SIMULATED MICROGRAVITY AND RADIATION EXPOSURE EFFECTS ON THE REGULATION OF SKELETAL MUSCLE PROTEIN SYNTHESIS

A Dissertation

by

MICHAEL PARKER WIGGS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2011

Major Subject: Kinesiology

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Approved by:

Chair of Committee,	James D. Fluckey
Committee Members,	John M. Lawler
	Steven E. Riechman
	Stephen B. Smith
Head of Department,	Richard B. Krieder

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ABSTRACT

Simulated Microgravity and Radiation Exposure Effects on the Regulation of Skeletal Muscle Protein Synthesis. (August 2011)

Michael Parker Wiggs, B.S., Texas A&M University

Chair of Advisory Committee: Dr. James Fluckey

Long duration spaceflight missions out of lower earth orbit, back to the lunar surface, or possibly to Mars highlight the importance of preserving muscle mass and function. Muscle atrophy occurs within days of exposure to microgravity and prevailing thought is that a primary mechanism for muscle atrophy is a reduction in skeletal muscle protein synthesis. This dissertation examines the ability of skeletal muscle to recover muscle protein synthesis with slight perturbation, such as ambulatory reloading during disuse as well as partial loading, similar to body mass seen on the moon or Mars. We use traditional precursor-product labeling to measure protein synthesis, but use a relatively novel tracer, deuterium oxide, in order to make cumulative measures of protein synthesis over 24 h. The overarching goal of this dissertation is to define the response of skeletal muscle protein synthesis to different loading parameters in order to better understand the contribution of protein synthesis to skeletal muscle mass during disuse.

In the first study, we demonstrate that muscle atrophy during 5 days of hindlimb unloading is in part due to a decrease in protein synthesis. We also highlight the ability of skeletal muscle to adapt by allowing two 1 h ambulatory reloading sessions on days 2

and 4. Although this countermeasure is able to rescue protein synthesis in soleus and gastrocnemius, it is unable attenuate any losses in muscle mass.

In the second study, we compare partial weight loading to traditional hindlimb unloading. Weight bearing of $1/3^{rd}$ or $1/6^{th}$ body weight is able to attenuate losses in muscle mass seen with unloading. Protein synthesis is maintained after 21 days of the experimental protocol, suggesting that protein synthesis is responsive to load and is likely not the only mechanism for determining muscle mass.

In the final study, the effects of < 1 Gy x-ray exposure and partial weight suspension are measured to better understand the complex space environment, which includes a wide variety of radiation. Surprisingly, we found no effects of radiation on muscle protein synthesis in 1 G or partial loading.

Targeting only protein synthesis may not be enough of a stimulus as evidenced by the data in this dissertation. Future plans should use a multiple-systems approach to counteract atrophy by increasing protein synthesis to maintain/elevate muscle mass during periods when it is otherwise compromised.

DEDICATION

To my beautiful wife, Kelsey Paige, and my always supportive family: Beth Wiggs, Elizabeth Sealey, Michael Sealey, and Libby Sealey, and my grandparents, the late Jefferson and Bettye Pringle.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Since human spaceflight missions began in the 1960's, scientists have researched how and why muscle atrophies during bouts of weightlessness (microgravity). Currently, long duration missions, such as six months on the International Space Station or even longer periods for the planned explorations out of lower Earth orbit, have provided the impetus for experiments related to the maintenance musculoskeletal health, which is of great importance to the overall health of astronauts and success of the mission. Overloading skeletal muscle with resistance exercise have been proposed and used as countermeasures to spaceflight induced atrophy since the 1970s. Ground based studies have demonstrated attenuation in losses in muscle mass in animals (41, 52, 67) and humans (24, 100, 169), but have yet to be fully effective at maintaining muscle mass (167). Not only is the loss of muscle mass significant on a macrolevel, but close examination of single muscle fibers show astounding decrements in force production (50).

Recently, NASA has developed an extended plan for long duration spaceflight back to the moon, our first attempt since 1972. In a speech given at Kennedy Space Center on April 15th, 2010, President Barak Obama predicted that, "By the mid-2030s, I believe we can send humans to orbit Mars and return them safely to Earth. And a landing

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This dissertation follows the style of *American Journal of Physiology - Endocrinology and Metabolism*.

on Mars will follow. And I expect to be around to see it" (128). It is estimated that a trip to Mars, 6 mo of exploration, and then the trip back would take an estimated three years to accomplish. Currently, the European Space Agency (ESA) experiments entitled Mars 500 is a ground based study to determine psychological and other assorted health issues during a simulation of activities that could occur over a proposed three year mission to Mars.

While the Mars500 study will seek to understand if humans can withstand the physiological stress of 3 years in a space vessel, the long term effects of partial gravitational loading and space radiation are difficult to determine in human subjects due to difficulties in study design and ethical considerations. This dissertation will focus on the effects of partial gravity and simulated space radiation exposure on muscle mass and protein metabolism in rodents using ground based methods for simulating microgravity.

Effects of Microgravity on Skeletal Muscle

Rapid muscle atrophy with microgravity

Muscle responses to microgravity environments have been well documented with data from spaceflight and ground based studies. The most noticeable and important factor is resulting from those studies is the rapid and appreciable loss of muscle mass, particularly extensor muscles in lower extremities and flexor muscles in upper extremities, which leads to deficits in muscle strength and power. Although there is considerable variability in human spaceflight data, the overall results are extremely consistent regarding the effect of microgravity on loss of muscle mass, whether it be

three days of unilateral lower limb suspension (66) or six months aboard the international space station (50). The rate of muscle atrophy appears to be linear (**Figure 1**), but stabilizes after a prolonged period of time when a new equilibrium is reached (141).

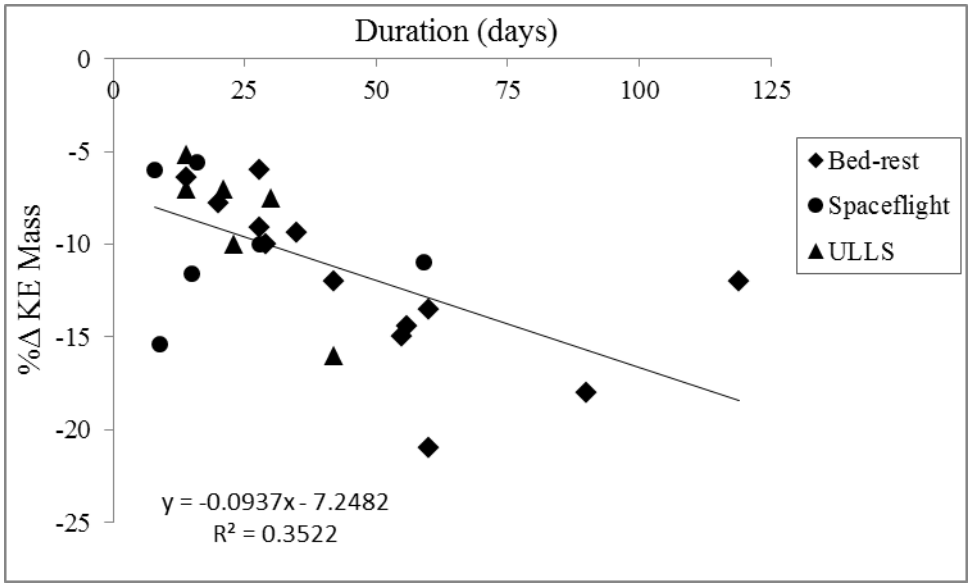

Fig 1. Muscle atrophy during spaceflight or simulated microgravity. Muscle atrophy is linear in humans during spaceflight, bed rest and ULLS. Compilation of MRI muscle volume changes in the vastus lateralis of humans during spaceflight or simulated microgravity with bed rest or ULLS (3, 4, 6, 16, 17, 26, 33, 37, 96, 97, 117, 121, 132, 146, 147, 169).

Figure 1 shows a timeline of muscle mass loss in a variety of human disuse models. Representative values show a 3% decrease after 7 days of bed-rest (48), a 6.3% decrease on astronauts aboard STS-47 (96), a 6-11% decrease after 20 days of bed-rest (insert citation), a 5-11% decrease after 30 days calf volume, 14-17% after 42 days of bed rest, 25% loss after 90 days and plateau value of 23% after 16-28 wk in astronauts

aboard Skylab/MIR (94). These data suggest that the plateau of muscle atrophy occurs at or around 90 days. It is to be noted that spaceflight data should not be considered strictly microgravity. Since the beginning of spaceflight, space agencies have understood the effect of disuse on muscle mass and therefore have implemented countermeasures in attempts to attenuate muscle atrophy. These countermeasures include resistance exercise with numerous different devices and aerobic exercise training with treadmill and cycle ergometer designed to increase dynamic loading. To date, these countermeasures have been unsuccessful in fully preventing atrophy during long duration spaceflight (167). Somewhat primitive exercise devices were used in the first capsule-base flights consisting of stretching rubber bands for muscle contractions (personal communication with astronaut Joseph Kerwin). Current data using resistance training on ISS is done with the interim resistance exercise device, allowing astronauts to perform dynamic resistive exercise (i.e. squats, calf raises, (145). Approximately 180 days aboard ISS with a combination of these countermeasures averaged a loss of 13% of their muscle volume of the gastrocnemius and soleus (167). While this may represent considerable improvements related to the losses of muscle, it also underscores that the prevention of muscle atrophy and muscle function is still a major concern to long duration spaceflight missions.

A consequence of muscle atrophy is the concomitant decrease in strength. Comparing knee extensor strength following a wide range of bed rest studies, 14 days led to a 9% decreased in force (14), 20 days produced 19-26% decrease (57), 30 days resulted in a 19% decrease (38), and 42 days showed 25-30% decrease in muscle force

(17). Finally, there was a 36% decrease in force following 120 days of bed rest (88). Skeletal muscle atrophy is directly related to strength changes. **Figure 2** illustrates that for every 1% change in the cross-sectional area, there is approximately a 1.19% loss of muscle strength. Therefore the loss in muscle quality, which is defined as strength divided by cross sectional area, is more severe. Due to the low number of astronauts in space each year and minimal experimental access to astronauts and flight crews during missions, ground based models mimicking spaceflight are commonly used.

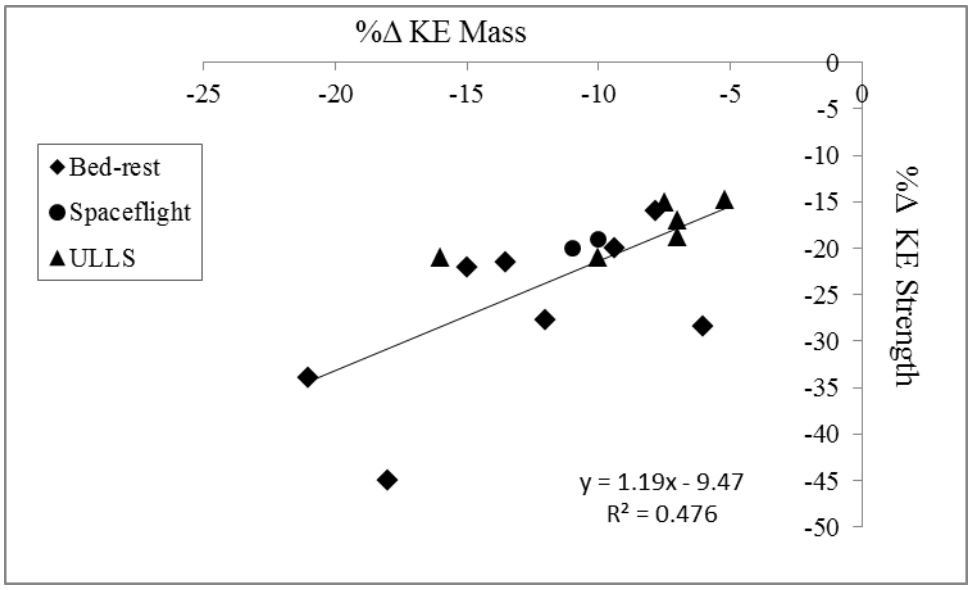

Fig 2. Percent chance in knee extensor strength vs. percent muscle lost. Muscle strength is lost at a higher rate than muscle mass. For every one percent of knee extensor (KE) mass lost, there is an estimated 1.19 percent decrease in KE strength. Data is compiled from both simulated and exposure to microgravity experiments (6, 17, 26, 33, 37, 97, 117, 121, 132, 146, 147, 169).

Bed rest and ULLS are two commonly used ground based models of microgravity

In humans, the two commonly used ground based models of spaceflight are bed rest and unilateral lower limb suspension (ULLS). Six degree head down tilt bed rest is used to accurately reflects the cardiovascular and skeletal muscle changes observed with spaceflight (170). The use of 6° head down tilt mimics the body fluid shift that is observed similar to the absence of a gravitational field in space. At 1G, blood pools in the venous side of the lower extremities; during microgravity, the blood is redistributed throughout the body, leading to increased central fluid volume. In a study by Trappe et al., a comparison of the physiological parameters of 4 astronauts on STS-78 on a 17-day mission paralleled with a ground base bed rest with respect to the cardiovascular and musculoskeletal changes (168). Specifically, these studies also demonstrated that exercise capacity was decreased to the same degree in spaceflight and 6° head down tilt bed rest.

In ULLS, the subject is fitted with a customized shoe with a ~6-inch platform added to the sole. Therefore, the body is elevated by the shoe on the loaded leg and the contralateral leg is unloaded (70). Ambulation is accomplished with the assistance of crutches. This is an upright model of unloading; therefore, a negative aspect of this model is the absence of a fluid shift as with 6° head down tilt bed rest. This model does allow for the subject to continue their activities of daily living, at least to a degree, and minimizes around-the-clock oversight (as with bed rest). Similar to spaceflight, ULLS causes preferential losses of the predominantly type I soleus compared to the mixed muscle gastrocnemius (180). Unfortunately, ULLS muscle atrophy does not mimic

spaceflight at the single fiber level. ULLS resulted in a even greater amount in force reduction and decreased shortening velocity, which is opposite of what is observed with bed rest and spaceflight (180). Regardless those results, ULLS provides an economical and a less resource-intensive method for understanding the effects of unloading on muscle protein synthesis as compared to bed rest studies.

Hindlimb unloading is the accepted rodent model of spaceflight

In order to investigate molecular mechanisms, typically requiring more invasive procedures, a hindlimb unloading model in rodents was developed (116) and later established as the gold standard for mimicking spaceflight (114). Hindlimb unloading results in a very rapid and dramatic decrease in skeletal muscle mass. Human models typically show a slower progression of atrophy than the rat model. For example, a seven days of bed rest resulted in a 7% change in muscle volume (48); whereas, 12 days of HU resulted in a 28% decrease in muscle volume in rats as determined by MRI (53). In similar studies, wet muscle mass lost during 12 days of HU were approximately 40% of ambulatory control animals (8, 106). These effects are suggestive that the amount of muscle lost before reaching equilibrium is larger in rats than humans. Rat studies have demonstrated up to a 60% decrease in muscle mass following 21 days of HU (43), while the greatest change in human skeletal muscle is around 35%.

Although the rate and quantity of muscle lost differs between species, it is important to note that skeletal muscle atrophy within rats is similar between HU models and rats flown on the 14 day Russian Cosmos 2044 mission (114). Soleus muscle mass in both flight and HU rats decreased by 25 and 34%, respectively (129). Despite the rate

and total amount of muscle mass lost, the mechanisms by which rats and humans lose muscle appear to be similar.

Table 1. Muscle mass atrophy and/or strength changes with hindlimb unloading

Study	HU Duration (days)	%Δ Soleus Mass	%Δ P_0
Arbogast S et al. (8)	3	-33	
Bajotto G et al. (13)	5.5	-27	
Arbogast et al. (8)	6	-38	
Hurst JE et al. (77)	7	-18	14
Wenke JC et al. (178)	10	-25	30
Arbogast et al. (8)	12	-40	37
Matuszczak Y et al. (106)	12	-44	64
Dupont E et al. (43)	14	-33	
Hanson AM et al. (68)	14	-26	25
Salazar JJ et al. (144)	14	-25	40
Fujino H et al. (55)	14	-33	48
Hurst JE et al. (77)	14	-31	43
Widrick JJ et al. (179)	15	-35	56
Dupont E et al. (43)	21	-48	
Dupont E et al. (43)	28	-60	

HU results in rapid muscle atrophy. Data compiled from a range of HU duration from 3 to 28 days in rodents demonstrates that muscle is lost rapidly at the onset of HU and continues atrophy until at least 28 days. Change in muscle strength (%Δ P_0) determined in some of the studies illustrated that the change in strength is equal to or greater than the change in muscle mass. Studies were selected to included a range of durations or if muscle strength was measured in the study.

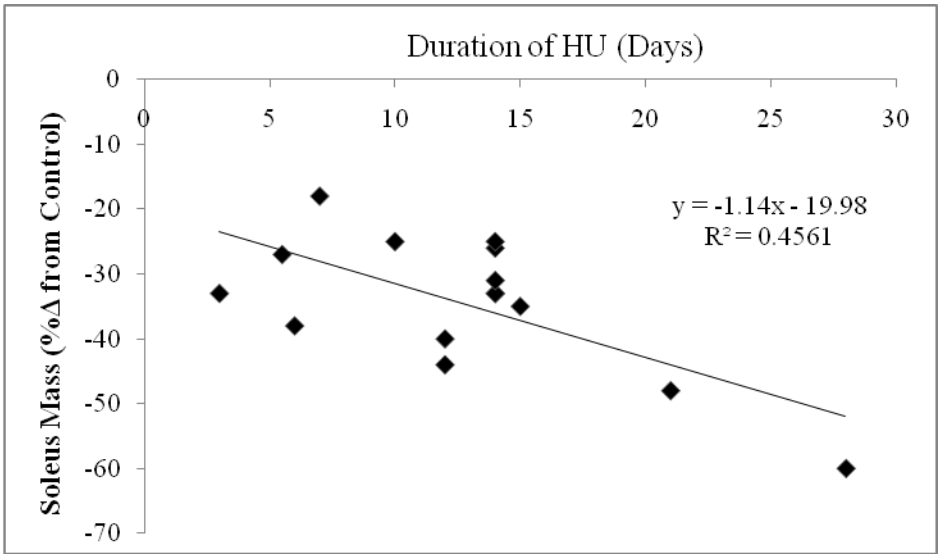

Fig 3. Soleus muscle atrophy is linear through 28 days. HU results in a linear decrease in muscle mass. Data from numerous studies highlight that HU result in a rapid decrease in muscle mass in the first few days, and then a linear decrease in muscle mass occurs throughout 28 days of HU which is considered long duration HU (8, 13, 43, 55, 68, 77, 106, 144, 178, 179).

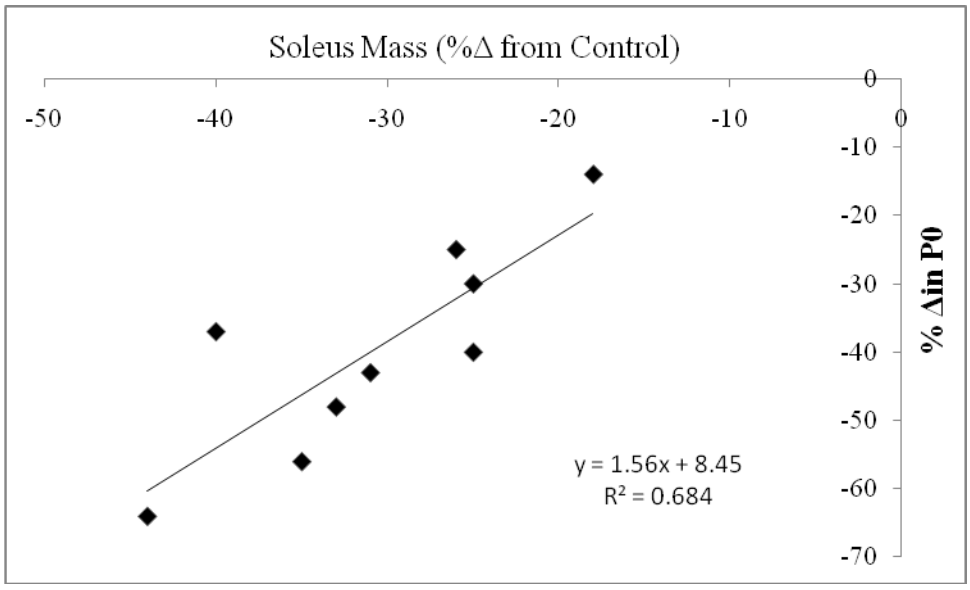

Fig 4. Soleus strength decreases at a greater rate than muscle mass.HU induced muscle atrophy results in greater loss in strength. Compiled data from rodent HU studies suggests for every 1% of muscle mass lost, there is a 1.5% reduction in isometric force (8, 55, 68, 77, 106, 144, 178, 179).

Similar to human data, muscle mass atrophy is linear during hindlimb unloading (**Table 1/Figure 3**), and strength loss is exacerbated on percentage to percentage basis when compared to muscle mass lost **(Figure 4)**.

Methods and results for muscle protein synthesis during unloading

Skeletal muscle mass is determined between the balance of protein synthesis and protein degradation. Unperturbed, muscle mass is does not change because protein synthesis and protein degradation are at an equilibrium. During disuse, skeletal muscle atrophy occurs because of an imbalance in this important process. Early immobilization studies that observed a significant increase in urinary nitrogen on the fifth day of bed rest (34). Proteins are the only macromolecules that contain nitrogen, so an increase in

urinary nitrogen suggests either an increase in protein degradation or dietary protein intake is not being used for the synthesis of proteins and, therefore, are excreted. Similarly, astronauts aboard the three NASA SKYLAB missions were found to be in net negative nitrogen balance throughout these long term missions (152).

In general, the measurement of protein synthesis is invasive and not very feasible in-flight. Therefore most of our knowledge and assumptions of protein synthesis during unloading are derived from ground based studies. Measurements are made using traditional precursor-product techniques (186). This method involves the introduction of a labeled (stable or radioactive) amino acid precursor in tracer amounts. Protein synthesis is then determined by measuring the protein bound isotopic enrichment of that amino acid in a specific tissue over a specified amount of time while accounting for the precursor enrichment.

The two most commonly used precursor labeling techniques are the primed-constant (or continuous) infusion (123) and the flooding dose (61). In the primed-constant infusion technique (**Figure 5a**), a bolus dose of the labeled amino acid is infused rapidly at the beginning of the protocol to increase the concentration of the amino acid and then a lower concentration dose is infused for the remainder of the protocol to keep the ratio of the tracer (labeled amino acid) and tracee (unlabeled amino acid) constant. The use of a priming dose sufficiently reduced the time needed to reach equilibrium, from approximately 6 h down to 2 h.

The flooding dose technique was designed to determine protein synthesis in a much shorter amount of time (59). Typical flooding dose protocols last between 10-45

minutes (**Figure 5b)**. Here, a supra-physiological dose of the tracer and tracee results in a rapid rise in the free amino acid pool in the tissues to values close to that in the plasma, followed by a slow, linear disappearance (59). This method is beneficial to capture the synthesis of short-lived half-life proteins.

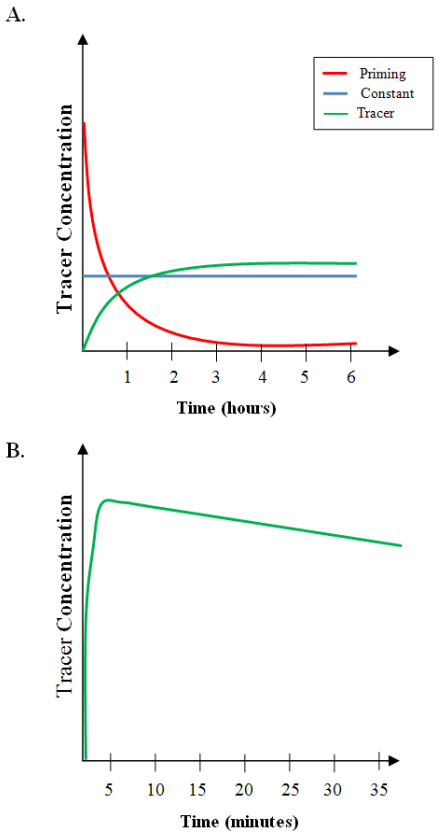

Fig 5. Representative tracer concentration in Primed-Constant Infusion and Flooding Dose. Representative amino acid tracer concentration in a) primed-constant infusion protocol and b) flooding dose technique. Duration of the primed constant infusion is much longer due to the need to reach a steady-state, which in this figure is at approximately 2hrs. Flooding dose measurements can be made in minutes due to the large dose of tracer/trace which saturates the amino acid pool.

Both flooding dose and primed constant infusion methods have been validated and both are well accepted. Generally speaking, flooding dose is used in rodent models because prolonged anesthesia decreases protein synthesis (45), and thus, requiring shorter-term techniques to assess the anabolic features of muscle. Primed-constant infusion is used most commonly in humans because of a smaller amount of tracer used and the ability to make repeated measurements of protein synthesis with perturbations during the infusion protocol.

Protein synthesis has been estimated in-flight aboard MIR by the consumption of ^{15}N glycine and the urinary excretion of ^{15}N ammonia (153, 176). Estimated, whole body protein synthesis over the long duration flights (mean of 143 days) was reduced by approximately 45% in-flight (153). Similar results have been demonstrated using the ground based models. Studies assessing protein synthesis following 21 days of bed rest studies demonstrated similar decreases of 48% (160). Ten days of ULLS resulted in a significant 10% decrease in protein synthesis (58). Hindlimb unloading in rodents produced similar reductions, averaging approximately 35% after 4 days of HU (51). Cumulatively, these representative data demonstrate that a deficit in muscle protein synthesis is a major contributor to muscle atrophy with unloading.

If skeletal muscle protein synthesis is the culprit for muscle atrophy, then it stands to reason that countermeasures targeted to increase protein synthesis should rescue the detrimental effects of microgravity on muscle mass. For this reason, overloading skeletal muscle by resistance exercise is the most commonly used countermeasure (2, 24, 52, 67, 100, 141, 169). Similarly, the used of artificial gravity has

been shown to maintain protein synthesis as well as muscle cross sectional area during bed rest (23, 160). Nutritional approaches stimulating protein synthesis by essential amino acids have shown mixed results on attenuating atrophy (133, 169). To date, countermeasures have been successful at maintaining skeletal muscle protein synthesis during simulated microgravity, but have yet to increase synthesis over and above control or baseline values as demonstrated with resistance exercise. Therefore, "maximally" stimulating protein synthesis may be the key to preventing disuse atrophy.

Deuterium oxide, a method to measure decrements in protein synthesis

Our lab has recently investigated the use of deuterium oxide (^2H_2O) to measure skeletal muscle protein synthesis (63, 64, 127). The concept of using ^2H_2O to determine protein renewal rate by measuring the incorporation of ^2H into protein was first originated by Hans H. Ussing in 1941 (172). To determine protein synthesis, it is possible to relate the incorporation of a ^2H-labeled amino acids into new proteins. Alanine was chosen as the amino acid tracer because of the rapid creation of tracer through intracellular transaminase reactions (131). Although transaminase reactions occur with numerous amino acids, it is the number of hydrogens and the simplicity in the structure of alanine that makes it easily quantifiable.

The use of deuterium has many benefits over the traditional uses of the flooding dose and primed-constant infusion methodologies. One of the advantages is the ability to measure of a long duration in a free living state. Flooding dose captures the rate of protein synthesis over minutes. Primed-constant infusion can be used to look at longer time points, but the subject is limited in the activities due to the invasiveness of the

procedure. Deuterium allows a free living state in rodents or humans over 24 h or longer if the study design dictates (64). Rates of muscle protein synthesis extrapolated out and expressed as a % per day, vary greatly from 3-20% in rats (60). A secondary benefit of the long duration is that it allows ample time for incorporation of the label into slow turnover proteins, such as the myofibrillar proteins of skeletal muscle (127).

A second major benefit of the deuterium method is that it does not use 'pre-labeled' amino acids. Deuterium (in the form of deuterium oxide) equilibrates with body water rapidly. Intracellular enzymatic transaminase reactions are therefore responsible fortracer creation. Dufner et al. determined that the percent of $^{2}H_{2}O$ in body water was identical with the percentage of ^{2}H-labeled alanine within 20 minutes after dosing the subject (40). This solves two problems; first, there is no need to administer exogenous amino acids, which must be pre-labeled and could actually alter protein synthesis (7) and second, eliminate the burden of the vascular compartment to deliver the labeled product which must subsequently rely on active transport across the cell membrane for potential incorporation into muscle proteins (109). A methodological limitation for the use of the primed-constant infusion technique is that it is typically done in the fasted state to be able to accurately predict the tracer to tracee ratio. The addition of specific substrates (for example, the traditionally used amino acid tracer leucine) facilitate protein synthesis because the amino acid may no longer be rate limiting, and in the case of leucine, actually stimulates a key modulator of translational apparatus (7). Although there have been a number of efforts to design methodologies to allow for the pre-labeling of ingested foods, generally, subjects must also refrain from feeding during the study

because of the dilution effect on the precursor pool. Being able to adequately describe the precursor pool during the labeling period is of paramount importance for interpretable assessments of protein synthesis. As stated above, the use of deuterium oxide allows for rapid equilibration with ^{2}H-labeled alanine (40), and since the labeling is actually being accomplished by cellular metabolism, the impact of feeding on the precursor pool is negligible.

Formation of translational apparatus for protein synthesis

Proteins are a polypeptide sequence of amino acids encoded by genes in our DNA (27). Each gene is transcribed into a single stranded message (message RNA, mRNA) that contains the antisense sequence. Each amino acid is encoded for by a sequence of 3 nucleotides (termed a codon), which yields a total of 64 possible codons, 3 of which are considered 'stop' codons. Every protein is a unique amino acid sequence created from a pool of 20 amino acids, therefore there is a redundancy in some of the codons as well as 3 stop codons which cause early termination and release of the polypeptide. Protein synthesis is a generic term for mRNA translation, which can be broken into three steps: initiation, elongation and termination (177).

Translation initiation is the primary rate–limiting step in mRNA translation (142) and is controlled by many proteins that are activated and/or inhibited by extracellular signals. The process can be divided into three stages: i) formation of the 43S preiniation complex which consists of the binding of initiator methionyl-tRNA (met-$tRNA_i$) to the 40S ribosomal subunit, ii) the binding of the mRNA to the 43S preinitiation complex to form 48S preinitiation complex by iii) the combining of a 60S ribosomal subunit to the

48S preinitiation complex to yield the functional 80S initiation complex, also known as the translation apparatus (82).

Formation of the ternary complex in the first step involves binding of eIF2 (GTPase), GTP (guanosine triphosphate) and met-tRNA$_i$ and attachment on the P site (binding site for the peptidyl-tRNA) of the 40S subunit with the assistance of eIF5B-GTP (GTPase) (20, 82, 104, 177). In addition, eIF1A (inhibits early hydrolysis of the ternary complex) and eIF3 (inhibits re-association with the 60S subunit) bind to the aminoacylated-tRNA site (A site) and the ejection site (E site) respectively (104, 177). Prior to formation of the 48S preinitiation complex, eIF4F formation must occur. eIF4F is a multi-subunit protein formed by the binding of eIF4E, eIF4G, and eIF4A as well as a few other proteins. The formation of eIF4F is tightly regulated by extracellular signals and will be discussed later. The three subunits each have their own function: eIF4E binds directly to the 5' cap structure, eIF4G is a scaffold protein, and eIF4A is an ATP-dependent RNA helicase that unwinds once activated by eIF4B (an RNA binding protein) (104, 112, 159). Next, eIF4F, eIF4B and the transcript can bind to the 43S preinitiation complex via an eIF4G and eIF3 interaction (104). Prior to 80S initiation complex formation, the 48S preinitiation complex performs an ATP dependent scan of the mRNA in the typical 5' to 3' direction for the universal methionine (AUG) start codon (177). Eukaryotes do not have a unique initiation sequence, therefore the presence of the 5' cap structure and subsequent scan prevents the functional ribosome from beginning translation at a miscellaneous AUG within the center of the mRNA (90). Identification of the start codon triggers a release of eIF2 and the corresponding GTP

hydrolysis, eIF3 and eIF4B allowing the 60S subunit to associate with the 48S complex forming a complete 80s (177). The formation of the 80S complex stimulates eIF5B-GTP hydrolysis resulting in the release of eI5B, eIF1A and the correct placement of met-$tRNA_i$, triggering translation initiation (177).The epsilon subunit of eIF2b is the guanine nucleotide exchange factor (GEF), which catalyzes the swap of the GDP bound to eIF2 with a GTP and another round of initiation can now occur (92).

Translation elongation is a three step process that leads to the decoding of the mRNA and the synthesis of the corresponding polypeptide chain by the 80s ribosome. The functional ribosome contains three RNA binding sites, designated A (binds aminoacyl-tRNA), P (binds a peptidyl-tRNA) and E (binds a free tRNA before it exits the ribosome) (177). At the beginning of elongation, the met-$tRNA_i$ is left in P site of the 80S ribosome following translation initiation. In the first step, the next appropriate amino acid charged tRNA (aminoacyl-tRNA) in the corresponding mRNA sequence is moved into the A site by eEF (eukaryotic elongation factor)1A-GTP (137). GTP is hydrolyzed to GDP and the resulting eEF1A-GDP is released and the aminoacyl-tRNA is moved to the P site (177). eEF1B is the GEF that catalyzes the recharge of eIF1A-GDP with a GTP (137). The second step is the formation of the peptide bond between the existing peptidyl-tRNA in the P site and the aminoacyl-tRNA in the A site (where the growing peptide is shifted to) and is catalyzed by peptidyl transferase (177). In the final step, the peptidyl-tRNA is translocated from the A site to the P site with the energy dependent eEF2-GTP (137). In addition, the ribosome moves by one codon relative to the mRNA with the previous peptidyl-tRNA moving into the E site.

The final step of translation is termination, which is a two step process occurring when the A site recognizes one of three stop codons (UAA, UAG, UGA) by eukaryotic release factor (eRF) 1 (177). Additionally, eRF1 possesses a unique glycine, glycine and glutamine motif that is located in close proximity to the 3' end of the peptidyl-tRNA, resulting in peptide hydrolysis (177). Step two involves the binding of eRF3-GDP to the eRF1 within the intact ribosome that still has two deacylated tRNAs bound to the P and E sites. eRF3-GDP and eRF1interaction results in the GDP to GTP exchange with eRF3 and subsequent hydrolysis and dissociation of both from the ribosome (177). After the polypeptide synthesis sequence is complete and has been release, the components of the ribosome are dissociated from the mRNA by ribosome recycling factor (RRF) and another round of translation can now occur (177).

Control of translation by mTOR and MAPK signaling

One of the key mechanistic questions on how to better understand and/or prevent microgravity-induced atrophy is to understand the control of protein synthesis. The synthesis of proteins is among the most energetically costly biological processes, with an estimated cost of approximately 495 kcal per day in physically active males (185). Processes that are as metabolically costly as protein synthesis are tightly regulated by the cell. The most energy efficient regulation occurs at the beginning of the process to avoid the waste of ATP. Therefore, the majority of regulatory steps are located in translation initiation and some regulation of elongation.

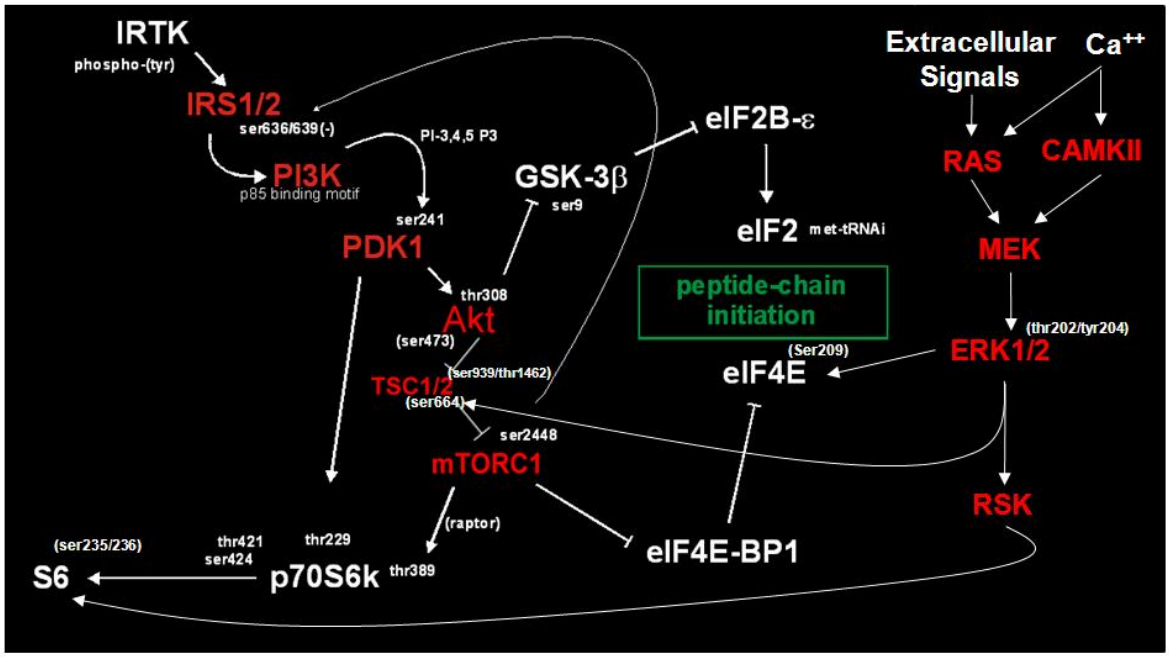

Fig 6. Signal transduction regulating protein synthesis. Akt/mTOR and ERK1/2 pathways work in parallel to control peptide chain initiation (the first step in translation). Key regulatory steps in the process controlled by mTOR include phosphorylation of 4E-BP1, releasing it from allosteric inhibition of eIF4E and phosphorylation of ribosomal protein s6, which increases preinitiation complex affinity for mRNA. ERK pathway can activate mTOR in an independent manner, as well as independent regulation of S6.

This process is effectively demonstrated by looking at the effects of feeding on protein synthesis. During the fasted state, limited substrate (amino acids) (87) and energy availability forces the cell minimize protein synthesis in an effort to conserve ATP (102). Feeding increases energy and amino acid availability, stimulating protein synthesis (182). As mentioned before, the amino acid, leucine, is singly able to stimulate protein synthesis (7), which serves to signal the cell that the organism is in the fed state.

These signal cascades that are largely responsible for the increase in translation initiation (**Figure 6**) operate through the Akt/mTOR (mammalian target of rapamycin)

pathway and extracellular regulated kinase (ERK) 1 and 2(19). In the mTOR pathway, upstream signals such as insulin (32, 54, 85) and IGF-1 (103) confer the signal which triggers the conversion of PIP_2 to PI3, which activates protein dependent kinase 1 (PDK1), whose kinase activity phosphorylates AKT^{Thr308} (5). Akt is an important regulator of skeletal muscle metabolism having a role in glucose disposal (i.e. GLUT-4 transporter translocation, glycogen synthesis) (157), protein degradation (156) , and protein synthesis (171).

One of the key regulatory translation initiation is formation of eIF4F complex which brings the mRNA to be translated to the 43s preinitiation complex. eIF4F is a complex of at least 5 subunits with eIF4E and eIF4G subunits being the targeted regulatory proteins (85). 4E-binding protein 1 (4E-BP1) is a translation repressor that shares eIF4G's binding on eIF4E (148). When 4E-BP1 is hyperphosphorylated, it dissociates from eIF4E thereby allowing eIF4F complex formation (56), leading to the eventual formation of the functional 80s ribosome. The phosphorylation state of 4E-BP1 is controlled by the activity of mTOR complex 1 (mTOR1), an AKT-dependent critical regulator or cell size (19). Its it thought that hyperphosphorylation of 4E-BP1 leads to muscle hypertrophy (19), while hypophosphorylation (99) leads to muscle atrophy. Indeed, if mTOR activity is inhibited by the potent competitive inhibitor rapamycin, 4E-BP1 is hypophosphorylated (83), and rates of synthesis are depressed. Therefore, a key marker of mTOR activity and protein synthesis is the phosphorylation state of 4E-BP1.

A second, yet semi-controversial downstream target of mTOR is p70S6 kinase (11). In cell culture, the mTOR inhibitor rapamycin reduces the phosphorylation of

p70S6K^{thr389}, suggests its importance in the Akt/mTOR pathway (79). Inhibition with rapamycin inhibited cap-dependent mRNA translation in cells (79). Interestingly, a p70S6k knockout mouse is still able to maximally activate ribosomal protein S6, the primary target of p70s6 kinase and an integral part in translation. Therefore, S6 activity may be more appropriate indicator of translation than p70s6K. However, while the enclosed studies may assess specific markers of mRNA translation, the primary outcome variable for all treatments is the actual measurement of protein synthesis.

Peptide-chain elongation is another highly regulated cell signal of mRNA translation. Increased cell stress or starvation results in phosphorylation of elongation factor 2^{thr56} (eEF2) limiting the rate of translation by decreasing its affinity to the ribosome (36, 76). Insulin, which activates protein synthesis in a wide range of cell types, induces rapid dephosphorylation of eEF2 allowing elongation to proceed (177). Although little data exists on microgravity effect on eEF2, increased eEF2 mRNA occurs during starvation (155) and hypophosphorylation of eEF2 has been demonstrated in humans following resistance exercise (113), further suggesting its role in protein synthesis (113). Current data suggests that p70 S6 kinase may be the link between mTOR signaling and ultimately eEF2 phosphorylation status (175).

Radiation exposure during spaceflight

Future long duration missions out of lower earth orbit brings the added risk of irradiation from numerous sources. Space radiation consists of a combination of low-linear energy transfer (LET) and high-LET radiations (125). Low-LET from x-ray and *γ-rays* have a reduced penetrating distance and do most of their damage from ionization,

while high-LET from high energy protons, neutrons or nuclei penetrate deep into tissues and create primary tracks of damage in addition to the ionizing effects of radiation (28). Space radiation, which differs from terrestrial radiation sources (consisting mostly of low-LET radiation), has more damaging effects on tissues because it includes high-energy protons found in solar particle events (SPE), galactic cosmic rays (GCR), and high-charge, high-energy nuclei (HZE, e.g., C, O, Ne, Si, Cl, and Fe) (125). Although it is difficult to calculate exact doses, it is predicted that in a long term mission to Mars, astronauts would receive a total radiation exposure higher than the current career maximum levels established by NASA (71). Very little research has been done on radiation's effect on skeletal muscle. Bandstra et al. showed a lower number of small cross-sectional area muscle fibers as well as an increase in central nuclei with simulated GCR (15), suggesting a change in skeletal muscle homeostasis. To date, it is unknown how these alterations impact the translational apparatus in muscle and its concomitant effect on muscle mass.

Despite a paucity of knowledge of radiation effects on muscle, we know HZE causes cellular damage by increasing reactive oxygen species (81). Therefore, we can make some assumptions on skeletal muscle protein synthesis based on antioxidant and reactive oxygen species. Supplementation with antioxidants have been shown to increase protein synthesis during conditions that lead to muscle atrophy (105), as well as restore AKT signaling (189). Although the aims of this study will not directly assess reactive oxygen species formation or damage, it will provide a preliminary evaluation of how

radiation affects skeletal muscle metabolism, which can then be followed up by future mechanistic study designs.

CHAPTER II

RELOADING INCREASES PROTEIN SYNTHESIS

Introduction

The responses to skeletal muscle disuse during hindlimb unloading (HU) have been well documented (30, 78, 114, 115, 139, 140, 162, 164, 165). Muscle atrophy occurs within the first few days (8, 13, 95, 155) corresponding with a loss in force equal to or greater than the percent loss of muscle mass (77). A generally recognizable contributor to muscle strength is the cross-sectional area of muscle mass, and the determinant of muscle mass is dependent on a balance between protein synthesis and degradation. We and others (52, 122, 161) have previously demonstrated that protein synthesis in skeletal muscle *in vitro* was ~35% lower following 4 d of HU (51). Thus, maintenance of muscle mass is prerequisite for optimal muscle performance in the face of microgravity, as a loss of mass and ultimately muscle strength will have deleterious effects on human performance, particularly upon reentry into gravitational fields. Designing effective countermeasures to maintain muscle mass during periods of disuse or microgravity is of paramount importance to the performance and overall safety of the individual.

A previous study in rats using 4 h per day of ambulatory reloading as an overload stimulus countermeasure have maintained muscle mass during 7 d of HU (111). Although (111), which is purported to be a critical determinant of muscle protein synthesis (19). We previously reported that the use of a high-intensity resistance exercise

countermeasure ameliorated microgravity-induced muscle atrophy, and this maintenance of muscle mass was likely due to augmented muscle protein synthesis (51). It has been reported that the increase in protein synthesis following an acute bout of resistance exercise can persist for at least 24 h, and up to 48 h (72, 187). Therefore, countermeasures designed to augment skeletal muscle protein synthesis should be effective in maintaining muscle mass during disuse if protein degradation is negligible.

It is unclear as to whether or not overload per se is sufficient to augment/maintain muscle protein synthesis and mass, or if there are temporal aspects to the countermeasure depending on the intensity employed (i.e., longer durations required for lower intensities). Given a need to reduce time commitment for a given countermeasure, the purpose of this study was to determine if intermittent ambulatory reloading on a short-term basis is adequate to maintain protein synthesis and muscle mass. We hypothesized that one hour a day, similar to a resistance exercise protocol, on days 2 and 4 during hindlimb unloading would elevate skeletal muscle protein synthesis; because skeletal muscle protein synthesis is a primary contributor to the maintenance of skeletal muscle mass (135), we expected an attenuation in the microgravity-induced loss of muscle mass.

Methods

Animals

To complete this study, 6-mo-old Male Sprague-Dawley rats were obtained from Harlan (Houston, TX) and individually housed in a climate-controlled room with a 12-h

light (2100–1100) and dark cycle (1100-2100) in an American Association for Accreditation of Laboratory Animal Care-accredited animal care facility. Water and standard rat chow were provided *ad libitum*. Animals were block-assigned by body mass to cage control (CC), hindlimb unloaded (HU), or hindlimb unloaded plus ambulatory reloading (HU+AR). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Hindlimb unloading and reloading

To simulate the effects of microgravity on skeletal muscle during 5 d of hindlimb unloading (HU), the traditional HU technique (115) was modified according to Knox et al. (86). For the harnessing procedure, all animals were anaesthetized with ketamine/xylazine (60 mg · kg^{-1} and 10 mg · kg^{-1} respectively). An 18 gauge stainless-steel needle was inserted on the ventral side of the tail between the third and fourth caudal vertebrae with special care taken to not injure the ventral artery and vein. With the needle in place, the bevel edge was cut, and a stainless-steel cable was strung through the needle. A thin layer of gauze was wrapped around the needle and encased by 2.54 cm casting tape (insert company name here) in order to hold the needle in place. The stainless steel wire was crossed over the tape, twisted and secured by clamping a grommet. This was secured by a second layer of cast. Animals were allowed to recovery from the procedure and anesthesia and surgical procedure. On day one of the study, the suspension cable was attached to a longitudinal bar across the top of the cage by a swivel and the animal was elevated so that the animal was at an approximate 30° head down tilt and the hindlimbs could not touch the floor.

Animals in the HU+AR were allowed their hindlimbs to be reloaded by normal ambulation for 1 h on days 2 and 4 at 11:00 (**Figure 7**).

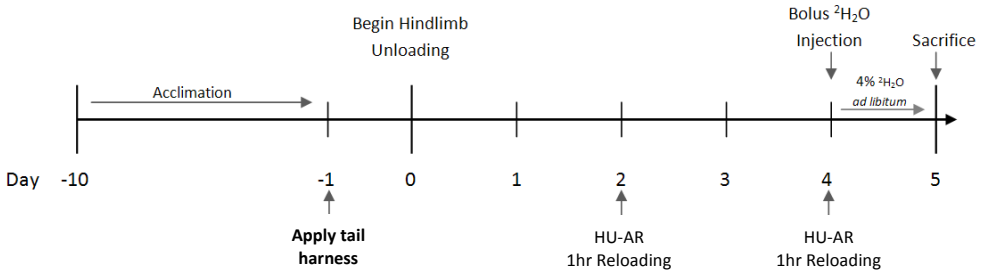

Fig 7. Schematic display of Chapter II study design. Following a 10 d acclimation period, tail suspension apparatus was affixed to the tail of the hindlimb unloaded animals (HU) and animals were allowed to become accustomed to the harness. Animals were unloaded the following day for duration of 5 d. Ambulatory reloading (HU-AR) animals were allowed 1 hour of reloading by allowing voluntary ambulation on days 2 and 4 during the study. For the study of protein synthesis, a bolus I.P. inject of deuterium oxide was given 24 h prior to tissue harvest and 4% deuterium was provided *ad libitum* in the drinking water to maintain a constant dosing.

After the five day hindlimb unloading period, the animals were anaesthetized with ketamine/xylazine (60 mg · kg^{-1} and 10 mg · kg^{-1} respectively). The soleus, plantaris and gastrocnemius muscles were dissected and weighed. The gastrocnemius muscle was visually dissected into the lateral white portion, red medial section, and mixed fiber types. All muscles were snap frozen in liquid nitrogen and stored at -80°C.

Fractional protein synthesis measurements

A common precursor-product methodology for determining skeletal muscle protein synthesis during atrophy in rodents can be accomplished using flooding dose procedures, either *in vivo* (7, 8) or *in vitro* (5). Flooding dose measurements typically

occur between 10 and 45 min (9, 12), and provide the investigator a ‘snap-shot’ of protein synthesis at the time of measure. Although these methods are useful, depending on the question, extrapolation of these shorter-term methods to infer longer-term anabolic states of the muscle can be problematic. To gain a better understanding of how shorter-term treatments affect longer term anabolic states, our lab has employed the administration of deuterium oxide (^2H_2O) to measure cumulative/integrative protein synthesis for periods up to 36 h (10-12, 21). To our knowledge, no studies exist that have examined total integrative responses of muscle protein synthesis in response to intermittent overload. To complete these studies, 24 h prior to tissue harvest, a bolus priming dose of 99.9% deuterium oxide (Cambridge Isotopes, Andover, MA) plus 0.9% NaCl (unless otherwise noted all chemicals are purchased from Sigma-Aldrich , St Louis, MO) w/v was administered intraperitoneal (IP) at a dose of 20.0 µl/g body weight. To maintain a constant introduction of deuterium oxide into the animal, a mixture of 4% ^2H_2O and tap water (v/v) was provided *ad libitum* in the drinking water.

Precursor/labeled products will be determined as previously described by our lab (64). Briefly, ^2H-labeling of body water (^2H_2O; E_{BW}) was measured by gas chromatography-mass spectroscopy (Agilent 7890 GC/5975MSD) following 24 h isotopic exchange between ^2H_2O-enriched plasma samples and acetone (insert reference) (188). Isotope exchange occurred by reacting 20.0 µl of plasma with 2.0 µl of 10 N NaOH and 4.0 µl of a 5% (vol/vol) acetone in acetonitrile. After 24 h, the reaction is stopped by the addition of 600 µl of chloroform and 0.5 g of $NaSO_4$. The following temperature program was used: 60ºC initial, increase by 20°C/min to 100ºC, increase by

50ºC/min to 220ºC, and hold for 1 min. The sample was injected at a spit ratio of 40:1 with a helium flow of 1 mL/min. Selective ion monitoring of mass-to-charge ratios (m/z) 58 (M) and 59 (M + 1) was conducted using a dwell time of 10 ms per ion. ^{2}H acetone elutes at approximately 1.7-1.85 min. The mass spectrometer is operated in electron impact mode (70eV).

Muscle tissue for the incorporated deuterated alanine was prepped by homogenizing 30 mg in 0.4 mL of 10% trichlorcetic acid (TCA) with a Polytron homogenizer (Brinkmann Lab Equipment, Westbury, NY). Samples were centrifuged at 5000 G for 15 min at 4°C. The supernatant containing free amino acid pool of alanine was decanted, 0.4mL of TCA was added to the pellet and the sample was vortexed to break up the pellet. These steps were repeated 3 times ensure the sample was void of free amino acids. A 0.4 mL aliquot of 6 N HCl was added to the pellet and samples were heated at 100°C for 24 h to hydrolyze the proteins into free amino acids. HCl was then evaporated off 0.05 mL of the hydrolysate and then amino acids were derivatized by methyl8 (Fisher Scientific, Waltham, MA). The ratio of protein-bound ^{2}H-alanine to alanine (E_A) in skeletal muscle was measured by gas chromatography-mass spectroscopy (Agilent 7890 GC/5975MSD) (39). The following temperature program was used: 90ºC initial, hold for 5 min, increase by 5°C/min to 130ºC, increase by 40ºC/min o 240ºC, and hold for 5 min. The sample was injected at a spit ratio of 20:1 (5:1 for human tissue) with a helium flow of 1 ml/min. The mass spectrometer was operated in electron impact mode (70eV). Selective ion monitoring of mass-to-charge ratios (*m/z*) 99 (M) and 100 (M + 1) was conducted using a dwell time of 10 ms per ion. ^{2}H alanine elutes at ~ 9 min.

Fractional Synthesis Rate (FSR) was calculated by the equation

$$E_A \times [E_{BW} \times 3.7 \times t\ (h)]^{-1} \times 100$$

where E_A represents amount of protein-bound ^2H alanine (mole % excess), E_{BW} is the quantity of ^2H_2O in body water (mole % excess), and 3.7 represents the exchange of ^2H between body water and alanine (39), and t is time in hours.

Western blotting

Approximated 20-30 mg of pulverized skeletal muscle tissue was with Polytron homogenizer in 0.4mL of lysis buffer (25 mM Hepes, 25mM benzamidine, 10 mM MgCl2, 5mM beta-glycerophosphate, 4 mM EDTA, 2 mM PMSF, .2 mM ATP, 0.5% protease inhibitor cocktail (v/v), 0.1% Triton-X 100, 10 mM activated Na3VO4, and 100 mM NaF, pH 7.4). Samples were placed on ice for 1 hr, then centrifuged at 10,000 G at 4°C for 30 minutes. Supernatant was collected for western blotting. Protein concentration will be determined by bicinchoninic acid assay (150) in order to load similar amounts of total protein per well. Proteins were separated by SDS-PAGE using traditional discontinuous tris-glycine buffer system (31, 93). Separated proteins will then be transferred to nitrocellulose membranes using a semi-dry transfer. Membranes will be blocked using 1% non-fat dry milk/tris-buffered saline (TBS; w/v) for one hour, and then primary antibodies were incubated per manufacturer's instructions. Primary antibodies included Akt, p70 S6 kinase, 4E-BP1, eEF2, eEF2K and ERK1/2 from Cell Signaling Technology (Beverly, MA). Membranes will then be washed 3X in TBS for 5 minutes each, then incubated with appropriate secondary antibody (typically diluted

1:2000) for 1 h at room temperature. Finally, membranes were washed 3X in TBS for five minutes each, then imaged using enhanced chemiluminescence (ECL).

Statistics

All statistics were performed using SigmaStat version 3.5 (Systat software, Chicago, IL). A one-way ANOVA was used to compare the three groups. When significant F-ratios were present, a Student-Newman-Keuls (SNK) post hoc procedure was used to evaluate differences among group means. The significance level was predetermined at $\alpha \leq 0.05$.

Results

Muscle mass is not maintained with intermittent reloading

Five days of hindlimb unloading resulted in a decrease in body weight and muscle mass (**Table 2**). The pre to post change in body mass was significantly different in HU animals compared to cage control (CC) ($p<0.05$), while ambulatory reloading (HU+AR) body mass was significantly less than CC ($p<0.05$) but greater than HU alone ($p<0.05$). There was a significant reduction of total mass in all plantar flexor muscles with HU ($p<0.05$, see **Table 1**) when compared to CC hindlimbs, and intermittent reloading (HU+AR) did not rescue this loss. There were no differences in any of the muscle masses between HU and HU+AR.

Table 2. Change in body and muscle mass following hindlimb unloading and reloading.

	CC	HU	HU+AR
Δ Body Weight (g)	6.50 ± 3.51	-40.80 ± 3.98*	-24.20 ± 5.61*†
Soleus (g)	0.1763 ± 0.004	0.1424 ± 0.006*	0.1486 ± 0.009*
Plantaris (g)	0.4481 ± 0.007	0.3663 ± 0.005*	0.3559 ± 0.019*
Gastrocnemius (g)	2.2383 ± 0.075	1.7633 ± 0.068*	1.7144 ± 0.036*

Values are as means ± SE (CC n=6, HU and HU+AR n=5). Body mass is expressed as a change in body mass from pre to post analyses. Muscle mass (soleus, plantaris, and gastrocnemius) is expressed as wet mass. * Significantly different from CC ($p \leq 0.05$).† Significantly different from HU and CC ($p \leq 0.05$).

Intermittent, ambulatory reloading normalizes fractional protein synthesis during periods of simulated microgravity

The combination of the bolus and drinking water dose of ^2H_2O resulted in plasma ^2H_2O enrichments that were similar among groups. Averages for plasma percent enrichment were 2.25 ± 0.17, 2.64 ± 0.21, 2.64 ± 0.19 in CC, HU, and HU+AR, respectively.

The cumulative daily/integrative FSR in the predominantly slow twitch soleus muscle (**Figure 8**) were significantly lower in the HU group (0.216 ± 0.025) than the CC group (0.381 ± 0.018, $p<0.05$), which is consistent with shorter term measures extrapolating that hindlimb unloading results in an overall reduction of muscle protein synthesis. The addition of intermittent ambulatory reloading (HU+AR) resulted in a rescue of muscle protein synthesis (0.319 ± 0.053) that was comparable to CC ($p>0.05$). FSR in the predominantly fast twitch plantaris muscle (**Figure 9**) were not significantly different among groups ($p>0.05$; CC: 0.286 ± 0.015; HU: 0.243 ± 0.031, and HU+AR: 0.324 ± 0.061).

Similar to results in the soleus muscle, the predominant fast muscle, the gastrocnemius, exhibited reductions of FSR in response to HU, and was completely rescued with the addition of intermittent ambulatory reloading (**Figure 10**). In order to determine if this 'mixed' response in gastrocnemius was regionally or fiber morphologically responsive, the muscle was partitioned into the the lateral/superficial white or medial/deep red portions during the tissue harvest. Consistent with the mixed portions, FSR from these morphologically/regionally distinct regions exhibited similar results to the mixed portions.

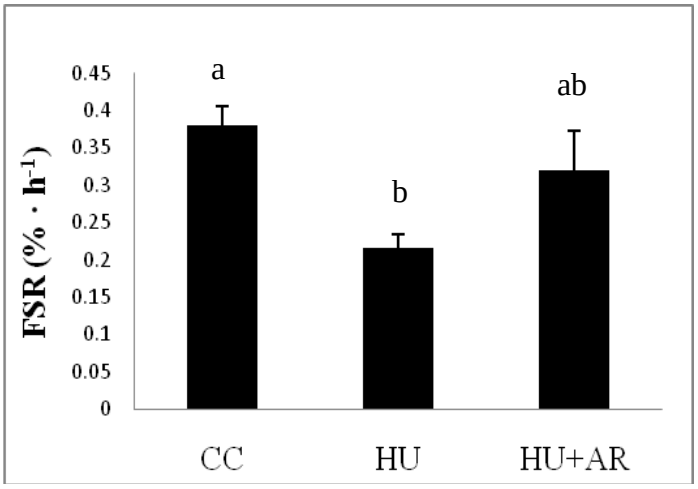

Fig 8. Mixed FSR in the soleus. Mixed frational protein synthesis rates (FSR) of soleus muscle during HU and ambulatory reloading. HU significantly lower that CC ($p<0.05$). HU+AR was not significantly different from CC or HU. Groups not sharing the same letter are significantly different one another. Values are means ± SEM (CC n=6, HU and HU+AR n=5).

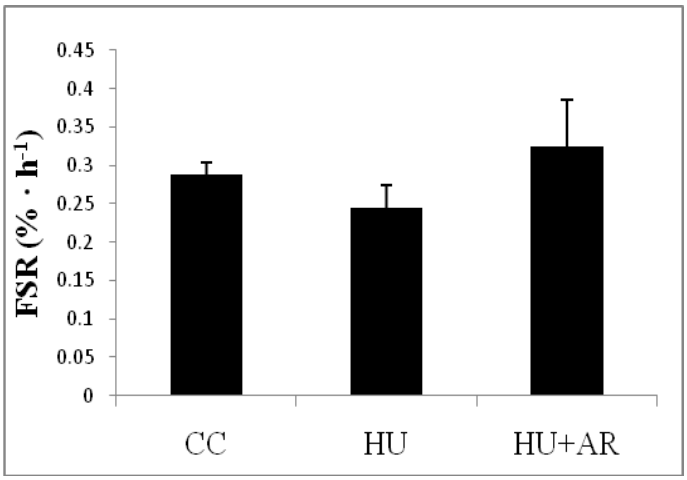

Fig 9. Mixed FSR in the plantaris. Mixed frational protein synthesis rates (FSR) of the plantaris muscle. There were no sigificant differences among groups cage control (CC), unloaded (HU), or reloaded (HU+AR). Values are means ± SEM (CC n=6, HU and HURE n=5).

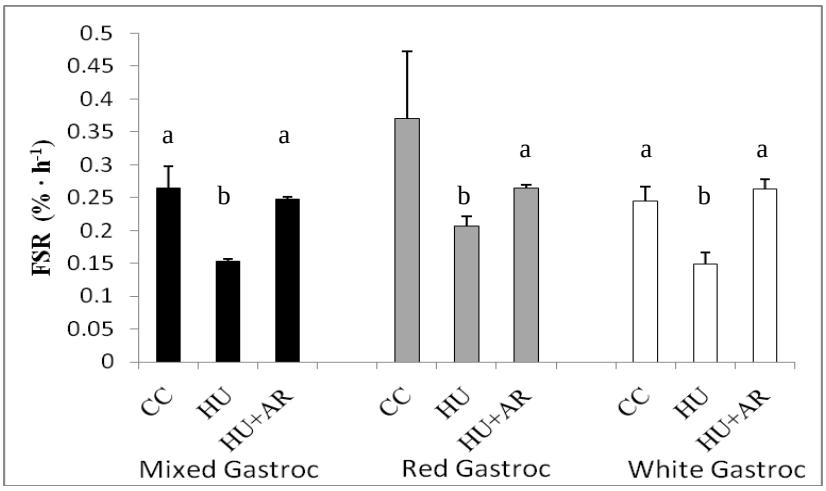

Fig 10. Mixed FSR in mixed, red and white gastrocnemius muscle. FSR supressed following hindlimb unloading (HU) from cage control (CC) in all fiber types that were visually differentiated at tissue harvest. In all types, ambulatory reloading (HU+AR) rescued FSR. Groups not sharing the same letter are significantly different one another. Values are means ± SEM (CC n=6, HU and HU+AR n=5).

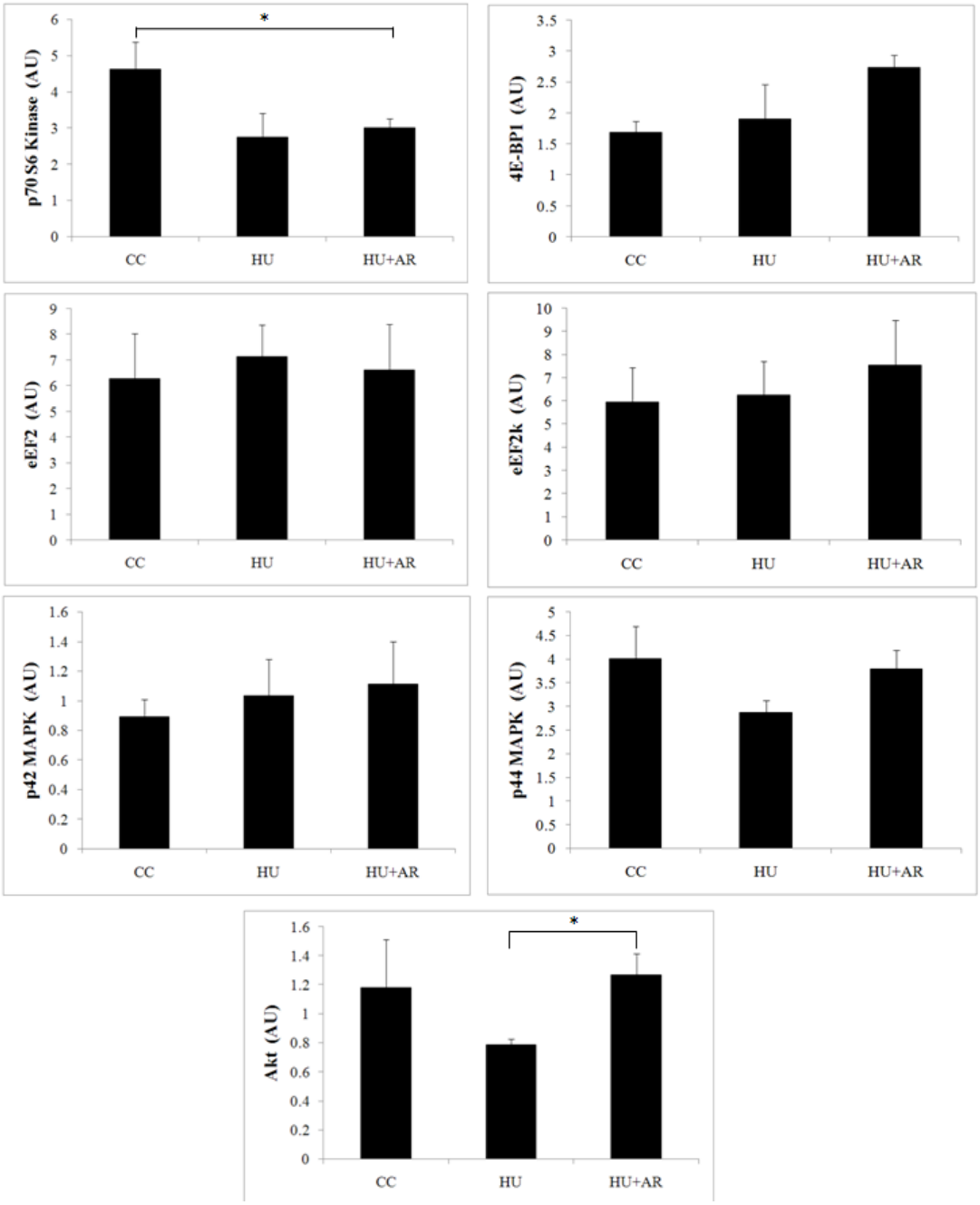

Fig 11. Anabolic signaling markers in the soleus. Akt/mTOR and ERK anabolic signaling pathways are affected by hindlimb unloading and reloading in the soleus. Total protein content in mTOR signaling cascade (Akt, 4E-BP1, p70 S6 kinase, eEF2, and eEF2k) and ERK1/2 (p44 and p42 MAPK) was determined. Significant differences were evident in total Akt content with HU, and were rescued by ambulatory reloading, as well as in p70 S6 kinase there was a significant difference between CC and HU+AR. * denote significant difference ($p < 0.05$). Values are means ± SEM (CC n=6, HU and HU+AR n=5).

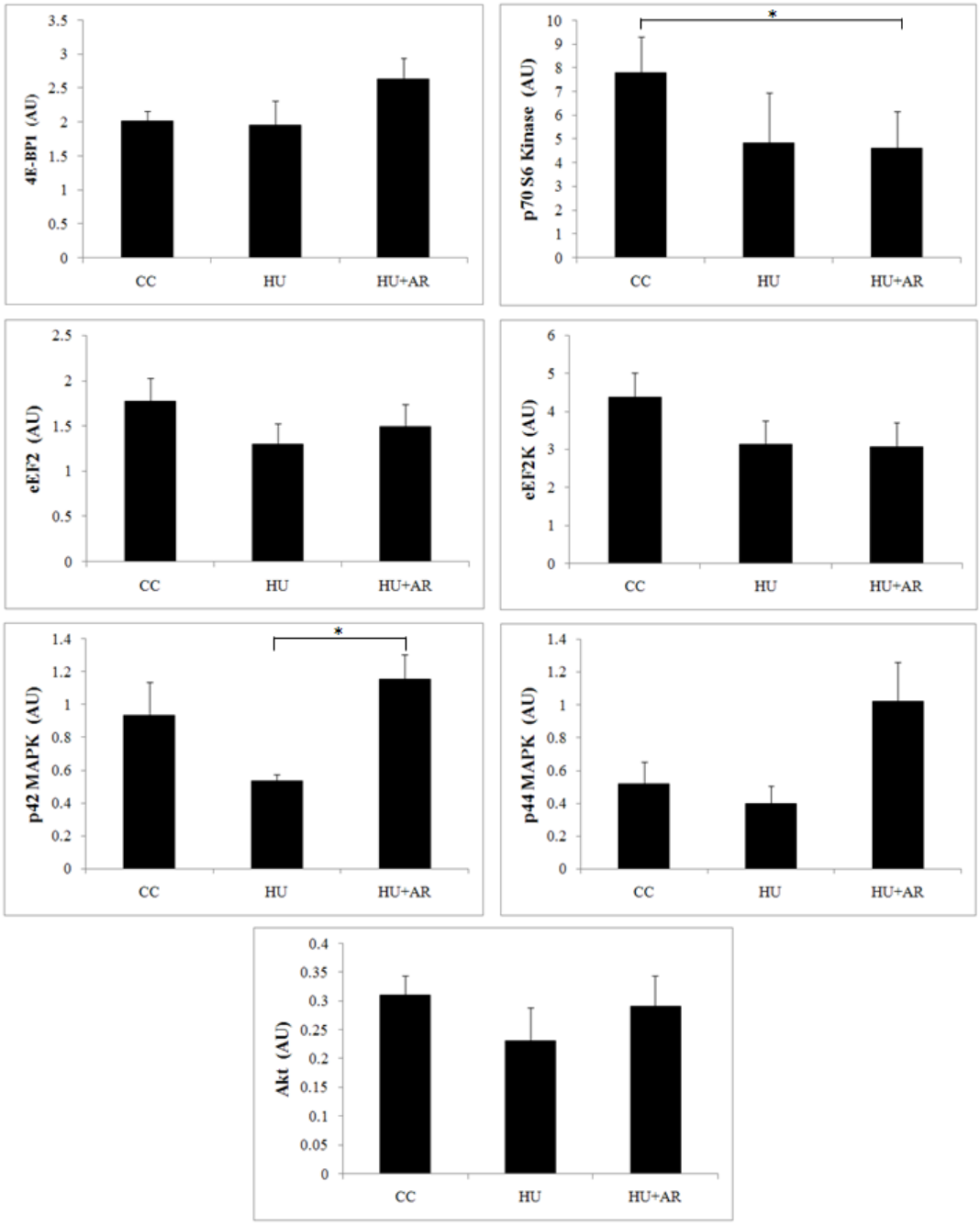

Fig 12. Anabolic signaling markers in the plantaris. Akt/mTOR and ERK anabolic signaling pathways are affected by hindlimb unloading and reloading in the plantaris. Total protein content in mTOR signaling cascade (Akt, 4E-BP1, p70 S6 kinase, eEF2, and eEF2k) and ERK1/2 (p44 and p42 MAPK) was determined. Significant decrements were were evident in total p70 S6 kinase with reloading (HU+AR). A significant increase in ERK signaling between unloading (HU) and HU+AR.* denote significant difference ($p < 0.05$). Values are means ± SEM (CC n=6, HU and HU+AR n=5).

Anabolic signaling potential in the soleus and plantaris muscle suggested decreased potential in HU and HU+AR.

Total protein content in Akt/mTOR and ERK signaling pathways were determined by western blot. In the soleus (**Figure 11**), total protein content of Akt lower in HU than HU+AR ($p<0.05$), which suggests a decrease in anabolic signaling. Conversely, p70s6 kinase, a downstream target of both Akt and mTOR that correlates with protein synthesis, was decreased in HU+AR compared to CC ($p<0.05$), but not significantly different from HU ($p>0.05$). There were no differences in 4E-BP1, eEF2, eEF2k, p44/42 MAPK ($p>0.05$).

In the plantaris (**Figure 12**), there was a decrement in p70s6 kinase protein content in HU+AR compared to CC ($p<0.05$). Akt protein expression was lower in HU compared to HU+AR ($p<0.05$). There were no differences in 4E-BP1, eEF2, eEF2k, p44/42 MAPK ($p>0.05$). A similar pattern of expression can noted between protein expression in soleus and plantaris.

Discussion

The most important finding of this study was that we observed that hindlimb unloading results in decreased integrative/cumulative protein synthesis, which is consistent with previous studies using flooding dose-type *in vitro* (51, 52) and *in vivo* (13, 64, 80, 103) measurements collected during shorter-term periods in rats. The strength of using the deuterium oxide methodology is that the tracer incorporation occurred over the entire 24-h period prior to the harvest of tissues, which included the

period of time where the animal was reloaded and/or unloaded. To our knowledge, these studies represent the first time that integrative responses of mixed muscle FSR have been made in conscious rats during periods of hindlimb unloading. Furthermore, although we elevations of muscle protein synthesis were observed in most muscles with intermittent ambulatory reloading, these improvements were not adequate to maintain/restore mass during that period of time. These findings provide important insight regarding the control of muscle mass as it relates to muscle protein synthesis during disuse muscle atrophy.

There was a fiber type specific difference in protein synthesis, in that predominantly slow twitch soleus muscle and medial red gastrocnemius had average FSR values of 0.375 $\%\cdot h^{-1}$ and 0.370 $\%\cdot h^{-1}$, respectively. The primarily fast twitch plantaris and lateral white gastrocnemius averaged FSR values of 0.28%$\cdot h^{-1}$ and 0.26%$\cdot h^{-1}$, respectively. Dickenson et al. showed a similar response in humans, in that there was 33% difference in FSR between type 1 and type IIa fibers in vastus lateralis biopsies (35). Here we demonstrate an ~25% difference between muscle groups largely consisting of a similar fiber type (9).This is likely due to activation and use of muscle as the slow twitch fibers are typically active at rest and fast fiber types are only recruited for strenuous activity (130).

Despite increasing protein synthesis in soleus and superficial, deep and mixed gastrocnemius muscles, wet mass was not rescued. The ambulatory reloading likely created an overload situation which normalized the response of protein synthesis, and this response was persistent throughout the 24 h period. However, it is equally plausible

that the hindlimb unloading days that were interspersed throughout the protocol resulted in the reduced FSR, effectively negating the positive effects of intermittent reloading. This supports the work of D'Aunno et al. (29), who used four 15 minute sessions per day of ambulatory reloading over 7 d of hindlimb suspension and observed only a 10% decrease in muscle mass as compared to hindlimb unloaded control animals that lost an average of 26%. To expand on that work, 4 h/d of ambulatory reloading was able to fully mitigate muscle mass lost over 7 d (111) and 28 d of unloading (191); however, this represented a considerable time commitment away from the hindlimb unloaded environment, which may not be readily generalizable in studies simulating microgravity. Although the 7 d study (111) did not measure protein synthesis, they determined AKT/mTOR signaling corresponded to muscle mass. Based on observations from the present study, we predict that repeated sessions of ambulatory reloading facilitated an elevation/normalization of FSR, which was almost adequate to maintain mass.

Interestingly, in the earlier study (29), overload placed on the muscles with accelerated gravity by centrifugation resulted in a greater degree of atrophy (16%) than loading alone, which was possibly due to the increased stress of centrifugation. We base this opinion on the basis of prior work from our laboratory which demonstrated augmented rates of synthesis in comparison to control and a complete mitigation of muscle atrophy when high intensity exercise was briefly performed only twice over a 5 d period (51). However, it is unknown if the augmented rates of synthesis persisted over a prolonged period time due to the short-duration of the measure (*in vitro*), and the impetus, at least in part, for the development of longer term assessments (62). More

work is warranted to systematically assess the influence of intermittent reloading on muscle protein synthesis to determine whether the lack of sufficient recovery/maintenance of muscle mass is due to the transient nature of the muscle anabolic response to gravity or some other advantage that muscle overload provides in addition to muscle protein synthesis.

One possible advantage to overload is its purported effects muscle protein degradation. The current study provides a unique instance in which protein synthesis was elevated, but muscle mass was not, suggesting the possibility that the continued loss of muscle mass could result from an elevation of degradative pathways (108, 118). We previously demonstrated that high intensity overload markedly reduces several of the hindlimb-induced elevations of muscle protein degradation (41). Although not systematically addressed using proteolytic measures, those results suggested that high-intensity muscle overload had a protective effect on muscle proteins. While protein degradation may provides a compelling hypothesis for why muscle mass was not maintained/augmented with intermittent reambulation, these results would also indicate that intermittent reambulation also facilitated degradation as well. This study did not include measures of protein degradation, but it is unlikely that normal ambulation facilitated an elevation of muscle protein degradation to the same degree that rates of synthesis were facilitated, making it difficult to reconcile why the elevated rates of synthesis observed over the entire 24 h period on days including reamblation were not sufficient to elevate mass above HU alone.

In conclusion, we demonstrated that cumulative muscle protein synthesis measured by deuterium oxide was decreased during hindlimb unloading. We were able to increase protein synthesis to the level of control animals using a simplistic approach of intermittent ambulatory reloading, but this did not result in attenuation of atrophy. Therefore, while muscle protein synthesis may be a critical component of skeletal muscle adaptation, other components leading to the maintenance of muscle mass are likely to contribute.

CHAPTER III

PARTIAL GRAVITY LOADING OVER 21 DAYS ATTENUATES MUSCLE ATROPHY IN COMPARISON TO UNLOADING

Introduction

It has been almost three decades since man last walked on the moon; however, aspirations for another lunar mission and eventual transport to Mars are being considered (128). Since the beginning of the manned space exploration, scientists have investigated physiological mechanisms associated with the consequences of microgravity (21). One detrimental adaptation is rapid muscle atrophy of knee extensors and foot plantar flexors (167). For example, 14 d of simulated microgravity with hindlimb unloading in rodents have resulted in a ~25-35% decrease in soleus muscle mass (43, 55, 68, 77, 144), which can partially be attributed to decreased mixed muscle protein synthesis (51). Conversely, protein synthesis is increased during overload with acute resistance exercise (53, 54, 136) and chronic training results in hypertrophy (163). The effects of partial weight bearing, similar to that of the moon (16% of Earth's gravity) and Mars (38% of Earth's gravity) on skeletal muscle metabolism, have not been examined.

Skeletal muscle protein synthesis is controlled primarily by AKT/mTOR and MAPK signaling pathways, both of which are responsive to mechanical load. mTOR has been shown to be sensitive to mechanical load (74) and stretch (73), and the activation of the mTOR pathway results in phosphorylation of 4E-BP1 and ribosomal protein s6 which increases translation initiation (84). Extracellular regulated kinase (ERK1/2),

substrates of MAPK, are not typically activated when the muscle is quiescent, but up-regulated following periods of muscle activity (51, 53). Phosphorylation of the ERKs has been shown to be decreased during hindlimb unloading (43). Therefore reductions of muscle activity may result in reduced mTOR and ERK signaling, leading to decrements of muscle protein synthesis, and ultimately, muscle mass, It is unknown how these important signaling mechanisms are affected by chronic partial loading.

Skeletal muscle is responsive to low load during disuse. Four bouts totaling one hour per day of acceleration at 1.2xG in a centrifuge or ambulatory reloading or attenuated muscle loss during 7 d of hindlimb unloading (29). Similarly, 4 h/d over the same timeframe was able to completely mitigate muscle atrophy (111), but 1 h/d of intermittent reloading was not adequate to maintain muscle mass (see Chapter II), suggesting that increased exposure, at least at 1xG, may be necessary for attenuating losses of muscle mass with microgravity. However, muscle responses to chronic low load are unknown. Recently, Wagner et al. developed a murine model allowing the ability titrate body weight based loading for partial weight suspension (174). Mars-analogue loading (~38% of Earth's gravitational field) resulted in a 23% decrease in gastrocnemius muscle of mice (174), but partial weight bearing was not compared to hindlimb unloading, nor were multiple loading percentages investigated to determine if muscle mass atrophy is linear over a range of loads.

The purpose of this experiment was to investigate the effect of chronic partial weight suspension on skeletal muscle mass and protein synthesis. We hypothesized that decrements in gravitational load would lead to linear decreases in muscle mass. Since

rates of protein synthesis are purported to be coupled to muscle mass, we hypothesized that alterations of muscle mass in response to chronic loading would be accompanied by concomitant changes in muscle protein synthesis.

Methods

Animals

Four mo-old female BALB/cByJ were obtained from Harlan (Houston, TX) and individually housed in a climate-controlled room with a 12:12 h light-dark cycle. Water and standard rodent chow were provided *ad libitum*. This research project was approved by the Institutional Animal Care and Use Committee of Texas A&M University. All procedures on animals were performed in accordance with institutional guidelines for the care and use of laboratory animals.

Following a 14 d acclimation period, mice were block-assigned by body mass into four loading groups: ambulatory control (1 G), one-third body weight gravitational load (G/3), one sixth body weight gravitational load (G/6), and non-weight bearing hindlimb unloaded (0 G). Animals were suspended for 21 d in order to understand the long term effects on unloading on skeletal muscle.

Partial and traditional suspension

G/3 and G/6 were suspended in a specially-designed, full body, horizontal passive suspension devise, previously described by Wagner et al (174). The harness apparatus was fitted onto the with mice while anaesthetized by 2% isofluorane (US Pharmacopeia, Rockville, MD) with 0.8-1 mL/min oxygen flow rate. To achieve partial

suspension, the forelimbs were placed in a tight fitting mole-skin jacket that was secured by Velcro at the scapula. An adhesive steri-strip (3M, St. Paul, MN) bandage was wrapped around the base of the tail to minimize skin irritation and covered by athletic tape to create loop, with could be used to attach the tail to the harness. The base of the tail and jacket were then connected by a stainless steel bar that was positioned parallel to the vertebrae. Animals were then suspended by attaching the harness apparatus to a variable tension spring (k ~1.6 N/m, Statico, San Carlos, CA). Tension on the spring was adjusted by connecting the spring to a eye-loop screw. Subtle manipulations of load were accomplished by loosening or tightening the screw. The overall harness system was connected to a steel rod that supported a small nylon wheel and a low-friction bearing to provide linear freedom of motion for the suspended animals, but prevented the animal from reaching the side-walls of the cage.

The cage was constructed using 30.48 cm x 30.48 cm Plexiglas with perforated floors, used to provide mice easier quadripedal ambulation as reported by Wagner et al (174). Body weight was checked and adjusted daily by weighing the animals on a suspended platform to prevent full loading (115). Body mass was titrated to within 1% of calculated weight by adjustment of the spring. Coefficient of variation for partial gravity titration for G/6 was ± 0.16% and G/3 was ± 0.34%.

Animals used to simulate 0 G animals were suspended by the traditional hindlimb suspension technique first developed and updated by Morey-Holton et al (114). Briefly, mice were anaesthetized with isofluorane, and a loop was created by gluing a thin strip of cloth the entire length of the lateral sides of the tail. Animals were raised by

the tail until the back legs were could no longer touch the cage floor, resulting in ~30° head down tilt. The loop at the tip of the tail was connected to a swivel and cable which attached to a similar horizontal bar and low friction wheel, as described above. 0 G (cube) and 1G (shoebox style) cages also used the perforated floor to maintain consistency among groups.

Following the 21 d experimental protocol, animals were anaesthetized with ketamine/xylazine 60 mg · kg^{-1} and 10 mg · kg^{-1} respectively). Approximately 0.75 mL of blood was drawn via cardiac puncture, allowed to clot and then centrifuged at 2000 x g for collection of blood serum. Gastrocnemius muscle was excised, immediately weighed for wet muscle mass, and then snap-frozen in liquid nitrogen. Samples were stored at -80°C until analyses. Gastrocnemius muscle was used for measurements due to the quantity of tissue (~20 mg) needed for FSR measurements.

Determining cumulative fractional protein synthesis (FSR)

Cumulative FSR in the gastrocnemius was determined using deuterium oxide as previously published by our lab (63-65, 127). Twenty-four hours prior to tissue harvest, a bolus priming dose of 99.9% ‘salinized’ (0.9% NaCl) deuterium oxide (Cambridge Isotopes, Andover, MA) was administered intraperitoneal (IP) at a dose of 20 µl/g body weight (w/v) to establish a deuterium oxide enrichment at 4% of the total body water. To maintain a constant enrichment of deuterium oxide, drinking water was subsequently enriched to 4% $^{2}H_2O$ and provided *ad libitum*. Unless otherwise noted all chemicals were purchased from Sigma-Aldrich (St Louis, MO).

^{2}H-labeling of body water ($^{2}H_2O$; E_{BW}) was measured by gas chromatography-mass spectroscopy (Agilent 7890 GC/5975MSD, Santa Clara, CA), following a 24 h isotopic exchange between $^{2}H_2O$-enriched serum samples and acetone (188). Isotope exchange occurred by reacting 0.02 mL of plasma with 0.002 mL of 10 N NaOH and 0.004 mL of 5% (vol/vol) acetone in acetonitrile. After 24 h, the reaction was stopped by the addition of 0.6 ml of chloroform and 0.5 g of $NaSO_4$. Samples (0.1 mL) were applied to a gas chromatography column and separated by size using helium as the carrier gas. Temperature settings were as follows: temperature was initially set at 60ºC, increased by 20°C/min to 100ºC, increased by 50ºC/min to 220ºC thereafter with a 'hold' for 1 min. The sample was injected at a split ratio of 40:1 with a helium flow of 1 mL/min. Samples were then subjected to mass spectrometry, which determines mass-to-charge ratios of compounds and can differentiate between deuterated and non-deuterated compounds. Selective ion monitoring of mass-to-charge ratios (m/z) 58 (M, acetone) and 59 (M + 1, deuterated acetone) was conducted using a dwell time of 10 ms per ion. ^{2}H acetone elutes at approximately 1.7-1.85 min. The mass spectrometer was operated in electron impact mode (70eV).

Muscle protein synthesis was assessed by measuring the incorporation of deuterated-alanine into acid-precipitable skeletal muscle proteins, as described previously (64). Briefly, muscle tissue was prepped by homogenizing 30mg in 0.4mL of 10% trichlorocetic acid (TCA) with a Polytron homogenizer (Brinkmann Lab Equipment, Westbury, NY). Samples were centrifuged at 5000 g for 15 mins at 4°C. The supernate containing free amino acid pool of alanine was decanted (discarded), 0.4 mL

of TCA was added to the pellet and the sample was vortexed to break up the pellet. These steps were repeated three times to ensure the sample was void of free amino acids. 0.4 mL of 6 N HCl was added to the pellet and samples were heated at 100°C for 24 h to hydrolyze the proteins into free amino acids. HCl was then evaporated off 0.05 mL of the hydrolysate. The resulting amino acids were derivatized by methyl-8 (Fisher Scientific, Waltham, MA) just prior to analyses. The ratio of protein-bound ^{2}H-alanine to unlabeled alanine (E_A) in skeletal muscle was measured by GC/MS (Agilent 7890 GC/5975MSD, Santa Clara, CA) (39). The following temperature program was used for separation: 90ºC initial (held for 5 min), increased by 5°C/min to 130ºC, and then increased by 40ºC/min to 240ºC, and held for 5 min. The sample was injected at a split ratio of 20:1 with a helium flow of 1 ml/min. The mass spectrometer was operated in electron impact mode (70eV). Selective ion monitoring of mass-to-charge ratios (*m/z*) 99 (M, alanine) and 100 (M + 1, deuterated alanine) was conducted using a dwell time of 10 ms per ion. ^{2}H-alanine eluted at ~ 9 min.

FSR was calculated by the equation:

$$E_A \times [E_{BW} \times 3.7 \times t\ (h)]^{-1} \times 100$$

where E_A represents amount of protein-bound ^{2}H alanine (mole % excess), E_{BW} is the quantity of $^{2}H_2O$ in body water (mole % excess), and 3.7 represents the exchange of ^{2}H between body water and alanine (39), and t is time in hours.

Western blotting

Approximately 30 mg of pulverized skeletal muscle tissue were homogenized with Polytron homogenizer in 0.4 mL of lysis buffer (25 mM Hepes, 25mM

benzamidine, 10 mM $MgCl_2$, 5 mM beta-glycerophosphate, 4 mM EDTA, 2 mM PMSF, 0.2 mM ATP, 0.5% protease inhibitor cocktail (v/v), 0.1% Triton-X 100, 10 mM activated Na_3VO_4, and 100 mM NaF, pH 7.4). Samples were placed on ice for 1 h, and then centrifuged at 10,000 G at 4°C for 30 min. Supernate was collected for western blotting analyses. Protein concentration was determined by bicinchoninic acid assay (150). Proteins were separated by SDS-PAGE using traditional discontinuous Tris-glycine buffer system. Separated proteins were transferred to nitrocellulose membranes using a semi-dry transfer. Membranes were blocked using 1% non-fat dry milk/tris-buffered saline (TBS; w/v) for 1 h, and then primary antibodies were incubated per manufacturer's recommendations. Phospho/total rpS6, phospho/total 4E-BP1, phospho/total eEF2k, and phospho/total ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA). Membranes were washed three times in TBS for 5 min each, then incubated with appropriate secondary antibody (typically diluted 1:2000) for 1 h at room temperature. Finally, membranes were washed 3X in TBS for five minutes each, then imaged using enhanced chemiluminescence (ECL). Blots were analyzed using an imaging system from AlphaInnotech using Fluorochem software (San Leandro, CA).

Statistics

Data were analyzed using SigmaStat (v3.5, Systat software, Chicago, IL). A one-way ANOVA was used to compare groups. When significant F-ratios were present, a Student-Newman-Keuls (SNK) post hoc procedure was used to evaluate differences among group means. The significance level was predetermined at $\alpha<0.05$.

Results

Body and muscle mass

G/3 and G/6 resulted in a 4.2% and 4.1% decrease in body mass but partial suspension maintained muscle mass throughout the 21 d duration (**Table 3**). There were no differences between G/3 or G/6 at any time point ($p>0.05$). In 0 G, body mass was significantly decreased after 14 d ($p<0.05$) and resulted in a overall 7% decrease in body mass after 21 d ($p<0.01$). Body mass was significantly different at day 14 between partial suspension groups and 0 G ($p<0.05$), but not different after 21 d ($p>0.05$). 1 G animals experiences a non-significant 4.7% weight gain during the study, but this resulted in significantly larger body mass than the three suspension groups ($p<0.05$).

Suspension (G/3, G/6, and 0 G) resulted in a lower plantar flexor muscle mass, made up of the soleus, plantaris, and gastrocnemius, compared to 1 G. Individually, muscle masses were as follows: soleus, a muscle known to be very sensitive to unweighting (165), displayed a linear response over the four loading groups in that G/3, G/6, and 0 G were significantly less than 1 G, G/3 was significantly greater than 0 G, and G/6 was not significantly different from G/3 or 0 G. Suspension in the plantaris resulted in significantly lower in suspension groups, but not different among the suspension groups. Gastrocnemius muscle exhibited a 20% and 17.3% difference between 1G in G/3 and G/6 respectively and a 28.5% difference in 0 G. Therefore, G/3 and G/6 were significantly lower than 1 G ($p<0.01$), but not different from each other ($p>0.05$), and both were greater than 0 G ($p<0.01$).

Table 3. Body and muscle mass with 21 d of 1 G, G/3, G/6 and 0 G loading

	1 G (n=11)	G/3 (n=11)	G/6 (n=11)	0 G (n=10)
Body Mass (g)				
Day 0	23.08 ± 0.31^{b}	22.63 ± 0.49^{b}	22.47 ± 0.36^{b}	22.57 ± 0.29^{b}
Day 7	24.27 ± 0.45^{a}	22.62 ± 0.52^{bc}	22.95 ± 0.50^{b}	21.41 ± 0.41^{c}
Day 14	$24.53 \pm 0.31^{*a}$	22.09 ± 0.54^{b}	22.20 ± 0.38^{b}	$20.49 \pm 0.54^{*c}$
Day 21	24.16 ± 0.43^{a}	21.76 ± 0.55^{b}	21.56 ± 0.37^{b}	$20.81 \pm 0.46^{*b}$
Body Mass Change (g)	1.09 ± 0.22^{a}	-0.87 ± 0.32^{o}	-0.91 ± 0.23^{o}	-1.76 ± 0.59^{o}
Plantarflexor Muscle Masses (mg)				
Soleus	5.89 ± 0.25^{a}	4.41 ± 0.26^{b}	3.86 ± 0.16^{bc}	3.16 ± 0.20^{c}
Plantaris	14.85 ± 0.38^{a}	12.74 ± 0.58^{b}	12.73 ± 0.31^{b}	12.21 ± 0.50^{b}
Gastrocnemius	107.65 ± 2.04^{a}	86.14 ± 2.73^{b}	88.93 ± 2.10^{b}	76.94 ± 3.60^{c}
Total Plantar Flexor Mass	128.39 ± 2.32^{a}	103.29 ± 3.01^{b}	105.52 ± 2.08^{b}	92.30 ± 3.66^{c}
Relative Total Mass (mg/g BM)	5.32 ± 0.10^{a}	4.75 ± 0.07^{b}	4.90 ± 0.09^{b}	4.43 ± 0.11^{c}
Gastrocnemius Protein Content (mg)	2.294 ± 2.04^{a}	2.078 ± 1.65^{a}	2.323 ± 2.03^{a}	1.949 ± 1.08^{a}
Gastrocnemius Protein Concentration (ug/wet mass)	114.54 ± 13.36^{a}	105.50 ± 24.24^{a}	119.34 ± 17.56^{a}	108.26 ± 5.28^{a}

Body mass was measured 0, 7, 14 and 21 d in partial 1 G, G/3 and G/6 partial suspension and 0 G unloading. In 1 G, there was gain in body mass over the 21 days experiment. Both partial suspension groups maintained mass throughout the study, so that by the end of 21 d, they were not different from 1 G or 0 G, despite the latter losing body mass. Muscle mass of the plantar flexors (soleus, plantaris, and gastrocnemius) measured at the end of the study demonstrated an attenuation of muscle lost compared to 0 G unloaded animals. Values are mean ± SEM. * denotes body mass was significantly different from day 0 within each group. Values not sharing the same letter are significantly different from each other.

Cumulative muscle protein synthesis

$^{2}H_2O$ plasma enrichment was significantly different among groups (data not shown). G/3 and G/6, 2.21 and 2.22%, respectively were significantly lower ($p<0.05$) than 1G (2.36%) and 0 G (2.57%), which were not different from each other ($p>0.05$). Mixed muscle homogenate protein synthesis (FSR) in the gastrocnemius measured over 24 h resulted in significantly lower protein synthesis in 0 G only (**Figure 13**). Compared to 1 G (0.217%·$h^{-1)}$, FSR for G/3 and G/6 (0.248 and 0.226 %·h^{-1}, respectively) were not significantly different ($p>0.05$), but 0 G (0.158%·h^{-1}) was significantly lower than the three other groups ($p<0.01$).

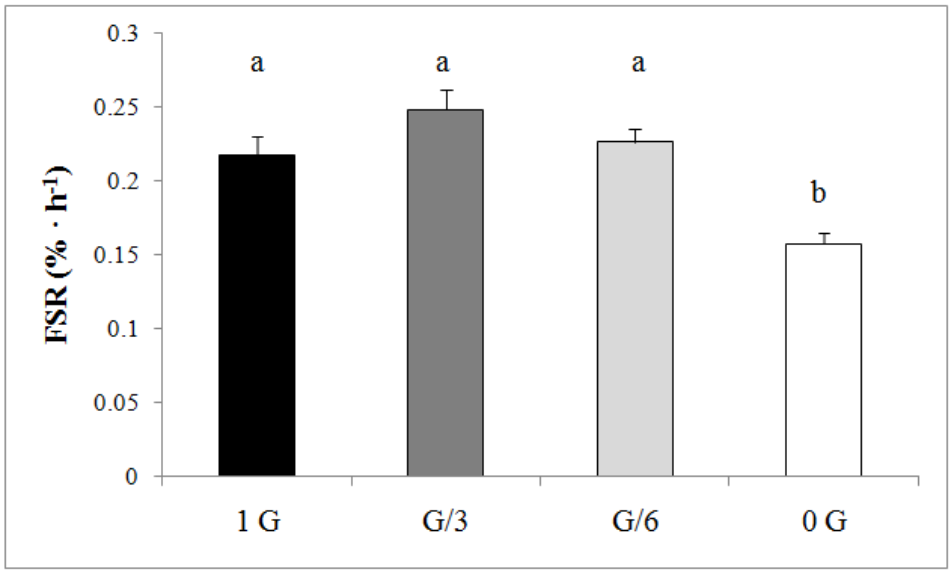

Fig 13. Gastrocnemius mixed muscle FSR across different levels of load. Cumulative muscle protein synthesis (FSR) in 1 G loading (n=11), partial weight suspension at G/3 (n=8) and G/6 (n=11) and 0 G unloading (n=7). FSR is suppressed following 21 days of 0 G unloading compared to partial unloading. Partial loading at G/3 and G/6 resulted in similar FSR responses when compared to 1 G. Values are mean ± SEM. Groups not sharing the same letter are different from each other ($p<0.05$).

The myofibrillar rich fraction of gastrocnemius muscle was analyzed to understand FSR of proteins primarily associated with the contractile apparatus. Comparisons of data from these groups did not achieve statistical significance (p=0.055, **Figure 14**) in the overall model or using pair-wise comparisons. Further, the data presented clearly indicate that myofibrillar FSR is not suppressed with 0G, as 0G and 1G values are similar (p>0.05), suggesting that myofibrillar fractional synthesis is remarkably conserved during periods of chronic unloading.

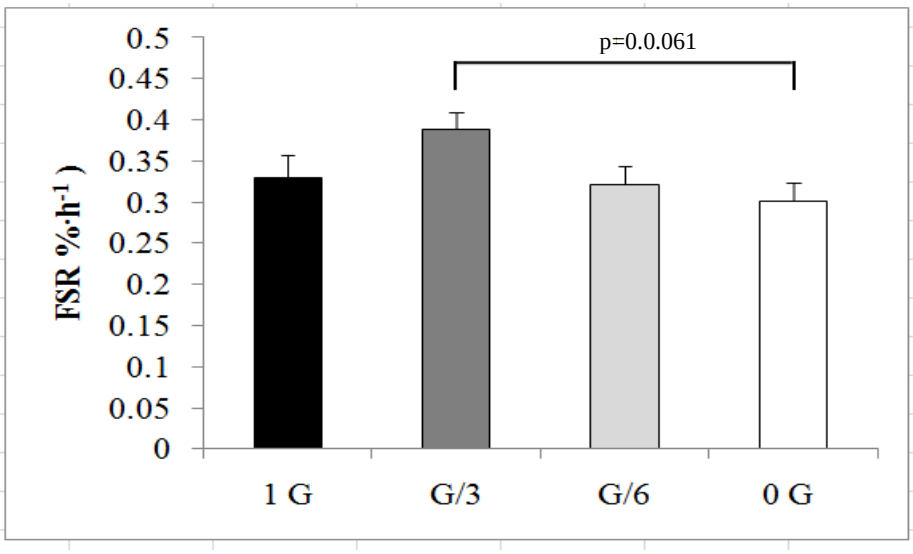

Fig 14. Gastrocnemius myofibrillar FSR with different levels of load. Cumulative myofibrillar protein synthesis (FSR) in 1 G loading (n=8), partial weight suspension at G/3 (n=11) and G/6 (n=8) and 0 G unloading (n=7). One way ANOVA was used to compare groups (p=0.55). FSR for each was not different for each group when compared to 1 G. Values are mean ± SEM.

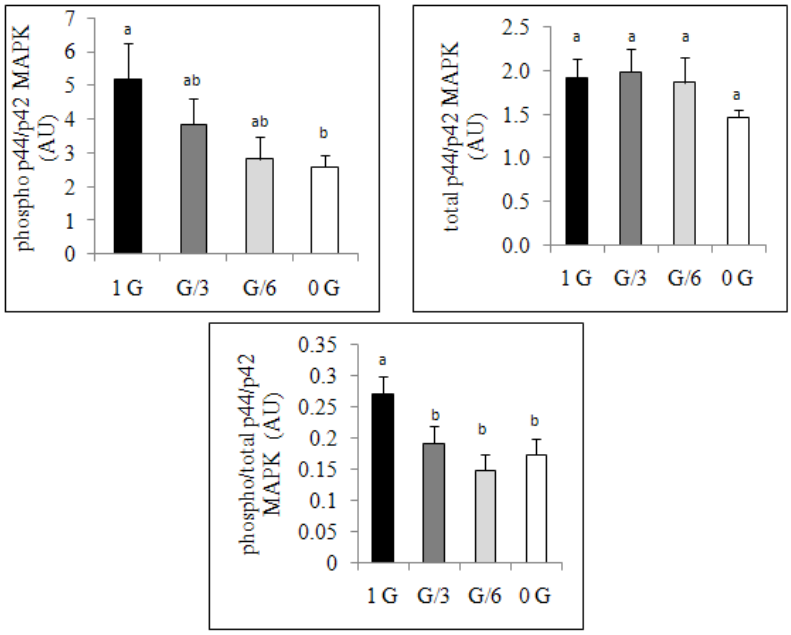

Fig 15. Anabolic signaling markers in the MAPK pathway. ERK1/2 signaling is down regulated with load. Phosphorylated ERK1/2 was decreased in 0 G unloaded animals, while total protein content was not altered with unloading. The ratio of phospho/total protein was significantly greater in 1 G than any of the partial loading or 0 G. Values are means ± SEM. Groups not sharing the same letter are different from each other.

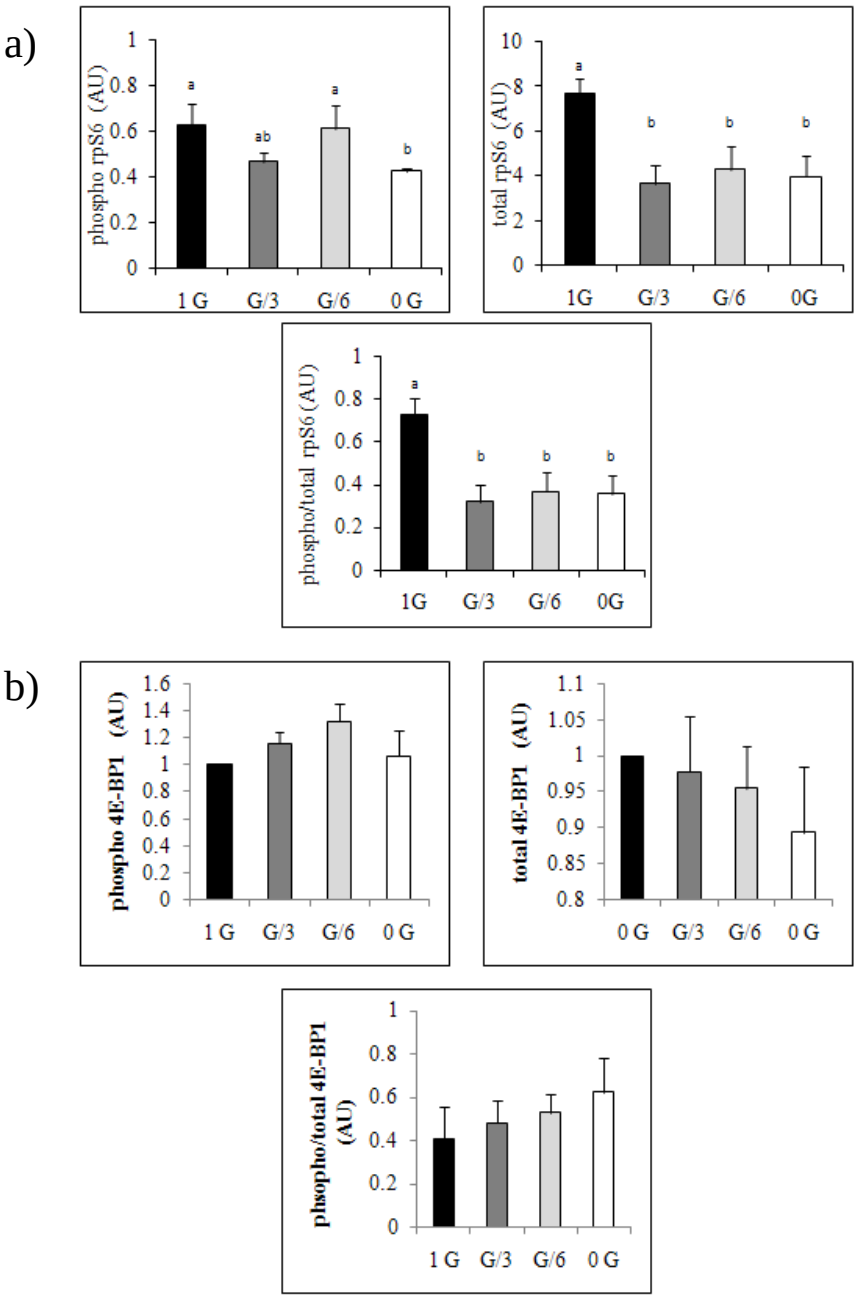

Fig 16. Anabolic signaling markers in Akt/mTor pathway. Protein content of a) Ribosomal protein S6 (rpS6) and b) 4E-BP1 are select downstream markers of mTOR signaling. Phosphorylated S6 was greater in 1 G and G/6 than 0 G. There was a down regulation of protein content in G/3, G/6, and 0 G, and the ratio shows a similar pattern. There were no significant differences in phospho, total, or the ratio between the two in 4E-BP1. Values are represented as means ± SEM. Groups not sharing the same letter are different from each other.

Protein markers of protein synthesis

ERK1/2 was down-regulated in unloading (**Figure 15**). Specifically, 1 G was significantly different from 0 G. G/3 and G/6 phosphorylation state was in between 1 G and 0 G such that they were not different from either. Total protein content was significantly decreased only in 0 G. Overall, partial or unloading resulted in decreased phosphor to total ratio.

mTOR signaling was determined by two downstream targets ribosomal protein S6 and 4E-BP1 (**Figure 16**). rpS6 phosphorylation was decreased in 0 G compared to 1 G and G/6. G/3 phosphorylation state was not different from any group. There was a decrease in total protein content in all three unloading groups, which results in a significantly larger phospho/total ratio in 1 G. There was no significant difference with 4E-BP1.

Discussion

For the first time, we present data on skeletal muscle mass over a range of chronic loading gravitational parameters, and demonstrate partial loading at 1/3rd of body weight is not a sufficient stimulus to maintain muscle mass. Gastrocnemius muscle mass data compares favorably to original model during 21 d of partial loading (174). In that study, 21 d of G/3 resulted in a 23% decrement in gastrocnemius muscle mass (174), while we observed a 20% decrease in the present study. There were no differences in body weight, muscle mass, or protein synthesis between G/3 and G/6. This may suggest that there are threshold levels for muscle adaptation when dealing with forces less than 1

G, particularly since it appears that the forces applied between G/3 and G/6 were not different enough to further attenuate muscle mass. If a threshold of load is required to maintain muscle mass, future studies examining higher loader could provide a minimal percent load required for maintenance of muscle mass. A second possibility is there are distinct levels of load for determining muscle mass, and in these experiments G/3 and G/6 were on similar levels; perhaps 50% loading may have further attenuated muscle mass loss. A final possibility is the sensitivity of the model did not allow detection of subtle changes between these two partial G environments. Wagner et al. showed an overnight drift average of 2.5 ± 4.9% true body mass (174). Peak vertical ground reaction forces in this model for G/3 are similar to predicted values for G/6 (174). Future studies, therefore should examine the effects of a wider range of loading on muscle. Regardless, the present study demonstrated that while partial G environments are adequate to attenuate losses of muscle mass when compared to simulated 0 G, chronic loading at partial G was not sufficient to maintain muscle mass at 1G levels. These results have important implications related to long-term space flight where astronauts may be repeatedly exposed to partial, negligible or 1 G gravitational environment, as well as the remarkable capacity of muscle to adjust to its loading environment.

Skeletal muscle protein synthesis is indicated as a primary cause of skeletal muscle atrophy during disuse and disease (135) and has been implicated as the root cause of disuse atrophy in humans (33, 138). Despite the dispute over the causes of muscle atrophy, it is generally agreed that changes (or lack thereof) in skeletal muscle protein synthesis are enough to affect muscle mass. Therefore, we set out to determine if

skeletal muscle protein synthesis is affected by partial loading and to compare partial loading to hindlimb unloading. As expected, FSR was lower in 0 G animals than 1 G animals, which has been purported to be the underlying cause in the substantial decrease in muscle mass during simulated microgravity. Cumulative muscle protein synthesis measured in the final 24 h of hindlimb unloading in mice compared favorably to FSR measurements we have previously repeated in rats (51, 52), in that there was a 27.5% decrement in protein synthesis with hindlimb unloading in mice. We have previously reported up to a 43% decrease in protein synthesis with rats following 5 d of hindlimb unloading (Chapter II). The discrepancy may be due to the duration of the studies (21 vs. 5 d), and that protein synthesis rapidly decreases at the onset of unloading and then rebounds slightly before reaching a new equilibrium (165). These data compare favorably to a 21 d study by Tailander et al., which showed a 33% decrease in protein synthesis (161).

This study, we demonstrated a disconnect between mixed muscle FSR and muscle mass in G/3 and G/6 groups, which is similar to the effects seen with intermittent reloading (Chapter II). This highlights the plasticity of skeletal muscle and the ability for it to respond to even low amounts of loading. This response may be to do the active stretch and contraction of the muscle which is not present in during hindlimb unloading (140). Stretch is enough to activate AKT/mTOR and ERK signaling in cell culture (10, 75). Evidence from previous studies suggest that muscle contractions by chronic electrical stimulation alone are not able to attenuate losses in muscle mass (43, 101). Similarly, passive stretch and/or electrical stimulation and stretch have been shown

ineffective as a countermeasure to disuse atrophy (143). Allowing support for plantar flexors during hindlimb unloading was successful in attenuation muscle atrophy(126). Therefore it appears loading is the primary stimulus for determining muscle homeostasis.

We (53), and others (110, 183) have previously demonstrated that the ERKs are related to load and responsive to perturbations. Consitent with this theory, our data suggest ERK activity as determined by phosphorylation, is depedent on load.There also appears to be a clear relationship between load and phosphorylation state. mTOR signaling as determined by ribosomal protein S6, a target of p70 S6 kinase, mimicks the response seen with ERK signaling suggesting mTOR and ERKs work in parallel.

Skeletal muscle mass is a result of balance between of protein synthesis and protein degradation. A change in the rate of either process results in a change in muscle mass (108). Because mixed-muscle protein synthesis was completely rescued during partial loading, elevated protein degradation may be a responsible for losses of muscle mass with chronic partial loading. Multiple pathways are involved in protein degradation during rapid muscle atrophy. Muscle specific e3 ubuiqutin ligases MAFbx and Murf-1, part of the proteasome degradation pathway, have been shown to be elevated during hindlimb unloading (18, 98) and inhibition of protein degradation attenuated muscle atrophy during 7 d of hindlimb unloading (118). Calcium-dependent protein degradation via calpains, target z-disk proteins and may be the first step in breakdown of the contractile properties that are further dismantled by the proteasome (149, 151). A third pathway of degradation during disuse atrophy is apoptosis (190), which is increased

during unloading (49). Therefore, the evidence for proteolytic activity during disuse suggests that degradation is a key component of unloading and partial loading rodent models of simulated space fllght. Future studies should focus on proteolytic mechanisms to test this hypothesis.

A potential limitation in comparing partial G to 30° head-down tilt hindlimb unloading is the lack of cephalic fluid shift and subsequent cardiovascular de-conditioning (69). There was no change in protein concentration following 21 d in either protocol, suggesting that at least by the end of the study, the muscle has reached homeostasis. It may be difficult to cause cardiovascular de-conditioning in a partial gravity model, limit stress to the animal, and maintain locomotion. Thus, this must be accepted as a limitation to the study design.

In conclusion, this study demonstrates a relationship between load and skeletal muscle mass. We saw no difference in muscle mass or protein synthesis between 1/3th and $1/6^{th}$ percent body weight-loaded mice. Partial loading at the corresponding body weights resulted in a ~9-10% attenuation of muscle atrophy compared to non-weight-bearing hindlimbs using traditional tail suspension. The 20% decrease in muscle atrophy could not be fully explained by differences in protein synthesis.

CHAPTER IV

EFFECTS OF RADIATION AND PARTIAL LOADING ON SKELETAL MUSCLE

Introduction

The future of space exploration is moving away from short-term shuttle missions and lower earth orbit, with emphasis on landing on the moon again for the first time in nearly three decades. Since the beginning of spaceflight we have been mostly concerned with the acute effects of microgravity including, but not limited to fluid shifts, neurovestibular effects, immune dysfunction, psychosocial effects, and muscluloskeletal health (181). For skeletal muscle, rapid atrophy occurs within days, and persists in a linear fashion (141). Combined aerobic and resistance exercise countermeasures have been marginally effective at mitigating atrophy in ground based studies, at least with losses associated with the knee extensors, but not plantar flexor losses (24, 169). However, astronauts on International Space Station (ISS) lost on average 10-15% of muscle mass despite rigorous exercise countermeasures (167). Possibly the greatest unknown regarding human space exploration outside of lower earth orbit is the radiation exposure accumulated over long duration flight (12, 44). Considering the amount of muscle mass lost during spaceflight alone, the complex interaction between radiation and microgravity could potentiate greater muscle lost and greater risk to the individual.

Terrestrial radiation is composed of low-linear energy transfer sources (low-LET) such as x- and γ-rays, have a reduced penetrating distance and do most of their damage from ionization and generation of reactive oxygen species (125). In contrast,

space radiation consists of a combination of low-linear energy transfer (low-LET) and high-LET radiations (125). High-LET particles, including solar particle events (SPE), galactic cosmic rays (GCR), and high-charge, high-energy nuclei (HZE, e.g., C, O, Ne, Si, Cl, and Fe,(125), penetrate deep in to tissues and creates primary tracks of damage in addition to the ionizing radiation effects. Access to high-LET generating sources is limited and requires the use of particle accelerators to produce; therefore, the present study used x-ray radiation to mimic potential radiation exposures during spaceflight. It is possible to relate damage to tissue by high- and low-LET using relative biological effectiveness (RBE). Space radiation is estimated to cause an approximately 10-fold greater effect than low-LET. Notably, RBE dose/response curves have not been established in muscle.

The effects of irradiation on skeletal muscle are not completely known. Extremely high doses of radiation (25 Gray; Gy) have been used to eliminate satellite cells *in vivo* to understand their function in muscle hypertrophy (1), but the effect of low doses <2 Gy are not yet understood. Gray units are used to describe absorbed dose of ionizing radiation and is equivalent to the absorption of 1 joule of energy. Although, muscle is generally thought to be post-mitotic and therefore may be less radiosensitive to mutations when compared to highly proliferative cells, this does not reflect the ability for radiation to alter protein metabolism.

One mechanism for the effects of irradiation on protein synthesis is DNA methylation of promoter region sequences (166), which may physically block RNA polymerase from binding to DNA. Methylation also may lead to the recruitment of

histone deacytlase (173), which forms compact heterochromatin, again creating a physical barrier to transcription. Low dose radiation (50 cGy) to increases methylation in skeletal muscle (89). Specifically, 10 fractionated doses of 5 cGy were more harmful to muscle than one acute dose of 50 cGy (89). Radiation and unloading may work in tandem in that unloading also leads to histone modifications that down regulate the expression of myosin heavy chain (134).

The purpose of this experiment was twofold. First, we wanted to determine the effect of ionizing radiation on skeletal muscle protein synthesis after 3 and 21 d post x-ray exposure. We hypothesized that protein metabolism would be affected 3 d post exposure, but not after 21 d. Radiation doses of 0.1, 0.5, and 1.0 Gy were used. Second, after determining the most effective dose on for changes to the musculoskeletal system, we wanted to determine if changes in muscle mass and protein synthesis are exacerbated with partial loading. We hypothesized that the muscle atrophy would be exacerbated by combining partial loading and x-ray exposure. To determine if the acute dose was fractionated into 3 doses on days 0, 7, and 14. We hypothesized that fractionated doses would have a greater effect on muscle protein metabolism.

Methods

Animals and experimental design

Similar to Chapter III, 4-mo-old female BALB/cByJ (Harlan Laboratories, Houston, TX) and individually housed in a climate-controlled room with a 12:12 h light-dark cycle. Water and standard rodent chow were provided *ad libitum*. This research

project was approved by the Institutional Animal Care and Use Committee of Texas A&M University in accordance with institutional guidelines for the care and use of laboratory animals.

This set of experiments was divided into two studies. In the first study, a dose response to 0, 0.1, 0.5, and 1.0 Gy x-ray radiation on normal ambulatory animals(1 G) was established. Following a 14 d acclimation period, mice were block-assigned by body mass into four dose groups: sham exposure (0 Gy) in which the animals were placed in the x-ray beam, but were not exposed to radiation; 0.1 Gy; 0.5 Gy; and 1.0 Gy. Whole-body irradiation was accomplished with the use of a 50 keV x-ray machine (Norelco MG300 x-ray industrial radiograph) at a dose rate of 1.85 $Gy \cdot min^{-1}$. Total dose was determined by time of exposure.

Based on the most effective dose at altering metabolic markers of the musculoskeletal system, the most appropriate dose was chosen for the second study. Animals were block assigned by body mass into groups. To determine the effects of radiation exposure and loading, animals were divided into two groups: 1 G ambulatory loading ;or G/6 partial suspension. The two groups were sub-divided into sham, 1 acute dose on day 0, and 3 fractionated doses on days 0, 7, 14 to accumulate the total acute dose to determine if smaller, fractionated doses affect skeletal muscle more than a larger acute does.

Partial suspension

G/6 animals were suspended in a specially-designed, full body, horizontal passive suspension devise, first described by Wagner et al. (174), and described in full in Chapter III. To achieve partial suspension, a harness was created by placing the forelimbs in a tight fitting mole skin jacket that was secured by Velcro at the scapula. An adhesive steri-strip (3M, St. Paul, MN) bandage was wrapped around the base of the tail to minimize skin irritation and covered by athletic tape to create loop, with could be used to attach the tail to the harness. The base of the tail and jacket were then connected by a stainless steel bar that was positioned parallel to the vertebrae. The harness was fitted onto the with mice while anaesthetized by 2% isoflurane (US Pharmacopeia, Rockville, MD) with 0.8-1mL/min oxygen flow rate. Animals were then suspended by attaching the harness apparatus to a variable tension spring (k ~1.6 N/m, Statico, San Carlos, CA). Tension on the spring was adjusted by connecting the spring to a eye-loop screw. Subtle manipulations of load were accomplished by loosening or tightening the screw. The overall harness system was connected to a steel rod that supported a small nylon wheel and a low-friction bearing to provide linear freedom of motion for the suspended animals.

The cage was constructed using 30.48 cm x 30.48 cm Plexiglas with perforated floors, used to provide mice easier quadripedal ambulation as reported by Wagner et al. (174). Body weight was checked and adjusted daily by weighing the animals on a suspended platform to prevent full loading (115). Body mass was titrated to within 1% of calculated weight by adjustment of the spring.

Following the 21 d experimental protocol, animals were anaesthetized with ketamine/xylazine 60 mg · kg^{-1} and 10 mg · kg^{-1} respectively). Approximately 0.75 mL of blood was drawn via cardiac puncture, allowed to clot and then centrifuged at 2000 x g for collection of blood serum. Gastrocnemius muscle was excised, immediately weighed for wet muscle mass, and then snap-frozen in liquid nitrogen. Samples were stored at -80°C until analyses.

Determining cumulative fractional protein synthesis (FSR)

Cumulative FSR in the gastrocnemius was determined using deuterium oxide as previously published by our lab (63-65, 127). Twenty-four hours prior to tissue harvest, a bolus priming dose of 99.9% 'salinized' (0.9% NaCl) deuterium oxide (Cambridge Isotopes, Andover, MA) was administered intraperitoneal (IP) at a dose of 20 µl/g body weight (w/v) to establish a deuterium oxide enrichment at 4% of the total body water. To maintain a constant enrichment of deuterium oxide, drinking water was subsequently enriched to 4% $^{2}H_2O$ and provided *ad libitum*. Unless otherwise noted all chemicals were purchased from Sigma-Aldrich (St Louis, MO).

^{2}H-labeling of body water ($^{2}H_2O$; E_{BW}) was measured by gas chromatography-mass spectroscopy (Agilent 7890 GC/5975MSD, Santa Clara, CA), following a 24-h isotopic exchange between $^{2}H_2O$-enriched serum samples and acetone (188). Isotope exchange occurred by reacting 0.02 mL of plasma with 0.002 mL of 10 N NaOH and 0.004 mL of 5% (vol/vol) acetone in acetonitrile. After 24 h, the reaction was stopped by the addition of 0.6 ml of chloroform and 0.5 g of $NaSO_4$. A 0.1 mL aliquot of sample was used to inject 1 µl into the gas chromatograph and separated by size using helium as

the carrier gas. Temperature settings were as follows: temperature was initially set at 60ºC, increased by 20°C/min to 100ºC, increased by 50ºC/min to 220ºC thereafter with a 'hold' for 1 min. The sample was injected at a split ratio of 40:1 with a helium flow of 1 mL/min. Samples were then injected into the mass spectrometer which determines mass-to-charge ratios of compounds and can differentiated between deuterated and non-deuterated compounds. Selective ion monitoring of mass-to-charge ratios (m/z) 58 (M, acetone) and 59 (M + 1, deuterated acetone) was conducted using a dwell time of 10 ms per ion. ^{2}H acetone elutes at approximately 1.7-1.85 min. The mass spectrometer was operated in electron impact mode (70eV).

Muscle protein synthesis was assessed by measuring the incorporation of deuterated-alanine into acid-precipitable skeletal muscle proteins, as described previously (64). Briefly, muscle tissue was prepped by homogenizing 30 mg in 0.4 mL of 10% trichlorocetic acid (TCA) with a Polytron homogenizer. Samples were centrifuged at 5000 x g for 15 min at 4°C. The supernate containing free amino acid pool of alanine was decanted (discarded), 0.4mL of TCA was added to the pellet and the sample was vortexed to break up the pellet. These steps were repeated three times to ensure the sample was void of free amino acids. An aliquot of 0.4mL of 6 N HCl was added to the pellet and samples were heated at 100°C for 24 h to hydrolyze the proteins into free amino acids. HCl was then evaporated off 0.05 mL of the hydrolysate. The resulting amino acids were derivatized by methyl-8 (Fisher Scientific, Waltham, MA) just prior to analyses. The ratio of protein-bound ^{2}H-alanine to unlabeled alanine (E_A) in skeletal muscle was measured by GC/MS (Agilent 7890 GC/5975MSD, Santa Clara,

CA) (39). The following temperature program was used for separation: 90ºC initial (held for 5 min), increased by 5°C/min to 130ºC, and then increased by 40ºC/min to 240ºC, and held for 5 min. The sample was injected at a split ratio of 20:1 with a helium flow of 1 ml/min. The mass spectrometer was operated in electron impact mode (70eV). Selective ion monitoring of mass-to-charge ratios (*m/z*) 99 (M, alanine) and 100 (M + 1, deuterated alanine) was conducted using a dwell time of 10 ms per ion. ^{2}H-alanine eluted at ~ 9 min.

FSR was calculated by the equation:

$$E_A \times [E_{BW} \times 3.7 \times t\ (h)]^{-1} \times 100$$

where E_A represents amount of protein-bound ^{2}H alanine (mole % excess), E_{BW} is the quantity of $^{2}H_2O$ in body water (mole % excess), and 3.7 represents the exchange of ^{2}H between body water and alanine (39), and t is time in h.

Statistics

Data were analyzed using SigmaStat (v3.5, Systat software, Chicago, IL). In the first study, a one-way ANOVA was used to compare groups. In the first study, the effect of radiation doses was determined using a one way ANOVA. When significant F-ratios were present, a Student-Newman-Keuls (SNK) post hoc procedure was used to evaluate differences among group means. The significance level was predetermined at $\alpha<0.05$. For the second study, the effects of gravitational load (1 G vs. Lun) and radiation dose (sham vs. 1RE vs. 3RE) by a 2x3 ANOVA will be used on all outcome measurements. A SNK post-hoc test was used for pairwise comparisons if main effects are found to be different.

Results

X-Ray irradiation effect on muscle mass and protein synthesis

Muscle mass was examined in the gastrocnemius, the primary muscle of plantar flexors. For the first phase of this experiment, independent of partial gravity, there were no differences in wet mass in 3 or 21 d post irradiation within cage control groups. Similarly, muscle protein synthesis was not affected by x-ray radiation of doses less than 1.0 Gy (**Table 4**). These results suggest that neither shorter nor longer intervals between radiation exposure and recovery affect muscle mass, per se.

Table 4. Muscle mass and FSR in gastrocnemius 3 or 21 d following radiation exposure

	Sham	0.17 Gy	0.5 Gy	1.0 Gy
3 days post radiation exposure	(n=9)	(n=9)	(n=9)	(n=9)
Gastrocnemius mass (mg)	98.2 ± 4.8	106.1 ± 2.3	105.3 ± 3.4	97.6 ± 2.9
FSR (% · h^{-1})	0.209 ± 0.0149	0.231 ± 0.17	0.187 ± 0.009	0.185 ± 0.008
21 days post radiation exposure	(n=9)	(n=7)	(n=9)	(n=8)
Gastrocnemius mass (mg)	109.4 ± 2.8	106.9 ± 2.9	101.5 ± 6.2	108.3 ± 3.1
FSR (% · h-1)	0.184 ± 0.006	0.219 ± 0.009	0.205 ± 0.009	0.205 ± 0.006

Skeletal muscle mass and protein synthesis were not affected by x-ray radiation of doses less than 1.0 Gy. Measurements were made in the gastrocnemius muscle of mice 3 or 21 d post x-ray exposures of 0, 0.17, 0.5, or 1.0 Gy. There were no significant differences between any groups. Values are presented as mean ± SEM.

Combined effects of partial loading and radiation exposure

Due to the collaborative nature of this muscluloskeletal project, the greatest effect of irradiation on catabolic bone markers was seen at 0.5 Gy, and therefore this dose was chose as the acute radiation exposure (1RE). Therefore, 3RE received three fractionated dose of 0.17 Gy on days 0, 7, and 14 of the 21 d study.

Body mass was maintained in all partial loading groups ($p>0.05$), while the 1 G animals all gained body mass from day 0 ($p<0.05$, **Table 5**). Statistically, there was no signficant interaction between loading and radiation exposure, nor was there a main effect of radiation on muscle mass. A main effect was observed for loading and pairwise comparison revealed all G/6 muscle mass were less than their 1 G counterpart (**Table 5**).

Mixed (**Figure 17**) and myofibrillar (**Figure 18**) protein synthesis also did not have a significant interaction or main effect of radiaiton, but again there was a main effect of load. In the mixed fraction, the only significant pairwise comparison was between 1 G sham and G/6 sham ($p=0.05$). In the myofibrillar-rich fraction, the only significant comparison was between 1 G 1RE and G/6 3RE ($p<0.05$).

Table 5. Body and muscle mass changes with partial suspension and radiation exposure

	1 G Sham (n=10)	1 G RE (n=10)	1 G 3RE (n=10)	G/6 Sham (n=14)	G/6 RE (n=12)	G/6 3RE (n=11)
Body Mass (g)						
Day 0	23.44 ± 0.63	23.14 ± 0.64	22.97 ± 0.47	23.23 ± 0.48	23.07 ± 0.48	22.68 ± 0.38
Day 7	24.63 ± 0.69	23.94 ± 0.50	23.73 ± 0.31	23.16 ± 0.35	22.77 ± 0.44	22.74 ± 0.39
Day 14	24.81 ± 0.88	24.37 ± 0.57	23.989 ± 0.38	22.71 ± 0.38	22.81 ± 0.46	22.33 ± 0.38
Day 21	24.42 ± 0.79	24.70 ± 0.58	23.77 ± 0.33	22.54 ± 0.40	22.73 ± 0.44	22.35 ± 0.38
Body Mass Change (g)	0.98 ± 0.37	1.55 ± 0.37	0.80 ± 0.33	-0.70 ± 0.40	-0.34 ± 0.33	-0.33 ± 0.35
Plantarflexor Muscle Masses (mg)						
Soleus	4.64 ± 0.50	4.36 ± 0.44	4.46 ± 0.21	3.49 ± 0.35 *	3.00 ± 0.26 *	3.28 ± 0.24 *
Plantaris	13.58 ± 0.64	14.98 ± 0.60	14.33 ± 0.46	11.93 ± 0.72 *	11.10 ± 0.46 *	10.98 ± 0.64 *
Gastrocnemius	106.09 ± 1.95	107.97 ± 2.58	105.58 ± 3.13	87.62 ± 1.79 *	83.44 ± 4.35 *	90.09 ± 1.15 *
Total Plantar Flexor Mass	124.31 ± 2.94	127.31 ± 3.45	124.36 ± 3.32	103.04 ± 2.35 *	97.55 ± 4.74 *	104.35 ± 1.66 *

Skeletal muscle mass of the plantar flexors are reduced with partial loading at 1/6th body weight (G/6). Body mass was maintained over the 21 d partial loading paradigm ($p>0.05$). There is a main effect of load ($p<0.05$), but no effect of radiation or interaction ($p>0.05$). Values are means ± SEM. * denotes different from 1 G pairwise comparison ($p<0.05$).

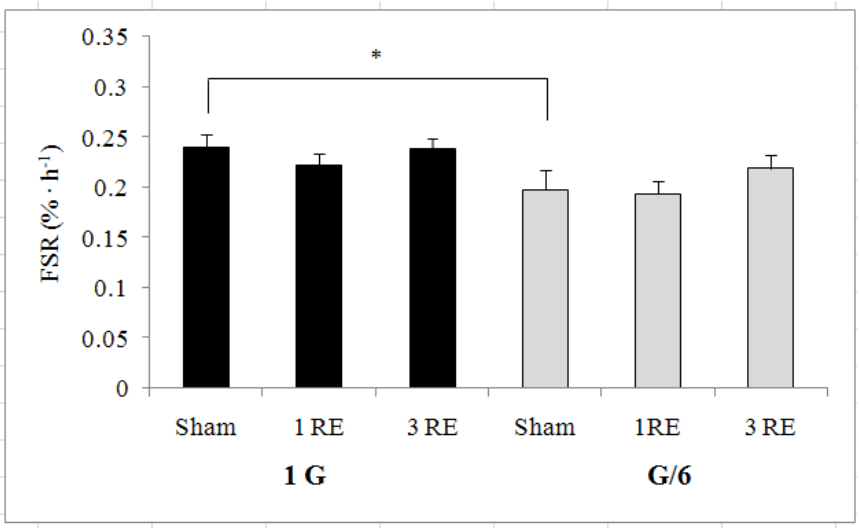

Fig 17. Mixed fraction FSR in the gastrocnemius with partial load and radiation. Gastrocnemius mixed fraction protein synthesis following an acute dose (1 RE) and three small fractionated doses exhibited a main effect of loading, but no effect of radiation. Sham G/6 was lower than 1 G sham animals, while there was no statistical difference in the other pairwise comparisons. Values are means ± SEM. * denotes different from 1 G.

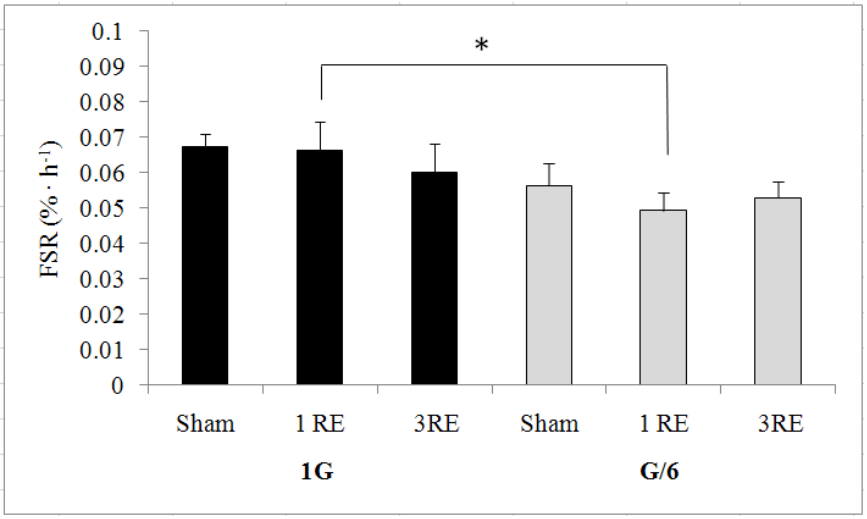

Fig 18. Myofibrillar rich fraction FSR in the gastrocnemius with partial load and radiation. Myofibrillar rich fraction FSR in gastrocnemius following an acute dose (1 RE) and 3 small fractionated doses exhibited a main effect of loading, but no effect of radiation. G/6 was lower than 1 G 1 RE animals, while there was no statistical difference in the other pairwise comparisons. Values are means ± SEM. * denotes different from 1 G.

Discussion

With a renewed interest in longer term, extra-orbital space flight missions in the near future, research efforts have begun to study effects on the musculoskeletal system. Results from the current experiments provide preliminary evidence that short or long term effects of x-ray radiation are negligible on muscle mass and/or protein synthesis at exposures below 1 Gy. However, 21 d is a relatively short period of time, when it is possible that effects from radiation exposure may take months to manifest (44). Thus, it is possible that detectable differences resulting from x-ray radiation could influence muscle mass or metabolism after a longer duration of exposure.

To our knowledge, this is the first study to demonstrate the combined effects of radiation and reduced loading on skeletal muscle. We hypothesized that skeletal muscle would be more susceptible to changes in muscle metabolism when combining reduced weight bearing and irradiation, but results did not suggest an interaction. Because of recent insight into intermittent radiation exposures and their negative effects on biological systems, this study also examined the effects of three fractionated doses (totaling 1 Gy), however this approach yielded similar results as the acute dose. Therefore, radiation exposures, either single or fractionated, do not result in negative outcomes related to maintenance of muscle mass or protein metabolism.

While it may be possible to conclude that low level radiation exposure, at least using x-rays, are not deleterious to muscle mass and/or protein metabolism, such a conclusion should be approached with caution. In the first study, it is possible that we may have missed the window in which damage and repair is occurring. Most DNA repair is thought to occur within the first 48 h (124), and despite our ability to measure incorporation of tracer over 24 h, our measurements were made between 48 and 72 hours. It is also plausible that there could be histological responses that are detectible using our more general measures of wet muscle mass or protein synthesis. Bandstra et al. observed a decrease in the number small cross sectional area (CSA) fibers with no change in large CSA fibers with 1 GeV/nucleon $^{56}Fe^{26+}$ (15). These results suggest there could be a change in pool size which, as noted earlier, would not be detectable with muscle mass or protein synthesis measurements. Future studies hoping to model space radiation exposure on muscle should use high-LET radiation because of its tendency to

create cluster damage, which is more difficult to repair and results in a larger number of chromosomal aberrations (158).

In conclusion, there were no apparent effects of x-ray radiation on muscle mass and mixed or myofibrillar fraction protein synthesis of the gastrocnemius muscle. These effects were non-existent in four different scenarios: 3 d, 21 d, combine with another stressor (partial gravity), and finally fractionating a single acute dose of 0.5 Gy into three small doses over a 21 d experiment. These results suggest that either skeletal muscle adapts quickly in response to radiation exposure or that x-rays are not a suitable form of radiation to understand the effect of radiation with partial gravity. Future studies using galactic cosmic radiation should be conducted in order to clarify these outcomes.

CHAPTER V

CONCLUSIONS

Overall Conclusions

Adaptability of skeletal muscle

Skeletal muscle is a highly adaptable tissue that responds to its environment rapidly. The studies in this dissertation demonstrate the sensitivity and ability of skeletal muscle to alter its metabolism to changes in load. Here on earth, theses adaptations are beneficial as we are able to maintain muscle mass relatively easily as well as increase muscle mass by resistance exercise training. But as with the old adage “use it or lose it”, disuse manifests into rapid muscle atrophy in situations such as microgravity or clinical disuse.

Specifically, each chapter of this dissertation highlights different aspects of adaptability. First, in Chapter II, we highlight that one of the key mechanisms for skeletal muscle to reach new equilibrium appropriate for load is by decreasing skeletal muscle protein synthesis. Hindlimb unloading resulted in an almost 50% reduction in muscle protein synthesis, which coincides with human bed rest models (46, 135, 168). We demonstrate that ambulatory reloading 2 h during a period of 120 total h was able to rescue protein synthesis across predominantly red, white, and mixed fiber muscles, but intriguingly, the normalized rates of synthesis (measured over a prolonged period of time) were not adequate to attenuate the losses of muscle mass with microgravity.

In Chapter III, we demonstrate partial loading at ~1/3rd and 1/6th of the animal's body weight was enough to attenuate muscle atrophy in plantar flexor muscles. In fact, our data strongly support the notion that muscle mass is uniquely tuned with the chronic forces imposed upon it, and in some muscles, the relationship of muscle mass to 'gravitational loading' is linear. Although we are confident that losses of muscle mass with microgravity are, in part, due to diminished rates of synthesis, consistent with our findings with intermittent reloading (Chapter II), daily anabolic responses to chronic partial gravity exposure rescued FSR to levels of cage controls, suggesting that there is a disconnect between muscle mass and FSR under specific conditions. Further perplexing was that rates of synthesis of the myofibrillar fraction, consisting of >50% of all cellular proteins and important for mechanical function, was relatively conserved regardless of loading paradigm, suggesting that proteins governing the mechanical properties of the cell remain intact, even when the total mixed fraction is compromised. Thus, it is possible that reductions in total mass are due to elevated rates of protein degradation; otherwise, one would suspect that the maintained FSR in the myofibrillar fraction would be adequate to maintain mass and function in the face of microgravity.

It is important to note, however, that the FSR measures presented in this study are derived from intact proteins, and cannot account for complete losses of myofibrillar proteins from that fraction. So while the intact proteins are being manufactured at comparable rates during each of the loading paradigms, it is possible that the overall protein abundance of this fraction was compromised by other factors, such as loss of nuclei and associated proteins (42). Regardless, for the first time, we present data that

indicates that the chronic exposure to partial or microgravity does not impact the fractional synthesis rates of the myofibrillar fraction.

In Chapter IV, we present FSR data that are inconsistent with Chapter III. While potential causes a lack of effect of radiation on muscle protein synthesis will be addressed in more detail later, it provides an interesting point that integrates information from the previous two chapters. In this study, partial suspension resulted in protein synthesis that was lower than control animals. Although we have developed a method that integrates protein synthesis over 24 h, slight perturbations with animals can result in 'normalized' protein synthesis similar to what was observed with 1 h of reloading 16 h before tissue harvest. Ultimately, it questions the convention that alterations of FSR are of critical importance for the control of muscle mass with disuse or reduced loading.

Taking results to a clinical setting

An important mission of NASA is to have its research agendas relate to the general population. The data from this dissertation highlight the importance of loading, no matter how much, on skeletal muscle metabolism. Passive and active movements are being prescribed in ICU to shorten hospital stay, improve functionality after discharge, and improve survival (22, 91). We suggest that even small amounts of load throughout the day totaling an hour can make a huge impact on whole body metabolism, and therefore patients could greatly benefit from brief periods of load and activity, such as isometric contractions, could serve as a great benefit for patients.

Conflicting results of partial loading on FSR

The experiments make a direct comparison on the data between 1 G and G/6 in

Chapter III and 1 G sham and G/6 sham in Chapter IV. To compare muscle atrophy, G/6 lost ~ 17.4% and 17.5% of gastrocnemius muscle respectively, but interestingly FSR for G/6 groups exhibited different responses at the level of the mixed fraction. To date, this reason is unknown, but will warrant further investigation. It is possible that our ability to titrate the animals during chronic G/6 exposure was more efficient and less disruptive to the animal as we became more experienced with the technique. As described in Chapter II, even subtle exposure to greater loads can dramatically affect muscle protein synthesis without apparently affecting the over dynamic of muscle mass. Although we did not measure the myofibrillar fraction in the radiation set of experiments, we are confident that partial-load dependent reduction of protein synthesis in the mixed fraction was not at the expense of the robustly conserved synthesis of the contractile proteins.

Our lab first implemented the used of deuterium oxide to measure protein synthesis because of the ability to make long term measurements (62). Our first study demonstrated that measuring cumulative muscle protein synthesis over 36 h revealed an increase in muscle protein synthesis following chronic resistance exercise that was not detectable with traditional flooding dose technique (63). Despite extending our measurements out to 24 h and not extrapolating daily FSR, we must still be cautious on taking a snapshot at the end of the study. A great example of this in muscle literature is using amino acids to stimulate protein synthesis (7, 105, 113). Acutely, protein synthesis is stimulated, but this signal has not been shown to result in hypertrophy of the skeletal muscle. These data have been used to design atrophy countermeasures entirely around

nutrition (47, 100, 133, 154, 169). Further experiments should be done to validate which FSR is best representative of chronic partial is loading.

X-ray radiation effect on skeletal muscle

The work contained herein presents novel data on the effects of low dose ionizing radiation exposure on muscle; but it is clear that more data are needed to explain the potential effects of ionizing radiation on skeletal muscle. Potential future studies should examine doses greater than 1 Gy and/or to isolate skeletal muscle and concentrate the dose instead of using whole body radiation as in this study. Unfortunately, there were no detectable effects of radiation at the time points studied in this dissertation, but this does not exclude the possibility that x-ray radiation can influence muscle mass or metabolism. Key radiosensitive targets are satellite cells, which serve as 'stem' cells for skeletal muscle (107). At these low doses and the low-LET of x-ray exposure, it is unlikely that satellite cells were affected. Satellite cells comprise ~2% of the total number of nuclei in skeletal muscle (25), resulting in small probability of ions hitting the satellite cells. Even if damages were to occur, x-ray radiation does most of its damage via reactive oxygen species and cause very little cluster damage (28). Therefore it is plausible that damage was not permanent and could be repaired. Alternatively, if the nucleus was irreparably harmed, it is feasible that both the nucleus and the protein associated with that genetic material, would be absent from the cell. Again, our measures of FSR can only describe protein synthesis of intact proteins of functioning cellular domains, and not sensitive to losses of DNA units

resulting from radiation exposure. Regardless, it may be appropriate to use higher-LET ions if our goal is to predict results of space radiation.

A second concern is the chronic exposure to radiation see with space travel. We used a fractionated dose of radiation, but only split the larger acute dose into three fractions which may not have been enough to perturb the system. New data are now emerging that fractionated doses can be worse those acute doses (personal communication with Jerry Shay). This goes against many scientists' long standing hypothesis that a single large dose would cause a larger quantity of damage than a single small dose, whose chance of creating a mutation was much less just based on the probability of a ion tract going through DNA and creating cluster damage. Mechanisms for how smaller fractionated doses can be more harmful than an acute dose are yet to be determined. Unfortunately, these studies did not address a chronic exposure to low doses, and more work should be done to accurately reflect daily radiation exposure.

Furthermore, the interaction of unloading/partial loading and radiation on muscle's regeneration ability should further be explored. It has been shown that unloading reduces satellite cell number (30) and delays recovery(119), and can even prevent future growth (120) in rats, which likely won't be impacted with 'normalized' rates of synthesis. The combination of radiation damage and reduced proliferative ability of satellite cells could limit an astronaut's ability to recover from a long duration spaceflight.

Final Thought

Skeletal muscle is often under-appreciated (184), but like many things, you don't miss it until its gone. The earliest space mission understood the importance of maintaining muscle mass for function and performance, but have yet to fully attenuate muscle atrophy, especially in type I fibers. Most of these countermeasures rely on the increasing skeletal muscle protein synthesis, which is a major culprit in causing muscle atrophy. Targeting only protein synthesis may not be enough of a stimulus as evidence by the data in this dissertation. It will likely take a multiple systems approach to counteract the loss in protein synthesis and increase in protein synthesis for us to maintain/elevate muscle mass during periods when it is otherwise compromised. As for future exploration out of lower earth orbit, our data suggest that a new equilibrium for muscle mass is reached and that partial loading protective of muscle mass, although the mechanism by which the equilibrium is reached is still unclear.

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VITA

Michael Parker Wiggs
Texas A&M University
Department of Health and Kinesiology MS 4243
College Station, TX 77843-4243
mpw82@hlkn.tamu.edu

Education

2005	B.S.	Texas A&M University	Applied Exercise Physiology
2011	Ph.D.	Texas A&M University	Kinesiology

Selected Honors

2008-2010	Texas Space Grant Consortium Fellowship
2010	American Physiological Society - Environmental & Exercise Physiology (EEP)/NSBRI Predoctoral Award.

Professional Memberships

American Physiological Society
American College of Sports Medicine
Texas Chapter of the American College of Sports Medicine

Research Interests and Experience

Current research interests include the effects of protein metabolism as it relates to disuse, aging, nutritional control, and exercise. My goal is to determine optimal strategies for maintaining and increasing muscle mass via measurements of protein synthesis and signaling cascades causing these metabolic changes. These research goals are accomplished using a variety of techniques including,, but not limited to: measurements of protein synthesis using stable and radioactive isotopic tracers determinable by gas chromatograph-mass spectrophotometer (GC/MS) or liquid scintillation, respectively, measuring protein content and activity with western blotting, and ELISA. Surgical techniques include rat hemi-corpus hindlimb perfusion, carotid artery and jugular vein cannulation, and survival surgery with implantation of time release drug capsules. With the use of rats and mice, I am trained in common animal handling techniques such as phlebotomy, injections, and tissue harvest, hindlimb suspension, full body suspension in mice for altered gravity (1/3 or 1/6 G), as well as operant conditioning rats to voluntarily perform ‘squat like’ resistance exercise.