Pretranslational Markers of Contractile Protein Expression in Human Skeletal Muscle: Effect of Limb Unloading Plus Resistance Exercise

F. Haddad¹, K. M. Baldwin¹ and P.A. Tesch²

¹Department of Physiology and Biophysics
University of California, Irvine
Irvine, CA 92697

²Department of Physiology and Pharmacology
Karolinska Institute
Stockholm, Sweden

Running Title: Molecular Markers of Unloaded Human Muscle

Send Correspondence To:

Dr. F. Haddad
Department of Physiology and Biophysics
University of California, Irvine
Irvine, CA 92697
Phone: (949) 824-4289
Fax: (949) 824-8540
Email: fhaddad@uci.edu
ABSTRACT

Previously it has been shown that the human ground based model consisting of unilateral limb suspension (ULLS) induces atrophy and reduced strength of the affected quadriceps muscle group. Resistance exercise (RE) involving concentric-eccentric actions, in the face of ULLS, is effective in ameliorating these deficits. The goal of the present study was to determine if alterations in contractile protein gene expression, e.g., myosin heavy chain (MHC) and actin, as studied at the pre-translational level, provide molecular markers concerning the deficits that occur in muscle mass/volume during ULLS, as well as its maintenance in response to ULLS plus RE. Muscle biopsies were obtained from m. vastus lateralis of 31 middle-aged men and women before and after five weeks of ULLS, ULLS plus RE or RE only. The RE paradigm comprised 12 sessions of four sets of seven concentric-eccentric knee extensions. Our findings show that there were net deficits in total RNA, total mRNA, and actin and MHC mRNA levels of expression following ULLS (P< 0.05); whereas, these alterations were blunted in the two groups receiving RE. Additional observations involving IGF-I and its associated receptor and binding proteins suggest that RE postures the skeletal muscle for signaling processes favoring a greater anabolic state relative to that observed in the ULLS group. Collectively these findings suggest that molecular markers of contractile protein gene expression serve as useful subcellular indicators for ascertaining the underlying mechanisms regulating alterations in muscle mass in human subjects in response to altered loading states.

Key words: muscle atrophy; mRNA, myosin, actin; insulin like growth factor-I
INTRODUCTION

Previous studies clearly show that skeletal muscle of human and of animal subjects undergoes adaptive changes in both fiber size and contractile phenotype in response to conditions of altered loading states. In studies on human subjects exposed to spaceflight and ground based analogues, e.g., bed rest, lower limb unloading of varying duration, it is well known that both muscle mass and voluntary strength are significantly reduced (7, 10, 13, 18, 19, 24, 25, 32-34). Additional studies suggest that slow-twitch fibers are transformed into hybrid fibers expressing both a fast and slow phenotype in response to chronic unloading (2). These changes occur even when various types of exercise intervention are imposed (7, 10, 13, 18, 19, 24, 25, 32-34). In contrast, when human subjects are challenged with exercise paradigms of the heavy resistance type, the opposite adaptive changes occur, e.g., muscle fibers enlarge (31, 35) and the phenotype is shifted to a slower profile (6, 31). Interestingly, the same adaptive patterns as seen in humans also occur when small animals (e.g., rodents) are exposed to either a state of unloading (including spaceflight) or chronic functional overload and/or resistance training (reviewed by Baldwin and Haddad (11, 12)).

In an attempt to ascertain the underlying mechanisms responsible for these changes in muscle structure and function, investigators have focused on a variety of cell and molecular markers in order to gain insight concerning the primary factors involved in these adaptive processes (17, 26). Recently, Haddad et al (28, 29) used an animal model of near complete muscle inactivation/unloading, referred to as spinal isolation (SI), in order to dissect the subcellular events that are involved in the marked atrophy that occurs
in this powerful model. These investigators demonstrated that factors affecting transcriptional, pre-translational, translational, and post-translational processes were operative thereby reducing total myosin heavy chain (MHC) and actin mRNA and protein levels; and these alterations were centrally involved in the rapid muscle wasting process (28, 29). Additional observations indicated that total RNA concentration (the bulk of which is ribosomal, which normally provides the primary machinery for protein translation) was significantly reduced in the atrophying muscles, especially in the early phases. Interestingly, there was little evidence to suggest that gene expression of growth factors such as insulin-like growth factor-I (IGF-I) was down regulated to contribute to the catabolic state that defined the SI state. On the other hand, other studies focusing on the anabolic processes that define the hypertrophic response to muscle overload, also show that these same processes are impacted (3). However, under these latter conditions the responses are directionally affected to provide an anabolic state to augment protein accumulation in the muscle fibers (3).

Given the importance of a) maintaining muscle structural and functional homeostasis in the face of conditions that cause muscle deterioration such as during chronic states of muscle unloading, and b) preventing such a process via exercise of the resistance type, we conducted a study in which adult human subjects were exposed to the conditions of unilateral limb suspension (ULLS), a recognized model of inducing muscle atrophy (13, 31), ULLS plus resistance exercise (ULLS+RE), and RE alone in order to ascertain if some of the same cellular/molecular markers indicative of muscle mass homeostasis that are operative in animal models are also occurring in humans. Therefore, we tested the
working hypothesis that ULLS would induce reductions in MHC and actin mRNA expression as well as total RNA (ribosomal) concentration thereby negatively impacting protein translational capacity; whereas, resistance training (RT) of the concentric-eccentric type would blunt such alterations in the face of ULLS. The findings reported herein are largely consistent with this hypothesis. Moreover, the collective findings are in support of resistance training for space travelers, and further studies of molecular markers can be useful in deriving the mechanisms controlling atrophy and hypertrophy. Portions of this report have been reported elsewhere (36, 38).

METHODS

General Design.

Thirty-one men and women, recruited from the greater Little Rock, AR. area, volunteered for this study. Whereas twenty-one individuals were subjected to unilateral unloading of the lower limb (see below) for five weeks, ten (7 men; 3 women) of these subjects in addition, performed resistance exercise every third day using the unloaded limb, (ULLS+RE) throughout the five weeks experimental period for a total of 12 training sessions. The eleven subjects (7 men; 4 women), who performed unloading (ULLS) only, refrained from any physical activity beyond their basic needs throughout the study. Ten ambulatory subjects (7 men; 3 women) carried out resistance exercise only (RE) using the same exercise regimen followed by the ULLS+RE group. Prior to this, subjects participated in three sessions over two weeks to practice and become familiar with the different procedures, unloading, resistance training and strength tests. To establish baseline data, all subjects then performed strength tests one day prior to initiating the
different interventions. With the use of magnetic resonance imaging (MRI), the volume of knee extensor muscles was measured before and upon completion of the three different interventions. These measurements were always carried out before the muscle function tests. The resistance exercise program was completed about 72 hrs before any post measurement. Muscle biopsies from vastus lateralis (mVL) were obtained before and after any intervention immediately after the MRI’s and prior to reloading. Samples were obtained from both the right leg (which served as an independent control) and the left leg (serving as the experimental, e.g., ULLS, ULLS+RE, and RE intervention). Changes in muscle function and volume have been reported in detail elsewhere (36, 38).

Subjects.

Subjects were healthy men (N=21) and women (N=10), age 30-56 years (Table 1). At the time of being recruited the subjects did not participate in any regular training program. A medical examination excluded women who were on contraceptives or hormone replacement therapy, as well as individuals who were hypertensive or reported past or present knee pathology. Subjects were then matched by gender, body size, age and knee extensor strength, and were assigned to ULLS, ULLS+RE, or RE. All subjects complied with the prescribed unloading and resistance training protocols (see below).

A written consent was given after the procedures, risks and premises associated with participation in the study had been explained. The experimental protocol was approved by the Institutional Review Board at the University of Arkansas Program for Medical Sciences, Little Rock, AR. The study was conducted in accordance with the Declaration of Helsinki.
Unilateral Lower Limb Unloading.

Twenty-four hr a day unilateral unloading of the left lower limb was accomplished using the model introduced in our laboratory (13), and subsequently modified (30, 38). In brief, in performing any upright or ambulatory activity short-length crutches, aided by handgrip and forearm support distal to the elbow (Swereco Rehab AB, Sollentuna, Sweden) was used. The right foot was equipped with a shoe, which has a 10-cm thick sole, which removes weight bearing from the left unloaded limb.

As prophylaxis to venous thromboembolism, subjects received a daily dose of 325 mg Aspirin®. In addition, they wore knee length, compression stockings during any ambulatory activity. As reported elsewhere (38), three men assigned to the ULLS group, were admitted to ultrasound-doppler examinations due to acute leg pain. Clinical assessments did however exclude suspected deep venous thrombosis. Daily injections of low molecular weight heparin (Fragmin® 2500 IE) were then administered and the reported symptoms subsided. All subjects completed the study without any disruption.

Resistance Exercise.

Unilateral resistance exercise, using the left limb, was performed at regular 3-day intervals spanning twelve exercise sessions. A flywheel resistance exercise system (14) constructed for the seated knee extension (YoYo® Technology Inc., Stockholm, Sweden) and a protocol, described in detail elsewhere (36), was employed. In brief, following a warm up, each session consisted of four sets of seven maximal, coupled concentric and
eccentric knee extensions from approximately a 90° knee joint angle to almost full extension. An electrogoniometer, affixed onto and aligned with the rotational center of the machine lever arm, and a miniature compression load cell (Model 276A, K-toyo, Seoul, Korea) measured joint angle and force during each exercise session. Subjects received verbal encouragement and, by producing force and joint angle signals on a PC display, instant performance feedback was provided during exercise. An A/D converting system (MuscleLab™, Ergotest AS, Langesund, Norway), which was interfaced to the PC, integrated these signals and logged performance during training.

_Percutaneous Muscle Biopsies._

Tissue samples were obtained from the lateral aspect of the right and left vastii muscle using the percutaneous needle biopsy technique (15) before and after any intervention and before subjects resumed weight bearing. Samples were immediately frozen and subsequently stored in liquid nitrogen until analyzed.

_Molecular Analyses:_

_Total RNA Isolation._ Total RNA was extracted from pre-weighed frozen muscle samples using the TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the company’s protocol, which is based on the method described by Chomczynski (20). Extracted RNA was precipitated from the aqueous phase with isopropanol, and after washing with ethanol, dried and suspended in a known volume of nuclease-free water. The RNA concentration was determined by optical density at 260 nm (using an OD 260 unit equivalent to 40 µg/ml). The muscle total RNA concentration was calculated based
on total RNA yield and the weight of the analyzed sample. The RNA samples were stored frozen at -80°C and were used subsequently in determining total mRNA (polyA), total MHC mRNA, and α skeletal actin mRNA expression using slot blotting procedures, and IGF-I, IGF-I receptor, and IGF-I binding protein 4 mRNAs using RT-PCR methods (see below).

**RNA Slot Blotting.** One microgram of total RNA was placed in 20 µl of denaturing buffer (10% formaldehyde, 67% formamide, and 0.5xMOPS pH 7) at 60°C for 15 min. Samples were brought up to 100 µl volume with 6 x SSC and were applied onto a positively charged nylon membrane (GeneScreen plus, NEN) using a slot blot apparatus (Schleisher and Schuell). Each sample was loaded in duplicates on two separate membranes. After UV fixation, these membranes were hybridized with 3 different probes consecutively as follows. 1) An antisense α-skeletal actin mRNA probe was used to determine α-skeletal actin mRNA expression, or a common antisense MHC mRNA probe was used to determine the total MHC mRNA expression. The MHC probe is complementary to the coding region ~500 nucleotides upstream from the stop codon of type I MHC mRNA. This region is 100% identical in all the MHC isoforms and the obtained signal corresponds to the total population of MHC mRNA expressed in human muscle. 2) An oligo dT probe (12-18 mer, Life Technology) was used to detect poly A RNA (total mRNA population). 3) An antisense 18S ribosomal RNA probe, in which the signal is directly proportional to the amount of total RNA that was used in order to normalize for possible variability in the amount of loaded RNA per slot. Probes were 5’ end-labeled with P32 using γ ATP and T4 polynucleotide kinase. Hybridization and
washing procedures were carried out as described previously (27). Hybridization signals were detected and analyzed using a phosphorimager and Image Quant analysis software (Molecular Dynamics). For each sample, the MHC mRNA, actin mRNA, and dT (polyA) signals were normalized to the corresponding 18S signal. The slot blot hybridization signal for these probes was strongly correlated with the amount of loaded total RNA ranging from 0.25 to 2 µg per slot. The sequence of oligonucleotides probes used for hybridization is reported in table 2. Total MHC mRNA, total α-skeletal actin mRNA and total mRNA were expressed as concentration per gram of muscle in arbitrary units (AU). This latter value was obtained based on total RNA concentration and the specific signal generated relative to the corresponding 18S per 1 µg total RNA.

**Reverse Transcription (RT).** One µg of total RNA was reverse transcribed for each muscle sample using the SuperScript II RT from Gibco BRL and a mix of oligo dT (100 ng/reaction) and random primers (200 ng/reaction) in a 20µl total reaction volume at 45°C for 50 minutes, according to the provided protocol. At the end of the RT reaction, the tubes were heated at 90°C for 5 minutes to stop the reaction and then were stored at -80°C until used in the PCR reactions for specific mRNA analyses (see below).
**Polymerase Chain Reaction (PCR).** A relative RT-PCR method, using 18S as an internal standard (Ambion, Austin, TX), was applied to study the expression of specific mRNAs for IGF-I, IGF binding protein-4 (IGF BP-4), and the IGF-I receptor (IGF-R). The sequences for the various primers used for the specific target mRNAs are shown in Table 2, and these primers were purchased from Life Technology (Invitrogen, Carlsbad, California). In each PCR reaction, the 18S ribosomal RNA was co-amplified with the target cDNA (mRNA) to serve as an internal standard and to allow correction for any differences in starting amounts of total RNA. PCR procedure and quantification methods were conducted as described previously (16, 40).

**Statistics.**

All values are presented as mean ± SE. For all parameters, the post value was compared to its corresponding pre value within a group using a paired t-test. This approach tested if the manipulation was able to induce a change per-se in the studied parameter. In addition, the post to pre change for a given parameter was compared among groups using one way analyses of variance followed by a Newman Keuls post hoc test. These latter comparisons enabled us to detect differential response of a parameter due to the different manipulations (ULL, ULL+RE, and RE). Differences were considered significant when p<0.05.

Although the number of women in each group was small (N=3 or 4/group), based on gender means within groups, there appeared to be no influence of gender on the responses. Thus, determinations on male and female subjects were combined.
RESULTS

Muscle Volume and Strength.

Changes in muscle volume and strength have been reported elsewhere (36, 38). Since these findings serve as important background information relative to the biochemical analyses presented below, they are briefly summarized as follows. Quadriceps muscle volume of the left limb decreased 8.8% (p<0.05) in ULLS group; whereas the ULLS+RE group showed a 7.7% increase (p<0.05) (36, 38). The RE alone produced a 6.1% increase (p<0.05) as reported previously (36, 38). In neither group did volume of the weight-bearing right limb change (p>0.05) (36, 38).

The average force generated during concentric and eccentric actions increased by 11% (p<0.05) over the twelve exercise sessions in both the RE and ULLS+RE groups (36, 38). Muscle strength of the left limb as determined based on the maximal voluntary isometric force decreased by about 25% (p<0.05) in the ULLS group, and was maintained in the ULLS+RE group (36, 38). RE alone was associated with ~ 11% increase (p<0.05) in muscle strength (36, 38). In all groups, the right limb showed no change in strength (p>0.05) in post vs. pre measurements (36, 38).

 Responses of Cellular/Molecular Markers

Total RNA and mRNA

Total extractable muscle RNA was used as an index representing the protein translational capacity of the muscle, since over 85% of the total RNA pool consists of ribosomal RNA. As shown in Figure 1A, the ULLS group (left leg) showed a significant reduction in total RNA concentration relative to the pre-experimental condition (e.g., before unloading).
Contrary to this response, in both the ULLS+RE and RE groups there was an increase in RNA concentration indicating that those muscles exposed to the resistance exercise stimulus were able to either maintain or augment the total RNA pool relative to the pre-training state. Pre versus post sample analyses for the right (control side) were not impacted statistically (Figure 1A). Determinations of the total poly A mRNA (e.g., the total message pool) showed similar responses as that seen for the total RNA (Figure 1B). ANOVA analyses to compare the changes among the 3 groups show that the change in total RNA and mRNA (polyA) in the ULLS were significantly different from changes occurring in the ULLS+RE and RE groups in the experimental leg. In contrast, the changes in the right (control) leg were not different from each other for the 3 groups.

**Total MHC and Actin mRNA.**

MHC and actin protein accounts for ~60-70% of the protein pool comprising the myofibril fraction, which, in turn, accounts for ~50% of the total protein pool of the muscle fibers. Thus, these two structural/regulatory proteins clearly represent the most abundant proteins expressed in muscle fibers. Since, in steady state, there is a strong agreement between the level of MHC and actin protein that is expressed relative to their respective abundance of mRNA expression within the muscle (28, 29), it would appear that the mRNA levels for these two proteins represent two critical pre-translational markers for maintaining the mass of the fiber. In the ULLS experimental muscle (left leg), total MHC and actin mRNA expression were reduced (P< 0.05) in the post state as shown in Figure 2. In contrast, in both the ULLS+RE and RE groups, MHC and actin mRNA concentrations were not different from the pre training values. However, it was
somewhat surprising that the actin and MHC post value (especially for the total MHC) for the control, right leg, were clearly lower relative to the pre values for all three experimental groups. At present we do not have an explanation for this peculiar response. One-way ANOVA analyses to compare the changes among the 3 groups show that the change in MHC and actin mRNA expression in the ULLS were significantly different for the changes occurring in the ULLS+RE and RE groups in the experimental leg. In contrast, the changes in right (control) leg were not different from each other across the experimental groups.

*IGF-I, IGF Receptor, and IGF BP4*

Previous studies have shown that IGF-I, in and of itself, is a potent growth factor that can induce muscle hypertrophy (8), and this factor is up-regulated at both the mRNA and peptide level under loading conditions that result in muscle hypertrophy (1, 4, 22). However, when animal muscles become unloaded, IGF-I mRNA expression and peptide levels do not appear to be reduced (9, 29). In the context of these observations it was of interest to examine the IGF-I mRNA profiles in the muscles of the three experimental groups. As shown in Figure 3A, Muscle IGF-I mRNA expression was not altered by ULLS or ULLS+RE, but was increased in the RE group (p<0.05). This observation was however confounded by the anomaly whereby the control muscles, e.g., right leg of the ULLS and ULLS+RE groups also demonstrated high levels. The reason for this is not readily apparent (see discussion). In the context of these alterations in IGF-I expression, we examined also expression of the IGF-I receptor (IGF-R). Our findings, presented in Figure 3B, suggest an up regulation of the receptor under the condition of ULLS, which
might represent a negative feedback response to the unloading state, since previous studies on unloaded-inactivated rodent skeletal muscle show the same response (29). With regard to the IGF-I axis, we examined the expression of IGF-I binding protein-4 mRNA, which has been shown to be increased in response to overloading stimuli (9). Our data presented in figure 3C show that IGF BP4 mRNA response was similar to that of IGF-I mRNA, i.e., increased in the RE group only. The exact mechanism of how IGF binding protein interacts with IGF-I remains poorly understood (21, 23).

Comparing changes that occurred for each variable in each group using ANOVA analyses showed that the alterations in IGF-I, IGF-R, and IGF BP4 were not significantly different among the 3 different groups.

Taken together the results presented herein suggest that the differential loading conditions imposed on the vastus muscle of the three test groups (left leg) reflect changes in gene expression of key contractile proteins coupled with changes in total RNA concentration that could impact key processes, e.g., translational processes. These responses could impact the differential changes in muscle volume and strength that occurred across the three experimental groups. In contrast, the changes in the IGF-I system are not clear-cut and suggest a more complex role in the regulation of this system as discussed below.
DISCUSSION

The ULLS Model of Muscle Unloading in Human

As described herein and elsewhere (2, 13, 37), the unilateral limb suspension (ULLS) model represents a unique approach to unloading the muscles of the lower limb in human subjects whereby one limb is prevented from weight bearing with the use of crutches. Effective muscle unloading results in a loss of muscle mass and strength. Resistance exercise is a common countermeasure and is designed to prevent the loss in muscle function. The goal of this study was to determine if the effects of ULLS and RE can also be observed at the molecular level with muscle biopsy analyses for total RNA and contractile protein mRNA.

In the ULLS experimental design, the unloading suspension is designed to affect only the left leg, and theoretically the right leg should serve as untreated control. However, with the use of the crutches, the ground reaction forces become attenuated on the control right limb so that it cannot in reality be considered as a normal control. With the possibility of obtaining biopsies before (pre) and after (post) the 5 week treatment program, we were able to compare pre to post measurement in both left and right leg. Although the right leg did not appear to be significantly different in post to pre comparisons based on volume or strength measurements (5, 13, 30, 38), our results show that the right leg underwent some subtle post treatment changes which makes it not suitable to be used as a control.

Consequently, our experimental design, from the biochemical perspective, treated each limb as a separate entity with each limb, more or less, being analyzed independently of one another. The reason for this response in the right untreated leg for some of the molecular measurements is unclear at present, but such a pattern may reflect the fact that
normal weight bearing activity was compromised by the use of the crutches when the subject performed any ambulatory movements. Nevertheless, these subtle changes at the cellular level should not confound the findings observed and the conclusion made concerning results on the biopsy samples of the test limb.

**Cellular Markers Impacting the Homeostasis of Muscle Mass**

It is well known that muscle unloading leads to muscle atrophy as a result of a negative protein balance; however, it is not clear how this protein imbalance is achieved and how it impacts structural muscle proteins particularly the total MHC and actin expression at the pre-translational level. Protein expression can be generally regulated at several levels in the cascade linking a gene’s transcriptional activity to its protein product. These include a) transcriptional/ post transcriptional/ pretranslational processes that impact the quantitative level of expression of mRNA; b) translational processes that modulate the conversion of the mRNA blueprint into protein; and c) post-translational/degradative processes, which impact the stability or half-life of the protein product. In a recent study, Bickel et al (16) reported that muscle of long term spinal cord injured (SCI) patients express less total RNA per unit muscle mass, which is an indicative of a lower anabolic potential. Interestingly, two 30-min bouts of resistance training in form of electro-myostimulation were effective in ameliorating the RNA deficit in these SCI muscles (16). However, that study did not examine the expression of specific muscle protein mRNA such as actin and MHC. In this study we have examined the MHC and actin mRNA concentrations as markers for pretranslational processes in regulating muscle protein homeostasis, as well as the total RNA concentration as a general marker for the protein
translation capacity of the cell. Our results show that following 5 weeks of unloading (ULLS) the muscle total RNA concentration is decreased by 13% (P<0.05), total MHC mRNA concentration was decreased by 41% (P<0.05) while actin mRNA concentration was decreased by 28% (P<0.05). These observations are consistent with the idea that protein imbalance is due in part, to a decrease in pre-translational and translational processes. Specifically, the reduction in total RNA, the majority of which is ribosomal, represents a reduction in the machinery that is necessary for translating mRNA into protein. This deficiency is further worsened with the decrease in the message substrates encoding the two most abundant proteins expressed in skeletal muscle, e.g., MHC and actin. Resistance exercise was effective in completely blunting those molecular alterations (Fig 2), and this response is in agreement with muscle volume and strength data reported previously (36, 38).

Findings Compared to Animal Models of Unloading

In rodent studies involving limb unloading coupled to markedly reducing neuromuscular activity of the muscle by the intervention of SI, one of the hallmarks of the adaptive muscle wasting that occurs involves both a reduction in total RNA concentration along with a reduced transcriptional activity (and corresponding pre-and mature mRNA levels) for both actin and myosin (29). These two alterations likely contribute to the rapid loss in both MHC and actin protein in this model of muscle wasting. During SI, while there is no clear cut evidence that there is repression of growth factor expression such as IGF-I (29), the prevailing evidence suggests that the IGF-I signaling pathway cannot compensate for the reduced capacity for protein synthesis and the likely augmentation of protein
degradation (based on analyses of protein degradation markers), the collection of which causes the net catabolic state of the muscle. The observations obtained on the ULLS group is consistent with this mechanism of catabolism, because the IGF signaling cascade was not elevated to the levels seen in the RE groups. In the context of the above mentioned alterations, it is important to note that when rodent skeletal muscle is chronically overloaded by synergistic ablation, IGF-I expression, at both the mRNA and peptide levels of expression are markedly elevated to relative levels that are significantly greater than those reported herein for the human ULLS and RE subjects as well as those reported on unloaded animal muscles (29). Thus, the maintained level of IGF-R gene expression on the contralateral right leg and the increase in the IGF-R expression in the ULLS group may be part of a compensatory response to maintain an anabolic response in the face of an insufficient chronic weight bearing state. Clearly more research is needed on this interesting phenomenon.

**Historical Perspectives**

From a historical perspective, the findings in this study add insight into the underlying mechanism(s) thought to impact muscle protein balance during unloading states. For example, in 1990, Thomason and Booth (39) published a classical working model of muscle atrophy. The essence of that model detailed a process whereby there was an initial transient decrease in protein translation, which was subsequently followed by an increase in degradation until the muscle reached a new steady state of reduced mass. These findings suggested that the atrophy process was temporally linked to both an initial contribution of decreased protein synthesis followed by increased protein degradation. Subsequently, Haddad et al (28) demonstrated that in the robust atrophy model of spinal
isolation (SI), pretranslational processes impacting key sarcomeric proteins, e.g., MHC and actin mRNAs occurred throughout the unloading process until a new steady state was reached. These latter observations suggested that pretranslational processes likely contribute significantly to loss of protein throughout the process of atrophy until a new steady state is reached. Thus, in agreement with the observation of Haddad et al (28), the present study extends the above findings on animal models by demonstrating that after 5 weeks of unloading (ULLS) a reduction in total RNA, MHC and actin mRNA is maintained throughout the period of unloading. Thus these findings suggest that human muscle responds similarly to atrophy stimuli as that of animals when weight bearing activity is reduced.

Collectively, the findings reported herein suggest three important findings at the subcellular/molecular level concerning the effects of altered loading states imposed on human skeletal that are consistent with previous findings seen in animal models: 1) unloading of skeletal muscle reduces gene expression of the two primary contractile proteins that make up the contractile apparatus of skeletal muscle cells at a strategic level of regulation; 2) such an adaptive process is impacted further by the reduction in total RNA (and ribosomal), which should further impose a reduced translational capacity of the unloaded muscle; and 3) a training paradigm consisting of concentric-eccentric actions spaced apart at regular three-day intervals, is capable of reversing these alterations at the molecular level thereby affecting key processes likely involved in ameliorating the deficits in muscle mass/volume occurring in these subjects. These conclusions would have been strengthened if additional markers were examined;
however, this was precluded by the amount of muscle available for analyses. Thus, it is apparent from the present study that molecular analyses of human muscle biopsies can provide insight into the underlying mechanisms that impact skeletal muscle during adaptive change in response to altered loading states and to resistance exercise. Finally, the findings reported herein clearly support in-flight resistance training for space travelers and that molecular markers may provide insight in fine-tuning such in-flight exercise protocols.
ACKNOWLEDGEMENTS

We thank all volunteers who endured the unloading and training interventions. The technical support by Anneli Ekberg, MD, Jay Trieschmann, BSc., Todd Trappe, PhD, Anqi Qin, Ming Zeng, and Li-Ying Zhang, is greatly appreciated.

This project was supported in part by a NASA National Biomedical Research Institute Grant NCC9-58-70 (KMB), NASA Grant 5286 (PT) administered by the University of Arkansas for Medical Sciences, the Swedish National Space Board (PT) and Swedish National Centre for Research in Sports (PT).
REFERENCES


Table 1. *Subject characteristics.*

<table>
<thead>
<tr>
<th></th>
<th>ULLS (n=11)</th>
<th>ULLS+RE (n=10)</th>
<th>RE (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>40±3</td>
<td>42±3</td>
<td>39±3</td>
</tr>
<tr>
<td>Height, cm</td>
<td>176±3</td>
<td>179±3</td>
<td>175±2</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>80±4</td>
<td>79±4</td>
<td>79±4</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>16.5±2.8</td>
<td>14.8±1.3</td>
<td>14.3±1.3</td>
</tr>
<tr>
<td>Strength, N</td>
<td>355±34</td>
<td>369±27</td>
<td>418±56</td>
</tr>
</tbody>
</table>

Values are mean ± SE. Strength is combined right and left maximal voluntary force during knee extension at 90º knee joint, prior to ULLS, ULLS+RE, or RE.
Table 2:

Oligonucleotides sequence for the probes used in slot blotting, and primers for PCR analyses.

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>Sequence 5’→3’</th>
<th>GenBank Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>All MHC isoforms</td>
<td>TGGGCGCTGGGTGTCCTG</td>
<td>NM_000257</td>
</tr>
<tr>
<td>α skeletal Actin</td>
<td>CGCCGATCCACACCGAGTATT</td>
<td>NM_001100</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Fwd: AGTGCTGCTTTTGTGATTCTTT Rev: CAATACATCTCCAGCCTCTTA</td>
<td>NM_000618</td>
</tr>
<tr>
<td>IGF-I receptor</td>
<td>Fwd: ACAAGGGCCATCGTTCATCC Rev: GCACACAGGGGCAGTCC</td>
<td>NM_000875</td>
</tr>
<tr>
<td>IGF-I BP-4</td>
<td>Fwd: CTTCATCCCCCATCCCCAACTG Rev: CTGCTGGCAGGCTACTCT</td>
<td>NM_001552</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1.
Total RNA (A) and poly A RNA (mRNA) (B) concentration in mVL muscles. Poly A RNA concentration is determined by slot blotting after hybridization with the oligo dT probe as explained in the methods. Left side is the actual experimental side subjected to ULLS and RE. *: post is significantly different from pre value

Figure 2.
Total MHC (A) and α skeletal actin (B) mRNA concentration in mVL. Both were determined via slot blotting hybridization. *: post is significantly different from pre value

Figure 3.
IGF-I (A), IGF-Receptor (IGF-R, (B)) and IGF BP4 (C) mRNA expression in the mVL in the various experimental goups. These were obtained via relative RT-PCR methods.
*: post is significantly different from pre value
Figure 1

A

RNA Conc. mg/g

ULLS  ULLS+RE  RE  ULLS  ULLS+RE  RE

Pre  Post  Pre  Post  Pre  Post

* * p=0.07

B

poly A RNA Conc. AU/g

ULLS  ULLS+RE  RE  ULLS  ULLS+RE  RE

Left leg  Right Leg
Figure 2

(A) MHC mRNA

(B) α sk Actin mRNA

Left leg

Right Leg
Figure 3

A

IGF-I mRNA vs 18S

ULLS ULLS+RE RE ULLS ULLS+RE RE

B

IGF-I R mRNA vs 18S

ULLS ULLS+RE RE ULLS ULLS+RE RE

C

IGF-BP4 mRNA vs 18S

ULLS ULLS+RE RE ULLS ULLS+RE RE

Left leg Right Leg