THE EFFECTS OF HINDLIMB UNWEIGHTING AND BETA 2-AGONIST ON THE UBIQUITIN-PROTEASOME PATHWAY AND INSULIN-LIKE GROWTH FACTOR I

By

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Dedicated to my grandfather

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The ubiquitin-proteasome pathway is the primary system responsible for myofibrillar protein degradation that is associated with muscle wasting seen in several catabolic conditions including hindlimb unweighting (HU). To attenuate protein degradation would obviously be beneficial to many muscle-wasting conditions. In that regard, beta-agonists such as clenbuterol (CB) are known to induce muscle hypertrophy and attenuate or reverse muscle atrophy due to disuse or inactivity. However, the molecular mechanism by which this drug exerts such effects remains poorly understood. Thus, the aim of this study was to investigate whether CB could attenuate HU-induced atrophy through an inhibition of the ubiquitin-proteasome pathway in both the slow (soleus) and fast (plantaris and tibiaris anterior) muscles, and whether insulin-like growth factor I (IGF-1) was involved in the modulation of these changes. Rats were randomly assigned to either weight-bearing control (CON), 14-day CB-treated (CB), 14-day HU

(HU), or CB+HU groups. The results showed that HU-induced atrophy is associated with increased proteolysis and the up-regulation of key components of the ubiquitin-proteasome pathway (i.e., the levels of ubiquitin conjugates, ubiquitin conjugating enzyme E2-14kDa, and 20S proteasome activity) in both fast and slow muscles. Clenbuterol induced hypertrophy in all muscles; however, CB+HU effectively attenuated the HU-induced atrophy only in the fast plantaris and tibiaris anterior, but not in the slow soleus muscle. These effects were accompanied by a down-regulation and/or by attenuation of the activation of ubiquitin-proteasome proteolysis seen during HU. None of the above changes correlated with an alteration in skeletal muscle IGF-I content. These results suggest that CB induces hypertrophy and alleviates the HU-induced atrophy particularly in the fast muscles, at least in part, through inhibiting the ubiquitin-proteasome pathway, and these effects are not mediated by the local production of IGF-I in skeletal muscle.

CHAPTER 1
INTRODUCTION

Background

Muscle atrophy due to inactivity is a serious problem associated with space flight or prolonged periods of bed rest due to illness or orthopedic injury. Hindlimb unweighting (HU) is one of the most frequently used models to study the effects of disuse and space flight (Baldwin and Haddad, 2002). Hindlimb unweighting results in a rapid loss of skeletal muscle mass, and the effect is more pronounced in the slow-twitch than in the fast-twitch muscle (Fitt et al. 2000; Edgerton and Roy, 1996). However, the precise mechanism by which HU leads to muscle atrophy remains unclear. Recent studies indicate that this loss of muscle mass and protein content mainly results from increased protein degradation, although decreased protein synthesis has been suggested (Thomason and Booth, 1990; Booth and Criswell, 1997). Like other cells, skeletal muscle contains the multiple pathways of protein degradation. These include a lysosomal, a Ca2+-dependent, and an ATP-dependent ubiquitin-proteasome pathway. Of the three pathways, the ubiquitin-proteasome pathway is the critical system responsible for the bulk of muscle proteolysis including the major contractile proteins actin and myosin (Solomon et al. 1996; Attaix et al. 1998; Jagoe and Goldberg, 2001). This system is also involved in the degradation of oxidized proteins resulting from oxidative stress which otherwise disrupts cellular homeostasis (Davies, 2001). Interestingly, HU has been shown to increase oxidative stress and decrease antioxidant capacity in skeletal muscle (Koesterer et al. 2002; Lawler et al. 2003).

The importance of the ubiquitin-proteasome pathway in muscle wasting during unweighting has recently been demonstrated (Demartino and Ordway, 1998; Taillandeir et al. 1996; Ikemoto et al. 2001). Taillandier et al. (1996) reported that the proteasome system accounts for most of the enhanced protein degradation seen in incubated soleus muscle from unweighted rat, while the lysosomal and Ca2+-dependent pathway were responsible for only a small fraction (15-20%) of total protein degradation. These changes have been correlated with an increase in mRNAs for polyubiquitin, E2-14kDa ubiquitin conjugating enzyme, and subunits of proteasome in such muscles. These observations were further supported by a recent study demonstrating that there was a time-dependent increase in mRNAs for various components of the ubiquitin pathway and protein ubiquitination in the muscle from animals submitted to space flight and tail suspension (Ikemoto et al. 2001). Since the modulations of mRNA expression are only suggestive for the activation of this system, it is not clear whether these changes in mRNAs would result in changes in the activity and protein levels of this proteolytic pathway during hindlimb unweighting. Moreover, there is now substantial evidence that activation of the system is muscle fiber-type dependent during hyperinsulinemia (Larbaud et al. 2001), burn injury (Fang et al. 1998), and muscle transformation (Sultan et al. 2001). Whether this activation specificity is present during unweighting has not been investigated.

Clenbuterol has been described as one of the most potent growth promoting β2-agonists. Administration of clenbuterol has been shown to increase muscle mass (Yang and McElligot, 1989) and reduced muscle atrophy in different animal models of muscle wasting including muscular dystrophy (Hayes and Williams, 1998), denervation (Zeman,

1987), immobilization (Babij and Booth, 1988), and hindlimb suspension (Dodd and Koesterer, 2002; Von Deutsch et al. 2001). However, the precise mechanism(s) through which this compound exerted their anabolic and anti-atrophy effects on skeletal muscle remains unclear. To date, there is still a debate regarding whether CB increases skeletal muscle mass through increased protein synthesis and/or reduced protein degradation. Recently, however, evidence suggests that β2-agonist clenbuterol exerts such effects primarily through a reduction in protein degradation (Sillence et al. 2000, Navegantes et al. 2002). Although the precise biochemical mechanism by which β2-agonist mediates its anabolic effects on skeletal muscle is unknown, a reduction in muscle protease has been proposed. So far, there have been few studies examining the effects of β2-agonists on the protease activity and the results are somewhat contradictory (Mantle and Preedy, 2002). In addition, to our knowledge, there is little information regarding the effect of β2-agonists on the activity of the ubiquitin-proteasome pathway, which is believed to provide a principal mechanism for the majority of protein breakdown in skeletal muscle, under normal and hindlimb unweighting conditions.

Although β2-agonists presumably exert their action by binding to β2 receptors and activating the synthesis of cyclic adenosine monophosphate (cAMP) (Barnes, 1995; Yang and McElligot, 1989), the possibility that β2-agonists act indirectly leading to an increase in the production of other factors and hormones that cause muscle hypertrophy by another mechanism has not been ruled out. Of various growth-stimulating factors, IGF-1 is known to play a role not only in myogenesis but also in the regulation of postnatal skeletal muscle growth (Florini et al. 1991, 1996). Interestingly, it has recently been reported that muscle hypertrophy induced by clenbuterol is associated with an increase in

muscle IGF-I expression and protein content (Awede et al. 2002). Several recent studies show that IGF-1 administration could inhibit muscle proteolysis in several catabolic states, possibly by suppressing the upregulation of the ubiquitin-proteasome pathway (Fang et al. 1998; Chrysis and Underwood, 1999). However, the exact role that IGF-1 plays in skeletal muscle hypertrophy/atrophy induced by β2-agonists remains unknown. Thus, the present study was designed to examine whether β2-agonists such as clenbuterol treatment would induce hypertrophy and/or attenuate muscle disuse atrophy caused by hindlimb unweighting by reducing the ubiquitin-proteasome proteolysis and whether muscle IGF-1 content may be involved in this process.

Questions

1. Does HU result in muscle atrophy in association with the increased activity and protein expression of the ubiquitin-proteasome pathway in the slow-twitch and fast-twitch skeletal muscle?

2. Does a β2-agonist attenuate hindlimb unweighting-induced muscle atrophy by suppressing the activation of the ubiquitin-proteasome pathway and are these effects fiber-type dependent?

3. Are these changes associated with an alteration in muscle IGF-1?

Hypotheses

1. Hindlimb unweighting results in muscle atrophy and increased protein degradation and these changes are associated with the increased activity and protein expression of the ubiquitin-proteasome pathway in both slow-twitch and fast-twitch skeletal muscle.

2. A β2-agonist will attenuate hindlimb unweighting-induced muscle atrophy by suppressing the activation of the ubiquitin-proteasome pathway; and these effects are fiber-type dependent.

3. These changes are associated with an alteration in muscle IGF-1 content.

CHAPTER 2
REVIEW OF LITERATURE

Hindlimb Unweighting Model

One unique feature of skeletal muscle is its adaptability. Skeletal muscle fibers are capable of changing their morphological, physiological, and biochemical properties in response to altered functional demands. For example, increased contractile activities such as chronic electrical stimulation and resistance exercise training result in muscle hypertrophy, whereas inactivity due to disuse typically lead to marked atrophy (Baldwin and Haddad, 2002). In the latter, several animal models for muscle disuse including denervation, limb immobilization and hindlimb suspension (HU) have been developed. Among these models, HU is one of the most frequently used models to study the adaptation of skeletal muscle to inactivity. This HU model has also been used as a ground-based model for space flight and microgravity because it mimics some aspects of exposure to microgravity, and producing similar patterns of muscle atrophy to those found in both animals and humans during space flight (Morey-Holton and Globus, 2002). In this model, the hindlimb of animals are suspended and non-weightbearing, but the animals are capable of moving around the cage using their forelimbs.

Hindlimb Unweighting and Muscle Mass

Unweighting of skeletal muscle via microgravity and hindlimb suspension in rats has been shown to cause severe muscle atrophy. This atrophic response occurs rapidly and is muscle and fiber type specific (Fitts et al. 2000; Edgerton and Roy, 1996). The extensor muscles that are comprised primarily of slow muscle fibers, such as the soleus,

show greater atrophy than the predominantly fast flexor muscles such as the tibialis anterior and extensor digitorum longus. Interestingly, the mixed fiber type muscles such as gastrocnemius show moderate atrophy with hindlimb unweighting. Muscle disuse atrophy is characterized by a decrease in muscle fiber cross-sectional area and not by changes in fiber number (Templeton et al. 1988). This is associated with a selective loss of contractile proteins (particularly myofibrillar proteins) that constitutes approximately 70% of total protein content. Moreover, HU is accompanied by a slow-to-fast transformation in both contractile and regulatory proteins (Thomason and Booth, 1990; Fitts et al. 2000). These adaptations may be responsible for, or contribute to, muscle dysfunction observed during microgravity and HU.

Mechanisms Responsible for Skeletal Muscle Protein Loss

Proteins in skeletal muscle, as in other tissues, are in a dynamic state of turnover. That is, they are continually degraded and replaced by new synthesis even when the overall muscle protein mass remains fairly constant. Thus, the loss of muscle mass during certain catabolic conditions including HU could result either from decreased protein synthesis, from increased protein degradation, or from a combination of the two. The effect of HU on rates of protein turnover in the soleus muscle of adult rats has recently been reviewed (Thomason and Booth, 1990; Booth and Kirby, 1992). According to the triphasic time course model, the alterations in protein turnover during HU can be subdivided into three phases: phase I, a rapid reduction in protein synthesis (0-3 days of HU); phase II, a slow increase in protein degradation rate and peak after 14 days of HU; phase III, rate of protein degradation decline to below control levels at 24 days of HU while the rate of synthesis remains lower than normal. Based on these data,

it was suggested that muscle wasting observed during HU is primarily due to increased protein breakdown although a decrease in protein synthesis has been implicated.

Role of protein synthesis

As pointed out by Booth and Criswell (1997), the protein synthesis rate in the unweighted soleus muscle is decreased rapidly within hours of suspension. In young growing rats, the mixed protein synthesis rate was decrease by 20% over a 3-5 day period of unweighting (Goldspink et al. 1986; Loughna et al. 1986). In older nongrowing rats, however, mixed and myofibrillar proteins were decreased up to 46% and 59%, respectively, within a week of hindlimb unweighting (Thomason et al. 1989). Thus, the mature rats showed a greater decrease in protein synthesis rates in unweighted muscle compared to their young counterparts. Since this reduction in protein synthesis occurs without a concomitant decrease in mRNAs coding for either α-actin or β-myosin heavy chain over this time period, it was thus suggested that atrophy occurring during HU is likely due to a decrease of translation (Thomason and Booth, 1990).

A recent study by Ku and Thomason (1994) showed that polyribosomes isolated from soleus muscle after 18h of suspension were larger than polyribosomes from control animals. This increased polyribosome density was probably due to a piling up of protein on the ribosome machinery, which was caused by a slowing in nascent polypeptide elongation rate. This in turn slowed the translation rate and subsequent protein synthesis. More recently, Hornberger et al. (2001) reported that an inhibition of protein synthesis occurs during the initiation of translation. Indeed, there was a significant reduction in ribosomal S6 kinase (p70S6k) phosphorylation, a key step in regulating the translation initiation, in soleus muscles either at 12 hrs or 7 days after HU. Therefore, it was

suggested that a decrease in phosphorylation of this protein kinase during HU would lead to a decrease in global protein synthesis.

Role of protein degradation

In contrast to protein synthesis, the mechanisms and regulation of intracellular protein degradation are still not completely understood. There are two potential reasons for this. First, protein degradation is more difficult to determine with precision *in vivo* than that of synthesis. Second, while there is only one pathway for protein synthesis, there are multiple pathways of protein degradation and these pathways are not as well understood as the protein synthesis pathway.

The rate of protein degradation can be determined by several means. First, rates of protein degradation are generally determined by following the net release of tyrosine from muscles in the media in the presence of cycloheximide, a protein synthesis inhibitor. Since muscle can neither degrade nor synthesize this amino acid, its accumulation directly reflects the net breakdown of protein in skeletal muscle. Tyrosine can be easily assayed fluorometrically (Waalkes and Udenfriend, 1965). Using this in vivo approach, several studies (Jasper et al. 1988; Jasper et al. 1985) showed that protein degradation rate increased up to 55% over a 7 day period of HU.

Secondly, the fractional rate of protein degradation can be estimated by subtracting the rate of protein loss from the rate of protein synthesis, since the protein content is regulated by the relative rates of protein synthesis and degradation. Goldspink et al. (1986) reported that the protein degradation rate was increased 340% after 5 days of HU. Other studies showed that the rate constant of myofibril degradation was increased by 200% after 15 days of HU (Thomason et al. 1987).

It should be pointed out that the measurement of rates of tyrosine release might reflect the breakdown of all cellular proteins since this amino acid is present in all tissues. Thus, this measurement does not distinguish between the breakdown of myofibrillar and nonmyofibrillar proteins. Alternatively, rates of myofibrillar protein breakdown can be estimated by measuring the release of 3-methylhistidine (3-MH). This posttranslational modified amino acid, which is found exclusively in actin and myosin, cannot be degraded and reutilized for proteins synthesis. Thus, its appearance is a good indication of the rate of myofibrillar protein degradation. In skeletal muscle, the rate of myofibrillar protein breakdown is estimated to be approximately 25% of the rate of total protein degradation (Sugden and Fuller, 1991). This value is, however, smaller (< 10%) in the heart muscle.

Pathway of Protein Degradation in Skeletal Muscle

It is generally accepted that proteolytic enzymes are responsible for degradation of proteins during metabolic turnover. Skeletal muscle, like other mammalian tissues, contains multiple proteolytic pathways that are presumably responsible for the breakdown of intracellular proteins. The best known proteolytic system in mammalian cells is the lysosomal pathway. The membrane-enclosed lysosome contains the cysteine protease cathepsins B, H, and L and the aspartate protease cathepsins (Dice, 1990). Although the lysosomes are responsible for the proteolysis of soluble and extracellular proteins, this system is not involved in the degradation of myofibrillar proteins in skeletal muscle (Lowell et al. 1986; Furno et al. 1990).

Skeletal muscle also contains two major cytosolic proteolytic pathways, calcium-dependent and ATP-ubiquitin-dependent proteolytic pathways. Ca2+-dependent proteases are ubiquitous enzymes, composed of μ-calpain and m-calpain that differ in

their affinity for Ca2+ (Murachi et al. 1981). The activity of calpain is modulated not only by Ca2+, but also by calpastatin, their physiological endogenous inhibitors. Recently, a third calpain, p94, has been identified; however, its physiological role is still unclear. Several studies provide evidence that calpains do not contribute significantly to increased muscle proteolysis observed in various muscle-wasting conditions (Furno et al. 1990), and they cannot degrade myofibrillar (actin and myosin) proteins into amino acids. Their primary role appears to be one of limited cleavage of some specific proteins, leading to a release of myofilaments from the myofibrils (Goll et al. 1992). Consequently, this limited cleavage by calpain may render myofibrillar proteins more susceptible to degradation by other enzymes (especially the ubiquitin-proteasome pathway). Recently, it was proposed that calpains might play a role in the initiation of muscle protein degradation (Attaix and Taillandier, 1998; Hasselgren and Fisher, 2001). However, since the potent synthetic calpain inhibitors have failed to inhibit protein breakdown in vitro, the possible involvement of calpains in myofibrillar protein degradation remains to be investigated. The ubiquitin-proteasome pathway is the principal mechanism of the degradation of proteins in the eukaryotic cells. This system is responsible for the breakdown of most intracellular proteins that are involved in a wide range of biological processes. Among these are regulation of cell cycle and division, differentiation and development, transcriptional control, signal transduction, receptor down-regulation, and antigen processing (Hershko and Ciechanover, 1998). Moreover, it was shown that this proteolytic pathway is involved in the degradation of abnormal proteins resulting from oxidative stress (Davies, 2001). The ubiquitin-proteasome pathway is also the primary system responsible for myofibrillar protein degradation that

is associated with muscle wasting seen in several physiological and pathophysiological conditions (Attaix and Taillandier, 1998; Mitch and Goldberg, 1996; Lecker et al. 1999).

Ubiquitin-Proteasome Pathway

Degradation of proteins via the ubiquitin-proteasome system can be divided into two distinct steps: (1) covalent attachment of polyubiquitin chain to the protein; and (2) degradation of the targeted protein by 26S proteasome.

Ubiquitin system

Ubiquitination is a multi-step process, involving the action of at least three different enzymes; ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin protein ligase (E3). The first step in protein ubiquitination involves the formation of a high-energy thiol-ester bond (requiring ATP) between the C-terminal glycine of ubiquitin, a highly conserved 76-residue polypeptide, and cysteine in the active site of the E1 enzyme. After activation, the ubiquitin is transferred to one of several E2 enzymes. The final step of ubiquitination, the transfer of ubiquitin to the epsilon-amino group of lysine residue in the substrate target, is catalyzed either by E3 ligases or, sometimes occurs directly from E2-ubiquitin conjugates. This process is repeated several times to generate a long polyubiquitin chain that marks the protein for degradation by the 26S proteasome.

Although only E1 is responsible for ubiquitin activation, there are a large number of distinct E2 enzymes that are thought to increase substrate specificity of the ubiquitin system. So far, three E2 enzymes have been found in rodent skeletal muscles, including the 14kDa, 17kDa, and 20kDa species. Among theses species, only the 14kDa was found to play a significant role in muscle wasting (Wing and Banville, 1994).

The number and diversity of E3 ligases in the cell are even greater than those of E2 enzymes. Since E3 enzymes specifically bind protein substrates, either alone or in conjunction with E2, it was suggested that they play a major role in recognition and selection of proteins for conjugation and subsequent degradation. At least three E3-ligases have now been identified (Weissman, 2001; Hershko and Ciechanover, 1998). The first ubiquitin ligase discovered, E3α, is one of the best-characterized ubiquitin ligases to date. This ligase is believed to mediate the direct transfer of activated ubiquitin from E2 (14 kDa E2) to the substrate (Joazeiro et al. 2000). Ubiquitin ligase E3α recognizes the destabilizing residues of the N-end rule substrates. The N-end rule degradation signal consists of a number of amino acids that (when located in the N-terminus) mark the protein for degradation by the proteasome system (Varshavsky, 1996). This enzyme has two distinct and independent binding sites for basic and bulky hydrophobic N-terminal residues. Interestingly, E3α can also recognize denature and N-acetylated proteins, non-N-end rule protein substrates (Scheffner et al. 1995). The second family of E3 enzymes is the hect (homologous to the E6-AP COOH terminus) domain family. The prototype of this family is the E6-AP mammalian protein involved in the degradation of the tumor suppressor p53 in cells infected with the human papilloma virus. Most hect-domain proteins are likely E3 enzymes or parts of multiprotein complexes that contain E3-like activity. These HECT-domain E3s have a unique mode of action: they catalyze ubiquitin transfer to the substrate through an intermediate thio-lester between ubiquitin and a conserved cysteine in the hect-domain. The enzymes can also interact with a variety of different E2s and have been demonstrated to target several cellular proteins including p53 for ubiquitination and degradation. The third family of

E3s, the SCF complex, consists of a Skp1, a Cullin, and F-box proteins. Among these, F-box proteins are thought to carry the substrate recognition site of the complex. Although there is no specific consensus on degradation signals among the substrates for SCF complexes, it appears that phosphorylation of substrates are required for all SFC-substrate interactions (Wiessman, 2001).

More recently, a fourth component of the ubiquitin system has been proposed: E4 (Koegl et al. 1999). The E4 is a protein required for ubiquitin chain elongation: it binds to short ubiquitin chain, and in the presence of E1, E2, and E3, catalyzes ubiquitin chain elongation, which can be recognized by the proteasome.

Deubiquitination

Ubiquitination is a reversible process in which ubiquitin is released from substrate protein and can be reutilized by the next proteolytic cycle. This process is catalyzed by deubiquitinating enzyme (DUBs). To date, a large number of DUBs have been identified and can be divided into two families, based on protein structure and activity. The ubiquitin C-terminal hydrolase (UCHs) are generally small (200-300 amino acids.) and are responsible for uncoupling ubiquitin from small peptides or larger substrates. The ubiquitin-specific processing enzymes (Ubps), on the other hand, have a wide range of sizes (500-3000 amino acids) and are responsible for removing the ubiquitin from larger proteins and disassembling the polyubiquitin chain. Recent studies reveal that the deubiquitination may influence the breakdown of ubiquitin conjugates (Wilkinson, 2000).

The 26S proteasome

The second major step in the ubiquitin-proteasome pathway is the degradation of polyubiquitinylated proteins by 26S proteasome complex with release of free and reutilizable ubiquitin. The 26S proteasome is a large multicatalytic proteinase (2000

kDa), consisting of two subcomplexes: a catalytic core or 20S proteasome and two 19S regulatory particles. The 20S proteasome is a cylinder-shaped structure consisting of four stacked rings, forming a central chamber that runs through the stack from top to bottom. Each ring is composed of seven subunits, which may be classified into two groups: the α-subunits comprise the outer two rings; and the β-subunits comprise the inner two rings. The catalytic sites of 20S proteasome are located within the internal cavity of three β- subunits (β1, β2, β5). Thus, substrates must enter the catalytic chamber in order to be degraded into peptides. The 20S proteasome is characterized by three distinct proteolytic activities against short synthetic peptides: the chymotrysin-like (ChT-L), the trypsin-like (T-L), and the peptidylglutamyl-peptide-hydrolyzing (PGPH). These activities preferentially cleave peptide bonds after hydrophobic, basic, and acidic amino acid residues, respectively. In addition to these well-characterized peptidase activities, the 20S proteasome is also known to possess two other endopeptidase activities, i.e., branched chain amino acid preferring (BrAAP), and small neutral amino acid preferring (SNAAP) activities (Orlowski et al. 1993).

Degradation mediated by the proteasome is processive meaning that protein substrates are cleaved at multiple sites and generate short peptides of 3-20 residues (Coux et al. 1996), which are further hydrolyzed by other cytosolic peptidases (Tomkinson et al. 1999). In this process, the protein substrates are first cleaved by the ChT-L activity, a rate-limiting step, and the two other peptidase activities (i.e., T-L and PGPH) could be regulated in an allosteric manner as proposed in the “bite-chew” model proposed by Kisselev et al. (1999).

Since the 20 proteasome exists in an active or latent form in the cell, the function and activity of this protease can be modulated through the interaction with regulatory proteins; PA700 (also known as 19S regulator) and PA28 (also known as 11S) complexes. The PA700 is composed of 20 different subunits that can be divided into a base subcomplex and a lid subcomplex. The PA700 can reversibly bind to both ends of the central catalytic core in an ATP-dependent manner and form the 26S proteasome. Also, PA700 contains ATPase activity and is believed to be responsible for binding and unfolding of substrates as they enter the proteolytic core. The PA700 also processes ubiquitin-specific protease activity and might function to edit substrates that are targeted to the proteasome.

The PA28 is another activator of the 20S proteasome. In contrast with the assembly of the 26S proteasome, the association of 11S with the 20S proteasome does not require ATP. The protein PA28 is composed of three related proteins, termed PA28α, PA28β, and PA28γ. PA28α and PA28β associate into a heterohexameric complex with alternating α and β subunits (i.e., PA28 (αβ) 3). Although this complex can stimulate multiple peptidase activities of 20S proteasome, it appears that this activator does not play a central role in the initial cleavage of protein substrates, but has a stimulating effect on the degradation of polypeptides of intermediate size that are generated by the 26S proteasome (Coux et al. 1996). Since PA28 can be induced by interferon-γ, it was suggested that it plays a role in the antigen processing function by trimming large peptides that were generated by the 26S proteasome to the precise antigenic epitopes recognized by the class I MHC complex (Rock et al. 2002).

Role of Ubiquitin-Proteasome Pathway in Muscle Wasting

There is ample evidence that muscle wasting in various catabolic conditions including sepsis, cancer, and burn injury, is associated with up-regulation of the ubiquitin-proteasome pathway (Attaix & Taillandier, 1998; Mitch and Goldberg, 1996; Lecker et al. 1999). The involvement of this proteolytic system in muscle wasting is supported by the finding that increased proteolysis seen in various catabolic processes is ATP-dependent and is suppressed by proteasome inhibitors. For example, Temparis et al. (1994) reported that inhibitors of lysosomal and Ca2+-dependent proteases did not attenuate increased rates of proteolysis in the atrophying muscle from tumor-bearing rats. By contrast, ATP depletion and/or proteasome inhibitors completely blocked the enhanced rates of protein breakdown (Hobler et al. 1997)

The importance of the ubiquitin-proteasome pathway in muscle wasting due to unweighting has recently been demonstrated (Demartino and Ordway, 1998; Attaix et al. 1998, Taillandeir et al. 1996). Taillandeir et al. (1996) reported that the proteasome system accounts for most of the enhanced protein degradation (~80%) seen in atrophying muscles, whereas the lysosomal and Ca2+-dependent pathway are responsible for only a small fraction (15-20%) of total protein degradation. This increased proteolysis was associated with an increase in mRNAs for several components of the ubiquitin-proteasome system in atrophying muscle. For example, the mRNA levels for the C2 and C9 proteasome subunits were increased by 133% and 61%, respectively, after 9 days of unweighting in atrophying soleus muscle. Similarly, mRNA levels for ubiquitin and 14kDa-E2 were also reported to increase (482% and 342%, respectively) under these conditions.

The involvement of the ubiquitin-proteasome system in HU-induced atrophy has been further substantiated in a recent study by Ikemoto et al. (2001). These investigators have demonstrated that there are time-dependent increases in mRNAs for polyubiquitin, E2-14kDa and protein ubiquitination, with little or no changes in the level of mRNAs for calpain and cathepsin proteinase in the muscle from animals subjected to space flight and tail suspension. Since the increased protein ubiquitination is associated with oxidative stress, as evidenced by altered glutathione status and markers of superoxide and hydrogen production, and can be attenuated by cysteine supplementation, it was concluded that the upregulation of the ubiquitin-proteasome system may be mediated via oxidative stress in skeletal muscle (Ikemoto et al. 2001). Surprisingly, more recent studies (Ikemoto et al. 2002; Koesterer et al. 2002) showed that a relative high dose of vitamin E or antioxidant supplementation did not suppress the ubiquitination of proteins and attenuate muscle atrophy observed during unweighting. Clearly, additional experiments are necessary to determine if oxidative stress plays a role in mediating muscle atrophy caused by unweighting.

Regulation of the Ubiquitin-Proteasome Pathway

The mechanisms of regulation of the ubiquitin-proteasome system remain elusive. The activity of this pathway could be regulated either at the level of substrate ubiquitination or proteasome activity. A number of studies have provided evidence that the rate-limiting step in this pathway is ubiquitin conjugation. For example, the levels of ubiquitin-protein conjugates were reported to increase in a wide variety of catabolic states and these changes have been correlated with the increased overall proteolysis observed in atrophying muscles (Baracos et al. 1995, Wing et al. 1995; Taillandier et al. 1996; Mansoor et al. 1996). This increased ubiquitination could be due to the activation of the

ubiquitin system, or it may result from a post-translational modification or a structural change of substrate that render it susceptible to recognition by certain E2s or E3s. As pointed out by Attiax et al. (1998), it is less likely that the expression of ubiquitin would become a rate-limiting step for ubiquitination since the amount of free ubiquitin in normal muscles are much higher than the dissociation constant for E1, the enzyme involved in the first step of ubiquitination. Wing and Banville (1994) proposed that the activity of 14kDa-E2 could be a rate-determining step in the regulation of the ubiquitin pathway. Later, a number of studies have shown the increased expression of E2-14k in various muscle wasting conditions including fasting, sepsis, diabetes and disuse atrophy (Attiax et al. 1998, Jagoe and Goldberg, 2001). Further, recent studies show that the rate of ubiquitin conjugation increases in atrophying muscles largely due to the activation of the N-end rule pathway that involves E2-14k and E3α (Jagoe and Goldberg, 2001; Solomon et al. 1998).

More recently, two novel skeletal muscle-specific ubiquitin ligases (i.e. muscle RING finger 1 (Murf1) and muscle atrophy F-box (MAFbx)) which are thought to be responsible for skeletal muscle atrophy, have been identified (Bodine et al. 2001; Gomes et al. 2001). These proteins are upregulated in several models of disuse including immobilization, denervation and hindlimb unweighting. Moreover, knockout mice lacking MAFbx and/or MuRF1 proteins show a significant reduction in the loss of muscle mass by 36-56% after 14 days of denervation. Since the knockout of these proteins partially attenuated the atrophy response, it seems likely that there are additional pathways or regulators involved in this protein degradation process.

Besides, it is possible that the degradation of ubiquitin conjugate by the proteasome is the rate-limiting step. This hypothesis is supported by the observation that there was an increased expression of 20S proteasome subunits in atrophying muscle and these changes were paralleled by the enhanced protein breakdown seen in a wide variety of catabolic conditions (Taillandier et al. 1996; Temparis et al. 1994; Tiao et al. 1997). Furthermore, the mRNA levels for subunits of 19S complex and of the PA28 activator, the regulators of the proteasome, were reported to increase in at least two different muscle wasting conditions e.g. hindlimb suspension and cancer, in rats (Attaix et al. 1997). Whether these changes in mRNAs would result in changes in protein levels and the peptidase activities of the proteasome in skeletal muscle remain unknown.

Factors that Cause Muscle Atrophy

Several hormones have been implicated in the regulation of muscle protein degradation in catabolic conditions. The following hormones are discussed because of the evidence for their apparent involvement in this process.

Glucocorticoids

There is considerable evidence that glucocorticoids may be an important mediator of muscle proteolysis in various catabolic conditions (Mitch and Goldberg, 1996; Hasselgren and Fisher, 2001). For example, treatment of septic and burned rats with RU38486, a selective glucocorticoids antagonist, completely suppressed the increased protein breakdown (Tiao et al. 1996, Fang et al. 1995). Furthermore, treatment of experimental animals with high doses of glucocorticoids has been shown to enhance muscle protein degradation (Tiao et al. 1996).

Although the precise cellular mechanism by which glucocorticoids induced muscle catabolism remains unknown, it has been suggested that glucocorticoids-induced muscle

atrophy is associated with the increase in level of mRNAs encoding ubiquitin and proteasome subunits (Tiao et al. 1996; Wing and Goldberg, 1993). In addition to stimulating protein degradation, glucocorticoids have also been reported to inhibit protein synthesis by decreasing translation of mRNAs for muscle proteins and suppressing entry into muscle (Lecker et al. 1999).

Unweighting of skeletal muscle was associated with increased plasma glucocorticoids especially for the first three days (Thomason and Booth, 1990). However, plasma glucocorticoids had returned to control value by the 7^{th} day of unweighting. Since treatment of suspended rats with RU38486 did not prevent a marked increase in protein breakdown at this same period (Tischler, 1994). Thus, it is unlikely that glucocorticoid-induced atrophy is an important component in the loss of muscle mass during unweighting.

Tumor nuclear factors alpha (TNF-α)

TNF-α is a proinflammatory cytokine that is thought to play a major role in the skeletal muscle catabolism. This cytokine was found to be upregulated in many disease states associated with muscle wasting (Espat et al. 1994; Ried and Li, 2001). The mechanism by which TNF-α stimulates muscle catabolism in vivo is still unclear. One possibility is that TNF-α acts directly on skeletal muscle to induce muscle catabolism. Indeed, TNF-α has been reported to inhibit protein synthesis and myogenic differentiation (Langen et al. 2001). TNF-α can also stimulate the proteolysis of muscle-specific proteins, including myosin heavy chain in cell culture (Li et al. 2000). Another possibility is that TNF-α can induce apoptosis in murine skeletal myoblast (Meadows et al. 2000). Alternatively, It has been shown that TNF-α can act indirectly to stimulate

muscle catabolism by modifying hormones that regulate protein turnover i.e. cytokines (Tracey and Cerami, 1993).

Recent studies suggest that TNF-α can stimulate muscle catabolism by activating the ubiquitin-proteasome system via the transcription factor nuclear factor κB (NF-κB) signaling pathway (Reid and Li, 2001; Li et al. 2003). Administration of TNF-α stimulates UbcH2, a ubiquitin carrier protein, expression (Li et al. 2003), and causes an increase in both free and conjugated ubiquitin as well as ubiquitin mRNA in skeletal muscles both in vivo and in vitro (Garcia-Martinez et al. 1993; Llovera et al. 1997).

Although TNF-α has been reported to stimulate muscle atrophy observed in various diseases including cancer cachexia, muscle unweighting via microgravity or hindlimb suspension, however, is a non-pathological model of muscle wasting which is not associated increased TNF-α production. Indeed, there is evidence that hindlimb suspension does not create lesions in hindlimb muscle, and there is no evidence of macrophages or monocyte invasion in these muscles (Krippendorf and Riley, 1994). Thus, it seems unlikely that TNF-α plays a major role in muscle wasting under these conditions.

Insulin-like growth factors (IGF-1)

Protein turnover in skeletal muscle is also regulated by growth factors (GH, insulin and IGF-I). One of the most interesting growth factors that has received much attention is insulin-like growth factor I (IGF-I). IGF-I is a 70-amino acid single chain polypeptide, which has 62% homology with proinsulin. IGF-I is synthesized by the liver under the influence of growth hormone secretion from the pituitary gland. This IGF-I is known to have an essential role for normal growth and development (Stewart and Rotwein, 1993).

In cell culture, IGF-I has been shown to stimulate satellite cell proliferation (Allen and Boxhorn, 1989), increase myonuclei number and myofibril size (Vandenburgh et al. 1991), increase glucose and amino acid uptake, and suppress protein degradation (Florini, 1987). In addition to hepatic IGF-I, there is local synthesis of IGF-I in other tissues including skeletal muscle. This local production of IGF-I has been shown to play an important role in the maintenance of skeletal muscle mass, possibly through the autocrine/paracrine action (Adams, 1998). IGF-I expression was reported to increase during compensatory hypertrophy induced by mechanical overloading and stretch (Yang et al. 1997; Adams and Haddad, 1996), whereas unweighting of hindlimb muscle is associated with a lowering of IGF-I mRNA and peptide levels (Awede et al. 1999; Suliman et al. 1999). In addition, localized infusion of IGF-I results in skeletal muscle atrophy (Adams and McCue, 1998). Other evidence that IGF-I is important in determining muscle mass has been obtained from transgenic mice. In this model, the IGF-IRs were knocked out or the IGF-I was truncated. Liu et al. (1993) found that mice lacking the IGF-IR were smaller than wide-type animals, and die after birth because of respiratory failure. In contrast, transgenic mice that overexpressed IGF-I in skeletal muscle exhibit a twofold increase in muscle mass over that seen in normal mice (Musaro et al. 2001; Coleman et al. 1995). Overexpression of IGF-I has also been shown to prevent the age-related loss in muscle mass (Barton-Davis et al. 1998). In spite of these effects, the mechanism of action of IGF-I in muscle hypertrophy and in the attenuation of muscle atrophy is not clearly elucidated.

It is well documented that IGF-I treatment not only increases protein synthesis but also inhibits the rate of protein degradation in skeletal muscle. In doing so, IGF-I binds

to a high affinity IGF-I receptor, a multimeric transmembrane glycoprotein composed of two alpha and two beta subunits. Binding of IGF-I to its receptor stimulates the intrinsic tyrosine kinase activity and activates the PI (3) K/Akt/mTOR signalling cascade (Bodine et al. 2001). Recent evidence suggests that PI (3) kinase may play a role in the activation of downstream targets i.e., p70S6K and PHAS-1/4E-BP1, which have been shown to promote protein synthesis through increases in translation initiation and elongation (Adams, 1998). Furthermore, it has recently been reported that IGF-1 is able to induce muscle fiber hypertrophy through the activation of calcineurin signaling pathway (Musaro et al. 2001).

In addition to stimulating protein synthesis, IGF-I also plays a role in the proliferation and differentiation into myoblasts of the satellite cells. Satellite cells are small mononucleate precursor cells located between the basal lamina and sacrolemma of muscle fibers. These cells are normally mitotically inactive but are activated in response to mechanical loading or damage (Adams, 1998). Once activated, these cells proliferate and differentiate into myoblasts, which then fuse with existing fibers, providing additional nuclei required for muscle growth.

Administration of IGF-I has also been shown to inhibit muscle proteolysis in burn injury and glucocorticoid-treated rats. These responses to IGF-I are associated with reduced gene expression of ubiquitin, E12-14k ubiquitin conjugating enzyme as well as some subunits of proteasome, suggesting that IGF-I inhibited the activation of the ubiquitin-proteasome system under these conditions (Fang et al. 1998; Chrysis and Underwood, 1999). Furthermore, in cell culture, IGF-I treatment has been reported to

reduce the stability of the 14 kDa E2 mRNA, a rate limiting step of the ubiquitin pathway (Wing and Bedard, 1996).

In addition to IGF-I itself, the action of IGF-I can be regulated by IGF binding proteins (IGFBPs). At least six different IGFBPs, designated IGFBP-1 to –6, have been characterized (Hwa et al. 1999). These proteins bind IGF-I with high affinity and act as carrier proteins in the blood stream and control efflux of IGFs from the vascular space. Of the six IGF binding proteins, IGFBP-3 is primarily responsible for maintaining IGF-I levels in the circulation together with another protein called acid labile subunit. In addition, some IGFBPs, e.g. IGFBP-5, have been shown to have biological actions that are IGF-independent (Schneider et al. 2002). Recently, there is evidence that IGFBPs mRNA and protein levels in skeletal muscles are altered during unloading or overloading (Awede et al. 1998; Spangenburgh et al. 2003). This suggests that IGFBPs may play a role in skeletal muscle adaptation in response to altered loading.

Beta 2-Agonist

A beta agonist is a compound that stimulates the β-receptors. Stimulation of these receptors by beta agonists such as norepinephrine or epinephrine results in activation of adenylase cyclase and increased cAMP, a major second messenger of β-receptor. There are a variety of β-agonists including isoproterenol, albuterol, cimaterol, salmaterol, fenoterol, isoprotelenol and clenbuterol (Burgess et al. 1997). Among them clenbuterol is one of the most potent growth-promoting β2-agonist. This compound is highly active on β2-receptor but has little effect on β1-receptor.

Although primarily used to treat asthma and related bronchospasm, high-doses of clenbuterol have been shown to increase muscle mass in many species of animals (Yang

and McElligot, 1989). It has been reported that treatment with clenbuterol induces a 10-20% increase in muscle mass in normal rats (Yang and McElligot, 1989), and reduce muscle wasting due to denervation (Zeman et al. 1988), and attenuate muscle atrophy associated with disuse i.e., immobilization and hindlimb unloading (Babij and Booth, 1988; Dodd and Koesterer, 2002; Von Deutsch et al. 2001). Although the anabolic effects of β2-agonists on skeletal muscle could be due to increased muscle protein synthesis and/or reduce muscle protein degradation, recent studies suggest that the effect of clenbuterol on muscle mass is mainly due to its ability to inhibit protein degradation (Reeds et al. 1986; Forsberg et al. 1989; Benson et al. 1991; Sillence et al. 2000; Navegantes et al. 2002).

Beta 2-Agonist and Muscle Proteases

Although the precise biochemical mechanism by which β2-agonists mediate their anabolic effect on skeletal muscle is unknown, a reduction in muscle protease has been proposed. There have been a few studies examining the effects of β2-agonist on protease activity and the results are somewhat contradictory. It has been reported that clenbuterol did not alter the cathepsin B and D activity in the EDL or gastrocnemius muscles but increased their activities in the soleus muscle of rats after 1 week of treatment (McElligot et al. 1987). Furthermore, after 2 weeks of treatment, cathepsin B and D activity in the EDL muscle decreased, while that in the soleus muscle increased (McElligot et al. 1989). However, Kretchmar et al. (1989) found that β-agonist treatment increased the activity of calpain II (40%) and calpastatin (65%) and decreased cathepsin B activity (30%) in lamb muscle. In contrast, Mantle et al. (1990) found no gross changes in the activities of a

wide range of proteolytic enzymes including acid, neutral and alkaline proteinase and peptidases from both innervated and denervated muscles following clenbuterol treatment.

Desensitization of β-Adrenergic Receptor

One unique characteristic of β-agonists is the desensitization and down-regulation of receptors after long-term treatment. Several studies (McElligot et al. 1987; Yang and McElligot, 1989) showed that β-agonist such as clenbuterol and cimaterol exert their anabolic effect during the first 7-10 days of treatment, after which the response is attenuated. There are several mechanisms involved in desensitization of β-receptor (Barnes, 1995). Short-term desensitization involves phosphorylation of the receptor, which results in uncoupling from Gs proteins. By contrast, a longer-term mechanism is the downregulation of surface receptors, a process that involves internalization of the receptor and its subsequence degradation. However, more chronic effects of β-agonists appear to involve changes in gene transcription and β-receptors synthesis (Barnes, 1995).

Effect of β2-Agonist on Muscle Size and Fiber Type Composition

Although it is generally agreed that β2-agonist induce muscle hypertrophy of fast-twitch type II fibers, there are conflicting observations about the effects of β2-agonists on slow-twitch type I fibers (Maltin et al. 1986; Zeman et al. 1988). Several studies reported an increase in cross-sectional area of the slow soleus muscle after β2-agonist administration, whereas others show little or no change in muscle fiber size (Zeman et al. 1988). In other studies, clenbuterol has been shown to produce hypertrophy of both type I and type II fibers (Apseloff et al. 1993; Steven et al. 2000), but the hypertrophy is less in type I fibers (Wineski et al. 2002; Von Deutsch et al. 2001). The mechanism that leads to this disparity is still unknown.

In addition to the effect on muscle growth, clenbuterol has been shown to produce a slow-to-fast fiber transition characterized by increased expression of MHC IIa and IId/x in slow soleus muscle (Criswell et al. 1997; Lynch et al. 1996; Ricart-Firinga et al. 2000). Chronic clenbuterol treatment also causes a type IIa to IId/x to IIb transition in the gastrocnemius muscle (Steven et al. 2000). Interestingly, clenbuterol had no significant effect on the MHC patterns in the EDL, the predominant expression of MHC IId/x and IIb fast muscle. In general, the transition in MHC isoforms induced by clenbuterol followed the well-established sequence i.e., I ↔ IIa↔ IId/x ↔ IIb (Pette and Staron, 2000). In addition to changes in MHCs, Steven et al. (2000) reported that clenbuterol also induced a slow to fast transition in myosin light chain in both soleus and gastrocnemius muscles.

Cellular Mechanisms of β2-Agonist

Anabolic actions of β2-agonists on skeletal muscle can be exerted in different ways. One potential mechanism is that β2-agonists may act directly on skeletal muscle and induces hypertrophy. Indeed, it has been reported that β2-agonists, including clenbuterol, selectively bind to β2 adrenergic receptors on the surface membrane (McLennon et al. 1989; Choo et al. 1992; Hinkle et al. 2002). Activation of β2-receptors results in an increase in cAMP, activation of protein kinase A (PKA), which then phosphorylates several key proteins in the cell leading to cellular responses (Barnes, 1995; Yang and McElligot, 1989). The subsequent step results in increased protein synthesis and reduced protein degradation rates. Activation of β-adrenergic receptors has also been shown to stimulate glucose uptake and metabolism, contractility and heat production in skeletal muscle (Yang and McElligot, 1989). The cAMP response element

binding protein (CREB), a transcription factor, is also phosphorylated by PKA. Once phosphorylated, the CREB binds to a cAMP response element on a target gene and stimulates the transcription of genes (Barnes, 1995).

The second mechanism involved the increased blood flow to skeletal muscle after β-agonist treatment, which may provide the extra substrate and energy for muscle growth. It has been reported that clenbuterol treatment results in increased blood flow in the hind limb muscle of animals (Mersmann, 1998). However, this effect was diminished after long-term treatment (Rothwell et al. 1987). Since there was a temporary increase in blood flow after β2-agonist treatment, it was suggested that β-agonists might not exert an effect on muscle growth through an increase in blood flow (Yang and McElligot, 1989).

The third mechanism is that β-agonists can act indirectly to stimulate muscle growth through modifying hormones that regulate protein turnover. One of the most interesting growth-stimulating factors is IGF-1. Indeed, recent studies have reported that muscle hypertrophy induced by clenbuterol is not associated with circulating IGF-I (Young et al. 1989) but an increase in muscle IGF-I expression and its binding proteins (Awede et al. 2002). This suggests that clenbuterol-induced muscle hypertrophy could be, in part, mediated by the autocrine/paracrine action of IGF-I. Thus, the focus of the proposed work is also to examine the role of IGF-1 in skeletal muscle growth and an attenuation of muscle wasting induced by β2-agonist clenbuterol.

CHAPTER 3
MATERIALS AND METHODS

Pre-Experimental Period

Animal and Experimental Design

Adult, female Sprague-Dawley rats, weighing ~200 grams, were obtained from Harlan Sprague Dawley (Indianapolis, IN). Upon arrival, animals were housed in a temperature-controlled room maintained on a 12-hour light/dark cycle. They were given standard food and water ad libitum and handled daily in order to reduce contact stress during a 7 -day acclimation period. Animals were then assigned randomly to one of four groups; control (C, n=8), clenbuterol-treated (CB, n=9), hindlimb unweighted (HU, n=9), and CB combined with hindlimb unweighting (HU-CB, n=9). The animals in the control and CB groups were housed individually in a conventional rat cage. The animals in HU and HU-CB groups were housed separately in standard rabbit cages to provide increased area for movement.

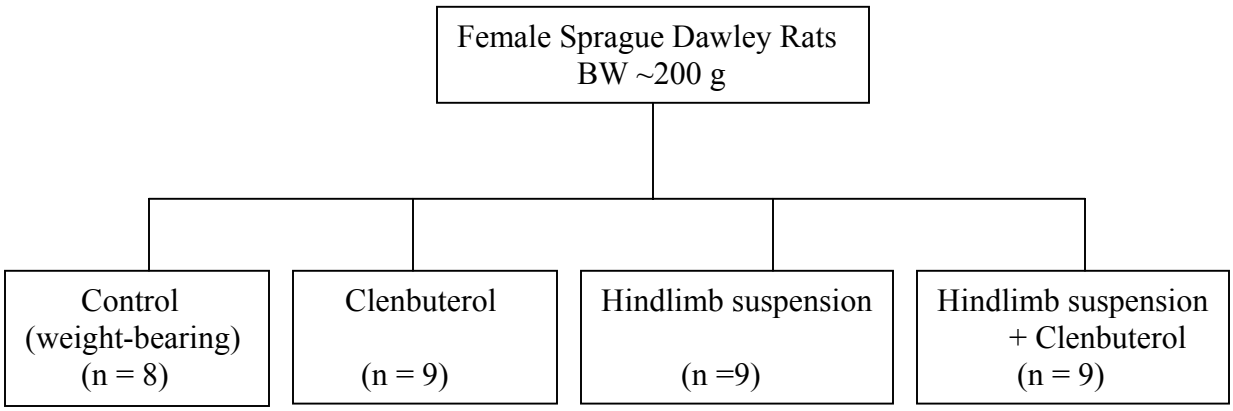

Figure 1. Experimental design.

Hindlimb Unweighting

The animals undergoing hindlimb unweighting each received the following preparation. The tails of the rats were wrapped with an orthopedic tape and connected to a swivel for suspension similar to the procedure described by Roer and Dillaman (1994) for 14 days. The rats were suspended from the top of a cage such that the hindlimbs are 1-2 cm above the cage floor, and have complete (360^0) range of motion by use of the forelimbs. This affords the animals free range of motion around the floor of the cage, but prevents climbing on the sides of the cage.

Clenbuterol Treatment

CB-treated animals were supplemented with clenbuterol (30 mg/L, Sigma, St. Louis, MO, U.S.A.) in their drinking water for 14 days, and the water intake was recorded every the other day. The average daily of clenbuterol intake of each animal was 0.9 mg/day during the entire experimental period. This drug concentration has been shown to be effective in promoting maximal growth of several types of muscles (Stevens et al. 2000; Lynce et al. 1996). The drug was routinely replaced every three days to prevent oxidation. This manner of administering the drug was chosen 1) because it enables us to avoid manipulation of the HU animals to prevent stress and 2) because its absorption by this route has been demonstrated to be as effective as injection.

Post-Experimental Data Collection

In Vitro Study of Protein Degradation

At the end of the treatment, all animals were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg). The soleus muscle was carefully dissected with intact tendon and mounted on plexiglass supports at resting length and immediately placed in a vial containing Krebs-Henseleit bicarbonate buffer, pH 7.4, 10 Mm glucose,

0.2 mM valine, 0.17 mM leucine, 0.1 mM isoleucine, and saturated with 95%O_2/5%CO_2. After a 1 h incubation period at 37°C in a shaking water bath, the muscle was transferred to a fresh medium containing 0.5 mM cycloheximide and incubated for 2 h. This concentration of cycloheximide has been shown to inhibit 95% of protein synthesis without having an effect on protein degradation in incubated muscle (Tischler et al., 1982). After incubation, the muscle was blotted, weighted, and frozen in liquid nitrogen and stored at -80°C for further analysis. The incubation media will be stored at -20°C until analyzed. The soleus muscle was selected for total in vitro protein degradation because of its small size and previous studies of HU in rats reported this muscle to be most affected by hindlimb unweighting.

Tissue Removal

At the end of the treatment period, animals were anesthetized with pentobarbital (60 mg/kg, IP) and the soleus (SOL), plantaris (PA), and tibialis anterior (TA) muscles are removed, weighed, and frozen for subsequent analysis.

Preparation of Myofibrillar and Cytosolic Fractions

A portion (~100 mg) of frozen muscle were minced and homogenized in 10 volumes of ice-cold buffer (50 mM Tris-HCl pH 8.0, 1 mM EGTA, 1 mM EDTA, 10 μM E-64, 1 μM pepstatin A, 1 mM PMSF, 10% glycerol) using a ground base glass homoginizer (Kontes). The homogenate was first centrifuged at 1,500g for 10 min at 4°C to pellet myofibrillar proteins, which were then washed three times in the same buffer containing 1% Triton X-100. Myofibrillar proteins were then resuspended in 8 M Urea, 50 mM Tris-HCl, at pH 7.5. The cytosolic fraction was prepared by centrifugation at

10,000 g for 10 min and then at 100,000g (4°C) for 1 h. The resulting supernatant (cytosolic fraction) was stored at -80° C for further analysis.

Biochemical Analysis

Measurement of tyrosine release

The rate of protein degradation was assayed by measuring tyrosine level in the media using a fluorometric method as previously described (Waalkes and Undenfriend, 1957). Briefly, the frozen incubation media was thawed and tricarboxilic acid (TCA) added to precipitated proteins. After centrifugation (800×g, 4C°, 10 min), 750 µl of the supernatant was transferred to a tube containing 750 µl of 5% TCA, 750 µl of 1% nitrosonapthol (w/v), and 750 µl of 20 % nitric acid containing 2.5% of NaN_2 (w/v). The sample was incubated at 55°C in a water bath for 30 minutes, and then a 7.5 ml of dichloroethane was added. After centrifugation (800 × g, 10 min, 4°C), the sample was allowed to cool down at room temperature and the content of tyrosine in the supernatant was determined by spectrofluorometry at excitation and emission wavelength 450 and 550, respectively. The rate of tyrosine release was expressed as nmol/g muscle weight/2h.

Protein concentration determination

The amount of protein in the homogenate and myofibrillar and cytosolic fractions was determined in duplicate by the Lowry method using the Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Proteasome activity assay

The trypsin-like and chymotrypsin-like activities of the proteasome were measured using 100 µM Suc-LLVY-AMC and 100 µM Boc-LRR-AMC, respectively, as

previously described by Stein et al. (1996) with some modifications. The enzymatic activity was determined in triplicate by incubating 10 μg of protein (cytosolic fraction) with 50 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, and 1 mM DTT, in the total volume of 200 μl, in the presence or absence of 10 mM lactacystin (BostonBiochem, USA). The proteolytic activity was monitored continuously by the release of the fluorescent group 7-amido-4-methyl-coumarin (AMC) with a temperature-controlled microplate fluorometric reader (SpectraMax-GeminiXS, Molecular Devices) during 30 min at 37° C using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The standard curve was established using a free-AMC. The difference between peptidase activities in the reaction medium, with and without proteasome inhibitor, is calculated and the data were expressed as nmol/min/μg protein. In our preliminary study, lactacystin caused a significant suppression (>90%) of the total trypsin and chymotrypsin activities if the cytosolic activity was based on proteasome function.

Immunoblotting analysis

Quantification of 20Sproteasome α- and β-subunits, 14kDa E2, and the amount of conjugated ubiquitin were determined by Western blotting. Aliquot of proteins from cytosolic (for 20S proteasome and 14kDa E2) and myofibrillar (for ubiquitin conjugates) were separated on a 4-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membrane (0.45 μm, Millipore, Bedford, MA, USA). The membranes were blocked overnight at 4°C in TBS-T (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat dry milk. The membranes were incubated with primary antibodies for 2 h at room temperature. The membranes were then washed in TBS-T buffer for 3 x 10 min and incubated for 1 h at

room temperature with secondary antibodies (anti-mouse or rabbit IgG conjugated to horseradish peroxidase, 1:5,000, Amersham, UK). After washing in TBS-T, the blots were exposed to the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, UK) for 1 min and to X-ray film for 2 min. Density of Bands were scanned and analyzed using NIH Image 1.6 software. Samples from all four groups were electrophoresed on the same gel and the data were expressed as a percentage of control value. The following antibodies were used for immunoblotting: a mouse antibody to 20S proteasome α- and β-subunits (1:1,000, Calbiochem, CA), and ubiquitin (1:100, SantaCruz Biotechnology, CA), and a rabbit polyclonal to E2-14kDa (1:1,000, BostonBiochem, MA).

Skeletal muscle IGF-I content

IGF-1 peptide was measured using radioimmunoassay as previously described (Fan et al., 2001). Briefly, IGF-1 was extracted from frozen plantaris and tibialis muscles in acid/methanol solution. Aliquots (~150 μl) of the supernatant were freezing dried and resuspended in assay buffer, and assayed using [^{125}I] [N-Met^{-}] human IGF-1 and IGF-1 anti-sera obtained from the National Institutes of Health. Bound and free [^{125}I]-IGF-1 were separated by incubation with goat anti-rabbit IgG and normal rabbit serum. IGF-1 peptide is expressed as ng/g muscle wet weight.

Statistical Analysis

All values were reported as means ± S.E.M. Otherwise stated, a two-way analysis of variance (ANOVA) followed by a Newman-Keuls test post-hoc was used for statistical analysis. Statistical significance was set at $p<0.05$.

CHAPTER 4
RESULTS

Effects of Hindlimb Unweighting and Clenbuterol on Body and Muscle Weights

There was no significant difference in initial body weights among groups. At the end of the experiment, body weight was significantly increased by 8.5% in CON animals and by 19 and 6.8% in CB and HU+CB animals, respectively, whereas the body weight of HU animals was decreased by 5.2% compared with the initial body weight. As expected, soleus, plantaris, and tibialis anterior muscle weights were significantly reduced (46.3, 24.2, and 11.8%, respectively) in HU animals after 14 days of unweighting compared with CON (Table 1). Clenbuterol treatment, however, resulted in an increase in muscle weight in all three muscles. The absolute muscle weight was increased by 35.5% in the TA, and by 28.3 and 17.4% in the PA and SOL muscle, respectively. Additionally, treatment of HU animals with CB attenuated ($p<0.05$) the loss of muscle weight associated with unweighting in both the PA and TA muscle; however, this inhibition was not observed in the SOL muscle. The magnitude of HU-induced loss of muscle mass in PA and TA changed from –24.2 and -11.8% to –9.1 and +13.1%, respectively, following 14 d of CB treatment. Similar results were obtained for changes in muscle weight/body weight ratio, with HU significantly reduced muscle weight/ body weight in both SOL and PA as compared with CON, while the ratio of muscle weight/body weight in TA muscle was unaffected. Clenbuterol treatment increased the muscle weight/body weight ratio in both PA and TA muscle; however, such change was not detected in the SOL muscle. Clenbuterol treatment of HU animals

significantly improved the muscle weight/body weight ratio only in the TA but not in the PA and SOL muscle (Table 1).

Table 1. Effects of hindlimb unweighting and clenbuterol on body weight (BW), muscle weight (MW), and muscle weight per body weight ratio (MW/BW).

	CON n=8	HU n=9	CB n=9	HU+CB n=9
Initial body weight(g)	226.6 ± 2.3	227.0 ± 2.4	226.0 ± 2.4	230.0 ± 2.6
Final body weight(g)	247.0 ± 3.2$^{\#}$	215.0 ± 2.5*$^{,\#}$	269.0 ± 2.3*$^{,\#}$	246.5 ± 5.5$^{\dagger,\#}$
Soleus muscle				
MW(mg)	108.3 ± 3.3	58.3 ± 1.8*	127.2 ± 4.0*	60.8 ± 2.4*
MW/BW(mg/g)	0.43 ± 0.01	0.26 ± 0.07*	0.46 ± 0.04	0.24 ± 0.0*
Plantaris muscle				
MW(mg)	305.1± 5.9	231.2 ± 5.6*	391.5 ± 5.5*	277.2 ± 13.6*,†
MW/BW(mg/g)	1.23 ± 0.01	1.06 ± 0.02*	1.45 ± 0.01*	1.12 ± 0.04*
Tibialis anterior muscle				
MW(mg)	499.7 ± 12.1	440.4 ± 11.5*	677.2± 6.2*	565.5 ± 7.5*,†
MW/BW(mg/g)	2.02 ± 0.04	2.04 ± 0.04	2.51 ± 0.07*	2.28 ± 0.0*,†

Values are mean ± S.E.M; n, number of animals/group; CON, control animals; HU, hindlimb-unweighted animals; CB, clenbuterol-treated animals; HU+CB, hindlimb-unweighted and clenbuterol-treated animals; * Significantly difference from CON ($p<0.05$); †, Significantly difference from HU ($p<0.05$); # Significantly from the initial body weight ($p<0.05$).

Effects of Hindlimb Unweighting and Clenbuterol on Total and Myofibrillar Protein Content and Concentration

Changes in total and myofibrillar protein content and concentration in soleus, plantaris, and tibialis anterior are presented in Table 2. Hindlimb unweighting caused a significant decline ($P<0.05$) in total and myofibrillar protein contents in all three muscles compared with CON. This reduction in protein content was higher in the SOL (-53%)

than in the PA (-29.9%) and TA (-20%) muscles. Clenbuterol treatment significantly increased ($p<0.05$) total and myofibrillar protein contents in all three muscles when compared to CON. Total and myofibrillar protein contents of the TA muscle were increased by 45.2% and 49.1 %, respectively. The percent increase was, however, relatively smaller for the PA (+30.2 and +30.3%, respectively), and for the SOL muscle (+33.3 and +30.1%, respectively). Treatment of HU animals with CB prevented the HU-induced reduce in total and myofibrillar protein contents in both the PA and TA muscle, respectively; however, this inhibition was not observed in the SOL muscle. Compared with CON, HU caused a significant reduction ($p<0.05$) in total and myofibrillar protein concentrations in both the SOL (23%) and PA (11%) muscle; however, such differences were not detected in the TA muscle. Clenbuterol treatment produced a significant increase in both total and myofibrillar protein concentrations in the TA, but not in the PA and SOL muscle. Treatment of HU animals with CB attenuated the HU-induced decrease in myofibrillar protein concentration in the PA, but had no additional effect on protein concentrations in either the SOL or the TA muscle.

Effects of Hindlimb Unweighting and Clenbuterol on Protein Degradation Rate

To determine whether a reduction in protein content observed in atrophied muscle was accompanied by increased protein degradation, an *in vitro* rate of tyrosine release was determined as a marker of total protein breakdown in the incubated muscle. The rate of protein degradation was analyzed only in the soleus muscle because of its small size and previous studies of HU in rats reported this muscle to be most affected by HU. Compared with CON, HU caused a significant increase (+69%) in protein degradation rate of the soleus muscle (Figure 2). However, CB treatment failed to attenuate the increased proteolysis observed in such muscle.

Table 2. Effects of hindlimb unweighting and clenbuterol on myofibrillar and total protein contents and concentrations.

Muscle	Group	Total protein mg	Total protein mg/g	Myofibrillar protein mg	Myofibrillar protein mg/g
SOL	CON	13.2 ± 0.7	135.7 ± 8.4	8.3 ± 0.3	86.9 ± 3.1
	HU	6.2 ± 0.2*	106.8 ± 3.9*	3.9 ± 0.2*	66.7 ± 2.8*
	CB	17.6 ± 1.6*	141.6 ± 9.9	10.8 ± 0.9*	87.1 ± 2.4
	HU+CB	6.3 ± 0.3*	106.2 ± 3.2*	4.1 ± 0.2*	67.2 ± 2.8*
PA	CON	44.7 ± 1.7	152.6 ± 4.5	34.3 ± 1.1	115.6 ± 2.7
	HU	31.2 ± 0.8*	134.4 ± 1.8*	24.1 ± 1.0*	104.2 ± 2.7*
	CB	58.2 ± 2.7*	148.6 ± 5.7	44.7 ± 1.5*	114.0 ± 3.4
	HU+CB	37.2 ± 1.8*,†	125.7 ± 3.0*	31.0 ± 1.8†	110.3 ± 3.5
TA	CON	81.4 ± 1.5	161.5 ± 3.4	54.5 ± 1.9	108.2 ± 3.4
	HU	66.6 ± 1.7*	151.6 ± 4.3	43.4 ± 2.7*	100.3 ± 4.7
	CB	118.2 ± 2.6*	172.4 ± 4.6*	81.3 ± 3.2*	120.0 ± 4.2*
	HU+CB	92.8 ± 6.0†	160.9 ± 5.9	63.0 ± 4.6†	110.0 ± 3.5

Values are mean ± S.E.M. (n=7-9/group); CON, control animals; HU, hindlimb-unloaded animals; CB, clenbuterol-treated animals; HU+CB, hindlimb-unloaded and clenbuterol-treated animals; SOL, soleus muscle; PA, plantaris muscle; TA, tibialis anterior muscle. * Significantly difference from CON ($p<0.05$); † Significantly difference from HU ($p<0.05$).

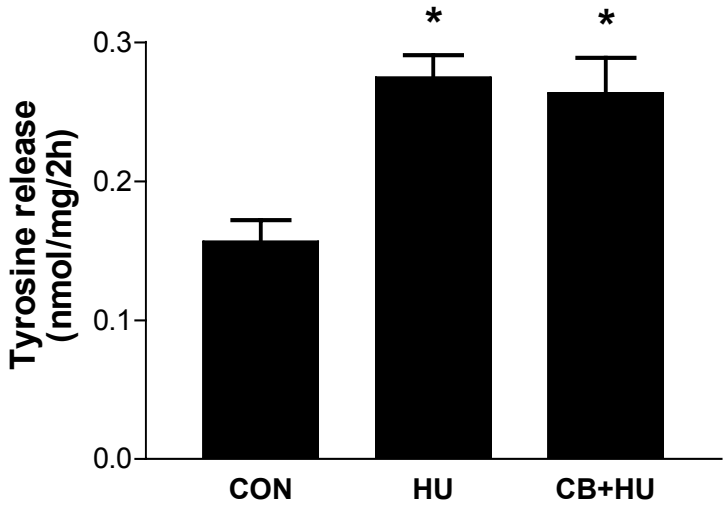

Figure 2. Effects of hindlimb unweighting and clenbuterol on protein degradation rates as measured by the rate of tyrosine release from the soleus muscle. Values are mean ± S.E.M. of 5-6 animals. Statistical difference was assessed using one way ANOVA. * significantly different from CON ($P<0.05$).

Effects of Hindlimb Unweighting and Clenbuterol on the Components of the Ubiquitin-Proteasome Pathway

To examine whether the HU-induced muscle atrophy was associated with the activation of the ubiquitin-proteasome pathway, we measured changes in activity and protein expression of the individual components of the ubiquitin-proteasome pathway in both fast- and slow-twitch muscles. In all muscles, HU induced a significant increase ($p<0.05$) in high-molecular weight (HMW) ubiquitin-conjugates, as quantified by immunoblotting, compared with CON (Figure 3.). The levels of HMW-ubiquitin-conjugates were increased by 255% in the SOL, and by 157%, and 135% in the PA and TA muscle, respectively. Clenbuterol treatment significantly reduced the amount of ubiquitin-conjugates in the PA and TA, but not in the SOL muscle. Also, treatment of HU animals with CB completely prevented or reversed the HU- induced increased accumulation of ubiquitin-conjugates in the PA and TA muscle. This inhibition was not detected in the SOL muscle.

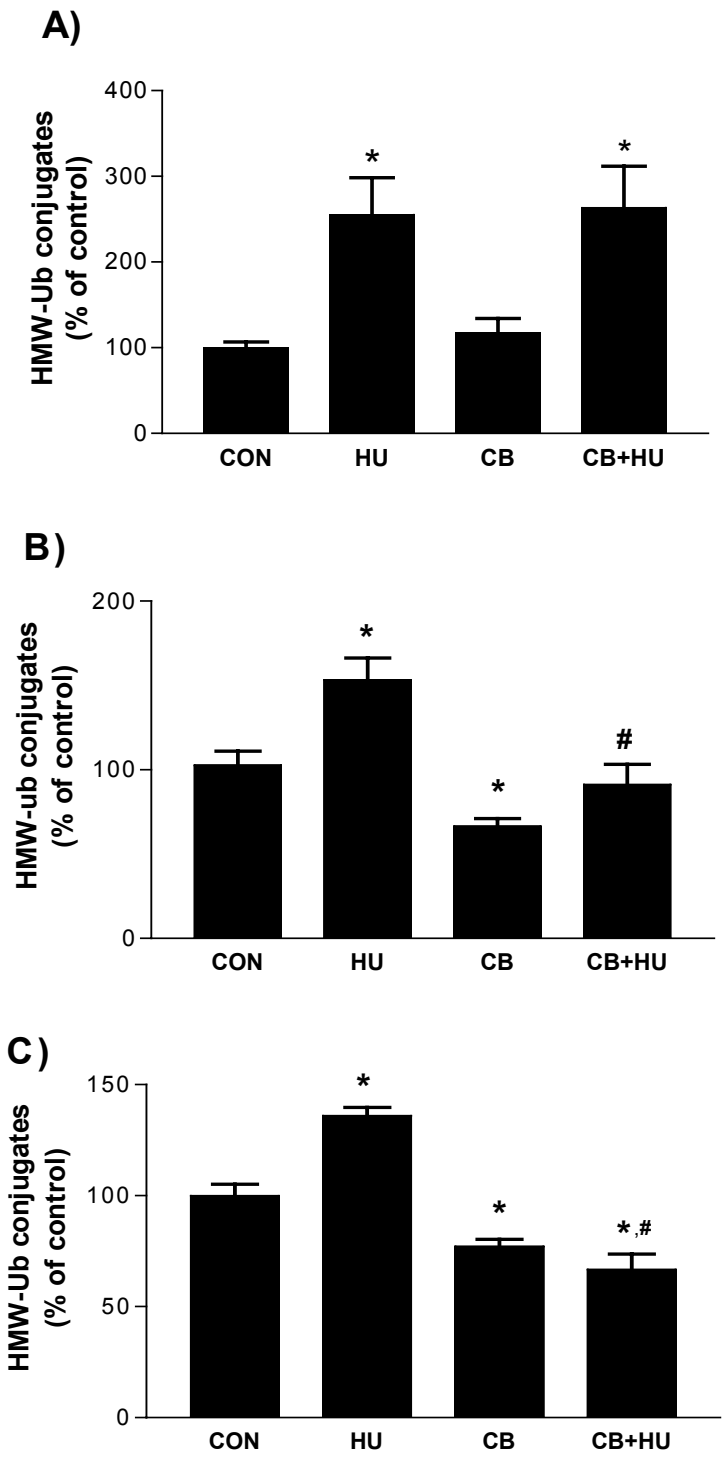

Figure 3. Effects of hindlimb unweighting and clenbuterol on the level of high-molecular weight (HMW) ubiquitin conjugates in soleus (A), plantaris (B), and tibialis anterior (C) muscles. Values are mean ± S.E.M. of 6-8 animals and are expressed as % of control animals. * significantly different from CON ($p<0.05$); # significantly different from HU ($p<0.05$).

We next determined whether the increased ubiquitin conjugation was paralleled by the increased expression of ubiquitin conjugating enzyme, i.e.14kDa E2, a rate determining- step of the ubiquitin pathway. Our results showed that HU caused a significant increase in expression level of ubiquitin conjugating 14kDa E2 protein in the PA (20%, $p<0.05$) and TA (31%, $p<0.05$), but not in the SOL muscle (20%, $P>0.05$) when compared to CON animals (Figure 4.). With respect to CON, CB treatment induced a significant increase ($p<0.05$) in the expression level of this enzyme in the TA, but not in the PA and SOL muscle. There were no differences between CON and CB+HU animals in expression level of 14kDa E2 protein in all three muscles.

Another marker of the ubiquitin proteasome pathway is the 26S proteasome, which contains a catalytic core (20S proteasome). In this study, the chymotrypsin-like (Ch-L) and trypsin-like (T-L) activities of the 20S proteasome were determined in crude muscle extract using fluorogenic substrates, Suc-LLVY-AMC and Boc-LRR-AMC, respectively. Our results showed that HU significantly increased ($p<0.05$) both Ch-L (+34%) and T-L (+32%) activity of 20S proteasome in the TA, but not in the PA and SOL muscles, when compared to CON (Figures 5 and 6). Clenbuterol treatment to CON animals had no effect on the activity level of 20S proteasome in all three different muscles. However, treatment of HU animals with CB effectively suppressed the HU-induced increased activity level of chymotrysin-like activity in both PA and TA muscles. The level of trypsin-like activity of HU animals was also significantly reduced in the TA muscle but not in the PA or SOL muscles following CB treatment.

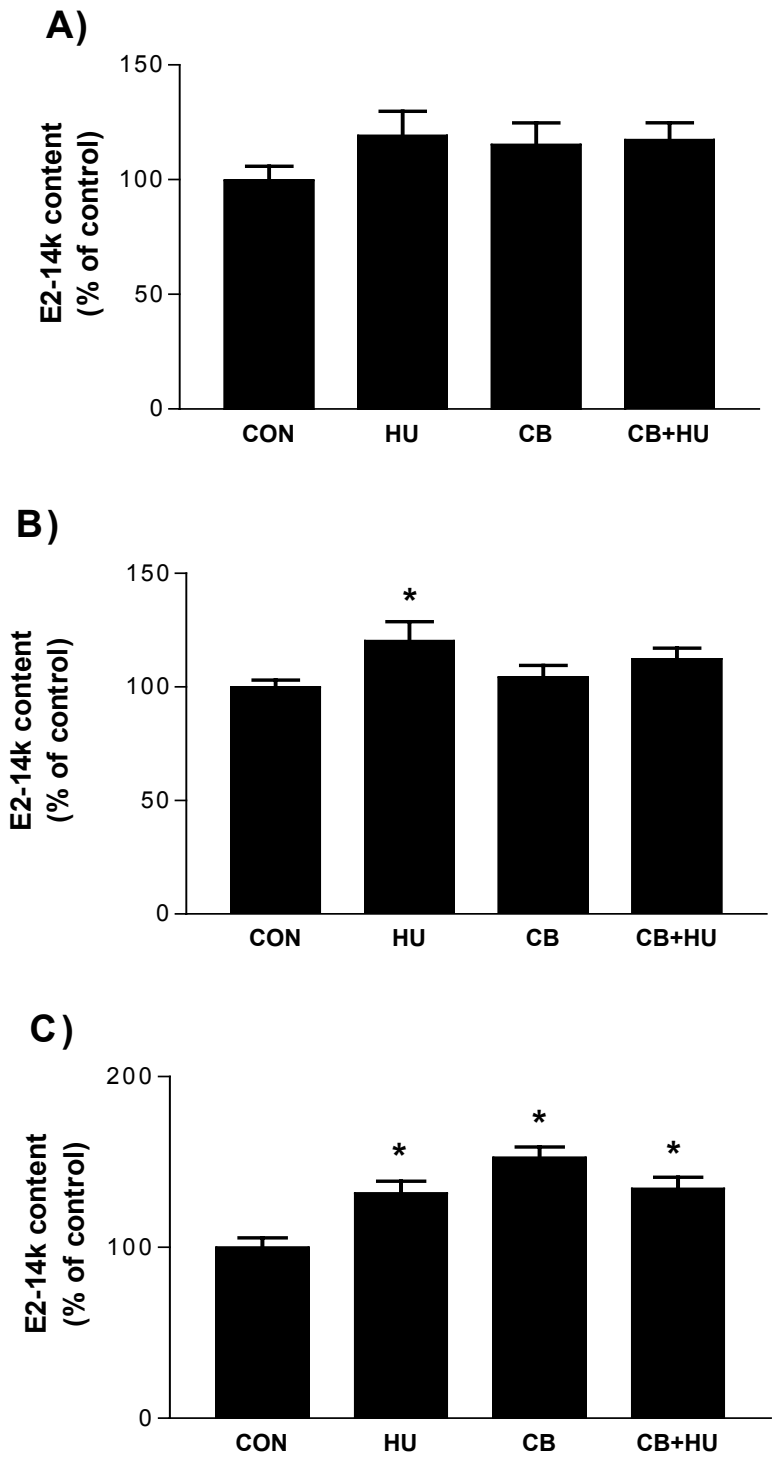

Figure 4. Effects of hindlimb unweighting and clenbuterol on the level of ubiquitin conjugating enzyme, E2-14K, in soleus (A), plantaris (B), and tibialis anterior (C) muscles. Values are mean ± S.E.M. of 6-8 animals and are expressed as % of control animals. * significantly different from CON ($p<0.05$).

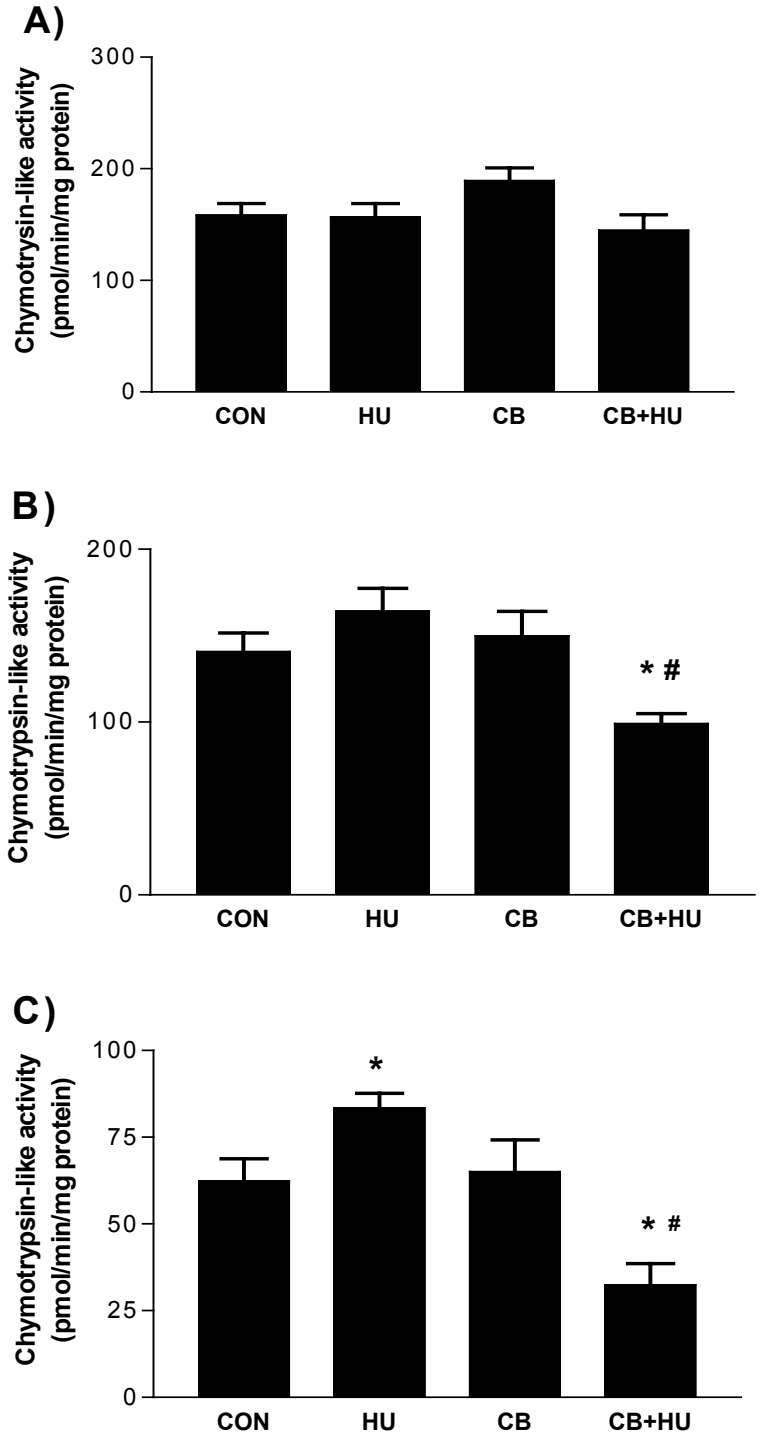

Figure 5. Effects of hindlimb unweighting and clenbuterol on the chymotrypsin-like activity of proteasome in soleus (A), plantaris (B), and tibialis anterior (C) muscles. Values are mean ± S.E.M. of 6-8 animals; * significantly different from CON ($p<0.05$); # significantly different from HU ($p<0.05$).

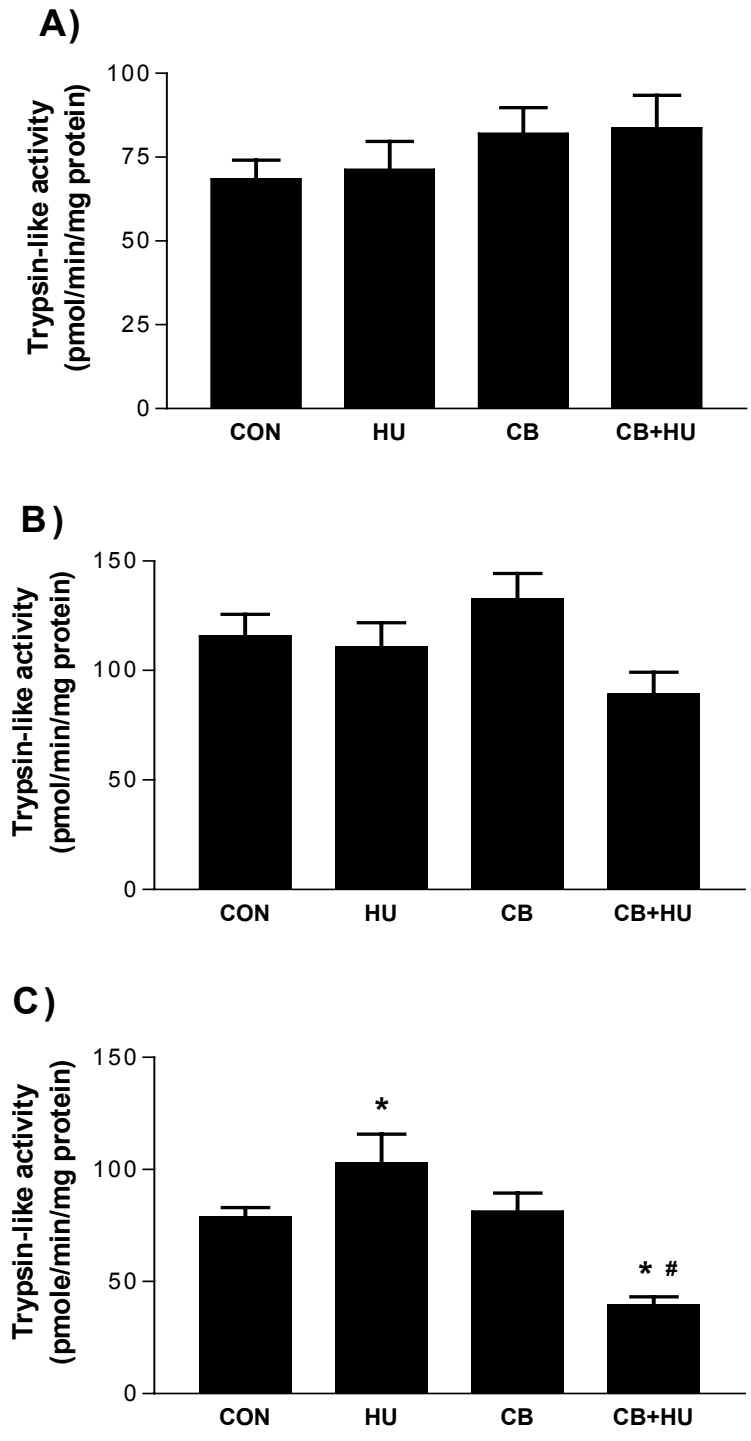

Figure 6. Effects of hindlimb unweighting and clenbuterol on the trypsin-like activity of proteasome in soleus (A), plantaris (B), and tibialis anterior (C) muscles. Values are mean ± S.E.M. of 6-8 animals. * significantly different from CON ($p<.05$); # significantly different from HU ($p<0.05$).

Figures 7 and 8 show the cellular expression of catalytic β- subunit and non-catalytic α-subunit of 20S proteasome in three different muscles in HU, CB, and CB+HU groups. In all muscles, there were no significant differences among groups in expression level of β-subunit of 20S proteasome. Neither HU nor CB treatments had a significant impact on the level of 20S proteasome α-subunit content in both PA and TA muscles compared with CON. However, the level of 20S proteasome α-subunit content of SOL muscle was significantly lower ($p<0.05$) in the HU than in the CON animals. Clenbuterol treatment to CON and/or HU animals did not affect the soleus 20S proteasome α-subunit content.

Effects of Hindlimb Unweighting and Clenbuterol on Skeletal Muscle IGF-1 Content

To further examine the mechanism underlying the induction of hypertrophy and /or the attenuation of HU-induced atrophy after CB treatment, skeletal muscle IGF-1 content was measured in this study in two different fast-twitch muscles. Figure 9 showed that HU cause a significant reduction in IGF-1 content in the PA, but not in TA muscle compared with the CON. Surprisingly, CB treatment produced a significant decline in the level of IGF-1 protein in the PA; however, such change was not observed in the TA muscle. Clenbuterol treatment to HU animals did not affect the IGF-1 content in both PA and TA muscles.

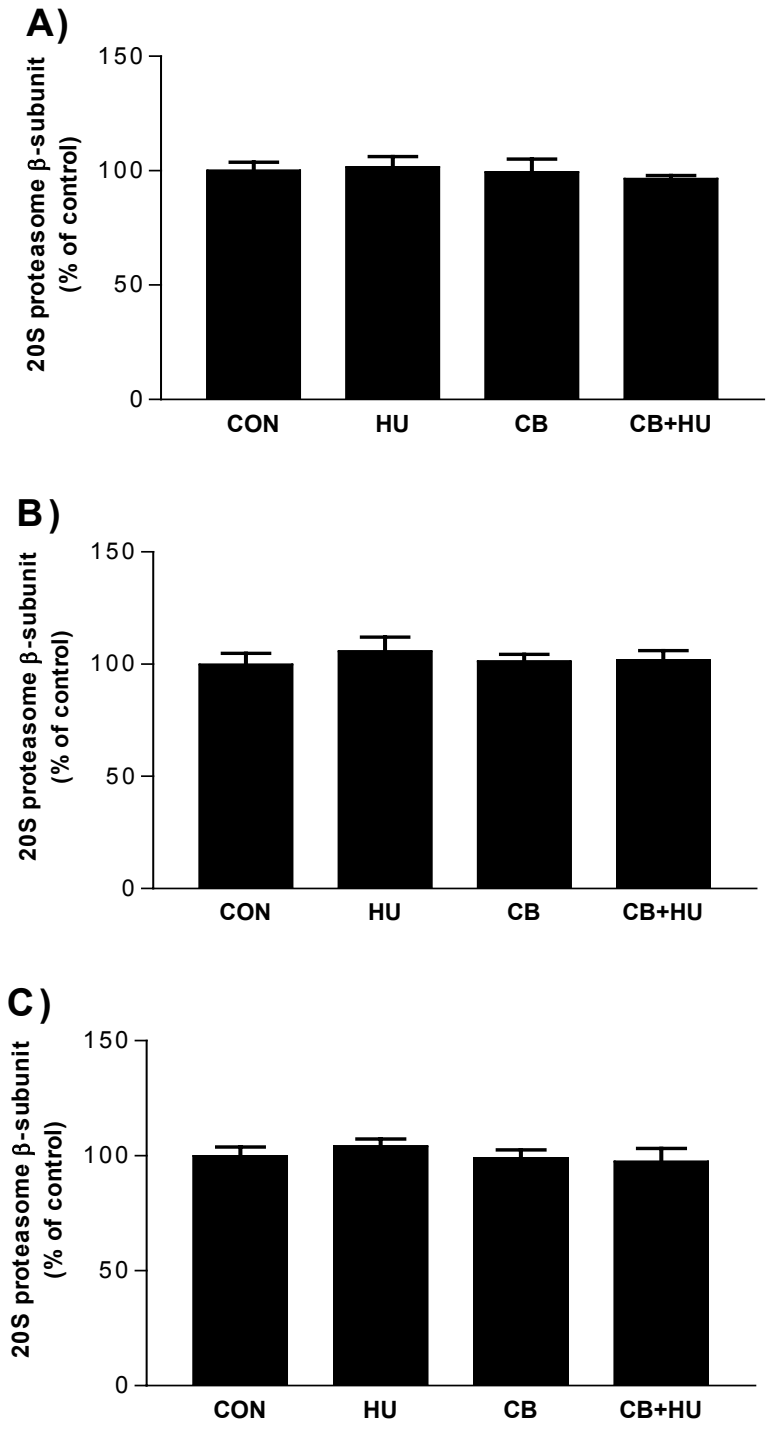

Figure 7. Effects of hindlimb unweighting and clenbuterol on 20S proteasome β-subunit content in soleus (A), plantaris (B), and tibialis anterior (C) muscles. Values are mean ± S.E.M. of 6-8 animals and are expressed as % of control animals.

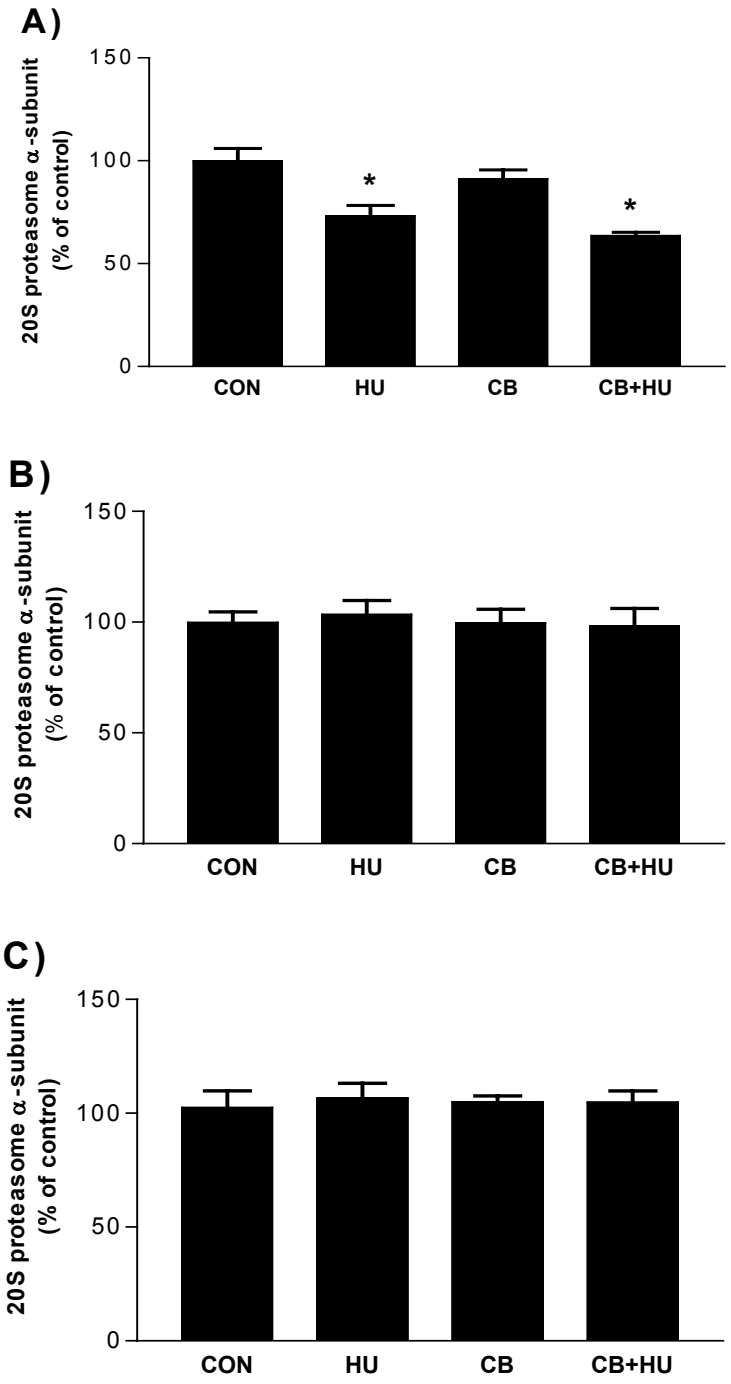

Figure 8. Effects of hindlimb unweighting and clenbuterol on 20S proteasome α-subunit content in soleus (A), plantaris (B), and tibialis anterior (C) muscles. Values are mean ± S.E.M. of 6-8 animals and are expressed as % of control animals; * significantly different from CON ($p<0.05$).

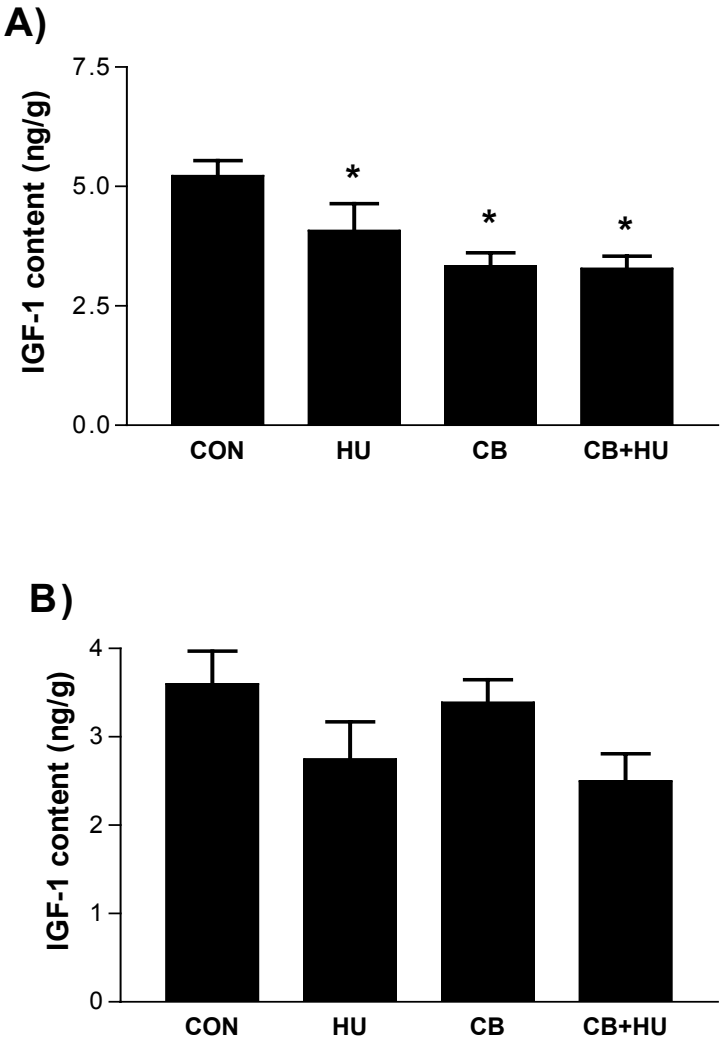

Figure 9. Effects of hindlimb unweighting and clenbuterol on muscle IGF-1 content in plantaris (A), and tibialis anterior (B) muscles. Values are mean ± S.E.M. of 4-6 animals; * significantly different from CON ($p<0.05$).

CHAPTER 5
DISCUSSION

Overview of Major Findings

The major findings of this study were that HU induced atrophy of all three muscles, with the extent of atrophy greater in the predominantly slow-twitch soleus. These changes were associated with increased protein expression of several components of the ubiquitin-proteasome proteolytic pathway. Clenbuterol treatment not only induced muscle hypertrophy but also attenuated or reversed the HU-induced atrophic responses, possibly through an inhibition of the ubiquitin-proteasome system in a fiber-type specific fashion. All of these effects did not correlate with an alteration in muscle IGF-1 content. A detailed discussion and interpretation of these data follows.

Hindlimb Unweighting Effects on Muscle Weight and Protein Contents

Consistent with previous studies (Riley et al. 1990; Agbenyega et al. 1990; Apseloff et al. 1993; Wineski et al. 2000), the present experiment demonstrated that HU resulted in a significant reduction in muscle weight in both slow- and fast-twitch muscles. This atrophic effect was more pronounced in the slow soleus muscle than in the fast PA and TA muscles. This reduced muscle weight was associated with a reduction in total and myofibrillar protein contents in these muscles. Because the myofibrillar proteins constitute approximately 75% of total protein content in the muscle (Wineski et al. 2001), the protein loss observed during unweighting was more likely due to the loss in myofibrillar proteins. The precise mechanism responsible for HU-induced muscle atrophy is not well understood. However, it is now apparent that this loss of protein

could be due to a decreased rate of protein synthesis and an increased rate of protein degradation. In the current study, the protein degradation rates of unweighted soleus were increased by~60% after 14 d of HU and were in the range of other studies (Jasper et al. 1988; Taillandier et al. 1996). Of interest was the finding that CB treatment did not attenuate this increase. Since the rate of protein synthesis was not measured in the present study, the relative contribution of decreased protein synthesis to HU-induced muscle atrophy could not be quantified. Nonetheless, our findings strongly support the hypotheses that HU induced muscle atrophy is due, in part, to an increased rate of protein degradation.

Clenbuterol Effects on Muscle Weight and Protein Contents

In this study, we have demonstrated that CB alone induced hypertrophy of all three muscles examined; however, the magnitude of effect was greater in the fast-twitch TA and PA muscles than in a slow soleus muscle. This change in muscle weight was accompanied by a marked increase in protein contents (e.g. total and myofibrillar proteins) in all muscles. These findings were in agreement with previous studies (Maltin et al. 1986; Zeman et al. 1988) showing CB had a preferential effect on the predominantly fast-twitch muscle, while it had no or minimal effect on the predominantly slow-twitch muscle. Moreover, our data demonstrated that CB treatment effectively attenuated or reversed the HU-induced loss of muscle mass and protein contents in both fast-twitch muscles, with the slow soleus muscle being unaffected. This finding was consistent with the finding of others (Zeman et al. 1988; Wineski et al. 2000; Von Deutsch et al. 2001; Stevens et al. 2000).

Despite these known effects of β2-agonists on skeletal muscle, the mechanism(s) through which this compound exerted such effects on skeletal muscle remains unclear. Changes in muscle mass may be due to an increased rate of protein synthesis or a reduced rate of protein degradation. Recently, however, there has been increasing evidence from both *in vivo* and *in vitro* experiments that these anabolic effects of clenbuterol treatment are mainly due to its ability to inhibit protein degradation (Reeds et al. 1986; Forsberg et al. 1989; Benson et al. 1991; Navegantes et al. 2002).

Hindlimb Unweighting Activates the Ubiquitin-Proteasome Pathway of Fast- and Slow-Twitch Muscles

Like other cells, skeletal muscle contains several distinct proteolytic pathways. These include the lysosomal, calcium-dependent, and ubiquitin-proteasome system. Of the three pathways, the ubiquitin-proteasome is a critical system responsible for the majority of intracellular protein breakdown including myofibrillar proteins (Solomon et al. 1996; Attaix et al. 1998; Jagoe and Goldberg, 2001). There are two major steps in the ubiquitin-proteasome pathway: 1) ubiquitin conjugation that involves the 14 kDa E2 and E3-α, and 2) the subsequent degradation of ubiquitin-conjugates by the 26S proteasome. Our data showed that HU-induced muscle atrophy was associated with the increased accumulation of ubiquitinated proteins and expression level of 14kDa E2 protein in all three muscles. These results are consistent with previous reports showing a significant increase of the level of ubiquitinated proteins in atrophied muscle in association with enhanced muscle proteolysis in several catabolic states including starvation, denervation (Wing et al. 1995), burn-injury (Solomon et al. 2000), and hindlimb suspension (Ikemoto et al. 2001). These findings strongly support the involvement of the ubiquitin-

proteasome proteolytic pathway in the development of muscle wasting during unweighting.

Central to this pathway is the degradation of ubiquitinated proteins by the 26S proteasome, which contains a catalytic core 20S proteasome. Unfortunately, we did not observe any significant alteration in either the activity or expression level of the 20S proteasome β-subunits in the soleus muscle. This is unexpected since a number of studies reported that HU resulted in increased gene expression of several components of the ubiquitin-proteasome system, including several subunits of the 20S proteasome (Taillandier et al. 1996; Ikemoto et al. 2001, Stevenson et al. 2003). The reason for the lack of the coordinated changes between mRNA and protein levels is not known in the present study. Nevertheless, it appears that the increased mRNA level of 20S proteasome observed in these muscles may not necessary reflect the changes in the activity and protein levels under unweighting condition. Therefore, the interpretation of these data, based on only the indirect measurement of mRNAs, should be viewed with caution. In the present study, we also demonstrated that the expression level of non-catalytic α-subunit was markedly reduced in soleus muscle following HU compared with the control. The mechanism causing this reduction, though intriguing, is not known, but it may reflect differential regulation of expression of distinct α- and β- subunits of the 20S proteasome as a result of hindlimb unweighting. Clearly, an additional study is needed to determine the site (s) of regulation of this proteolytic pathway during unweighting condition.

In contrast to the response observed in soleus muscle, our finding that the activity of the 20S proteasome in the fast-twitch TA was elevated following HU, suggesting differential response in the activation of the ubiquitin-proteasome system in different

muscle type. This finding was supported by the results of several studies showing that the fast twitch-muscle being more sensitive to catabolism than those in the slow twitch-muscle in a wide variety of muscle wasting conditions including hyperinsulinemia (Larbaud et al. 2001), endotoxemia (Chai et al. 2003), and burn injury (Fang et al. 1998). Since the expression level of 20S proteasome α- and β-subunits in these muscles were not changed during unweighting, thus the increased proteasome activity found in this study is likely resulted from changes in the specificity of 20 S proteasome. The activity of 20S proteasome can be activated by a PA28 activator (Coux et al. 1996). However, this is not the case because our results showed that the increased activity of 20S proteasome did not correlate with changes in the amount of PA28 in the crude muscle extract from HU animals (unpublished data). Another possibility is that the activity of 20S proteasome may be modulated by post-translational modifications such as phosphorylation (Mason et al. 1996; Bose et al. 1999). Mason et al. (1996) showed that certain proteasome subunits (C8 and C9) can be phosphorylated in vivo and dephosphorylation of these subunits results in small but significant decreases in the peptidase activity of 20S proteasome.

Of interest, our data demonstrated that the plantaris muscle exhibited a pattern of responses similar to those observed in the soleus with respect to the activation of the ubiquitin-proteasome system (i.e. no alterations in either the activity or expression level of proteasome subunits content). This is unexpected given that the plantaris contains mainly the fast-twitch fibers, whereas the soleus mainly consists of slow-twitch fibers. The mechanism(s) underlying these similar effects is not known but it could be related to the function of muscle. Both the soleus and plantaris are plantar-flexors and their

function is to plantarflex the ankle. During hindlimb unloading, the hind limb experiences a plantar-flexed posture that resulted in shortening these muscles, whereas lengthening the TA (Riley et al. 1990). This may affect the adaptive response of skeletal muscles caused by hindlimb unweighting. Collectively, these data suggest that the activation of the ubiquitin-proteasome system during unweighting is not only fiber type specific but also muscle specific. Further studies are required to better understand the regulation of this proteolytic system at the molecular level in different muscle phenotype.

Clenbuterol Inhibits the Ubiquitin-Proteasome Proteolysis in the Fast-Twitch Muscles of Control Rats

In this study, we have demonstrated that CB alone did not inhibit the activity and expression of key proteins involved in the ubiquitin-proteasome system in soleus muscles from the control animals. This is not unexpected given that CB treatment has a preferential effect on fast-twitch muscle and CB exerts its anabolic effects largely through reduced protein degradation as previously described (Reeds et al. 1986; Benson et al. 1991; Navegantes et al. 2002). However, since the soleus muscle weight and protein contents found in this study were elevated following CB treatment, this suggests that protein synthesis rate was also increased and may account for increased protein accretion observed in soleus muscle under this condition. On the other hand, the finding that the level of ubiquitin conjugates from myofibrillar fraction was significantly reduced in both TA and PA following CB treatment as compared with the control, indicating that CB preferentially inhibits the ubiquitin-proteasome pathway in the fast-twitch muscles. Interestingly, since CB did not affect the activity and protein levels of 20S proteasome subunits in these muscles, this suggests that CB had a specific inhibitory effect on the ubiquitin conjugation. This finding is in keeping with a previous report that CB

treatment reduced the ubiquitin mRNAs expression in the control animals (Costelli et al. 1995). As above, the present study did not allow us to calculate the relative contribution between the increased protein synthesis and decreased protein degradation to CB-induced hypertrophy of these muscles. Nevertheless, recent studies (Reed et al. 1986; Costelli et al. 1995) have shown that, in normal control rats, rates of protein degradation in a fast-twitch muscle e.g. gastrocnemius were markedly reduced, while protein synthesis rates were not affected after CB treatment. Taken together, these findings suggest that CB exerts its hypertrophic effect, at least in part, by reducing muscle proteolysis through an inhibition of the ubiquitin-proteasome pathway in the fast-twitch PA and TA, but not in the slow-twitch soleus muscle.

Clenbuterol Attenuates the Upregulation of Ubiquitin-Proteasome Proteolysis in the Fast-Twitch Skeletal Muscles of Hindlimb-Unweighted Rats

Beta-agonists such as CB and cimaterol have been shown to increase muscle mass (Yang and McElligot, 1989) and to reduce muscle atrophy induced by denervation (Zeman et al. 1987) and hindlimb suspension (Dodd and Koesterer, 2002; Deutsch et al. 2001; Wineski et al. 2002). However, the precise mechanism (s) by which this compound mediates its anabolic and anti-atrophy effects remain unclear. In the present study, we have demonstrated that CB treatment effectively attenuated the HU-induced increased activity of 20S proteasome and the accumulation of ubiquitin conjugates in the myofibrillar fraction in both the fast-twitch muscles, with the magnitude of effect greater in the TA than the PA muscle. Moreover, it should be noted that the magnitude of these measures was below the basal control level. These data clearly indicate that CB treatment attenuates the HU-induced muscle atrophy through suppressing the activation of the ubiquitin-proteasome pathway in the fast-twitch muscle. This finding is in keeping

with a previous study in which the mRNAs level for ubiquitin was significantly reduced in the tumor-bearing animals, another animal model of muscle wasting, after CB treatment (Costelli et al. 1995). Interestingly, however, the finding that the expression level of 14 kDa E2 protein, a potential rate-limiting step of the ubiquitin pathway, in these unweighted muscles was not affected by CB, and in fact the 14kDa E_2 content remained elevated in the TA muscle, suggests that the activity and expression level of 14kDa E_2 protein is not crucial for the activation of the ubiquitin-proteasome pathway during unweighting. This finding was supported by a recent study (Adegoke et al. 2002) showing that mice-lacking 14kDa E_2 gene did not prevent muscle atrophy as compared with wild-type animal in response to fasting. More recently, two novel skeletal muscle-specific ubiquitin ligases i.e. muscle RING finger 1 (Murf1) and muscle atrophy F-box (MAFbx), which are thought to be responsible for skeletal muscle atrophy, have been identified (Bodine et al. 2001; Gomes et al. 2001). These proteins are upregulated in several models of disuse including immobilization, denervation and hindlimb unweighting. In addition, knockout mice lacking these proteins show a significant reduction in the loss of muscle mass caused by denervation. It is not known whether CB treatment does affect the expression level of these E3 ligases under these conditions. On the other hand, we also demonstrated that CB treatment of HU animals unsuccessfully inhibited the protein markers for the activation of ubiquitin-proteasome system and the accelerated muscle proteolysis in the soleus muscle (Figure 2). Taken together, these results suggest that CB treatment attenuates muscle atrophy associated with unweighting, possibly through a downregulation of the ubiquitin-proteasome proteolysis in the fast-twitch muscle only.

IGF-1 as a Potential Mediator for β2-agonist Action

The intracellular mechanism by which the β2-agonist clenbuterol mediates its anabolic and anti-atrophy effects remains unclear. β2-agonists presumably exert their action by binding to β2 receptors and activating the synthesis of cyclic adenosine monophosphate (cAMP) (Barnes, 1995; Yang and McElligot, 1989). However, the possibility still exists that β2-agonists may act indirectly leading to an increase in the production of other factors and hormones that cause muscle hypertrophy. Of various growth-stimulating factors, IGF-1 is known to play a role not only in myogenesis but also in the regulation of postnatal skeletal muscle growth (Florini et al. 1991, 1996). Interestingly, there is recent evidence that CB-induced hypertrophy is associated with muscle IGF-1 content (Awede et al. 2002). Also, hindlimb unweighting has been associated with a lowering of IGF-1 expression in skeletal muscle (Awede et al. 1999). Therefore, we hypothesized that CB treatment would increase IGF-1 expression and provide a protection against the HU- induced atrophic responses in skeletal muscle. In the present study, we demonstrate that hindlimb unweighting significantly reduces muscle IGF-1 content in the PA but not in the TA. This finding was consistent with the findings of others (Awede et al. 1999; Suliman et al. 1999) and supports our hypothesis. The reason for differential expression of IGF-1 peptide between the two muscles, however, is not known but it may reflect the differential regulation of IGF-1 protein synthesis between muscles. Although the induction of muscle hypertrophy by CB could be due to an increase in muscle IGF-1 content, as previously reported (Awede et al. 2002), our current data did not reflect this possibility, as CB treatment for 14 days indeed reduced the PA muscle IGF-1 content compared with the control. Nevertheless, this

finding was not inconsistent with a previous report (Awede et al. 2002) that IGF-1 peptide in the soleus muscle was significantly increased at 3 days after CB treatment; however, the level returned to baseline at 9 days. Moreover, our results showed that treatment of HU animals with CB had no additional effect on IGF-expression in both muscles. These data indicate that a local production of IGF-1 is not directly involved in stimulating skeletal muscle growth caused by CB. Based on these finding, we suggest that the hypertrophic and anti-atrophy effects of β2-agonist clenbuterol are probably not mediated by an alteration in muscle IGF-1 peptide. However, the early response to this drug could not be excluded.

Summary and conclusions

In the present study, we demonstrated that HU-induced muscle atrophy is associated with the increased activity of the ubiquitin-proteasome system in both slow-twitch and fast-twitch muscle, although with a different degree. CB treatment attenuated these effects by reducing the activation of ubiquitin –proteasome proteolysis in the predominantly fast-twitch PA and TA muscle, but not in the slow-twitch soleus muscle. This anti-atrophy effect of CB is probably not mediated by IGF-1 peptide in skeletal muscle.

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BIOGRAPHICAL SKETCH

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