Analysis of the \( \gamma \) isoform of 4E-BP1 was performed with a site-specific antibody for phosphorylation at Thr 37/46, an mTOR-dependent process thought to serve as a priming event for additional subsequent phosphorylation events that result in release of bound eIF4E (56). Following five days of immobilization, there was no difference in the \( \gamma \) form between casted and control muscle under basal conditions (Fig. 2-5A). Additionally, both muscles displayed similar increases in phosphorylation following growth factor treatment (Fig. 2-5A).

The proportion of eIF4E associated with 4E-BP1 (Fig. 2-5B) and eIF4G (Fig. 2-5C) was not different in gastrocnemius from casted and control limbs under basal conditions. Treatment with IGF-I caused a dissociation of eIF4E with 4E-BP1 and a concomitant increase in association of eIF4E with eIF4G (Figs. 2-5B and 2-5C, respectively) that was comparable in both groups and correlated with the above mentioned growth-factor induced phosphorylation of 4E-BP1 (Fig. 2-5A).

There was no difference in the extent of basal Ser 1108 phosphorylation of eIF4G between immobilized and control muscle after five days of immobilization (Fig. 2-5D). Both muscles remained responsive to the actions of IGF-I, although the increment in the muscle from the casted limb was significantly lower than that seen in control muscle. These observations were independent of a change in the total amount of eIF4G.

Finally, the phosphorylation of mTOR at Ser 2448 and 2481 was not significantly different in saline-treated immobilized and control muscle under basal conditions. Furthermore,
stimulation with IGF-I resulted in equivalent increases in the phosphorylation at both residues, regardless of muscle load (data not shown).

2.3.5 Subunit-specific changes in the guanine nucleotide-exchange factor eIF2B

The largest subunit of eIF2B, ε, serves as a substrate for several protein kinases and its activity can be modulated by these phosphorylation events (185). There were no significant differences in eIF2Bε phosphorylation at Ser 535 between any treatment following 5 days of hindlimb immobilization (data not shown). The five subunits of eIF2B associate to form two distinct subcomplexes whereby the ε and γ subunits form a catalytic subcomplex and the α, β, and δ subunits form a regulatory subcomplex (139). Immobilization induced a subcomplex-dependent increase in total subunit expression. The casted muscle exhibited an increase in total eIF2Bε protein when compared to control + saline values (1153 ± 224 vs. 1829 ± 180 arbitrary units, control vs. immobilized muscle, $P < 0.05$). This same augmentation was not observed for the β subunit, where no change in total protein was seen under any condition (data not shown). An additional mechanism of translation initiation control involves the phosphorylation of the α subunit of eIF2 at Ser 51, which effectively alters its role from substrate to a competitive inhibitor for the guanine nucleotide exchange factor activity of eIF2B (section 1.3.1.1). No change in phosphorylation or total amount of eIF2α was noted with immobilization or IGF-I treatment (data not shown).

2.3.6 Eukaryotic elongation factors

Translocation activity of eEF2 is modulated by phosphorylation at Thr 56 by a calcium and calmodulin dependent kinase, eEF2k. This phosphorylation event inactivates eEF2 by
preventing its binding to the ribosome (145). The phosphorylation state of eEF2 was not altered in response to immobilization or IGF-I treatment (data not shown). Additionally, no change in protein content was seen for either eEF2 or eEF1A in any treatment condition.

2.3.7 Protein synthesis and translational efficiency

When mixed muscle protein synthesis was assessed directly, no difference was observed between any of the experimental conditions (Table 2-1). Though the total RNA content of the casted gastrocnemius decreased, when normalized to muscle wet weight the total RNA concentration was found to be equivalent to that of control muscle (Table 2-1). Translational efficiency, was not significantly reduced in immobilized muscle (Table 2-1). No change in any of the above parameters was detected following 20 minutes of IGF-I stimulation.

2.3.8 Effect of insulin on regulators of translational initiation

Since the observed decrement in wet weight with casting could be a result of resistance to humoral anabolic cues, the acute effects of insulin on regulatory proteins implicated in 5'-TOP and cap-dependent translation were also investigated following five days of immobilization. Casted and control gastrocnemius muscle remained equally responsive to the stimulatory effects of insulin and displayed qualitatively similar results to those obtained following IGF-I administration regarding phosphorylation of Akt, S6k1, rpS6, 4E-BP1, eIF4G, and mTOR (data not shown).
2.3.9 Gene expression of MAFbx/Atrogin-1, MuRF1, and polyubiquitin

Immobilization resulted in a rapid and persistent increase in both MAFbx/Atrogin-1 (Fig. 2-6A) and MuRF1 (Fig. 2-6B) mRNA in the casted muscle as compared to control. For both genes, expression was significantly upregulated at day 1, attained a peak at day 3, and returned to day 1 values at day 5. It is noteworthy that the maximum expression of these genes is associated with immobilization-induced atrophy (compare day 3, Figs. 2-1, 2-6A, and 2-6B), while growth failure coincides with the lower level of induction (compare day 5, Figs. 2-1, 2-6A, and 2-6B).

The time course of induction for the polyubiquitin gene family (106) in response to unilateral unloading was identical to that determined for the ubiquitin ligases (Fig. 2-7A). This stimulation of the polyubiquitin gene concurs with a qualitative assessment of increased ubiquitin conjugates in immobilized and control muscle over 1, 3, and 5 days (Fig. 2-7B). Ubiquitin antibody immunoreactivity is increased in immobilized muscle homogenates (compare immobilized vs. control conditions) and was greatest at day 3, allied with peak mRNA expression.

2.3.10 Gene expression of IGF-I, myostatin, and pro-inflammatory cytokines

Muscle loss during immobilization may also be a consequence of altered expression of other positive or negative regulators of growth and tissue mass. In this regard, five days of immobilization did not result in a statistically significant change in IGF-I, myostatin, TNFα, IL-6, or IL-1α mRNA between casted and control muscle (data not shown).
2.3.11 Immobilization-induced atrophy is glucocorticoid-independent

To determine if an altered sensitivity of casted muscle to glucocorticoids was a causal factor in the observed wasting and gene expression for the E3 ligases MAFbx/Atrogin-1 and MuRF1, rats were subjected to unilateral hindlimb immobilization for 3 days while concomitantly treated with vehicle or the glucocorticoid receptor antagonist RU-486. There was no significant difference in skeletal muscle wet weight or mRNA expression of MAFbx/Atrogin-1 and MuRF1 between vehicle and RU-486 treated immobilized muscle, as was the case for vehicle and antagonist-treated control muscle (data not shown).

2.3.12 Immobilization-induced atrophy is proteasome-dependent

In order to better define the proteolytic systems potentially responsible for atrophy during immobilization and to determine if inhibition of proteasome function could potentially rescue weight loss, the peptide boronate Velcade was administered in vivo. Gastrocnemius wet weight was not significantly different between saline- and Velcade-treated contralateral control muscle. Critically, injection of the proteasome inhibitor prevented ~53% of the wet weight loss of the immobilized muscle relative to the saline-treated contralateral control muscle (Fig. 2-8).

2.4 Discussion

The accepted progression of disuse atrophy is rooted in adaptations in protein metabolism that involve both a reduction in synthesis and an increase in degradation (section 2.1). To investigate the signaling pathways likely to underlie these changes we performed a systematic analysis of mediators of translational initiation and elongation as well as components
of the ubiquitin-tagging process in a well-defined model of unilateral hindlimb casting. Five days of unilateral immobilization drastically reduced gastrocnemius wet weight relative to the contralateral control muscle, which was an outcome of both atrophy and growth failure (section 2.3.1). In stark contrast to results from previous investigations of disuse, we observed no defect in the status of or intracellular signaling to critical regulators of the translational machinery, including Akt, mTOR, S6k1, 4E-BP1, and eIFs and eEFs under basal and growth factor-stimulated conditions at day 5 (sections 2.3.2 - 2.3.4 and 2.3.6). In accord with these findings, there was no alteration in the binding behavior of eIF4E under basal and stimulated conditions in the casted muscle (section 2.3.4). Furthermore, the phosphorylation of rpS6 and protein content of eIF2Be was augmented in immobilized gastrocnemius (sections 2.3.3 and 2.3.5), though the significance of these changes is unclear. When protein synthesis was assessed directly in vivo, no difference in synthetic measurements or translational efficiency was evident between casted and control muscle (section 2.3.7). In contradistinction, the mRNA content for polyubiquitin, MAFbx/Atrogin-1, and MuRF1 was elevated throughout the time course of immobilization and functionally reflected in the increased content of ubiquitin-conjugates in whole muscle homogenates from casted gastrocnemius (section 2.3.9). Moreover, in vivo administration of the proteasome inhibitor Velcade abrogated a significant portion of weight loss in immobilized muscle (section 2.3.12). Therefore, these studies describe a unique case of disuse remodeling wherein the reduction in gastrocnemius size after a moderate duration of unloading is likely attributable to elevated proteolysis, independent of decreases in protein synthesis and responsiveness of hypertrophic signaling cascades – observations which represent a considerable departure from conclusions reached previously with other models of disuse including hindlimb suspension and bilateral hindlimb immobilization.

Combinatorial atrophy and/or impairment of growth of “mixed” and fast muscle types (e.g. tibialis anterior, EDL, plantaris, and gastrocnemius) during unloading has been noted with other models, particularly hindlimb suspension (61, 129). This phenomenon is mostly apparent
when rapidly-growing juvenile animals are used and makes interpretation of data generated by a single time-point comparison to any cohort of normally growing animals problematic. In such cases it is difficult to demonstrate that the growth failure is a product of disuse itself and not a general characteristic of the model due to depression of overall animal growth which may occur (129, 179). However, the coordinate sampling of wet weight at multiple time points and use of an internal contralateral control plainly demonstrate in young rats that the secondary growth failure response of the gastrocnemius is a direct consequence of disuse and not a general response of the organism per se (section 2.3.1).

Several lines of evidence support the conclusion that a repression of protein synthesis is not necessary for the decline in gastrocnemius wet weight following five days of unilateral immobilization. First, total RNA concentration was preserved in the casted muscle. Accordingly, the capacity for translation appears to be coordinately regulated with tissue size under conditions of immobilization and cannot account for the wasted phenotype. Second, a thorough analysis of translational control factors failed to detect any abnormality in regulators tasked with determining global rates of translation as well as translation of specific species of mRNA - those with 5'TOPs and highly structured untranslated regions (UTRs). Third, intracellular signaling to and from several strategically located kinases that control protein synthetic responses, including PDK1, Akt, and mTOR (as assessed by phosphorylation events of known downstream targets), was preserved in the casted gastrocnemius muscle. Fourth, and most importantly, a direct in vivo measurement of protein synthesis and translational efficiency following an overnight fast did not indicate any depression of either synthetic index between immobilized and control gastrocnemius.

In contrast to the results of the present study, in a bilateral hindlimb immobilization model, casting produced a rapid (within 6 hours) and persistent (up to 7 days) decrease in rates of protein synthesis (15, 180). This discrepancy in the responses of protein synthesis and translational control pathways may be a consequence of animal age. The present results were
obtained with young, rapidly growing rats, whereas the response of adult animals (used in the aforementioned reports with bilateral immobilization) following unilateral immobilization may differ owing to the slower rate of protein accretion in mature muscle relative to its younger counterpart. Alternatively, this incongruity may reflect a fundamental difference in the models (i.e. there is a decrease in synthesis with bilateral but not unilateral casting). There is little data to support the contention that fasting imposed here masked a protein synthetic defect in the immobilized limb. First, in other catabolic conditions a reduction in protein synthesis is readily apparent in fasted animals, and second, unilateral casting produced comparable changes in the phosphorylation of key regulators of translation (e.g mTOR, 4E-BP1, S6k1, eIF2α, and eEF2) independent of prandial state (data not shown). Though the data presented here argue against the contribution of a defect in protein synthesis to the immobilization-induced atrophy at a time point where it has been observed in previous investigations, they do not exclude the possibility that a transient depression of synthesis or defect in signaling through pathways of translational control occurred at an earlier time point. Furthermore, the global rate of protein synthesis determined here may not be representative of the modulation of any one specific protein. It is possible that the reduced synthesis of one or more key regulatory protein(s) may underlie the wasting observed here. Regardless, the results presented here indicate that defective signaling to regulators of initiation and/or elongation and a decrease in protein synthesis are not prerequisites for moderate duration disuse-mediated atrophy, despite the widely accepted dogma that states otherwise.

Three main observations support the conclusion that unilateral immobilization upregulates ubiquitin-proteasome dependent protein degradation. First, unilateral immobilization increased the mRNA for several components of the UPP, including polyubiquitin and the E3 ligases MAFbx/Atrogin-1 and MuRF1. This response of the polyubiquitin gene is an archetypal marker of proteolytic conditions (including cancer cachexia, denervation, glucocorticoid excess, fasting, hindlimb suspension, and sepsis) that changes coordinately with
rates of ATP-dependent proteolysis (5, 122, 171, 177). In addition, the overall pattern of gene expression for both ubiquitin ligases is virtually identical to results obtained in the gastrocnemius following external fixation and similar to those seen with hindlimb suspension for a comparable time course (12). Second, the corroborating increases in ubiquitin immunoreactivity in muscle homogenates of unilaterally immobilized skeletal muscle is comparable to results obtained by immunoblot analysis in other well-characterized catabolic states (5, 50, 177, 189). Though these data do not represent a direct measurement of conjugating activity, they nevertheless support the notion that unilateral immobilization induces the ubiquitination of muscle proteins and argues for involvement of energy-dependent proteolysis by the proteasome during hindlimb casting. Finally, the injection of Velcade (also known as PS-341 or bortezomib) (1) prevented a significant loss of gastrocnemius wet weight following 3 days of immobilization, thereby directly implicating the proteasome in catalyzing a portion of the muscle wasting occurring in this model of disuse. While not a direct measurement of proteolysis, the anti-catabolic effect of this proteasome inhibitor provides strong physiological evidence that increased protein degradation causes significant gastrocnemius weight loss with immobilization. Altogether, these coordinated changes in gene expression are consistent with upregulated ubiquitin conjugation and consequent proteasomal targeting of skeletal muscle protein as major causal factors in mediating immobilization-induced atrophy.

The catabolic role of glucocorticoids is well-supported by in vivo reports using adrenalectomized rats or administration of the type II glucocorticoid receptor antagonist RU-486 (125, 177), treatments which have a muscle-sparing effect. Critically, RU-486 treatment averted MAFbx/Atrogin-1 and MuRF1 transcription induced by experimental sepsis (193). In studies presented here, RU-486 neither prevented immobilization-induced gastrocnemius atrophy and growth failure nor the upregulation of MAFbx/Atrogin-1 and MuRF1 at any time point. These results are consistent with prior investigations that found adrenalectomized rats or those treated with RU-486 at doses greater than used here had only a minor sparing in unloading-induced
atrophy of the soleus (83, 179). While glucocorticoids are necessary for MAFbx/Atrogin-1 and MuRF1 induction in some catabolic states, they do not appear to be so in unilateral immobilization.

Though one published study has administered the proteasome inhibitor PSI to prevent acute sepsis-induced protein degradation and 3-methylhisitidine release measured in vitro (50), the data here represent the first demonstration of the ability of a proteasome inhibitor to attenuate in vivo muscle wasting resulting from a catabolic state. The reduction in muscle catabolism apparent with Velcade treatment is striking, yet this antagonism was not complete. This raises the possibility that other proteolytic pathways, such as the lysosomal or calcium-dependent systems, are responsible for the remaining loss in gastrocnemius weight. Furthermore, the relative contributions of energy-dependent versus energy-independent proteolysis by the proteasome cannot be determined with this approach. The sparing effect reported here is likely to be under-reported because of the comparison to the contralateral control muscle, which continues to grow throughout the 3-day period of immobilization. Moreover, it is possible that a greater dose of Velcade could have a more pronounced effect. Nevertheless, these data provide proof that the proteasome is responsible for increased muscle loss during unilateral immobilization. Subsequent to this work, others have reported that in vivo administration of Velcade can also partially prevent the loss of muscle mass seen in response to thermal injury (102).

In conclusion, this chapter details a model apposite for the study of disuse muscle wasting using unilateral hindlimb immobilization. In the gastrocnemius, the prominent loss of muscle mass after a moderate duration of unloading results from cooperative atrophy and growth failure that is independent of a decrease in protein synthesis and appears at least partially contingent upon an increase in protein degradation. Mechanistically, the implementation of a defined transcriptional program, by a stimulus other than glucocorticoids, may accelerate proteolysis in the unloaded state. This response induces polyubiquitin,
MAFbx/Atrogin-1, and MuRF1 gene expression and results in increased ubiquitin-conjugate formation in the wasted gastrocnemius. Antagonism of proteasome-dependent proteolysis averts a portion of skeletal muscle loss, an observation consistent with the role of the ubiquitin-proteasome system as a primary effector of immobilization-induced skeletal muscle atrophy.
Fig. 2-1: Effect of one, three, and five days immobilization on gastrocnemius wet weight. The value from the contralateral control gastrocnemius of rats subject to one day immobilization is set at 100%. Data are presented as a line graph of the mean percentage of gastrocnemius weight in comparison to the one day control ± SE for 10 animals per group. Means with different letters are statistically different from one another ($P < 0.05$). Average immobilized and contralateral control gastrocnemius wet weights (in grams) were 1.01 and 1.05 after one day, 0.92 and 1.12 after three days, and 0.93 and 1.32 after five days, respectively.
Fig. 2-2: **Effect of five days immobilization on phosphorylation of Akt.** *Top:* Densitometric analysis of Akt phosphorylation at Thr$^{308}$. The value from the contralateral control gastrocnemius of rats given saline (iv) is set at 1.0 A.U. Data are presented as a bar graph of the mean ± SE for 12 animals per group. Means with different letters are statistically different from one another ($P < 0.05$). *Bottom:* Representative immunoblots of phosphorylation at the Thr$^{308}$ site of Akt and total Akt, respectively.
Fig. 2-3: Effect of five days immobilization on phosphorylation of S6k1.  

A: Representative immunoblot of total S6k1 phosphorylation. Presence of greater phosphorylation states is indicative of its activation by IGF-I.  

B, Top: Densitometric analysis of S6k1 phosphorylation at Thr^{389}. The value from the contralateral control gastrocnemius of rats given saline (iv) is set at 1.0 A.U. Data are presented as a bar graph of the mean ± SE for 12 animals per group. Means with different letters are statistically different from one another (P < 0.05).  

B, Bottom: Representative immunoblot of phosphorylation at the Thr^{389} site of S6k1.
Fig. 2-4: Effect of five days immobilization on phosphorylation of rpS6.  A, Top: Densitometric analysis of rpS6 phosphorylation at Ser\textsuperscript{235} and Ser\textsuperscript{236}. The value from the contralateral control gastrocnemius of rats given saline (iv) is set at 1.0 A.U.  A, Bottom: Representative immunoblots of phosphorylation at the Ser\textsuperscript{235} and Ser\textsuperscript{236} sites of rpS6 and total rpS6, respectively.  B, Top: Densitometric analysis of rpS6 phosphorylation at Ser\textsuperscript{240} and Ser\textsuperscript{244}. The value from the contralateral control gastrocnemius of rats given saline (iv) is set at 1.0 A.U.  B, Bottom: Representative immunoblots of phosphorylation at the Ser\textsuperscript{240} and Ser\textsuperscript{244} sites of rpS6 and total rpS6, respectively.  Data are presented as a bar graph of the mean ± SE for 12 animals per group. Means with different letters are statistically different from one another (P < 0.05).
Fig. 2-5: Effect of five days immobilization on regulators of cap-dependent translation.  
A, Top: Densitometric analysis of the hyperphosphorylated γ-isoform of 4E–BP1 phosphorylation at Thr$^{37}$ and Thr$^{46}$. The value from the contralateral control gastrocnemius of rats given saline (iv) is set at 1.0 A.U.  A, Bottom: Representative immunoblot of phosphorylation at Thr$^{37}$ and Thr$^{46}$ sites of 4E–BP1. β- and γ- isoforms are indicated.  B, Top: Densitometric analysis of 4E–BP1 associated with eIF4E assessed through immunoprecipitation. The value from the contralateral control gastrocnemius of rats given saline (iv) is set at 1.0 A.U.  B, Bottom: Representative immunoblots of 4E–BP1 bound to eIF4E and total eIF4E, respectively.  C, Top: Densitometric analysis of eIF4G associated with eIF4E assessed through immunoprecipitation. The value from the contralateral control gastrocnemius of rats given saline (iv) is set at 1.0 A.U.  C, Bottom: Representative immunoblots of eIF4G bound to eIF4E and total eIF4E, respectively.  D, Top: Densitometric analysis of eIF4G phosphorylation at Ser$^{1108}$. The value from contralateral control gastrocnemius of rats given saline (iv) is set at 1.0 A.U.  D, Bottom: Representative immunoblots of phosphorylation at the Ser$^{1108}$ site of eIF4G and total eIF4G, respectively. Data are presented as a bar graph of the mean ± SE for 12 animals per group. Means with different letters are statistically different from one another ($P < 0.05$).
Table 2-1: Effect of five days hindlimb immobilization on gastrocnemius mixed muscle protein synthesis, RNA content, and translational efficiency

<table>
<thead>
<tr>
<th></th>
<th>Control + Saline</th>
<th>Control + IGF I</th>
<th>Immobile + Saline</th>
<th>Immobile + IGF I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of mixed muscle protein synthesis (nmol Phe/mg protein/hour)</td>
<td>8.92 ± 0.53</td>
<td>6.37 ± 0.46</td>
<td>7.00 ± 0.49</td>
<td>6.97 ± 0.65</td>
</tr>
<tr>
<td>Muscle RNA concentration (mg RNA/g wet weight)</td>
<td>1.23 ± 0.06</td>
<td>1.09 ± 0.05</td>
<td>1.13 ± 0.03</td>
<td>1.10 ± 0.02</td>
</tr>
<tr>
<td>Muscle RNA content (mg RNA/muscle)</td>
<td>2.03 ± 0.10</td>
<td>1.90 ± 0.05</td>
<td>1.548 * ± 0.09</td>
<td>1.594 * ± 0.09</td>
</tr>
<tr>
<td>Translational efficiency (nmol Phe/mg RNA/hour)</td>
<td>1051 ± 96</td>
<td>1031 ± 96</td>
<td>1015 ± 68</td>
<td>1032 ± 129</td>
</tr>
</tbody>
</table>

Values are means ± SE. Averages for protein synthesis, RNA content, RNA concentration, and translational efficiency were calculated with \( n = 8 \) to 10 animals per group. Means with * are statistically different from control + saline values (\( P < 0.05 \)).
Fig. 2-6: Effect of one, three, and five days immobilization on mRNA content of MAFbx/Atrogin-1 and MuRF1. 

A, Top: Quantitation of MAFbx/Atrogin-1 mRNA. Changes for all transcript sizes were qualitatively similar; data are shown for the predominant transcript. The value for the contralateral control gastrocnemius of rats subjected to one day immobilization is set at 1.0 A.U. 

A, Bottom: Representative autoradiographs of MAFbx/Atrogin-1 and 18S mRNA, respectively. 

B, Top: Quantitation of MuRF1 mRNA. The value for the contralateral control gastrocnemius of rats subject to one day immobilization is set at 1.0 A.U. 

B, Bottom: Representative autoradiographs of MuRF1 and 18S mRNA, respectively. Data are presented as a line graph of the mean ± SE for 10 animals per group. Means with different letters are statistically different from one another ($P < 0.05$).
Fig. 2-7: Effect of one, three, and five days immobilization on mRNA content of polyubiquitin and content of ubiquitin conjugates. A, Top: Quantitation of polyubiquitin mRNA. Changes for all transcript sizes were qualitatively similar; data are shown for the predominant transcript. The value for the contralateral control gastrocnemius of rats subject to one day immobilization is set at 1.0 A.U. Data are presented as a line graph of the mean ± SE for 10 animals per group. Means with different letters are statistically different from one another (P < 0.05). A, Bottom: Representative autoradiographs of polyubiquitin and 18S mRNA, respectively. B: Representative immunoblot of ubiquitin conjugates.
Fig. 2-8: Effect of Velcade injection on gastrocnemius wet weight following three days of immobilization. The value from the contralateral control gastrocnemius of rats given saline vehicle (ip) is set at 100%. Data are presented as a bar graph of the mean percentage of gastrocnemius weight ± SE for 10 to 11 animals per group. Means with different letters are statistically different from one another ($P < 0.05$). The average contralateral control gastrocnemius weight (in grams) of vehicle-treated rats was 1.08 ± .03.
Chapter 3

The muscle-specific ubiquitin ligases MAFbx/Atrogin-1 and MuRF1 as determinants of the skeletal muscle atrophic phenotype

3.1 Introduction

Arguably the most intriguing results gleaned from the unilateral immobilization experiments involve the potential role of ubiquitin conjugation and proteasomal degradation in determining the variable wet weight response expressed by the casted gastrocnemius. In particular, the results detailed in sections 2.3.1 and 2.3.9 establish a strong inverse correlation between muscle weight and the mRNA expression of the E3s MAFbx/Atrogin-1 (hereafter referred to solely as MAFbx) and MuRF1, suggesting these ligases have some contributory role to the immobilization-induced loss of muscle mass. These data, in combination with evidence obtained in other investigations (mentioned previously in section 2.1 and discussed below), suggest the significance of these specific E3s as principal regulators of skeletal and cardiac muscle size. Given their potential importance in unilateral immobilization and many other models of muscle wasting, a closer examination of their regulation and functional properties and is warranted.
3.2 MAFbx: An F-box component of the SCF ubiquitin ligase machinery

3.2.1 Discovery and characterization of MAFbx as an E3

MAFbx was initially discovered by two independent laboratories via high-throughput screening methods as a means to identify potential gene markers of atrophying muscle (12, 62). Subsequent cloning and analysis of the open reading frame of the gene predicted a protein product of roughly 41 kDa for which homo- and ortho-logs exist in humans, *Drosophila melanogaster*, and *Caenorhabditis elegans* (62). Further investigation for functional domains indicated the presence of an F-box domain. F-box domain-containing proteins most often function as the F-box component (the critical substrate recognition component) of a Skp1-Cullin/Cdc53-F-box (SCF) ubiquitin ligase (30, 187). SCF ligases themselves do not possess inherent catalytic/ubquitin conjugation activity, instead relying on individual functions of its subunits as constituents of the holocomplex to both bind substrates (i.e. proteins) targeted for degradation and bring them into close proximity of an accompanying E2 conjugating enzyme (30, 187) (section 1.3.2.1). A role in substrate recognition was initially suggested for MAFbx on the basis of its ability to physically interact with the individual components of a SCF ligase. Both yeast two-hybrid analysis (12) and co-immunoprecipitations using epitope-tagged proteins (12, 62) confirmed that MAFbx was capable of interacting with Skp1 (dependent on the presence of an intact, complete F-box domain). The accumulation of more definitive proof (including identification of its specific substrates, detailed below in section 3.2.2) has been limited despite the disproportionate attention MAFbx currently receives.
3.2.2 MAFbx expression patterns and known functions

MAFbx mRNA expression was found to be exclusive to muscle and responsive to several specific insults which cause skeletal muscle atrophy (12, 62); numerous subsequent studies have both confirmed and expanded the contexts in which the mRNA content for this gene increases significantly (section 2.1). This correlational relationship is the primary basis for the assumption that increases in MAFbx gene expression are required for wasting of skeletal muscle during these states. To date, there are few experimental data justifying such a generalization. While homozygous MAFbx knockout mice are indeed partially resistant to denervation-induced atrophy (12), such an occurrence has not been demonstrated for any other catabolic condition where mRNA content of the E3 is known to increase. Additionally no direct evidence demonstrates that MAFbx expression is necessary and/or sufficient for basal proteolysis or the amplified proteasomal degradation at the muscle level (i.e. non-specific degradation) that occurs during marked atrophy.

The known repertoire of specific MAFbx targets is no more developed than the understanding of its general function in various (patho)physiological states. A single investigation determined that the basic helix-loop-helix transcription factor MyoD is a direct target of the SCF complex containing MAFbx, ultimately resulting in the ubiquitination and degradation of the myogenic regulatory factor (178). Such a finding offers a plausible explanation for the loss of skeletal muscle during wasting – the destruction of MyoD could be hypothesized to severely impair the regenerative capacity and sustainability of muscle mass by preventing the complete differentiation of progenitor (satellite) cells into mature fibers. However, no such cause-and-effect relationship has been reproduced in vivo as of yet. The most well-established substrate of the SCF^{MAFbx} ligase is calcineurin in cardiac tissue (110), where MAFbx acts to suppress development of cardiac hypertrophy by preventing calcineurin transactivation and NFATc4 translocation to the nucleus. Whether or not calcineurin signaling is also
antagonized in skeletal muscle is unknown. The universality of such an effect in all muscle types may be unlikely due to the contrasting functions MAFbx appears to possess in skeletal muscle (where the available evidence suggests it has pro-atrophic properties) versus cardiac muscle (where it is decidedly anti-hypertrophic).

3.3 MuRF1: An RBCC family ubiquitin ligase

3.3.1 Discovery and characterization of MuRF1 as an E3

In contrast to MAFbx (which was identified based on its direct association with muscle atrophy, section 3.2.1), the discovery of MuRF1 was the product of a yeast two-hybrid search for putative regulators of the titin kinase domain (22) and an independent data-mining of a human EST database for sequence similarity to a known RING domain amino acid sequence (33). Further studies established MuRF1 as one of three very similar MuRF family proteins capable of homo- and hetero-oligomerization (63). The full length cDNA of MuRF1 predicts a protein of 38 kDa containing an N-terminal RING domain and a zinc-binding B-box domain (22, 33) - features common to all MuRFs (63). These domains establish the MuRF proteins as members of a more expansive RING-B-box-coiled-coiled (RBCC) family whose members can, among other functions, ubiquitinate proteins for degradation by the proteasome (63). Such a function was established for recombinant MuRF1 specifically using an in vitro assay system which reconstitutes ubiquitin ligase activity by controlling the presence or absence of the requisite E1 and E2 enzymes and ATP (12). A more detailed analysis of its ubiquitin ligase activity, complete with the identification of an in vivo substrate, has been performed in cardiomyocytes (section 3.3.2 below).
3.3.2 MuRF1 expression patterns and known functions

Like MAFbx, MuRF1 mRNA expression was found to be muscle-specific (22, 33) and remarkably responsive to conditions that induce wasting (section 2.1). Also, similarly to MAFbx, there is little evidentiary validity to the concept that an increase in MuRF1 is responsible for either elevated global proteolysis or muscle loss occurring during all the catabolic conditions where its mRNA content has been reported to be increased. There are two notable instances where disruption of the MuRF1 gene offers partial resistance to atrophic stimuli: during denervation-induced atrophy (12) and under conditions of transgenic activation of the NF-κB pathway (20).

Given its discovery as a binding partner of titin, one of the more well-established functions of MuRF1 surrounds its role in associating with and regulating portions of the cytoskeletal architecture of muscle fibers, particularly M-line integrity (120). Intriguingly, MuRF1 also localizes to the nucleus (33, 120) and associates with a number of ancillary proteins involved in diverse signaling processes, including SUMOylation (120). This has led to the hypothesis that the E3 may act to link the titin kinase domain to gene expression and protein ubiquitination/degradation in skeletal muscle (63). Like MAFbx, MuRF1 also appears to possess anti-hypertrophic functions in cardiomyocytes. Yeast two-hybrid screens have revealed an association between MuRF1 and troponin I (92). The latter was proven to be an in vivo ubiquitinated substrate of the ligase, leading to its proteasomal-dependent degradation. MuRF1 overexpression itself was sufficient to reduce the force and frequency of contractile activity in these cells (92). In separate studies conducted by the same laboratory, MuRF1 was found to antagonize phenylephrine-induced cardiomyocyte hypertrophy by interfering with PKCε translocation to and formation of focal adhesions (3). These observations have yet to be reproduced in any skeletal muscle tissue or culture model.
3.4 Cellular signaling and transcriptional control of MAFbx and MuRF1 gene expression

Although both MAFbx and MuRF1 have received considerable attention since their respective discoveries in 2001, relatively few fundamental questions about their function at an organ level and in specific cellular processes have been answered. Among the areas most hotly researched in this burgeoning field are the identification of specific ubiquitinated substrates for each ligase (the progress of which has been outlined above in the previous sections 3.2.2 and 3.3.2) and the cellular signaling mechanisms accountable for the accumulation of their mRNAs observed during muscle atrophy.

Extensive investigation aimed at identifying both an initiating signal and a unified gene expression profile common to many distinct atrophic conditions (as well as triggers and genes capable of reversing such catabolism), has led to several notable discoveries. First, glucocorticoids and inflammatory stimuli/cytokines are central initiating stimuli for increases in MAFbx and MuRF1 expression. In specific instances, these effects appear to depend on the actions of the glucocorticoid receptor (193) and NF-κB (20) or p38 (111) signaling, respectively. Second, elevations in expression of these E3s are antagonized by insulin and IGF-I action through PI3K signal transduction to both Akt (35, 152, 153, 169) and/or mTOR (104). Finally, the reduction in mRNA content of these ligases in response to growth factor treatment appears to depend on suppression of their transcriptional synthesis and is not a consequence of increased mRNA degradation (35, 152). In hindsight, the arrival at the first two conclusions is somewhat expected. The opposing functional relationship between insulin and the counter-regulatory hormone response induced by starvation or stress, of which glucocorticoids play a key role, is one of the best-characterized circuits of metabolic control. Furthermore, manipulations of the various components of the insulin signaling cascade control organ and cell size in virtually every organism, while many of the wasting conditions exhibiting increases in
MAFbx and MuRF1 are also associated with insulin resistance as well as decreases in circulating insulin and/or IGF-I.

Therefore the available data suggest the transcriptional regulation of MAFbx and MuRF1 is exquisitely sensitive to situations entailing an increased production of catabolic factors in tandem with the absence or repression of signaling to growth-promoting kinases (including those discussed in section 1.4), and would be predicted to favor increased ligase expression in situations such as starvation, infection, disuse, or diabetes (as is clearly the case in the equivalent experimental models).

3.5 AMPK: A link between energy insufficiency and MAFbx and MuRF1 expression?

Despite several key advancements in the collective understanding of MAFbx and MuRF1 regulation, unilateral immobilization appears to be an instance where this regulatory paradigm is incapable of explaining the increased expression of these ubiquitin ligases. Akt has emerged as the preeminent negative regulator of MAFbx and MuRF1 expression, yet a deficit in basal or growth factor-induced signaling to this kinase is not observed in the casted gastrocnemius (section 2.3.2). While glucocorticoids have been established as a major stimulus for increased muscle-specific E3 expression, the administration of the glucocorticoid receptor antagonist RU-486 failed to prevent augmented MAFbx and MuRF1 expression during unloading (section 2.3.11). It is also unlikely that the “traditional” NF-κB pathway is singularly responsible for the ligase upregulation seen in the present studies, as its genetic activation in muscle resulted in the specific upregulation of MuRF1 and not MAFbx (20), while casting resulted in increases in both MAFbx and MuRF1 (section 2.3.9). Though these observations do not definitively exclude the influence of these pathways during unilateral immobilization, they suggest additional regulatory mechanisms must exist that control expression of these ligases.
In that regard, the findings which demonstrate sensitivity of one or both of their gene expression(s) to conditions of nutrient and energy deprivation in vivo (12, 62, 106) and in vitro (153) are of particular interest. One predicted consequence of nutrient limitation is cellular energy deprivation (i.e. ATP depletion). mTOR has previously been shown to control the expression of MAFbx (104) (section 3.4) and act as an energy sensor for levels of cellular ATP (36); therefore it is reasonable to hypothesize that exhaustion of cellular energy stores during starvation would inhibit mTOR and lead to increases in expression of MAFbx (and perhaps MuRF1). However, the sensitivity of mTOR to ATP depletion is poor relative to intracellular concentrations of ATP, requiring drastic reductions in energy stores before mTOR activity becomes inhibited (145). A more likely candidate for controlling energy-dependent expression of MAFbx and MuRF1 is AMPK. Briefly, AMPK responds to a disruption of the “energy charge” of a cell (discussed in more detail in the following chapter). AMPK has established effects on the expression of a numerous genes involved in energy metabolism in multiple tissues, including skeletal muscle (section 4.1). However, its ability to control expression of genes associated with protein catabolism or proteolysis is unknown. Therefore studies were undertaken with the following purpose and working hypothesis.

3.5.1 Purpose

The purpose of these investigations was to expand upon the understanding of the cellular signaling events regulating the expression of MAFbx and MuRF1, two muscle-specific ubiquitin ligases identified by the preceding work on hindlimb immobilization as potential contributors to the phenotype(s) displayed by atrophied skeletal muscle.
3.5.2 Working Hypothesis

The working hypothesis tested by this research was that activation of the AMPK signaling cascade in both *in vitro* and *in vivo* models would result in an increase in the mRNA content of MAFbx and MuRF1.
Chapter 4

AMP-activated protein kinase agonists increase mRNA content of the muscle-specific ubiquitin ligases MAFbx and MuRF1 in C2C12 cells

Data and text within this chapter have been re-printed from the following published journal article (used with permission):


4.1 Introduction

Proper growth and survival of all organisms require means of responding to diverse inputs originating from a discontinuous environmental milieu. Failure to both properly integrate these signals and accordingly adjust metabolism within favorable physiological limits may ultimately result in maladaptation, disease, and death. One critical component of this control system in mammals is AMPK. AMPK functions as a heterotrimer (consisting of a catalytic α subunit and regulatory β and γ subunits) that senses alterations in the AMP/ATP ratio within cells (69, 87, 117). In response to energy-depleting stressors (which consequently increase cellular AMP concentrations) AMPK acts to balance energy consumption with production by suppressing ATP-expensive processes and activating ATP-repleting ones. Regulation of its
activity is achieved through several AMP-dependent mechanisms (69). Binding of AMP to the γ-subunit allosterically activates the kinase, makes the kinase a better substrate for upstream AMPK kinases (AMPKKs), and prevents dephosphorylation of the kinase by phosphatase activity. Conversely, high ATP concentrations inhibit these stimulatory effects of AMP, perhaps via competitive and mutually exclusive binding of AMP versus ATP to the γ-subunit (157). These mechanisms consort to make AMPK sensitive to a critical threshold AMP concentration (70), above which it endeavors to drive the system back towards energetic homeostasis.

AMPK has proven to be an essential intermediate in the control of fundamental cellular processes such as growth (80, 81), proliferation (86), and survival (32, 81). Additionally AMPK is equally important in orchestrating multiple signaling pathways controlling nutrient uptake and fuel metabolism in many tissue types (69, 87, 117). Furthermore, AMPK plays a crucial underlying role in more complex physiological and behavioral phenomena, such as inter-organ communication via various cyto- and adipo-kines (87, 117), and control of feeding behavior (87, 117), voluntary energy expenditure (130), and cognitive ability (32). AMPK induces these and other effects at a cellular level primarily through two means: direct phosphorylation of rate-limiting or otherwise strategic components involved in pathways of metabolic control and through a less-well understood control of gene expression.

The outcome of AMPK activation on the expression of specific genes is, in general, consistent with the corresponding metabolic responses and tissue adaptations observed after acute and chronic activation of the kinase. The preponderance of data has focused on either the repression of gluconeogenic (9, 97, 116, 160) and lipogenic (90, 160, 192, 197) genes in the liver, or the induction of genes involved in mitochondrial biogenesis (10, 132, 188, 198), glucose and lipid metabolism (132, 146, 170), and glucose transport (74, 196) in skeletal muscle. However, despite its established role in altering protein balance by promoting catabolism through its suppression of both mTOR signaling (14, 80, 81) (section 1.4.2) and translation
elongation (77) (section 1.3.1.2), and its stimulation of macroautophagy (124), little is known regarding the ability of AMPK to influence the representation of specific genes involved in protein breakdown (acknowledged previously in section 3.5).

Despite evidence implicating MAFbx and MuRF1 as potential contributing factors to the (patho)physiological response of skeletal muscle to many atrophic stimuli (sections 2.1, 3.2, and 3.3), a complete understanding of the initiating stimuli and cellular signaling underlying their induction is still lacking. Given the pivotal roles AMPK plays in the adaptive responses to energy insufficiency it is reasonable to suspect its activation may be capable of regulating the expression of one or more of these genes. Therefore the purpose of these studies (outlined in section 3.5.1) was to investigate the working hypothesis proposed in the previous chapter (section 3.5.2) by examining changes in mRNA content of specific ubiquitin ligases complicit in skeletal muscle atrophy in response to AMPK activation.

4.2 Materials and Methods

4.2.1 Cell Culture

C2C12 myoblasts (American Type Culture Collection, Manassas, VA) were maintained in Eagle Minimum Essential Media (EMEM) supplemented with 10% fetal bovine serum, penicillin (100 I.U./ml), streptomycin (100 µg/ml), and amphotericin (250 ng/ml) (all from Mediatech, Herndon, VA) under 5% CO₂ at 37°C. For experimental treatments myoblasts were subcultured into 6-well tissue culture plates (Grenier Bio-One, Frickenhausen, Germany). At ~100% confluence the cells were switched to media consisting of EMEM with the above antibiotics/antimycotics and 10% bovine calf serum (Hyclone, Logan, UT) to promote myoblast fusion and differentiation to myotubes. Cells were allowed to differentiate for 4 days prior to experimental manipulation. Myotubes were provided with fresh differentiation media for 2 hours
immediately preceding treatment on the 5th day. All experiments were performed using serum-
free EMEM plus antibiotics/antimycotics. 5-aminoimidazol-4-carboximide ribonucleoside
(AICAR; Toronto Research Chemicals, Ontario, Canada), 1-1 dimethylbiguanide hydrochloride
(metformin), 2-deoxy-D-glucose, D-mannitol, dexamethasone (all from Sigma-Aldrich), and/or
Compound C (Calbiochem, San Diego, CA) were administered singly or in combination at
concentrations and times specified in the figures and text. Dexamethasone and Compound C
were solubilized in 100% ethanol and 100 mM HCl, respectively. Under no condition did the
volume of solvent exceed 0.25% of the volume of the culture media and have a significant effect
on the parameters investigated (data not shown).

4.2.2 Animals

All experiments were performed in adherence with the National Institutes of Health
Guide for Care and Use of Laboratory Animals and with the approval of The Pennsylvania State
University College of Medicine Institutional Animal Care and Use Committee. C57BL/6 mice
were obtained from Charles Rivers Laboratories. All mice were housed in a controlled
environment and provided water and standard rodent chow (Harlan Teklad) ad libitum for 1
week before use. At the time of the study, mice were 8 - 9 weeks of age and weighed 22.8 ±
0.4 g. On the morning of study, fed mice were injected intraperitoneally with AICAR (1 mg/g
body weight; 0.5 ml/mouse) or an equivalent volume of isotonic saline. Six hours after the
injection of AICAR or saline mice were anesthetized with ketamine/xylazine (90 and 9 mg/kg,
respectively), and the gastrocnemius/plantarum complex was excised and frozen in liquid
nitrogen.
4.2.3 Multi-Probe template production for Ribonuclease Protection Assay

Primer selection for mouse genes of interest was determined with the help of Genefisher software (55). The lengths of amplified regions were chosen to allow distinct resolution during electrophoretic separation. Primers were synthesized (IDT, Coralville, IA) with restriction sites for EcoRI or KpnI at the 5’ end and with three extra bases at the extreme 5’ end as follows: 

**MAFbx/Atrogin-1** - *Forward* (5’-GCA GAA TTC CAC ATC CTT ATG CAC ACT GGT GCA-3’), *Reverse* (5’-GCA GGT ACC GGT ACT GGC AGA GTC TCT TCC ACA-3’), **MuRF1** - *Forward* (5’-GCA GAA TTC AGT GTG TCT TCT CTC TGC TCA GAG A-3’), *Reverse* (5’-GCA GGT ACC AGA CCC AGC CCT CCC ACC AA-3’), **UBR1/E3αI** - *Forward* (5’-GCA GAA TTC CCC TAA CCC AGC ACA GAG GGA A-3’), *Reverse* (5’-GCA GGT ACC ACT TGC AGA GCG GGC ATA GGT A-3’), **UBR2/E3αII** - *Forward* (5’-GCA GAA TTC CTG AAG TGC ATG CAG GGA ATG GA-3’), *Reverse* (5’-GCA GGT ACC CCA CCG AGT GTC CAC AAA TAC TGA-3’), **L32** - *Forward* (5’-GCA GAA TTC CGG CCT CTG GTG AAG CCC AA-3’), *Reverse* (5’-GCA GGT ACC CCT TCT CCG CAC CCT GTT GTC A-3’). 

PCR was conducted using HotStarTaq DNA Polymerase (Qiagen, Valencia, CA) and mouse total RNA reverse-transcribed with Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). PCR products were phenol:chloroform extracted, ethanol precipitated, and sequentially digested with KpnI and EcoRI (Promega, Madison, WI). Digested products were gel-purified, re-extracted, and cloned into KpnI/EcoRI-digested pBluescript II SK+ (Stratagene, La Jolla, CA). Plasmid DNA was isolated with both QIAprep® Spin Miniprep and Plasmid Maxi Kits (Qiagen). Plasmids with inserts were verified by sequencing in the Pennsylvania State College of Medicine Molecular Genetics Core Facility. Final constructs were linearized with EcoRI, gel-purified, and quantitated spectrophotometrically. The template was prepared so that a 2 µl aliquot contained 10 ng of MAFbx/Atrogin-1, 30 ng of MuRF1, 10 ng of UBR1/E3αI, 10 ng of UBR2/E3αII, and 20 ng of L32.
4.2.4 RNA extraction and Ribonuclease Protection Assay

Total RNA was extracted from cells or powdered muscle tissue using TRI Reagent (Molecular Research Center). Cell and tissue mRNA expression was determined by RPA. A 2 µl aliquot of template was prepared using T7 Polymerase with buffer (Fermentas, Hanover, MD), NTPs and tRNA (Sigma-Aldrich), RNaisin and DNase (Promega), and $^{32}$P-UTP (Amersham Biosciences). Unless otherwise noted, the entire RPA procedure including labeling conditions, component concentrations, sample preparation, and gel electrophoresis was performed as published (BD Pharmingen) and utilized previously (section 2.2.6). Hybridization buffer was 80% formamide and 20% stock buffer (200 mM Pipes pH 6.4, 2 M NaCl, and 5 mM EDTA). Hybridization proceeded overnight at 56°C in a dry bath incubator (Fisher Scientific, Pittsburgh, PA) without the use of mineral oil. Samples were treated with RNase A+T$_1$ (Sigma-Aldrich) in 1x RNase buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA, and 300 mM NaCl) followed by Proteinase K (Fisher Scientific) in 1x Proteinase K buffer (50mM Tris pH 8.0, 1 mM EDTA, 1% Tween-20). Following ethanol precipitation, samples were resuspended in 5 µl of loading buffer (98% formamide (v/v), 0.05% xylene cyanol (w/v), 0.05% bromphenol blue (w/v), and 10 mM EDTA). 34 x 45 cm polyacrylamide gels were run at 75 watts for 70 minutes in an S3S Sequencing System (Owl Separation Systems, Portsmouth, NH), transferred to chromatography paper, and dried for 10 minutes at 80°C (FB GD 45 Gel Dryer, Fisher Scientific). Gels were exposed and analyzed as previously described (section 2.2.6). Signal densities for mRNAs were normalized to densities for L32 mRNA.

4.2.5 Immunoblot Analysis

Following drug treatment cells were rinsed with cold Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen) and collected on ice in lysis buffer (20 mM HEPES, 50 mM β-
glycerophosphate, 1% Triton X-100, 100 mM KCl, 2 mM EDTA, 50 mM NaF, 1 mM DTT, 0.5 mM PMSF, 1 mM benzamidine, 1 mM sodium orthovanadate, and 2 µg/ml leupeptin). Lysates were then passed several times through a 27 gauge needle and centrifuged at 1500 g for 10 minutes at 4°C. A portion of the resulting cell supernatant was used to determine protein concentration via a bicinchoninic acid assay kit (Pierce; Rockford, IL). 5X sample buffer was added to an aliquot of supernatant.

Samples were loaded according to total protein content (20 µg) on polyacrylamide gels for separation by SDS-PAGE as detailed previously (section 2.2.5). Membranes were incubated overnight at 4°C with phospho-specific antibodies for AMPKα (Thr172) and acetyl-CoA carboxylase (ACC; Ser79) (both from Cell Signaling Technology), developed, exposed, and antibody removed as described previously (section 2.2.5). Blots were then blocked with nonfat dry milk and incubated overnight at 4°C with antibodies for AMPKα and ACC (both from Cell Signaling Technology). An antibody against β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) served as a control for equal protein loading of samples.

4.2.6 Statistics

Results for individual cell experiments were replicated in at least three independent experiments and (when applicable) are presented as means ± SE calculated from the pooled data. Statistical analysis and demonstration of significance in two- and multi-group comparisons was performed as previously indicated (section 2.2.7).
4.3 Results

4.3.1 AMPK agonists AICAR and metformin induce muscle-specific ubiquitin ligase mRNA content in a dose-dependent fashion

AICAR is a pharmacological agent commonly used to artificially activate AMPK (28). Once taken up by intact cells AICAR is phosphorylated to form 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate (ZMP), which is capable of producing stimulatory effects identical to those of AMP on AMPK, but in the absence of detectable changes in adenine-nucleotide levels. In order to assess the sensitivity of ubiquitin ligase mRNA to AMPK signaling, C2C12 cells were exposed to increasing concentrations of AICAR for 24 hours (Fig. 4-1). Both MAFbx (Fig. 4-1A) and MuRF1 (Fig. 4-1B) increased dose-dependently in response to increasing concentrations of AICAR up to 1 mM; exposure of cells to concentrations greater than 2 mM resulted in substantial loss of cell viability (unpublished observations). Effective doses of AICAR concomitantly induced phosphorylation of the α subunit of AMPK at the Thr 172 residue (Fig. 4-1C), a signaling event shown to be requisite for nearly all AMPK activity (166). Activation of AMPK in response to AICAR was further corroborated by a slight gel-shift in an immunoblot for total AMPKα protein (Fig. 4-1C). Furthermore, the phosphorylation of ACC, a well-established target of AMPK, is frequently used as an indirect measure of AMPK activity. Phosphorylation of ACC was increased at all doses capable of inducing ligase expression (Fig. 4-1C) in a manner parallel with AMPKα phosphorylation. In both cases maximal expression was achieved using a concentration of 1 mM, which was subsequently used for all further studies involving AICAR.

The conclusiveness of data obtained regarding AMPK-dependent responses to AICAR are limited by the potential non-AMPK specific “side effects” of the treatment (28). To confirm the role of AMPK in modulating ligase expression cells were also treated with metformin (Fig. 4-2). Metformin is an anti-diabetic biguanide that activates AMPK in a mechanistically distinct
manner from that of AICAR (see section 4.4 below). Similarly to AICAR treatment, exposure of myocytes to increasing concentrations of metformin up to 2 mM dose-dependently increased mRNA for MAFbx (Fig. 4-2A). Doses above 2 mM compromised cell survival (unpublished observations). MuRF1 expression, which was only induced with the highest tolerated concentration of metformin (Fig. 4-2B), appeared to be the notable exception to this recurring pattern of dose-dependence observed in AICAR- and metformin-treated cells. Effective doses again correlated with Thr 172 phosphorylation of AMPKα, gel-shifting of native AMPK, and Ser 79 phosphorylation of ACC (Fig. 4-2C). Maximal expression for both MAFbx and MuRF1 was achieved with a concentration of 2 mM; consequently all further studies utilizing metformin were performed with this dose.

4.3.2 AICAR increases MAFbx and MuRF1 mRNA content time-dependently in a biphasic pattern

Given the substantial induction of MAFbx and MuRF1 following prolonged exposure to activators of AMPK, the acute and intermediate kinetics of these agents on ligase expression were examined. Cells were stimulated with AICAR (1 mM) for incremental periods of time up to and including 24 hours and compared with time-matched control cells (Figs. 4-3 and 4-4). Treatment beyond 24 hours resulted in significant cell death (unpublished observations). Serum deprivation alone of control cells increased MAFbx mRNA (Fig. 4-3A), which peaked at 2 hours ($P < 0.05$) and remained elevated above 1 hour baseline values thereafter. Additionally this deprivation appeared to slightly induce AMPK and ACC phosphorylation rapidly (within 1 to 2 hours) and persistently (through 24 hours, Fig. 4-5). The presence of AICAR initially repressed MAFbx (at 1 and 2 hours, Fig. 4-3B), while more protracted exposure led to an eventual increase in expression at later time points with an apparent maxima at 24 hours. Signaling to ($\alpha$
subunit phosphorylation) and from (ACC phosphorylation) AMPK in response to AICAR appeared to be competent at all examined times (Fig. 4-5).

Serum deprivation also increased MuRF1 mRNA content (Fig. 4-4A), which climbed steadily until reaching a peak at 16 hours ($P < 0.05$). Similarly to MAFbx, MuRF1 mRNA also exhibited an acute repression (at 2 hours) followed by a robust increase in response to AICAR (Fig. 4-4B). However, for MuRF1 this induction occurred earlier and by 8 hours a maximal response had already been achieved, which was sustained throughout the remainder of the time course.

### 4.3.3 AICAR and dexamethasone act synergistically to induce MAFbx and MuRF1 mRNA content

Dexamethasone induces MAFbx both in culture (104, 152, 153, 169) and in animals (section 2.1). Here dexamethasone reduced expression of the ligase acutely (at 1 and 2 hours, Fig. 4-3B), a result which has not been reported in previous investigations. By 4 hours dexamethasone had reciprocally increased MAFbx mRNA, an effect which persisted throughout the remainder of the time course (Fig. 4-3B). This induction occurred in the absence of alterations in phosphorylation of AMPK$\alpha$ and ACC when compared with time-matched controls (data not shown), suggesting dexamethasone treatment alone does not activate the AMPK signaling cascade.

In order to examine the potential interactive effects between AMPK activity and glucocorticoid action on MAFbx and MuRF1 mRNA content, myocytes were incubated with both AICAR and dexamethasone together (Figs. 4-3 and 4-4). Acutely this drug combination behaved similarly to AICAR alone (at 1 and 2 hours, Fig. 4-3B) with respect to MAFbx mRNA. Interestingly, this repression appeared to be extended through 4 hours – a response distinct from that for either AICAR or dexamethasone treatment alone. By 8 hours, the dual treatment
had begun to induce expression of the ligase at an intermediate level between that of AICAR or dexamethasone administered singly. At later time points (16 and 24 hours, Fig. 4-3B) the drug combination dramatically stimulated MAFbx expression in a synergistic manner. These effects could not be attributed to dexamethasone-dependent alterations in AMPK signaling, as AMPK\textsubscript{\alpha} and ACC were phosphorylated to a similar extent between AICAR alone and the combination treatment at all time points (Fig. 4-5).

While dexamethasone has been shown to stimulate MuRF1 expression \textit{in vitro} (104, 152, 169), such an effect was not observed in the current study. Though dexamethasone appeared to slightly induce the ligase, this increase did not reach statistical significance (Fig. 4-4B). The pattern of MuRF1 mRNA expression in response to a combination of AICAR and dexamethasone was reminiscent of that for MAFbx. Acutely MuRF1 expression in response to the dual treatment mirrored that for AICAR alone (2 hours), while the two treatments produced a synergistic response at all time points where cells had been previously shown to be responsive to AICAR alone (8, 16, and 24 hours, Fig. 4-4B).

### 4.3.4 Metformin-induced alterations in MAFbx and MuRF1 mRNA content

Time course studies using metformin were conducted as a second independent means for exploring a causal role of AMPK activation in increasing ubiquitin ligase mRNA expression (Fig. 4-6). Metformin treatment at later time points produced alterations in mRNA content of MAFbx (Fig. 4-6A) and MuRF1 (Fig. 4-6B) that were qualitatively similar to those achieved with AICAR administration. Additionally, as with AICAR, the combination of metformin and dexamethasone synergistically induced both ligases. The above results correlated with increases in phosphorylation of AMPK\textsubscript{\alpha} and ACC caused by metformin, independent of dexamethasone co-treatment (data not shown).
4.3.5 Disruption of cellular energy homeostasis reproduces the bi-phasic pattern of MAFbx and MuRF1 mRNA content

While both AICAR and metformin are accepted pharmacological activators of AMPK, neither activates the kinase by directly disturbing the energy status of the cell. In an effort to determine if muscle-specific ubiquitin ligase mRNA expression was sensitive to cellular energy starvation, C2C12 cells were incubated with 2-deoxy-D-glucose (2-DG) (Fig. 4-7). 2-DG is a D-glucose analog that is phosphorylated but not further metabolized post-uptake. As a result, import of 2-DG inhibits hexokinase action through a negative feedback mechanism and ultimately restricts cellular glucose utilization (54). Cells were incubated with 25 mM 2-DG for time periods up to 16 hours (beyond which considerable lethality resulted, unpublished observations). To account for any potential effect(s) of osmotic stress (an environmental stimulus itself capable of stimulating AMPK (51)) independent of energy depletion, equimolar amounts of D-mannitol were added to control cells. The presence of 25 mM mannitol did not appreciably shift the expression profile of MAFbx or MuRF1 during serum deprivation (data not shown). Furthermore, there was no notable increase in phosphorylation of ACC in mannitol-treated controls (Fig. 4-7C), which argues against significant activation of AMPK in response to the presence of the osmolite.

Exposure to 2-DG rapidly decreased MAFbx mRNA at early time points (1 and 2 hours, Fig. 4-7A). This effect had reversed completely by more intermediate times, where a small but significant increase in mRNA content had occurred (4 and 8 hours, Fig. 4-7A). MuRF1 mRNA expression demonstrated a comparable pattern of regulation, where 2-DG acted as a potent inducer at later times (particularly at 8 hours, Fig. 4-7B). AMPK signaling appeared to be active at all time points examined (Fig. 4-7C).

Dual treatment with 2-DG and dexamethasone augmented expression of MAFbx (Fig. 4-7A) and MuRF1 (Fig. 4-7B) synergistically, albeit not as strikingly as observed earlier with AICAR. However, for MAFbx, this interaction was transient and was not present at 16 hours,
where 2-DG had a slightly antagonistic effect on the ability of dexamethasone to increase ligase expression (Fig. 4-7A). Identical to results with AICAR and metformin outlined above, dexamethasone had no effect on 2-DG induced increases in AMPK signaling events (Fig. 4-7C).

4.3.6 AMPK activation does not increase the mRNA content of the UBR box family ligases UBR1/E3αI and UBR2/E3αII

UBR box family ubiquitin ligases (173) serve as the E3 recognition factors for the N-end rule pathway, a ubiquitous process found in all cells whereby specific proteins bearing destabilizing N-terminal residues are marked for degradation by the proteasome. Several members of this E3 family have been implicated in the pathogenesis of skeletal muscle atrophy observed during cachexia, sepsis, and diabetes (99, 107, 164). To determine if activation of AMPK results in an accumulation of mRNA content for all ubiquitin ligases implicated in skeletal muscle proteolysis, the expression of UBR1/E3αI and UBR2/E3αII in response to AICAR was determined (Fig. 4-8). In contradistinction to the responses of the muscle-specific ligases detailed above, both UBR1/E3αI and UBR2/E3αII were depressed in response to AICAR in a dose- (data not shown) and time-dependent manner (Figs. 4-8A and 4-8B, respectively). Furthermore, at no time was this effect synergistic or additive with that of dexamethasone. While having a similar effect on both, the mechanism by which AMPK suppressed expression appears distinct for each ligase. For UBR1/E3αI, the time course suggested that AMPK activation decreased mRNA content of the E3, while for UBR2/E3αII AICAR prevented a serum deprivation-induced increase in expression (data not shown). Similar results were obtained in cells treated with metformin and 2-DG (data not shown).
4.3.7 AMPK inhibition prevents serum deprivation-, dexamethasone-, and AICAR-induced increases in MAFbx and MuRF1 mRNA content

To address the specificity of the treatments used previously to activate AMPK, cells were co-treated with AMPK agonists and Compound C (Fig. 4-9), a small molecule reversible inhibitor of AMPK (197). Initial dose response experiments determined that a concentration of 20 µM was optimal on the basis that it achieved maximal inhibition of ligase mRNA content while not significantly impacting cell viability during extended culture times of up to 16 hours, after which the inhibitor was cytotoxic (data not shown). Addition of Compound C effectively prevented the increase in mRNA of both MAFbx and MuRF1 in response to treatment with AICAR (Fig. 4-9). Interestingly, the inhibitor also blocked the induction of the ligases by dexamethasone (whether added individually or in combination with AICAR). In all cases, including control cells treated with Compound C, MAFbx and MuRF1 mRNA were reduced below vehicle-treated control levels, demonstrating that the serum deprivation-induced increase for each ligase noted previously during time course studies (Figs. 4-3 and 4-4) was antagonized by AMPK inhibition. Similar results were obtained in cells exposed to 2-DG and Compound C for 8 hours (data not shown).

4.3.8 AMPK activation in vivo stimulates expression of MAFbx and MuRF1 in murine skeletal muscle

In light of the mounting body of evidence suggesting that AMPK activation increases muscle-specific ubiquitin ligase mRNA in vitro, it was desirable to determine if similar effects could be reproduced in intact skeletal muscle. To establish if activation of AMPK in vivo would increase MAFbx and MuRF1 mRNA, mice were injected intraperitoneally with AICAR. The mRNA content of both ligases increased in the gastrocnemius of AICAR-treated mice relative to saline-treated controls 6 hours post-injection (Fig. 4-10). Correspondingly, AICAR
administration significantly increased Thr 172 phosphorylation of AMPKα in skeletal muscle (~70%; 4484 ± 286 vs. 2618 ± 206 arbitrary units, AICAR vs. control muscle, P < 0.05).

4.4 Discussion

A wealth of investigation has highlighted the importance of AMPK as a master regulator of cellular metabolism during times of energetic famine induced by a variety of physiological and pathological stimuli. In several of these contexts (particularly nutritional starvation) there is an established acceleration of proteolysis that occurs selectively in skeletal muscle which, if left unchecked, leads to debilitating wasting (125). As AMPK has proven to be an obligate effector of many of the responses of an organism to nutrient limitation, it was reasonable to suspect its activation may contribute to the development and progression of skeletal muscle atrophy. To address this possibility we analyzed the genetic signature of an in vitro model of murine skeletal muscle after exposure to agonists of AMPK for changes in mRNA expression of E3 ubiquitin ligases previously identified as high-fidelity markers of the atrophic process (sections 2.1, 3.2, and 3.3). Treatment of C2C12 cells with several AMPK activators resulted in a dose- and time-dependent modulation of mRNA content of the muscle-specific ubiquitin ligases MAFbx and MuRF1, characterized by an acute repression preceding a sustained induction (sections 4.3.1 - 4.3.5). The stimulatory effect was potently synergistic with the synthetic glucocorticoid dexamethasone, which was not a result of amplified signaling through the AMPK cascade (section 4.3.3). This response was selective and did not extend to the ubiquitin ligases UBR1/E3αI and UBR2/E3αII, both of which were decreased (section 4.3.6). Inhibition of AMPK signaling prevented increases in MAFbx and MuRF1 mRNA content in response to AMPK agonists and dexamethasone (section 4.3.7). Finally, in vivo activation of AMPK via AICAR injection recapitulated the stimulation of MAFbx and MuRF1 expression in skeletal muscle (section 4.3.8). Therefore, activation of AMPK results in discriminatory gene regulation of E3s
in skeletal muscle, whereby muscle-specific ligases are strongly induced by persistent AMPK activity while those with more ubiquitous tissue expression patterns are suppressed. These observations begin to delineate a heretofore unknown relationship between AMPK and UPP-dependent protein degradation in skeletal muscle and represent the first implication of the AMPK cascade as an intracellular signaling pathway that may contribute to the etiology of the proteolysis-associated aspects of skeletal muscle atrophy.

Here the role of AMPK in provoking E3 expression was demonstrated using three independent agents capable of activating AMPK in distinctive manners - AICAR, metformin, and 2-DG. While AICAR and 2-DG activate the kinase by acting as an AMP-mimetic or placing cells directly under energetic duress (respectively), metformin is one of a few select stimuli which appears to activate the kinase without detectably perturbing levels of AMP or ATP (51, 71), perhaps involving an alternative pathway of activation involving mitochondrial-derived reactive nitrogen species and the function of protein kinase Cζ (PKCζ) as an AMPKK kinase (AMPKKK) towards LKB1 (194). While none of these reagents is entirely specific in stimulating AMPK, that similar results were achieved using each treatment (albeit with varying time courses and intensities) suggests that changes in gene expression for these ubiquitin ligases are indeed AMPK-dependent and not a function of some alternative kinase or signaling pathway. Furthermore the stimulatory effects of these treatments were wholly antagonized by the addition of Compound C, a previously characterized inhibitor of AMPK. The specificity of Compound C itself has been demonstrated against select structurally-related kinases (197), though an alternate independent analysis has revealed that the application of the inhibitor concomitantly with AICAR may interfere with AICAR uptake itself (and therefore may not antagonize AMPK directly) (52). However, that virtually identical results were achieved with inhibition during 2-DG treatment reiterates that data generated with Compound C demonstrate a causal role for AMPK itself in controlling muscle-specific E3 expression. Collectively, this evidence supports the
notion that AMPK is indeed a *bona fide* modulator of both MAFbx and MuRF1 mRNA content in skeletal muscle.

The interaction between dexamethasone and AMPK function on E3 gene expression is also noteworthy. The synergy between these two stimuli has not been previously reported in skeletal muscle, and is in fact contrary to the known effects of AMPK activity on dexamethasone-induced gene expression of two key gluconeogenic genes - glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) - in rat hepatoma cells (9, 116). One possible explanation for this interaction evidenced here could be a positive regulation of AMPK expression or activity in response to glucocorticoid administration. Such an outcome has been reported in hepatic (183) and cardiac (147) tissue, though the authors of the former study cite unpublished data that this effect was not observed in skeletal muscle. Results here examining native AMPKα expression and activity (sections 4.3.3 and 4.3.5) are in accord with that latter finding, and thus the synergistic effect of the two agents on muscle-specific ligase expression cannot be attributed to an increased abundance of or signal transduction through AMPK. Importantly, inhibition of AMPK ablated the stimulatory effects of dexamethasone, indicating that AMPK signaling not only cooperates with the glucocorticoid in upregulating MAFbx and MuRF1 but is required for dexamethasone-dependent increases in both E3s. These results suggest AMPK exerts a dichotomous influence on glucocorticoid-induced gene expression which is likely tissue- and gene-dependent, and raises the possibility that AMPK may be an important determinant of glucocorticoid sensitivity in skeletal muscle.

There are several potential mechanisms through which AMPK activity could be acting individually and in cooperation with dexamethasone to produce these effects on ubiquitin ligase expression. Numerous studies have highlighted the impact of AMPK signaling events on proteins, particularly transcription factors and transcriptional co-activators and repressors, which control various aspects of gene expression (109). Several well-established examples of this regulation include the ability of the kinase to disrupt the gluconeogenic gene program by
controlling the subcellular localization of the CREB coactivator TORC2 (97) and the stability of the transcription factor HNF4α (76) through phosphorylation-mediated mechanisms. Likewise, AMPK is able to restrain lipogenic gene expression by repressing mRNA expression of the transcription factor SREBP-1 (197) and by interfering with the DNA binding activity of the co-activator ChREBP (90). Suppression of transcription- and/or co-factor activity is not the only consequence of AMPK activation. Expression of the GLUT4 gene by the kinase appears to be driven by positive regulation and redistribution of GEF and MEF2A to the nucleus (74), while AMPK-induced mitochondrial biogenesis coincides with increased DNA binding of NRF-1 (10) and the upregulation of the PGC-1α and CaMK IV genes (198). As well as these transcriptional mechanisms, AMPK is also able to regulate gene expression via post-transcriptional means involving control of mRNA stability through the nuclear import of mRNA binding protein HuR (184). Additionally, SNF1 (the yeast equivalent of AMPKα) is capable of influencing chromatin configuration via histone phosphorylation, which results in acetylation of particular promoters by the acetyltransferase GNC5 (115) (though such a role has not been confirmed for mammalian AMPK). Therefore, AMPK is capable of influencing mRNA abundance by regulating the intracellular partitioning, stability, mRNA expression, and DNA binding capacity of specific coactivators or transcription factors, the stability of transcriptional end-products themselves, and perhaps through epigenetic gene regulation. Further studies will be needed to determine which mechanisms are requisite for the control of MAFbx and MuRF1 gene expression.

In conclusion, these data describe the role of AMPK in controlling the mRNA content of select muscle-specific ubiquitin ligases using both in vitro and in vivo experimental approaches. Exposure of differentiated C2C12 cells to agonists of AMPK causes dose- and time-dependent increases in mRNA of MAFbx and MuRF1. This stimulatory effect synergizes with dexamethasone, an occurrence that cannot be accounted for by either altered signaling through or protein expression of AMPK itself. Accordingly signal transduction through the AMPK
pathway appears necessary for the induction of these muscle-specific ligases in response to several stimuli, including glucocorticoid treatment and energetic starvation. Lastly, pharmacological activation of AMPK in vivo reproduces the stimulation of MAFbx and MuRF1 mRNA expression demonstrated in vitro. The studies herein elucidate a key piece of the regulatory puzzle that governs elements of the atrophic gene expression response and will likely control other phenotypes associated with skeletal muscle catabolism.
Fig. 4-1: AICAR increases MAFbx and MuRF1 mRNA in a dose-dependent manner.  

A, Top: Quantitation of MAFbx mRNA. The value for the AICAR-free control is set at 1.0 A.U.  
A, Bottom: Representative autoradiographs of MAFbx and L32, respectively.  

B, Top: Quantitation of MuRF1 mRNA. The value for the AICAR-free control is set at 1.0 A.U.  
B, Bottom: Representative autoradiographs of MuRF1 and L32, respectively. Data are represented as a bar graph of the mean ± SE for n = 6 per group. Means with different letters are statistically different from one another (P < 0.05).  

C: Representative immunoblots of phosphorylation at the Thr^{172} site of AMPKα, total AMPKα, phosphorylation at the Ser^{79} site of ACC, total ACC, and β-tubulin, respectively.
Fig. 4-2: Metformin increases MAFbx and MuRF1 mRNA in a dose-dependent manner.  

A, Top: Quantitation of MAFbx mRNA. The value for the metformin-free control is set at 1.0 A.U.  
A, Bottom: Representative autoradiographs of MAFbx and L32, respectively.  

B, Top: Quantitation of MuRF1 mRNA. The value for the metformin-free control is set at 1.0 A.U.  
B, Bottom: Representative autoradiographs of MuRF1 and L32, respectively. Data are represented as a bar graph of the mean ± SE for n = 8 to 9 per group. Means with different letters are statistically different from one another (P < 0.05).  

C: Representative immunoblots of phosphorylation at the Thr$^{172}$ site of AMPKα, total AMPKα, phosphorylation at the Ser$^{79}$ site of ACC, total ACC, and β-tubulin, respectively.
**Fig. 4-3: Time dependence of AICAR-induced MAFbx mRNA expression.**  

*A:* Inter-time point quantitation of MAFbx mRNA. The value for the 1 hour AICAR-free control is set at 1.0 A.U. All other control and treatment times are compared to the 1 hour AICAR-free control. Data are represented as a line graph of the mean ± SE for n = 8 to 9 per time point per treatment. The presence of solvent in control treatments did not have a statistically significant effect on the indices measured; therefore control groups from all individual experiments were pooled into a single group for this and all subsequent analyses. Here control data are represented as a line graph of the mean ± SE for n = 25 to 27 per time point.  

*B:* Intra-time point quantitation of MAFbx mRNA. The value for each AICAR-free time-matched control is set at 0.0 A.U. Treatments at each time are compared to their corresponding time-matched control. Data are represented as a bar graph of the mean ± SE for n = 25 to 27 for control and n = 8 to 9 per group. Means with different letters are statistically different from one another (P < 0.05). Statistics comparing control treatment over time are not graphically represented but cited in the text.  

*C:* Representative autoradiographs of MAFbx and L32, respectively, under indicated treatment conditions.
Fig. 4-4: Time dependence of AICAR-induced MuRF1 mRNA expression.  
A: Inter-time point quantitation of MuRF1 mRNA. The value for the 1 hour AICAR-free control is set at 1.0 A.U. All other control and treatment times are compared to the 1 hour AICAR-free control. Data are represented as a line graph of the mean ± SE for n = 25 to 27 for control and n = 8 to 9 per time point per treatment. 
B: Intra-time point quantitation of MuRF1 mRNA. The value for each AICAR-free time-matched control is set at 0.0 A.U. Treatments at each time are compared to their corresponding time-matched control. Data are represented as a bar graph of the mean ± SE for n = 25 to 27 for control and n = 8 to 9 per group. Means with different letters are statistically different from one another (P < 0.05). Statistics comparing control treatment over time are not graphically represented but cited in the text. 
C: Representative autoradiographs of MuRF1 and L32, respectively, under indicated treatment conditions.
Fig. 4-5: Phosphorylation of AMPK and ACC in response to AICAR treatment. Time course of phosphorylation events in response to treatment with AICAR singly (1 mM) or in combination with dexamethasone (10 µM). Representative immunoblots of phosphorylation at the Thr₁ seventy-two site of AMPKα, total AMPKα, phosphorylation at the Ser seventy-nine site of ACC, total ACC, and β-tubulin are presented.
Fig. 4-6: Time dependence of metformin-induced MAFbx and MuRF1 mRNA expression. 
A: Inter-time point quantitation of MAFbx mRNA. The value for the 1 hour metformin-free control is set at 1.0 A.U. All other control and treatment times are compared to the 1 hour metformin-free control. Data are represented as a line graph of the mean ± SE for n = 23 to 27 for control and n = 8 to 9 per time point per treatment. 
B: Inter-time point quantitation of MuRF1 mRNA. The value for the 1 hour metformin-free control is set at 1.0 A.U. All other control and treatment times are compared to the 1 hour metformin-free control. Data are represented as a line graph of the mean ± SE for n = 24 to 26 for control and n = 8 to 9 per time point per treatment.
Fig. 4-7: Time dependence of 2-deoxyglucose-induced MAFbx and MuRF1 mRNA expression.  

A: Intra-time point quantitation of MAFbx mRNA. The value for each mannitol-treated time-matched control is set at 0.0 A.U. Treatments at each time are compared to the corresponding time-matched control. Data are represented as a bar graph of the mean ± SE for $n = 17$ to 18 for control and $n = 8$ to 9 per group.  

B: Intra-time point quantitation of MuRF1 mRNA. The value for each mannitol-treated time-matched control is set at 0.0 A.U. Treatments at each time are compared to the corresponding time-matched control. Data are represented as a bar graph of the mean ± SE for $n = 17$ to 18 for control and $n = 8$ to 9 per group. Note that in both A and B, data for the dexamethasone (Dex) only treatment has been re-plotted from Figures 3 (for MAFbx) and 4 (for MuRF1) for comparison purposes. Means with different letters are statistically different from one another ($P < 0.05$).  

C: Time course of AMPK and ACC phosphorylation events in response to treatment with 2-deoxyglucose singly (25 mM) or in combination with dexamethasone (10 µM). Representative immunoblots of phosphorylation at the Thr$^{172}$ site of AMPKα, total AMPKα, phosphorylation at the Ser$^{79}$ site of ACC, total ACC, and β-tubulin are presented.
Fig. 4-8: AICAR decreases mRNA expression of the UBR box family ligases UBR1/E3αI and UBR2/E3αII.  A: Intra-time point quantitation of UBR1/E3αI mRNA.  The value for each AICAR-free time-matched control is set at 0.0 A.U.  Treatments at each time point are compared to their corresponding time-matched control.  Data are represented as a bar graph of the mean ± SE for n = 26 to 27 for control and n = 8 to 9 per group.  B: Intra-time point quantitation of UBR2/E3αII mRNA.  The value for each AICAR-free time-matched control is set at 0.0 A.U.  Treatments at each time point are compared to their corresponding time-matched control.  Data are represented as a bar graph of the mean ± SE for n = 24 to 27 for control and n = 8 to 9 per group.  Means with different letters are statistically different from one another (P < 0.05).
Fig. 4-9: Compound C antagonizes the stimulatory effects of AICAR and dexamethasone on MAFbx and MuRF1 mRNA expression. Cells were pre-treated with Compound C (20 µM) for 30 minutes preceding the switch to treatment media containing the reagents specified in the figure with re-addition of the inhibitor. Representative autoradiographs of MAFbx, MuRF1, and L32, respectively, in response to the indicated treatments singly or in combination with Compound C are presented.
Fig. 4-10: AICAR injection increases MAFbx and MuRF1 mRNA in vivo. Top: Quantitation of MAFbx and MuRF1 mRNA isolated from the gastrocnemius/plantaris complex. The value for control mice given saline (ip) is set at 1.0 A.U. Data are presented as a bar graph of the mean ± SE for $n = 10$ for control and $n = 5$ for treatment animals per group. * $P < 0.05$ compared to the respective control value. Bottom: Representative autoradiographs of MAFbx, MuRF1, and L32, respectively.
Chapter 5

Summary and concluding remarks

5.1 The control of protein balance in atrophic skeletal muscle during unilateral hindlimb immobilization

The working hypothesis proposed in Chapter 1 (section 1.5.2) theorized that atrophy induced by muscle immobilization would be a consequence of changes in the main components of protein balance – protein synthesis and degradation. Studies detailed in Chapter 2 have directly addressed this hypothesis, and proven it to be partially accurate. This analysis has achieved the purpose earlier set forth (section 1.5.1) by defining the synthetic and proteolytic characteristics of this model using traditional and innovative approaches.

Despite the rapid and marked atrophic response of the gastrocnemius to immobilization in a shortened position, there was no accompanying change in protein synthesis or translational efficiency; data obtained regarding the status of signal transduction cascades and factors controlling translation corroborate those observations (as thoroughly discussed in section 2.4). This finding is the most controversial, as it is contrary to data generated using other similar models of disuse atrophy. One possible explanation for this disparity is that an alteration in protein synthesis may have occurred temporally at an earlier point and that by five days the atrophic gastrocnemius has achieved a new baseline or set point with respect to synthesis and/or translational efficiency (section 2.4). This is an especially salient possibility given the wet weight response of the casted gastrocnemius, where genuine atrophy occurred prior to the onset of growth failure by three days time (section 2.3.1). To address this possibility directly, many of the same translation-related endpoints were examined following one day of hindlimb
immobilization, a time immediately preceding atrophy of the gastrocnemius. Similarly to the results detailed for five days of immobilization (sections 2.3.2 - 2.3.6), there was no discernable defect exhibited by unloaded muscle in phosphorylation state or protein expression of these indices in comparison to contralateral control muscle (data not shown). Though additional analysis directly measuring the status of eIFs and eEFs as well as synthesis at all time points would strengthen this conclusion, the results obtained from these acute studies in combination with those from Chapter 2 do not support the element of the working hypothesis stating that protein synthesis and the process of translation would be impaired in this model of skeletal muscle unloading.

The data examining the role of protein degradation using the proteasome inhibitor Velcade as a novel in vivo method confirm that proteasomal function (and protein degradation by extension) is a primary contributor to immobilization-induced skeletal muscle atrophy (section 2.3.12). Furthermore, the increase in mRNA content of the ubiquitin ligases MAFbx and MuRF1 is consistent with the belief that upregulation of components of the UPP contributes to the accelerated proteolysis observed in wasting states (section 2.1). Therefore, in contrast to conclusions reached regarding protein synthesis, these results support the contention of the working hypothesis that protein degradation contributes to muscle atrophy caused by muscle immobilization. In addition to providing this experimental evidence, these data present interesting therapeutic implications as well. Currently Velcade is used clinically as an antineoplastic agent in the treatment of multiple myeloma (16). Intensive efforts continue to focus on discovering therapeutics for the pronounced muscle catabolism which accompanies many physiological and pathological conditions. These data presented here raise the possibility that the proteasome, while historically eschewed as a therapeutic candidate due to the potential for non-specific side effects, is itself a viable target in antagonizing skeletal muscle loss and that Velcade and perhaps other proteasome inhibitors could be efficacious in the management of catabolic conditions.
In other models of skeletal muscle disuse (such as denervation, hindlimb suspension, and bilateral hindlimb casting) there is a decrease in protein synthesis that occurs concomitantly with an increase in protein degradation (section 2.1). These alterations are a rational physiological response on the part of muscle, as changes to both determinants of protein balance would decrease cell size in a maximally efficient manner. In this regard the preservation of protein synthesis observed during unilateral immobilization seems uneconomical, given its cost in “natural resources” (i.e. cellular stores of energy and basic metabolic substrates), and ultimately counter-productive with respect to atrophy of the gastrocnemius. A likely explanation for this occurrence involves the age of the animals used for these studies (discussed briefly in section 2.4). The rats used in these experiments were adolescent and still experiencing rapid tissue and body growth prior to full adulthood. It could be hypothesized that an insult of muscle disuse by immobilization is insufficient to reduce protein synthesis in this developmental milieu, perhaps due to the increased quantity or effectiveness of hypertrophic factors associated with the maturation process. Additionally, it is possible that the loss of gastrocnemius size with unilateral immobilization could result from a failure of protein synthesis to elevate in response to anabolic cues, such as feeding itself and/or the hormonal responses implicit with such an activity. Here, twenty minutes of IGF-I treatment was insufficient to stimulate protein synthesis under these conditions (section 2.3.7), and as a result the responsiveness of the casted muscle to growth-factor stimulation was not directly assessed. Thus, while the age-dependent growth state of the animals is sufficient to preserve unstimulated protein synthesis, a failure to increase synthesis in response to anabolic cues could contribute to the loss of muscle mass with hindlimb immobilization. These points highlight the potential importance of animal age, a factor frequently undiscussed or underemphasized in many model systems, as a major contributor to experimental phenotypes.
5.2 Protein metabolism during immobilization: Unresolved questions and future directions

As an increase in protein degradation appears to be the principal cause of the skeletal muscle atrophy produced by unilateral immobilization in Chapter 2, determining the exact mechanisms by which this breakdown is achieved becomes paramount to understanding this adaptive process and its relation to the catabolic response imposed during other wasting conditions. Though Velcade has been used here as a unique approach to prove the necessity of proteasome-dependent proteolysis, technological limitations of this model prevent the utilization of traditional \textit{in vitro} methods used as direct assessments of the contributions of other proteolytic systems referred to previously in Chapter 1 (section 1.3.2). Related studies in hindlimb suspension have demonstrated an increased expression or activity of several different components of these proteolytic pathways during muscle disuse (171). Though previous investigations have reported a minimal involvement of these systems (e.g. the calcium-dependent and lysosomal systems) to indices of global degradation using these \textit{in vitro} assays, more recent studies have challenged this notion and suggested that contributions by these pathways may be crucial to the breakdown of skeletal muscle (8, 29, 126). Furthermore, recent investigations have suggested the importance of the apoptotic system and caspase-3 action as a proximal step in the UPP-dependent proteolysis of myofibrillar proteins (40, 108). Therefore the atrophy resulting from hindlimb casting is likely a product of a unified response of these and other potential mechanisms - a theory that has defined a rapidly emerging area of research interest. The advent and application of more advanced methodologies, particularly a combination of \textit{in vivo} imaging of proteolytic pathways and genetic manipulation (114, 118, 126), would provide more definitive evidence detailing the process of skeletal muscle atrophy in this and other model systems.

One product of the attention paid to the UPP in skeletal muscle has been the identification of numerous E3 ubiquitin ligases that are induced under catabolic conditions.
These E3s have received the lion’s share of these efforts due to their status as the most specific and selective components of the UPP (section 1.3.2.1), and therefore may control the larger proteolytic process of muscle through the ubiquitination of their respective substrates. As discussed in Chapter 3 (sections 3.2 and 3.3), there is a glaring omission from the current literature base regarding specific functions of MAFbx and MuRF1 during skeletal muscle wasting. This considerably limits the interpretation of their importance in nearly all conditions causing atrophy, including immobilization. In many of these models the examination of these muscle-specific ubiquitin ligases is limited to the measurement of their mRNA content (sections 3.2.2 and 3.3.2). Therefore, the analysis of these genes is restricted to the transcriptional and post-transcriptional stages and does not demonstrate that their respective protein products are also increased or that there are functional consequences to the accumulation of their mRNAs during catabolic conditions. The lack of high-quality, commercially obtainable reagents severely impairs assessment of the protein content of these E3s. All of the primary antibodies for MAFbx and MuRF1 tested during the completion of work in Chapters 2 and 4 demonstrated poor specificity when used for immunoblotting (data not shown). This problem is compounded by the inavailability of proper controls (e.g. lysates from skeletal muscle tissue or cells where these genes have been overexpressed or disrupted), which are necessary for a definitive determination of the protein signal corresponding to these E3s. The inability to reliably measure MAFbx and MuRF1 protein in skeletal muscle is a recognized limitation in this field and represents one of the most obvious and immediate areas for advancement in their study.

In addition to the limitations regarding assessment of protein expression of MAFbx and MuRF1 during immobilization, the relationship between the size of the casted muscle (section 2.3.1) and the fluctuating levels of these E3s (section 2.3.9) is strictly correlative. As a result, the level of analysis completed here does not provide demonstrable proof that either the atrophic phenotype of the muscle or the proteolysis inhibited by Velcade depends on ubiquitin conjugation reactions that are facilitated by the ligase activity of these E3s. While the majority
of published studies concerning MAFbx and MuRF1 in skeletal muscle adopt a similar speculative approach, the advancement of the field both experimentally and clinically demands more targeted and mechanistic approaches be taken to define the precise physiological roles these ligases play in relation to control of skeletal muscle size. Several elegant studies (discussed in greater detail in sections 3.2 and 3.3) completed in both skeletal and cardiac muscle tissue or cells provide examples of experimental methods to accomplish this. These studies have directly manipulated the expression of these ubiquitin ligases to determine their necessity for the progression of skeletal muscle atrophy (12, 20) or retardation of cardiac muscle hypertrophy (3, 110) in response to various stimuli. The use of these gene manipulation techniques (i.e. overexpression, knockdown, or knockout) in combination with various methods of construct delivery (transgenically or with intramuscular injection of viral or plasmid-based expression vectors, the latter in conjunction with in vivo electroporation) offer powerful avenues that could be applied not just to unilateral immobilization, but to virtually any of the catabolic models for which expression of MAFbx and/or MuRF1 is associated with the incidence of skeletal muscle atrophy. Such experiments would pointedly resolve much of the speculation surrounding the role of these E3s in controlling the various phenotypes displayed by skeletal muscle during the wasting process.

5.3 AMPK as a new entity in the control of atrophic signaling in skeletal muscle

The completion of the experiments within Chapter 4 directly addressed the working hypothesis formulated at the conclusion of Chapter 3 (section 3.5.2) - that activation of AMPK in skeletal muscle would increase the mRNA content of MAFbx and MuRF1. These studies have supported this hypothesis and have fulfilled the stated purpose (section 3.5.1) by defining a previously unknown regulatory role for the AMPK pathway in the positive regulation of both ubiquitin ligases in skeletal muscle both in vitro and in vivo.
Within the contemporary model of atrophic gene regulation outlined in Chapter 3 (section 3.4), the finding that AMPK activation is a positive regulator of ubiquitin ligase expression makes sound contextual and teleological sense. AMPK has an acknowledged role in antagonizing mTORC1 signaling by stimulating the GAP activity of tuberin (78) towards Rheb, an important activator of mTOR (156). Furthermore, emerging evidence suggests Akt may directly regulate the activity of AMPK (66), thereby linking growth factor and energetic signaling upstream of tuberin. Thus in the context of atrophic signaling AMPK could serve to monitor the energetic state of skeletal muscle and provide this information as an input to modify anti-atrophic signals derived from mitogenic (receptor-tyrosine kinase/Akt-dependent) and nutritional (mTORC-dependent) sensors while amplifying pro-atrophic signals originating from circulating glucocorticoids, all to better control mass in response to environmental cues. This would afford skeletal muscle a greater level of flexibility in responding to environmental stress, as AMPK would offer an avenue through which muscle could execute the proposed atrophic transcriptional response (section 2.1) in reaction to energy deprivation despite the presence of anabolic cues. If such a model is born out by further experimentation, AMPK would become a comprehensive regulator of skeletal muscle size by acting on distal effectors of both synthesis and degradation. Moreover, the work here raises the possibility that MAFbx and MuRF1 expression may be increased during perturbations that lie outside of the traditionally studied realm of muscle wasting, and therefore may have functions beyond those relating to atrophy or excessive muscle proteolysis.

5.4 Induction of MAFbx and MuRF1 during immobilization: AMPK-dependent signal transduction at work?

The finding that activation of the AMPK signaling cascade alone is sufficient to increase expression of both MAFbx and MuRF1 offers a possible explanation for the alterations in E3
mRNA content noted during unilateral immobilization (an insult that seems to rely on an undefined mechanism(s) to induce these genes, as described in section 3.5). If the casting of the hindlimb induced activation of AMPK in the gastrocnemius, then the increase in muscle-specific ubiquitin ligase expression may be attributable to this pathway in a manner independent of Akt activity and the presence of endogenous glucocorticoids. The existing evidence examining this possibility is conflicting. During the completion of the studies in Chapter 4, a link between muscle unloading and increased AMPK phosphorylation was reported for the soleus muscle of rats subjected to prolonged hindlimb suspension (73). Conversely, the only investigation examining this response in casting immobilization found that prolonged muscle inactivity did not result in a change in AMPK expression or phosphorylation in human vastus lateralis muscle (44).

If AMPK activation were produced in the gastrocnemius in the model of unilateral immobilization detailed in Chapter 2, then it would be reasonable to posit that the kinase may be responsible for the upregulation of MAFbx and MuRF1. However, such a relationship would be contingent upon the resolution of an obvious confounding issue: if the AMPK cascade was activated during unilateral immobilization, then one would expect to observe a decrease in protein synthesis and an antagonism of mTOR-dependent signaling (events which have been previously demonstrated in skeletal muscle) (14) in the atrophic muscle. It is possible that the preservation of Akt function in the casted muscle is sufficient to counteract the inhibitory influence of AMPK on protein synthesis through control of tuberin phosphorylation (sections 1.4.1 and 2.3.2), while the manner in which AMPK increases ligase expression occurs by some other distinct mechanism (of which many possibilities are listed in section 4.4). Clearly additional studies are needed to determine the status of the AMPK signaling cascade in immobilized muscle and the precise method by which the kinase controls MAFbx and MuRF1 expression.
5.5 A global viewpoint of the control of skeletal muscle size by cellular signaling and protein metabolism

The understanding of the intracellular signaling and molecular mechanisms underpinning skeletal muscle atrophy has traditionally lagged behind those regulating hypertrophy. It has only recently become appreciated that many of the canonical growth-controlling pathways which promote or suppress protein accretion are also intimately involved in controlling the presentation of classic markers of wasting muscle and/or the atrophic process itself (section 3.4). This functional quality adds dramatically to the complex paradigm of skeletal muscle size control, as these pathways (and presumably a number of their ultimate targets) are capable of influencing both aspects of cellular size adaptation. Though many of the specific constituents of these cascades have been identified and their functions examined as they relate to muscle hypertrophy (section 1.4), the identity and role of singular effectors of muscle atrophy remain largely unknown. Topical investigations continuously seek to unravel these quantities and place them into existing frameworks that model the flow of information from an initiating stimulus through the appropriate signaling pathways to the protein balance machinery and the resultant change in muscle cell size.

The discovery and ongoing characterization of the muscle-specific ubiquitin ligases MAFbx and MuRF1 illustrate this principal well (sections 3.2 and 3.3). The current state of research regarding these E3s indicates their potential involvement in both cytosolic and nuclear processes linked with calcineurin, NF-κB, PI3K, mTOR, and PKCε signaling, determinations of cellular organization and cell and organ size in response to a myriad of growth-related cues, and resultant effects on functional parameters (such as contractility) of cardiac and/or skeletal muscle tissue. The work completed within this thesis has extended this body of knowledge to include an additional model system of muscle disuse (unilateral hindlimb immobilization, Chapter 2) and identified the influence of yet another intracellular signaling cascade, traditionally associated with the control of protein synthesis (the AMPK cascade, Chapter 4), which
influences their expression. As both MAFbx and MuRF1 seem acutely sensitive to both atrophic and hypertrophic signals, they may serve as influential points of co-regulation in muscle size adaptation.

The accumulation of cited and unique data within this text provide proof-of-principle that the state of trophy in skeletal muscle is determined by execution of anabolic and catabolic “programs” that determine fluctuations in protein balance, are in many instances mutually exclusive, and are regulated by the same intracellular kinase machinery. In essence, this establishes a metabolic switch that is loosely analogous to the one turned “on” or “off” in response to feeding or fasting (i.e. the rival influences of the insulin and counter-regulatory responses, referred to previously in section 3.4) that ultimately regulates the blood glucose concentration. This system is simultaneously exceedingly complex in design and execution (being capable of controlling both the global and specific machinations of protein synthesis and degradation), yet elegantly simple in its size-control response (hypertrophy or atrophy of skeletal muscle). The operation of this circuit would control growth-promoting or -suppressing states in muscle to match its size with the demands imposed upon the tissue. This response, as part of a coordinated adaptation of physiological systems within the host, would encourage the continued survival of an organism in response to environmental stress.
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Bachelor of Science in Kinesiology, May 2001
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PUBLICATIONS

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Abstracts


AWARDS AND HONORS

2001  University Graduate Fellowship, The Pennsylvania State University

2002  -  Ruth L. Kirchstein National Research Service Award
2005  Institutional Training Grant, The Pennsylvania State University

2005  Mead Johnson Award for Outstanding Research, Experimental Biology 2005 Endocrinology and Metabolism Section of the American Physiology Society