SKELETAL MUSCLE GROWTH FACTOR RESPONSE TO CUTANEOUS STIMULATION OF THE PLANTAR SURFACE OF THE FOOT

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A Dissertation Presented to

The Faculty of the Department

of Health and Human Performance

University of Houston

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In Partial Fulfillment

Of the Requirements for the Degree of

Doctor of Philosophy

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By

Stuart Matthew Clark Lee

May 2015

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ABSTRACT

Skeletal muscle atrophy, deconditioning, and decreased function are consequences of skeletal muscle unloading induced by reduced physical activity, bed rest, and space flight. Reduced sensory input, particularly from the plantar surfaces of the feet, is one of the earliest consequences of unloading. This has been hypothesized to be a key factor in modulating the negative effects of unloading, especially those observed in the anti-gravity muscles of the lower limbs. Mechanical stimulation of the plantar surface during unloading to provide sensory input has been investigated as a potential means of attenuating unloading-induced muscle deconditioning. Results from human and animal models, including space flight, dry immersion, and hindlimb suspension, demonstrated that plantar stimulation evokes an increase in neuro-sensory activity and attenuates some elements of skeletal muscle deconditioning. The physiological mechanism(s) responsible for the protective effects of plantar stimulation in unloaded muscle, especially its anti-atrophic effects, have yet to be fully elucidated. Previous studies suggest that plantar stimulation might result in the release of growth hormone (GH) in response to increased afferent sensory nerve traffic induced by plantar stimulation. Since increased levels of circulating GH results in the activation of several different hypertrophic signaling pathways within skeletal muscle, it is possible that the anti-atrophic effects of plantar stimulation during unloading are mediated by GH.

The purpose of this dissertation project was to develop a system that delivers plantar stimulation to human subjects in a controlled manner and to investigate whether plantar stimulation results in the release of GH during acute skeletal muscle unloading

(supine rest). Specifically, we hypothesized that GH concentrations would increase during acute unloading when subjects received 10 min of plantar stimulation to the right forefoot. Further, we hypothesized that the GH response would be augmented when subjects received plantar stimulation that activated muscle and joint proprioceptors as well as cutaneous mechanoreceptors compared to plantar pressures which stimulated only cutaneous mechanoreceptors. Thirteen healthy subjects participated in three sessions in random order: (1) control (or sham) condition in which the subjects received no stimulation; (2) 10-min plantar stimulation to the right forefoot with no support for the feet (stimulating cutaneous mechanoreceptors only); and (3) 10-min plantar stimulation to the right forefoot with the feet supported with the ankle at ~90° (stimulating cutaneous mechanoreceptors and muscle/joint proprioceptors). After 20 min of rest, subjects received either no stimulation or 10 min of stimulation. During the stimulation periods, force equivalent to 25% of body weight was applied cyclically by inflating and deflating an air bladder situated under the foot. Within each cycle, the bladder was inflated for 0.4 sec and deflated for 1.2 sec to simulate the frequency of forces applied during a normal walking pace (5.6 km/h). Force at the feet (pressure sensing insoles), muscle activity of the lower leg (surface electromyography, sEMG), and circulating immuno-assayable GH (venipuncture) were measured before, during, and after the plantar stimulation or sham period. Plantar stimulation with or without foot support did not affect immuno-assayable GH concentration or an aggregate measure of muscle activity (root mean square, RMS) compared to the control condition. Thus, immuno-assayable GH and sEMG appear not to have a role in the prevention of muscle atrophy during unloading. Other skeletal

muscle growth factors should be considered. Continued work to understand the mechanism(s) responsible for the anti-atrophic effects of plantar stimulation may aid in the treatment of subjects who are bedridden, individuals who cannot regularly perform exercise due to an orthopedic or other medical condition and astronauts during space flight either as a supplement to current countermeasures or in place of them when countermeasure hardware is not available.

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It is rare that an individual succeeds in any endeavor in vacuum, devoid of support and influence from individuals and organizations. An effort to create an exhaustive list all persons influential on my development as a person and as a researcher could not be undertaken successfully, and therefore I apologize to anyone not included here. I am thankful for the support of my family and friends who provided support before, during and (I assume) after my PhD program. This includes my daughter Anna, who unknowingly provided her own source of inspiration, particularly during her deployment in Afghanistan; my parents (Joy Lee and John Lee) who fostered my interest in science and education from an early age; my sister Jo-Anne who challenged me both inside and outside of the classroom; Dr. Suzanne Schneider, who was my first, and perhaps most influential, science mentor and who also was the first to seriously encourage me to return to graduate school for my PhD; Robert and Gwen Fraser, who provided support to both my wife and me through this process; my Wyle supervisors, Elisa Allen and Jeannie Nillen, and my co-workers who provided encouragement and allowed me to adjust my schedule to attend classes and conduct this work; Helen Hall Library staff and volunteers, in whose library I spent much of my time writing the proposal and dissertation; Todd Boutte, Graduate Program Administrator, who fielded so many of my questions and requests over the years; Igor Kofman, Dr. Brian Peters, and Dr. Michael Stenger, friends and colleagues who participated in brainstorming activities and helped turn my concepts about the valve control system and data analysis programs into reality; Boaz Blake who shared many of his experiences regarding his work with Dr. Paloski that contributed to the study design and implementation,

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Table of Contents

CHAPTER 1: INTRODUCTION

1.1 Overview

Skeletal muscle size, which is intrinsically linked to skeletal muscle function, is largely determined by the balance of protein synthesis and degradation, particularly of myofibrillar proteins. Many factors influence anabolism and catabolism in skeletal muscle (e.g. loading state, activity level, diet, physiological stress), and the proportional activity of regulatory pathways associated with these physiological states dictates whether muscle is eutrophic, hypertrophic, or atrophic. Proteins in skeletal muscle are in constant flux (i.e. turnover), and overall skeletal muscle size is determined by whether anabolism or catabolism predominates within the tissue (Phillips, Glover, & Rennie, 2009). It generally is accepted that skeletal muscle responds to the loading environment in which it operates; acute adaptations result from changes in the protein synthesis and degradation of a wide range of intracellular and extracellular proteins, and chronic loading results in structural adaptations such as alterations in myofibril sarcomere number, myofiber cross-sectional area, neuromuscular junction (NMJ) number and complexity and capillary vascular bed tortuosity. The strength and frequency of muscle contractions, controlled by activation of both voluntary and involuntary neural pathways innervating skeletal myofibers, contribute to factors which lead to the increase (i.e. hypertrophy) or decrease (i.e. atrophy) of skeletal muscle size.

Skeletal muscle unloading, common during extended convalescence in bed rest, experimental conditions, and weightlessness associated with space flight, causes

muscle atrophy and decrements in muscle function (Adams, Caiozzo, & Baldwin, 2003). These forms of unloading primarily affect the lower body and postural musculature, particularly extensor muscles (LeBlanc et al., 2000; Trappe et al., 2009) that support the body during locomotion and in the upright posture (Adams et al., 2003; Narici & de Boer, 2011). Decreased muscle tone, or tension, and alterations in neuromuscular activity (from tonic to phasic activity) during unloading, particularly in the fatigue-resistant postural muscles (Kozlovskaya et al., 2007), precede muscle atrophy. Muscle atrophy and the concomitant decrease in muscle strength and endurance have the potential to negatively impact function, health, and safety in individuals attempting to return to normal activities after injury, bed rest, and during and after space flight (Adams et al., 2003; Layne & Forth, 2008).

A logical method to combat the physiological de-conditioning observed in skeletal muscle during unloading is to increase the level of muscle activity and/or replace the load on the muscle. In patients who can tolerate the upright posture, the solution would include simple ambulation and other rehabilitation activities. However, in patients who cannot tolerate normal upright standing or ambulation for extended periods of time and in astronauts for whom gravity replacement cannot be achieved without the application of external loading (Genc et al., 2010) or artificial gravity (Pavy-Le Traon, Heer, Narici, Rittweger, & Vernikos, 2007), more intense but shorter duration exercise paradigms have been employed. Resistive exercise has been demonstrated to prevent bed-rest induced decrements in muscle mass and function (Akima et al., 2000; Hiroshi Akima et al., 2003; Alkner & Tesch, 2004; Alkner & Tesch, 2004; Bamman et al., 1998;

Bamman, Hunter, Stevens, Guilliams, & Greenisen, 1997; Shackelford et al., 2004; Trappe, Burd, Louis, Lee, & Trappe, 2007), and now is a routine part of the exercise countermeasures employed on the International Space Station (ISS) (Lee et al., 2014; Loehr et al., 2011; Moore, Lee, Stenger, & Platts, 2010; Schneider et al., 2003; Trappe et al., 2009).

While traditional exercise paradigms may be appropriate in many situations, an alternative or a supplemental modality would be beneficial in individuals who cannot tolerate exercise or for whom exercise is not completely effective. Work by Layne and colleagues (Forth & Layne, 2008; Layne et al., 1998, 2001; Layne & Forth, 2008; Layne & Spooner, 1990) concerning the effectiveness of sensory stimulation of the plantar surface of the foot to manipulate neuromotor function during space flight and space flight analogs has led to the exploration of plantar stimulation of the surface of the foot as a countermeasure to muscle de-conditioning. Plantar stimulation has been shown to increase neuromuscular activity in lower limb skeletal muscle (De-Doncker, Picquet, & Falempin, 2000; Layne, Forth, & Abercromby, 2005; Vinogradova, Popov, Saenko, & Kozlovskaya, 2002), and it has been suggested that this increased muscle activity can protect against muscle atrophy during unloading (Kozlovskaya et al., 2007; Layne & Forth, 2008).

Experimental results from both animal and human studies on muscle mass and function during skeletal muscle unloading support the efficacy of this countermeasure. For example, Kyparos et al. (Kyparos, Feeback, Layne, Martinez, & Clarke, 2005) preserved

the cross-sectional area of Type I soleus and medial gastrocnemius muscle fibers during 10 d of hind limb unloading (HLU) in rats through the daily application of dynamic foot pressure. Similarly, Russian investigators at the Institute of Biomedical Problems demonstrated that daily application of plantar surface pressure (20 min/h for 6 h/d) during 7 d of dry immersion in human subjects prevented soleus Type I myofiber atrophy and decreased unloading-induced decrements in knee extensor and knee flexor muscle strength (Moukhina et al., 2004; Netreba, Khusnutdinova, Vinogradova, & Kozlovskaya, 2004).

However, the underlying mechanism(s) linking the effects of plantar stimulation and the protection of muscle mass and function during unloading are unclear. The general consequences of exercise/muscle activation under normal loading conditions suggest that unloading may inhibit a variety of mechano-sensitive signaling pathways involved in the maintenance of muscle mass and protein balance. Several biochemical/growth factor pathways participate in the regulation of muscle mass in response to mechanical loading levels, but growth hormone (GH) and insulin-like growth factor-1 (IGF-1) may be particularly important. GH (which stimulates the release of IGF-1 from the liver and acts directly in skeletal muscle) and IGF-1 are both potent stimulators of myofiber protein synthesis induced by an intracellular pathway that includes Protein Kinase B (also known as Akt) and mammalian Target of Rapamycin (mTOR). The Akt/mTOR pathway is considered the major hypertrophic signaling pathway operating within skeletal myofibers (Tidball, 2005). Circulating levels of GH and IGF-1 are known to acutely increase in response to exercise (Kraemer & Ratamess, 2005; Spiering et al.,

2008), resulting in an elevation of protein synthesis and inhibition of protein degradation within skeletal myofibers. This hypertrophic effect is mediated via stimulation of GH/IGF-1 receptors located on the myofiber sarcolemma which in turn induce the activation of the intra-myofiber Akt/mTOR pathway. Conversely, while the effects of unloading on the circulating levels of GH/IGF-1 remain unclear, expression of intra-myofiber signaling elements of the Akt/mTOR pathway are reduced during unloading, suggesting that unloading impacts the function of one or more signaling elements along the Akt/mTOR pathway (Bodine, Stitt, et al., 2001; Hornberger, Hunter, Kandarian, & Esser, 2001; Reid, Judge, & Bodine, 2014; Reynolds, Bodine, & Lawrence, 2002). Thus, it is not surprising that exercise during skeletal muscle unloading can attenuate or prevent skeletal muscle atrophy (Adams et al., 2003; Adams, Haddad, Bodell, Tran, & Baldwin, 2007; Hiroshi Akima et al., 2003; Alkner & Tesch, 2004; Alkner & Tesch, 2004; Bamman et al., 1998, 1997; Shackelford et al., 2004) by stimulating one or more signaling elements of the hypertrophic Akt/mTOR pathway, thereby balancing or overcoming the atrophic effect of unloading.

While plantar stimulation has been shown to increase muscle activity and muscle tone in both the animal and human models (De-Doncker et al., 2000; Kozlovskaya et al., 2007; Kyparos et al., 2005; Moukhina et al., 2004; Netreba et al., 2004), it is not readily apparent how such a “neural” stimulus prevents muscle de-conditioning/myofiber atrophy during unloading. Plantar stimulation of the foot results in a modest transient increase in lower limb muscle electrical activity induced via activation of neural reflex loops modified within the integration centers of the spinal cord. Such increased

electrical activity occurs without increases in muscle tension or external loading (De-Doncker et al., 2000; Layne et al., 2005; Vinogradova et al., 2002) that would be comparable to that generated in response to normal exercise. However, it is clear that plantar stimulation is capable of preventing or ameliorating myofiber atrophy during unloading suggesting that such a stimulus modifies or interacts with the biochemical pathways responsible for protein synthesis and degradation within the myofiber. Since circulating levels of GH and IGF-1 (responsible for the activation of the myofiber Akt/mTOR pathway) appear to be the primary biochemical effectors of myofiber protein synthesis it is logical to postulate that the anti-atrophic effects of plantar stimulation during unloading may be related to the actions of these growth factors on the myofibers in unloaded muscle.

Interactions between the neurosensory system and the endocrine system are not without precedent (McCall et al., 2001). Anatomical studies have demonstrated direct connections at the cellular level between neurons involved in peripheral proprioceptive pathways activated during exercise and spinal cord neurons that project into the anterior pituitary. Edgerton and colleagues (Gosselink et al., 1998, 2000, 2004; McCall et al., 1997, 1999; McCall, Grindeland, Roy, & Edgerton, 2000) conducted a number of studies in animals and humans that demonstrated that the control of GH secretion from the pituitary, in particular bio-assayable growth hormone (bGH, a specific isoform of growth hormone exhibiting enhanced biological activity), is modulated by afferent nerve traffic from nerves involved in muscle and joint proprioception in the lower limb. Additionally, while total resting circulating levels of GH are not affected during chronic

skeletal muscle unloading for a period of days, the secretion of bGH in response to plantar flexor exercise is suppressed (McCall et al., 1997, 1999). As such, an increase in neuromuscular activity resulting from plantar stimulation during exercise under normal gravitational loading might be result from activation of both plantar mechanoreceptors and intra-muscle proprioceptors, which in turn stimulates the release of GH, especially bGH, from the pituitary. Therefore, the up-regulation of muscle protein synthesis observed in response to normal exercise may be in part mediated by the release of pituitary GH elicited by increased neuronal traffic within/between neurons located in such proprioceptive pathways and neurons within the spinal cord/central nervous system that terminate in the anterior pituitary. Thus, a link may exist between afferent nerve traffic induced by plantar surface stimulation, subsequent reflex muscle activity induced in the lower limb, and the preservation of muscle mass due to activation of the Akt/mTOR pathway in unloaded myofibers by increased levels of circulating GH. However, studies designed to mechanistically investigate whether such a relationship exists between plantar stimulation, modulation of the GH/IGF-1 axis, and prevention of unloading induced atrophy in humans have yet to be performed.

While a link between afferent nerve traffic from the lower limb and release of GH from the anterior pituitary has been implied from anatomical studies, it is likely that the endocrine response would be modified or enhanced by the integration of afferent signals from different proprioceptors in the lower body. In humans, stimulation of muscle spindles in the tibialis anterior by tendon tap has been shown to elicit an increase in circulating bGH, but this effect is absent, and results in a decrease in bGH,

when the same stimulation protocol is applied to the soleus muscle (McCall et al., 2000). However, bGH is increased during plantarflexion exercise in humans (McCall et al., 1997, 1999) when the muscle proprioceptor activity is combined muscle activity (gastrocnemius and soleus) and cutaneous receptor stimulation. Similarly in rats, direct stimulation of afferent nerves from cutaneous receptors (sural nerve) evokes no change in circulating levels of bGH, while increased afferent nerve traffic from the soleus (Gosselink et al., 1998, 2000) causes a decrease in circulating bGH. However, bGH levels are increased with treadmill running (Bigbee, Gosselink, Roy, Grindeland, & Edgerton, 2000) an activity that results in the activation of a multitude of afferent pathways. Integration of afferent nerve traffic from different lower limb proprioceptors appears to result in discrepant physiological effects, perhaps reflecting different reflex neural responses. For example, cutaneous stimulation applied to the plantar surface of the foot combined with mild joint and muscle loading (seated with foot on the ground) evokes an increase in muscle activity (Layne et al., 2005), but this effect is absent when cutaneous stimulation is applied without joint and muscle proprioceptor activation (Stuart Lee, unpublished observations). This may result from an altered level of spinal cord excitability associated with the integration of an increasing amount of information within the spinal cord caused by the activation of lower limb proprioceptors. Recent data from our laboratory demonstrates that spinal cord excitability is greater when only cutaneous receptors are stimulated in comparison to the concurrent stimulation of cutaneous, joint, and muscle receptors (Boaz Blake, unpublished observations).

While studies have demonstrated that plantar stimulation during unloading can prevent or ameliorate the myofiber atrophic effects of unloading in rodents and humans (De-Doncker et al., 2000; Kyparos et al., 2005; Moukhina et al., 2004), little work has been carried out to define the optimal parameters for this type of atrophy countermeasure, particularly in humans. Much like an exercise prescription for maintaining or increasing muscle mass in which duration, frequency, intensity, pattern of stimulation, and periodization are important factors in eliciting the optimal response, the most favorable conditions for application of plantar stimulation as an atrophy countermeasure have yet to be systematically explored. It was the intent of this dissertation first to investigate the possible relationship between plantar stimulation and activation of the GH/IGF-1 axis, and secondly, to determine whether any such relationship is impacted by the level of spinal cord excitability (i.e. in the absence or presence of joint/muscle proprioceptor activation) that exists during plantar stimulation (Boaz Blake, unpublished results). In addition, it is unclear whether or not the neural signaling pathways activated during plantar stimulation result in concurrent changes in the level of neuromuscular activity (i.e. electrical activity and/or muscle tone) in the postural muscles of the unloaded limb suggesting some anatomically distinct neural pathway between the mechanoreceptors of the feet and the neurons innervating the muscles of the lower limb. As such, a secondary aim of this dissertation was to investigate whether any relationship identified between plantar stimulation and activation of the GH/IGF-1 axis during acute unloading is related to alterations in concurrent neuromuscular activity in the postural muscles of the unloaded limb. If plantar stimulation during acute unloading activates the GH/IGF-1 axis and this

response is paralleled by concurrent modulation of neuromuscular activity (i.e. electrical activity and/or muscle tone) in the postural muscles of the unloaded limb, such an observation will provide supporting evidence for the concept that the GH/IGF-1 neuro-endocrine response is mediated via activation of anatomically distinct neural connections emanating from mechanoreceptors located in the feet and neural connections terminating in the muscle. Conversely, if concurrent neuromuscular activity in the postural muscles of the unloaded limb is absent, this would suggest that this neuro-endocrine response is mediated by plantar stimulation-induced neural activity arising elsewhere, such as in the higher centers of the central nervous system. If successful, this study will provide direct experimental evidence that the anti-atrophic mechanism elicited by plantar stimulation during unloading is mediated via activation of the GH/IGF-1 pathway and provide initial information on the optimal conditions for eliciting such a protective effect while shedding light on the underlying neural connections involved in activation of the GH/IGF-1 axis by plantar stimulation.

1.2 Problem Statement

This project was part of a larger effort to characterize the effects of peripheral mechanoreceptor stimulation on neuromuscular activity and control of muscle mass during skeletal muscle unloading. Pressure applied to the plantar surface of the foot has been shown previously to partially or completely prevent the loss of muscle mass and function during unloading in animal and human models. While the regulatory pathways associated with muscle atrophy (or negative protein balance) have been investigated in other studies, our understanding of the underlying biochemical mechanisms linking

plantar stimulation to the prevention of muscle atrophy during unloading remains unclear. It was the premise of this research that plantar stimulation and increased afferent neural traffic affects muscle hypertrophic signaling pathways, specifically elements of the GH/IGF-1 axis, which in turn are responsible for preventing or attenuating the myofiber atrophic process induced during unloading.

Afferent nerve traffic is increased in response to peripheral mechanoreceptor stimulation (i.e. increased muscle spindle and cutaneous mechanoreceptor stimulation). Mechanoreceptor activation in the lower limbs also can increase neuromuscular activity in the musculature of the lower limbs by activation of reflex neural pathways such that electromyographic activity in the soleus, gastrocnemius, and tibialis anterior is increased. In aggregate, increased afferent nerve traffic and neuromuscular activity appears to be related to the secretion of at least one hypertrophic regulator of muscle mass, GH, from the pituitary. Overall, this dissertation addresses the potential causal relationship between plantar surface stimulation, the neuromuscular response to plantar stimulation in human subjects, the circulating levels of GH and the possible modulating effect of spinal cord excitability on this relationship during acute bouts of unloading in healthy individuals. Specifically, this dissertation investigates whether plantar stimulation and the resulting neuromuscular response in the presence or absence of joint/muscle proprioceptor activation (i.e. modulation of spinal cord excitability) alters circulating levels of GH (both total and bio-assayable growth hormone) during acute unloading. It also addresses whether or not any relationship between plantar stimulation and growth factor release is related to the

induction of concurrent neuromuscular activity (i.e. alterations in surface electromyography (sEMG) activity and/or muscle tone) in the postural muscles of the lower limb. Understanding whether a relationship exists between plantar stimulation, GH release and neuromuscular activity of the postural muscles of the lower limb during acute unloading will shed light on the underlying neural and biochemical mechanisms involved in the protective effects of plantar stimulation in unloading-induced muscle atrophy.

1.3 Specific Aims and Research Hypotheses

Specific Aim 1

To determine whether plantar stimulation without activation of muscle and joint proprioceptors during acute unloading results in increased levels of circulating GH in normal, healthy subjects.

Hypothesis 1: During a 10-min period of plantar stimulation applied during acute unloading without ankle joint/muscle proprioceptor activation, circulating levels of GH will be increase over the baseline levels detected immediately prior to the beginning of plantar stimulation.

Specific Aim 2

To determine whether plantar stimulation with activation of muscle and joint proprioceptors during acute unloading results in increased levels of circulating GH in normal, healthy subjects.

Hypothesis 2: During a 10-min period of plantar stimulation applied during acute unloading with ankle joint/muscle proprioceptor activation, circulating levels of GH will increase over the baseline levels detected immediately prior to the beginning of plantar stimulation.

Specific Aim 3

To determine whether plantar stimulation with and without joint and muscle proprioceptor activation during acute unloading results in increased levels of surface electromyographic (sEMG) activity in the anti-gravity muscles of the lower leg of normal healthy subjects.

Hypothesis 3: Integrated sEMG activity in the anti-gravity muscles of the lower leg (soleus, gastrocnemius, tibialis anterior) of normal healthy subjects will be reduced after 20 min of acute unloading and will subsequently increase during 10 min of plantar stimulation when the joint and muscle proprioceptors are activated, but will be remain unchanged during 10 min of plantar stimulation when the joint and muscle proprioceptors are inactive.

1.4 Potential Contributions

This work has specific relevance to care of individuals after injury, during prolonged bed rest, and to space exploration missions. In people who are unable to ambulate or bear their own weight during bed rest, pressure applied to the plantar surface of the foot may be an easy, safe, and effective way to attenuate or prevent muscle atrophy and loss of muscle strength and function. This would allow the individual to recover and

return to normal activity levels more quickly after bed rest. Individuals who participate in countermeasures to muscle deconditioning during bed rest anecdotally report that they feel more able to return to normal levels of activity at a faster rate (Watenpaugh et al., 2000). Also, the exercise countermeasure hardware and prescriptions currently employed by astronauts on ISS are impractical for exploration class missions to the moon, an asteroid, or Mars. The present hardware is too large to be used in the planned exploration space capsule (Loehr et al., 2011; Moore et al., 2010), the duration of exercise requires too much crew time, and the additional consumption of oxygen, food, and water by the crew in association with the performance of these exercise countermeasures will impact crew and vehicle supplies for the mission (Cowell, Stocks, Evans, Simonson, & Greenleaf, 2002). A countermeasure to muscle atrophy and decreased function that can be employed with minimal impact to the astronaut with a small volume of equipment would be ideal (Layne & Forth, 2008).

CHAPTER 2: REVIEW OF LITERATURE

Skeletal muscle mass is inherently linked to muscle performance and ability to perform normal daily activities. Maintenance of muscle mass during unloading conditions, as a result of injury, disease, or spaceflight, can be particularly challenging; replacing the loading associated with a normal ambulation and other activities in a limited amount of time (therapy or programmed exercise sessions) is difficult. While traditional exercise modalities that stimulate hypertrophy in healthy, ambulatory subjects have been the mainstay of countermeasures to unloading-induced muscle atrophy, additional or supplementary anti-atrophic effects might be achieved through stimulation of the neuro-endocrine system via stimulation of the plantar surface of the foot. Mechano- and proprioceptors in the lower leg and foot have been linked to the release of an isoform of growth hormone from the anterior pituitary gland that might participate in stimulation of protein synthesis and inhibition of protein degradation in skeletal muscle. When the lower limb mechano- and proprioceptors are not stimulated during unloading in animals and humans, muscle atrophies and muscle strength is reduced. Conversely, when the plantar surface of the foot is stimulated during unloading, even though there is not a significant increase in muscle tension, skeletal muscle atrophy is attenuated or prevented, particularly in the Type I myofibers. However, while the release of a growth factor in response to plantar stimulation may be responsible, the mechanism explaining the anti-atrophic effects of plantar stimulation has not been systematically studied.

2.1.0 Muscle mass and protein balance

Skeletal muscle has the ability to respond and adapt to the acute and chronic loading environment (magnitude, frequency, and duration of loading) in which it functions. It is well known that increased loading, such as through programmed resistive exercise, increases muscle mass and strength. Conversely, skeletal muscle unloading, due to decreased levels of activity, immobilization, spinal cord injury, bed rest due to injury or illness, or exposure to the weightless environment of space, results in muscle atrophy and reduced muscle function.

Modulation of skeletal muscle size in response to different loading levels results from alterations in the size of individual myofibers rather than an increase or decrease in the number of muscle fibers (i.e. hyperplasia or apoptosis) (Glass, 2005). Changes in muscle fiber size primarily result from the addition or subtraction of myofibrils and their associated sarcomeric proteins (**Figure 1**). When loading levels increase, myofibers hypertophy through the addition of myofibrils (Spiering et al., 2008), while myofibrils are removed from the myofiber during atrophy (Fitts, Riley, & Widrick, 2000). This alters the number of myofibrils acting in parallel within the myofiber and accounts for the changes in potential force production associated with hypertrophy and atrophy.

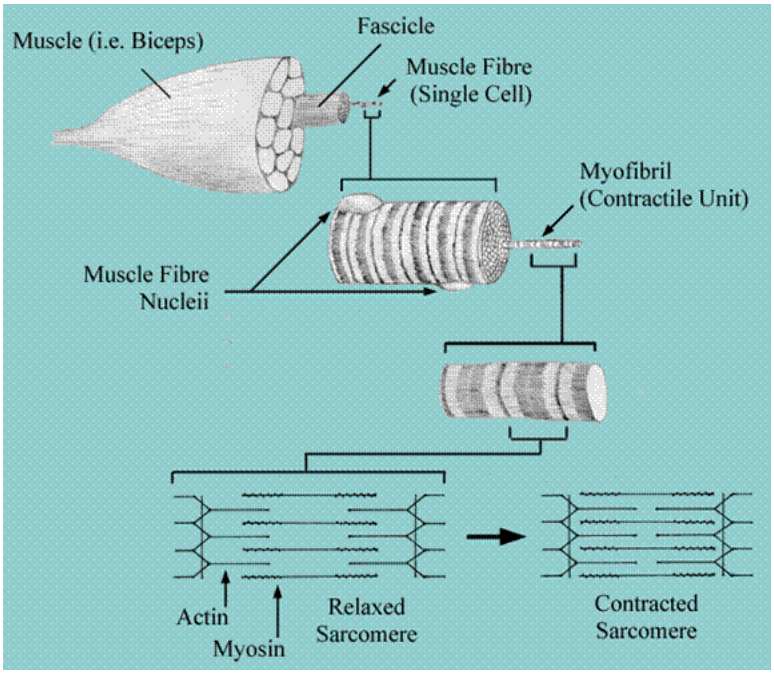

Figure 1. Exploding diagram showing skeletal muscle architecture and organization, demonstrating that skeletal muscle is composed of bundles (or fascicles) of muscle cells (myofibers), each containing highly organized groups of myofibrils. Myofibrils are composed of repeating elements, called sarcomeres, which contain the contractile proteins actin and myosin. (Source: http://grants.hhp.coe.uh.edu/clayne/6397/Unit3.htm)

Overall, changes in muscle size and strength are related to net protein balance (Greenhaff, 2006) within the individual myofibers, particularly factors influencing the synthesis or degradation of sarcomeric proteins. Although protein synthesis and degradation are continuous, ongoing processes in skeletal muscle, the relative balance of the two opposing forces determines whether myofiber size increases or decreases (**Figure 2**) (Phillips et al., 2009). When synthesis and degradation are in balance (i.e. eutrophic state), there is no net change in muscle size although individual proteins may be degraded and replaced. However, when protein synthesis predominates, muscle

hypertrophies and when protein degradation is proportionally higher, muscle atrophies.

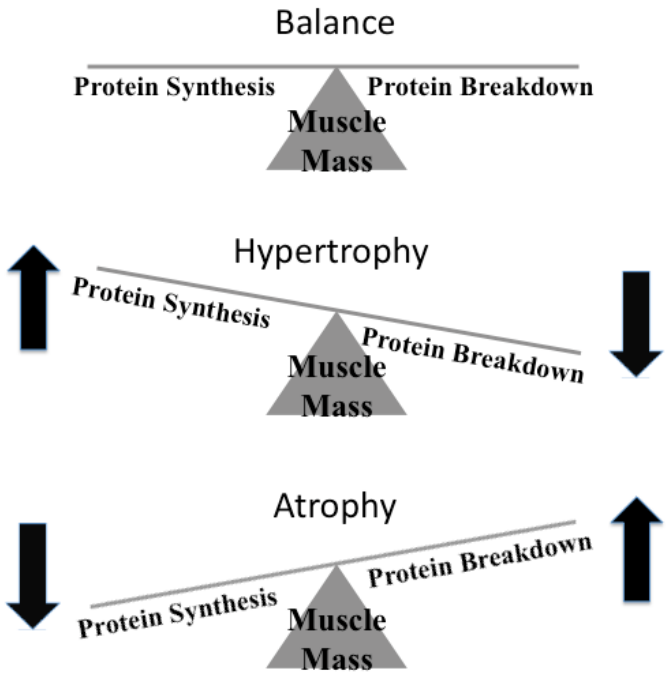

Figure 2. Relation between protein synthesis and degradation during muscle eutrophy (or balance), hypertrophy, and atrophy.

Regulation of protein synthesis, protein degradation, and ultimately muscle mass and size depends upon several regulatory pathways which can act independently, yet in some cases influence one another (**Figure 3**) (Glass, 2005; Tidball, 2005). Emerging research demonstrates interactions between hypertrophic and atrophic pathways. It is through the manipulation of these pathways that muscle hypertrophy and atrophy is induced by changes in net protein balance within the muscle. Each pathway represents a process through which either mechanical stimuli, due to physical loading, neuromuscular activity in response to loading or a combination of both, are transduced

to chemical messages that affect growth and adaptation of skeletal muscle (i.e. mechano-transduction pathways).

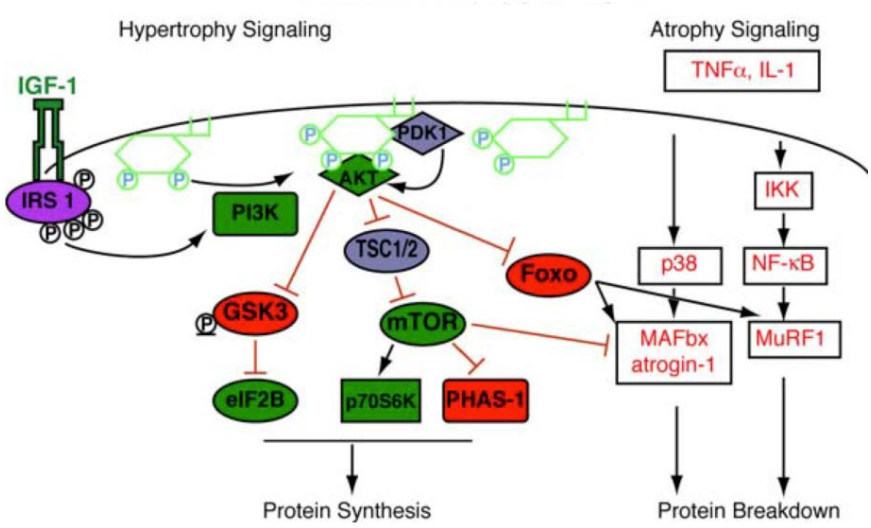

Figure 3. Molecular signaling pathways involved in muscle hypertrophy and atrophy. (Glass, 2005). Many of the details of these pathways are described in subsequent sections

2.1.1 Muscle Hypertrophy

Muscle hypertrophies in response to repeated bouts of increased loading, such as through programmed exercise or through increased activities of daily living. Acute exercise increases the uptake of amino acids and elevates protein synthesis for up to 48 h (Biolo, Maggi, Williams, Tipton, & Wolfe, 1995). There also is a transient increase in protein degradation, but with adequate nutrition this effect is small compared to the increased rate of protein synthesis. Continued regular application of loading with the appropriate rest intervals (Spiering et al., 2008) produces a net elevation of protein

synthesis over time. The initial increase in protein synthesis in hypertrophying muscle results from an increase in translational efficiency of intramyofiber ribosomes but later is the consequence of increased translational capacity through the addition of myonuclei (Adams, Caiozzo, Haddad, & Baldwin, 2002; Bodine, Stitt, et al., 2001). Myonuclei number is increased (Roy, Monke, Allen, & Edgerton, 1999) through donation of nuclei from mononuclear progenitor cells called satellite cells, which are normally quiescent and lie between the sarcolemma and the basal lamina (Hawke & Garry, 2001). In response to loading, satellite cells are activated, proliferate, and later fuse with existing myofibers. The addition of myonuclei during hypertrophy, as described by the myonuclear domain theory (Van der Meer, Jaspers, & Degens, 2011), is an important step because each nucleus regulates protein production for a finite volume of the myofiber. When satellite cell proliferation and donation of nuclei are prevented through irradiation, for example, muscle hypertrophy is inhibited (Adams et al., 2002; Mitchell & Pavlath, 2001; Rosenblatt, Yong, & Parry, 1994).

Several theories have been advanced which suggest that hypertrophy can be induced by direct mechanical stimulation of the muscle cells. Vandenburgh and colleagues performed a series of tissue culture studies in which deformation of the growth substratum upon which myotubes adhered resulted in muscle cell hypertrophy, increased protein synthesis, and inhibited proteolysis (Vandenburgh, Hatfaludy, Sohar, & Shansky, 1990; Vandenburgh & Kaufman, 1979). These studies demonstrated that muscle cells could respond to loading independent of other cells and tissues. While the mechanism for direct mechanically-stimulated muscle hypertrophy remains unclear,

several possibilities have been suggested. For example, up-regulation of protein synthesis may be stimulated in response to attempts by the muscle cell to maintain its homeostatic ion concentrations; an ion flux through a mechanical force-gated ion channel and the resulting increased ion concentration or the cell activity required to correct it might induce an adaptation of the muscle fiber. One potential candidate for such an effect is the voltage-sensitive of Na^+ channel. Mechanical stretch of skeletal muscle myotubes increases amino acid transport into cells, but this response is blocked by the Na^+ channel blockers ouabain or tetrodotoxin. It has been suggested that the increased transport of amino acid by myofibers in response to mechanical loading might be an attempt by the myofiber to re-establish resting membrane potential associated with increased Na^+ pump activity. In contrast, denervation, which is associated with reduced myofiber Na^+ flux and pump activity, reduces protein uptake (Goldberg & Goodman, 1969).

Muscle hypertrophy also might be induced by the flux of calcium (Ca^{2+}), another important ion involved in many aspects of skeletal muscle function. Many intra-myofiber signaling pathways involved in the control of myofiber protein balance have specific proteins located in their signaling cascade whose biological activity is directly related to Ca^{2+}concentration. Once such hypertrophic pathway, the calcineurin pathway, is sensitive to changes in intra-myofiber Ca^{2+} levels brought about by myofiber contraction. Calcineurin is dephosphorylated and activated by Ca^{2+} bound to calmodulin, and is thus sensitive to changes in intracellular Ca^{2+} concentrations in response to muscle contraction. In turn, calcineurin dephosphorylates NFAT (nuclear

factor of activated T cells), which subsequently translocates to the nucleus where it binds the NFAT response element to promote expression of specific genes, including transcription factors myocyte enhancer-2 (MEF2) and MyoD (Friday, Mitchell, Kegley, & Pavlath, 2003). The hypertrophic influence of calcineurin has been demonstrated by several studies. Inhibition of calcineurin activity by cyclosporine A prevents hypertrophy (45%) of mouse plantaris muscle during overload induced by synergist ablation (Dunn, Burns, & Michel, 1999) and reduces normal growth of soleus and plantaris muscles in healthy ambulatory rats (Bigard et al., 2000). Calcineurin expression also may influence myofiber type (i.e. myosin heavy chain- MHC- protein expression). Inhibition of calcineurin with cyclosporin results in a shift towards a "faster" Type II myofiber phenotype (i.e. MHC II expression) within muscle (Bigard et al., 2000; Dunn et al., 1999) and over-expression of calcineurin produces a shift towards a "slower" Type I myofiber phenotype (i.e. MHC I expression) and selective hypertrophy of muscles with predominantly slow fibers (Naya et al., 2000; Talmadge et al., 2004).

Another pathway that might participate in muscle hypertrophy involves fibroblast growth factor. Fibroblast growth factor (FGF), both the acidic and basic isoforms (aFGF and bFGF), is stored in the sarcoplasm of skeletal muscle cells and is believed to participate in the proliferation and differentiation of satellite cells in response to exercise, leading to skeletal muscle hypertrophy (Hawke & Garry, 2001). Mechanical loading is linked to the amount of muscle damage (Yamada, Buffinger, DiMario, & Strohman, 1989) and muscle membrane injury or damage is proportional to the amount of FGF released (Clarke, Khakee, & McNeil, 1993). Micro-tears in the sarcolemma,

particularly due to eccentric exercise, result in the release of fibroblast growth factor from the sarcoplasm that can then act locally at FGF receptors on the external surface of the myofiber. A causal link between mechanically-induced myofiber damage, FGF release, and muscle hypertrophy has been demonstrated in cell culture using a mechanically-active tissue culture system. Specifically, mechanical loading of culture human myotubes induces a load-dependent increase in myotube membrane damage paralleled by an increase in myotube protein synthesis, an effect which is inhibited by the presence of either heparin (known to bind both FGF and IGF-1) or an antibody specific to FGF in the tissue culture medium (Clarke & Feeback, 1996). In humans undergoing bed rest, serum levels of FGF and creatine kinase (a marker of muscle membrane damage) decrease during skeletal muscle unloading with concomitant myofiber atrophy, while both markers are increased in bed rest subjects performing resistive exercise to preserve muscle mass (Clarke, Bamman, & Feeback, 1998). In addition, the level of myofiber membrane damage caused by eccentric exercise in rodent muscle has been directly related to the amount of load applied (Knoblauch, O Connor, & Clarke, 2012; Yamada et al., 1989) while simultaneously causing a "dose-dependent" loss of intra-myofiber FGF from such damaged myofibers (Clarke et al., 1993).

However, it is generally accepted that the most influential regulator of muscle hypertrophy is the IGF-1/Akt/mTOR pathway (Tidball, 2005). This pathway can be activated in three different ways. First, muscle contraction by itself has been suggested to produce an autocrine/paracrine response of IGF-1 and a mechano-sensitive isoform

of IGF-1 called mechano-growth factor (Adams, 2002). Mechanical stimulation of muscle cells *in vitro* results in the release of IGF-1 within 1 h of stimulation but it is not detectable after 24-72 h (Perrone, Fenwick-Smith, & Vandenburgh, 1995). Second, GH released from the anterior pituitary stimulates the release of IGF-1 from the liver, which subsequently binds to the sarcolemmal IGF-1 receptor. In both cases described thus far, IGF-1 bound to the IGF-1 receptor activates the kinase activity of the receptor, resulting in the serial activation of insulin response substrate-1 (IRS-1), phosphatidyl-inositol-3 kinase (PI3K), and Akt (also known as Protein B). Alternatively, circulating GH can attach to the GH receptor on the sarcolemma, which in turn activates Janus kinase 2 (JAK2), a tyrosine kinase. JAK2 then activates PI3K and the cascade continues in a similar manner as the two other pathways. In each case, Akt phosphorylates and activates mTOR (Navé, Ouwens, Withers, Alessi, & Shepherd, 1999) and activates $p70^{S6K}$ (Rommel et al., 2001). Activation of $p70^{S6k}$ particularly important because it enhances the translation of mRNAs encoding ribosomal proteins and elongation factors (Adams, 2002). Activation of this pathway also inhibits glycogen synthase kinase 3B, a serine-threonine kinase that can inhibit translation that is initiated by eukaryotic initiation factor 2B (Hardt & Sadoshima, 2002). Also, IGF-1 and IGF-II are known to stimulate satellite cell proliferation (Hawke & Garry, 2001).

The importance of the Akt/mTOR pathway in muscle hypertrophy has been demonstrated in models of muscle overload in rats. Surgical ablation of the synergistic soleus and gastrocnemius results in a four-fold increase in total levels of Akt in hypertrophying plantaris and a nine-fold increase in the phosphorylated/activated

form of Akt. This increase in activated Akt, and subsequent increase in activated mTOR levels, leads to downstream events such as the phosphorylation and deactivation of GSK3B, phosphorylation and activation of $p70^{S6k}$ and release of PHAS 1/4-BP1 from eIF4E allowing its binding to eIF4G. Further, over-expression of constitutively active forms of Akt or PI3K results in larger tibialis anterior muscle fibers in ambulatory adult mice compared to controls not receiving treatment. However, treatment with rapamycin, a specific inhibitor of mTOR, prevents activation of $p70^{S6K}$, inhibits the release of PHAS 1/4-BP1, and prevents muscle hypertrophy (Bodine, Stitt, et al., 2001). mTOR signaling also can be activated in the absence of Akt phosphorylation. In animals in which PI3K is inhibited or with a null mutation for Akt, passive stretching of mouse extensor digitorum longus activates mTOR (Hornberger et al., 2004).

Myostatin, known as growth and differentiation factor-8 (Sharma, Langley, Bass, & Kambadur, 2001), also may participate in the control of muscle hypertrophy but as an inhibitor of muscle hypertrophy, rather than a promoter, and acts through the regulation of protein synthesis and satellite cell proliferation and differentiation (Hawke & Garry, 2001). Myostatin is perhaps best known for its association with hyperplasia and hypertrophy in mice null for the myostatin gene (McPherron, Lawler, & Lee, 1997), in cattle (the Belgian Blue and Piedmontese breeds) that have mutations of the myostatin gene sequence leading to a “double muscling” effect (Kambadur, Sharma, Smith, & Bass, 1997), and loss of gene function in a human child (Schuelke et al., 2004). In normal conditions, an acute bout of resistance exercise training decreases myostatin

mRNA expression (Garma et al., 2007) and long-term training reduces myostatin expression.

2.1.2 Muscle Atrophy

Muscle atrophies due to skeletal muscle unloading (without injury or disease), probably as a means to conserve metabolic resources (Lin, Hershey, Mattoon, & Robbins, 2012). Muscle atrophy is the consequences of a net decrease in protein synthesis or a net increase in protein degradation. The decrease in myofiber cross-section, particularly in humans, is due to decreased protein synthesis (Ferrando, Paddon-Jones, & Wolfe, 2002; Paddon-Jones et al., 2004; Phillips & McGlory, 2014; Symons, Sheffield-Moore, Chinkes, Ferrando, & Paddon-Jones, 2009) without a loss of muscle cell number, although increased protein degradation also likely contributes (Reid et al., 2014). Several lines of evidence support this contention based upon alterations in regulatory pathways associated with protein synthesis. Although it appears that circulating levels of GH and IGF-1 are not decreased during skeletal muscle unloading, IRS-1 and the Akt/mTOR pathway activity is reduced (Haddad, Adams, Bodell, & Baldwin, 2006). Akt phosphorylation is decreased, $p70^{S6K}$ activation is reduced, and the binding of PHAS 1/4-BP1 is higher compared to ambulatory controls (Bodine, Stitt, et al., 2001). Also, circulating levels of FGF are reduced during bed rest (Clarke et al., 1998). The result is that there is a decrease in total RNA in skeletal muscle cells, which is primarily ribosomal, and the reduction would be consistent with a decrease in total capacity to synthesize protein (Haddad, Roy, Zhong, Edgerton, & Baldwin, 2003). Additionally, the

number of myonuclei within each cell (Zhang et al., 2010) decreases with muscle fiber atrophy, perhaps to maintain the myonuclear/sarcoplasm volume ratio, although this finding has been challenged (Bruusgaard et al., 2012; Wada, Takahashi, Katsuta, & Soya, 2002). The time course of myofiber atrophy and myonuclei loss are not the same, such that atrophy precedes the decrease in myonuclei number (Gundersen & Bruusgaard, 2008).

Skeletal muscle atrophy during unloading also could involve activation of catabolic pathways leading to protein degradation (Glass, 2005; Reid et al., 2014). Three catabolic pathways are involved in protein degradation in muscle: lysosomal, calpain, and ubiquitin-proteasome pathways (Kandarian & Stevenson, 2002). Lysosomes, membrane-vesicles containing proteolytic enzymes, are associated with degradation of damaged proteins but are not believed to contribute to disuse-associated atrophy. Rather, sarcomeric protein degradation is believed to result from the sequential activation of the calpain and proteasome pathways (Greenhaff, 2006). Calpains are a family of calcium-activated proteases, including calpain-1, calpain-2, and calpain-3, that are concentrated at the z-disks (Huang & Forsberg, 1998) and appear to be involved in the initial atrophic responses to unloading. Calpains do not act directly on myosin or actin but are considered integral to the disassembly of the large sarcomeric proteins by targeting titin, viniculin, dystrophin, and desmin (Greenhaff, 2006; Kandarian & Stevenson, 2002). Calpain-1 and calpain-2 mRNA expression is transiently increased with unloading, increasing in the first 24 h of immobilization but decreases thereafter (Jones et al., 2004), and soleus muscle atrophy can be largely inhibited *in vitro* in the

first few days of unloading with either a caplain inhibitor or an antagonist against calcium release from the sarcoplasmic reticulum (Tischler et al., 1990). Muscle atrophy also is reduced by 30% during HLU in rats induced to over-express calpastatin, a calpain inhibitor (Tidball & Spencer, 2002).

The disassembly of sarcomeric proteins by calpains is the critical step which then allows for contractile protein degradation in skeletal muscle by the ATP-dependent ubiquitin-proteosome pathway (Phillips et al., 2009). The ubiquitin-proteasome pathway cannot directly degrade intact myofibrils, but can degrade individual actin and myosin molecules. Degradation begins with the attachment of multiple ubiquitin molecules to targeted proteins, one ubiquitin molecule at a time, in three-step, ATP-dependent process involving three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3). Ubiquitin is first attached to E1 in the presence of ATP, then transferred to E2, and then bound to the protein targeted for degradation by E3. Once four or more ubiquitin molecules have been attached to the target protein, it is then moved to the proteasome where it is rapidly degraded. The importance of the ubiquitin-proteasome pathway in muscle atrophy has been demonstrated by the direct inhibition of the proteasome. Proteolysis in incubated rat muscles is reduced by 50-70% by peptide aldehyde inhibitors (Tawa, Odessey, & Goldberg, 1997). However, the overall contribution of the ubiquitin-proteasome pathway to muscle atrophy during unloading may be a species-specific effect. E3 ligases tend to be up-regulated during bed rest in humans (Caiozzo et al.,

2009), but there is not an accompanying increase in the rate of protein breakdown (Symons et al., 2009).

Although E1 and E2 ligases are important, it is the E3 ligases that confer specificity to the degradation of proteins in the proteosomal pathway. Two E3 ligases appear to be particularly important to the atrophic process, Murine Ring Finger-1 (MuRF-1) and Muscle atrophy F-box (MAFbx or atrogin-1) (Bodine, Latres, et al., 2001; Gomes, Lecker, Jagoe, Navon, & Goldberg, 2001). Immobilization, denervation, and unloading in rats alter the expression of many genes, but the upregulation of MuRF-1 and MAFbx is common across all three models of muscle atrophy. Additionally, mice deficient in MAFbx and MuRF-1 experience 56% and 36% less muscle atrophy than wild-type mice after 14 d of denervation of the gastrocnemius (Bodine, Latres, et al., 2001). Further, 5 d of HLU increases MuRF-1 and MAFbx mRNA by 140% and 230%, respectively, while there is concomitant absolute decrease in medial gastrocnemius muscle weight (Haddad et al., 2006). However, isometric exercise during HLU that normalizes MuRF-1 and MAFbx mRNA levels to pre-HLU levels does not fully prevent muscle atrophy. There are likely to be other E3 ligases important to muscle atrophy, but these have not been fully investigated (Glass, 2005).

The effects of the up-regulation of MuRF-1 and MAFbx can be attenuated through treatment with IGF-1 acting through the PI3K/Akt pathway (Sandri et al., 2004; Stitt et al., 2004), demonstrating the interaction between regulators of protein synthesis and

degradation. Akt phosphorylation inhibits the activation and translocation of FOXO1, a member of the forkhead family of transcription factors that is necessary for the expression of MAFbx and MuRF-1. However, prevention of muscle atrophy during HLU may require a countermeasure that does more than normalize MuRF-1 and MAFbx mRNA to levels similar to normal muscle loading. An exercise countermeasure which included concentric, eccentric, and isometric components and maintained muscle mass during HLU resulted in IGF-1 mRNA and MGF mRNA levels that were higher than that observed in normal ambulatory controls yet atrogin mRNA levels also were elevated (Adams et al., 2007).

Myostatin has been implicated as a contributor to muscle atrophy as a method by which protein synthesis is inhibited, but its effects are not clear. There are no detectable levels of myostatin in the soleus muscle in mice either while weight bearing or during HLU, but levels are increased on day 1 of HLU in the gastrocnemius and plantaris muscles (Carlson, Booth, & Gordon, 1999). This occurs even though the soleus atrophy is greater after HLU than in either the gastrocnemius or the plantaris (Wehling, Cai, & Tidball, 2000). Additionally, while elevated levels of myostatin mRNA during HLU can be prevented by electrically-induced muscle contractions (Adams et al., 2007) and by rigorous isometric exercise (Haddad et al., 2006), muscle mass is not consistently protected. Further, 30 min of daily loading during HLU prevents muscle atrophy even though myostatin mRNA increased by 55% during HLU (Wehling et al., 2000). More recently, activation of the myostatin/activin IIB receptor pathway has been shown to down-regulate the Akt/mTOR pathway by reducing the level of Akt/mTOR

phosphorylation without the up-regulation of protein degradation in cell culture (Trendelenburg et al., 2009) but in pathological conditions inactivation of the Akt/mTOR pathway leads to the up-regulation of enzymes in the ubiquitin-proteasome pathway (Sandri et al., 2004).

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) also may contribute to skeletal muscle unloading-induced muscle atrophy through increased expression of MURF-1 (Cai et al., 2004). NF-kB appears to be expressed in a fiber specific manner during unloading that corresponds to the pattern of muscle atrophy. NF-kB increases 10-fold during 7 d of HLU in the soleus muscle of rats, which is susceptible to atrophy, but not in extensor digitorum longus, which is resistant to atrophy (Hunter et al., 2002). While several NF-kB family members are up-regulated during HLU, the standard indicators for cytokine-induced activation of NF-kB observed during disease- and inflammation-induced muscle wasting are not apparent during skeletal muscle unloading, suggesting an alternative pathway for NF-kB increased expression.

2.1.3 Models of Skeletal Muscle Unloading

There are many forms of skeletal muscle disuse and unloading that can lead to muscle atrophy and decreased muscle performance. Muscle disuse and unloading result from decreased levels of physical activity (detraining) or are the result of injury or illness (e.g. casting of a broken bone, prolonged bed rest, spinal cord injury). Also, skeletal

muscle unloading can be induced as an experimental condition that is imposed upon otherwise healthy individuals as means to study the control of muscle mass and function. These models of muscle disuse and unloading include denervation, hindlimb unloading (HLU), spinal cord transection, and limb immobilization in animals and bed rest, unilateral limb suspension, dry immersion, and limb immobilization by casting in humans. Also, both animals and humans exposed to the weightlessness environment of space flight (without countermeasures) experience muscle atrophy and deconditioning.

Each form of skeletal muscle unloading is characterized by changes in neuromuscular activity, muscle atrophy, and decreased muscle performance. Although the results of each form of unloading are similar in many ways, the degree and type of skeletal muscle unloading ultimately determines the magnitude and mechanisms of muscle atrophy (Adams et al., 2003; Navasiolava et al., 2011). Describing the characteristics of each is beyond the scope of this literature review, therefore this review will focus on models of skeletal muscle unloading in animals and humans in which a specific countermeasure to muscle atrophy, pressure applied to the plantar surface of the foot, has been tested: HLU in rodents, bed rest in humans, space flight exposure in humans and rodents, and dry immersion in humans.

Skeletal muscle unloading in rodents in accomplished using a methodology called hindlimb unloading (HLU; **Figure 4**). The rodents are suspended in their cage such that the forelimbs touch the cage bottom, but the hindlimbs are lifted and do not contact the cage bottom. This model of unloading was developed to mimic the effects of space flight,

including cephalad shift of body fluids and atrophy of the lower limbs, (particularly the extensor muscles) without surgical intervention or animal restraint as had been previously employed (Morey-Holton & Globus, 2002). The animals are suspended at angle of 30°, such that 50% of the animals body weight is supported through the forelimbs and no weight is applied to the hindlimbs (Hargens, Steskal, Johansson, & Tipton, 1984). The first methods of HLU used a full body harness (Morey, Sabelman, Turner, & Baylink, 1979; Musacchia, Deavers, Meininger, & Davis, 1980). Later investigators used an attachment to the tail to suspend the animal, which appears to be less stressful than a whole body harness. Attachment to the tail once was accomplished by a plaster cast, but more recently investigators use highly adhesive traction tape applied to the base of the tail. The tape holds a metal clip, which then is attached to a fishline swivel suspended from a bearing that can travel along a bar at the top of the cage. The bar also has bearings on either end that allow it to travel the length of the cage. Thus, the rodent can have access to all parts of the cage and rotate their body through 360° by use of their forelimbs (Morey-Holton & Globus, 2002).

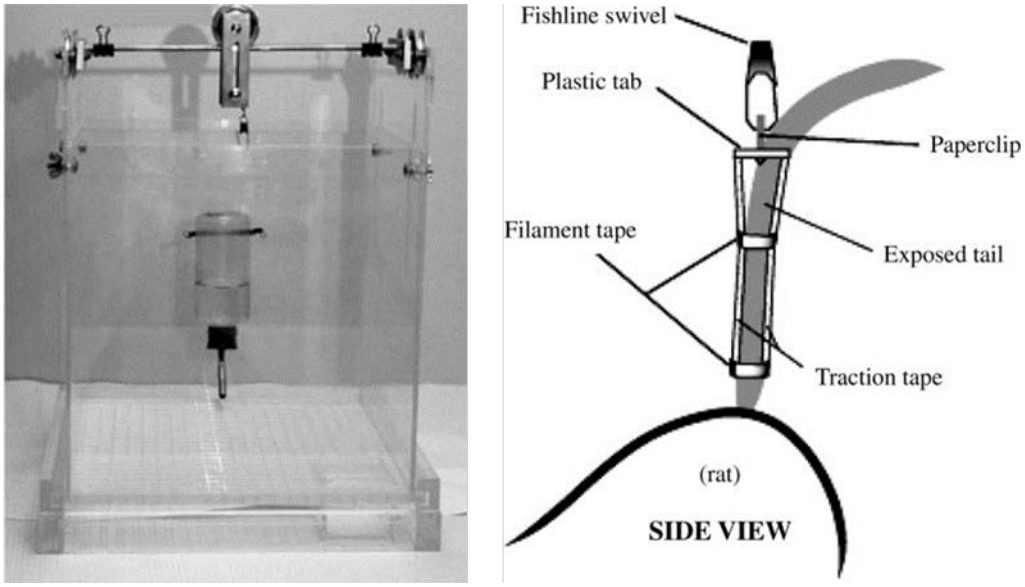

Figure 4. Picture of one of style of cage used to hindlimb unloaded rats (left panel) and a schematic of one method to use the rat's tail for suspension. (Morey-Holton & Globus, 2002)

Bed rest is the most commonly employed experimental method to assess the effect of unloading in humans. Once routinely used in medical management of disease because it was thought that the forced rest would aid in recovery, bed rest later became a favorite modality to simulate the effects of space flight (Pavy-Le Traon et al., 2007). Experimental subjects remain in bed for 24 h/day in this model of unloading (Meck, Dreyer, & Warren, 2009), with the bed maintained in a horizontal or head-down position (**Figure 5**). Some studies which are designed specifically to test the effects of unloading on skeletal muscle have used the horizontal bed position, but often subjects are placed in a 6° head-down tilt bed to simulate the effects of space flight on other physiological systems, particularly the cardiovascular system (Schneider & Convertino, 2011). Bed rest effectively minimizes the skeletal muscle loading associated with the upright posture (minimized z-axis loading), reduces overall activity and metabolism

(Adams et al., 2003), and decreases proprioceptive stimulation and overall sensory stimulation (Pavy-Le Traon et al., 2007). As in other models of skeletal muscle unloading, the greatest effects are realized in the lower body of humans (Adams et al., 2003; LeBlanc et al., 1992).

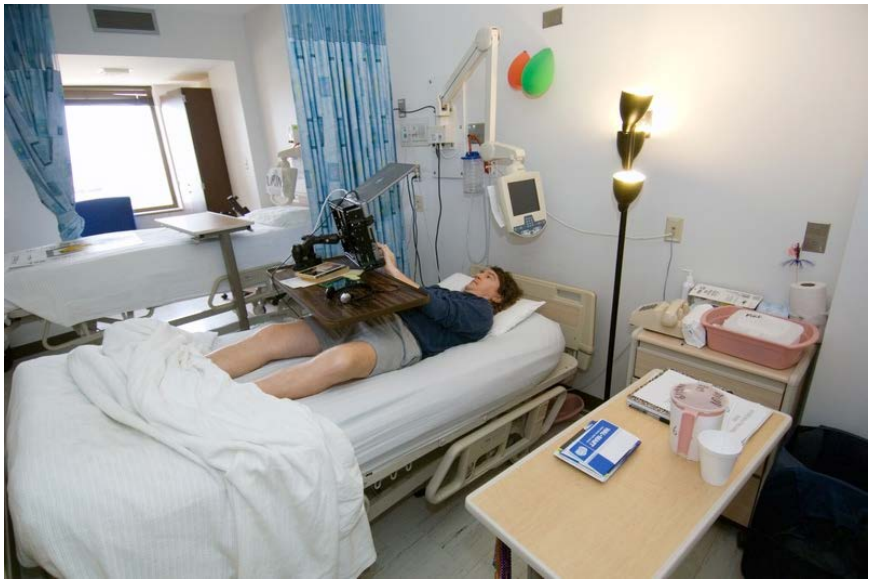

Figure 5. Subject participating in the National Aeronautics and Space Administration's 6° head-down tilt bed rest at the University of Texas Medical Branch in Galveston, TX. (Source: http://www.wired.com/wiredscience/2008/05/nasa-bed-rest-l/)

Skeletal muscle unloading during space flight (**Figure 6**) is novel in that no tissues experience a significant acceleration due to gravity (Adams et al., 2003); space flight is the only condition in which both z-axis and x-axis loading are eliminated (Pavy-Le Traon et al., 2007). Exposure to the weightless environment of space flight is an effective means to induce skeletal muscle unloading in rodents, producing significant muscle atrophy (Fitts et al., 2000), but there has been some question as to whether the amount of muscle atrophy and deconditioning measured in astronauts fully represents

the deconditioning effects of space flight. The magnitude of the skeletal muscle atrophy in space flight is comparable to bed rest (Adams et al., 2003), but astronauts routinely participate in some form of exercise countermeasures to combat the space flight-induced deconditioning. Countermeasures to deconditioning are indicated and considered a necessity because astronauts experience an abrupt change in skeletal muscle loading, from no-load to full load upon return to Earth (Adams et al., 2003) and may be expected to work at or near maximal levels in the event of an emergency (Bishop et al., 1999). Thus, space flight is not a pure model of skeletal muscle unloading as astronauts must perform work during the course of their mission and participate in exercise countermeasures to attenuate or prevent the deleterious effects of space flight (Adams et al., 2003; Edgerton et al., 2001; Trappe et al., 2009; Trappe et al., 2001).

Figure 6. Astronauts onboard the International Space Station experience skeletal muscle unloading in the weightless environment of space.

Immersion of a human subject in a tank of water was thought to be the best model of unloading in ground-based studies, particularly to mimic the effects of space flight, but more than one day of immersion in water is considered to be particularly inconvenient and uncomfortable (Pavy-Le Traon et al., 2007). Thus, while many researchers utilized bed rest to study skeletal muscle unloading, an alternative called dry immersion was developed during the mid-1970's. In this model, the subject is placed in the middle of a waterproof sheet and slowly lowered into the water to the level of the clavicles such that the material wraps the subject and keeps the subject dry (**Figure 7**). The subjects are allowed to have their arms above the surface of the water to read, work, and perform experiments and are permitted to get out of the immersion tank once per day for 15 min to perform personal hygiene activities. It has been suggested that dry immersion may be the most effective model of unloading, particularly for the neuromuscular system, because it effectively eliminates mechanical support from all surfaces of the body (Navasiolava et al., 2011). The duration of dry immersion studies has varied from just a few hours to up to 56 d, although the most common duration is 3-7 d (Navasiolava et al., 2011).

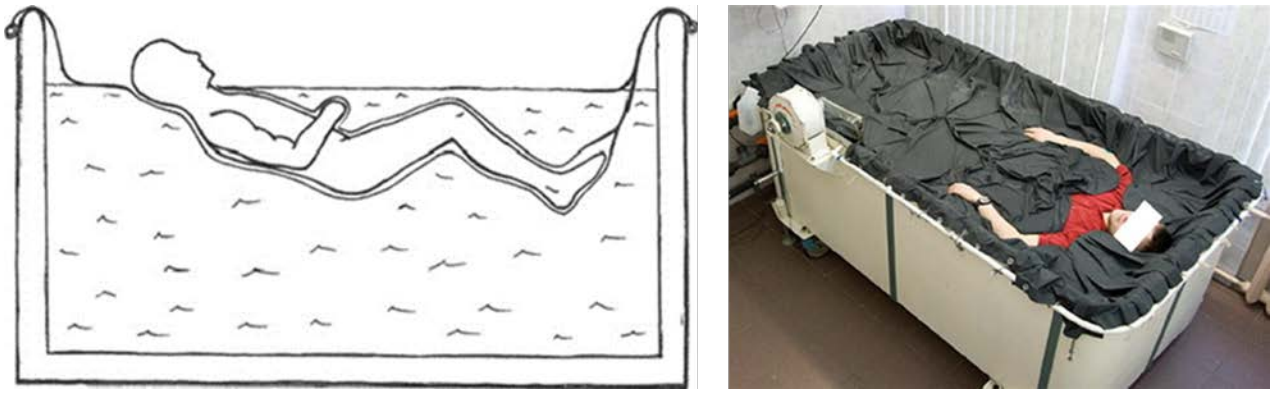

Figure 7. Schematic (left panel) and photograph (right panel) of dry immersion model of skeletal muscle unloading in which the subject is suspended in water but separated from it by a heavy, waterproof material (Navasiolava et al., 2011)

2.1.4 Effect of Unloading on Skeletal Muscle

Exposure to skeletal muscle unloading is associated with adaptation of the body's physiological systems. In the musculoskeletal system, muscle tissue atrophies, muscle strength and endurance decreases, and individual myofibers shift towards a "faster" phenotype (Adams et al., 2003; Phillips et al., 2009). Despite the breadth of investigations performed, the mechanisms responsible for such adaptations to skeletal muscle unloading have yet to be fully elucidated (Narici & de Boer, 2011). Myofiber atrophy in response to unloading is of particular concern because it is one of the prime determinants of an unloading-associated decrease in muscle force, power, and endurance (di Prampero & Narici, 2003) affecting overall human performance.

As in all cells, the amount of protein in skeletal muscle cells at any time is the consequence of the relative balance between protein synthesis and protein degradation. During muscle atrophy protein synthesis and degradation are not in balance, resulting

in loss of skeletal muscle protein, particularly contractile proteins. In rats, it has been suggested that after an initial decrease in protein synthesis during unloading, protein degradation is upregulated after about 3 d (Thomason, Biggs, & Booth, 1989). In contrast, human models of skeletal muscle unloading, in conditions in which inflammatory processes do not predominate, the unloading-induced skeletal muscle atrophy is generally accepted to result from a down-regulation of protein synthesis while protein degradation is unchanged (Ferrando et al., 2002; Phillips et al., 2009; Stein, Leskiw, Schluter, Donaldson, & Larina, 1999). Recent data suggest that protein synthesis is reduced both in the fasting state and in the fed state (Glover et al., 2008), with a combined effect of a ~58% reduction in protein synthesis each day during unloading (Phillips et al., 2009).

The time course of unloading-induced muscle atrophy generally is not considered to be linear. In rats, much of the muscle atrophy occurs with the first week of unloading, with as much as 37% loss of soleus muscle mass during the first week of space flight (Fitts et al., 2000), and with the greatest losses occurring in the slow Type I fibers (Jiang et al., 1992; Ohira et al., 1992). In humans, the decrease in muscle mass and cross-sectional areas also is initially rapid (0.6-1.0% per week) (Narici & de Boer, 2011), but the rate slows after about one month (Phillips et al., 2009) and appears to reach a new steady state after several months if unloading is prolonged (LeBlanc et al., 2000). Most of the reduction in muscle mass and strength occurs within the first 2-3 weeks of bed rest, with a slower rate of loss thereafter (Adams et al., 2003) in most studies, although a linear decline in quadriceps cross-sectional area has been reported during 8 wk of bed

rest (Mulder et al., 2006). Whether there is a fiber type-specific decrease cross-sectional area during unloading in humans is less clear (Fitts et al., 2000), but there appears to be a trend across studies for a greater loss in Type II fibers (Edgerton et al., 1995; Widrick et al., 1999).

2.1.5 Neuromuscular Activation in Control of Muscle Mass

Skeletal muscle hypertrophy and atrophy are modulated by loading and neuromuscular activity. In general, skeletal muscle adapts to an increase in neuromuscular activity and loading by increasing muscle mass. This effectively reduces the mechanical and metabolic stress per unit of muscle in response to the stimulus. This is the expected response to chronic increases in muscle work or exercise. Conversely, when neuromuscular activity is decreased, muscle atrophies to lessen the metabolic cost of maintaining muscle mass that is not in use (Lin et al., 2012). Changes in neuromuscular activity, in general, are considered to be a specific modifier of muscle mass (Falempin & Mounier, 1998) since muscle function and structure are influenced by neural input (Adams et al., 2003).

As an example of the relation between muscle mass and neural input, the magnitude and rate of muscle atrophy is not consistent across muscle groups and may be related to the normal levels of neuromuscular activation in the upright, normal gravity environment. Although the exposure to unloading is experienced throughout the body in humans, the alterations in skeletal muscle occur primarily in the lower body in what have been called the anti-gravity muscles (Adams et al., 2003; Fitts et al., 2000; Fitts,

Riley, & Widrick, 2001; LeBlanc et al., 1992). Muscle atrophy in the lower body may be more readily apparent earlier during unloading because of either greater muscle mass to lose, the changes are large enough so as to be more reliably measured, and/or there is a greater change in loading levels experienced by these muscles from normal gravity to the unloaded condition. With respect to loading and muscle activity, the lower body is responsible for supporting, carrying, and transporting the whole body throughout the day in normal gravity, and this is largely lost during unloading. Normally, muscle loading occurs in the static condition, as in standing, and during dynamic situations, such as rising from a bed or chair, walking, running, etc. These activities are not performed during unloading, unless performed as part of an exercise countermeasure or scientific investigation. Decreased muscle mass and strength in the plantarflexor muscles of the ankle, in particular, may be of great importance to function during locomotion in normal upright activities on Earth because they experience proportionally greater loading (Narici & de Boer, 2011) and higher metabolic stress (Lee et al., 2011).

In contrast, changes in muscle size, structure, and function occur in the upper body but to a lesser degree and at a slower rate (Convertino, 1996). Perhaps this is because the muscle mass in the upper body is smaller so it is more difficult to measure changes reliably or there is less to lose to achieve new baseline. An alternative explanation is that the upper body muscles are proportionally more active than the lower body in some models of unloading (i.e. bed rest and space flight) than in normal ambulatory humans. Bed rest subjects use the arms and upper torso are used to support themselves

during eating and other activities, and the arms and upper torso become a means of locomotion and attachment during space flight (LeBlanc et al., 1992).

While neuromuscular activity contributes to the control of muscle mass, attempts to mimic neuromuscular activity experienced during normal stance and ambulation using electrical stimulation during skeletal muscle unloading have not been particularly effective in preventing muscle atrophy. Chronic low frequency stimulation (CLFS) has been applied in a manner designed to mimic the tonic muscle activity normally associated with the postural muscles of the lower limb. To-date, this form of muscle stimulation independent of loading has been only partially protective of muscle mass (Leterme & Falempin, 1994), particularly in slow muscle fibers (Canon, Bigard, Merino, Lienhard, & Guezennec, 1995). However, CLFS appears to protect against some of the fiber type shifting which is characteristic of unloading (Furby, Mounier, Stevens, Leterme, & Falempin, 1993) and some parameters of muscle performance, such as time to peak tension and half-relaxation time (Leterme & Falempin, 1994). Similarly, manipulation of muscle activity by Achilles tendon vibration only attenuates muscle atrophy and the decrease in muscle function (Falempin & In-Albon, 1999).

The overall importance of muscle activity during unloading and its effect on muscle performance upon reloading perhaps is best summarized by Kozlovskaya et al. (Kozlovskaia, Grigor'eva, & Gevlich, 1984). These investigators have suggested that the decrease in muscle tone, an indirect measure of random and spontaneous muscle contractions, during exposure to skeletal muscle unloading is correlated with the

decrements in muscle strength as well as the relative amount of unloading achieved (e.g. bed rest vs. dry immersion). They suggest that the loss of chronic muscle tone is the primary determinant of decreased muscle mass and function during unloading, where a reduction in neural activation of the unloaded muscle is a key stimulus in the subsequent atrophic pathways activated within the unloaded myofibers.

2.2.0 Neuromuscular Response to Skeletal Muscle Unloading

One might assume that skeletal muscle unloading results in rapid and prolonged decreases in muscle activity. While this appears to be the case acutely (Alford, Roy, Hodgson, & Edgerton, 1987; Blewett & Elder, 1993; Kawano, Nomura, Ishihara, Nonaka, & Ohira, 2002; Layne et al., 2005), it is unclear what level of neuromuscular activity is present over days, weeks, and months of skeletal muscle unloading. However, it is believed that the changes in neuromuscular activity (reduced amount and/or shift from tonic to phasic activity) are the precursors to altered neuromuscular control during and after prolonged unloading and potentially contribute to decreased muscle mass and function (Kozlovskaya et al., 2007; Layne & Forth, 2008).

2.2.1 Neuromuscular Response to Skeletal Muscle Unloading in Animal Models

A consistent observation is that the initiation of skeletal muscle unloading results in a rapid alteration in efferent and afferent neuromuscular activity in rats. That is, EMG activity in plantarflexor muscles decreases upon exposure to skeletal muscle unloading produced by parabolic flight (**Figure 8**) (Kawano et al., 2002; Leterme & Falempin, 1998) and at the initiation of HLU in rats (Alford et al., 1987; Blewett & Elder, 1993; De-

Doncker, Kasri, Picquet, & Falempin, 2005; Falempin & In-Albon, 1999), although ankle flexor activity increases. The initiation of skeletal muscle unloading also is accompanied by a decreased in afferent neural activity in rodents, measured by encapsulating the L5 dorsal root in a silicone tube with electrodes to detect neural signals (De-Doncker et al., 2005; Kawano et al., 2004, 2002). EMG activity and afferent neural activity returns to the level of activity corresponding to normal gravity conditions when gravity is restored or acute HLU is terminated.

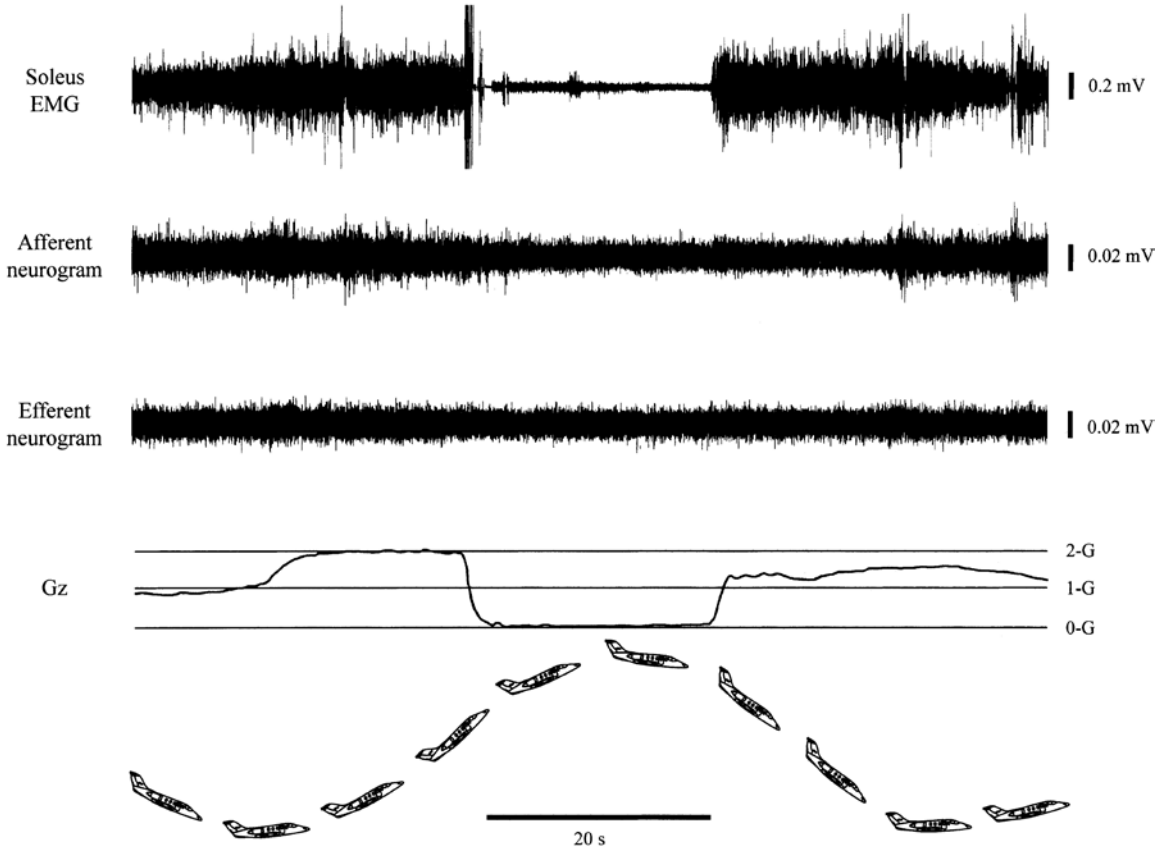

Figure 8. Representative EMG tracing from the soleus (top) and a neurogram recording at L5 (second from top) in a rat during normal gravity (1-G), hypergravity (2-G), and microgravity (0-G) produced during parabolic flight of an airplane (bottom). (Kawano et al., 2002)

While an immediate effect of unloading on lower limb EMG activity and the afferent nerve traffic in rodents is not disputed, there appears to be no clear consensus as to whether these remain depressed during prolonged unloading. Some authors observed that EMG activity remains depressed throughout HLU (Blewett & Elder, 1993; De-Doncker et al., 2000) while others (Alford et al., 1987; De-Doncker et al., 2005; Ohira et al., 2002) have reported that EMG activity of the plantarflexors increases and of the tibialis anterior decreases to pre-HLU within the first two weeks (**Figure 9**). Afferent nerve activity also recovers in this time frame (De-Doncker et al., 2005). However, after 4 weeks of HLU, EMG activity in the plantarflexors gradually declines until at least 9 weeks of HLU (Ohira et al., 2002). However, even if muscle EMG and the afferent neurogram are normalized to or greater than pre-HLU levels, soleus and medial gastrocnemius muscle mass and function decreases during HLU (Winiarski, Roy, Alford, Chiang, & Edgerton, 1987).

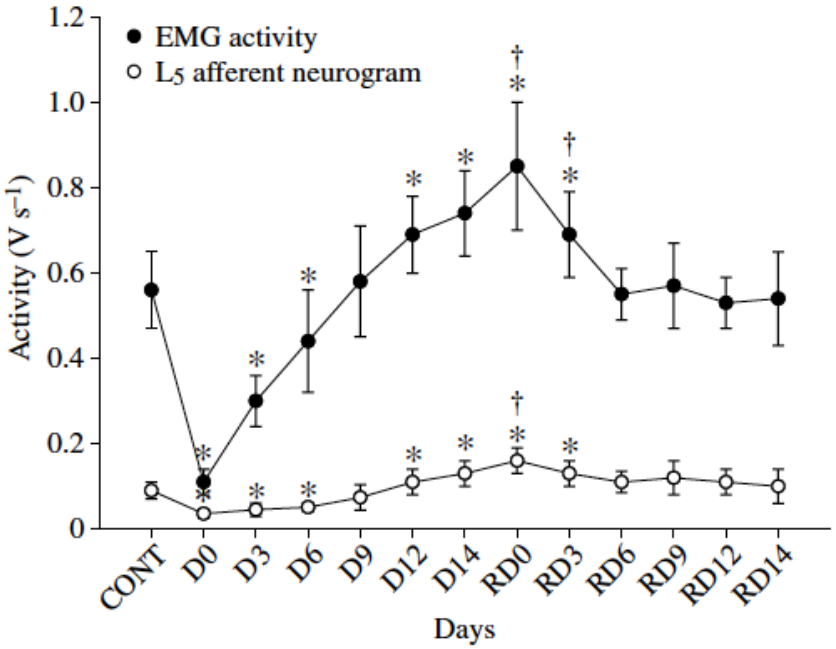

*Figure 9. Mean soleus EMG activity and afferent neural traffic before (CONT), during (D0-D14), and while recovering from (R0-R14) 14 d of HLU (De-Doncker et al., 2005). *Significantly different than pre-HLU (CONT). Dagger represents significantly different than pre-HLU (CONT). Dagger represents significantly different than 14th day of HLU (D14)*

Besides changes in total EMG activity of the plantarflexors, the qualities of EMG activity may be altered during HLU (De-Doncker et al., 2005), which has been described as a shift from tonic to phasic muscle activity. The duration of EMG activity (average train duration) decreases and the time between EMG bursts is prolonged (Riley et al., 1990). Perhaps these changes may be related to the decrease in afferent neural activity from the cutaneous mechanoreceptors and the muscle spindles. Cutaneous receptors in the feet normally transmit information to the central nervous system and thus participate in reflexes that contribute to the stabilization of the feet and posture (Aniss, Gandevia, & Burke, 1992; Kavounoudias, Roll, & Roll, 1998). Neural input from the feet would be expected to be minimal to nonexistent during muscle unloading in HLU and spaceflight,

for example, since the soles of the feet do not touch the ground (De-Doncker et al., 2005, 2000).

Stimulation of the muscle spindles (muscle proprioception) also is likely to be decreased during unloading since the muscles typically are in a shortened position and the spindles normally react to muscle stretch. The ankles of rats exposed to unloading during microgravity simulated by parabolic flight immediately assume a plantarflexed position (Kawano et al., 2002; Leterme & Falempin, 1998; Riley et al., 1990). These ankle positions are associated acutely with lower levels of passive muscle tension and decreased EMG activity and chronically with decreased fiber length, reduced number of sarcomeres (Kawano et al., 2004), and increased muscle atrophy (Baker & Matsumoto, 1988; Jaspers, Fagan, Satarug, Cook, & Tischler, 1988; Ohira, Yasui, Roy, & Edgerton, 1997). The plantarflexed position is common in rats during HLU despite the elevated EMG activity in the tibialis anterior and the depressed EMG activity in the plantarflexors (Ohira et al., 2002). Interestingly, astronauts have been observed to assume a similar a plantarflexed ankle position during space flight (Barratt & Pool, 2008; Nicogossian, Huntoon, & Pool, 1994).

Prolonged skeletal muscle unloading also appears to result in neuromuscular reorganization during muscle contractions (Ohira et al., 2002). After 9 weeks of HLU, rats have difficulty in standing while EMG activity in the plantarflexors is persistently low and tibialis anterior EMG activity is elevated. After prolonged unloading, rats are unable to maintain a stable position while resting on the floor, perhaps because the

knees and ankles remain in a plantarflexed position. The plantarflexed ankle joint probably does not result from active muscle contraction, as EMG activities of the soleus and the plantaris are 86 and 95% lower than that measured in ambulatory controls, but may due to the shortened muscle fiber length. The rats' stance and the EMG activities of the muscle surrounding the ankle joint gradually return to pre-HLU conditions over the course of a week. Reorganization of the neuromuscular activity also has been observed in primates during and following space flight. Soleus muscle activity decreases while medial gastrocnemius activity increases when performing a pedal pushing task (Hodgson et al., 1991; Roy et al., 1996). Changes in the relative contributions of individual muscles to joint torques and gross motor movements likely contribute to decreased muscle strength after skeletal muscle unloading.

2.2.2 Neuromuscular Response to Skeletal Muscle Unloading on in Humans

Comparatively, there are few reports regarding chronic muscle activity measured by EMG in humans participating in skeletal muscle unloading. One would expect a significant decrease in plantarflexor muscle activity compare to standing (Masani, Sayenko, & Vette, 2013) but this has not been clearly demonstrated. The available data from short-duration space flight do not appear to agree with observations in animal models. Twenty four-hour EMG activity of the tibialis anterior is increased in astronauts during space flight (~10 fold) as it is in HLU rats but to a larger extent. However unlike the observations in rats during early HLU, EMG activity in the soleus also is increased while EMG activity in the medial gastrocnemius is unchanged (Edgerton et al., 2001). Increased soleus and tibialis anterior EMG activity during space flight in astronauts

might be explained by the nature of the muscle work required to perform routine tasks during space flight, including the use of reciprocal contractions of the plantar and dorsiflexors to control body positions with foot loops used to position crew members during activities. Soleus EMG activity in these particular astronauts also may have been elevated due to the nature of the mission during which these data were collected; this was a space flight mission dedicated to scientific investigations, many involving muscle performance tests (Edgerton et al., 2001). Thus, these neuromuscular activity data do not represent a pure model of skeletal muscle unloading in humans.

Similarly, space flight may not provide complete unloading the cutaneous mechanoreceptors in the sole of the foot. Although acute unloading results in an immediate cessation of afferent signals from cutaneous receptors (Kennedy & Inglis, 2002), it is unknown if this persists as unloading continues over a long period of time. Afferent nerve activity recovers in rats during the first week of HLU (De-Doncker et al., 2005) but is unknown whether this also occurs in humans and proportionately how much of the afferent activity originates from the proprioceptors and mechanoreceptors in the lower leg and foot. Interpretation of these data, like that of sEMG results, likely is confounded in astronauts due to their normal daily activities and participation in science investigations and countermeasures (Edgerton et al., 2001).

However, skeletal muscle unloading during space flight may result in neuromuscular reorganization. EMG activity in the soleus, anterior tibilias, quadriceps, and biceps femoris muscles were monitored in two cosmonauts, one novice and one veteran flyer,

while attempting to maintain a standing-like posture before, during, and after space flight (Clément, Gurfinkel, Lestienne, Lipshits, & Popov, 1984, 1985). In the novice cosmonaut, muscle EMG activity was reorganized early in flight (flight day 2), suggested by a reduction in soleus EMG activity and an increase in tibialis anterior EMG activity compared to pre-flight, similar to the response observed in the rat during HLU and parabolic flight. During the course of the mission this slowly, but not completely, trended towards the pre-flight condition. EMG activity was not completely resolved to pre-flight conditions until three days after landing. Unfortunately, there were no in-flight data collected for the veteran cosmonaut except for that collected on the fifth day of the mission. The expected reorganization was not readily apparent in this individual, perhaps due to resolution over the course of the mission, individual variability, or alternatively a learned response from previous space flight missions.

Some data are available, however, from long-duration bed rest that support neuromuscular alterations during chronic unloading in humans, specifically a change from tonic to phasic activity (Belavy, Richardson, Wilson, Felsenberg, & Rittweger, 2007). Muscle activation during a controlled motor task, repetitive knee extension in the prone position in a manner meant to require tonic muscle activation to stabilize body positioning, is altered such that the ratio of the burst-to-tonic EMG activity increases. Interestingly, the largest shift in neuromuscular activity corresponded to the region of the musculature that also experienced the greatest amount of atrophy, the lumbar extensor spinae (Hides et al., 2007). These findings parallel previous reports in rats in HLU; changes in tonic-to-phasic activity shift during HLU is greatest in the soleus

muscle which also has the greatest proportion of muscle atrophy (Blewett & Elder, 1993).

The combination of these findings in humans and rats may suggest a link between changes in afferent stimulation from muscle spindles and cutaneous receptors (proprioception) with changes in neuromuscular activation patterns and muscle atrophy (Belavy et al., 2007). That is, deep muscles which have higher densities of muscle spindles, such as the soleus in the rat and the lumbar erector spinae in humans, also may be more likely to experience muscle atrophy and have a relatively larger shift in metabolic profile from slow to fast twitch. Therefore, a decrease in muscle spindle activity resulting in a shift from tonic-to-phasic activity may be related to biochemical adaptations and atrophy (Riley et al., 1990). A potential weakness in this argument is that in this study lumbar erector spinae muscle atrophy is reversed after 6 months of reambulation, but the shift from tonic-to-phasic EMG activity persists in this muscle (Belavy et al., 2007).

2.3.0 GH/IGF-1 axis in Normal Skeletal Muscle

Human GH is a family of polypeptides secreted into the blood by the anterior pituitary in a pulsatile manner following a circadian pattern. Generally, GH is secreted in 6-12 pulses per day with the largest pulse occurring approximately one hour after sleep (Godfrey, Madgwick, & Whyte, 2003), although the magnitude and the timing of GH pulses can be modified in response to various stimuli. Normal circulating levels of GH are essential for normal growth and maturation, particularly of the musculoskeletal

system. The importance of GH with regard to normal growth and development is illustrated by GH-deficient individuals and the effects of GH replacement therapy. GH-deficient subjects exhibit lower muscle mass and strength which can be normalized through long-term GH replacement (Götherström et al., 2009; Svensson, Sunnerhagen, & Johannsson, 2003).

One external stimulus shown to consistently induce changes in GH release is exercise. In general, GH secretion is elevated in response to the increased metabolic stress associated with a bout of exercise (Kraemer & Ratamess, 2005), although the exact mechanism stimulating GH release after exercise is unclear (Gibney, Healy, & Sönksen, 2007). Potential stimuli for GH secretion due to exercise include elevated body temperature, elevated circulating catecholamines, increases in blood lactate or nitric oxide levels, and changes in blood acid-base balance (Gibney et al., 2007; Godfrey et al., 2003). In general, GH secretion is related to the intensity and duration of exercise (Felsing, Brasel, & Cooper, 1992) which has been reported to positively influence the amount of GH secreted in each pulse, rather than the frequency and duration of each pulse (Pritzlaff et al., 1999; Laurie Wideman et al., 2006). Additionally, some investigators have suggested that peripheral mechanoreceptors in the lower body also may stimulate GH release independent of a metabolic or exercise stimulus through direct stimulation with the hypothalamus or anterior pituitary by afferent nerve traffic (McCall et al., 2001).

In skeletal muscle, GH is thought to act through two different pathways to affect skeletal hypertrophy (**Figure 10**). In one pathway, GH released from the anterior pituitary into the general circulation binds to GH receptors in the liver, stimulating the production and release of IGF-1 from the liver into the systemic circulation (Kraemer & Ratamess, 2005). Circulating IGF-1 then acts through the IGF-1 receptor on the myofiber plasma membrane (i.e. the sarcolemma) and activates the Akt/mTOR pathway, which ultimately results in an increase in translational efficiency of intra-myofiber ribosomes and an inhibition of the formation of proteolytic ubiquitin ligases (Adams, 1998; Adams, 2002; Spiering et al., 2008). Inhibition of the Akt/mTOR pathway with rapamycin has been shown to inhibit muscle hypertrophy, whereas over-expression of Akt during HLU prevents muscle atrophy (Bodine, Stitt, et al., 2001). IGF-1 also operates through negative feedback as an inhibitor of growth hormone release from the anterior pituitary through two pathways. IGF-1 directly inhibits the anterior pituitary from releasing more GH, and IGF-1 influences the release of GH releasing hormone (GHRH) and somatostatin from the hypothalamus (Godfrey et al., 2003). However, exercise stimulus counteracts negative feedback from IGF-1 to allow for increasing levels of GH secretion during exercise (Godfrey et al., 2003; L. Wideman, Weltman, Hartman, Veldhuis, & Weltman, 2002) through inhibition of somatostatin activity (de Vries et al., 2004).

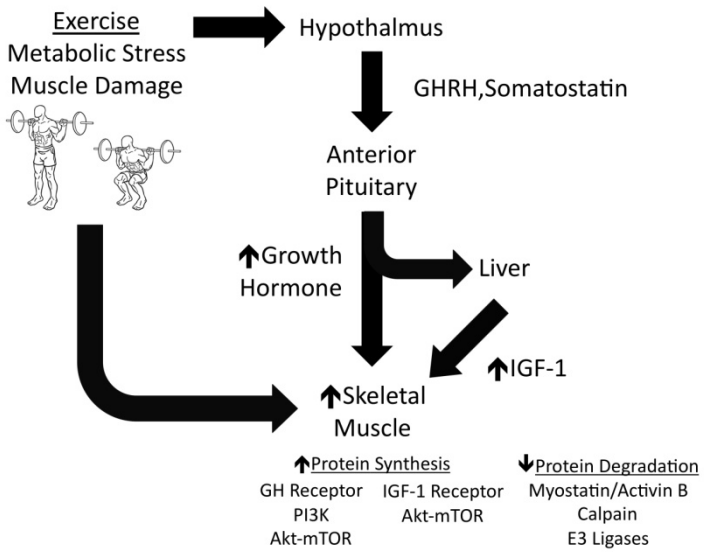

Figure 10. GH/IGF-1 response to an acute bout of resistive exercise and the influence on skeletal muscle.

In the second pathway, circulating GH released from the anterior pituitary can have a direct effect on skeletal muscle through activation of GH receptors located on the sarcolemma of individual myofibers which in turn activates JAK2, a tyrosine kinase, located within the myofiber sarcoplasm. Activation of JAK2 signaling activates phosphatidylinositol-3 kinase (PI-3K), also resulting in activation of the Akt/mTOR pathway (Argetsinger et al., 1993). The resulting increase in translational efficiency at the ribosomal level and suppression of protein catabolism within the myofiber is the same as that seen with direct stimulation of the Akt/mTOR pathway by activation of IGF-1 receptors. However, it is generally agreed that the increase in myofiber protein synthesis as a result of GH secretion (Fryburg & Barrett, 1993; Fryburg, Gelfand, & Barrett, 1991; Fujino et al., 2009) is driven primarily by liver-derived IGF-1 and subsequent myofiber IGF-1 receptor stimulation with only a moderate contribution

from the direct effect of GH through specific GH receptors located on skeletal myofibers (Godfrey et al., 2003).

GH secretion in response to exercise is a well-documented promoter of skeletal muscle hypertrophy, but surprisingly resting circulating levels of GH and the exercise-induced increase in GH do not appear to be sensitive to chronic loading states. For example, resting levels of GH are not changed and the exercise-induced GH release is unchanged or only increases slightly from before to after participation in chronic resistive exercise training (Häkkinen, Pakarinen, Alén, & Komi, 1985; Kraemer et al., 2006; Kraemer & Ratamess, 2005). Detraining also does not appear to affect resting levels of GH, although the acute response to an exercise bout may be at least transiently blunted (Häkkinen, Alén, & Komi, 1985; Kraemer et al., 2001). However, with regard to exercise training, the measured response of GH to changes in chronic loading might be dependent upon the assay used and thus isoform of GH measured (Kraemer et al., 2006).

Many different forms of GH exist with more than 100 variants being reported (Baumann, 1991). Of the many different forms of GH, the 22 kDa isoform is the most commonly studied (Kraemer & Ratamess, 2005) probably because it is the isoform most often used to produce monoclonal and polyclonal antibodies (Kraemer et al., 2006) and hence can be most easily measured using standard immunoassays (Thomas et al., 2011). Other biologically active isoforms of GH exist (i.e. 5 kDa, 17kDa and 20kDa isoforms), probably as a result of post-translational protein modifications or mRNA splicing (Lewis, 1984; Smith & Norman, 1990), as well as protein-bound GH and

aggregates of GH (Kraemer & Ratamess, 2005). Variants of GH are believed to have similar physiological function, but the biological activity level may differ depending upon their ability to dimerize the GH receptor (Kraemer et al., 2006).

One variant of GH (perhaps a growth hormone dimer (Hymer et al., 2001) or aggregate), believed to be 60-80 kDa in size, is not measured normally by standard immuno-assay techniques but is rather measured using a whole animal tibial growth plate bioassay. This larger GH variant, termed bio-assayable growth hormone (bGH), is estimated to have a biological activity 200-300 times greater than the 22 kDa immuno-assayable growth hormone (Ellis, Vodian, & Grindeland, 1978). bGH also appears to have a different stimulus for release from the pituitary. Immuno-assayable growth hormone (iGH) is released during or after relatively intense large muscle group exercise. In contrast, bGH appears to be released in response to moderate exercise in small muscle groups or in response to stimulation of afferent receptors such as muscle spindles and Golgi tendon organs (**Figure 11**) (McCall et al., 2001). Interestingly, although bGH does not increase in response to an acute bout of intense resistive exercise, an increase in resting and higher post-exercise levels are evident after 24 weeks of resistive exercise training. The increased levels of bGH suggests a specific explanation by the GH/IGF-1 pathway for increased muscle mass with exercise training (Kraemer et al., 2006).

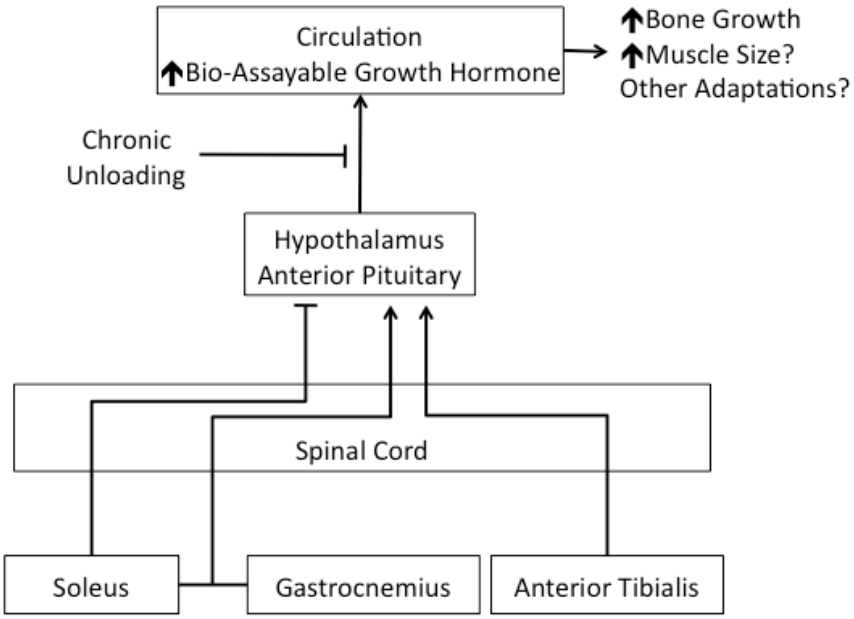

Figure 11. Mechanism of control for the control of bio-assayable growth hormone (bGH) from the anterior pituitary in response to afferent nerve traffic proposed by McCall et al. (McCall et al., 2001). Stimulation of afferent nerves arising from the gastrocnemius and tibialis anterior muscles stimulates the release of bio-assayable growth hormone from the anterior pituitary into the general circulation. Once in the general circulation, bGH stimulates bone growth and also may positively affect muscle mass and other physiological functions. In contrast, stimulation of afferent nerves arising from the soleus reduces bGH, and skeletal muscle unloading inhibits its release in response to either peripheral mechanoreceptors or exercise.

2.3.1 Altered GH/IGF-1 Axis in Skeletal Muscle Unloading in Animals

Whereas pituitary size and structure in rats do not appear to be affected by skeletal muscle unloading after space flight there are indications that pituitary secretory function may be altered. Pituitary cells harvested from space flight-flown animals contain twice as much iGH and bGH as ground control animals (before and after incubation) and over a period of 6 d of incubation in cell culture post-flight release half as much as cells from ground-based control animals (Grindeland et al., 1987). Further, the epiphyseal plate of hypophysectomized rats implanted with pituitary cells from the

space-flown rats grows half that measured in rats that are implanted with cells from ground control animals after 15 d. These data indicate that GH release in rats is reduced in response to unloading induced by space flight, but this may not be true across all isoforms of GH during HLU. Plasma levels of iGH do not change after HLU, while basal plasma levels of bGH are reduced after 1 and 8 wk of HLU (Bigbee et al., 2006). Skeletal muscle unloading may alter the basal levels of GH variants in rats in different ways.

Whether or not circulating GH levels are reduced by skeletal muscle unloading, it is apparent that GH supplementation (immuno-reactive variants), especially when combined with exercise, can help prevent muscle atrophy in rats induced by HLU. Hypophysetomized rats experience significant muscle atrophy during HLU despite the performance of a daily exercise countermeasure (5 climbs up a ladder carrying ~20% of body weight, three times per day), while administration of recombinant GH (immuno-reactive type, 22kDa isoform) alone prevents muscle atrophy in slow muscles and the combination of GH and exercise prevents muscle atrophy in both slow and fast muscles (Grindeland et al., 1994). A similar pattern of results has been observed in rats that are not hypophysectomized (Linderman, Gosselink, Booth, Mukku, & Grindeland, 1994). The participation of the GH/IGF-1 axis in regulation of skeletal muscle atrophy also might be inferred from the observation that when rats are HLU for 14 d, Akt phosphorylation is decreased, $p70^{S6K}$ activation is reduced, and the binding of PHAS 1/4-BP1 is higher compared to controls (Bodine, Stitt, et al., 2001). This apparent down-regulation of the Akt/mTOR pathway can be prevented by muscle exercise

during HLU (Haddad et al., 2006), although this does not completely prevent muscle atrophy unless the exercise intensity is high (Adams et al., 2007).

Although foot pressure protocols stimulate peripheral receptors in muscle and skin and are protective against skeletal muscle atrophy, the effectiveness of afferent nerve traffic to produce an elevation in bGH may be compromised during HLU (Bigbee et al., 2006). Tibial nerve stimulation previously shown to elicit an increase in circulating bGH and a concomitant decrease in pituitary bGH in ambulatory controls paradoxically results in a decrease in plasma bGH and an increase in pituitary bGH in rats at 4 and 8 weeks of HLU. Three explanations for this response have been suggested (Bigbee et al., 2006). First, the HLU rats' pituitary production of bGH is increased in response to tibial nerve stimulation but the release from the pituitary is inhibited. Second, HLU results in an alternative form of released bGH that is not detected by the standard bio-assay procedures. Third, the nerve itself or nerve transmission responsible for GH release is altered by HLU.

2.3.2 GH/IGF-1 Axis in Skeletal Muscle Unloading in Humans

A consistent finding in human subjects is that circulating iGH levels are not decreased during de-training in ambulatory subjects (Kraemer et al., 2002) or during skeletal muscle unloading by bed rest (McCall et al., 1997; Schmitt et al., 2000) and space flight (McCall et al., 1999), although circulating IGF-1 levels (presumably liver-derived) have been reported to be reduced during detraining (-11%) (Izquierdo et al., 2007). Urinary GH levels also are not affected by skeletal muscle unloading in space flight nor are

urinary levels of IGF-1 (Stein, Schluter, & Moldawer, 1999). Similarly, resting levels of circulating bGH are not reduced by bed rest or space flight in humans (McCall et al., 1997, 1999). However, the increase in bHG normally observed in response to a standardized plantarflexor exercise protocol is absent during bed rest and space flight, although it recovers within one week of reloading (**Figure 12**). These data suggest that some element of the exercise response mechanism resulting in bGH release is absent in the unloaded state.

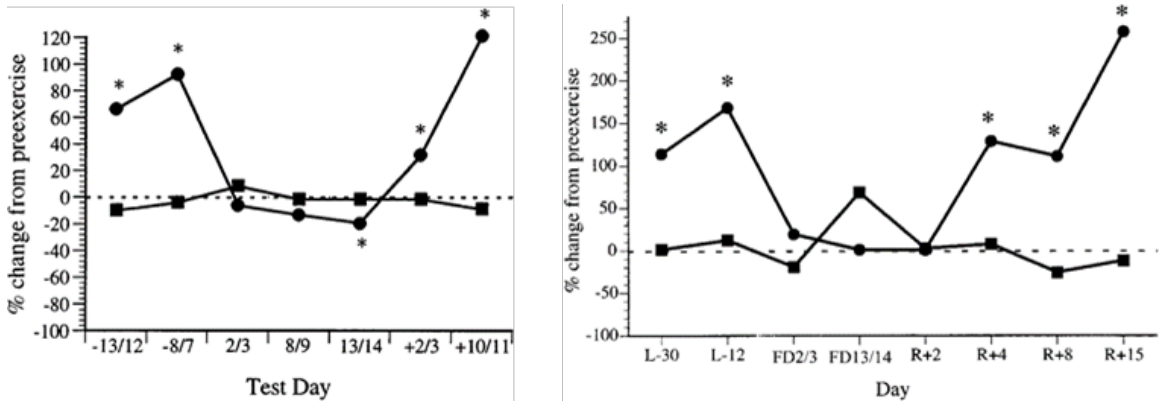

Figure 12. Left panel: Exercise-induced increase in bio-assayable (circles) and immuno-assayable growth hormone (squares) before (-13/12, -8/7), during (2/3, 8/9, 13/14) and after a 17-d bed rest (+2/3, +10/11), from McCall et al. (McCall et al., 1997). Right panel: Exercise-induced increase in bio-assayable (circles) and immuno-assayable growth hormone (squares) before (L-30, L-12), during (FD2/3, FD 13/14), and after (R+2, R+4, R+8, R+15) a 17-d Space Shuttle mission (STS-78), from McCall et al. (McCall et al., 1999).

Taken together, the bed rest and space flight studies in humans demonstrate the altered GH response during prolonged periods of unloading. While changes in the exercise-induced bGH response may be the result of decreased loading and the subsequent reduced afferent neuro-sensory input, this effect also might result from decreased ability of the pituitary gland to secrete bGH. This could be modulated by decrease in

GHRH. Evidence to suggest that space flight may impact the ability of the pituitary gland to release GH comes from studies which indicate that there is a decrease in the amount of GHRH present in the hypothalamus as detected by immuno-staining, and a reduction in the expression of GHRH mRNA (Sawchenko, Arias, Krasnov, Grindeland, & Vale, 1992) in pituitary cells derived from space flown rats, while the amount of bGH secretion in response to exogenous GHRH stimulation is also reduced in pituitary cell cultures derived from space flown rats (Hymer et al., 1996).

2.4.0 Afferent Nervous Activity Affects Growth Hormone

Both anatomical and physiological studies suggest an effect of afferent nerve traffic on hypothalamic and pituitary function. For example, afferent neuronal projections appear to extend from the spinal cord to the hypothalamus. Injection of Fluoro-Gold, a retrograde tracer, into the hypothalamus of rats under anesthesia that then are allowed to ambulate freely for 4-5 d revealed labeled neurons at all levels of the spinal cord in the dorsal horn (Burstein, Cliffer, & Giesler, 1990; Burstein, Dado, & Giesler, 1990). Additionally, a substantial number of afferent nerve endings appear to extend into the anterior pituitary of humans, monkeys, dogs, and rats, with a large proportion of them found near gland cells. The close proximity of the nerve endings to these cells suggests that activation of the neurons will cause a response in the gland cells (Ju, 1999).

2.4.1 Afferent Nervous Activity Affects Growth Hormone in Animal Models

The possibility that there is a link between afferent nerve traffic and a pituitary release of GH has been advanced by several studies in animals (McCall et al., 2001). Specifically,

afferent nerve traffic from peripheral receptors, including muscle spindles, appears to modulate release of bGH from the anterior pituitary (Gosselink et al., 1998, 2000, 2004) while release of iGH appears to result from muscle activity sufficient to cause a significant metabolic disturbance (Kraemer & Ratamess, 2005). Stimulation of the proximal end of severed nerves (tibial, peroneal, sciatic nerves) arising from muscles which are considered to have a primarily "fast" metabolic profile, increases plasma levels of bGH by 200-250%, while pituitary levels decrease 50-70% (Gosselink et al., 1998). In contrast, iGH levels do not respond to afferent nerve traffic (Gosselink et al., 2000), but iGH does increase when muscles contract in response to the stimulation of the distal ends of the same severed nerves (+140%) (Gosselink et al., 1998). Further evidence of a link between afferent nerve traffic and GH response is provided by the observation that stimulation of the proximal end of the severed tibial nerve in hypophysectomized rats does not increase circulating bGH levels (Gosselink et al., 1998).

While afferent traffic from muscle spindles in "fast" muscle has been shown to increase circulating levels of bGH in rats, the response to stimulation from other peripheral receptors may be inhibitory or non-existent in rodents. For example, afferent nerve traffic from muscles with a "slow" metabolic profile (soleus) decreases circulating bHG by ~60% while pituitary levels of bGH increase 30-50% (Gosselink et al., 2000). Additionally, afferent nerve traffic from cutaneous receptors (sural nerve) has no effect on either circulating or pituitary levels of bGH (Gosselink et al., 1998, 2000). However, the bGH response to afferent traffic from "fast" muscle types overrides these effects. In

rats that exercise for 15 min on treadmill, during which cutaneous and mechanoreceptors in both muscle types would be stimulated, bGH increases 300% greater while pituitary bGH decreases 50%. In contrast, plasma and pituitary iGH do not change in response to the same stimulus (Bigbee et al., 2000). Thus, there is a degree of difference between the effects of the stimulation of peripheral mechano-receptor on secretion of different variants of GH based upon the afferent nerves from which the signal arises, but the response of bGH to afferent nerve traffic from muscles with predominantly fast-twitch characteristics predominate in rats.

2.4.2 Afferent Nerve Activity Affects Growth Hormone in Humans

The relationship between afferent neural activity and GH secretion can be extended to humans. Similar to previous experiments in rats (Gosselink et al., 2000), stimulation of the muscle spindles with vibration over the tibialis anterior increases circulating bGH by >90%, while stimulation of the soleus (predominantly slow muscle) tends to decrease circulating levels of bGH (McCall et al., 2000). While there appears to be a muscle fiber type-specific effect of afferent nerve traffic in rats (tibialis anterior, fast twitch; soleus, slow twitch) (Gosselink et al., 1998, 2000), this explanation does not appear to be true in humans since fiber composition of the tibialis anterior and soleus are similar (75-80% slow twitch) (Gollnick, Sjödin, Karlsson, Jansson, & Saltin, 1974). Thus, the relationship between afferent activity and bGH secretion in humans may be related to muscle function rather than to the specific myofiber composition of the muscle stimulated (McCall et al., 2000). As previously observed (Gosselink et al., 1998, 2000), afferent nerve activity does not affect circulating iGH in human subjects.

Like the exercise-induced response of bGH in rats, there also is an effect of exercise in humans. That is, plasma bGH levels increases rapidly after as little as 5 min of isometric plantar flexor exercise while iGH is not affected (McCall et al., 1997, 1999). Circulating bGH is elevated 2 min after the completion of the exercise protocol, peaks by 5 minutes post-exercise, and is not different than pre-exercise by 30 minutes post-exercise. Contraction of the plantarflexors might be expected to reduce circulating bGH (McCall et al., 2000), so the elevated bGH following exercise protocol may have resulted from co-contraction of the tibialis anterior during the isometric exercise and the activation of the spindles during transitions between muscle lengths between muscle contractions and relaxations (McCall et al., 2000). Alternatively, as suggested in rats after a single bout of treadmill exercise (Bigbee et al., 2000), perhaps the stimulatory effect of the tibialis anterior muscle spindles is superior to the inhibitory effect of the afferent traffic from the soleus.

2.5.0 Foot Pressure Increases or Alters Neuromuscular Activation

When maintaining stable posture while standing on a horizontal surface, the soleus muscle is tonically active, and other muscles of the lower leg (tibialis anterior, gastrocnemius) are only intermittently active (Aniss et al., 1992; Masani et al., 2013). Muscle activity in the lower leg is adjusted and fine-tuned to maintain a stable posture through input from peripheral receptors, including cutaneous mechanoreceptors and muscle spindles.

Cutaneous stimulation alone alters neuromuscular activity in the lower leg, perhaps as a sensor for changes in center of pressure in the foot that might be used for an anticipatory response to maintain balance or to prepare muscle activity in the sequence of ambulation. That is, the location on the foot where pressure is applied has a significant effect on the strength of the neuromuscular response to plantar surface stimulation (Layne et al., 2005). Based upon evidence gathered using a device to provide discrete stimulations to the sole of the foot (the heel, lateral ridge and medial metatarsal), stimulation of the lateral ridge results in the greatest increase in reflex muscle activity particularly in the plantarflexor muscles (soleus and lateral gastrocnemius) while stimulation of the medial metatarsal region results in a similar but reduced response to that induced by lateral ridge stimulation (**Figure 13**). In contrast, cutaneous stimulation of the heel results in an EMG response that is not different than resting baseline. The relative effectiveness of these single stimulation sites likely is related anatomically to the density and threshold level of cutaneous receptors in the sole of the foot. For example, the lateral ridge of the sole has a higher density of cutaneous receptors than the medial aspect of the foot or the heel (Kennedy & Inglis, 2002).

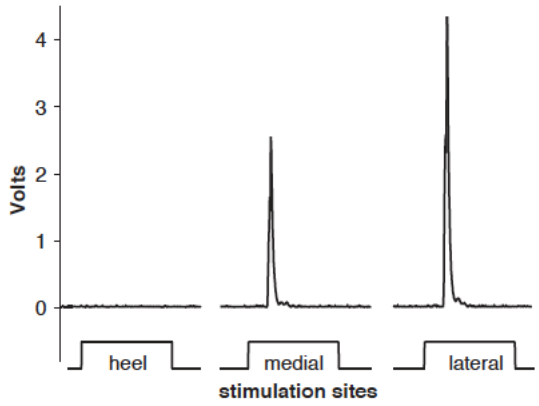

Figure 13. Typical response in sEMG from the soleus in muscle resulting from plantar surface stimulation in discrete locations (Charles S Layne et al., 2005). Soleus muscle activity is greater in response to forefoot stimulation in comparison to stimulation of the heel, which is largely non-existent.

Muscle length also is a modulator of neuromuscular activity during cutaneous stimulation. Changing the angle of the platform during standing, which influences muscle length and muscle spindle activity, alters muscle recruitment as subjects seek to maintain a vertical posture (Aniss et al., 1992; Burke, Dickson, & Skuse, 1991; Masani et al., 2013). In particular, cutaneous stimulation of the sole of the foot in the region with the greatest resulting reflex muscle activity, the lateral ridge, demonstrates that muscle stretch affects the magnitude of the response in a graded fashion (Layne et al., 2005). The response to stimulation is highest in the plantarflexors when the ankle is dorsiflexed (ankle angle of 70°), lower in the neutral position (90°), and lowest when the ankle is plantarflexed (110°). The magnitude of the response relative to ankle positioning is opposite to this in the tibialis anterior. Increasing rates of muscle spindle firing, induced by muscle length, is assumed to augment the response to cutaneous stimulation.

Background muscle activity is another factor that can alter neuromuscular activity in response to cutaneous stimulation. Aniss et al. (Aniss et al., 1992) demonstrated that manipulation of afferent nerve traffic through stimulation of the sural and tibial nerve modifies muscle activity of the lower leg. Stimulation of nerves innervating the foot modulate EMG activity in specific muscles of the ankle, but only when the muscles are already active, perhaps as a way to fine tune muscle activity during stance and walking (Aniss et al., 1992). The voluntary muscle contractions may enhance the excitability of the interneurons in reflex pathway so that α-motorneuron receives enhanced stimulation. Others (Layne, Forth, Baxter, & Houser, 2002) also have observed that EMG activity is enhanced when the voluntary muscle activation is paired with the stimulation and that the increased activity is proportional to the level of background muscle activity (Forth & Layne, 2007). Interestingly, with or without voluntary muscle contraction, EMG activity in the soleus in response to cutaneous stimulation appears to reach levels that are at least 80% of maximal EMG activity and that trials with 80% MVC result in %EMG that are equal to or greater than 100% EMG during 100% MVC. Thus, Forth and Layne (Forth & Layne, 2007) suggest that cutaneous stimulation activates a large number of motor units and may be helpful in individuals who have difficulty recruiting motor units.

2.5.1 Neuromuscular Responses to Plantar Surface Foot Pressure During Unloading in Animals

Only one study has measured the neuromuscular responses in rats to the stimulation of the plantar surface of the foot during HLU. During 14 d of HLU, DeDoncker et al. (De-Doncker et al., 2000) stimulated the soles of the rat's feet simultaneously by alternatively inflating (5 sec) and deflating (10 sec) a latex balloon to 40 mmHg using a sphygmomanometer, a level of pressure the authors believed would result in stimulation of the cutaneous receptors (Merkel disks, Meissner corpuscles, Ruffini endings, Pacinian corpuscles). Fourteen days of HLU resulted in an 88% decrease in mean EMG (0.37 ± 0.03 mV/s) compared to the ambulatory control animals (2.96 ± 0.05mV/s), but inflation of the bladder in the boots worn by HLU rats transiently increased mean EMG (6.2 ± 0.1 mV/sec). However, the plantar stimulation protocol used in this study likely resulted in a phasic neuromuscular stimulation rather than a tonic level of activity normally associated with postural muscles like the soleus, and thus was not completely effective (De-Doncker et al., 2000).

2.5.2 Neuromuscular Responses to Plantar Surface Foot Pressure during Unloading in Humans

Changes in muscle tone, measured by transverse muscle stiffness, occur rapidly during skeletal muscle unloading induced by dry immersion and persist throughout but these can be at least partially prevented by foot pressure stimulation. Within a single day of dry immersion, transverse stiffness in the soleus decreases by 25-30% and tibialis anterior stiffness increases by ~15-20% (Kozlovskaya et al., 2007; Miller, Saenko,

Popov, Vinogradova, & Kozlovskaya, 2004; Popov, Saenko, Vinogradov, & Kozlovskaya, 2003; Vinogradova et al., 2002). Similarly, EMG activity decreases in the soleus (59%) and increases in the tibialis anterior during the first 24 h of dry immersion. However, stiffness of the soleus is essentially unchanged after 24 h and 72 h of immersion when subjects receive dynamic foot pressure as a countermeasure, although transverse stiffness still is decreased by ~17% on day 6. Transverse stiffness of the tibialis anterior gradually decreases during dry immersion in subjects receiving plantar stimulation, reaching a low of ~19% below pre-immersion levels by 6 d of immersion. Integrated EMG levels in subjects receiving plantar surface stimulation parallel muscle stiffness measures.

Plantar stimulation during unloading also may protect against unloading-induced changes in neuromuscular control during locomotion. EMG activity in the soleus and gastrocnemius are greater after 7 d of dry immersion in control subjects, and there is a greater contribution by the gastrocnemius during walking based upon the ratio of the EMG activity between the two muscles (Kozlovskaya et al., 2007). However, while the overall EMG activity in the two muscles also increases in subjects who receive plantar surface stimulation during dry immersion, the ratio of the activity between the two muscles is not changed from pre- to post-immersion. These results suggest that plantar surface stimulation can prevent neuromuscular reorganization during muscle unloading.

Interestingly, a cosmonaut who wore the special boots to provide plantar stimulation while in microgravity reported that their use was associated with the disappearance of the illusion of being upside down, and the sensation of body inversion returned within 1-2 h after removing the boots (Hernández Corvo, Kozlovskaia, Kreĭdich, Martínez Fernández, & Rakhamanov, 1983). However, higher pressure was required to prevent the inversion illusion later in the flight, and the cosmonaut increased pressure and length of time that he wore the countermeasure boots. Thus, the effectiveness of the boots to prevent these sensations may decrease over repeated days of use. The effectiveness of this countermeasure to prevent skeletal muscle deconditioning also may be limited in long duration space flight, although this hypothesis has not been directly tested. Alternatively, the prolonged wearing of the pressure-loading boots within a day may have resulted in sensory habituation such that short, frequent wearing of the boots may be more effective.

2.6.0 Plantar Surface Foot Pressure Protects Skeletal Muscle During Unloading

Stimulation of the plantar surface of the foot results in a reflex increase in neuromuscular activity in the muscles of the lower leg and stimulation of peripheral mechanoreceptors from skeletal muscle promotes the secretion of bGH from the anterior pituitary. Alone or in combination, these responses may represent a hypertrophic response, up-regulation of protein synthesis, which may counter muscle atrophy during skeletal muscle unloading.

2.6.1 Plantar Surface Pressure Protects Skeletal Muscle During Unloading in Rats

The most compelling evidence that cutaneous stimulation of the plantar surface of the foot can protect against unloading-related muscle atrophy comes from studies of rats during HLU. De-Doncker et al. (De-Doncker et al., 2000) used a slow dynamic stimulation of the plantar surfaces of the feet in rats during 14 d of HLU to counteract most of the changes observed in control animals. A boot was fitted to the animals while anaesthetized and inflated to 40 mmHg in alternating cycles of 5 sec inflation and 10 sec of deflation for just 10 min/d with the ankle fixed in a neutral position (90°) during stimulation. Compared to HLU rats, the mean muscle weight and muscle cross-sectional area were 40 and 41% higher, respectively, in the HLU rats that received the countermeasure. Further, while HLU rats experienced decreases of 64% and 80% in peak twitch tension and peak tension, respectively, HLU rats with stimulation experienced decreases of only 44 and 60%, respectively. However, the plantar stimulation protocol used in this study failed to protect against shifts in the expression of myosin heavy chains and alterations in fiber type resulting from HLU (De-Doncker et al., 2000).

Kyparos et al. (Kyparos et al., 2005) subsequently conducted a 10-d HLU study in rats which produced more promising results than previously observed by De Doncker et al. (De-Doncker et al., 2000). The primary observation was that soleus Type I muscle fiber cross-sectional was not significant different between rats receiving active plantar stimulation during HLU and ambulatory controls, and the plantar surface countermeasure also attenuated the muscle atrophy in medial gastrocnemius Type I

muscle fibers. Type I cross-sectional area in the medial gastrocnemius in the rats receiving plantar stimulation was significantly less than the ambulatory controls, but was significantly larger than the Type I cross-sectional area in than the HLU animals. Unfortunately, the plantar stimulation did not similarly protect either the soleus Type IIA or Type IIB fibers. Similarly, Type IIA fibers in the medial gastrocnemius were only partially protected while there was no protection of Type IIB fibers.

There were several differences in the design of the foot pressure countermeasure from the study by De-Doncker et al. (De-Doncker et al., 2000) that might explain the improved results. First, the plantar stimulation of Kyparos et al. (Kyparos et al., 2005) used a higher pressure (104 vs. 40 mmHg), was longer per application (cycles of 5-sec inflation and 5-sec deflation for 20 min vs. cycles of 5 sec inflation and 10 sec of deflation for 10 min), and was applied more frequently (repeated eight times over 4-h period vs. once per day) than the previous study of De-Donker et al. (De-Doncker et al., 2000). Additionally, the animals were not anaesthetized during the foot pressure stimulus in the event that this might have impaired the effectiveness of the countermeasure. Kyparos et al. (Kyparos et al., 2005) anesthetized the rats using fast-acting inhalation anesthesia each day to fit the boot on the animals, but the animals were allowed to recover and were conscious during the plantar surface stimulation protocol.

Kyparos et al. (Kyparos et al., 2005) reported two additional important findings**.** First, the contralateral leg, the limb not receiving stimulation, did not receive any level of

protection from the countermeasure in the HLU rats receiving plantar stimulation. This might be explained two ways. First, the plantar stimulation countermeasure does not produce a systemic response that by itself protected the unloaded leg. Alternatively, muscle activity is a necessary component of the countermeasure, in combination with any potential systemic responses, and would have been inhibited in the contralateral limb during plantar stimulation (Kyparos et al., 2005). Second, rats that wore a boot which was not inflated (n=10) experienced a level of protection against muscle atrophy that was not different than that achieved in rats when the boot was inflated. Perhaps this resulted from an increase in neuromuscular activity associated with wearing the boot, whether inflated or not. That is, while wearing the boot, with or without inflation, animals were observed to extend both hindlimbs. Thus, movement of the ankle joint inside this semi-rigid boot would have resulted in pressure exerted between the plantar surface of the foot and the un-inflated bladder, a combination of plantar surface stimulation plus muscle activity (Kyparos et al., 2005). In contrast, rats that wore the boot without a plantar surface did not experience protection against muscle atrophy. Muscle atrophy was not prevented in rats wearing a boot without stimulation studied by De Doncker et al. (De-Doncker et al., 2000), but those animals were anaesthetized while wearing the boot and likely were not exhibiting the same behaviors. The reflex muscle activity resulting from cutaneous stimulation may have been potentiated in already active muscles in rats that were conscious (Aniss et al., 1992; Forth & Layne, 2008).

The observation that muscle atrophy could be prevented by plantar surface stimulation without significant muscle loading, as observed by De Doncker et al. (De-Doncker et al., 2000) and Kyparos et al. (Kyparos et al., 2005), is challenged by the findings of Nemirovskaya and Shenkman (Nemirovskaya & Shenkman, 2002). Soleus muscle atrophy was prevented in rats whose right limb received support by an adjustable platform but not immobilized (plantar stimulation plus muscle contraction) during 14 d of HLU. In these animals, soleus muscle mass, myofiber CSA, and percentage of slow twitch fibers was not different than that of the ambulatory controls. In contrast, soleus muscle mass in a separate group of rats whose right limb received support but the leg was immobilized with an ankle angle of 90° (plantar stimulation without muscle contraction) was less than in the ambulatory control animals and smaller than in the rats whose limb was supported without immobilization. Thus, support of the leg (plantar stimulation) was ineffective unless combined with active muscle contractions (Nemirovskaya & Shenkman, 2002). An alternative interpretation is that shortened muscle length in the limb-immobilized animals prevented any potential benefit in the supported limb.

The importance of afferent nerve traffic from sources other than cutaneous receptors, such as the muscle spindles, also has been demonstrated by partial protection against muscle atrophy during HLU (Falempin & In-Albon, 1999). Twice daily bouts of Achilles tendon vibration with the soleus muscle stretched during 14 d of HLU in rats reduced by muscle and myofiber atrophy by ~50%; the muscle wet weight and myofiber CSA in the countermeasure group after HLU was significantly greater than in the HLU only rats,

but significantly less than in the ambulatory controls. Stretching the soleus increased mean EMG more than three-fold, and the tendon vibration enhanced mean EMG by 16-fold. The peak twitch tension and peak tetanic tension relative to muscle weight, measured by electrical stimulation of the sciatic nerve, were preserved by the tendon vibration protocol, but the countermeasure failed to prevent the fiber type shifting and altered expression of MHC observed in the HLU only group. While Falempin and In-Albon (Falempin & In-Albon, 1999) characterized this combination of tendon vibration with muscle stretch as eccentric exercise, McCall et al. (McCall et al., 2000) challenged this interpretation and suggested that vibration of Achilles tendon partially preserved soleus muscle during HLU (Falempin & In-Albon, 1999) through muscle spindle activation and subsequent secretion of bGH.

2.6.2 Plantar Surface Pressure Protects Skeletal Muscle during Unloading in Humans

While previous reports of the effectiveness of the plantar stimulation protocols in rats are encouraging, it is important to determine whether similar results can be obtained in human subjects during skeletal muscle unloading. Russian investigators have since sought to replicate findings from ground-based animal studies and from the initial space flight study in humans (Hernández Corvo et al., 1983) by utilizing the controlled environment afforded by the dry immersion model of skeletal muscle unloading (Kozlovskaya et al., 2007; Navasiolava et al., 2011). In a series of studies, some reports apparently utilizing the same subjects, the effectiveness of plantar surface stimulation (**Figure 14**) on the preservation of muscle mass and function during dry immersion has been demonstrated (Kozlovskaya et al., 2007; Navasiolava et al., 2011). The acute drop

in afferent nerve traffic from and the decrease in muscle tone in the lower body is an important component of dry immersion (Navasiolava et al., 2011) that makes it a suitable condition in which to test the effectiveness of plantar surface stimulation relative to prolonged modification of afferent nerve activity on muscle mass. The countermeasure protocol was similar across the series of studies conducted and consisted of daily applications of a 20-min stimulation protocol consisting of alternating heel and forefoot pressures (0.5 ± 0.1 kg·cm^{-2}) split into two 10-min periods. One 10-min period simulated slow walking at 75 steps·min^{-1}, and the other 10-min period simulated a fast walk at 120 steps·min^{-1}. The protocol was conducted once per hour for 6 h each day of dry immersion. Within a stimulation cycle, the investigators reported that stimulation of the forefoot evoked ankle, and sometimes knee, extensor muscle contractions, while stimulation of the heel resulted in contraction of the tibialis anterior (Kozlovskaya et al., 2007; Vinogradova et al., 2002).

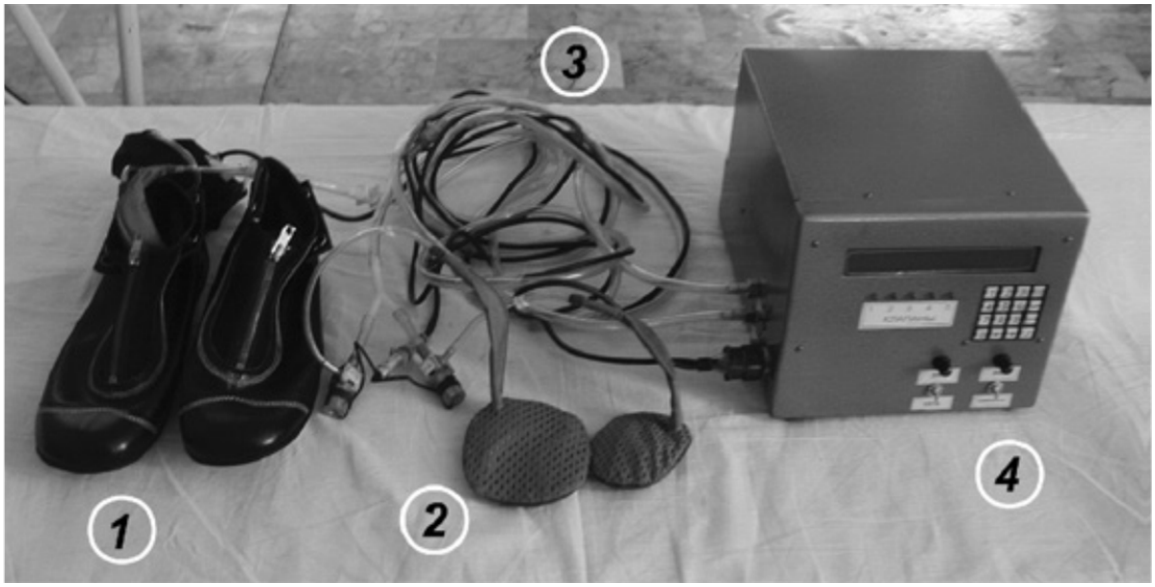

Figure 14. Dynamic foot pressure hardware (Compensator of Support Unloading, Zvezda Co., Moscow, Russia) used during dry immersion studies, including footwear (1), compressed-air insoles (2), air tubes (3), compressor and control unit. From Kovlovskaya, 2007.

The measurable effects of short-duration dry immersion without countermeasures on whole muscle mass is small, eliciting 1.5-2.5% decrease in muscle cross-sectional area after 3 d and ~5% decrease after 7 d (Navasiolava et al., 2011), and no studies have reported on the effect of the foot pressure countermeasure on whole muscle cross-sectional area. However, there are other indications that muscle deconditioning is attenuated or prevented. For example, the proportion of type I fibers in a biopsy of the soleus decreases (~6%) and the type I fiber cross-sectional area is reduced (~24%) in the control subjects during 7d of dry immersion, but these reductions appear to be largely prevented in the countermeasure subjects (Moukhina et al., 2004). Additionally, decreases in the calcium-stimulated maximal tension in skinned fiber and reduced ratios of titin:MHC and nebulin:MHC after dry immersion are prevented in the subjects

using the countermeasure protocol (Litvinova, Vikhlyantsev, Kozlovskaya, Podlubnaya, & Shenkman, 2004; B S Shenkman et al., 2004). Further, reductions in isokinetic ankle and knee extensor strength were protected during 7 d of dry immersion by the countermeasure in comparison to subjects who served as controls (Khusnutdinova, Netreba, & Kozlovskaya, 2004; Kozlovskaya et al., 2007; Netreba et al., 2004).

To our knowledge, only one study has been conducted describing the effects of foot pressure during unloading on muscle function after space flight (Hernández Corvo et al., 1983). During 7 d of space flight, one cosmonaut did not participate in the foot pressure countermeasure while the other wore specially designed sandals with "spring-loaded" insoles and a pressure bladder that could be inflated up to 60 mmHg (1.2 psi; 8 kPa). On the first day, pressure in the boot was maintained in the boots at 20 mmHg (0.39 psi; 2.67 kPa) for 4 h but on subsequent days the treatment was increased to 60 mmHg for 6 $h \cdot d^{-1}$. Pre- to post-flight muscle mass was not measured, but muscle function was assessed. In the cosmonaut who did not wear the boots, isokinetic plantarflexor strength decreased by ~10-25% and maximal EMG amplitude increased by 50% across all speeds tested. In contrast, plantarflexor and dorsiflexor strength increased by ~20% in the cosmonaut receiving the plantar surface pressure treatment, and there was no change in EMG amplitude.

2.7.0 Modulation of Spinal Cord Excitability by Joint Proprioception

Recent data from our laboratory (Boaz Blake, unpublished observations) suggests that spinal cord excitability, measured by Hoffman reflex (H-reflex) responses, may be

modulated by stimulation of different proprioceptors in the lower leg. Specifically, spinal cord excitability appears to be reduced when both joint proprioceptors and cutaneous mechanoreceptors are stimulated in comparison to the condition in which only cutaneous mechanoreceptors are stimulated (**Figure 15**). This difference appears to be greatest at lower levels of simulated body weight loading (25% of body weight was the lowest level investigated in that study). If this modifies the magnitude or direction of afferent sensory nerve traffic, this may explain some differences in the results reported by different animal studies discussed previously. For example, enhanced spinal cord excitability resulting in increased sensory nerve traffic terminating in the pituitary gland in response to cutaneous mechanoreceptor stimulation alone might clarify why the protocol performed by Kyparos et al. (Kyparos et al., 2005) during HLU appeared to be superior to the protocol employed by De Doncker et al. (De-Doncker et al., 2000). Animals studied by Kyparos et al. (Kyparos et al., 2005) did not have their ankles fixed during plantar stimulation (unloaded joint receptors) while the ankle joint was fixed (partial loaded joint receptors) in animals studied by De Doncker et al. (De-Doncker et al., 2000). Supporting the concept that spinal cord excitability modulated by proprioceptors may have an influence on muscle mass during unloading, previous studies have demonstrated that the H-reflex is reduced after bed rest (Mulder et al., 2011; Yamanaka et al., 1999).

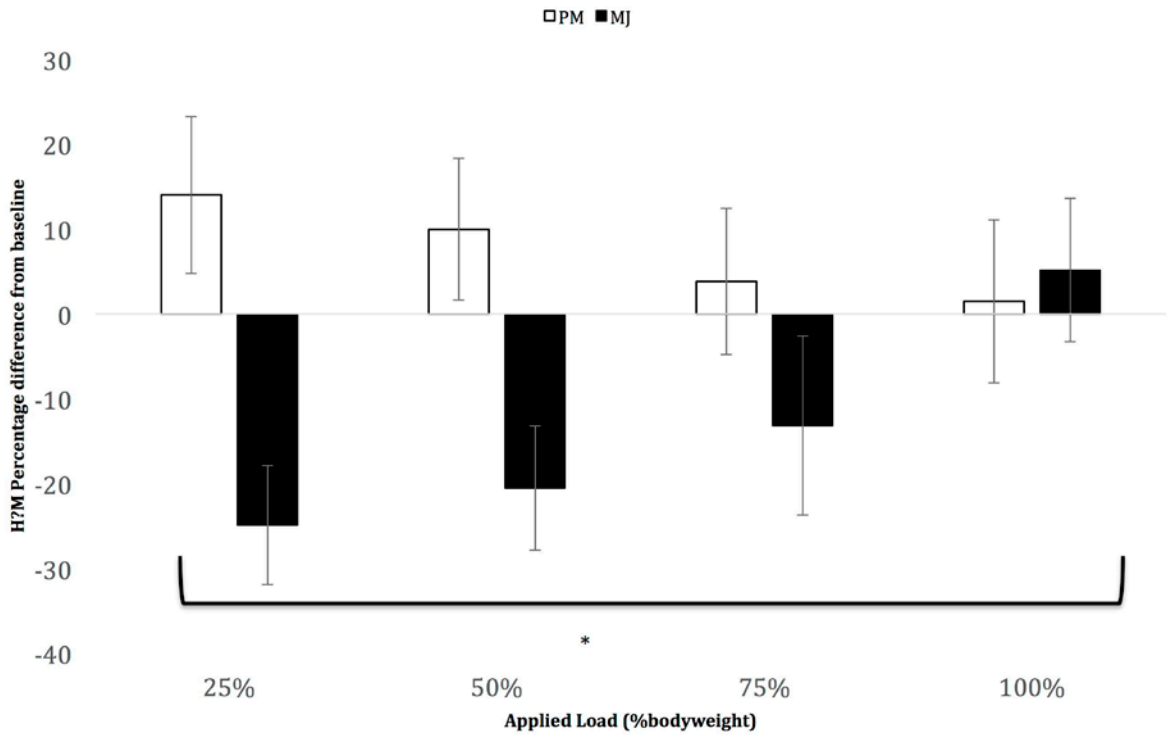

Figure 15. The percent change in the ration of the H-reflex to the M-wave compared to the baseline condition (0% BW) when loading the plantar mechanoreceptors (□, PM) and the muscle and joint receptors (■, MJ). From Boaz Blake et al., unpublished results.

2.8.0 Summary of the Literature

Skeletal muscle atrophies during unloading due to changes in protein balance that favors loss of contractile proteins from myofibers. In humans, this shift is believed to be primarily the result of a decrease in protein synthesis, rather than protein degradation (Ferrando et al., 2002; Phillips et al., 2009; Phillips & McGlory, 2014; Symons et al., 2009), suggesting that pathways that participate in muscle hypertrophy are preferentially down-regulated. Models of skeletal muscle unloading, including bed rest, dry immersion, space flight, and HLU in rats, are characterized by decreased muscle tension, reduced metabolic loading, and altered neuromuscular activity. Thus, it is not

surprising that countermeasures and modalities, such as exercise and periodic re-loading, that increase muscle tension and neuromuscular activity are at least partially protective against muscle atrophy during unloading.

While the underlying mechanisms that protect against muscle atrophy during unloading are better understood in countermeasures which induce a hypertrophic response in normal ambulatory subjects (i.e. resistive exercise and activation of the GH/IF-1 axis), the mechanism(s) by which pressure applied to the plantar surface of the foot prevents myofiber atrophy during unloading are still unclear and have not been specifically investigated. Plantar surface pressure increases neuromuscular activity during unloading (as measured by changes in EMG and muscle tone) and attenuates muscle atrophy and loss of muscle strength, but the specific pathways linking plantar stimulation and the biochemical signals preventing myofiber atrophy are as yet unknown. Although plantar stimulation can acutely increase neuromuscular activity and more chronically prevents a shift from tonic to phasic activity in unloaded muscle, stimulation of peripheral mechanoreceptors may also play a role in the anti-atrophic effects of plantar stimulation by inducing the release of GH from the anterior pituitary. Anatomical studies have suggested a physical link between afferent nerve projections from the lumbar region of the spine to the hypothalamus (responsible for production of GHRH acting on the pituitary), while stimulation of afferent nerves associated with lower limb muscles causes a pulsatile release of GH from the pituitary. While other, as yet unknown mechanisms may contribute, the available experimental evidence suggests that a combination of increased neuromuscular activity and/or afferent nerve

traffic associated with plantar stimulation is related to up-regulation of the GH/IGF-1 pathway is responsible for the protective effect of plantar surface pressure against unloading-induced lower limb skeletal muscle atrophy (**Figure 16**).

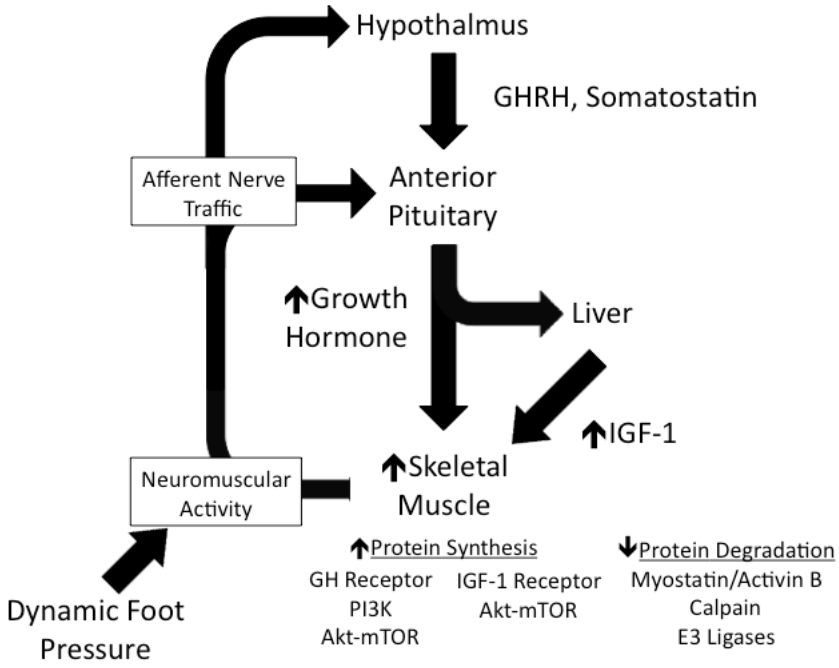

Figure 16. Potential pathways through which dynamic foot pressure might influence the GH/IGF-1 axis to preserve skeletal muscle during unloading.

This dissertation will address the potential causal relationship between plantar surface stimulation, spinal cord excitability, the neuromuscular response to plantar stimulation in human subjects, and the circulating levels of GH and IGF-1 during acute unloading in healthy individuals. Specifically, this dissertation sought investigate the relation between plantar stimulation and circulating levels of circulating GH (both iGH and bGH) and IGF-1 by altering the level of spinal cord excitability during plantar stimulation with acute bouts of unloading. Understanding whether a relationship exists between plantar

stimulation and GH/IGF-1 release during acute bouts of unloading will shed light on the underlying biochemical mechanisms involved in the protective effects of plantar stimulation in unloading-induced muscle atrophy.

2.9.0 Research Objectives and Significance

This dissertation investigated the potential relationship between activation of peripheral mechanoreceptors and modulation of circulating growth factors, specifically GH and IGF-1, involved in the control of skeletal muscle mass. Since the hypertrophic effects of GH and IGF-1 are coupled (i.e. pituitary-derived GH causes the release of IGF-1 from the liver) and IGF-1 is considered the major hypertrophic factor acting on skeletal muscle any stimulus which impacts circulating levels of either growth factor can potentially impact protein synthesis within skeletal muscle. Previous investigations have suggested that there is a link between afferent neural traffic arising from the lower limb in response to mechanoreceptor stimulation induced by exercise and the release of GH from the anterior pituitary. Anatomical studies have demonstrated direct connections at the cellular level between neurons involved in peripheral proprioceptive pathways activated during exercise and spinal cord neurons that project into the anterior pituitary. As such, it has been suggested that the up-regulation of muscle protein synthesis observed in response to exercise may in part be mediated by the release of pituitary GH elicited by increased sensory traffic within/between neurons located in such proprioceptive pathways and neurons within the spinal cord/central nervous system that terminate in the anterior pituitary. Separately, studies in both animals and humans have demonstrated that stimulation of peripheral

mechanoreceptors of the plantar surface of the feet results in prevention/amelioration of skeletal myofiber atrophy induced as a consequence of musculoskeletal unloading. However, the underlying signaling mechanism(s) linking the protective effect of plantar stimulation against mechanical unloading-induced muscle atrophy in the lower limbs has not been specifically investigated. In addition, it is unclear whether or not the signaling pathways activated during plantar stimulation result in simultaneous changes in the level of neuromuscular activity in the muscles of the unloaded limb leading to growth factor release or if the putative link between plantar stimulation and growth factor release is mediated by connections elsewhere, such as in the spinal cord. This study was designed to examine the neuro-endocrine response to the stimulation of the plantar surface of the foot during acute unloading in human subjects as a first step in understanding the link between peripheral mechanoreceptor stimulation, GH/IGF-1 release and the control of skeletal muscle mass. Resolution of this knowledge gap may aid in the development of a non-exercise countermeasure to muscle atrophy that could be applied in injured or bedridden patients who cannot tolerate or participate in exercise training and may enhance current countermeasures for astronauts during space flight for whom exercise time is limited.

Acute skeletal muscle unloading results in a rapid decrease in overall neuromuscular activity observed in the muscle, whereas chronic unloading causes a shift in muscle electrical burst activity from the normal tonic pattern to a phasic pattern. These unloading-induced alterations in neuromuscular activity, resulting at least in part to decreased stimulation of peripheral mechanoreceptors located beneath the skin and

within the muscle tissue itself (i.e. propioceptive pathways), are paralleled by decreases in muscle tone thought to correspond to a reduction in number of motor units which are randomly activated within the unloaded muscle. While reductions in muscle tone are a hallmark of muscle atrophy associated with chronic unloading, it is still unclear if a reduction in muscle tone initiates myofiber atrophy or is rather a consequence of myofiber atrophy. Conversely, stimulation of peripheral mechanoreceptors during both acute and chronic unloading appears under some circumstances to transiently increase neuromuscular activity, increase muscle tone, and/or induce the release of endocrine growth factors such as GH into the circulation. However, while stimulation of mechanoreceptors on the plantar surface during chronic unloading is paralleled by prevention of myofiber atrophy of at least certain myofiber types (i.e. Type I myofibers), a causal link between the two has yet to be definitively established. While increased mechanical loading during exercise induces the release of GH from the anterior pituitary, it is still unclear whether or not the anti-atrophic effect of plantar stimulation during chronic unloading involves the activation of similar signaling pathways (e.g. the GH/IGF-1 axis) to that observed during exercise loading. Other than the ability of acute plantar stimulation to elicit direct neuromuscular activation in the lower limb, coupled with limited evidence to suggest that there is an increase in overall muscle tone in response to foot pressure during chronic unloading, there is little direct evidence linking the effects of plantar stimulation, increased afferent neural/neuromuscular activity and the activation of the GH/IGF-1 axis to explain the anti-atrophic effect of plantar stimulation during chronic unloading.

It was the specific aim of this dissertation to measure circulating levels of both GH in response to stimulation of the cutaneous receptors in the plantar surface of the foot with and without loading of joint proprioceptors during acute unloading. We hoped to determine if plantar stimulation promotes GH release when joint loading (G_z axis) is absent and spinal cord excitability is enhanced compared to plantar stimulation plus joint loading, which we expect to suppress, relatively, spinal cord excitability. Determination of such a relationship between plantar stimulation, sensory nerve afferent pathway activity, and the release of GH/IFG-1 into the circulation will provide direct experimental evidence that a causal link exists between the protective effects of plantar stimulation on unloading-induced myofiber atrophy and the already established hypertrophic effects of GH/IGF-1 on skeletal muscle. In addition, in order to investigate whether or not any changes observed in circulating growth factors in response to plantar stimulation may be mediated by concurrent alterations in neuromuscular activity of the lower limb muscles, the effect of plantar stimulation during acute unloading on neuromuscular activation in important postural muscles of the lower leg (i.e. soleus, gastrocnemius, and tibialis anterior) also was investigated. If plantar stimulation resulted in release of circulating growth factors with or without concurrent changes in neuromuscular activity of the lower limb, this information would shed light on the anatomical nature of the underlying neural pathways activated in this neuro-endocrine response. For the purposes of this dissertation, acute mechanical unloading of the lower limbs was achieved by placing the subject in a supine position for 20 min followed by mechanical stimulation of the plantar surface for 10 min using an inflatable bladder applied to the forefoot. This anatomical location was chosen for the application

of plantar stimulation as this area of the foot (i.e. the forefoot) had been previously shown to produce the greatest proprioceptive-initiated neuromuscular response in the muscles of the lower limb (Layne et al., 2005). In one condition, the subject's foot was supported by an immovable object such that bladder inflation initiates joint movement and stimulated joint proprioceptors. In the other condition, the subject's foot was not be supported.

2.9.1 Expected Significance

This research sought to investigate the relationship between plantar stimulation, neural/neuromuscular activity in the skeletal muscle of the lower limbs, spinal cord excitability, and activation of the GH/IGF-1 axis during acute unloading as a first step in understanding the anti-atrophic effect of plantar stimulation during chronic unloading. Specifically, this dissertation addresses whether the anti-atrophic effect achieved during chronic muscle unloading by the application of pressure to the soles of the foot may in part be the result of an increase in circulating growth factors known to have hypertrophic effects in skeletal muscle. In addition, this study also tested whether or not any increases in circulating levels of GH elicited by plantar stimulation are linked to acute changes in neuromuscular activity/muscle tone within the unloaded muscle, thereby providing additional information in understanding the underlying neural and biochemical signaling mechanisms linking mechanical loading of the musculoskeletal system and the GH/IGF-1 axis in the control of muscle mass. Muscle atrophy and decreased muscle performance resulting from skeletal muscle unloading impacts the ability to return safely to activities of daily living after injury or prolonged bed rest and

may impact the safety of space exploration after long-duration exposures to microgravity. Knowledge of the underlying signaling mechanisms involved in the protective effects of plantar surface stimulation during unloading will improve the design of a countermeasure for these populations and also might be effective in treatment of other groups suffering from similar muscle pathologies, such as an elderly suffering from the effects of sarcopenia or spinal cord-injured individuals.

CHAPTER 3: METHODOLOGY

The objectives of this project was to deliver a consistent level of pressure, scaled to the individual's body weight, to the subject's forefoot at a frequency and duration which simulated normal walking while the lower leg was unloaded and to measure to neural and endocrine responses to this stimulation pattern. To achieve these objectives, a pneumatic system was designed and tested to apply the foot pressure, foot pressure and electromyography measurement (EMG) systems were integrated, and methodology to quantify growth hormone responses were tested (See Appendix for details). The result was an experimental set-up and methodology designed to address the specific aims and hypotheses of this dissertation project.

3.1 Overall Study Design and Session Descriptions

The dissertation project used a repeated measures design in which each subject participated in three separate experimental sessions in randomized order. The three experimental sessions were: control with no plantar stimulation (sham condition), plantar stimulation for cutaneous mechanoreceptor stimulation without foot support/joint and muscle proprioceptor activation, and plantar stimulation providing cutaneous stimulation combined with foot support/joint and muscle proprioceptor activation. The same measurements (blood draws for plasma and serum samples; surface electromyography of the soleus, lateral gastrocnemius, and tibialis anterior; pressure at the plantar surface of the foot) were obtained in each session following the same timeline (**Figure 17**). Although we originally planned to include myotonometry

as an index muscle tone in this study, the hardware failed before data collection began and could not be repaired (manufacturer was out of business).

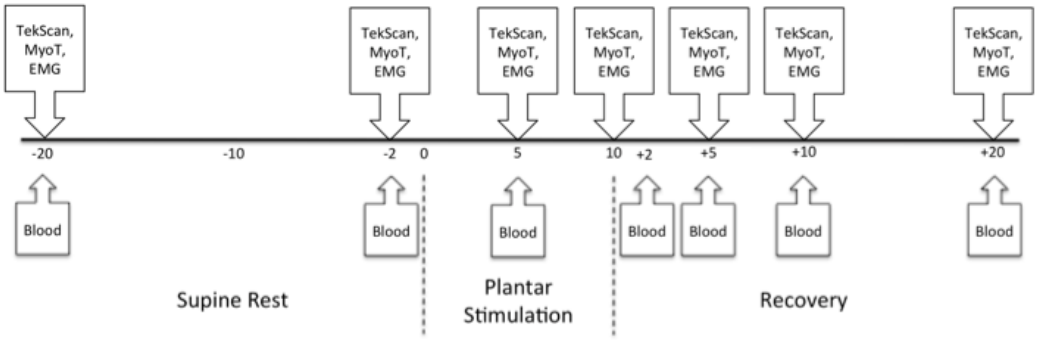

Figure 17. Session timeline. Blood = venous blood samples; TekScan = pressure insole measurements; MyoT = myotonometer measure of muscle compliance (planned but not executed in this study); EMG = surface electromyography of six muscles in the right and left lower legs

In each session, the subjects donned standardized nylon socks (thin trouser socks to maximize stimulation to the plantar surface of the foot) and were weighed using a standard bathroom scale. Subjects then donned the laboratory-provided exercise shoes with the inflatable forefoot bladder and pressure-sensing insoles already inside. The subject was encouraged to don the shoes carefully so as not to wrinkle the pressure-sensing insoles. The placement of the foot bladder was verified through inflation of the bladder using a sphygmomanometer. If the subject reported that the bladder was not pressing against the forefoot along the line connecting the first and fifth metatarsophalangeal articulations when inflated, the shoe was removed, the bladder was repositioned, and the location of the bladder was verified again once the subject donned the shoe. After the location of the bladder was deemed to be appropriate, subjects were instrumented for surface EMG (sEMG; tibialis anterior, lateral

gastrocnemius, and soleus; right and left lower leg); placement of the sEMG electrodes was verified through simple movements (ankle plantarflexion and dorsiflexion) while standing. The pressure insoles were calibrated using the subject's own body weight per the manufacturer's standard procedures. Subjects stood on one foot at a time while pressure from the insole was recorded. If subjects were unable to maintain their balance, creating large variations in pressure across the insoles, an error was reported and the calibration protocol was repeated. After the calibration of the insoles, for many but not all sessions, subjects then stood quietly on both feet and then balanced on only the right foot for 15 sec in each posture while pressure and sEMG were recorded. Standing on two feet was meant to provide data representative of normal stance. Standing on one foot was meant to provide information about the calibration drift of the pressure insoles that became apparent after the first few testing sessions.

Following calibration of the pressure insoles and data recording while standing, subjects assumed a supine, resting posture on a padded table with their knees supported but their feet extending beyond the end of the table. The subject's heels rested on padded shelf constructed to be at the same height as the padded table (**Figure 18**). Thus, the sEMG electrodes were not pressed between the subjects' legs and the table, minimizing the likelihood movement artifact. Subjects were allowed a small amount of padding behind their knees to prevent hyperextension and behind the small of their back to facilitate their comfort throughout the duration of the protocol. In the control condition and stimulation without foot support/joint and muscle proprioceptor activation condition, the subject's foot was positioned on the padded shelf so that with

the lower leg relaxed, the subject's foot did not touch the wall. In the stimulation with foot support/joint and muscle proprioceptor activation condition, the sole of the exercise shoe rested lightly against the wall. The subjects were instructed to position themselves so that the foot was supported but so they did not perceive any appreciable pressure from the foot against the wall. Subjects were repositioned when the foot pressure was detected from the pressure sensing insoles prior to bladder pressure-foot force calibration.

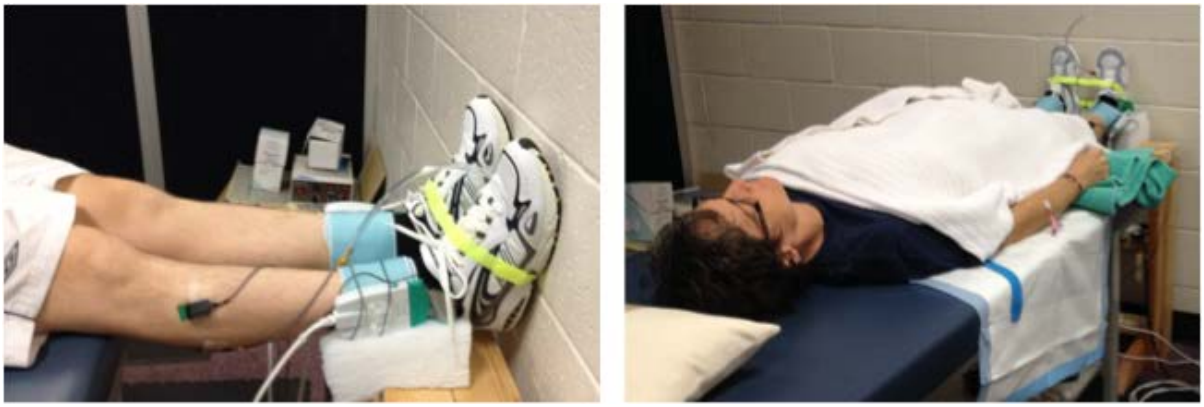

Figure 18. Pictures demonstrating experimental set-up. Left panel: The subject's knees were supported by the padded table and the ankles and feet were supported by a separate table, preventing pressure on the sEMG electrodes. In this example, the subject's feet are resting lightly on the wall (stimulation with support condition). Right panel: Photograph showing subject positioning from the perspective of the whole body.

Although the subjects were instructed to lace the shoes snugly, it was not possible to standardize how tightly the shoes were laced in a reproducible manner. Therefore, several trials were performed on the test days when stimulation was to be applied to determine the appropriate bladder pressure required to generate the desired force at the forefoot. While the subjects were supine, with their feet supported or not depending

upon the test condition that day, the bladder was inflated and deflated in 2-sec cycles for a period of 16 sec. The pressure data were exported to a text format, analyzed, and then reviewed. A bladder pressure that produced forefoot loading within 1 kg (~2 lb) of the target load (25% of the subject's body weight) was determined to be acceptable and used for subsequent stimulation periods during the test protocol. These calibration procedures were completed before the start of the 20-min rest period.

After the forefoot loading verification protocol was performed, a baseline blood sample was collected and baseline measures of sEMG activity and foot pressure were obtained. Subjects then rested quietly for 20 min before a second blood sample was collected and sEMG and foot pressure measures were repeated. At the conclusion of this 20 min baseline period, subjects continued to rest quietly with no dynamic foot pressure stimulation (control or sham condition) or received plantar surface foot pressure for 10 minutes with or without foot support (with or without stimulation of muscle and joint proprioceptors). When the bladder was inflated, plantar pressure equivalent to 25% of body weight was applied unilaterally to the right forefoot for 10 min at a rate simulating a fast walking speed (75 steps/min) (Miller et al., 2004; Popov et al., 2003), such that the forefoot bladder was inflated ~37.5 times/min. Assuming that the forefoot is in contact with the ground ~1/2 of the duration of each step in this simulated walking pattern, the bladder was inflated for 0.4 sec and deflated for 1.2 sec. The forefoot was chosen for this procedure because it elicits the greatest sEMG response to acute plantar surface stimulation (Layne et al., 2005). Measures of sEMG activity and foot pressure were repeated at 5 and 10 min of foot stimulation and at 2, 5, 10, and 20

min after foot stimulation (recovery). Blood sampling was repeated at 5 min of foot stimulation, within 1 min after stimulation, and 5, 10, and 20 minutes post-stimulation.

Each session was performed at the same time of day within a subject to minimize circadian effects on GH. The subjects abstained from exercise for at least 12 h before testing, and subjects fasted for at least 8 h (overnight fast) to control for any potential dietary effects on GH levels. Each session lasted 1.5-2.0 h: instrumentation (30-40 min), catheter insertion (5-10 min), pre-stimulation rest period and blood draw (20 min), plantar stimulation and blood draws (10 min), post-stimulation recovery and blood draws (20 min), and de-instrumentation (5 min). In most subjects, the catheter was inserted and was patent without incident on the first attempt, but in a few subjects a second insertion was necessary. A small amount of sterile saline (3 ml) was used to flush the catheter after catheter insertion and between blood draws to reduce the chance of clotting. Subjects were instructed to find a comfortable position that they could maintain with no or little movement for the duration of the time from the start of the 20-min rest period to the end of the 20 min recovery period. Most subjects laid quietly for this period, but a couple had to reposition themselves to relieve their discomfort from laying in one spot at a time. Subjects were allowed to talk during the testing, particularly to report any concerns or ask questions. Subjects were instructed not to resist bladder inflations during the stimulation period, and observation of the sEMG during this time confirmed compliance. No subjects reported discomfort from the bladder inflation during the stimulation period.

3.2 Subjects

Subjects for this study were normal healthy subjects, drawn from the student, faculty, and staff of the University of Houston and the general public. Subjects were in good health, according to self-report and judged by a standard health history questionnaire (Physical Activity Readiness Questionnaire, PAR-Q) (Warburton et al., 2011), without known neurological, endocrine, metabolic, or musculoskeletal disorders that might interfere with their participation in this study. Subjects served as their own experimental controls, thus only one group of subjects was required.

Subjects first received verbal and written explanation of the study design, the expectations for their participation, and a description of the risks and benefits of their participation. Subjects were encouraged to ask questions of the investigators and were informed that they could terminate their participation at any time. After providing written informed consent, the subjects were offered a signed copy of the informed consent form and were scheduled to participate in the three test sessions occurring in random order. The initial plan was to schedule all three test sessions weekly across a three-week period so as to minimize any changes in health status, but this was not possible for all individuals. Subjects were fitted for the appropriate size shoes (generally ½ size smaller than their normal size to minimize extra space in the shoe), and pressure insoles (Model 3000E, F-Scan, TekScan, Inc., South Boston, MA) were cut to size. Subjects used the same insoles across all three testing conditions unless the insoles were damaged; they were stored flat in between sessions, as recommended by the manufacturer but repeated use caused wrinkling of the insoles in some subjects that

cause the sensors to malfunction. Subjects also were familiarized with the other experimental hardware: the foot bladder, the EMG system, the foot pressure measurement system and blood draw equipment. In most cases, the familiarization immediately proceeded the first data collection session.

3.3 Foot Pressure Bladder

Foot pressure was applied to the right forefoot using a custom-designed inflatable bladder (Jack's Plastic Welding, Inc., Aztec, NM; **Figure 19**) inserted into a standard exercise shoe. Several different configurations were investigated (see Pilot Studies below), but the final location for the foot bladder was overlying the shoe insole and below the pressure-measuring insole (**Figure 20**). The bladder was inflated using a pressurized gas tank with two calibrated regulators. Overall pressure from the tank was controlled with a standard two-step regulator. This regulator was used to control bladder pressures in excess of 30 psi. Pressures less than 30 psi were controlled with a second in-line regulator (In-line regulator, 0-30 psi, Numatics, Inc., Novi, Michigan; **Figure 21**). Air flowed into and out of the bladder through Tygon® tubing (United States Plastics Corporation, Lima, Ohio) that was routed through two solenoid valves (12 VDC single solenoid valves with spring return, Numatics, Inc., Novi, Michigan). The opening and closing of the valves to inflate and deflate the bladder was computer-controlled using a customized program developed in LabView (National Instruments Corporation, Austin, TX) (**Figure 22**).

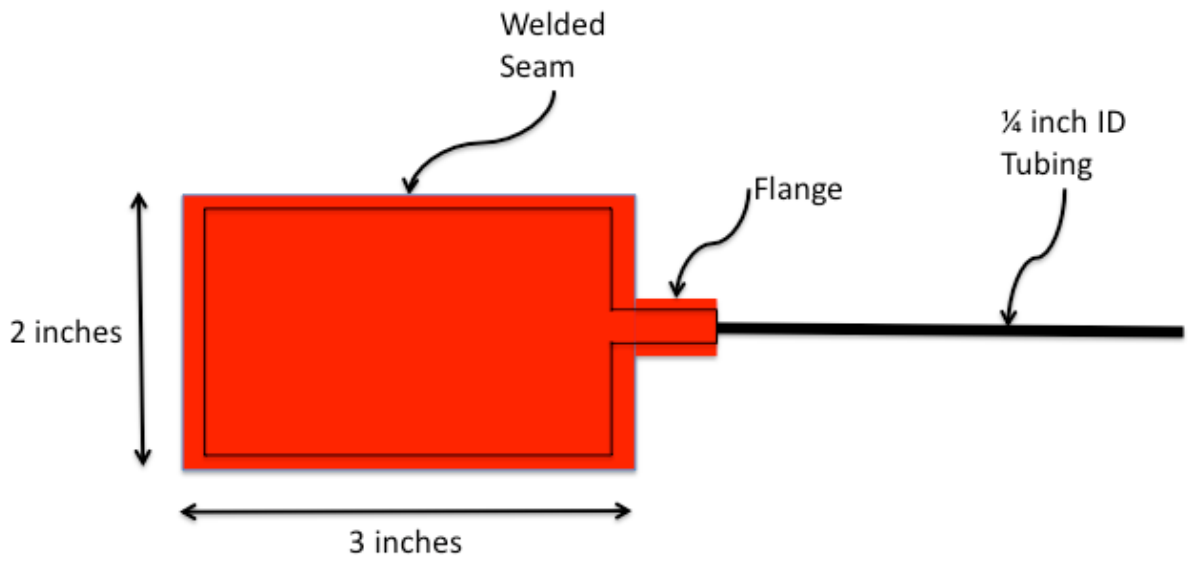

Figure 19. General design of the bladder placed under the forefoot of the test subjects. Bladder was placed inside a standard exercise shoe between the insole of the shoe and pressure sensing insole beneath the foot.

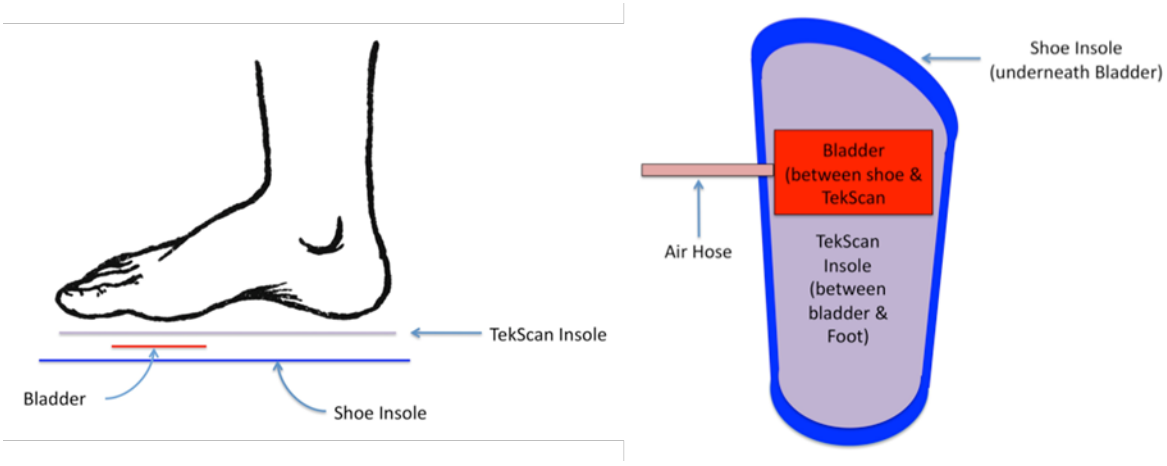

Figure 20. Diagram showing placement of the pressure bladder relative to the shoe's insole, the TekScan pressure sensor (cut and fitted to the size of the shoe), and the subject's foot (left panel). Diagram showing placement of the bladder within the shoe, situated under the forefoot of the subject (right panel).

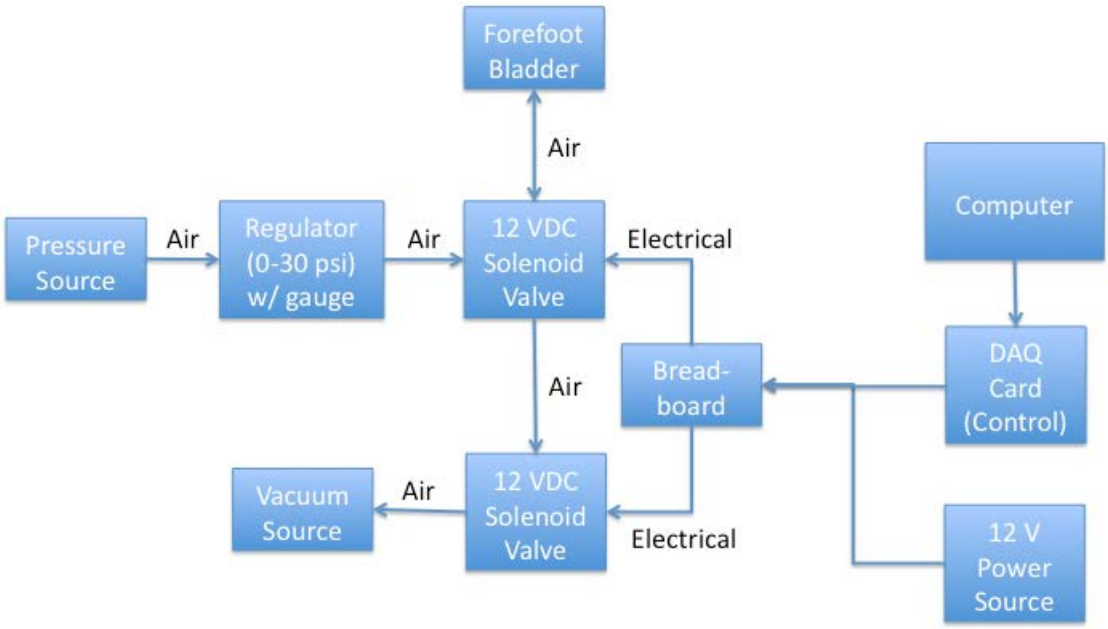

Figure 21. Schematic of the computer-controlled air flow to inflate and deflate the forefoot bladder to provide plantar surface stimulation.

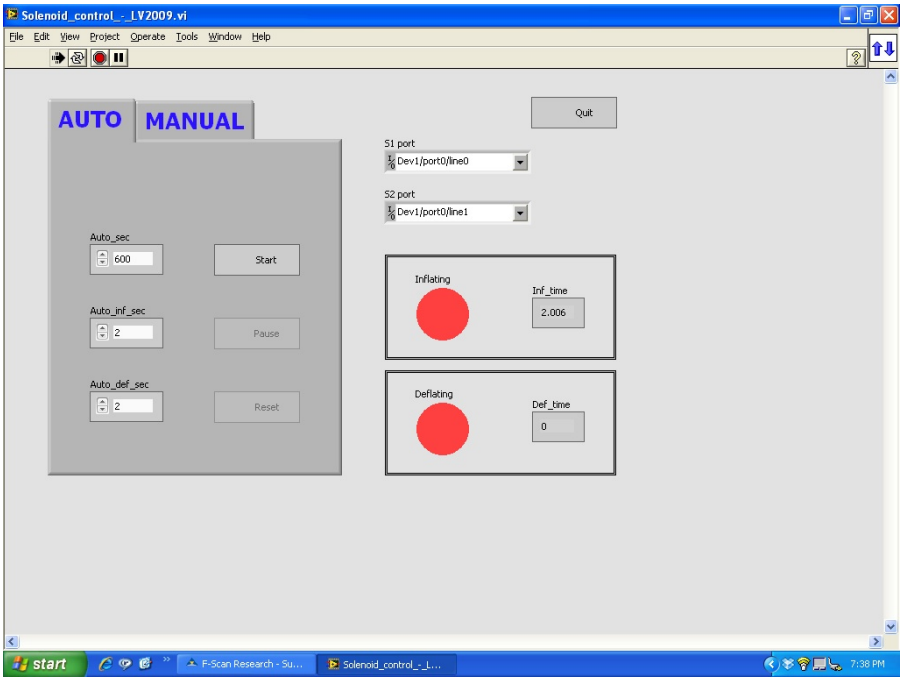

Figure 22. Screenshot of custom-designed air-flow control software used to inflate and deflate the bladder. Time of inflation, time of deflation, and total time of the stimulation period can be set individually, depending upon the desired frequency and duration of

bladder inflation. Bladder pressure is set separately using pressure regulators in line with the pressurized gas tank.

3.4 Surface EMG

Surface EMG (sEMG) of the tibialis anterior, lateral gastrocnemius, and soleus was measured in both legs using bipolar electrodes (DE-2.1, Delsys Inc., Boston, MA). sEMG signals were amplified 1000 x (Bagnoli-8, Delsys Inc., Boston, MA) and recorded at 1000 Hz using a standard data acquisition system (PowerLab 8/35, AD Instruments Inc., Colorado Springs, CO), software program (LabChart v7.2.5, AD Instruments Inc., Colorado Springs, CO), and a laptop computer (MacBook Pro, Apple Inc., Cupertino, CA). To reduce electrical resistance and improve adhesion of the surface electrodes to the skin, subjects refrained from using any lotion or oil on the morning of testing, excess hair was removed at the site of electrode placement, a pumas stone was used to lightly abrade skin and remove dead skin cells, the skin was cleansed with an alcohol pad, and the area was wiped with a sterile gauze pad. The skin surface was allowed to dry before the electrodes were adhered to the skin using manufacturer-provided double-sided adhesives.

The sEMG electrode for the tibialis anterior was placed on the anterior aspect of the lower leg, approximately one-third of the distance below the head of the fibula and the lateral malleolus. The sEMG electrode for the lateral gastrocnemius was placed on the posterior aspect of the lower leg, midway between the lateral condyle of the femur and the junction between the gastrocnemius and the calcaneal, or Achilles, tendon. The sEMG electrode for the soleus was placed on the posterior aspect of the lower leg

approximately two inches below the lower edge of the bifurcation of the lateral and medial gastrocnemius. The ground electrode was placed on the lateral malleolus of the right ankle. Appropriate location of the EMG electrodes was verified prior to data collection when the subjects contracted specific muscles and an increased sEMG was observed. If sEMG activity was not apparent during controlled muscle contractions, the EMG electrode was removed, the skin surface was cleaned again, and the electrode was reapplied. Only one of two operators applied EMG electrodes, and the same test operator applied sEMG electrodes across all testing sessions within a subject. Graduate student experts from the Center for Neuromotor and Biomechanics Research provided training for placement of the sEMG electrodes and sEMG data collection.

3.5 Foot Pressure Measurement

Foot pressure was measured before, during, and after plantar surface stimulation using pressure insoles (Model 3000E, F-Scan, TekScan, Inc., South Boston, MA, **Figure 23**) inserted between the bladder in the shoe and sole of the foot. The insole has 4 sensels per cm^2 and measures pressures in a range of 517-862 kPa (75-125 psi). The pressure insoles were calibrated before each session using the manufacturer's two-point calibration procedure (no load and 100% of the subject's body weight [standing on one foot at a time]). Foot pressure data were recorded with the vendor-provided software (F-Scan Research v6.62, TekScan, Inc., South Boston, MA) for 30 sec at each of the measurement time points. A 3-volt pulse, generated by the TekScan software at the initiation of the pressure recordings, was routed to the sEMG data acquisition system and served as a trigger for the start of the EMG recordings. Thus, foot pressure and

sEMG data were synchronized. Pressure data for the entire 30-sec period were saved and exported as text files for later analysis by a custom MATLAB program (MathWorks, Inc., Natick, MA). The program was designed to calculate peak pressure, average pressure, total pressure, total area of pressure application, peak force, average force, and total force applied to the forefoot during testing.

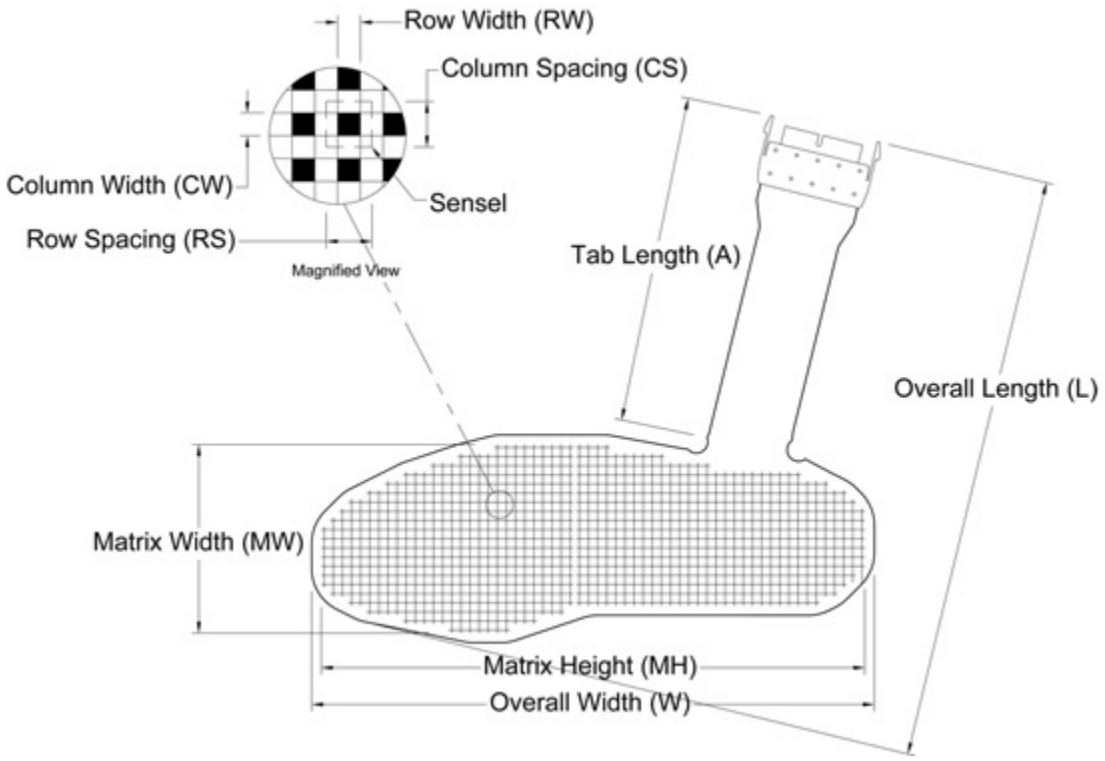

Figure 23. Pressure measuring insole (Model 3000E, F-Scan, TekScan, Inc., South Boston, MA) that was inserted between the inflation bladder in the shoe and the sole of the foot (Source: http://www.tekscan.com/3000E-pressure-sensor)

3.6 Blood Sampling

Venous blood samples were collected using a standard clinical closed-catheter system (Nexiva, 20 gauge needle with 1.25 in extension, BD Medical, Sandy, UT) attached to a Vacutainer Leur-Lok Access Device (BD Medical, Sandy, UT). Before each blood draw,

the sample line was cleared by drawing blood into a 3-ml waste tube. Thereafter, samples were collected into an EDTA-treated blood collection tube and a serum separator tube. EDTA tubes were inverted at least 10 times and stored in an ice chest until tubes could be centrifuged for 15 min at 1000-g. Serum separator tubes were inverted at least 5 times and then stored at room temperature for at least 30 minutes to allowing for clotting before spinning for 15 min at 1000-g. The plasma and serum were removed, separated into aliquots (250-1000 µl), and frozen at -80 °C until assay. Approximately 7 ml of blood was collected at each of seven time points for a total of ~35 ml per session.

3.7.0 Data Reduction and Sample Analysis

3.7.1 Foot Pressure Analysis

Pilot data demonstrated that ~0.1 sec was required from the time that bladder inflation began until the desired foot pressure was achieved. Therefore, the total force applied with each bladder inflation was quantified in two ways. First, the total force (total pressure x area of pressure application) was calculated from the onset of the bladder inflation (defined using the total foot pressure in excess of 200 psi of the baseline pressure as in the latency calculation) to the termination of bladder inflation (when pressure measured by the insoles fell below the threshold). These calculations included the forces experienced during the time that the bladder was expanding (lower force), the time when the desired force was achieved, and the time when the bladder was deflating (lower force). While this represents the total force applied to the bottom of the foot, it is not representative of whether a target force was achieved and how

consistently the force was applied. Therefore, force applied to the bottom of the also was calculated in a 200-msec window spanning 100 msec from the onset of onset of bladder inflation.

3.7.2 Surface EMG Analysis

Root mean square (RMS) was calculated for each 30-sec segment for each muscle at each time point as an aggregate measure of muscle activity during supine rest, plantar stimulation (or sham), and recovery from stimulation. RMS was calculated independent of the number of bladder inflations during the 30-sec data collection period.

Visual review of each sEMG record suggested that the application of foot pressure during the 10-min stimulation period did not produce sustained muscle activity but single reflex-like responses were observed in some muscles under some conditions, specifically during stimulation with foot support. These appeared as a momentary spike in sEMG activity paired with the onset of bladder inflation. To identify these and measure the latency of the onset from the time of pressure application, the sEMG data were processed with custom analysis code (MATLAB, The MathWorks, Inc., Natick, MA).

Multiple attempts were made to filter the raw data to remove the presence of 60 Hz noise in the signal. None of these was deemed acceptable because they attenuated the sEMG response to a level that was then undetectable. Instead, a process to manually subtract the electrical noise was applied. In order to subtract the noise, a sine wave with similar characteristics to that observed in the signal was created. A Fast-Fourier

Transform was performed on the entire data trial to provide the best estimate of the actual noise frequency, with the wave amplitude determined by creating a histogram of the signal. As expected from a largely sinusoidal signal, the result of this histogram was bimodal and the distance between the two values where the peaks occurred was used as the amplitude of the sine wave. The phase of the wave was determined from the lag of the peak correlation from a cross-correlation calculation. Propagating errors from slight differences in the actual and calculated frequencies were observed when the artificial noise wave was subtracted from the entire raw sEMG signal. To combat this, the signal was broken into 30 epochs, ~1 second each, and the cross-correlation technique was used to determine the phase of the signal for each epoch.

With a significant portion of the electrical noise removed, the process of identifying individual responses to the foot pressure perturbation was improved. A RMS calculation was applied to the cleaner EMG signal using a sliding 3-msec window. All of the instances when the resulting signal exceeded 6 SD from the mean were identified. Only instances when the response occurred within 200 msec of the stimulus onset were considered; EMG bursts occurring outside of this window were not considered to be a consequence of the plantar stimulation. Stimulus onset was identified as the time at which the total pressure measured using the foot pressure insoles exceeded 200 psi above the baseline pressure, or 3.6 kg (~8 lb) of the baseline force. In this way, the ratio of EMG bursts per stimulation (measure of consistency of the response) and the latency of the response (from stimulation to onset of EMG activity, if present) were calculated.

3.7.3 Growth Hormone Assay

Duplicate plasma samples from time points corresponding to 20 min of rest, immediate post-stimulation, and 20 min post-stimulation were analyzed for iGH concentration using a 4.5 h solid-phase sandwich ELISA (Quantikine, R&D Systems Inc., Minneapolis, MN; **Figure 24**).

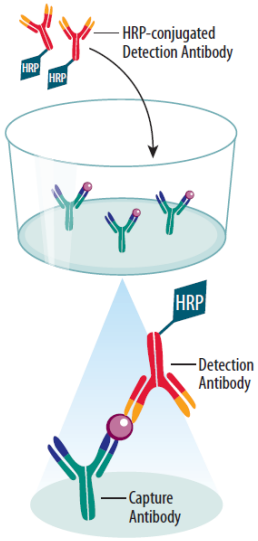

Figure 24. In a sandwich ELISA, the 96-well plate is pre-coated with a monoclonal capture antibody specific to the protein of interest (i.e. iGH). The target analyte is captured during the incubation period with the standard or sample, and then the analyte conjugated with a second antibody (a horseradish peroxidase (HRP)-labeled polyclonal detection antibody) that attaches to a different epitope on the analyte. A substrate solution containing tetramethylbenzadine (TMB) then is added, which produces a blue color signal when enzymatically altered by HRP. Finally, this reaction is halted with the addition of a Stop Solution, which alters the pH of the solution inside the well and changes the color to yellow in a manner that is proportional to the concentration of the analyte. The intensity of the resulting color is read with a standard spectrophotometer. (Source: ELISA Reference Guide and Catalog, R& D Systems, http://www.rndsystems.com/resources/images/6836.pdf)

Standards for GH (1600, 800, 400, 200, 100, 50, and 25 pg/mL) were prepared using the manufacturer-suggested dilution protocol (calibrator diluent RD6-15, R&D Systems Inc., Minneapolis, MN). GH standards (R&D Systems Inc., Minneapolis, MN) were highly purified E. coli-expressed recombinant human GH that had been calibrated to the NIBSC/WHO 2nd International Standard 98/574, a recombinant DNA-derived human GH. Plasma samples from test subjects were thawed prior to assay and allowed to reach room temperature per the manufacturer's instructions. Assay diluent (100 µL RD1-57; R&D Systems Inc., Minneapolis, MN) was pipetted to each well of a 96-well plate, and then 50 µL of either standard or sample was added. The plate was covered and allowed to incubate at room temperature for 2 h. Each well then was aspirated and filled with 400 µL of wash buffer (R&D Systems Inc., Minneapolis, MN). These steps were completed three more times for a total of 4 washes. GH conjugate (200 µL) was added to each well, allowed to incubate for 2 h at room temperature, and then the aspiration/wash steps were repeated four times as before. Substrate solution (200 µL, R&D Systems Inc., Minneapolis, MN) was added to each well and allowed to incubate for 30 min before the Stop Solution (50 µL, R&D Systems Inc., Minneapolis, MN) was added. Absorbance for each standard and sample was determined using a standard spectrophotometer (Spectra Max 190 Microplate Reader, Molecular Devices, LLC, Sunnyvale, CA) using vendor-provided software (Softmax Pro, Molecular Devices, Molecular Devices, LLC, Sunnyvale, CA). Optical density of the standards and samples was calculated by subtracting the absorbance at 540 nm from 450 nm to correct for optical imperfections of the 96-well plate. The averages of the duplicate standards and samples were calculated. A calibration curve was constructed from the standards and

applied to the plasma samples to calculate iGH concentration. Three 96-well plates were required to analyze plasma samples from all the subjects. In each case, the R^2 value for the calibration curves was >0.985.

3.7.4 Western Blots

Several primary and secondary antibodies were tested for their ability to detect high molecular weight isoforms of GH. Initially, the most promising primary antibody was a purified goat polyclonal antibody (sc-10365, Santa Cruz Biotechnology, Inc., Dallas, TX) used to detect a peptide near the C-terminus of human GH, in combination with the manufacturer-recommended secondary antibody (donkey anti-goat igG-HRP, sc2020, Santa Cruz Biotechnology, Inc., Dallas, TX). The ability of the primary and secondary antibodies to detect high molecular weight GH (~48 kDa) had been recently demonstrated in human lymphocytes when only molecular weight fractions of interest were considered (Weigent, 2011, 2013). However, secondary control testing in the albumin-depleted serum samples from this study demonstrated non-specific binding of the secondary antibody. Thus, other antibodies were tested. These included a mouse monoclonal IgG_{2a} (sc-166696, Santa Cruz Biotechnology, Inc., Dallas, TX) specific to an epitope between amino acids 40-75 near the N-terminus of human GH and a mouse monoclonal IgG_1 raised against recombinant human GH (sc-73289, Santa Cruz Biotechnology, Inc., Dallas, TX). For both of these primary antibodies, we used a goat anti-mouse IgG-HRP (sc-2005, Santa Cruz Biotechnology, Inc., Dallas, TX) as the secondary antibody. From the manufacturer-provided data sheets, both of these primary/secondary antibody combinations appeared to detect proteins in the regions

near 45 and 60 kDa, which correspond to dimers and trimers of GH (McCall et al., 2001; Weigent, 2011, 2013). In performing Western blotting, several iterations of the protocol were used but the final protocol is detailed below. Samples were prepared (albumin-depleted or serum diluted) with sample buffer on one day and frozen at -80 °C until the Western blotting, usually the next day.

Serum samples from two subjects known to be responders (sEMG response in the right lateral gastrocnemius during plantar stimulation with support) were thawed and prepared for assay. Samples from the end of the 20-min rest period and immediately after the 10 min-stimulation period were assayed with and without albumin depletion. Albumin was removed from serum samples using an albumin depletion kit (Pierce™ Albumin Depletion Kit, Catalog #85160, Thermo Fisher Scientific, Rockford, IL). Serum samples that were not albumin-depleted were diluted with Dulbecco`s Phosphate Buffered Saline (DPBS) at a ratio of 1:25 so that total protein in the sample could be measured and the lanes in the gel could be loaded with equivalent amounts of total protein across samples.

For the albumin depletion, the following steps were completed in accordance with the manufacturer's instructions. First, 400 µL of slurry, containing 200 µL of Cibracon Blue agarose resin, was added to a spin column using a wide-bore micropipette. The spin column was placed in a 2.0 mL Eppendorf tube and centrifuged at 12,000-g for 1 min. The fluid from the slurry was discarded, 200 µL of Binding/Wash buffer was added to the spin column, and then the spin column was centrifuged again within an Eppendorf

tube for 1 min at 12,000-g. This flow through was discarded, the spin column was placed in a new Eppendorf tube, 50 µL of serum sample was added to the column, and the column was spun again at 12,000-g for 1 min. The spin column was discarded, the flow through (albumin-depleted serum) was retained, and 200 µL of DPBS was added to the sample. Thus, the total protein concentration was within the range of measurement by a bicinchoninic assay (BCA) assay.

Total protein concentration of each sample, albumin-depleted and diluted samples, was measured using the Pierce™ BCA Protein Assay Kit (Catalog #23225, Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions. Standards were diluted from the provided ampule (2 mg/mL, Catalog # 23209, Thermo Fisher Scientific, 3747 N Meridian Rd, Rockford, IL) to produce a range of known concentrations of bovine serum albumin from 20 to 20,000 µg/mL. Standards and samples were pipetted in duplicate (25 µL each well) into a 96-well plate, and then 200 µL of working reagent (an alkaline medium containing bicinchoninic acid) was added to each well. The contents of the wells were gently mixed for 30 sec on an orbital shaker before the plate was covered and allowed to incubate for 30 min at 37 °C. After cooling to room temperature (~5 min), absorbance was measured at 562 nm using a standard spectrophotometer (Spectra Max 190 Microplate Reader, Molecular Devices, LLC, Sunnyvale, CA) using Softmax Pro software (Molecular Devices, Molecular Devices, LLC, Sunnyvale, CA). A calibration curve describing the relation between the known standards and the absorbance was constructed, and the resulting linear regression was applied to calculate the concentration of protein in each of the samples.

Based upon the concentration of protein in each sample, the volume of sample required containing 10 μg of protein, the corresponding amount of sample buffer (6x), and double-distilled water (ddH_2O) were calculated to generate samples of 15 μL. Sample, sample buffer, and ddH_2O were mixed in batches of 60 μL to minimize pipetting volume errors. The combined sample was vortexed and heated for 5 min at 85 °C to denature the protein to allow the sodium dodecyl sulfate (SDS) in the sample buffer to coat the protein so as to provide a negative charge. The samples were vortexed again, centrifuged for 2 min at 10,000-g, and then frozen at -80 °C.

On the day that the Western blot was started, the samples were thawed, vortexed, and centrifuged at 10,000-g so that they were well-mixed but any solid particulates that might interfere with movement of the proteins through the 10% polyacrylamide gel were collected at the bottom of the Eppendorf tube, not in the sample drawn up with the pipette. Electrophoresis was conducted using the Mini-PROTEN® Tetra Cell Assembly (Bio-Rad Laboratories, Hercules, CA). Two gel cassettes were inserted into the electrode assembly, the electrode assembly was placed in the mini tank, and running buffer (10x Tris/Tricine/SDS, catalog #161-0744, Bio-Rad Laboratories, Hercules, CA; diluted to 1x with ddH_2O) was added between the plates and in the mini tank to the appropriate level. Molecular weight markers (Precision Plus Protein™ Dual Xtra Standards, catalog #161-0377, Bio-Rad Laboratories, Hercules, CA) were loaded into lanes 1 and 6 of each gel. The serum samples were loaded into the rest of the gel so that later when the membrane was cut in half after blotting, the two halves would

contain the samples from the same subjects and time points (end of rest and immediate post-stimulation) in the same order (**Table 1**). That is, lanes 2 and 3 and lanes 7 and 8 contained albumin-depleted samples from one subject. Lanes 4 and 5 and lanes 9 and 10 contained albumin-depleted samples from the second subject. This pattern was repeated in the second gel into which diluted serum samples were added to the respective lanes. Once the gels were loaded, the lid was placed on the mini-tank, and the cables were attached to the power supply (PowerPac Basic™ Power Supply, Catalog #164-5050, Bio-Rad Laboratories, Hercules, CA). Electrophoresis ran for ~45 minutes at 150 V, or until the leading edge of the samples (dye front) neared the bottom of the gel.

Table 1. Depiction of gel loading plan prior to electrophoresis. MWM = molecular weight markers. Rest = sample from end of 20-min rest period. Stim = sample from immediate post-stimulation protocol (with wall support)

Lanes									
1	2	3	4	5	6	7	8	9	10
MWM	1Rest	1Stim	2Rest	2Stim	MWM	1Rest	1Stim	2Rest	2Stim

Two pieces of thick filter paper and a nitrocellulose membrane (Nitrocellulose/Filter Paper Sandwiches, 0.45 µm, Catalog #162-0215) for each gel, along with two sponges, were allowed to soak for ~10 min in transfer buffer (10x Tris/Glycine, catalog #161-0734, Bio-Rad Laboratories, Hercules, CA) in a small tub. The two pieces of glass making up the gel cassette were separated, and the ragged edges of the gel (stacking gel) were removed. The gel was then placed on the membrane, sandwiching the gel and the

membrane between the two pieces of filter paper. A glass tube was gently rolled over this sandwich while still in the transfer buffer to remove air bubbles. The filter paper/membrane sandwich was placed between the two sponges, closed within the Mini TransBlot® Cell Assembly (Bio-Rad Laboratories, Hercules, CA) and then inserted into the Mini TransBlot® Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA). The transfer cell was placed inside the mini-tank with a stirring bar and ice pack and placed on top of a stirring plate. The lid was closed on the mini-tank, and the cables were attached to the power supply, running at 100 V for 1 h, while the stirring bar was actuated.

At the end of 1 h, the power supply was turned off, and the cassettes were removed and opened. The filter paper and gel were discarded, and each membrane was placed in a small plastic box such that the side that had been next to the gel was facing upward. A small amount of Ponceau stain (~15 mL) was added to each box, and the boxes were placed on an orbital shaker for 5 min. The Ponceau stain was decanted, and then the membranes washed with DPBS (~15 mL) until the protein bands could be visualized. This step was performed to check for bubbles and loading of the lanes. Thereafter, the membranes were washed several times with DPBS until the Ponceau stain was removed.

Non-specific binding of the primary antibody to nitrocellulose was membrane was accomplished by adding a solution of 20 mL of DPBS with 1 g of non-fat dry milk, 400 µL of heat inactivated goat serum (HIGS), and 20 µL of 0.1% TWEEN-20 to each box.

The boxes were placed on an orbital shaker inside a 4 °C refrigeration unit overnight (for at least 8 h). Thereafter, the membranes were washed twice for 30 sec each time with DPBS alone before being washed three times for 10 min each time in a solution of 20 mL plus 60 µL of 0.1% TWEEN-20.

At the completion of these washing steps, each membrane was cut in half with a razor blade between lanes 5 and 6, using the molecular weight markers as a guide. One half of each membrane was placed in a separate container, so that one half could be incubated with the primary antibody (generally 1:200 dilution of primary antibody in 20 mL of DPBS with 1% HIGS and 20 µL of 0.1% TWEEN-20) and the other half without the primary antibody (20 mL DPBS with 1% HIGS and 20 µL of 0.1% TWEEN-20). The membranes were incubated in their respective solutions for 1 h at room temperature.

Thereafter, the membranes were washed twice for 30 sec each time with DPBS alone before being washed three times for 10 min each in a solution of 20 mL DPBS plus 60 µL of 0.1% TWEEN-20. Then each membrane was incubated with the secondary antibody (generally 1:2000 dilation of secondary antibody in 20 mL of DPBS with 1% HIGS and 20 µL of 0.1% TWEEN-20) on the orbital shaker at room temperature for 1 h.

The wash steps (as described above) were repeated again before detection of the protein bands. Membrane halves from the same gel (with and without primary antibody) were placed on the imaging surface of a charge-coupled device imaging system. Images were captured using Alpha Ease FC Software (v4.0.1, Alpha Innotech

Corporation, Santa Clara, CA) after incubating for 5 min with Amersham ECL Prime Western Blotting Detecting Agent (GE Health Care Life Sciences, Piscataway, NJ). Several images were collected, with image capture times at 2, 3, 4, and 5 min. Images were saved as a TIFF file for later review. Because no antibody combination provided acceptable imaging specific to the molecular weight ranges of interest, no further analysis was performed.

3.7.5 Power Analysis

The power analysis for this was were performed based upon bGH, the original primary variable of interest in this study. Because there are no studies in which bGH has been measured with this specific form of plantar stimulation, power analyses were conducted using data available from a tendon vibration protocol performed in humans by McCall et al. (McCall et al., 2000). With no prior knowledge about the amount of bGH change that could be expected in response to plantar stimulation, we chose to size our study based upon the assumption that mean differences between the responses to the two protocols implemented would have to be greater than twice the standard deviation of the resting values of bGH to be physiologically meaningful. From the study by McCall et al. (McCall et al., 2000), the standard deviation of the resting values of bGH was ~150 µg/L. Assuming that plantar stimulation would possibly increase (but not decrease) the mean bGH concentration relative to the control condition (and thus a one-sided test) by a mean difference of 300 µg/L (twice the SD), at least 12 subjects would need to participate in this study to achieve a statistical power of 0.80. The results of power analysis for this and three other hypothetical values of mean difference between the

two test protocols are displayed below (**Figure 25**), illustrating that smaller mean changes in bGH would require greater numbers of subjects to confidently detect differences between control and stimulation conditions. Unfortunately, later we would learn that the manner in which we intended to measure bGH would not work (no acceptable antibodies to detect bGH using Western blots), but this was determined during the sample analysis phase after data collection had been completed. Thus, only iGH was assayed in this dissertation project.

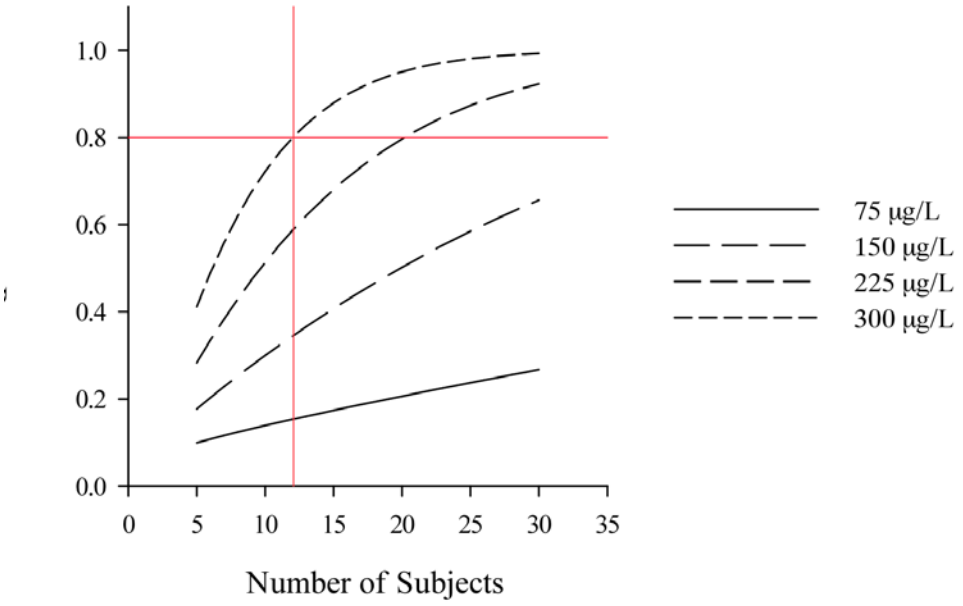

Figure 25. Statistical power vs. number of subjects for four hypothetical values of the mean difference in the bGH response to plantar stimulation

3.7.6 Statistical Analyses

Data were analyzed using a repeated measures statistical design in which time within a session (end of 20-min rest, end of stimulation (or sham), and end of 30-min recovery) and plantar stimulation (no plantar pressure [control], plantar pressure without muscle/joint proprioceptor activation [sole of foot not touching the wall], and plantar pressure with muscle/joint proprioceptor activation [sole of foot against the wall]) were the independent variables. Dependent variables were circulating GH concentration (Hypotheses 1 & 2) and integrated sEMG (Hypothesis 3). Muscle tone measured by myotonometry (Hypothesis 4) was not performed for main part of the dissertation project because the hardware failed and could not be repaired (after the pilot data collection sessions); the company that built this hardware no longer exists, and there was no one to service the equipment.

Shapiro-Wilkes tests for normality revealed that neither RMS nor GH concentration measures were normally distributed. RMS and GH concentration data could not be transformed in a manner that would produce a normal distribution, and therefore data were analyzed using two-way ANOVA on ranks within subjects in which time and condition were independent factors. In some subjects the measured GH concentration exceeded the range of standards provided with the ELISA kit, and therefore data were analyzed with and without values greater than 120% of highest standard. When data were analyzed without the GH measures that were outside of this range, the ranks were adjusted within these subjects so that the values that they could take on covered the same range as subjects with a complete data set. GH data also were analyzed separately

for the group of subjects who were responders to the dynamic plantar stimulation with support to determine whether GH secretion would be responsive to stimulation in these subjects. Statistical significance for the overall effects of time, treatment and their interaction was determined a priori at $p \leq 0.05$. When statistical analyses revealed a significant effect, contrasts were used post-hoc with control for multiple comparisons (Sidak). Analyses were conducted using Stata Data Analysis and Statistical Software (v12, StataCorp LP, College Station, TX). Data are expressed as mean ± standard deviation (SD), unless otherwise noted.

CHAPTER 4: RESULTS

4.1 Subjects

Thirteen volunteers (8 men, 5 women; 34±9 years; 174 ± 11 cm; 75.5 ± 11.2 kg; mean ± SD) participated in this investigation. The average time between sessions was 7 ± 2 d (Day 1 to Day 2: 8 ± 3 d; Day 2 to Day 3: 6 ± 2 d), such that the average time to complete the study from the first to the last session was 14 ± 3 d (Max: 20 d; Min: 10 d). No subject reported a significant change in his or her activity level, nor was there a significant change in body mass from Day 1 to Day 3 (Day 1: 75.5 ± 11.2; Day 3: 75.7 ± 11.5 kg; one-way ANOVA). All but one subject regularly participated in aerobic or resistive exercise weekly; five took vitamins daily; two regularly took antihistamines or other allergy medications; and three of the women took oral contraceptives.

4.2 Foot Pressure

Target force (25% of body weight) based upon measured body weight before each testing session was 18.9±2.9 kg. The forces measured during the calibration of the bladder pressure to the target forefoot pressures were 18.8±2.9 and 18.8±3.1 kg during the stimulation without support and stimulation with support conditions, respectively (**Figure 26**). The difference between the target force and the force measured during the bladder calibration was -0.1±0.7 and -0.2±0.5 kg during the stimulation without support and stimulation with support conditions, respectively. The average pressure and area of application were 0.8±0.2 kg/cm^2 and 26.3±6.4 cm^2 for the stimulation without support condition and 0.8±0.1 kg/cm^2 and 26.3±5.6 cm^2 for the stimulation

with support condition. The amount of pressure was approximately 60% greater than that used in previous studies (Kozlovskaya et al., 2007).

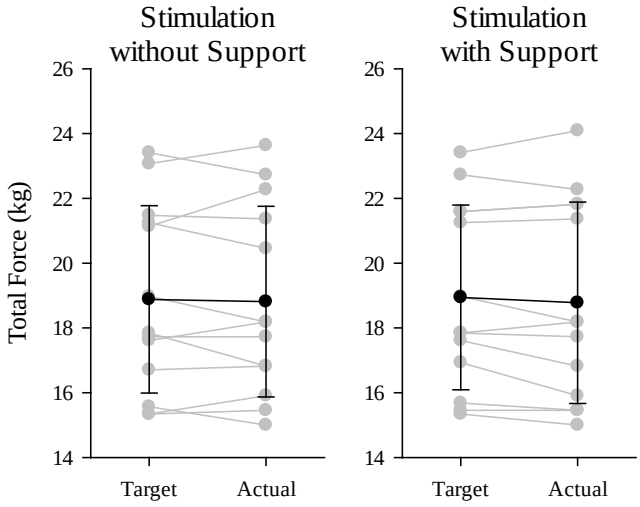

Figure 26. Individual (grey symbols) and mean (± SD; black symbols) target and measured total force at the forefoot measured with the pressure insoles during calibration of bladder pressure vs. the forefoot pressure

The total force that a subject received was consistent across the number of repetitions within a 30 sec recording (**Figure 27**). The mean (±SD) total force experienced during bladder inflation across all subjects (calculated within the defined 200 msec window) was measured to be 13.7±2.0, 12.5±2.1, 15.2±2.9, and 13.6±2.6 kg during the stimulation without support 4:30-5:00, stimulation without support 9:30-10:00, stimulation with support 4:30-5:00, and stimulation with support 9:30-10:00 periods, respectively. Within the trials across all subjects, the SD of the measured force was between 0.3 and 0.1 kg (data not shown). The measured total force was different across trials and across days, but this likely is an artifact of the drift associated with the pressure sensing insoles (see below).

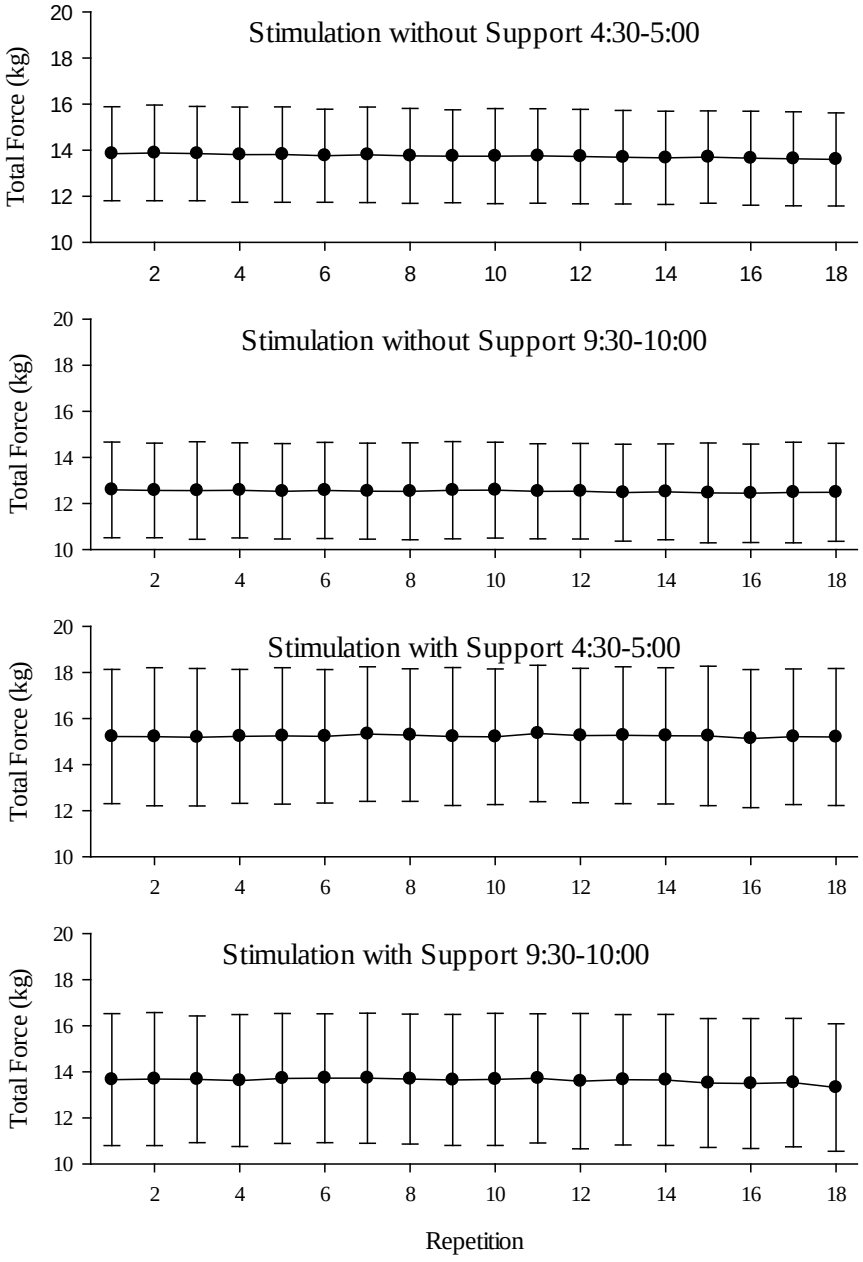

Figure 27. Mean (±SD) total force (kg) measured across all subjects in each of the measurement periods during the periods. Data were recorded for 30 sec at two times during the 10-min stimulation protocol (4:30-5:00 and 9:30-10:00).

When reviewing data from the first few subjects to complete the test protocol it was apparent that significant sensor drift occurred from the time of the insole calibration before testing started to the time that foot stimulation was applied. Although pilot testing demonstrated consistency of pressure application over several trials in close succession, this did not appear to be the case when plantar surface pressure was applied with foot bladder >20 minutes following the calibration of the bladder pressure vs. measured force applied to the forefoot. The average time between bladder calibration and data recording after 5 min of stimulation was 40±6 and 36±7 min during the stimulation without support and stimulation with support conditions, respectively. After differences between the target and measured force during the stimulation periods were observed, additional data were collected in an effort to determine whether sensor drift might contribute to this discrepancy. Subjects stood quietly on one foot for 15 sec before and after the test protocol so as to apply a known total force to the insoles to determine whether a calibration factor could be applied to the total force measured during the stimulation periods to account for the drift. Unfortunately, there was no consistent pattern in the change was observed. On average, the total force measured decreased from pre- to post-testing by 5.7 kg, but ranged from an increase of 1.1 kg to a decrease of 17.4 kg (**Figure 28**). The manufacturer confirmed that sensor drift is expected over time, which can be at least partially influenced by temperature of the sensors, frequency of pressure applied to the insoles, and/or number of trials (Personal communication, Bill Burns. TekScan, Inc.).

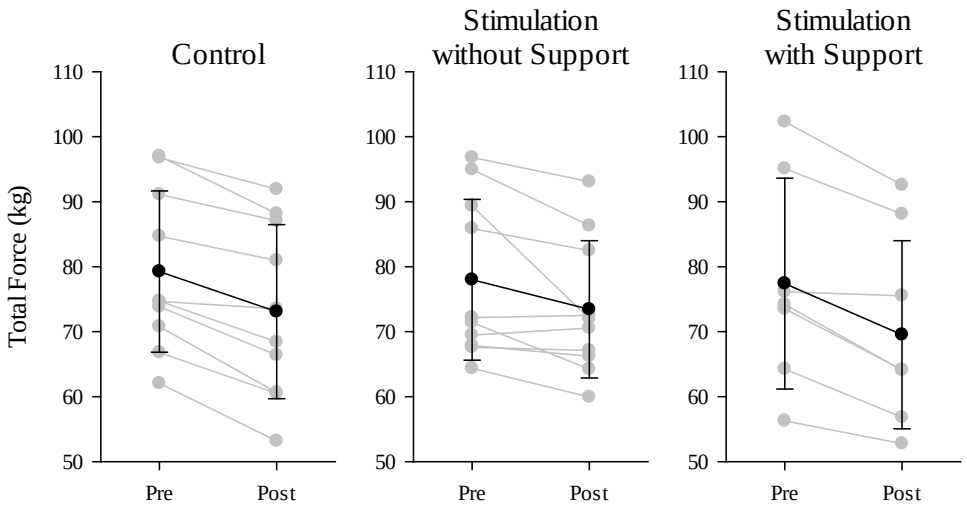

Figure 28. Individual (grey symbols) and mean (± SD; black symbols) total force measured with the pressure insoles while standing on one foot before and after testing, demonstrating the drift in the sensors over time, in the control (n=10), plantar stimulation without support (n=10), and stimulation with support (n=7) conditions.

4.3 Surface EMG

RMS was low in each of the muscles tested and remained low throughout the protocol independent of the condition (**Table 2**). Although there was a main effect of time on measured RMS in the RTA (p=0.02), RLG (p=0.006), LTA (p=0.0001), and LSOL (p=0.006), and there was a tendency for LLG to change (p=0.12), there was no interaction between time and condition. Thus, while the sEMG activity may have changed over time while subjects were unloaded, there was no effect of either plantar stimulation protocol.

Table 2. Average (±SD) Root mean square (RMS) for each muscle across all subjects in all conditions

Muscle	Pre Rest	End Rest	Stim 05	Stim10	Rec 02	Rec 05	Rec 10	Rec 20	Post Stand
					Control				
RTA	0.008	0.008	0.008	0.009	0.006	0.006	0.006	0.008	0.006
	(0.008)	(0.010)	(0.011)	(0.009)	(0.005)	(0.005)	(0.005)	(0.011)	(0.005)
RLG	0.013	0.014	0.014	0.014	0.015	0.014	0.013	0.014	0.020
	(0.013)	(0.013)	(0.013)	(0.013)	(0.013)	(0.013)	(0.012)	(0.013)	(0.010)
RSOL	0.012	0.011	0.011	0.011	0.010	0.011	0.011	0.011	0.014
	(0.014)	(0.013)	(0.013)	(0.013)	(0.013)	(0.013)	(0.012)	(0.013)	(0.014)
LTA	0.011	0.011	0.011	0.012	0.011	0.011	0.010	0.011	0.010
	(0.013)	(0.013)	(0.012)	(0.012)	(0.013)	(0.012)	(0.012)	(0.012)	(0.011)
LLG	0.008	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.017
	(0.006)	(0.007)	(0.007)	(0.007)	(0.007)	(0.007)	(0.007)	(0.007)	(0.009)
LSOL	0.006	0.006	0.006	0.006	0.007	0.006	0.006	0.006	0.010
	(0.006)	(0.006)	(0.006)	(0.006)	(0.006)	(0.006)	(0.006)	(0.006)	(0.008)
					Stimulation without Support				
RTA	0.007	0.007	0.009	0.011	0.009	0.008	0.007	0.008	0.010
	(0.008)	(0.009)	(0.011)	(0.012)	(0.010)	(0.009)	(0.009)	(0.009)	(0.011)
RLG	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.011	0.022
	(0.014)	(0.013)	(0.014)	(0.014)	(0.014)	(0.014)	(0.014)	(0.013)	(0.014)
RSOL	0.012	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.014
	(0.014)	(0.013)	(0.013)	(0.013)	(0.012)	(0.012)	(0.012)	(0.012)	(0.012)
LTA	0.014	0.014	0.013	0.014	0.014	0.013	0.013	0.013	0.015
	(0.013)	(0.014)	(0.015)	(0.014)	(0.014)	(0.014)	(0.014)	(0.014)	(0.014)
LLG	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.018
	(0.012)	(0.013)	(0.013)	(0.013)	(0.013)	(0.013)	(0.013)	(0.013)	(0.012)
LSOL	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.007
	(0.005)	(0.005)	(0.005)	(0.005)	(0.005)	(0.005)	(0.004)	(0.004)	(0.006)
					Stimulation without Support				
RTA	0.014	0.014	0.012	0.013	0.012	0.012	0.012	0.013	0.013
	(0.016)	(0.016)	(0.017)	(0.016)	(0.016)	(0.016)	(0.016)	(0.016)	(0.017)
RLG	0.009	0.009	0.010	0.009	0.009	0.009	0.009	0.009	0.018
	(0.008)	(0.007)	(0.007)	(0.007)	(0.007)	(0.007)	(0.007)	(0.007)	(0.009)
RSOL	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.007
	(0.006)	(0.006)	(0.006)	(0.006)	(0.006)	(0.006)	(0.007)	(0.006)	(0.007)
LTA	0.008	0.008	0.007	0.007	0.008	0.007	0.008	0.007	0.008
	(0.006)	(0.006)	(0.006)	(0.007)	(0.007)	(0.007)	(0.007)	(0.007)	(0.006)
LLG	0.011	0.010	0.011	0.010	0.010	0.010	0.010	0.011	0.021
	(0.015)	(0.015)	(0.015)	(0.015)	(0.015)	(0.015)	(0.015)	(0.015)	(0.014)
LSOL	0.010	0.009	0.010	0.009	0.009	0.009	0.009	0.009	0.012
	(0.011)	(0.010)	(0.010)	(0.009)	(0.009)	(0.009)	(0.009)	(0.009)	(0.009)

RTA: right tibialis anterior; RLG: right lateral gastrocnemius; RSOL: right soleus; LTA: left tibialis anterior; LLG: left lateral gastrocnemius; LSOL: left soleus; Pre Rest: start of supine rest period; End Rest: after 20 min of supine rest; Stim 05: at 5 min of dynamic plantar simulation (or sham condition); Stim 10: at 10 min of dynamic plantar stimulation (or

sham condition); Rec 02: 2 min after end of stimulation (or sham condition); Rec 05: 5 min after end of stimulation (or sham condition); Rec 10: 10 min after end of stimulation (or sham condition); Rec 20: 20 min after end of stimulation (or sham condition).

RTA RMS tended to increase (p=0.08) from end of rest to end of stimulation but significantly deceased (p=0.007) from end of stimulation to the end of the recovery period. RLG RMS did not change from end of rest to end of stimulation but decreased from end of rest to the end of recovery (p=0.004) and from end of stimulation to the end of recovery (p=0.0007). LTA RMS also did not change from end of rest to end of stimulation but decreased from end of rest to the end of recovery (p=0.0003) and from end of stimulation to the end of recovery (p=0.004). LSOL did not decrease from end of rest to end of stimulation but did decrease from the end of rest to the end of recovery (p=0.004) and from the end of stimulation to the end of recovery (p=0.01)

Based upon visual review of the data files, a sEMG response to plantar stimulation (bladder inflation) was observed in 8 of the 13 subjects studied in the stimulation with support condition (**Table 3**); only a few random spikes were observed during the stimulation without support condition. Subjects were labeled as Responders if >50% of the stimulations resulted in an EMG response. The sEMG response to bladder inflation (seen as a spike in sEMG; **Figure 29**) in the Responders occurred primarily in the right lateral gastrocnemius. The sEMG response also was observed in the right soleus muscle in a subset of the Responders. There were an equal number of men and women classified as Responders, but only one of the five Non-Responders was a woman. The

pattern observed with the visual review of the data was confirmed using the objective evaluation described in the Methods section (**Figure 30,** R=0.97, p<0.001). When analyzing the sEMG response to plantar stimulation, only complete repetitions (inflation to deflation) were analyzed. In 30-sec segments of force and sEMG data, in all but one case the number of dynamic foot pressure stimulations was between 17 and 19; in one case the foot pressure and sEMG data collection was initiated late so that data from only 14 stimulation cycles was available.

Table 3. Mean (±SD) percentage of responses per muscle by visual review and objective evaluation in responders (n=8; 4 men, 4 women) vs. non-responders (n=5; 4 men, 1 woman)

		RTA		RLG		RSOL	
Condition	Time	Visual	Objective	Visual	Objective	Visual	Objective
				Responders			
Wihout Support	4:30-5:00	1±4	0	1±2	0	0	0
	9:30-10:00	3±8	1±2	1±2	1±2	0	0
With Support	4:30-5:00	0	6±14	83±15	79±16	16±30	23±33
	9:30-10:00	1±4	5±8	70±35	67±31	0	27±34
				Non-Responders			
Wihout Support	4:30-5:00	16±35	0	2±5	0	0	0
	9:30-10:00	0	0	4±10	0	0	0
With Support	4:30-5:00	0	0	1±2	2±3	2±5	1±2
	9:30-10:00	0	0	0	0	1±2	0

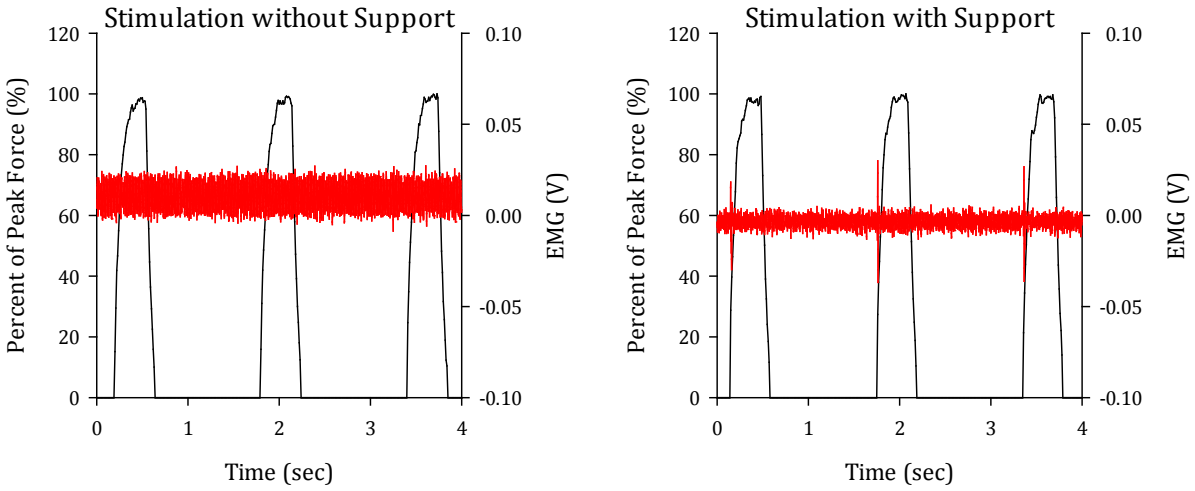

Figure 29. Example graphs from a representative subject (Responder) during stimulation without (left panel) and with support (right panel). Total force is displayed as percentage of maximal force measured within these trials to normalize the data across conditions rather than total force due to drift in the sensors which was not able to be reliably quantified.

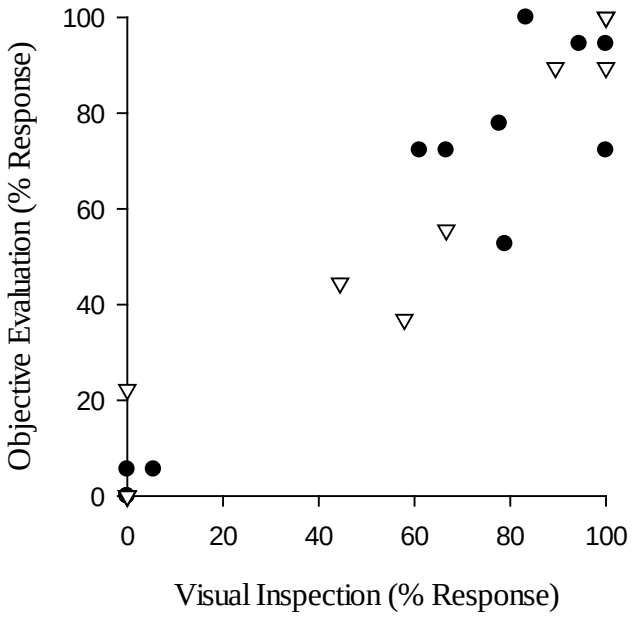

Figure 30. Comparison between the visual review and the objective detection of a sEMG response in the right lateral gastrocnemius after 5 min (solid circles) and 10 min (open diamond) of stimulation with the foot supported

Latency of the response, from onset of bladder inflation with foot support to the sEMG spike, was calculated only in the group of subjects who displayed a sEMG response. The mean (±SD) latency after 5 min of stimulation with support (4:30-5:00) was 29±14 msec in the right lateral gastrocnemius (n=8), 30±13 msec in the right soleus (n=4), and 15±14 msec in the right tibialis anterior (n=2). The mean latency after 10 min of stimulation (9:30-10:00) was 34±21 msec in the right lateral gastrocnemius (n=8), 24±8 msec in the right soleus (n=4), and 15±14 msec in the right tibialis anterior (n=3).

All but one subject failed to perceive any sensation of muscle contraction in the muscles during either plantar stimulation protocols, except during the muscle stretch associated with stimulation with foot support. One subject reported that they experienced thigh as well as lower leg muscle contraction during the stimulation with support. Reflexive contraction of the thigh and lower leg muscles was reported by some subjects in dry immersion studies (Kozlovskaya et al., 2007; Vinogradova et al., 2002) but was unique to this one individual in the current study.

4.4 Immuno-assayble Growth Hormone

In general, iGH concentration was not different between conditions (main effect), across time within conditions (main effect), and there was no interaction between condition and time (**Table 4**), whether all data were included in the analyses (**Figure 31**) or whether only data within the range of the ELISA standards were included (**Figure 32**). Similar results were obtained when only data from the responders to plantar

stimulation with support were evaluated. Results from data analyses that included only values in range of the ELISA are likely to be the most valid since linearity outside the range of the ELISA could not be verified, and analyses with ranks adjusted for missing data are likely to be the least biased.

Table 4. p-values for interactions and main effects from two-way repeated measures ANOVA on ranks for different combinations of data

Subjects	Data	Adjusted Ranks	Interaction	Condition	Time
All	All	NA	0.54	0.29	0.04
All	In Range	No	0.08	0.39	0.48
All	In Range	Yes	0.12	0.19	0.18
Responders	All	NA	0.76	0.10	0.45
Responders	In Range	No	0.25	0.25	0.90
Responders	In Range	No	0.24	0.22	0.78

In Range = data analyzed only from measured iGH concentrations within 120% of the highest calibration standard. Responders = subjects who demonstrated a brief neuromuscular (sEMG) response to plantar stimulation with foot support. Adjusted Ranks = ranks adjusted for subjects with missing data so that the values for ranks had the same range as subjects without missing data. NA = not applicable.

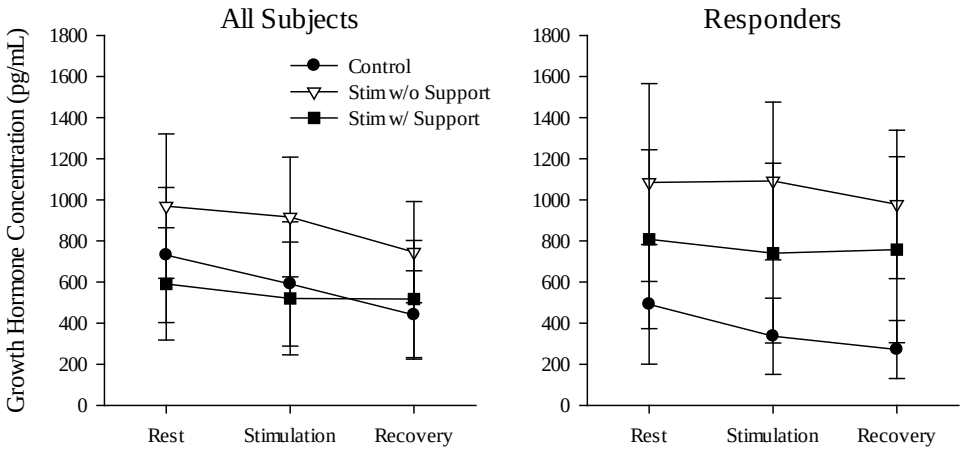

Figure 31. Measured iGH concentration across time (Rest = end of 20 min rest; Stimulation = end of 10 min stimulation or sham period; Recovery = 20 min after end of stimulation) and conditions (Control, Stimulation without foot support, Stimulation with foot support) in all subjects (n=13) and in Responders (n=8; subjects in whom a spike in sEMG was observed during the plantar stimulation with foot support). All data are shown. Data are mean ± standard error of the mean.

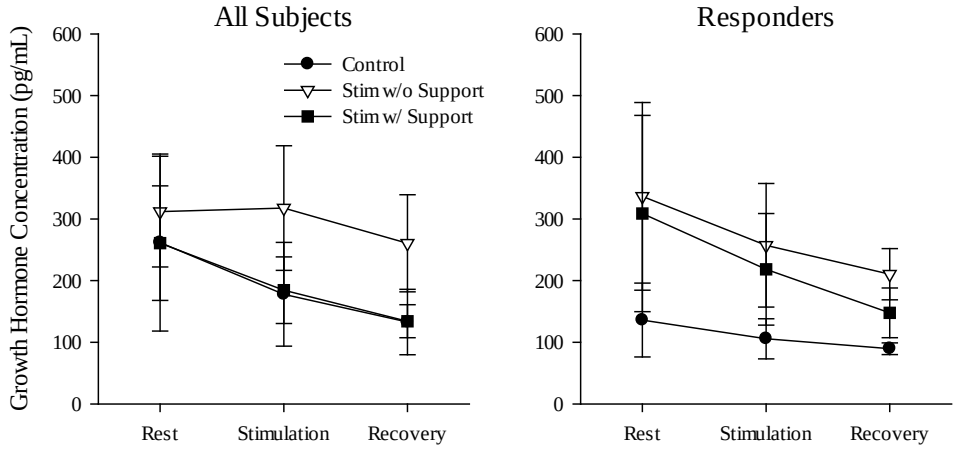

Figure 32. Measured iGH concentration across time (Rest = end of 20 min rest; Stimulation = end of 10 min stimulation or sham period; Recovery = 20 min after end of stimulation) and conditions (Control, Stimulation without foot support, Stimulation with foot support) in all subjects (n=9; 6 men, 3 women) and in Responders (n=5; 3 men, 2 women; subjects in whom a spike in sEMG was observed during the plantar stimulation with foot support) whose measured GH concentration was within 120% of the highest standard used in the ELISA. Data are mean ± standard error of the mean.

4.5 Western Blots

In all cases, the removal of serum albumin reduced the noise in the signal and improved the clarity of the images. However, with each set of antibodies, while bands appeared in the region of interest (48 and 60-80 kDa) (McCall et al., 2001; Weigent, 2011), these bands were present with or without the primary antibody, suggesting a non-specific binding of the secondary antibody to potential target proteins and thus complicating any analysis of the resulting images (**Figure 33**). Other antibodies must be identified or the Western blot protocol optimized for these GH variants before further work can be performed.

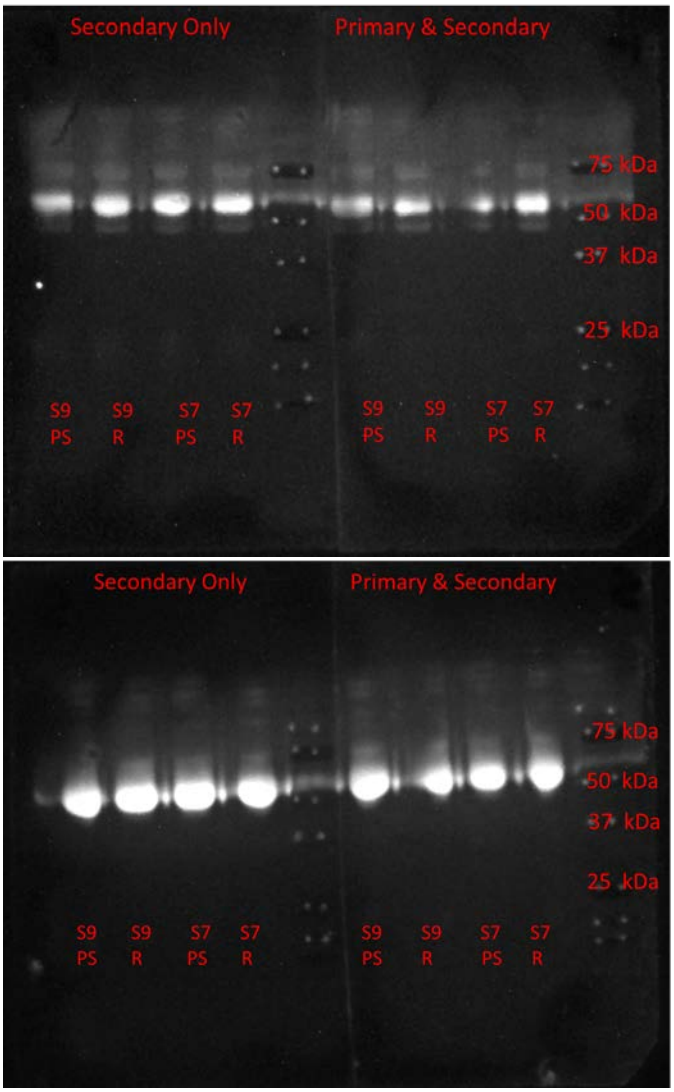

Figure 33. Western blot of albumin-depleted serum (top panel) and serum samples diluted 1:25 with DPBS (bottom panel) in two subjects (S7 and S9) who were responders to dynamic foot pressure (had a spike in sEMG activity in the right gastrocnemius in response to bladder inflation during stimulation with support condition). Primary antibody, 1:400. Secondary antibody, 1:4000. 5 min exposure time. R = End of 20-min rest. PS: Immediately post-stimulation

CHAPTER 5: DISCUSSION

5.1 Overall Results

These results obtained during an acute model of musculoskeletal unloading do not support a role for iGH in the anti-atrophic effect of plantar stimulation that previously has been observed during HLU in animal models or during dry immersion studies in humans. When plantar surface stimulation was applied in a manner that simulate the pressure on the forefoot associated with walking at a load equivalent to 25% of the subject's body weight, iGH did not increase above resting levels. This was true whether the foot forces were applied in a manner which stimulated primarily skin mechanoreceptors (Specific Aim 1) or the combination of skin mechanoreceptors with muscle and joint proprioceptors (Specific Aim 2). Further, forefoot plantar stimulation failed to increase RMS, whether with or without foot support (Specific Aim 3), and only caused a transient spike in sEMG activity that was associated with the onset of foot pressure. This transient spike in sEMG occurred in the majority of subjects (8 of 13) and primarily in the right gastrocnemius muscle, but was unlikely to have been of sufficient magnitude or frequency to provide any protective effects against adaptations associated with skeletal muscle unloading. These results do not preclude the participation of bGH or another growth factor in the prevention of muscle atrophy observed in previous HLU and dry immersion studies.

5.2 Growth Hormone in Response to Dynamic Foot Pressure

Previous unloading studies in animal (De-Doncker et al., 2000; Kyparos et al., 2005) and human models (Hernández Corvo et al., 1983; Moukhina et al., 2004; Navasiolava et al.,

2011; Netreba et al., 2004) demonstrated that dynamic foot pressure can prevent or attenuate muscle atrophy and decreased muscle strength, but the mechanism for this countermeasure largely has remained elusive. While plantar stimulation increased muscle tension in humans during dry immersion (Kozlovskaya et al., 2007; Vinogradova et al., 2002) and transiently elevated EMG activity in rats during HLU (De-Doncker et al., 2000), the level of muscle activity associated with dynamic foot pressures in the current study likely would have been insufficient to replace the total muscle activity comprising a whole day in normal ambulatory subjects. In this study, we were unable to stimulate meaningful increases in sEMG in the lower leg muscles using dynamic foot pressure; only a subset of subjects experienced a transient low level spike in sEMG with bladder inflation, similar to that observed by Layne et al. (Layne et al., 2005). Contrary to previous reports and experiences from our pilot studies, we observed this sEMG activity primarily in the lateral gastrocnemius with few subjects experiencing activation of the soleus muscle.

However, the low levels of "inducible" electrical activity reproducibly observed in the lateral gastrocnemius muscle of the leg receiving plantar stimulation suggests some underlying response to plantar stimulation mediated via a neural network. While the increase in electrical activity induced by plantar stimulation in the lateral gastrocnemius was small, it was reproducible across time within the same subject and was temporally linked to the stimulus. Conversely, this low level response was absent in the lateral gastrocnemius of the contra-lateral leg of the same subject which did not receive plantar stimulation. It remains unknown whether or not such low level

responses could affect other factors linked to reductions in muscle mass during unloading, such as the maintenance of muscle tension (Adams et al., 2003). However, since this study was in acute in nature (less than 1 h of unloading), any such effect of repeated bouts of plantar stimulation on the skeletal muscle atrophy response to unloading would need to be studied over a much longer period of unloading (days to weeks). However, similar to the sEMG response in this study, the protective effect of plantar stimulation in rats during HLU was specific to the leg that received the stimulation (Kyparos et al., 2005).

A pathway independent of muscle tension or work might stimulate hypertrophic and/or inhibition atrophic pathways in response to dynamic foot pressure during musculoskeletal unloading. GH, which acts directly on muscle through GH receptors or indirectly through hepatic release of IGF-1 to upregulate the activity of the AKT/mTOR pathway, is one candidate that might have beneficial effects on preventing muscle atrophy during unloading. Increased circulation of IGF-1 also may prevent muscle atrophy by promoting satellite cell proliferation that contributes to myofiber hypertrophy. At the same time, actions of GH on the AKT/mTOR pathway induce the phosphorylation of FOXO transcription factors, thereby inhibiting the production of E3 ligases, MuRF1 and MAFbx (Brooks & Myburgh, 2014). While data exist that suggest the down regulation of the Akt/mTOR pathway during skeletal muscle unloading (Bodine, Stitt, et al., 2001), there appears to be no concurrent decrease in iGH) in rats during HLU (Bigbee et al., 2006) or in humans during bed rest (McCall et al., 1997; Schmitt et al., 2000) or space flight (McCall et al., 1999). Additionally, in the current study, we

were unable to stimulate the release of iGH, and therefore it is unlikely that this variant participates in the protection against muscle atrophy afforded by dynamic foot stimulation during unloading. This 22kDa form of GH is most often measured (Kraemer & Ratamess, 2005), but over 100 different isoforms of GH have been suggested (Baumann, 1991).

Several lines of evidence suggest that high molecular weight form of GH (48 kDa or 60-80 kDa), referred to as bGH because it has been primarily measured through a measured of tibial growth rate in rats, may moderate skeletal muscle atrophy. First, resting levels of bGH decrease during HLU in rats concurrent with skeletal muscle atrophy (Bigbee et al., 2006). bGH secretion is stimulated by afferent nerve traffic (McCall et al., 2001), and there is a decrease in afferent nerve activity, particularly during the first week of unloading (De-Doncker et al., 2005) when skeletal muscle atrophy is most rapid (Adams et al., 2003; Thomason & Booth, 1990). Second, while bed rest and space flight do not affect resting levels of bGH in humans, the release of bGH in response to stimuli is impaired (McCall et al., 1997, 1999). Similarly, the release of bGH decreases with afferent stimulation after HLU in rats (Bigbee et al., 2006). Thus, even with intermittent stimulation of factors that would have increased bGH prior to unloading, muscle atrophy continues as the period of unloading progresses. Third, HLU rats who received a tendon vibration countermeasure twice daily, a protocol that would be expected to increase the secretion of bGH (Gosselink et al., 2004), experienced significantly less muscle atrophy during HLU compared to rats who did not receive this countermeasure (Falempin & In-Albon, 1999). bGH also is released in humans in

response to tendon vibration (McCall et al., 2000), and therefore a protocol which increases afferent nerve traffic such as dynamic foot pressure might contribute to the protection against muscle atrophy. If bGH is released in response to dynamic foot stimulation, its effect on preventing muscle atrophy could be dramatic; its estimated biological activity is believed to be 200-300 times greater than the 22 kDa iGH (Ellis et al., 1978).

The release of bGH is mediated by the stimulation of afferent nerve traffic primarily from muscle and joint proprioceptors, rather than from skin mechanoreceptors. When the distal ends of severed tibial or peroneal nerves (nerves with primarily motor-related functions) were electrically stimulated at levels expected to elicit responses in the Type I and Type II afferents associated with Golgi tendon organs and muscle spindles, plasma levels of bGH increased while pituitary levels decreased (Gosselink et al., 1998). Both De-Doncker et al. (De-Doncker et al., 2000) and Kyparos et al. (Kyparos et al., 2005) applied pressure to the whole foot using a boot which one assumes allowed some measure of ankle movement. The boot was not well-described by De-Doncker et al. (De-Doncker et al., 2000) but Kyparos et al. (Kyparos et al., 2005) clearly state their boot was made from material that was thin enough to allow normal range of motion. In both cases, it is not difficult to imagine the ankle briefly dorsiflexing when the bladder under the rat's foot was inflated and became more rigid, thus activating muscle spindles and ankle joint proprioceptors. A similar response might have occurred during forefoot bladder inflation in human subjects during dry immersion (Kozlovskaya et al., 2007);

while this device had both heel and forefoot bladders, they were stimulated in an alternating fashion to simulate the loading pattern of walking.

Arguing against the role of bGH during dynamic foot pressure during stimulation if only the cutaneous receptors are activated, electrical stimulation of the proximal end of the severed sural nerve does not elicit secretion of bGH (Gosselink et al., 1998). This would suggest that secretion of bGH would not explain the anti-atrophic effects of dynamic foot pressure during HLU if the ankle and foot do not move during bladder inflation during HLU and dry immersion studies. Further, rats who wore a boot without bladder inflation during HLU experienced similar levels of protection against muscle atrophy as those with bladder inflation even though presumably only cutaneous mechanoreceptors were stimulated in those animals (Kyparos et al., 2005). bGH release in response to sural nerve activity occurs only when the sural nerve is stimulated in combination with the peroneal and tibial nerves. However, neurally-mediated release of bGH might be elevated by modulation of spinal cord excitability. In our own lab, Blake et al. (unpublished observations) observed that spinal cord excitability was higher, as measured by the Hoffman reflex (H-reflex), when only cutaneous mechanoreceptors were stimulated than when both skin, muscle, and joint receptors were activated through external loading. Increased spinal cord excitability with decreased joint and muscle loading also has been previously reported by others (Abbruzzese, Rubino, & Schieppati, 1996; Conway & Knikou, 2008; Hwang, Jeon, Kwon, & Yi, 2011; Knikou, Angeli, Ferreira, & Harkema, 2009).

An alternative explanation for the protective effects of plantar stimulation during HLU and dry immersion might be an interruption of the atrophic events necessary for degradation of the sarcomeric proteins (Boris S. Shenkman & Nemirovskaya, 2008). Previous studies in rats with HLU have shown that maintenance of muscle stretch (foot fixed in the dorsifelxed position) can reduce the degree of muscle atrophy (Jaspers et al., 1988; Ohira et al., 1997), perhaps through a nitric oxide-mediated inhibition of calpain (Xu et al., 2012). Muscle atrophy induced by 14 d of HLU was attenuated in the plantarflexors of rats when the foot was fixed at 35° of dorsiflexion. In rats receiving this countermeasure, intracellular calcium levels and calpain activities were not elevated and degradation of desmin was not increased. Further, passive stretching of the mouse extensor digitorum longus has been shown to activate mTOR (Troy A Hornberger et al., 2004), which potentially would have both hypertrophic and anti-atrophic effects in HLU. Perhaps a muscle stretch during bladder inflation during plantar stimulation applied at regular intervals throughout the day as in previous HLU and dry immersion studies would have elicited similar effects.

5.3 Muscle Activity in Response to Dynamic Foot Pressure

The foot bladder for this study was positioned based on work by previous investigations that suggested the greatest neuromuscular response would occur when pressure was applied to the forefoot. Congruent with the work of Layne et al. (Layne et al., 2005), Inglis et al. (Inglis, Kennedy, Wells, & Chua, 2002) demonstrated that the vibrotactile thresholds were lowest at the medial ball of the foot and highest at the heel, with thresholds at the lateral ball and mid-ball intermediate to those. Similar data were

obtained by Lowrey et al. (Lowrey et al., 2014) although they tested only three sites, with the order of sensitivity decreasing from the head of the fifth metatarsal to the great toe and then to the heel.

In this study, we observed a spike in sEMG activity with the initiation of the plantar surface stimulation at the forefoot, an indication of a neuromuscular response. While it could be argued that this resulted from motion artifact due to movement of the sEMG electrodes when the forefoot bladder inflated, perhaps stretching the skin, we believe that we obviated this possibility by having the foot and knees (and rest of the body) supported by separate tables such that the skin of the lower leg where the electrodes were placed did not contact any hard surfaces. Additionally, two subjects who participated in both the pilot study and the main dissertation project had a sEMG response to forefoot bladder inflation independent of lower leg support. In pilot tests, the lower leg was supported by a table and the electrodes were pressed against the skin while in the main project the leg was not supported. Further, when a response occurred in the main study, within a subject the variation in the latency of the response was rather narrow and was similar to that of an H-reflex. However, Layne et al. (Layne et al., 2005) contended that the response that they observed was not stretch-reflex response because they observed a spike sEMG activity in the tibialis anterior in addition to the soleus. A stretch-reflex response due to plantar stimulation should have been observed in the plantarflexors, not in a dorsiflexor muscle. In contrast, we observed no consistent response to plantar stimulation in the tibialis anterior.

In the current study, the sEMG activity in response to bladder inflation was observed in only one condition: when subjects received plantar stimulation while the foot rested against the wall. Further, this response also was observed primarily in the lateral gastrocnemius and not in all subjects. The lack of a response when the foot was not supported might be somewhat surprising given that rats during HLU experienced a transient increase in EMG activity while receiving foot pressure (De-Doncker et al., 2000). Similarly, soleus muscle stiffness was maintained during the first 24 h of dry immersion when subjects receive dynamic foot pressure as a countermeasure while it decreased control subjects (Kozlovskaya et al., 2007; Miller et al., 2004; Popov et al., 2003; Vinogradova et al., 2002). However, our results are similar to Layne and colleagues in that they reported only brief pulses in sEMG activity in response to plantar stimulation in human subjects when the subjects were seated with their feet resting a platform (Layne et al., 2005).

The inability to detect a response in soleus muscle during this dissertation project might result from electrode placement compared to that of Layne et al. (Layne et al., 2005), but the same electrode placement in the main project was used as during the pilot study when soleus activity was detected in two subjects. Differences between observed sEMG of the soleus in response to plantar stimulation with the foot supported is more likely to be a factor of subject selection or differences in the stimulation protocol.

Several factors play a role in the sEMG response that may have affected our results with and without foot support. First, muscle length affects the strength of the response, perhaps as an augmentation or diminution of the sEMG activity in response to differing levels of muscle spindle firing (Layne et al., 2005). Specifically, the response to plantar stimulation is highest in the plantarflexor muscles when the ankle is dorsiflexed and lowest when the ankle is plantarflexed. When the subjects in the current study received plantar stimulation without the support of the wall, most relaxed their feet into a mildly plantarflexed position such that the strength of the sEMG response to plantar stimulation would have been diminished and perhaps not measurable in our subject population. In contrast, with the support of the wall against the bottom of the feet in the other condition, the ankle angle was maintained at ~90° at rest and the subjects experienced a brief period of dorsiflexion when the bladder inflated, both likely augmenting the response in comparison to the cutaneous stimulation-only, unsupported condition (Blackburn, Padua, & Guskiewicz, 2008).

Second, the spike in sEMG activity that was observed during plantar stimulation with support was largely limited to the lateral gastrocnemius. Although the spike in sEMG occurred in a few subjects, including those in the pilot studies, differences between subjects with the responder groups is unclear given that the same electrode locations were used across all subjects and within a subject the same operator applied the electrodes. The general lack of response in the soleus muscle also differs from that observed by Layne et al. (Layne et al., 2005). This may have resulted from differences in electrode placement or other equipment considerations. However, one potentially

important difference between the current study and that of Layne et al. (Layne et al., 2005) was the knee angle. In the current study, the knee was straight and the gastrocnemius muscle would have been in a longer, or stretched, position so that a larger proportion of the pull from the Achilles tendon would be transmitted to the gastrocnemius than when the knee was bent in the work by Layne and colleagues. Arguing against this, even with the knee bent at 90° and the gastrocnemius may have been in a relatively slackened condition, Layne et al. reported similar increases in sEMG activity in the soleus and gastrocnemius. Another explanation for the inability to observe soleus activity might be related to the stimulation of the broader area of the forefoot rather than discrete locations. Layne et al. (Layne et al., 2005) demonstrated that the spike in neuromuscular activity was reduced when stimulating either to medial or lateral aspect when the stimulation was preceded by stimulation of the other sites. It is unclear from previous work whether presumably simultaneous pressure application in the current student would affect the neuromuscular responses.

Third, background muscle activity augments the response to plantar surface stimulation in a graded fashion (Forth & Layne, 2007) but this is an unlikely explanation. Although not measured in these subjects (partially due to the pre-study failure of the myotonometer), our subjects could have had higher background muscle tension in the condition with their feet against the wall. But all subjects reported that they were relaxed, there were no indications that there was an increased sEMG activity at rest (even in the muscles of the responsive subjects), and the subjects did not report any muscle or joint discomfort due to the positioning of the foot. A foruth factor that

might contribute to different responses to the plantar stimulation is subject selection. In previous experiments not all subjects demonstrated an increase in sEMG activity in response to plantar stimulation (Personal communication, Charles S Layne). A similar, yet unknown, factor of subject selection may have played a role in our results; most but not all our subjects (8 of 13) were "responders" to this form of stimulation. Presently, there is no clear predictor of responders vs. non-responders. Future studies might screen for reduced responsiveness to a tendon-tap or similar maneuver that would activate muscle spindles in response to rapid stretch.

If this is a myotatic reflex response (a monosynaptic reflex in response to stretching of the muscle spindle) in response to the stretch of the gastrocnemius via the Achilles tendon when the forefoot bladder inflated (i.e. dorsiflexion as the foot moves away from the wall), then one would expect that the latency of the EMG activity relative to the bladder inflation would be ~50-70 msec (Fellows & Thilmann, 1989). In contrast, the latency measured in this study was closer to the average latency of the H-reflex: ~30 msec (Buschbacher, 1999). The latency of the sEMG response measured in this study appeared to be relatively low (~25-30 msec), but the nature of the stimulation made it difficult to determine the exact onset of the stimulation relative to the sEMG spike. In studies of the H-reflex, for example, there is a discrete time at which the electrical impulse is imparted to the skin and nerve. In other studies when a tendon tap occurs or the angle through which a joint is rotated can be quantified. In this report, latency of the sEMG response is reported relative to a threshold of inflation pressure, the only available objective source of data (e.g. joint angle was not measured). As such, the angle

of ankle dorsiflexion and amount and rate of muscle stretch induced by bladder inflation likely were different across different subjects even if the bladder inflation was the same due to such factors as foot length.

Although not specifically demonstrated by De-Doncker et al. (De-Doncker et al., 2000), there was a concern for the habituation of the neuromuscular response (Jackson, Gutierrez, & Kaminski, 2009) during the 10-min dynamic plantar stimulation which might have affected our iGH results, but this was not apparent from the sEMG recordings after 5 and 10 min of stimulation. Fatigue may reduce the amplitude of the response, and therefore it might have been more difficult to detect in the later phases of the stimulation protocol. However, in this study, in all but one subject who demonstrated a neuromuscular response to the plantar stimulation (Responders) after 5 min also demonstrated a response at 10 min of stimulation. Further, it is possible that some of the subjects that were labeled as non-responders actually did have a neuromuscular response at the start the 10-min stimulation protocol (when no measurements were made) but was absent by the time that the data were recorded at 5- and 10-min of stimulation. But in the few subjects who participated in both pilot testing and the main dissertation project, the neuromuscular response or lack thereof was consistent within subjects. Two subjects who had a sEMG spike during pilot tests had the same response during the subsequent data collections for the dissertation project. Conversely, the subject who did not have a sEMG response during pilot tests did not respond to plantar stimulation during the main study. Therefore, we believe that

subjects classified as non-responders did not have a spike in sEMG activity even at the beginning of the dynamic foot pressure protocol.

Although a neuromuscular response was observed in the majority of subjects (8 of 13) while the foot was supported against the wall (or the floor during seated pilot tests), overall it would be difficult to ascribe protection against muscle atrophy during unloading in animals or humans to dynamic plantar stimulation based upon the brief spikes in sEMG activity observed in this acute study. It is unlikely that a significant amount of muscle tension would have been developed and the frequency of muscle activity was low. Thus, any protective effect of this countermeasure likely would be the result of a skeletal muscle growth factor response independent of muscle tension. However, there was no evidence from this study that stimulation of the skin mechanoreceptors, with or without the activation of muscle and joint proprioceptors, induced a significant metabolic stressor that would result in the elevation in iGH. iGH was not increased due to dynamic plantar stimulation in this project and therefore, combined with previous information, is an unlikely candidate to explain the maintenance of muscle mass and performance in animal and human models of unloading. This does not exclude the participation of other GH variants, such as bGH, which have yet to be fully tested in this model of acute unloading or other longer duration unloading conditions.

5.4 Application of Dynamic Foot Pressure as a Muscle Atrophy Countermeasure

To date, the countermeasures that have most effectively prevented generalized muscle atrophy during bed rest and space flight to those that have included high levels of musculoskeletal loading through resistance exercise (Adams et al., 2003; Shackelford et al., 2004; SSmith et al., 2012). While this form of countermeasure has been widely accepted, the soleus muscle has been difficult to protect against muscle atrophy for unknown reasons (Krainski et al., 2014; Trappe et al., 2009; Trappe et al., 2007). Although some difficulties in protecting the soleus might arise from the exercise countermeasure prescription, duration of unloading (Bamman et al., 1997), or subject selection (Lee et al., 2014), perhaps the effectiveness of the current countermeasures might be enhanced through the use of dynamic foot pressure regularly throughout the day as a form of passive countermeasure (Layne et al., 1998, 2005). Bedridden patients who cannot ambulate or do so with difficulty and astronauts during space flight could wear dynamic foot pressure shoes with little interference with activities of daily living. Thus, patients could participate in a countermeasure that does not require specific time for nursing/rehabilitation team care, and astronauts would not require additional time in their normal daily schedule in which to participate in countermeasures during space flight. However, continued research is required to refine prescriptions for dynamic foot pressure countermeasures.

Assuming that the bGH is at least partially responsible for the protection against muscle atrophy, the dynamic foot pressure protocol should be applied regularly throughout the unloading period. After a period of unloading, the responsiveness of bGH to afferent

nerve traffic is diminished in rats (Bigbee et al., 2006), even though afferent nerve activity may be on the rise in the later stages of HLU (De-Doncker et al., 2005). bGH response also was diminished in humans after just 2-3 days of bed rest and space flight and remained depressed through the unloading period (McCall et al., 1997, 1999). This suggests that regular stimulation of the afferent pathways associated with bGH release may be necessary to realize its beneficial effects. Regular bouts of dynamic foot pressure might be required to maintain the sensitivity of the mechanoreceptors and proprioceptors (Lowrey et al., 2014), sustain a constant or cumulative level of bGH in the circulation, and/or prevent adaptations in the anterior pituitary that might inhibit release of bGH (Grindeland et al., 1987). Additionally, if bGH release is enhanced by increased levels of afferent activity from the muscle and joint proprioceptors, plantar stimulation with the ankle in the dorsiflexed position might be preferential since this elicits a large neuromuscular response compared to the normal plantarflexed position (Layne et al., 2005) that most astronauts (Barratt & Pool, 2008; Nicogossian et al., 1994) and rats (Ohira et al., 2002) assume during space flight. The reduced ability to secrete bGH, however, is recoverable after unloading, as observed over the course of several days with reambulation or return to a normal gravity environment (McCall et al., 1997, 1999).

Intermittent application of foot pressure may be preferential over constant foot pressure. A large percentage (~70%) of the cutaneous receptors in the sole of the foot are fast adapting (respond to initial application or removal of pressure) (Kennedy & Inglis, 2002), and therefore afferent sensory nerve activity might be limited if pressure

were constantly applied. As an example, the cosmonaut who wore the "Cuban boots" (spring loaded platforms inside the shoe to provide constant pressure) had to increase the level of pressure and duration of wearing the boots across days of space flight in order to reverse the perception of being upside down that had been provided initially by the countermeasure (Hernández Corvo et al., 1983). When dynamic foot pressure has been applied dry immersion studies and in one HLU investigation, the plantar stimulation was applied at regular intervals throughout the day, perhaps to model the loading animals and humans normally experience when ambulatory. There may be a habituation to a constant stimulus if dynamic or static foot pressure was applied continuously throughout the day (Layne & Forth, 2008). Further, early work demonstrated an impaired growth response in hypophysectomized rats when infusion of GH was constant in comparison to intermittent administration (Isgaard, Carlsson, Isaksson, & Jansson, 1988; Maiter, Underwood, Maes, Davenport, & Ketelslegers, 1988). Similarly, although not a direct measure of muscle size or strength but an indicator of muscle performance, walking for 2 h/d or 4 h/d in 15-min bouts (one time per hour) during 4 d of bed rest resulted in smaller decreases in maximal oxygen consumption than in standing alone (Vernikos et al., 1996). It is unclear, however, if the effectiveness of the dynamic foot pressure could be enhanced if applied in a different manner than has been tested previously. Just as in exercise prescriptions, factors that might be manipulated include the frequency, duration, amount of load, and rest between plantar stimulations as well as the regularity of countermeasure application within a day and the entirety of the unloading condition.

Unfortunately, there may be an age-related decreased in effectiveness of the plantar stimulation to prevent muscle atrophy (sarcopenia) and decreased strength and endurance. First, the sensitivity of the cutaneous mechanoreceptors might be reduced in older adults. In a cross-sectional study of aging, the vibrotactile thresholds were lower in older (n=6, mean age: 89 yr) than younger subjects (n=6, mean age: 26 yr) across a range of stimulation frequencies (Inglis et al., 2002). Further, if neuromuscular activity is an important component of the protective effects of plantar stimulation, the effectiveness of this countermeasure might be further impaired by age-induced reduction in stretch-reflex response (Chandrasekhar, Abu Osman, Tham, Lim, & Wan Abas, 2013).

5.5 Limitations of the Study

There were three primary limitations to this study. First, we were unable to find suitable primary and secondary antibodies to measure high-molecular weight bGH that likely would have been more responsive to this form of stimulation, particularly when involving muscle and joint proprioceptors (McCall et al., 2001). Secretion of bGH previously has been shown to be responsive to afferent nerve traffic while iGH has not. Assays that had been used successfully in the past by other groups were no longer available, investigators who had used the tibial growth length assay were unresponsive to inquiries, alternative antibodies that did not suffer from some methodological shortcomings were not identified, and budget constraints preempted the ability to conduct additional tests. Thus, our conclusions about the growth factors associated with the dynamic foot pressure countermeasure were limited. Second, both the iGH and

sEMG data were not normally distributed, and we were unable to find a transformation that produced a normal distribution. Therefore, we chose to use ANOVA by ranks within a subject to perform our analyses. A major drawback of using ranks in statistical analyses is that interpretation of the results is limited to indicating a change or no change, and no absolute measure of magnitude of change in the original units of measurement is available (Fields, 2005). Although we did observe significant change in sEMG in some muscles over time using ANOVA by ranks, the absolute change in sEMG (based upon the mean data) appears so small as to be physiologically insignificant. Third, the unloading protocol utilized was for this study was very short in comparison to the duration of unloading experienced in subjects who are injured, bedridden, or in space. If a bGH response to stimulation had been observed during this acute unloading period, it may not have been maintained during prolonged unloading; bGH secretion is blunted in humans after bed rest and space flight (McCall et al., 1997, 1999) and in rats after HLU (Bigbee et al., 2006). However, the time course of the degraded response and whether it is possible to maintain it through plantar stimulation during unloading have not been investigated.

Additional factors associated with this study may have affected its applicability and overall findings. First, the drift in the pressure sensing insoles did not allow us to verify that the force applied to the forefoot during the stimulation periods was the same as the amount of force we would have expected based upon the calibration of bladder pressure to force performed before the rest period. While we had hoped that the measurement of force when standing on one foot before and after the test protocol

would provide a correction factor, the seemingly random change in the measured force (total body weight on one foot) from pre- to post-testing precluded this. Second, the sizing of the shoes was problematic in that the size and dimensions of each subject's feet was not the same even in subjects wearing the same shoe size. Shoe size was determined primarily from foot length, and thus subjects with wider feet may not have received the same amount plantar stimulation relative to their foot width, although we expect differences were small. More even and/or predictable application of foot pressure could be achieved with customized shoes. Third, the top of the foot is pushed against the top of the shoe during bladder inflation, which can be uncomfortable and might elicit responses from other skin receptors besides those on the plantar surface of the foot (Kyparos et al., 2005). In pilot testing, we tried several different shoe configurations to maximize plantar pressure transmission from the bladder to the foot, improve individual adjustability, and minimize subject discomfort. Combined with previous experiences in an earlier companion study (Boaz Blake, personal communication) and pilot testing for this work, the method chosen to apply foot forces in this project appeared to be the best method to provide dynamic pressure in a reliable and comfortable manner. Fourth, we chose one level of pressure, one frequency of bladder inflation/deflation, and a single duration of stimulation, and it is possible that different combinations of these factors could have elicited a stronger and/or more consistent sEMG or GH response. However, although all subjects reported that they tolerated this level of plantar pressure over the 10-min stimulation period in the main part of the dissertation project, several indicated that they would not have tolerated higher pressures or longer stimulation times. Thus, modification to the shoe and/or

bladder hardware likely will be required to increase the foot forces, frequency of the inflation/deflation cycles, and/or the duration of the stimulation. Furthermore, while we attempted to maintain overall subject comfort while supine on the padded table, this might be improved for future projects so that the unloading time can be extended (if needed) and the likelihood increasing stress-induced levels of cortisol, and thereby GH, would be minimized. Some subjects were unable to lie still for long periods of time, which contributed to increased variability of sEMG activity during the rest periods and in at least one subjects made it difficult to separate a neuromuscular response to plantar stimulation from voluntary muscle contractions.

5.6 Areas for Improvement for Applying Dynamic Foot Pressure

While the standard exercise shoes used in this experiment were comfortable for use by all the subjects, there were several issues associated with the choice of footwear that likely impacted the study. As noted above, using a standardized shoe did not allow for proper sizing across all foot shapes. The shoelaces had to be pulled together tightly for subjects with narrow feet but less so for individuals with wider feet. Thus, the pressure applied to the subjects' feet may have been more consistent if the shoes were more individually-sized or custom-fitted to each person. Additionally, the pliable nature of the shoes' uppers may have made them more comfortable but the stretch of the material meant that the bladder had be inflated to higher pressures in some subjects to achieve the desired force on the plantar surface of the foot and may have affected the how evenly the pressure was applied. The next generation of these shoes might include more width sizing and rigid uppers, perhaps with a buckling system like that used in

cycling shoes and ski boots to provide consistent, reproducible adjustability. Other improvements to the footwear might include a bladder that is integrated into the sole of the shoe, rather than lying on top of the insole, so as to improve comfort, and a bladder that is sized individually to the width of each subject's feet. In this experiment, we used a single size bladder for all the subjects; the size of the bladder was chosen based on measurements of the sole of the foot of several potential test subjects across a range of foot sizes. As a result, application of the pressure may not have been consistent across subjects as a proportion of foot width and may have influenced our results.

Independent of this study and our experiences, a Russian company recently began marketing a new dynamic foot pressure device (http://www.diasled.net/korvit.html), touting positive results from scientific studies from Russia's Institute of Biomedical Problems. These plantar-stimulating boots, called "Korvit" (**Figure 34**), provide pressure using pneumatic bladders on the heel and forefoot inflated in a pattern to simulate walking, with three sizes available for children and three sizes available for adults. Reportedly clinical trials have been conducted using this device at the Russian Research Center of Neurology, Department of Neurology, Russian State Medical University, and other locations. The "Korvit" has been approved for use by Ministry of Health of the Russian Federation.

Figure 34. The "Korvit" shoes and controller currently available for use during rehabilitation from ischemic stroke, traumatic brain injury, and other motor control disabilities. (Source: http://www.diasled.net/korvit.html)

5.7 Conclusions

With these experimental results, we could provide no evidence that dynamic foot pressure, stimulating skin mechanoreceptors alone or in combination with muscle and joint proprioceptors, induces an increased secretion of iGH during acute unloading. In agreement with animal models that that showed no increase in iGH when isolated afferent neural pathways were stimulated, this would suggest that iGH likely does not contribute to the protective effects provided by plantar stimulation against chronic unloading-induced muscle atrophy. However, because we were unable to identify an antibody with sufficient specificity, we were unable to confirm or exclude the participation of bGH in the anti-atrophic mechanism associated with dynamic foot pressure. We also did not identify or exclude any other skeletal muscle growth or atrophic factor.

This study did determine that dynamic foot pressure using this protocol did not result in a muscle activity level that, by itself, likely would counter muscle atrophy during unloading. Muscle activity was lower than normal quiet standing in our acute model of musculoskeletal unloading, but was not increased by dynamic foot pressure. Dynamic foot pressure at the level of force and frequency used had no effect on muscle activity when plantar stimulation was applied when the foot and ankle was free to move (without support condition), stimulating mechanoreceptors in the foot. Plantar stimulation by bladder inflation when the foot was supported, however, did result in a brief spike in sEMG activity in the plantarflexors of most subjects (8 of 13). The strength of this response is unlikely to be sufficient to induce enough muscle tension to be protective, but animal and human studies have shown that stimulation of afferent pathways specific to muscle proprioception have resulted in the release of bGH. If bGH is released in response to dynamic foot stimulation, its effect on preventing muscle atrophy could be protective and warrants further investigation.

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APPENDIX A: COMMITTEE FOR THE PROTECTION OF HUMAN SUBJECTS

APPROVAL LETTER

UNIVERSITY of HOUSTON

DIVISION OF RESEARCH

March 18, 2014

Mr. Stuart Lee
c/o Dr. Mark Clarke
Health and Human Performance

Dear Mr. Stuart Lee,

The University of Houston Committee for the Protection of Human Subjects (1) reviewed your research proposal entitled "SKELETAL MUSCLE GROWTH FACTOR RESPONSE TO CUTANEOUS STIMULATION OF THE PLANTAR SURFACE OF THE FOOT" on January 17, 2014, according to federal regulations and institutional policies and procedures.

At that time, your project was granted approval contingent upon your agreement to modify your protocol as stipulated by the Committee. The changes you have made adequately fulfill the requested contingencies, and your project is now **APPROVED**.

- **Approval Date: March 18, 2014**
- **Expiration Date: March 17, 2015**

As required by federal regulations governing research in human subjects, research procedures (including recruitment, informed consent, intervention, data collection or data analysis) may not be conducted after the expiration date.

To ensure that no lapse in approval or ongoing research occurs, please ensure that your protocol is resubmitted in RAMP for renewal by the **deadline for the February 2015** CPHS meeting. Deadlines for submission are located on the CPHS website.

During the course of the research, the following must also be submitted to the CPHS:

- Any proposed changes to the approved protocol, prior to initiation; AND
- Any unanticipated events (including adverse events, injuries, or outcomes) involving possible risk to subjects or others, within 10 working days.

If you have any questions, please contact Alicia Vargas at (713) 743-9215.

Sincerely yours,

Daniel O'Connor

Dr. Daniel O'Connor, Chair
Committee for the Protection of Human Subjects (1)

PLEASE NOTE: All subjects must receive a copy of the informed consent document, if one is approved for use. All research data, including signed consent documents, must be retained according to the University of Houston Data Retention Policy (found on the CPHS website) as well as requirements of the FDA and external sponsor(s), if applicable. Faculty sponsors are responsible for retaining data for student projects on the UH campus for the required period of record retention.

Protocol Number: 14189-01 Full Review: X Expedited Review: ____

316 E. Cullen Building Houston, TX 77204-2015 (713) 743-9204 Fax: (713) 743-9577
COMMITTEES FOR THE PROTECTION OF HUMAN SUBJECTS

APPENDIX B: PILOT DATA

B.1 Devices for Applying Foot Pressure

The original intent of this project was to utilize existing, commercially-available shoes constructed with bladders in the soles to provide pressure to the feet. The shoes contained four separate bladders which inflated sequentially for ~10 sec each, proceeding in order from the heel to the forefoot (**Figure 35**). An external controller managed pressure and timing of bladder inflation. The sequencing and timing of the bladder inflation was mechanically controlled and was not able to be altered without significant modification.

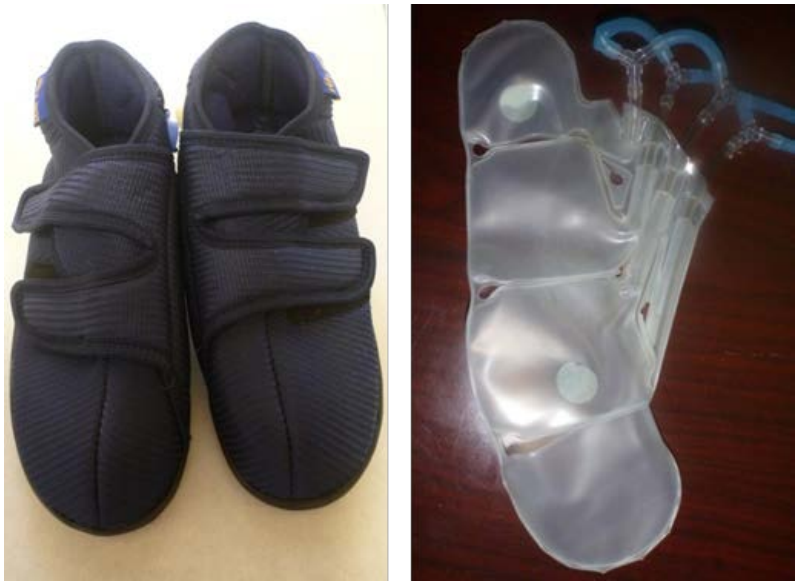


Figure 35. Commercially-available boots (left panel) and the four bladders (right panel) that are sequentially inflated.

Four volunteers participated in pilot tests using commercially-available shoes. These subjects were instrumented with sEMG electrodes on soleus, lateral gastrocnemius, and tibialis anterior of each lower leg. Muscle tone also was measured in the lateral

gastrocnemius and tibialis anterior using a myotonometer (Neurogenic Technologies, Inc., Missoula, Montana) previously shown to produce reliable measurements of muscle compliance (Kerins, Moore, Butterfield, McKeon, & Uhl, 2013) . Subjects rested in the recumbent position (phlebotomy chair) for 20 min and then received foot stimulation using the commercial shoes for 10 minutes. Measurements were obtained every 5-10 minutes during 60 min of recumbent rest, 10 min of plantar stimulation, and 10 min of resting recovery. While the resting position clearly decreased sEMG activity and muscle tone compared to standing, as expected (Masani et al., 2013), foot stimulation did not evoke a sEMG response or increased muscle tone (**Figure 36**). Concerns with the use of the commercial boots included the inability to control inflation pressure and timing. Also, the material from which the shoes were constructed stretched when the bladders were inflated such that sizing and Velcro closures of the shoes were critical to the pressure experienced by the subject. This was a significant impediment to the delivery of the same level of pressure between subjects and across trials since the shoe sizes were limited to whole numbers and the use of the two Velcro closures was too crude to be reproducible.

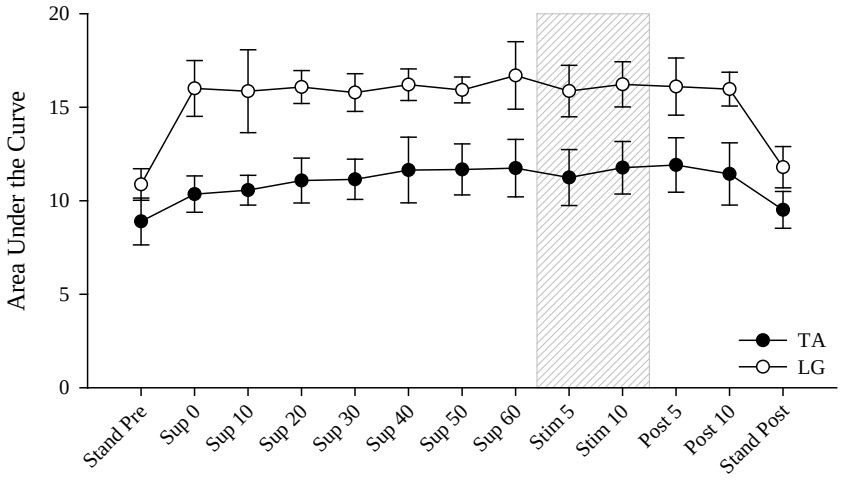


Figure 36. Muscle tone decreased from standing to supine (area under the curve describing the relation between force applied to the muscle and displacement increased) but does not appear to be affected by foot pressure stimulation. The hatched box designates the foot stimulation period.

Although attempts were made to provide sturdier and more consistent support external to the commercial shoe to improve the delivery of foot pressure, e.g. using an steel-toed overshoe, a foot brace (used for treatment of plantar fasciitis), or a walking boot, no method was found to be acceptable for the purposes of this project (**Figure 37**), largely due to comfort issues. Thus, we initiated efforts to design and build a custom bladder and computer-controlled valve and inflation system through which we could consistently deliver known pressures from a pressurized gas source controlled by inline regulators (**Figure 38**). This bladder inserted into a normal exercise shoe was used for all subsequent pilot tests and the main dissertation project.

Figure 37. Four alternative configurations examined during pilot testing to improve quality of foot pressure application, strapping against dynamometer plate (left panel), an overshoe with additional heel strap (left of center panel), using a plantar fasciitis brace (right of center panel), and a walking boot (right panel).

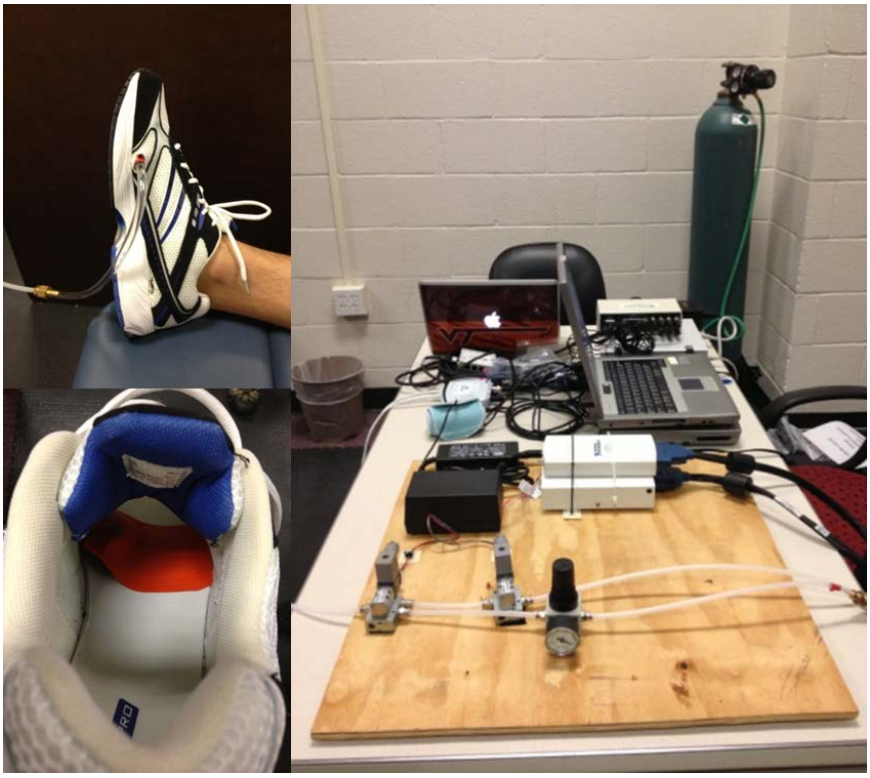


Figure 38. Photographs depicting the custom-designed bladder inserted through the side of a standard exercise shoe (top left panel) such that the bladder lies on top of the shoe's insole (bottom left panel; pressure-sensing insole not shown), and the computer controlled valve system (right panel) to inflate and deflate the bladder in a reliable manner. The large pressurized gas cylinder served as the air source, but pressure regulators upstream

of the valve control system controlled the gas pressure in the bladder and thus the plantar surface pressure experienced by the subject.

B.2 Pressure Bladder Placement

The inflatable foot pressure bladder had to be located below the foot and below the pressure insole, but it was unclear whether it would be preferable to locate the bladder underneath or above the shoe's insole. Testing was performed in both conditions across bladder inflation pressures that were similar to those anticipated for use in the main dissertation project (20, 25, and 30 psi). While the peak values for pressure and area across which pressure was sensed were similar independent of bladder position (above or below the insole), the pressure apparently was more evenly distributed with the bladder above the shoe insole such that the average pressure and total force were greater when the bladder was above the insole than when then bladder was placed below it (**Figure 39**). Thus, for the purposes of the dissertation project, the bladder was placed between the shoes insole and the pressure-sensing insole to maximize the area across which pressure is transmitted and increase the total force experienced by the subjects as a result of the bladder inflation.

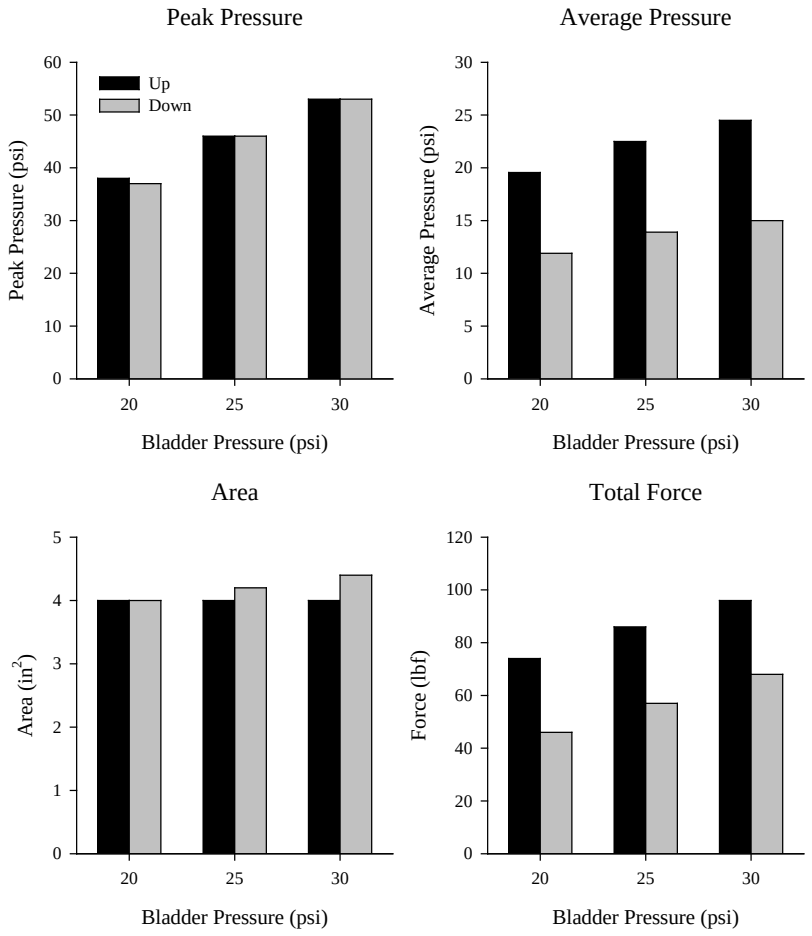


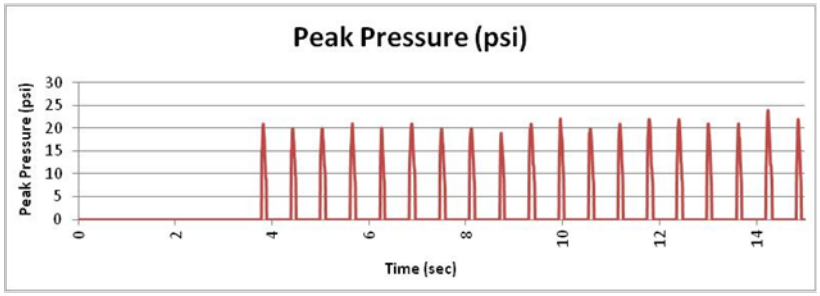
Figure 39. Plantar pressure levels measured using the pressure-sensing insole in one subject when the bladder was placed above the shoe insole (directly underneath the subject's plantar surface, black bars) and the bladder was placed under the shoe insole (gray bars). Peak pressure and area of stimulation appeared to be similar between the two conditions, but the bladder appeared to more uniformly apply pressure to the plantar surface of the foot when the bladder was placed above the shoe insole, based upon observations of higher average pressure and total force measurements.

B.3 Foot Bladder Inflation Frequency

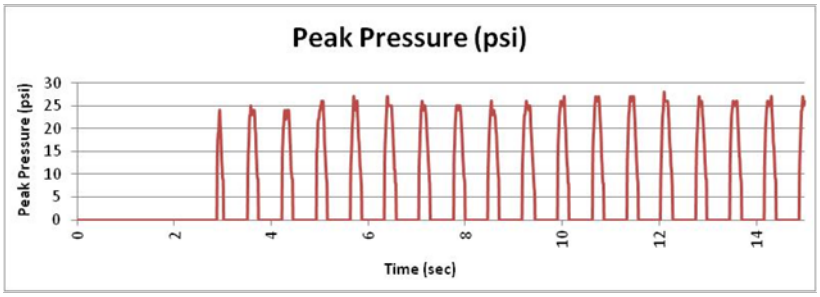
It was the intent of this project to deliver forefoot forces at frequencies representative of normal walking, similar to the protocols which have successfully attenuated muscle deconditioning during dry immersion (Khusnutdinova et al., 2004; Moukhina et al., 2004; Netreba et al., 2004). Therefore, trials were conducted to verify that the bladder

could be inflated to the intended pressure (**Figures 40**) and deflated to zero pressure (**Figure 41**) within the desired time cycles. Inflation-deflation cycles tested were as rapid as 0.1 sec inflation-0.1 sec deflation. Also, trials were conducted with and without a vacuum source to determine whether it was needed to deflate the bladder. From these trials it was apparent that at the shortest bladder inflation-deflation times there was insufficient time to increase bladder pressure before it was released and that bladder pressure did not always return to zero pressure before the bladder was re-inflated. Thus, the shortest duration of inflation or deflation of the bladder that could be reliably used was 0.2 sec, which is within the parameters of simulated walking.

Inflate 0.1 seconds, deflate 0.5 seconds



Inflate 0.2 seconds, deflate 0.5 seconds



Inflate 0.3 seconds, deflate 0.5 seconds

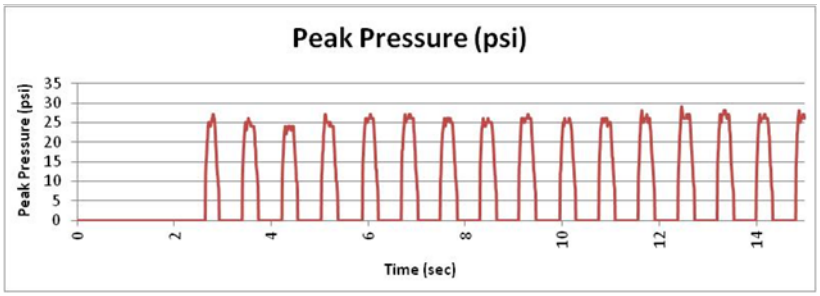


Figure 40. Force measurements at different durations of bladder inflation. Target pressure was 25 psi, and deflation time (0.5 seconds) was kept constant across trials.

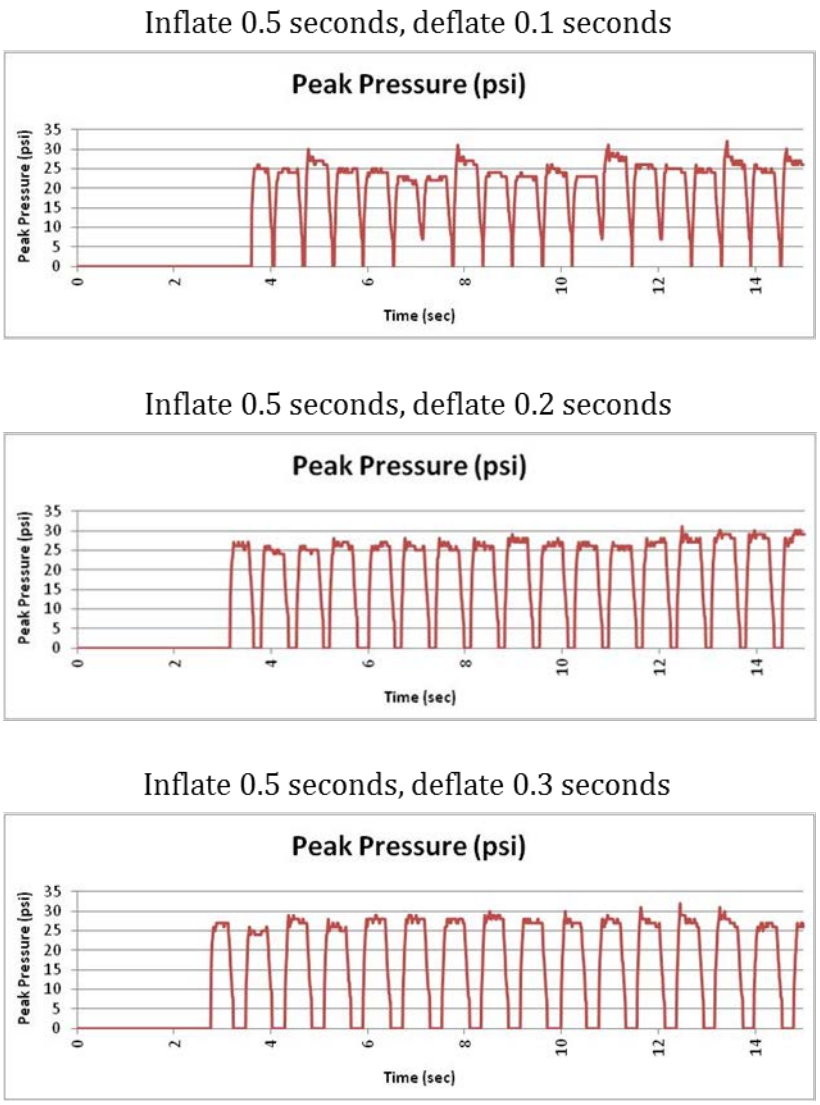


Figure 41. Force measurements during different durations of bladder deflation. Target pressure was 25 psi, and inflation time (0.5 seconds) was kept constant across trials.

B.4 Subject Posture

Layne and colleagues (Layne et al., 2005) reported a brief sEMG response immediately following foot pressure stimulation with a solenoid system that applied point pressures (2.5 cm^2). Several different configurations of the foot pressure applications were pilot tested for this dissertation project, using both the commercially-available boots as well

as the custom bladder inside a normal exercise shoe, with the subjects in the supine position. The supine position was the focus of these pilot tests because that was the posture planned for this dissertation as model of short-duration unloading. However, we were unable to replicate the sEMG findings previously reported by Layne and colleagues (Layne et al., 2005) when subjects were supine; we were able to induce an sEMG response in the lower leg only when subjects were seated during initial testing of the bladder design. Thus, we conducted two pilot studies to investigate the discrepancy between our experiences and the findings reported previously.

First, three subjects were tested while supine, while seated, and while supine with the feet elevated and supported such that the hip, knee, and ankle angles were ~90°, similar to the seated posture. Also, tests were conducted with and without a lower leg foot restraint (i.e. a walking boot) to determine whether increasing the area of foot pressure stimulation would increase the likelihood of the sEMG response. The walking boot restrained the movement of the foot during the bladder inflation so that more of the bladder contacted the bottom of the foot. In these subjects, the sEMG response to foot stimulation was observed only when the subjects were seated and only when the foot was not constrained by the walking boot (**Figure 42**). Thus, we tentatively concluded that movement of the foot about the ankle (stimulating joint and muscle proprioceptors) or preloading of the foot and ankle while seated (preloading plantar surface skin mechanoreceptors) was necessary to evoke a sEMG response.

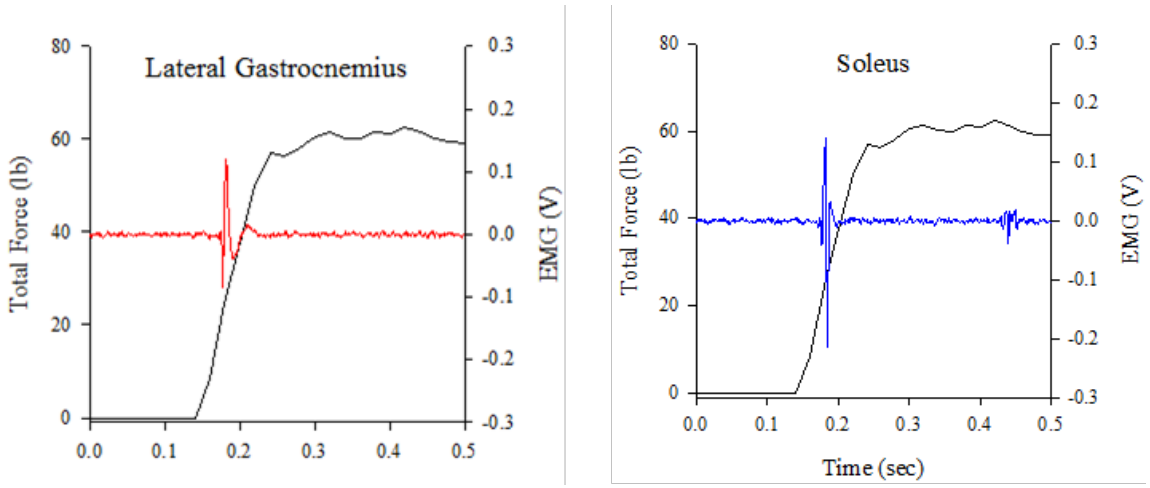


Figure 42. Representative evoked sEMG response in the lateral gastrocnemius (left panel, red tracing) and soleus (right panel, blue tracing) during plantar stimulation in the seated posture when the subject's foot was supported by an immovable object (floor). This replicated the posture and test set-up used by Layne and colleagues (Layne et al., 2005), except that the plantar stimulation was different (bladder inflation across the forefoot vs. single point stimulation). No EMG response was evoked when subjects were supine, supine with hip, knee, and ankle angles were similar to seated, or in any posture when the ankle and foot were restrained by a walking boot.

Second, four subjects were tested in the seated and supine positions (phlebotomy chair, **Figure 43**) with increasing levels of foot bladder pressure, ranging from 5 to 60 psi. These tests were conducted to test what previously appeared to be a posture effect on the sEMG response and to determine whether the posture effect, if it was present, was influenced by increasing foot pressure; in previous testing we were able to achieve higher foot pressures with bladder inflation when the subjects were seated with the foot resting on the ground. Subjects did not wear the walking boot as they had in the previous pilot tests. In these tests, we observed that sEMG responses in the lateral gastrocnemius and soleus muscles were present only when the subjects were seated, but sEMG responses were absent at the lowest bladder inflation pressures (≤10 psi). There was no sEMG response with foot bladder inflation when subjects reclined in the

phlebotomy chair and increasing plantar pressures (up to 60 psi) had no effect. In this same round of pilot tests, one subject also was tested while fully recumbent with their feet placed against the wall. In this way, the ankle angle was similar to that as when seated and the sole of the foot was supported by the wall during bladder inflation. We observed that the sEMG response was evoked with bladder inflation when the foot was placed against the wall, similar to the seated posture, and there appeared to be a threshold bladder pressure above which the response was present (~15 psi). Testing was repeated with this same subject in a separate session in two conditions: once while supine with the feet supported against the wall and once while supine with the feet unsupported. Using the same protocol of increasing bladder pressure across time, we observed no evoked sEMG response unless the foot was supported and only when the bladder pressure was >10 psi. Thus, the combination of the results from these experimental conditions suggest that the foot support is necessary to evoke a sEMG response and that this occurs at plantar pressures >10 psi independent of posture and joint angles at the knee and hip when ankle angle is ~90°. Layne and colleagues (Layne et al., 2005) previously demonstrated that the magnitude of the evoked EMG response is increased when the ankle angle is more acute and reduced when it is more obtuse, demonstrating the modulation by muscle spindle activation of the reflex EMG response to plantar stimulation.

Figure 43. Subjects recumbent in phlebotomy chair (left panel), and subject supine with base of the foot against the wall (right panel) during pilot testing.

Tentative conclusions from these pilot tests were that in order to evoke a sEMG response to foot pressure stimulation, there was a minimum bladder pressure required and the foot had to be supported in some fashion, either while seated or supine. One explanation for this observation was that the foot moved, the ankle joint was rotated, and the plantarflexor muscles are stretched when the bladder is inflated, resulting in a stretch-reflex response. The stimulation protocol used by Layne and colleagues (Layne et al., 2005) was unlikely to have produced similar movement about the ankle joint but may have stretched the plantar fascia on the base of the foot such that forces were transmitted through the Achilles tendon to the plantarflexors. The magnitude of the evoked sEMG response was greatest when Layne and colleagues (Layne et al., 2005) applied pressure to the distal portions of the foot, when the stretch of the fascia and Achilles tendon would have been greatest; there was little to no response when the heel alone was stimulated and fascia tensioning would be lower. An alternative explanation is that the support of the foot by the ground while seated or against the wall while

supine resulted in stimulation of proprioceptors in the joint, such as the Golgi type endings or the Ruffini endings, which are activated by the deformation of the ligaments or the joint capsule. Data recently collected in the laboratory suggests that spinal cord excitability is lower when both cutaneous and joint receptors are stimulated through external loading, in comparison to spinal cord excitability when only the cutaneous receptors are stimulated using a bladder inflated under the bottom of the foot (Boaz Blake, unpublished observations).

B.5 Growth Hormone and IGF-1 Responses to Walking in Place

As pilot testing for the neuro-endocrine portion of the dissertation project, four healthy subjects were recruited to perform a walking-in-place protocol with a similar overall timeline as the foot pressure protocol planned for this dissertation. Subjects reclined quietly in a phlebotomy chair for 20 min before blood samples were obtained. Then subjects walked in place at a cadence of 75 steps/min, the same step frequency modeled during dry immersion investigations (Khusnutdinova et al., 2004; Moukhina et al., 2004; Netreba et al., 2004). Thereafter, subjects returned to the recumbent posture, and a second blood sample was drawn within 2 minutes of the end of exercise, similar to the protocol used by others (McCall et al., 1997, 1999). Blood was drawn into EDTA-treated collection tubes and centrifuged for 15 minutes at 1000-g within 30 minutes of collection. Plasma was aliquotted and stored at -80 °C until assay.

IGF-1 was assayed in triplicate in plasma samples from all four subjects with ELISA according to the manufacturer's specifications (Quantikine Human IGF-I Immunoassay,

R&D Systems, Minneapolis, MN). GH samples were split, albumin was depleted from half of each aliquot, and the molecular weight separation was performed. Albumin was removed from samples by standard procedures (SwellGel® Blue Albumin Removal Kit, Thermo Fisher Scientific Inc., Rockford, IL), and proteins in solution were separated according to molecular weight (Amicon Ultra-0.5 mL Centrifugal Filters, EMD Millipore Corporation, Billerica, MA). Assuming that what other investigators have called i GH has a molecular weight of 22 kDa and bGH is a dimer with a molecular weight of ~60 kDa (Hymer et al., 2001), samples for GH assays were separated into three fractions: <30 kDa, 30-100 kDa, and >100 kDa. The protocols for these activities are depicted in **Figures 44** and **45**. A similar molecular weight separation protocol was utilized by Kraemer and colleagues (Kraemer et al., 2006). Each sample, in duplicate, then was applied to the ELISA plate for analysis.

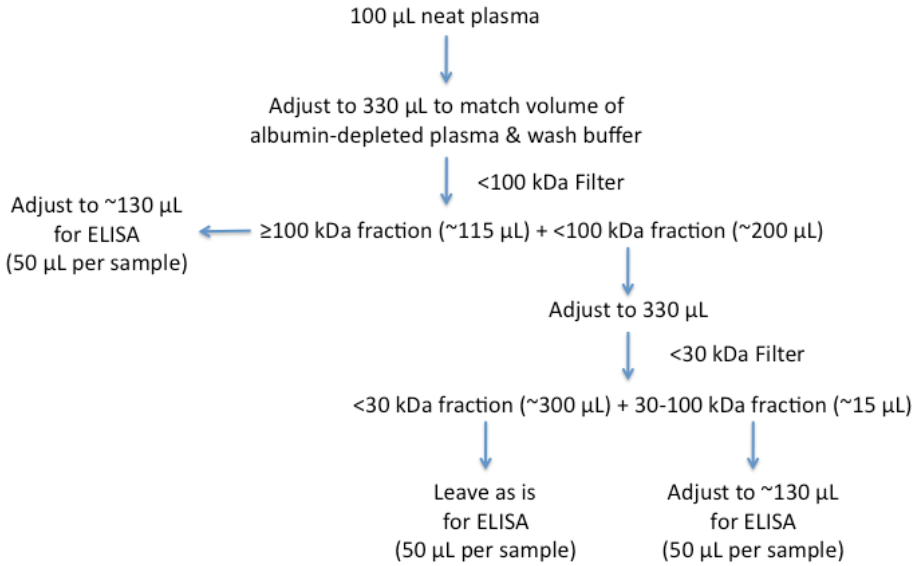


Figure 44. Protocol for molecular weight separation for plasma samples with albumin.

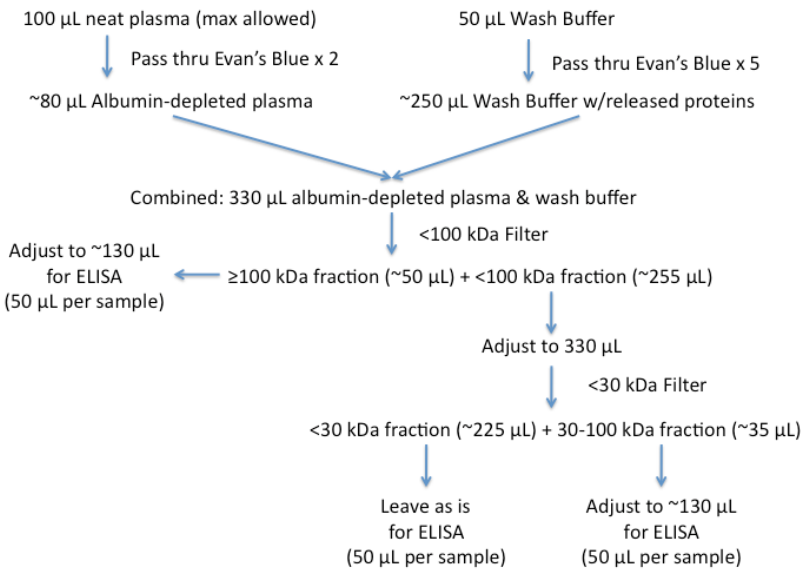


Figure 45. Protocol for albumin removal and subsequent molecular weight separation for plasma samples.

Duplicates of each sample from each time point were analyzed using standard ELISAs. GH concentration was measured using a 4.5 h solid-phase ELISA (Quantikine, R&D Systems Inc., Minneapolis, MN), and IGF-1 concentration was measured using 3.5 h solid-phase ELISA (Quantikine, R&D Systems Inc., Minneapolis, MN). Standards for GH and IGF-1 were analyzed at the same time as the samples to develop the appropriate calibration curves.

GH was assayed in neat plasma samples, as well albumin-depleted and normal samples separated by molecular weight. GH assays were possible only for three of the four subjects due to the large number of fractions to be tested using a single ELISA plate (**Figure 46**). Only neat plasma and >100 kDa fractions were observed to have

measureable quantities of GH. Subsequent to these findings, an alternative approach was planned for the final project.

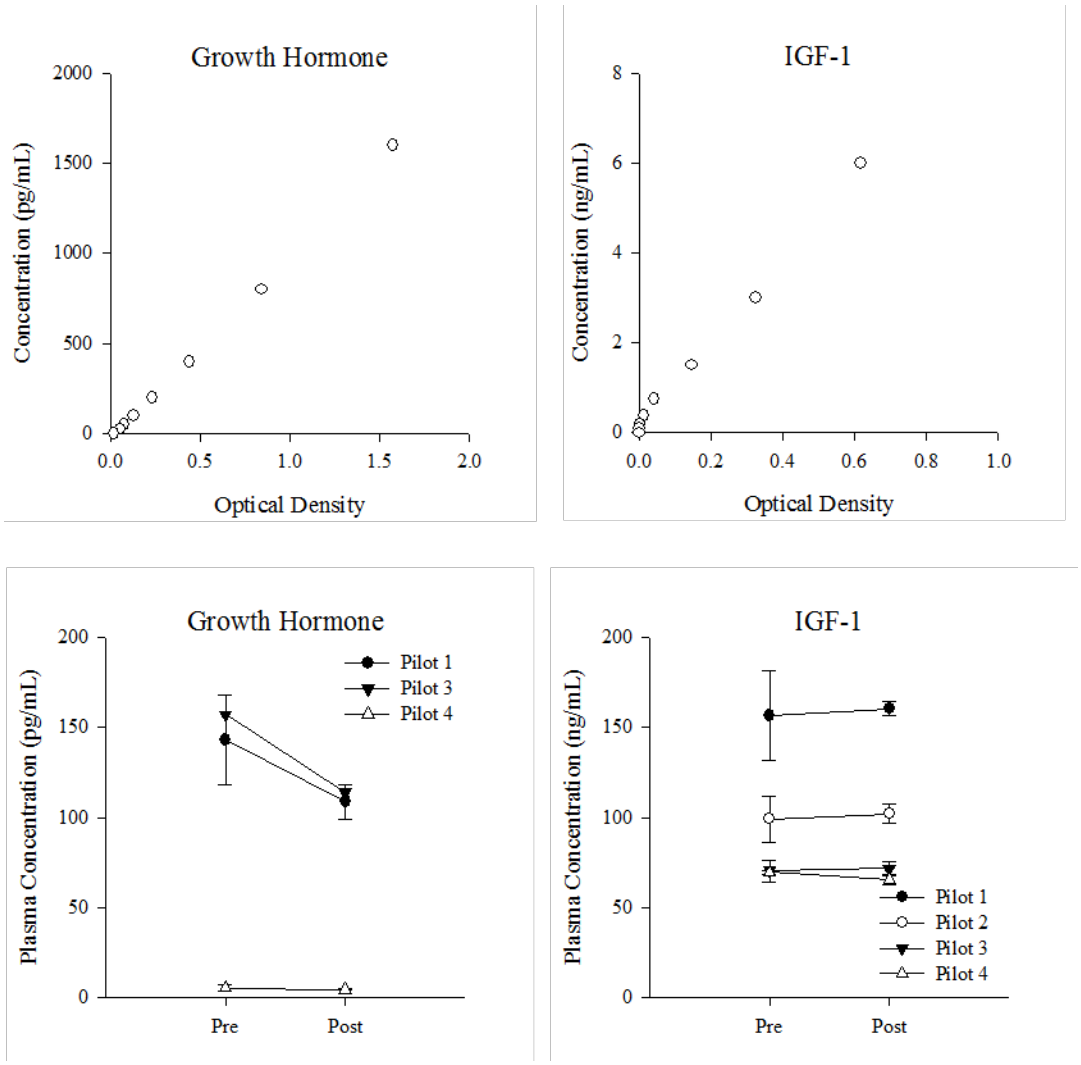


Figure 46. Top panels: Calibration curves for from ELISAs for GH and IGF-1. Bottom panels: Mean (±SD) GH (n=3, from neat plasma) and IGF-1 (n=4) levels measured before and after 10 minutes of walking place in pilot tests. These preliminary results suggest no effective of this mild exercise protocol on iGH and IGF-1 in plasma using standard ELISA protocols. No GH was detected in any other fractions after molecular weight separation, except for the >100 kDa fraction (data not shown), which followed a similar pattern as the plasma samples without molecular weight separation.

APPENDIX C: INDIVIDUAL DATA

Subject characteristics

Subject	Gender	Age (yr)	Height (cm)	Medications	Normal Exercise Habits
1	M	30	187	Daily Multivitamin	Lift, swim or cycle 2 d/wk
2	M	42	183	Multivitamin 1-3 d/wk	Run, bike, swim 3-4 d/wk
3	F	48	165	PRN: antihistamines, Protonix Daily multivitamin, oral contraceptive	Resistive exercise 2-3 d/wk; treadmill or cycle exercise 3-4 d/wk
4	M	48	188	None	Run 3-4 x/wk
5	M	27	171	Antihistamines	Aerobic exercise 2-3 d/wk
6	F	27	157	Oral contraceptive	Running or Crossfit 3-5 d/wk
7	M	34	183	Multivitamin	Resistive exercise 5-7 d/wk
8	M	28	178	No	Resistive or aerobic exercise 5-7 d/wk
9	F	23	168	Oral contraceptive	Aerobic exercise or Crossfit 5-7 d/wk
10	M	33	188	No	Aerobic exercise 5-7 d/wk
11	F	25	163	Vitamin C & B12 Supplement	Aerobic exercise 5 d/wk; Resistive exercise 3 d/wk
12	F	28	173	No	Aerobic exercise 3 d/wk
13	M	48	163	Daily Multivitamin	None

Subject characteristics by test day (Control)

Subject	Date	Weight (kg)	Health Change	Exercise in last 12 h?	Fasted	Sleep (hr)	Medications
1	4/12/14	75.9	No	No	Yes	7	No
2	5/8/14	84.5	No	No	Yes	7	No
3	4/27/14	61.4	No	No	Yes	8	Oral contraceptive
4	5/19/14	85.9	No	No	Yes	7	No
5	5/1/14	66.8	No	No	Yes	7	No
6	4/30/14	62.7	No	No	Yes	6	Oral contraceptive
7	5/14/14	90.5	No	No	Yes	7	No
8	4/11/14	85.0	No	No	Yes	6	No
9	5/29/14	71.8	No	No	Yes	8	Oral contraceptive
10	5/30/14	71.8	No	No	Yes	6	No
11	6/3/14	70.0	No	No	Yes	7	No
12	6/2/14	93.2	No	No	Yes	7	No
13	6/13/14	61.4	No	No	Yes	5.5	No

Subject characteristics by test day (Stimulation without support)

Subject	Date	Weight (kg)	Health Change	Exercise in last 12 h?	Fasted	Sleep (hr)	Medications
1	4/26/14	75.9	No	No	Yes	7	No
2	5/15/14	84.5	No	No	Yes	7	No
3	5/4/14	61.4	No	No	Yes	8	Oral contraceptive, Protonix, fiber supplement
4	5/22/14	85.9	No	No	Yes	7	None
5	4/24/14	66.8	No	No	Yes	6.5	Antihistamine
6	5/6/14	62.3	No	No	Yes	7	Oral contraceptive
7	5/19/14	92.3	No	No	Yes	6	No
8	4/22/14	85.0	No	No	Yes	6	No
9	6/12/14	70.9	No	No	Yes	7.5	Oral contraceptive
10	5/13/14	71.4	No	No	Yes	6	No
11	6/10/14	70.5	No	No	Yes	7	No
12	6/18/14	93.6	No	No	Yes	7	Anti-nausea medication
13	6/20/14	61.4	No	No	Yes	6	No

Subject characteristics by test day (Stimulation with support)

Subject	Date	Weight (kg)	Health Change	Exercise in last 12 h?	Fasted	Sleep (hr)	Medications
1	4/20/14	75.9	No	No	Yes	7	No
2	5/20/14	86.4	No	No	Yes	6	No
3	4/20/14	61.4	No	No	Yes	9	Oral contraceptive, Protonix, Advair
4	5/2/14	86.4	No	No	Yes	6	No
5	5/7/14	67.7	No	No	Yes	7	Antihistamine
6	4/23/14	62.7	No	No	Yes	6	Oral contraceptive
7	5/24/14	90.9	No	No	Yes	8	No
8	4/15/14	85.0	No	No	Yes	6	No
9	6/5/14	71.4	No	No	Yes	7.5	Oral contraceptive
10	5/20/14	71.4	No	No	Yes	6	No
11	5/27/14	70.5	No	No	Yes	8	No
12	6/11/14	93.6	No	No	Yes	7	No
13	6/6/14	61.8	No	No	Yes	6.5	No

Results of visual review of sEMG files: Response to stimulation without support, 4:30-5:00

				Number of Responses						Percent Response (%)					
Subject	Condition	Time	Reps	RTA	RLG	RSOL	LTA	LLG	LSOL	RTA	RLG	RSOL	LTA	LLG	LSOL
1	Stim	5	18	2	0	0	0	0	0	11	0	0	0	0	0
2	Stim	5	18	14	2	0	0	1	0	78	11	0	0	6	0
3	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
4	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
5	Stim	5	19	0	0	0	0	0	0	0	0	0	0	0	0
6	Stim	5	18	0	1	0	0	0	0	0	6	0	0	0	0
7	Stim	5	19	0	0	0	0	0	0	0	0	0	0	0	0
8	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
9	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
10	Stim	5	18	0	0	0	0	1	0	0	0	0	0	6	0
11	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
12	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
13	Stim	5	19	0	0	0	0	0	0	0	0	0	0	0	0
								All	Mean	7	1	0	0	1	0
									SD	22	3	0	0	2	0
								Responders	Mean	1	1	0	0	1	0
									SD	4	2	0	0	2	0
									Count	8	8	8	8	8	8
								Non-Responders	Mean	16	2	0	0	1	0
									SD	35	5	0	0	2	0
									Count	5	5	5	5	5	5

Results of visual review of sEMG files: Response to stimulation without support, 9:30-10:00

				Number of Responses						Percent Response (%)					
Subject	Condition	Time	Reps	RTA	RLG	RSOL	LTA	LLG	LSOL	RTA	RLG	RSOL	LTA	LLG	LSOL
1	Stim	10	18	4	0	0	0	0	0	22	0	0	0	0	0
2	Stim	10	18	0	4	0	1	1	0	0	22	0	6	6	0
3	Stim	10	14	0	0	0	0	0	0	0	0	0	0	0	0
4	Stim	10	18	0	0	0	0	0	0	0	0	0	0	0	0
5	Stim	10	18	0	0	0	0	0	0	0	0	0	0	0	0
6	Stim	10	18	0	1	0	0	0	0	0	6	0	0	0	0
7	Stim	10	19	1	0	0	0	1	0	5	0	0	0	5	0
8	Stim	10	19	0	0	0	0	0	0	0	0	0	0	0	0
9	Stim	10	19	0	0	0	0	0	0	0	0	0	0	0	0
10	Stim	10	18	0	0	0	0	1	2	0	0	0	0	6	11
11	Stim	10	19	0	0	0	0	0	0	0	0	0	0	0	0
12	Stim	10	19	0	0	0	0	0	0	0	0	0	0	0	0
13	Stim	10	18	0	0	0	0	0	0	0	0	0	0	0	0
								All	Mean	2	2	0	0	1	1
									SD	6	6	0	2	2	3
								Responders	Mean	3	1	0	0	1	1
									SD	8	2	0	0	3	4
										8	8	8	8	8	8
								Non-Responders	Mean	0	4	0	1	1	0
									SD	0	10	0	2	2	0
										5	5	5	5	5	5

Results of visual review of sEMG files: Response to stimulation with support, 4:30-5:00

				Number of Responses						Percent Response (%)					
Subject	Condition	Time	Reps	RTA	RLG	RSOL	LTA	LLG	LSOL	RTA	RLG	RSOL	LTA	LLG	LSOL
1	Wall	5	19	0	15	0	0	0	5	0	79	0	0	0	26
2	Wall	5	18	0	1	0	0	3	0	0	6	0	0	17	0
3	Wall	5	18	0	0	0	0	0	0	0	0	0	0	0	0
4	Wall	5	19	0	0	0	0	0	0	0	0	0	0	0	0
5	Wall	5	18	0	0	2	0	0	0	0	0	11	0	0	0
6	Wall	5	18	0	18	0	0	0	0	0	100	0	0	0	0
7	Wall	5	18	0	14	0	0	0	0	0	78	0	0	0	0
8	Wall	5	19	0	0	0	0	0	0	0	0	0	0	0	0
9	Wall	5	18	0	11	0	0	0	0	0	61	0	0	0	0
10	Wall	5	18	0	18	12	0	0	0	0	100	67	0	0	0
11	Wall	5	18	0	12	0	0	0	0	0	67	0	0	0	0
12	Wall	5	18	0	17	0	0	0	0	0	94	0	0	0	0
13	Wall	5	18	0	15	11	0	0	0	0	83	61	0	0	0
								All	Mean	0	51	11	0	1	2
									SD	0	43	24	0	5	7
								Responders	Mean	0	83	16	0	0	3
									SD	0	15	30	0	0	9
									Count	8	8	8	8	8	8
								Non-Responders	Mean	0	1	2	0	3	0
									SD	0	2	5	0	7	0
									Count	5	5	5	5	5	5

Results of visual review of sEMG files: Response to stimulation with support, 9:30-10:00

				Number of Responses						Percent Response (%)					
Subject	Condition	Time	Reps	RTA	RLG	RSOL	LTA	LLG	LSOL	RTA	RLG	RSOL	LTA	LLG	LSOL
1	Wall	10	18	0	0	0	0	0	4	0	0	0	0	0	22
2	Wall	10	19	0	0	0	1	4	0	0	0	0	5	21	0
3	Wall	10	18	0	0	0	0	0	0	0	0	0	0	0	0
4	Wall	10	18	0	0	0	0	0	0	0	0	0	0	0	0
5	Wall	10	18	0	0	0	0	0	0	0	0	0	0	0	0
6	Wall	10	18	0	12	0	0	0	0	0	67	0	0	0	0
7	Wall	10	19	0	11	0	0	0	0	0	58	0	0	0	0
8	Wall	10	17	0	0	0	0	0	0	0	0	0	0	0	0
9	Wall	10	19	0	17	6	0	0	0	0	89	32	0	0	0
10	Wall	10	18	0	18	6	0	0	0	0	100	33	0	0	0
11	Wall	10	18	0	8	0	0	0	0	0	44	0	0	0	0
12	Wall	10	19	0	19	0	0	0	0	0	100	0	0	0	0
13	Wall	10	18	2	18	12	0	0	0	11	100	67	0	0	0
								All	Mean	1	43	10	0	2	2
									SD	3	45	21	1	6	6
								Responders	Mean	1	70	16	0	0	3
									SD	4	35	25	0	0	8
									Count	8	8	8	8	8	8
								Non-Responders	Mean	0	0	0	1	4	0
									SD	0	0	0	2	9	0
									Count	5	5	5	5	5	5

Results of objective review of sEMG files: Response to stimulation without support, 4:30-5:00

				Number of Responses						Percent Response					
Subject	Condition	Time	Reps	RTA	RLG	RSOL	LTA	LLG	LSOL	RTA	RLG	RSOL	LTA	LLG	LSOL
1	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
2	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
3	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
4	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
5	Stim	5	19	0	0	0	1	0	0	0	0	0	5	0	0
6	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
7	Stim	5	19	0	0	0	0	0	0	0	0	0	0	0	0
8	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
9	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
10	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
11	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
12	Stim	5	18	0	0	0	0	0	0	0	0	0	0	0	0
13	Stim	5	19	0	0	0	0	0	0	0	0	0	0	0	0
								All	Mean	0	0	0	0	0	0
									SD	0	0	0	1	0	0
								Responders	Mean	0	0	0	0	0	0
									SD	0	0	0	0	0	0
										8	8	8	8	8	8
								Non-Responders	Mean	0	0	0	1	0	0
									SD	0	0	0	2	0	0
									Count	5	5	5	5	5	5

Results of objective review of sEMG files: Response to stimulation without support, 9:30-10:00

				Number of Responses						Percent Response					
Subject	Condition	Time	Reps	RTA	RLG	RSOL	LTA	LLG	LSOL	RTA	RLG	RSOL	LTA	LLG	LSOL
1	Stim	10	18	0	0	0	0	0	0	0	0	0	0	0	0
2	Stim	10	18	0	0	0	1	0	0	0	0	0	6	0	0
3	Stim	10	14	0	0	0	0	0	0	0	0	0	0	0	0
4	Stim	10	18	0	0	0	0	0	0	0	0	0	0	0	0
5	Stim	10	18	0	0	0	0	0	0	0	0	0	0	0	0
6	Stim	10	18	0	0	0	0	0	0	0	0	0	0	0	0
7	Stim	10	19	1	0	0	0	0	0	5	0	0	0	0	0
8	Stim	10	19	0	0	0	0	0	0	0	0	0	0	0	0
9	Stim	10	19	0	0	0	0	0	0	0	0	0	0	0	0
10	Stim	10	18	0	0	0	0	1	0	0	0	0	0	6	0
11	Stim	10	19	0	0	0	0	0	0	0	0	0	0	0	0
12	Stim	10	19	0	1	0	0	0	0	0	5	0	0	0	0
13	Stim	10	18	0	0	0	0	0	0	0	0	0	0	0	0
								All	Mean	0	0	0	0	0	0
									SD	1	1	0	2	2	0
								Responders	Mean	1	1	0	0	1	0
									SD	2	2	0	0	2	0
										8	8	8	8	8	8
								Non-Responders	Mean	0	0	0	1	0	0
									SD	0	0	0	2	0	0
										5	5	5	5	5	5

Results of objective review of sEMG files: Response to stimulation with support, 4:30-5:00

				Number of Responses						Percent Response					
Subject	Condition	Time	Reps	RTA	RLG	RSOL	LTA	LLG	LSOL	RTA	RLG	RSOL	LTA	LLG	LSOL
1	Wall	5	19	0	10	0	0	0	0	0	53	0	0	0	0
2	Wall	5	18	0	1	0	0	0	0	0	6	0	0	0	0
3	Wall	5	18	0	0	0	0	0	0	0	0	0	0	0	0
4	Wall	5	19	0	0	0	0	0	0	0	0	0	0	0	0
5	Wall	5	18	0	1	1	0	0	0	0	6	6	0	0	0
6	Wall	5	18	0	13	0	0	0	0	0	72	0	0	0	0
7	Wall	5	18	0	14	0	0	0	0	0	78	0	0	0	0
8	Wall	5	19	0	0	0	0	0	0	0	0	0	0	0	0
9	Wall	5	18	0	13	5	0	0	0	0	72	28	0	0	0
10	Wall	5	18	7	17	16	0	0	0	39	94	89	0	0	0
11	Wall	5	18	0	13	0	0	0	0	0	72	0	0	0	0
12	Wall	5	18	0	17	2	0	0	0	0	94	11	0	0	0
13	Wall	5	19	2	19	10	0	0	0	11	100	53	0	0	0
									All Mean	4	50	14	0	0	0
									SD	11	41	27	0	0	0
									Maximum	39	100	89	0	0	0
									Minimum	0	0	0	0	0	0
									Responders Mean	6	79	23	0	0	0
									SD	14	16	33	0	0	0
									Count	8	8	8	8	8	8
									Non-Responders Mean	0	2	1	0	0	0
									SD	0	3	2	0	0	0
									Count	5	5	5	5	5	5

Results of objective review of sEMG files: Response to stimulation with support, 9:30-10:00

				Number of Responses						Percent Response					
Subject	Condition	Time	Reps	RTA	RLG	RSOL	LTA	LLG	LSOL	RTA	RLG	RSOL	LTA	LLG	LSOL
1	Wall	10	18	0	4	0	0	0	0	0	22	0	0	0	0
2	Wall	10	19	0	0	0	0	0	0	0	0	0	0	0	0
3	Wall	10	18	0	0	0	0	0	0	0	0	0	0	0	0
4	Wall	10	18	0	0	0	0	0	0	0	0	0	0	0	0
5	Wall	10	18	0	0	0	0	0	0	0	0	0	0	0	0
6	Wall	10	18	1	10	0	0	0	0	6	56	0	0	0	0
7	Wall	10	19	0	7	0	0	0	0	0	37	0	0	0	0
8	Wall	10	17	0	0	0	0	0	0	0	0	0	0	0	0
9	Wall	10	19	0	17	9	0	0	0	0	89	47	0	0	0
10	Wall	10	18	0	18	14	0	1	0	0	100	78	0	6	0
11	Wall	10	18	0	8	0	0	0	0	0	44	0	0	0	0
12	Wall	10	19	4	17	3	0	0	0	21	89	16	0	0	0
13	Wall	10	18	2	18	13	0	0	0	11	100	72	0	0	0
									All	Mean	3	41	16	0	0	0
										SD	6	41	29	0	2	0
										Maximum	21	100	78	0	6	0
										Minimum	0	0	0	0	0	0
									Responders	Mean	5	67	27	0	1	0
										SD	8	31	34	0	2	0
										Count	8	8	8	8	8	8
									Non-Responders	Mean	0	0	0	0	0	0
										SD	0	0	0	0	0	0
										Count	5	5	5	5	5	5

Measured latency (sec) from onset of bladder inflation to sEMG response in stimulation without support

				RTA		RLG		RSOL		LTA		LLG		LTA	
Subject	Condition	Time	Reps	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	Stim	5	18	-	-	-	-	-	-	-	-	-	-	-	-
2	Stim	5	18	-	-	-	-	-	-	-	-	-	-	-	-
3	Stim	5	18	-	-	-	-	-	-	-	-	-	-	-	-
4	Stim	5	18	-	-	-	-	-	-	-	-	-	-	-	-
5	Stim	5	19	-	-	-	-	-	-	-	-	-	-	-	-
6	Stim	5	18	-	-	-	-	-	-	-	-	-	-	-	-
7	Stim	5	19	-	-	-	-	-	-	-	-	-	-	-	-
8	Stim	5	18	-	-	-	-	-	-	-	-	-	-	-	-
9	Stim	5	18	-	-	-	-	-	-	-	-	-	-	-	-
10	Stim	5	18	-	-	-	-	-	-	-	-	-	-	-	-
11	Stim	5	18	-	-	-	-	-	-	-	-	-	-	-	-
12	Stim	5	18	-	-	-	-	-	-	-	-	-	-	-	-
13	Stim	5	19	-	-	-	-	-	-	-	-	-	-	-	-

				RTA		RLG		RSOL		LTA		LLG		LTA	
Subject	Condition	Time	Reps	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	Stim	10	18	-	-	-	-	-	-	-	-	-	-	-	-
2	Stim	10	18	-	-	-	-	-	-	0.018	-	-	-	-	-
3	Stim	10	14	-	-	-	-	-	-	-	-	-	-	-	-
4	Stim	10	18	-	-	-	-	-	-	-	-	-	-	-	-
5	Stim	10	18	-	-	0.019	-	0.023	-	-	-	-	-	-	-
6	Stim	10	18	-	-	-	-	-	-	-	-	-	-	-	-
7	Stim	10	19	0.068	-	-	-	-	-	-	-	-	-	-	-
8	Stim	10	19	-	-	-	-	-	-	-	-	-	-	-	-
9	Stim	10	19	-	-	-	-	-	-	-	-	-	-	-	-
10	Stim	10	18	-	-	-	-	-	-	-	-	0.037	-	-	-
11	Stim	10	19	-	-	-	-	-	-	-	-	-	-	-	-
12	Stim	10	19	-	-	0.022	-	-	-	-	-	-	-	-	-
13	Stim	10	18	-	-	-	-	-	-	-	-	-	-	-	-

Measured latency (sec) from onset of bladder inflation to sEMG response in stimulation with support, 4:30-5:00

				RTA		RLG		RSOL		LTA		LLG		LTA	
Subject	Condition	Time	Reps	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	Wall	5	19	-	-	0.022	0.005	-	-	-	-	-	-	-	-
2	Wall	5	18	-	-	0.002	-	-	-	-	-	-	-	-	-
3	Wall	5	18	-	-	-	-	-	-	-	-	-	-	-	-
4	Wall	5	19	-	-	-	-	-	-	-	-	-	-	-	-
5	Wall	5	18	-	-	-	-	-	-	-	-	-	-	-	-
6	Wall	5	18	-	-	0.006	0.004	-	-	-	-	-	-	-	-
7	Wall	5	18	-	-	0.035	0.006	-	-	-	-	-	-	-	-
8	Wall	5	19	-	-	-	-	-	-	-	-	-	-	-	-
9	Wall	5	18	-	-	0.047	0.004	0.039	0.003	-	-	-	-	-	-
10	Wall	5	18	0.025	0.008	0.035	0.004	0.034	0.006	-	-	-	-	-	-
11	Wall	5	18	-	-	0.040	0.003	-	-	-	-	-	-	-	-
12	Wall	5	18	-	-	0.036	0.004	0.036	0.006	-	-	-	-	-	-
13	Wall	5	18	0.006	0.001	0.013	0.008	0.011	0.004	-	-	-	-	-	-
		Reponders	Mean	0.015		0.029		0.030							
			SD	0.014		0.014		0.013							
			Count	2		8		4							

Measured latency (sec) from onset of bladder inflation to sEMG response in stimulation with support, 9:30-10:00

				RTA		RLG		RSOL		LTA		LLG		LTA	
Subject	Condition	Time	Reps	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	Wall	10	18	-	-	0.076	0.028	-	-	-	-	-	-	-	-
2	Wall	10	19	-	-	-	-	-	-	-	-	-	-	-	-
3	Wall	10	18	-	-	-	-	-	-	-	-	-	-	-	-
4	Wall	10	18	-	-	-	-	-	-	-	-	-	-	-	-
5	Wall	10	18	-	-	-	-	-	-	-	-	-	-	-	-
6	Wall	10	18	0.000	-	0.006	0.006	-	-	-	-	-	-	-	-
7	Wall	10	19	-	-	0.040	0.014	-	-	-	-	-	-	-	-
8	Wall	10	17	-	-	-	-	-	-	-	-	-	-	-	-
9	Wall	10	19	-	-	0.039	0.005	0.033	0.004	-	-	-	-	-	-
10	Wall	10	18	-	-	0.024	0.010	0.015	0.007	-	-	-	-	-	-
11	Wall	10	18	-	-	0.040	0.004	-	-	-	-	-	-	-	-
12	Wall	10	19	0.029	0.002	0.033	0.005	0.028	0.002	-	-	-	-	-	-
13	Wall	10	18	0.017	0.001	0.018	0.005	0.019	0.006	-	-	-	-	-	-
		Reponders	Mean	0.015		0.034		0.024							
			SD	0.014		0.021		0.008							
			Count	3		8		4							

Body weight, target force, force measured at the forefoot during calibration of the bladder (bladder pressure vs. target force), and difference between the target and actual force before both stimulation conditions. All data are expressed in kg.

	Stimulation without Support				Stimulation with Support			
		Target Force	Bladder Calibration			Target Force	Bladder Calibration	
Subject	Body Mass	25% Body Weight	Force at Forefoor	Difference	Body Mass	25% Body Weight	Force at Forefoor	Difference
1	75.9	19.0	18.2	-0.8	75.9	19.0	18.2	-0.8
2	84.5	21.1	22.3	1.1	86.4	21.6	21.8	0.2
3	61.4	15.3	15.5	0.1	61.4	15.3	15.0	-0.3
4	85.9	21.5	21.4	-0.1	86.4	21.6	21.8	0.2
5	66.8	16.7	16.8	0.1	67.7	16.9	15.9	-1.0
6	62.3	15.6	15.0	-0.6	62.7	15.7	15.5	-0.2
7	92.3	23.1	23.6	0.6	90.9	22.7	22.3	-0.5
8	85.0	21.3	20.5	-0.8	85.0	21.3	21.4	0.1
9	70.9	17.7	17.7	0.0	71.4	17.8	18.2	0.3
10	71.4	17.8	16.8	-1.0	71.4	17.8	17.7	-0.1
11	70.5	17.6	18.2	0.6	70.5	17.6	16.8	-0.8
12	93.6	23.4	22.7	-0.7	93.6	23.4	24.1	0.7
13	61.4	15.3	15.9	0.6	61.8	15.5	15.5	0.0
AVG	75.5	18.9	18.8	-0.1	75.8	18.9	18.8	-0.2
SD	11.6	2.9	2.9	0.7	11.4	2.9	3.1	0.5

Mean and SD of measured total force (kg) applied to the forefoot with bladder inflation during the stimulation without and stimulation with support protocols. Total force was lower than target total force due to drift in pressure insole sensors.

	Stimulation without Support				Stimulation with Support			
	4:30-5:00		9:30-10:00		4:30-5:00		9:30-10:00	
Subject	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	10.8	0.2	7.3	0.1	15.3	0.4	11.5	0.1
2	17.3	0.2	15.8	0.1	16.5	0.1	16.5	0.1
3	12.9	0.1	13.5	0.1	11.9	0.2	11.5	0.1
4	15.9	0.3	14.1	0.1	17.8	0.1	14.3	0.1
5	11.9	0.1	10.7	0.1	11.2	0.2	9.3	0.1
6	13.2	0.1	13.3	0.1	11.1	0.2	9.9	0.1
7	15.2	0.2	12.8	0.1	18.7	0.2	16.6	0.1
8	14.9	0.2	13.2	0.1	18.2	0.1	16.9	0.3
9	12.8	0.1	12.2	0.1	17.9	0.2	15.9	0.1
10	13.2	0.1	12.9	0.1	13.1	0.2	11.0	0.3
11	12.8	0.1	12.2	0.1	16.4	0.1	15.7	0.1
12	16.5	0.1	14.2	0.2	18.0	0.1	16.2	0.1
13	11.1	0.1	10.9	0.1	12.1	0.2	11.8	0.3
Mean	13.7		12.5		15.2		13.6	
SD	2.0		2.1		2.9		2.8	

Average pressure measured with insoles before and after test protocol with subjects standing on one foot. Data were not recorded for all subjects in all conditions (cells without data are cases in which these data were not collected).

	Control				Stimulation without Support				Stimulation with Support			
	Pre		Post		Pre		Post		Pre		Post	
Subject	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD
1							72.2	1.8				
2	91.1	2.1	87.1	1.9	89.3	2.1	72.0	1.5	76.1	2.9	75.5	3.6
3					67.6	1.4	67.1	1.8				
4	84.7	2.1	81.0	2.1	85.9	2.8	82.5	3.1			74.8	3.0
5	66.8	1.8	60.5	2.0					74.2	1.2	64.1	1.4
6	70.8	2.0	60.6	1.7	64.4	2.7	60.0	1.4				
7	96.8	1.3	91.9	1.1	96.8	1.5	93.1	1.6	102.3	1.4	92.6	1.6
8												
9	74.7	1.1	68.4	0.8	71.4	0.9	64.2	0.7	56.3	1.5	52.8	2.5
10	74.7	1.4	73.5	1.6	69.5	1.9	70.6	1.7	73.5	1.8	64.2	2.0
11	73.8	1.9	66.4	1.1	72.2	2.7	72.5	1.8			62.2	0.9
12	97.0	1.9	88.2	0.9	95.0	1.6	86.3	1.3	95.1	2.2	88.1	2.1
13	62.1	1.3	53.2	1.5	67.9	1.4	66.2	1.5	64.2	1.9	56.8	1.1
AVG	79.2	1.7	73.1	1.5	78.0	1.9	73.3	1.7	77.4	1.8	70.1	2.0
SD	12.4	0.4	13.4	0.5	12.4	0.6	10.0	0.6	16.2	0.6	13.6	0.9
Count	10	10	10	10	10	10	11	11	7	7	9	9

Growth hormone concentration measured by ELISA across conditions and by responder vs. non-responder; all data

			Control		Stimulation without Support			Stimulation with Support		
Subject	Responder?	Rest	Stim	Rec	Rest	Stim	Rec	Rest	Stim	Rec
1	Y	157	175	328	418	1283	3085	440	286	269
2	N	72	61	60	242	171	100	102	91	83
3	N	1378	834	553	530	356	267	400	204	209
4	N	3899	3906	2772	2954	2489	1280	143	108	98
5	Y	61	54	58	287	975	845	22	20	36
6	Y	631	403	188	3708	3240	1646	3691	3690	3717
7	N	64	68	81	135	91	140	290	181	135
8	N	164	124	76	65	71	82	280	253	140
9	Y	2465	1600	1235	2714	2040	1336	1061	1018	1434
10	Y	68	58	79	77	109	245	125	164	180
11	Y	128	150	102	63	106	112	109	103	78
12	Y	51	40	67	771	591	347	929	572	285
13	Y	370	211	120	638	388	207	91	72	60
All	Mean	731	591	440	969	916	745	591	520	517
	SD	1185	1090	774	1267	1050	889	986	989	1028
Responders	Mean	491	336	272	1084	1092	978	808	741	758
	SD	822	524	399	1361	1085	1022	1232	1237	1281
	Count	8	8	8	8	8	8	8	8	8
Non-Responders	Mean	1115	999	709	785	636	374	243	167	133
	SD	1652	1658	1172	1226	1042	512	121	68	49
	Count	5	5	5	5	5	5	5	5	5

APPENDIX D: FORMS

UNIVERSITY of HOUSTON
A CARNEGIE-DESIGNATED TIER ONE PUBLIC RESEARCH UNIVERSITY

UNIVERSITY OF HOUSTON
CONSENT TO PARTICIPATE IN RESEARCH

PROJECT TITLE: SKELETAL MUSCLE GROWTH FACTOR RESPONSE TO CUTANEOUS STIMULATION OF THE PLANTAR SURFACE OF THE FOOT

You are being invited to take part in a research project conducted by Stuart Lee from the Department of Health and Human Performance at the University of Houston. This project is being conducted as the dissertation project of the principal investigator, Stuart Lee, under the supervision of his faculty sponsor, Dr. Mark Clarke.

NON-PARTICIPATION STATEMENT

Taking part in the research project is voluntary and you may refuse to take part or withdraw at any time without penalty or loss of benefits to which you are otherwise entitled. You may also refuse to answer any research-related questions that make you uncomfortable. *If you are a student, a decision to participate or not or to withdraw your participation will have no effect on your academic standing.*

PURPOSE OF THE STUDY

Conditions in which muscle activity is limited for long periods of time, such as bed rest and space flight (called unloading), leads to muscle size and strength decrements. As you might expect, the best way to prevent these adaptations is to perform exercise but there are cases in which exercise is not possible or is limited. Surprisingly, previous studies have shown that mild pressure applied periodically to the bottom of the feet, both in animals and humans, can protect against losses in muscle mass and strength during skeletal muscle unloading. However, the mechanism by which muscle is protected is still unclear but may be related to the release of a, growth hormone, from the anterior pituitary gland located in the base of the brain. Growth hormone is normally secreted into the bloodstream periodically throughout the day, particularly at night during sleep, but its release also can be stimulated by exercise and perhaps by stimulation of the bottom of foot. The purpose of this study is to determine whether mild foot pressure during supine rest can induce the release of growth hormone.

You are one of a maximum of 15 subjects who have volunteered to participate in this study. You will be required to attend three different testing sessions, each lasting approximately 1.5 hours. No less than 5 days will separate each of the sessions. Ideally these sessions will occur over the course of 2-3 weeks. Your total time commitment will be approximately 4.5 hours. The goal is to complete all the data collection for all subjects within 6 months from the start of the study.

PROCEDURES

This study will be conducted in the Laboratory for Integrative Physiology on the second floor of the Susanna Garrison Gymnasium at the University of Houston in the Department of Health and Human Performance. You will be one of approximately 15 subjects invited to take part in this project.

You will participate in three different testing sessions in random order. In each session, you will lie quietly on a padded table, and a catheter (a small plastic tube) will be inserted into a vein in your arm (similar to that which you might have in the doctor's office or in the hospital) from which blood samples can be taken. You will wear standard exercise shoes, which we will provide, with an air bladder that has been inserted through the side of the shoe and located under your foot. A thin insole also will be inside the shoe to measure foot pressure, particularly when the air bladder is inflated during the study. Sensors (electrodes) will be held on your skin with special double-sided tape and used to measure electrical (electromyograhic, EMG) activity in three muscles in your lower leg. Periodically, we will press a blunt metal probe (myotonometer) against two muscles in your lower leg, with no more then 4 kg (9 lb) of force to measure muscle tone.

In one session, you will lie quietly on the table and receive no foot pressure stimulation. This is called the control condition. In the other two sessions, you will receive 10 min of foot stimulation in which the air bladder will be inflated cyclically for 0.4 seconds and then deflated for 1.2 seconds. The bladder will be inflated to provide a force equal to approximately 25% of your body weight, applied to the forefoot of your right foot. You regularly apply more than 100% of your body weight to your forefoot during normal walking (~110%) and running (~250%). In one of these two conditions, the soles of your feet will rest against a wall during the session (foot support) and in the other they will not (no foot support).

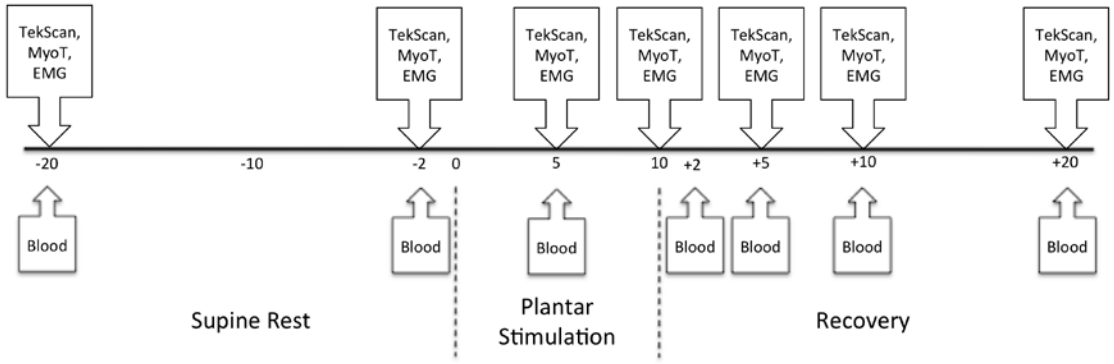
These test sessions will be scheduled in the morning. You will report to the laboratory in the fasted state, not having eaten or drank anything other than water for the previous 8 hours. Also, you will not have exercised on the day of testing before reporting to the laboratory. You will complete a brief questionnaire about your recent exercise and dietary habits prior to each testing session. If you have experienced any recent changes in your health status (since your last visit to the laboratory), please notify the investigators.

Upon arrival to the laboratory, the test operators will briefly describe the procedures to be performed during that testing session, and you will be encouraged to ask any questions before the testing proceeds. If this is the first session in which you are participating for this study, you will sign this informed consent form indicating that you understand the risks and benefits of participating in this study. Then you will put on the exercise shoes containing the air bladder and the pressure sensing soles inside the right shoe. The EMG electrodes will then be applied to your skin overlying the muscles of interest (on the front and back of the lower leg). Hair may be shaved in these areas using standard electric clippers, and the skin will be lightly abraded with a pumas stone and cleaned with alcohol. Once the test operators verify that the insoles and the EMG electrodes are working properly, you will lie comfortably on the padded table. The catheter will be inserted into the vein in your arm using standard aseptic techniques. A small amount of blood (approximately 5 ml; equivalent volume to a teaspoon) will be drawn into two separate tubes. One tube will contain a very small amount of blood (~1 ml) and will be discarded. The blood in the other tube (~4 ml) will be stored for later analysis. After this and each time that blood is drawn, a small amount of sterile saline (water with an electrolyte concentration similar to blood) will be used to flush the catheter line. This pushes blood out of the catheter so that the blood will not clot, blocking the catheter for subsequent blood samples. This is a standard procedure for use when drawing multiple blood samples with a catheter, and syringes are prefilled with saline by the manufacturer to maintain sterility. Then foot pressure will be measured using the insoles, and muscle activity will be recorded using the EMG electrodes for 30 seconds. You will not

feel anything from these measurements. Muscle tone will be measured using a device called the mytonometer. The myotonometer will be pushed three times against each of two muscles in your lower leg (one on the shin and one on the calf).

After 20 minutes of quiet rest, lying on your back, the blood draw, foot pressure, EMG activity, and muscle tone measurements will be repeated. For the next 10 minutes you either will or will not receive pressure applied to the forefoot of your right foot in the cyclical manner described above. During this 10-minute period, foot pressure, EMG activity, and muscle tone measurements will be repeated. Blood will be drawn within 2 minutes after the end of this 10-minute period.

You will continue to rest quietly on the table for 20 minutes. The blood draw, foot pressure, EMG, and muscle tone will be repeated at 5, 10, and 20 minutes during this period. The total volume of blood removed during each session will be ~35 ml. Then, the catheter will be removed, you will take off the exercise shoes, and the EMG sensors will be taken off your skin. You will be released from the testing session at this time. The diagram below describes the testing timeline.



Each of your sessions will be scheduled to occur at approximately the same time of day, you will be required to abstain from any exercise prior to the testing session, and you will be asked to consume the same diet on that day of testing. You will complete a brief questionnaire about your recent activities prior to each testing session.

CONFIDENTIALITY

Every effort will be made to maintain the confidentiality of your participation in this project. Each subject's name will be paired with a code number by the principal investigator. This code number will appear on all written materials. The list pairing the subject's name to the assigned code number will be kept separate from all research materials and will be available only to the principal investigator. Confidentiality will be maintained within legal limits.

RISKS/DISCOMFORTS

For research involving more than minimal risk, an explanation must be provided regarding whether any compensation or medical treatment is available in case of research-related injury.

1. **Blood Collection**: The risks associated with blood sampling are similar to those experienced when donating blood. These include light headiness, nausea, swelling,

edema, and/or bruising. Collection of the blood sample while you are in a seated position by a qualified technician will minimize these risks.

2. **Foot Pressure:** There is the chance that the mild pressure applied through the inflation of the air bladder will become uncomfortable over the course of 10 minutes in some subjects. Should the discomfort become unbearable, you always have the right to discontinue to the testing session.
3. **Back or Joint Discomfort:** In some subjects, you may experience discomfort from laying on your back for the moderate length of time associated with the data collection session. We will provide pillows and pads for you head and lower back to alleviate this discomfort as much as possible. Should the discomfort become unbearable, you always have the right to discontinue to the testing session.
4. **Skin Irritation:** The adhesive used to secure the sensors (EMG electrodes) to you skin on your lower leg may cause mild skin irritation in some subjects. The electrodes will be kept on your skin only for the time required to obtain measurements during the testing session, and you will be encouraged to wash your skin with soap and water as soon as possible after the session is completed.

Persons trained in cardiopulmonary resuscitation (CPR), first aid, and emergency response will be present during testing. Also, a telephone is available in the laboratory from which emergency personnel can be notified, if necessary. If you feel uncomfortable for any reason at all during the study, a researcher will be on hand to assist you.

BENEFITS

While you will not directly benefit from participation, your participation may help investigators better understand the way that the human body controls the amount of muscle mass in normal, healthy subjects and may improve the treatment of bedridden patients.

ALTERNATIVES

Participation in this project is voluntary and the only alternative to this project is non-participation.

COSTS

You are not expected to incur any costs or debits associated with your participation in this project.

INCENTIVES/REMUNERATION

No direct remuneration will be available through your participation in this study.

PUBLICATION STATEMENT

The results of this study may be published in scientific journals, professional publications, or educational presentations; however, no individual subject will be identified.

AGREEMENT FOR THE USE OF PHOTOGRAPHS AND VIDEO RECORDINGS

If you consent to take part in this study, please indicate whether you agree to be photographed and/or video recorded during the study by checking the appropriate box below. If you agree, please also indicate whether the photographs and/or video recordings can be used for publication/presentations.

_____ I agree to be photographed and/or video recorded during the study.

_____ I agree that the photographs and/or video recording(s) can be used in publication/presentations.

_____ I do not agree that the photographs and/or video recording(s) can be used in publications/presentations.

_____ I do not agree to be photographed and/or video recorded during the study.

If you decide not to participate in photography or video recording, this does not preclude you from participating in this study.

CIRCUMSTANCES FOR DISMISSAL FROM PROJECT

Your participation in this project may be terminated by the principal investigator:

- if you do not keep study appointments;
- if you do not follow the instructions you are given;
- if the principal investigator determines that staying in the project is harmful to your health or is not in your best interest
- if the study sponsor decides to stop or cancel the project

SUBJECT RIGHTS

1. I understand that informed consent is required of all persons participating in this project.

2. I have been told that I may refuse to participate or to stop my participation in this project at any time before or during the project. I may also refuse to answer any question.

3. Any risks and/or discomforts have been explained to me, as have any potential benefits.

4. I understand the protections in place to safeguard any personally identifiable information related to my participation.

5. I understand that, if I have any questions, I may contact Stuart Lee at (832) 541-5615 or slee38@uh.edu. I may also contact Dr. Mark Clarke, faculty sponsor, at (713) 743-9854 or (281) 450-3612.

6. **Any questions regarding my rights as a research subject may be addressed to the University of Houston Committee for the Protection of Human Subjects (713-743-9204). All research projects that are carried out by Investigators at the University of Houston are governed be requirements of the University and the federal government**.

SIGNATURES

I have read (or have had read to me) the contents of this consent form and have been encouraged to ask questions. I have received answers to my questions to my satisfaction. I give my consent to participate in this study, and have been provided with a copy of this form for my records and in case I have questions as the research progresses.

Study Subject (print name): _____

Signature of Study Subject: _____

Date:_____

I have read this form to the subject and/or the subject has read this form. An explanation of the research was provided and questions from the subject were solicited and answered to the subject's satisfaction. In my judgment, the subject has demonstrated comprehension of the information.

Principal Investigator (print name and title):_____

Signature of Principal Investigator: _____

Date:_____

PHYSICAL ACTIVITY READINESS QUESTIONNAIRE (PAR-Q): Modified for "Skeletal Muscle Growth Factor Response to Cutaneous Stimulation of the Plantar Surface of the Foot"

PAR-Q was designed to identify the small number of adults for whom physical activity might be inappropriate or those who should have medical advice concerning the type of activity most suitable for them. The use of the questionnaire for this study is to obtain a general statement of your overall health status. Additional questions have been added to the end of the PAR-Q which are specific to the study in which you have volunteered to participate.

Please read these questions carefully and mark the yes or no opposite the question as it applies to you

Yes☐	No☐	1) Has a physician ever said you have a heart condition and you should only do physical activity recommended by a physician?
Yes☐	No☐	2) When you do physical activity, do you feel pain in your chest?
Yes☐	No☐	3) When you were not doing physical activity, have you had chest pain in the past month?
Yes☐	No☐	4) Do you ever lose consciousness or do you lose your balance because of dizziness?
Yes☐	No☐	5) Do you have a joint or bone problem that may be made worse by a change in your physical activity?
Yes☐	No☐	6) Is a physician currently prescribing medications for your blood pressure or heart condition?
Yes☐	No☐	7) Are you pregnant?
Yes☐	No☐	8) Do you have diabetes?
Yes☐	No☐	9) Do you have any breathing difficulties or suffer from asthma?
Yes☐	No☐	10) Do you suffer from Epilepsy?
Age:____		11) How old are you?
M ☐	F ☐	12) What is your gender?
Yes☐	No☐	13) Do you exercise regularly? If yes, please specify
Yes☐	No☐	14) Are you or have you been sick or ill recently? If yes, please specify
Yes☐	No☐	15) Have you had a major operation? If yes, please specify

Yes☐	No☐	16) Do you have or have you ever had any muscle, bone, or joint injuries or illnesses? If yes, please specify
Yes☐	No☐	17) Do you have any neck, back, leg, or foot discomfort or pain from lying on your back with little or no movement for up to 1.5 hours? If yes, please specify.
Yes☐	No☐	18) Do you or have you ever suffered from any nervous system disorders, particularly one that would reduce or interfere with your sense of touch? If yes, please specify.
Yes☐	No☐	19) Do you experience any numbness or tingling in your lower body when sitting or lying for long periods of time in one position (1.5 hours)? If yes, please specify.
Yes☐	No☐	20) Do you have a fear of needles? If yes, please specify.
Yes☐	No☐	21) Do you get lightheaded, feel nauseous, or faint when someone draws your blood or you make a blood donation? If yes, please specify.
Yes☐	No☐	22) Do you experience excessive bruising when someone draws your blood or you make a blood donation? If yes, please specify.
Yes☐	No☐	23) Do you have a skin condition, particularly on your lower leg, which would make it uncomfortable or unadvisable to adhere electrodes to your skin? If yes, please specify.
Yes☐	No☐	24) Has your doctor or other medical professional advised you not to have electrodes placed on your skin? If yes, please specify.
Yes☐	No☐	25) Do you regularly take or have you taken today any medications? If yes, please specify.
		26) Describe your normal diet.
Yes☐	No☐	27) Do you regularly take any vitamins or nutritional supplements? If yes, please specify.
Yes☐	No☐	28) Do you know of any other reason why you should not participate in this study? If yes, please specify.

If you answered YES to any of the above questions, talk with your doctor by BEFORE you become more physically. If your health changes so you then answer YES to any of the above questions, seek guidance from a physician and immediately inform the investigators for this study immediately.

By submitting, you certify that you have read, understood and completed this questionnaire honestly.

Pre-Study Session Questionnaire

1) Have there been any recent changes to your health since the last time you were interviewed for this study? _____ Yes _____ No

If yes to either answer, please describe

---

2) Have you exercised today or in the last 12 hours? _____ Yes _____ No

If yes, please describe

---

3) Have you eaten or drank in the last four hours? _____ Yes _____ No

---

---

4) How many hours did you sleep last night? _____ hours

Is this a normal amount of sleep for you? _____ Yes _____ No

Do you feel well rested? _____ Yes _____ No

When did you wake up? _____ am/pm

5) Have you taken today any medications?
_____ Yes _____ No

If yes, please describe

Have you taken any vitamins or nutritional supplements today? _____ Yes _____ No

If yes, please describe