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HUMAN MUSCLE DISUSE ATROPHY AFTER 28 DAYS OF IMMOBILIZATION IN A LOWER-LIMB WALKING BOOT: A CASE STUDY

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ABSTRACT

HUMAN MUSCLE DISUSE ATROPHY AFTER 28 DAYS OF IMMOBILIZATION IN A LOWER-LIMB WALKING BOOT: A CASE STUDY. **Darryn S. Willoughby, Shawn Sultemeire, Melanie Brown**. **JEP**online 2003;6(2):88-95. This case study determined the effects of 28 days of lower limb immobilization on muscle atrophy and the changes in myofibrillar protein and DNA content, heat shock protein 72 (HSP-72), heat shock factor 1 (HSF-1), ubiquitin (UBI), ubiquitin conjugating enzyme (E2), ubiquitin 20S proteasome (20S), and myostatin mRNA and protein expression. One male immobilized the left gastrocnemius muscle in a passively shortened position by way of a lower limb walking boot. The boot was only removed every 7 days for measurements of lower limb girth. Gastrocnemius biopsies were obtained on day 1 and 28. Results showed decreases in lower limb girth, myofibrillar protein, and DNA content of ~16%, ~25%, and ~66%, respectively. Caspase-3 activity underwent an increase of ~582%. Myostatin mRNA and protein expression increased ~125% and ~71%, respectively. The mRNA expression of UBI, E2, and 20S mRNA expression increased ~96%, ~81%, and ~87%, respectively, while respective increases of ~131%, ~166%, and ~169% for UBI, E2, and 20S protein expression were observed. Respective increases of ~100% and ~79% were observed for HSP-72 mRNA and protein while HSF-1 increased ~110%. These results indicate that 28 days of muscle immobilization and disuse resulted in decreases in myofibrillar protein and subsequent muscle atrophy, likely a result of the proteolytic effects of apoptosis, elevated myostatin expression, and increased activity of the ubiquitin proteolytic pathway.

Key words: muscle atrophy, disuse, gene expression, proteolysis

INTRODUCTION

Skeletal muscle atrophy occurs as a consequence of immobilization and disuse (i.e., sarcopenia). The debilitating effects of muscle disuse due to immobilizing conditions such as those following orthopedic surgical procedures, musculoskeletal injury, bed rest, and aging can occur as a result of alterations to the contractile machinery and contractility of skeletal muscle (1,2), thereby negatively impacting muscle strength and function. The balance between muscle protein synthesis and degradation has a direct effect on the content (accretion) of

myofibrillar protein. Consequently, sarcopenia is known to occur as a result of muscle proteolysis and the subsequent degradation of myofibrillar protein (2,3). Several biochemical and molecular mechanisms such as apoptosis (DNA degradation), growth differentiating factor 8 (myostatin), and proteolysis mediated by the ubiquitin pathway have been implicated as playing a role in sarcopenia whereas heat shock protein-72 apparently attempts to eradicate muscle atrophy.

The ATP-dependent ubiquitin proteolytic pathway has been shown to be associated with muscle proteolysis that accompanies immobilization (4,5). During immobilization, protein denaturation and degradation through the ubiquitin proteolytic pathway plays a critical role in the cellular processes involved in myofibrillar degradation and muscle atrophy (5,6,7). The selection of myofibrillar proteins for ubiquitin-related degradation is mediated by signals predicated on post-translational mechanisms (4,8). In this pathway, multiple molecules of ubiquitin (UBI) are covalently linked to lysine residues on the denatured proteins, thereby "tagging" (poly-ubiquinating) the proteins with a ubiquitin-conjugating enzyme (E2), and then degraded by the catalytic 20S proteasome (5,9).

Muscle proteolysis has also been shown to increase the expression of the heat shock protein 72 (HSP-72), a molecular chaperone that seems to have an anti-proteolytic and anti-apoptotic effect by preventing protein degradation and/or instigating the repair of damaged proteins by facilitating re-folding and assembly (1). This stress-induced protein seems to be involved in preventing aggregation of denatured proteins and facilitates the refolding of denatured proteins (10,11,12). Therefore, it has been suggested that HSP-72 may play an essential role in maintaining myofibrillar integrity during periods of muscle disuse atrophy (10,11,13).

Growth differentiating factor-8 (i.e., myostatin) is a member of the transforming growth factor-β superfamily. It has been shown to act as negative regulator of muscle growth by inhibiting myoblast proliferation (14). When expressed in high levels, myostatin causes proteolysis and muscle atrophy (14,15) and has also been shown to be specifically associated with the atrophy of Type IIa and IIb (15) muscle fibers and the Type IIb myosin heavy chain isoform (16). The expression of myostatin mRNA in muscle fibers expressing more of the fast (Type IIa and IIb) phenotype may operate by way of a negative feedback mechanism to inhibit satellite cell proliferation, thereby inhibiting increases in muscle fiber size (16).

Apoptosis is known to play a pivotal role in the cell loss and atrophy of skeletal muscle during disuse (17). Apoptosis is known to occur as a result of the induction of the caspase cascade of proteases, in particular, increases in the activity of caspase-3 (18). Caspase activation leads to reorganization of the cytoskeleton, impairs DNA replication and repair, degrades DNA, disrupts the nuclear structure, and disintegrates the cell into apoptotic bodies, eventually destroying the cell (18).

Herein, we present a descriptive analysis of changes that occur in various mechanisms known to play a role in atrophy in human skeletal muscle in response to 28 days of disuse. The purpose of this case study was to examine the extent of selective muscle disuse atrophy that occurred in healthy skeletal muscle when voluntarily subjected to immobilization by way of passive shortening while, in all other respects, the subject maintained an active lifestyle. An additional purpose was to observe the pre- and post-translational behavior of the various atrophy mechanisms known to be play a role in sarcopenia.

METHODS

Participant

One apparently healthy male volunteer (24 years of age, 180.34 cm tall, and 72.72 kg body mass) completed a university-approved informed consent form, a medical history screening, and an exercise history questionnaire. The study was approved by the Institutional Review Board for the use of Humans in Research. The subject was briefed on the study's purpose, procedures, and possible risks prior to testing. The subject was instructed to

maintain a normal mixed diet and, within reason, to carry out his normal routine of daily activities. The study conformed to the ethical considerations of the Declaration of Helsinki.

Experimental Design

The subject of this study had his left gastrocnemius muscle immobilized in a passively shortened position using a lower-limb walking boot (Donjoy, Ultra-4 ROM Walker, Medco Sports Medicine, Tonawanda, NY) by stabilizing the talocrural joint at an angle of 120° of plantar flexion. The angle of the talocrural joint was verified through goniometric measurement prior to placing the lower limb into the walking boot and the corresponding angle of the boot adjusted accordingly. The immobilization boot was worn for a total of 28 days with muscle biopsies taken from the left gastrocnemius prior to and after immobilization on days 1 and 28, respectively. The cast was only taken off for measurements of lower limb girth.

Lower Limb Girth

Prior to placing the subject in the walking boot on day 1 and once the boot was finally removed on day 28, a measurement was taken of the girth of the subject's lower limb. Upon measurement on day 1, an indelible mark was made on the skin to indicate the location for the four subsequent measurements. Each measurement was made to the nearest 0.2 cm at the point of largest girth of the gastrocnemius. Three measurements were taken at each measurement period and the average was recorded. Also, at days 7, 14, and 21 the boot was briefly removed while the subject was in a supine position, the girth measurements were made, the appropriate angle of the talocrural joint determined by goniometric measurement, and the boot was then immediately replaced.

Muscle Biopsies

Percutaneous muscle biopsies (30-50 mg) were obtained from the subject on days 1 and 28. Muscle samples were taken from the mid-portion of the lateral head of the left gastrocnemius muscle. To ensure adequate sample sizes, the biopsies were obtained using a double-chop method combined with suction. The muscle samples were immediately frozen in liquid nitrogen and stored at –70°C until further analysis.

Total RNA and DNA Isolation

Total cellular RNA was extracted from the homogenate of biopsy samples with a monophasic solution of phenol and guanidine isothiocyanate (19) contained within the TRI-reagent (Sigma Chemical Co., St. Louis, MO). The RNA concentration was determined by optical density (OD) at 260 nm (by using an OD_{260} equivalent to 40 μg/mL), and the final concentration was adjusted to 1 ng/mL (20-23). Aliquots (5 μl) of total RNA samples were then separated with 1% agarose gel electrophoresis, ethidium bromide stained, and monitored under an ultraviolet light to verify RNA integrity and absence of RNA degradation. This procedure yielded non-degraded RNA, free of DNA and proteins, as indicated by prominent 28s and 18s ribosomal RNA bands, as well as an OD_{260}/OD_{280} ratio of approximately 2.0 (20-24). The RNA samples were stored at -70^{o}C until further analyses.

DNA was extracted from the organic phase remaining from the total RNA isolation procedure using ethanol, 0.1 M sodium citrate, and 8 mM sodium hydroxide. The DNA concentration was determined at an OD of 260 nm (by using an OD_{260} equivalent to 50 μg/mL), and the final concentration expressed relative to muscle wet-weight (24).

Reverse Transcription and cDNA Synthesis

Two hundred ng of total skeletal muscle RNA was reverse transcribed to synthesize cDNA based on previously described guidelines (20-23). A 20 μl reverse transcription (RT) reaction mixture was incubated at 42°C for 60 min, heated to 95°C for 10 min, and then quick-chilled on ice. Prior to the polymerase chain reaction procedure (PCR), template concentrations for each sample were standardized by adjusting the RT reactions for all samples to 200 ng, thereby ensuring amplification efficiency (20-23).

Oligonucleotide Primers for PCR

The following 5' sense and 3' antisense oligonucleotide primers were used to isolate HSP-72 mRNA (5' primer: bases 1801-1821, 3' primer: bases 2302-2282, GenEMBL AC M11717), UBI mRNA (5' primer: bases 435-455, 3' primer: bases 638-618, GenEMBL AC M26880), E2 mRNA (5' primer: bases 74-94, 3' primer: bases 555-575, GenEMBL AC U39318), 20S mRNA (5' primer: bases 305-325, 3' primer: bases 778-798, GenEMBL

AC D00759), and myostatin mRNA (5' primer: bases 1419-1439, 3' primer: bases 1898-1918, GenEMBL AF 104922). We have recently shown these primers to amplify PCR fragments of 500, 204, 494, 507, and 500 bp for HSP-72, UBI, E2, 20S, and myostatin, respectively (22).

Due to its consideration as being a constitutively expressed "housekeeping gene," glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an external reference standard for detecting the relative change in the quantity of HSP-72, UBI, E2, 20S, and myostatin mRNA using PCR. For GAPDH mRNA (5' primer: bases 616-636, 3' primer: bases 1189-1169, GenEMBL AC NM 002046), we have previously shown these primers to amplify a PCR fragment of 574 bp (20-23).

PCR Amplification

Two hundred ng of cDNA were added to each of the six 25 μl target PCR reactions for GAPDH, HSP-72, UBI, E2, 20S, and myostatin. A seventh absolute negative control reaction without cDNA was constructed to demonstrate the specificity of the PCR. The cocktail for the PCR reaction was established based on previous guidelines (20-23). To control for amplification efficiency variations, all PCR reactions were prepared from the same stock solution. The amplification profile involved a denaturation step at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and extension at 72°C for 60 sec. Thirty cycles were used to optimize the amplified signal on the linear portion of a plot, with the yield expressed as a function of the absorbance at OD_{260} and the number of cycles for the relative control standard and the other five target amplifications. To assess reliability between amplifications, two separate PCR amplifications were performed for each sample to control for systemic differences between samples that could affect amplification efficiencies.

mRNA Quantitation

Aliquots (20 μl) of the purified PCR reaction mixtures were electrophoresed in 1.5% agarose gels in 1X Tris-Acetate-EDTA (TAE) buffer to verify positive amplification of HSP-72, UBI, E2, 20S, and myostatin mRNA. The oligonucleotides within the gels were stained with ethidium bromide (present in the TAE buffer at 1 ng/ml) and illuminated with a UV trans-illuminator. Aliquots of each remaining purified PCR reaction were used to quantify mRNA spectrophotometrically at a wavelength of OD_{260} (21-24). The mRNA content was calculated and normalized relative to GAPDH based on a statistical method previously established (25). It should be noted, however, that this method of PCR quantitation determines relative mRNA concentration only and should not be interpreted as absolute concentration values.

Protein Quantitation

The protein contents of HSP-72, heat shock factor-1 (HSF-1), UBI, E2, 20S, and myostatin were determined with an enzyme-linked immunoabsorbent assay (22) using primary antibodies for anti-HSP-72, anti-HSF-1, anti-UBI, (Stressgen Biotechnologies, Victoria, BC, Canada), anti-myostatin (Santa Cruz Biotech, Santa Cruz, CA), anti-E2, and anti-20S (Affiniti Research Products, Mamhead, UK). The anti-UBI antibody has a high affinity for poly-ubiquinated proteins. The anti-20S antibody has a high affinity for the α and β subunits comprising the 20S proteasome. The secondary antibody (ICN Biomedical, Costa Mesa, CA) involved immunoglobulin-G (IgG) conjugated to the enzyme horseradish peroxidase. The control peptide used for the UBI assay (Sigma, St. Louis, MO) was UBI isolated from bovine erythrocytes. The control peptide for HSP-72, and HSF-1 (StressGen Biotech, Victoria, BC, Canada) was from heat-shocked human cervical adrenocarcinoma (HeLa) cells. The control peptide was myostatin isolated from bovine skeletal muscle (Santa Cruz Biotech, Santa Cruz, CA). All assays were run in duplicate and the protein concentrations were determined at an optical density of 450 nm with a microplate reader (Bio Rad, Hercules, CA). Protein concentrations were expressed relative to muscle wet-weight.

Myofibrillar Protein Quantitation

Total protein remaining from the total RNA isolation procedure was isolated with isopropanol, ethanol, and 0.3 M guanidine hydrochloride. Myofibrillar protein was further isolated with 1% SDS (20,22,23). Myofibrillar protein content was then determined spectrophotometrically based on the Bradford method (26) at a wavelength of 595 nm, using bovine serum albumin as the standard, and expressed relative to muscle wet-weight. All assays were performed in duplicate and the average concentrations reported.

Caspase-3 Activity

As an indicator of apoptosis, caspase-3 content (pmol), the relative change in fluorescence units [ΔFU (change in fluorescence units/min)], and caspase-3 enzyme specific activity [ESA (pmol AMC liberated/min/µg protein)] were determined with the CaspACE™ fluorometric assay system (Promega, Madison, WI) based on the manufacturer's guidelines. The assay was performed in duplicate with a fluorometer (VersaFluor, BioRad, Hercules, CA) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

RESULTS

After 28 days of immobilization, lower limb girth was shown to decrease by ~16% (Figure 1). In addition, a decrease in myofibrillar protein content of ~25% was observed (Figure 3). Myostatin mRNA and protein expression increased ~125% and ~71%, respectively (Figures 2 & 3). The content of DNA decreased by ~66%, whereas for apoptotic caspase-3 activity, the caspase-3 protein content, ΔFU, and ESA were shown to undergo ~70%, ~610%, and ~582% increases, respectively (Table 1). For components of the ubiquitin proteolytic pathway, UBI, E2, and 20S mRNA expression increased

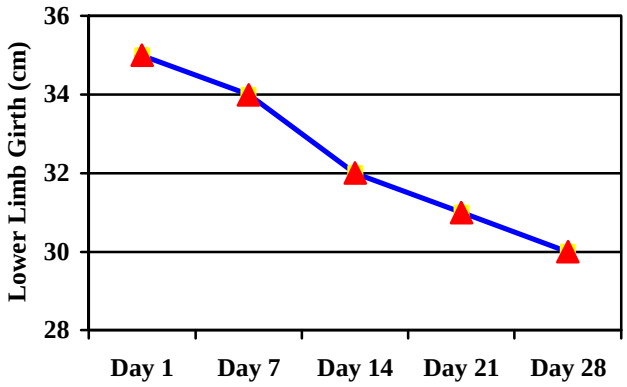

Figure 1. Lower limb girth (cm) at days 1, 7, 14, 21, and 28.

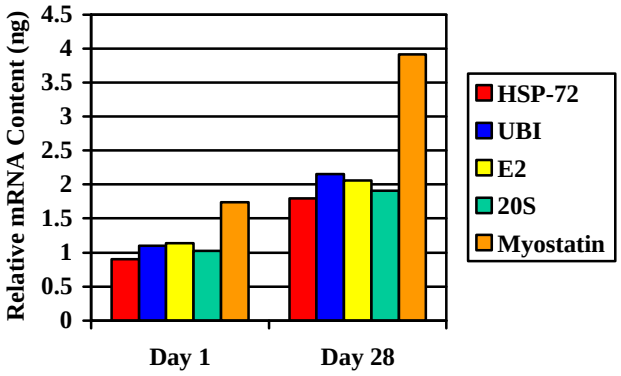

Figure 2. Relative mRNA content (ng) at day 1 and 28 for HSP-72, UBI, E2, 20S, and myostatin.

Table 1. Subject data at Day 1 and 28, and the percent change over the four-week period for caspase-3 activity.

Variable	*Day 1*	*Day 28*	*%D*
Caspase-3 (content)	95.49	141.27	70.01
Caspase-3 (DFU)	0.49	3.48	610.20
Caspase-3 (ESA)	9.41^{-6}	6.42^{-5}	582.22

caspase-3 content (pmol); caspase-3 ΔFU (change in fluorescence units/min);caspase-3 enzyme specific activity (pmol AMC liberated/min/µg protein)

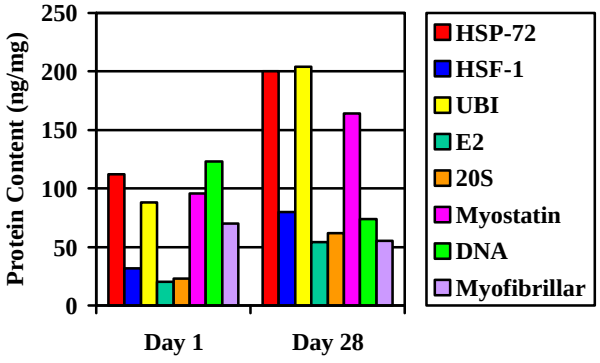

Figure 3. DNA content and protein content (mg/mg) for HSP-72, HSF-1,UBI, E2, 20S, and myostatin.

~96%, ~81%, and ~87%, respectively, while respective increases of ~131%, ~166%, and ~169% for UBI, E2, and 20S protein expression were observed (Figures 2 & 3). Respective increases of ~100% and ~79% occurred for HSP-72 mRNA and protein expression whereas HSF-1 increased ~110% (Figures 2 & 3).

DISCUSSION

A number of investigations have studied the response of skeletal muscle to immobilization and disuse under a variety of conditions (2,13,16,27). The present findings suggest the possible involvement of several atrophy

mechanisms in response to 28 days of muscle immobilization by way of passive shortening. Muscle immobilization causes the degradation of skeletal muscle fibers as a result of decreases in the myofibrillar protein concentration (1,2,3), thereby contributing to muscle atrophy.

It has been shown that the primary myofibrillar proteins degraded by the ubiquitin-proteolytic pathway during immobilization are the contractile and regulatory proteins such as myosin, actin, troponin, and tropomyosin (6,9). The magnitude of myofibrillar protein poly-ubiquination may be due to increased oxidative stress, which occurs during muscle atrophy (5,9). Studies have shown that oxidative stress modifies proteins thereby making them more susceptible to proteolysis (5,7,10). Muscle disuse is associated with increases in oxidative stress, thereby increasing proteolytic activity. The expression of HSP-72 may prevent or reduce the degradation of oxidatively modified proteins by binding to the protein and assisting in refolding (10,11,28). The mechanism mediating the induction of the HSP-72 gene is triggered by the activation and translocation of the transcription factor HSF-1, which is a DNA binding protein that initiates transcription of the HSP-72 gene following the induction of stress (29). It has been indicated that HSF-1 is activated by the accumulation of denatured and degraded proteins (4,30).

The present study suggests increases in apoptotic and proteolytic activity by way of increases in the activity of caspase-3 in addition to increases in ubiquitin and myostatin expression. Also, the mRNA and protein expression of HSP-72, as well as HSF-1 was also increased. Therefore, the increased HSF-1 activation and HSF-1 dependent HSP-72 transcription is likely attributed to the increased degradation of myofibrillar protein by way of apoptosis and proteolysis (28). It is conceivable that HSP-72 can play an essential role in the prevention of protein degradation following immobilization (13). Even though HSP-72 and HSF-1 expression were elevated in the present study, it is possible that any HSP-72-mediated anti-proteolytic effects were overshadowed by the overall increase in apoptosis, myostatin expression, and ubiquitin proteolysis. Even so, however, the collective function of HSP-72 is to maintain the integrity of cellular protein during proteolytic activity (10,13); therefore, the increased expression of HSP-72 with muscle atrophy observed in the present study may have served as a protective measure by the cell to reduce the overall amount of myofibrillar protein degradation occurring during muscle immobilization.

The present results also demonstrate an up-regulation in the mRNA and protein expression of myostatin in response to muscle disuse. It has previously been suggested that the mRNA expression of myostatin is a muscle-wasting factor in humans contributing to the atrophy of Type IIa and IIb muscle fibers after 5 days of disuse (15). Similarly, myostatin mRNA observed to be increased after 1, 3, and 7 days of hindlimb unloading in rodents and was also correlated to the selective expression of the Type IIb myosin heavy chain isoform (16). Myostatin has also been implicated as an attenuator of skeletal muscle growth in adult men and contributes to muscle wasting in HIV-infected males (31). Also, 10 days of hindlimb unloading in rodents resulted in a 16% decrease in muscle mass along with increases in myostatin mRNA and protein expression of 110% and 37%, respectively (32).

The present study demonstrated increases in the content and activity of the apoptotic protease caspase-3, along with decreases in DNA content. As such, it appears that the immobilization was contributing to apoptosis and muscle atrophy. During conditions of disuse, apoptosis is responsible for the elimination of myonuclei and subsequent muscle atrophy. Muscle disuse in humans (33) and rodents (34) has been shown to induce apoptosis through increases in caspase-3 and ubiquitin. As such, it appears that slow muscles are less prone to apoptosis than fast muscles and that the magnitude of myocyte apoptosis is associated with the severity of muscle disuse and the degree of muscle atrophy.

The myofibrillar protein degradation observed in the present study, following 28 days of immobilization, was likely a result of the combined increases in the activity of the ubiquitin-proteolytic pathway, apoptosis, and an

up-regulation in the expression of myostatin. All three mechanisms were possibly attenuated to some extent by the pre- and post-translation increases in the expression of HSP-72. The muscle atrophy accompanying disuse indicates that the loss of myofibrillar protein during immobilization is due to decreased myofibrillar protein synthesis, increased muscle degradation, or some relative combination of the two (1,3). More research is necessary to determine the relative contribution and involvement of each of the particular mechanisms in sarcopenia.

Practical Implications

The results of this case study should be interpreted with caution since they only represent the responses of one subject. However, the results are interesting in that they descriptively illustrate various mechanistic changes that occur in skeletal muscle during muscle immobilization/disuse while the subject maintained a relatively active lifestyle. Therefore, the design of the current study is unlike classic muscle disuse models employing either bed rest for humans or hind limb unloading in rodents where essentially all other forms of muscular activity are curtailed. Our results show, however, that even if activity levels are not curtailed, a marked amount of atrophy still occurs in the immobilized muscle. Therefore, these results may provide insight into the atrophy mechanisms likely involved during periods of muscle immobilization and disuse that typically occur post-injury (e.g., ankle and knee ligament sprains) and/or post-operatively (e.g., anterior cruciate ligament reconstruction) in athletes and non-athletes alike.

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