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Protein metabolism in unweighting atrophy

Muñoz, Kathryn Anne, Ph.D.
The University of Arizona, 1993
PROTEIN METABOLISM IN UNWEIGHTING ATROPHY

by

Kathryn Anne Muñoz

A Dissertation Submitted to the Faculty of the
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In the Graduate College
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1993
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Kathryn Anne Munoz entitled PROTEIN METABOLISM IN UNWEIGHTING ATROPHY and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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SIGNED: Kathryn Anne Martinez
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DEDICATION

I dedicate this dissertation to my wonderful parents Edward and Margaret Muñoz whose continuous love, support and guidance have given me the courage "to be the best I can". To my father, "I will sail my vessel till the ocean runs dry". To my mother, your love and laughter will always be with me. Thank you both for all the sacrifices you made to give me the opportunity to further my education. I Love you both.
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ABSTRACT

The weightless environment results in atrophy of the anti-gravity muscles. Hindlimb suspension is a model for weightlessness induced atrophy. This study evaluated the effects of hindlimb suspension, microgravity and exercise training followed by suspension on skeletal muscle. Soleus mass, myofibrillar and sarcoplasmic protein content were measured in one to four day hindlimb suspended animals. Protein synthesis was measured by intramuscular injection of $^3$H phenylalanine with correction for the difference between tRNA and intracellular specific activities. Myofibrillar protein loss was minimal after two days of unweighting but significant after three days. Although sarcoplasmic protein content showed no change, synthesis of both protein pools declined in parallel. Myofibrillar degradation increased during the first three days of unweighting, partially accounting for protein loss. The decline in degradation during day four explained the slower rate of protein loss at this time. Sarcoplasmic protein degradation increased slightly during the first two days of unweighting then declined sharply, thus explaining the sparing of sarcoplasmic proteins.

Animals exposed to weightlessness showed soleus atrophy similar to suspended animals. The plantaris and gastrocnemius had reduced growth while the extensor digitorum longus and tibialis anterior grew normally in flight and suspended animals. Insulin stimulated glucose uptake was enhanced in soleus, but not extensor digitorum longus of flight and suspended animals. In situ insulin and IGF-1 stimulated 2-deoxyglucose uptake was greater after six days of suspension.

Voluntary wheel training increased soleus mass, protein content and in
vivo protein synthesis which plateaued by three weeks. Suspended or trained-suspended animals showed reductions in soleus mass, protein content and synthesis compared to trained animals. However, trained-suspended animals showed higher values for protein content and synthesis compared to suspended animals.

In conclusion, these studies show that unweighting atrophy is characterized by decreased synthesis and increased degradation of myofibrillar proteins, and a sparing of sarcoplasmic proteins due to slower degradation. Tailcast hindlimb suspension may be used as a ground based model to mimic the effects of weightlessness on muscle proteins. Wheel training causes muscle hypertrophy; and although training prior to suspension provides some protection against protein loss, it not does prevent atrophy.
Manned exploration of space by the National Aeronautics and Space Administration has provided information about the weightless environment. Prior to the 1980's, the main biological emphasis of the flight program was to identify and prevent physiological changes which occur from exposure to microgravity. As technology has advanced from the initial step of sending man into space, to providing the opportunity to walk on the moon, so has our understanding of biological alterations which occur from exposure to weightlessness. With the development of the Shuttle Program, the opportunity to study these effects has increased and expanded to include a search for answers to health and medical problems on Earth as well. To continue the quest for knowledge through longer space flight and the permanent manned space station, it will be necessary to evaluate the physiological and biochemical alterations which occur from exposure to microgravity.

Biological alterations due to weightlessness include physical deconditioning of the cardiovascular and musculoskeletal systems. In the muscular system, the primary alteration which occurs is significant muscle atrophy of the lower limbs which are used for weight bearing activity in the presence of gravity.
Physiology of Normal Muscle

Mammalian skeletal muscle is composed of three distinct fiber types which are classified according to their oxidative and contractile properties. Slow-oxidative fibers (type I) are recruited for slow repetitive motion while fast contracting oxidative fibers (type IIa) and fast glycolytic (type IIb) fibers are adapted for quick movement (Saltin and Gollnick, 1982). Although most muscle groups contain fibers of each type, one fiber type usually predominates (Ariano et al., 1973). The rat soleus muscle is considered a postural, weight-bearing muscle and contains mainly type I fibers with some type IIa fibers, while the extensor digitorum longus, a non weight-bearing muscle is made up of type IIa and type IIb fibers. Although each muscle group contains a distinct pattern of fiber types, switching of fiber types may occur in response to immobilization, stretch, electrical stimulation or unweighting (Bouissou et al., 1985; Goldspink et al., 1991; Jaspers and Tischler, 1984; Ohira et al., 1992; Steffen and Musacchia, 1984; Taguchi et al., 1991).

Skeletal muscle fibers can undergo a variety of biochemical and physiological changes in response to disease or altered work load. In response to increased workload, muscle fibers undergo rapid growth or hypertrophy at which time there is an increase in fiber diameter, muscle mass and protein content (Goldberg et al., 1975; Goldspink et al., 1985). Muscle atrophy may occur from reduced-use as in bed rest, immobilization, unweighting or from denervation (Goldspink, 1976; Jaspers and Tischler, 1984; Simard et al., 1982). Although skeletal muscle responds readily to various stimuli, the biochemical and physiological processes which occur within the muscle are complex. Therefore, the pathways and mechanisms through which muscle alterations
occur vary depending on the model used (i.e. denervation versus unweighting). The determining factor for the muscle hypertrophic or atrophic response is the balance of rates of protein synthesis and degradation.

**Protein Metabolism in Normal Skeletal Muscle**

Skeletal muscle protein turnover is a cyclical process which is balanced through rates of protein synthesis and degradation. Rates of muscle protein accretion or loss are determined from protein synthesis and degradation rates (Millward et al., 1976) These rates are under strict metabolic control (Kettelhut et al., 1988).

Several proteolytic systems are involved in the degradation of proteins which occurs via enzymes contained either in the cytoplasm or lysosome (Goldberg and Dice, 1974). These systems include the lysosomal system, the secretory degradative system, the nonlysosomal system (cytosolic), the ATP dependent system and the calcium dependent system (Mayer and Dogerty, 1968). Increased lysosomal proteolysis is primarily responsible for increased degradation in mammals (Dice, 1987). Since the muscle cell contains large amounts of calcium, calcium dependent proteases (i.e. Calpains I and II) are important in muscle atrophy (Demartino and Croall, 1984). Myofibrillar protein degradation occurs through activation of the cytosolic system (Hare, 1988; Lowell et al., 1986a) whereas it is generally accepted that sarcoplasmic proteins are degraded through the lysosomal pathway (Mayer and Dogerty, 1968).

Skeletal muscle protein turnover is regulated hormonally (Goldberg et al., 1980) and possibly through regulatory amino acids (May and Buse, 1989). Hormonal regulation is primarily due to the activity of insulin, IGF-I, thyroid
hormone, glucocorticoids, and glucagon (Sugden and Fuller, 1991).

The role of insulin in regulating protein balance has been assessed under normal conditions (Goldberg and St John, 1976) and under metabolic disturbances such as diabetes (Fagan and Tischler, 1986). In vitro, physiological insulin concentrations have been shown to increase protein synthesis and inhibit protein degradation thereby regulating protein balance (Fulks et al., 1975; Fuller and Sugden, 1986). The importance of insulin in the regulation of protein balance can be observed in metabolic diseases such as diabetes where in the absence of insulin, muscle wasting occurs (Preedy and Garlick, 1986). The mechanism by which protein synthesis is increased by insulin appears to be at the level of translation (Kimball and Jefferson, 1988), whereas the mechanism of inhibition of protein degradation has not been clearly established (Sugden and Fuller, 1991).

The thyroid hormone, triodothyronine (T3) has also been shown to regulate protein turnover by increasing rates of protein synthesis through a pretranslational mechanism (Brown and Millward, 1983). However, differing concentrations of T3 show opposing effects on protein metabolism. At physiological levels, T3 promotes protein synthesis (Griffen and Goldberg, 1977), but in excessive amounts may lead to muscle wasting by increasing protein degradation (Griffen and Goldberg, 1977).

The glucocorticoids have been shown to have profound effects on protein metabolism. In contrast to insulin, the glucocorticoids promote the breakdown of muscle protein in the fasted state (Goldberg et al., 1980) and though still debatable in the fed state (Odedra et al., 1983). Glucocorticoids have been shown to inhibit protein and DNA synthesis (Goldberg et al., 1980) and specifically to
inhibit myofibrillar protein synthesis (Odedra, et al., 1983). The effects of glucocorticoids on protein degradation remain unclear as some investigations have shown increased degradation rates while others have shown either a decrease (Mcgrath and Goldspink, 1982) or no change in protein degradation (Rannels and Jefferson, 1980).

In addition to hormonal regulation, protein turnover may be regulated through the amino acids leucine and glutamine (May and Buse, 1989). Leucine has been shown to stimulate protein synthesis, but the mechanism remains unclear (Tischler et al., 1982). In vitro, glutamine has been shown to increase protein synthesis and inhibit degradation (Wu and Thompson, 1990). However, this effect has not been demonstrated in vivo (Wu and Thompson, 1990). Although some investigations have shown the ability of these amino acids to stimulate protein synthesis and inhibit degradation, the regulation by these amino acids remains unclear and debatable.

**Protein Metabolism in Unweighted Skeletal Muscle**

Many models have been used to study protein metabolism in reduced use. These models include denervation, immobilization and unweighting. In unweighting, muscles are free to move within a full range of motion while the hindlimbs are lifted off of the floor of the cage so they are unable to bear weight. The forelimbs may be used for locomotion and grooming. Several suspension models have been developed to accomplish this non weight bearing state. The model used in this laboratory is the tail cast suspension model developed by Jaspers and Tischler (1984). This model is a modification of the tail cast suspension model which was first developed by Morey-Holton (Morey-Holten
and Wronski, 1979) to study bone metabolism.

Tail cast or harness suspension results in preferential atrophy of the slow twitch, weight-bearing or posture supporting muscles in the hindlimb of the rat (Musacchia et al., 1983; Thomason et al., 1987a). These "antigravity" muscles include the soleus and to a lesser extent the plantaris and gastrocnemius (Jaspers and Tischler, 1984). The non-weight bearing anterior muscles, the extensor digitorum longus and tibialis anterior, do not undergo atrophy using this model (Jaspers and Tischler, 1984; Musacchia et al., 1983). Although other models of disuse (denervation and immobilization) have been used to study protein metabolism, this discussion will be limited to alterations in protein metabolism due to unweighting.

In rats which have been hindlimb suspended, soleus muscle mass and total protein content decline significantly within three days of unweighting (Goldspink et al., 1986; Jaspers et al., 1985; Jaspers and Tischler, 1984; Jaspers and Tischler, 1986; Loughna et al., 1986). Protein loss in the soleus has been reported to range from 26% by day three (Hauschka et al., 1987) to 40% by 14 days (Steffen and Musacchia, 1984). Although protein content decreases in the soleus, the protein concentration is unchanged after three to seven days of unweighting (Babij and Booth, 1988; Herbert et al., 1988; Jaspers and Tischler, 1986; Steffen and Musacchia, 1984).

In vivo and in vitro studies of protein metabolism have shown decreased total protein synthesis and faster degradation in unweighted soleus. After six days of unweighting the soleus of male rats show a 36% to 38% decline in protein synthesis when measured in vitro and a 26% decrease when measured in vivo (Jaspers et al., 1988). In addition, protein degradation increases by 55% at 4-6
days of unweighting. Decreased in vivo total mixed protein synthesis (19-21\%) and faster degradation are also observed in harness suspended young male rats (Goldspink et al., 1986). In vivo protein synthesis in young male harness suspended rats decreases by 21\% over 7 days of unweighting, while degradation increases by 100\% (Loughna et al., 1986). In adult female rats, there is a very rapid decline in total and myofibril protein synthesis after only 5 hours of suspension (Thomason et al., 1989). This rate continues to decline to about 50\% of its original value by day 7 of unweighting. Following the initial decline in protein synthesis there is an estimated slow, transiently increasing myofibril degradation which reaches a steady state after 24 days of unweighting. The changes in myofibrillar protein metabolism have also been demonstrated by other investigators (Thomason et al., 1987b).

Some studies have assessed the role of myofibrillar proteins in muscle atrophy from unweighting. After six days of unweighting, myofibrillar protein content in soleus muscle decreases approximately 20\%, while sarcoplasmic proteins showes no significant loss (Jaspers et al., 1988). After 28 and 42 days of unweighting, 80\% (Thomason et al., 1987a) and 63\% (Vailes et al., 1988) of myofibrillar proteins were lost in the soleus muscle. In addition, after 5 weeks of unweighting there is a 6\% decrease in volume density of myofibrils (Desplanches et al., 1990a). The preferential loss of myofibrillar protein leads to an increase in the ratio of sarcoplastic to myofibrillar proteins in soleus muscle. However, in non weight bearing muscle such as the tibialis or EDL, the ratio of proteins is not altered (Jaspers et al., 1988). Similar to findings in adult male rats (Thomason et al., 1989), myofibrillar protein synthesis is decreased by 59\% in suspended adult female rats (Thomason et al., 1989). In young, growing rats, in
In vitro myofibrillar synthesis has been shown to decrease by 7% (Jaspers and Tischler, 1984). In addition, enhanced myofibrillar protein degradation is evident in unweighted soleus of adult female rats (Thomason et al., 1989; Thomason et al., 1987b). Investigations conducted thus far indicate that prolonged myofibril protein loss is primarily due to decreased synthesis (Thomason et al., 1989). However, acute losses within the first six days of unweighting may be due to an increase in myofibrillar protein degradation (Jaspers et al., 1985; Jaspers and Tischler, 1984; Jaspers and Tischler, 1986).

The differing rates of total mixed or myofibrillar protein synthesis and degradation as discussed in the above sections may be due to the method used (in vivo versus in vitro), the age of the animal, and the type of suspension model used. It appears that synthesis rates are not as responsive to decline in young rats as they appear to be in adult rats. For example, at 3 and 5 days of unweighting, there is a 21% and 19% decrease respectively in protein synthesis rates of young rats (Goldspink et al., 1986; Loughna et al., 1986), but in adult female rats, protein synthesis rates decline by 46%. This discrepancy may be due to the effects of muscle growth on unweighting atrophy. The method of suspension may also affect protein metabolism. The influence of corticosterone on proteolysis in harness suspended animals may contribute to differences in rates of protein breakdown because this steroid is elevated in harness suspended animals. Although protein synthesis and degradation rates differ depending on experimental conditions, there is a consensus that protein degradation rates increase as synthesis rates decline in the first few days of unweighting.
Exercise Countermeasures to Unweighting Muscle Atrophy

There are several proposed countermeasures to prevent cardiovascular and musculoskeletal deconditioning which occurs from spaceflight. These protocols include electrical stimulation (Duvoisin et al., 1989), artificial gravity (Burton, 1989), pharmacological intervention, (Nicogossian, 1989) lower body negative pressure (Gruell et al., 1990) and exercise training (Convertino, 1990). These countermeasures are designed to prevent or diminish cardiovascular deconditioning and orthostatic alterations which occur during flight and are of limited value in preventing or attenuating muscular deconditioning.

Exercise training has been the most frequently used countermeasure to prevent the deleterious effects of weightlessness on muscle atrophy. Exercise regimens have been used by astronauts and cosmonauts in an attempt to prevent the detrimental effects of space travel on muscle mass (Kozlovskaya et al., 1981; Thorton and Rummel, 1974). In flight treadmill exercise and cycle ergometry have been utilized, but have not proven effective in preventing muscle atrophy from weightlessness (Convertino, 1990). Of the protocols tested, resistance training appears to be the most adequate in preventing muscle atrophy. However, these regimens require long time periods of up to two and one half hours per day to obtain any benefit. In addition, these regimens have been relatively unsuccessful as the atrophic response is only partially attenuated. In addition, different exercise protocols produce varying effects on muscle atrophy (Thorton and Rumme, 1974). This may be partially due to the nature of the exercise as most are designed to increase aerobic conditioning as opposed to muscular strength.

In hindlimb suspended animals, periodic high load exercise training has
been shown to attenuate the effects of unloading on the soleus muscle (Herbert et al., 1988). In addition, treadmill training consisting of 40 minutes per day during the suspension period ameliorated, but did not prevent muscle atrophy in seven day hindlimb suspended animals (Hauschka et al., 1988) Treadmill training after suspension appears to allow for faster muscle recovery as muscle weight and fiber cross sectional area are greater in trained versus sedentary muscle (Kasper et al., 1990) thereby offering some potential benefit. In addition, ground support and uphill running during long-term suspension (4 to 8 weeks) spares both myofibril and myosin protein when administered during the atrophic phase (Thomason et al., 1987a).

Taken together, these findings provide some much needed information concerning the role of exercise in the prevention of unweighting muscle atrophy. However, knowledge to date concerning the effectiveness of exercise as a countermeasure to unweighting atrophy remains very limited and an appropriate regimen for prevention of muscle atrophy has yet to be defined.

**Measurement of in Vivo Protein Metabolism**

Investigation of the role of protein synthesis and degradation in the regulation of muscle mass requires accurate, reliable techniques. Many techniques have been developed to measure in vivo rates of protein synthesis. These methods are usually based on the use of radiolabeled amino acids which are incorporated into proteins during synthesis thereby serving as tracers to measure amino acid incorporation into a protein (or a group of proteins) and it’s subsequent release (Hasselgren et al., 1988). Measurement of tRNA specific activity is considered to be the most reliable method because tRNA serves as the
immediate amino acid pool for protein synthesis (Sameral, 1991). However, this technique is technically difficult because tRNA concentration is low and rapidly turned over (Hasselgren et al., 1988). Therefore, large amounts of radioactivity and a large number of animals are needed to obtain accurate results. In addition, compartmentalization of the tRNA pool may occur between mitochondrial and cytosolic pools within the cell thereby leading to inaccurate results (Fern and Garlick, 1974). Because of the difficulties associated with the measurement of aminoacyl tRNA, alternative methods have been developed to approximate protein synthetic rates. The most frequently used methods include intravenous constant infusion and intraperitoneal injection of radiolabeled amino acid (Sameral, 1991). Constant infusion is a reliable method for measuring in vivo rates of synthesis. However, this method also requires a large dose of radioactivity in order to flood each amino acid pool. Therefore, this method was modified to entail intraperitoneal injection of radiolabeled amino acid along with a large dose of non radiolabeled amino acid (Garlick et al., 1980). Intraperitoneal injection of a flooding dose of amino acid allows for rapid equilibration of amino acid precursor pools. This allows the specific radioactivity of these pools to be approximately equivalent to the plasma specific radioactivity and therefore provides an accurate estimation of protein synthetic rates (Garlick et al., 1980).

Rates of protein degradation may be assessed using in vivo techniques or by indirect measurement. In vivo measurements are made by following tissue loss of radiolabeled protein (Hasselgren et al., 1988). However, due to recycling of proteins, degradation rates may be underestimated (Sameral, 1991). Indirect measurement involves estimation of the degradation rate by measuring protein synthesis and then using this measurement to calculate degradation (Jepson et
al., 1986). In the steady state, the rate of degradation is equal to the rate of synthesis. However, during muscle hypertrophy or atrophy these rates are not equal and therefore the rate of growth (or atrophy) of the muscle must be taken into account. During muscle hypertrophy or atrophy, the degradation rate is equal to the synthesis rate minus the growth (or atrophy) rate. Although there are inherent problems associated with indirect measurement of degradation rates, this method had been used effectively to measure protein metabolism (Garlick et al., 1980; Jepson et al., 1986).

**Glucose Metabolism in Normal Skeletal Muscle: Hormonal Regulation**

Although the major functions of skeletal muscle are support and locomotion, this metabolically active tissue is also responsible for maintenance of whole body glucose homeostasis (DeFronzo et al., 1983). The regulation of glucose uptake by skeletal muscle is influenced by muscle contraction and hormonal stimulation by insulin and catecholamines. Regulation of skeletal muscle glucose metabolism is a complex process involving glucose uptake, utilization and deposition as glycogen. The focus of this discussion will be limited to the uptake of glucose and its regulation.

Glucose is transported across the cell membrane by a carrier-mediated, facilitated diffusion mechanism (Baly and Horuk, 1988; James et al., 1985). After transport across the cell membrane, glucose is phosphorylated by hexokinase. It may then be stored as glycogen or used for cell energy. The protein responsible for transport is the glucose transporter protein. This transport protein is located intracellularly where it appears to be inactive and at the plasma membrane where it is involved in the transport of glucose (Wantabe et al., 1984). The
glucose transport protein is found in skeletal muscle (Klip and Marette, 1992) and adipocytes as well as a variety of other tissues (Pessin et al., 1982). Glucose transport may be regulated by altering the number of glucose transporters or by changing the intrinsic activity of the transporter (Klip and Marette, 1992). Insulin has been shown to regulate glucose transport in skeletal muscle by increasing the number and activity of transport proteins in the plasma membrane ((Sternlicht et al., 1988).

Contractile activity of muscle also affects glucose uptake independently of insulin (Wallberg-Henrikson and Holloszy, 1984). The mechanism by which glucose uptake is enhanced is similar to that of insulin stimulation; i.e. activation of the glucose transport system by increasing both the number and activity of these proteins (Fushiki et al., 1989). However, the pathways of activation of insulin stimulated versus contractile activity for glucose transport may differ (Wallberg-Henrikson et al., 1988).

**Glucose Metabolism in Unweighted Skeletal Muscle**

Changes in carbohydrate metabolism from unweighting atrophy include enhanced glucose uptake, increased glycogen deposition, and increased insulin sensitivity (Bonen et al., 1988; Henriksen and Tischler, 1988b; Henriksen et al., 1986). An increase in the response of glucose uptake in the presence of insulin and an increase in the binding capacity of insulin have been demonstrated in unweighted muscle. Henriksen and Tischler (Henriksen and Tischler, 1988b) studied in vitro glucose uptake in unweighted soleus and extensor digitorum longus muscle in the presence and absence of insulin. In the presence of insulin, the uptake of 2-deoxyglucose did not change after 24 hours of unweighting.
(Henriksen and Tischler, 1988b). In contrast, insulin stimulated glucose uptake was greatly enhanced after three (Henriksen and Tischler, 1988b) six (Henriksen et al., 1986) or 28 days of unweighting (Bonen et al., 1988). Extensor digitorum longus muscle of unweighted hindlimbs is unresponsive to unweighting atrophy and does not demonstrate increased glucose uptake (Bonen et al., 1988; Henriksen and Tischler, 1988b; Henriksen et al., 1986). In addition to enhanced glucose uptake, stimulation of glucose oxidation, incorporation into glycogen and glycolysis are also enhanced after three, six (Henriksen and Tischler, 1988b; Henriksen et al., 1986) and twenty eight days of unweighting (Bonen et al., 1988). It is suggested that enhancement of the response to insulin in unweighting atrophy is due to an increase in insulin sensitivity (Bonen et al., 1988; Henriksen and Tischler, 1988b; Henriksen et al., 1986).

One possible explanation for increased glucose uptake is enhanced insulin binding capacity. In fact, this has been shown after six (Henriksen et al., 1986) and 28 days of unweighting (Bonen et al., 1988). The increased binding capacity is due to an increase in the concentration of insulin receptors as the number of receptors does not decrease as the muscle atrophies.

Relationship Between Protein and Carbohydrate Metabolism in Unweighted Skeletal Muscle

Previous studies in this laboratory have evaluated the effects of unweighting on protein and carbohydrate metabolism. Evidence accumulated thus far has lead to the development of a model to describe the relationship between protein and carbohydrate metabolism in unweighting and denervation
atrophy. Although the model in it's entirety is shown in Fig. 1, this discussion will be limited to the responses of unweighted, atrophic muscle. In brief, studies showed that the insulin and β-adrenergic receptors do not undergo increased proteolysis in unweighting atrophy. Sparing of these membrane proteins may be attributed to suppression of the lysosomal protein degradation system since membrane proteins are degraded via the lysosome. These studies provide indirect evidence that sarcoplasmic protein are spared in unweighting atrophy. In addition they demonstrate a possible relationship between protein and carbohydrate metabolism in unweighting atrophy.

As previously described, skeletal muscle atrophy from unweighting has been attributed to protein loss through decreased protein synthesis and accelerated degradation (Jaspers et al., 1988; Jaspers and Tischler, 1984; Thomason et al., 1989; Tischler et al., 1990). In addition, a large portion of the total protein loss is due to a selective loss of myofibrillar proteins (Jaspers et al., 1989; Jaspers et al., 1988; Thomason et al., 1987b), with little or no sarcoplasmic protein loss. Since membrane proteins constitute a portion of the sarcoplasmic protein pool, it is hypothesized that membrane proteins such as the insulin receptor are spared during unweighting atrophy.

Sparing of membrane proteins during unweighting was first demonstrated in studies involving the insulin receptor (Henriksen et al., 1986). The sparing was characterized by an increased insulin binding capacity with insulin receptor number remaining constant (Bonen et al., 1988; Henriksen et al., 1986). The time at which insulin binding capacity increases, is commensurate
Fig 1. Proposed model of protein breakdown in muscle atrophy.
with the time course during which there is a significant amount of muscle atrophy, suggesting a possible relationship.

Further lines of evidence for a sparing of sarcoplasmic proteins were obtained recently from studies of the β adrenergic system (Kirby and Tischler, 1990) An increased response to a β agonist, isoproterenol was observed in soleus muscles which were unweighted for three to six days. This response to unweighting included increased glycogen breakdown, decreased glycogen formation, and an increased production of cyclic AMP. The response of the β adrenergic system was similar to the insulin receptor in that there was an increased receptor binding capacity with no loss of receptor number. This finding provides additional indirect evidence and reinforces the hypothesis that sarcoplasmic proteins are spared during unweighting.

The role of lysosomal and cytosolic proteolytic systems in protein degradation from unweighting was studied using various proteolytic inhibitors. Incubation with inhibitors of lysosomal (chloroquine, methylamine and leucine methyl ester) and cytosolic (mersalyl) proteolysis were used to evaluate pathways of proteolysis in muscle atrophy from unweighting (Tischler et al., 1990). The lysosomal inhibitors did not decrease in vitro proteolysis in unweighted muscle, while mersalyl abolished the difference in degradation between control and unweighted muscle. In addition, incubation with TMB-8, a calcium antagonist, caused attenuation of the difference in protein degradation between weight bearing and unweighted muscle. Proteolysis was further enhanced in unweighted soleus muscle when the calcium ionophore, A23187, was added to increase intracellular calcium. The increase in proteolysis demonstrated the role of cytosolic and possibly calcium dependent proteolysis in
To demonstrate physiological significance, some of these inhibitors were evaluated in vivo, by intramuscular injection. Chloroquine showed no effect while mersalyl not only prevented the loss of protein, but allowed for slight accretion. These changes in protein content were attributed to alterations in protein degradation.

These studies indicate that calcium dependent-cytosolic proteolysis is responsible for the increase in degradation observed in unweighted muscle, while contribution of the lysosomal process is minimal. This finding may explain the sparing of membrane proteins such as the insulin and β adrenergic receptors during unweighting atrophy since these proteins are degraded via a lysosomal mechanism.

Taken together the results of these various studies provide indirect evidence that sarcoplasmic proteins are spared during unweighting atrophy. However, direct evidence of this process remains limited and the time course during which this process occurs remains unclear.

Specific Aims of the Project

As discussed above, previous studies have indicated that sarcoplasmic proteins may be spared during unweighting atrophy, while myofibrillar proteins are degraded. However, there have been no studies which have evaluated the time course of acute alterations which occur in myofibrillar and sarcoplasmic proteins from unweighting atrophy. In addition, while spaceflight experiments have been conducted to evaluate the effects of weightlessness on muscle metabolism and the use of ground based models to study muscle metabolism,
these studies have concentrated on adult rather than juvenile animals. Lastly, although there is some evidence that exercise may attenuate muscle atrophy during unweighting, there are no studies which have evaluated the effect of exercise on protein metabolism in animals which are hindlimb suspended. Therefore, the goals of this study were:

1. To analyze the time course of alterations which occur in myofibrillar and sarcoplasmic proteins during unweighting atrophy.
2. To compare muscle mass, muscle protein content and insulin stimulated glucose transport in animals which were exposed to microgravity or hindlimb suspension.
3. To evaluate the effects of exercise on muscle mass, protein content and total protein synthesis animals which were endurance trained on a running wheel and then hindlimb suspended.
CHAPTER TWO

IN SITU MEASUREMENT OF PROTEIN METABOLISM:
METHODOLOGICAL APPROACH

Abstract

Intraperitoneal injection of a flooding dose of $^3$H phenylalanine (40 uCi/100 g body wt) can be used to measure protein synthesis. To avoid using a high dose and to allow for measurement of the small sarcoplasmic protein pool, an intramuscular injection technique was developed. To assess $^3$H phenylalanine distribution within the soluble compartments, injected muscles were trisected. The mid portion and ends contained 51% and 49% of the total protein, but 67% and 33% of $^3$H phenylalanine respectively indicating inadequate diffusion of the label to the ends of the muscle. Therefore, the middle portion of the muscle must be used. Since use of the appropriate specific activity value is critical for estimating protein synthesis, specific activities were compared for acyl-tRNA, intracellular, and total (intra plus extracellular) phenylalanine. Intracellular specific activity was measured after washing muscles with cycloleucine, an amino acid transport inhibitor, to trap phenylalanine within the cytoplasm. tRNA specific activity was 24% of the total specific activity compared to 50% of the total for the intracellular fraction. After normalization to total specific activity, the tRNA specific activity was 49.6% of the intracellular value. To account for the difference between intracellular and tRNA specific activity, a factor of 0.48 is applied to better estimate actual synthesis rates.
Introduction

Because of the role of protein turnover in muscle metabolism, it is important to develop accurate techniques to measure protein synthesis. Many methods for measuring in vivo protein synthesis have been reported. These methods typically utilize constant infusion of radiolabeled amino acid or intraperitoneal injection with a flooding dose of radiolabeled and non radiolabeled amino acid. When measuring protein synthetic rates, it is important to choose the appropriate amino acid compartment (i.e. extracellular, intracellular or tRNA) to assess the specific activity of the labeled precursor amino acid (Garlick et al., 1980). Since tRNA - bound amino acids serve as precursors for synthesis of protein, this compartment is the most appropriate for measuring the synthetic rate (Sameral, 1991). However, the tRNA pool is small in comparison to the other protein pools. Therefore, it is difficult to obtain accurate measurements. Although useful, intraperitoneal injection with a flooding dose and tRNA measurement require large amounts of radioactivity and a large number of animals to obtain accurate measurements of myofibrillar and sarcoplasmic protein synthetic rates. Therefore, the purpose of this study was to develop an intramuscular injection flooding dose technique to estimate in situ rates of myofibrillar and sarcoplasmic protein synthesis. This method utilizes fewer animals and less radiolabeled amino acid and thus facilitates measurement of the relatively small pool of sarcoplasmic proteins. This chapter describes the methods used to develop an intramuscular injection technique for measuring protein synthesis.
Methods

Treatment of Animals

Female Sprague Dawley rats (Sasco Sprague Dawley, Omaha, NE) were provided with food and water ad libitum and maintained under standard housing conditions. Animals were killed by cervical dislocation. All procedures described here and in subsequent chapters were approved by the University of Arizona Animal Care and Use Committee. During all potentially stressful procedures animals were tranquilized by forelimb muscle injection of 0.1 ml/100g body weight of 10% (v/v) Innovar-vet (4 ug sublimaze, 200 ug Inaspine; Pitmann-Moore, Washington Crossing, NJ) or pentobarbital sodium (50 mg/kg body weight).

Methodological Approach For Intramuscular (IM) Injection

The measurement of fractional synthesis rates of myofibrillar and sarcoplasmic proteins was problematic mainly due to the small size of the sarcoplasmic protein pool. Therefore, the flooding dose method was modified to entail IM rather than intraperitoneal (IP) injection. IM injection has been previously used in our laboratory to test in situ effects of protease inhibitors, and hormones (Tischler et al., 1990). The flooding dose technique is based on the IP method of Garlick (1980), while IM injection, is adapted from Gerard et al. (1977). as described previously (Tischler et al., 1990).

For IM injection, a 5 mm incision was made on the outer side of the shaved hindlimb of anesthetized animals. After the underlying fascia was cut,
the soleus muscle was gently exposed by hooking it with fine curved tip forceps. The belly of the muscle was injected using a hamilton syringe. The injection solution consisted of L-[sidechain-\(^3\)H] phenylalanine (50 mM; approximately 4 \(\mu\)Ci) contained in 150 mM NaCl. The volume injected depended on muscle size; 4 ul/100 g body weight for control, one or two day unweighted muscle, and 3.5 ul/100 g body weight for three or four day unweighted muscles. Average muscle sizes were obtained from studies in which only protein content was analyzed.

Distribution of \(^3\)H Phenylalanine

The distribution of radioactivity between the middle and ends of the muscle was analyzed since there was concern that the radioactive phenylalanine injected into the belly of the muscle might not diffuse rapidly to the far ends. To assess \(^3\)H distribution, both soleus muscles of six animals were injected with a flooding dose of \(^3\)H phenylalanine as described above, then excised after 15 minutes. The ends were separated from the middle and the data for the two muscles were averaged. The two ends and middle piece were homogenized separately in 1.5 ml of 5\% w/v trichloroacetic acid (TCA) and centrifuged at 2800 \(\times\) g for 15 minutes. The pellets were washed once with 1 ml of 5\% TCA, then solubilized in 2 ml 1 N NaOH. A 0.5 ml aliquot of bound protein was used for scintillation counting and 1 ml utilized for protein analysis using the Biuret procedure with bovine serum albumin as the standard (Gornall et al., 1949).

Measurement of tRNA Specific Activity

The ability to use a flooding dose method by direct IM injection provided
an opportunity to compare relative rates of total, intracellular, and aminoacyl tRNA specific activity. The three values could not be compared directly using the same muscle because of processing procedures. Instead, the specific activities were compared as a fraction of the total (intracellular and extracellular) specific activity of each group of muscles.

To determine tRNA specific activity, soleus muscles of 32 female rats (100-120g, Harlan Sprague Dawley, Indianapolis, IN) were injected with a flooding dose of 0.8 uCi of 50 mM ^3H phenylalanine. After seven minutes, both soleus muscles were excised, clamp frozen in liquid nitrogen and weighed. Muscles were divided into 5 groups of approximately 111 mg total mass per group. The specific activity of aminoacyl-tRNA was measured according to previously described methods (Airhart et al., 1979; Everett et al., 1981) with modification limited to substitution of l-[sidechain ^3H] phenylalanine for l-[^3H] leucine as described below. For RNA extraction, muscles were pulverized in liquid nitrogen then transferred to 2.5 ml of homogenizing buffer containing 5% methanol, 0.17% 2,5-napthelenedisulfide, 2 mM EDTA, 10 mM magnesium acetate 0.5% SDS, 200 mM sodium acetate, pH 4.0, and 2 ml water saturated phenol. After homogenization, samples were centrifuged at 2°C at 8000 x g. 0.8 ml phenol, 5.1% Sevag was added and the samples vortexed every 5 minutes for 30 minutes then recentrifuged as described above. RNA was precipitated overnight at 20°C in 7 ml of potassium acetate (1.2% w/v) in ethanol then centrifuged as above. Twenty ul of supernatant was added to 3 ml of scintillation cocktail for counting. The remaining RNA pellet was dissolved in 1.6 ml of 100 mM sodium acetate/100 mM magnesium acetate and 4 ml of 120 mM potassium acetate in ethanol was added and allowed to precipitate for 2
hours. The above precipitation process was repeated, then samples were dissolved in 1.6 ml sterile water. To precipitate tRNA's, 3 ml of 100% ETOH was added and allowed to stand overnight at 20°C. Samples were centrifuged as above, and .02 ml was removed for counting. The pellet was dried under nitrogen (g) then dissolved in 1 ml 50 mM NaCO3 and incubated for 90 minutes at 37°C to deacylate the amino acids. In order to precipitate remaining tRNA, 2 ml of 100% ETOH was added to the above solution and centrifuged as above. Samples were dried under nitrogen gas, then dansylated by addition of 50 mM NaCO3 to pH 10.5 and an equal volume of 0.1 DPM/pmole 14C dansyl chloride. Samples were heated for 30 minutes at 37°C and then 20 ul of ethylacetate was added. Samples were subsequently dried in a speed vac and further processed on 15 cm x 15 cm polyamide sheets (Cheng Chin Trading Co.) for two dimensional chromatography. Polyamide sheets were placed in tanks containing water/formic acid (100:3 v/v) for the first dimension and benzene acetate (9:1 v/v) for the second dimension. Dansylphenylalanine was identified by comparison with standards under ultraviolet light. The dansylated-phenylalanine spot was cut out of the polyacrylamide sheet and then placed in 10 ml of 4.57% NCS (Amersham Corp) and 4% liquiflour (New England Nuclear) in toluene for determination of radioactive phenylalanine. The specific activity of 3H phenylalanine in the tRNA and the total fractions were determined from the known specific activity of the 14C dansyl chloride and 3H:14C ratio of dansyl-phenylalanine.

Measurement of Total and Intracellular Specific Activities

To compare total and intracellular specific activities, muscles were injected
as described above then excised after 15 minutes. Total specific activity was measured in the muscle from one leg by immediately freezing the muscle in liquid nitrogen and processing as described below. To determine intracellular specific activity, the muscles were washed to remove residual interstitial phenylalanine. Muscles were washed twice for 10 minutes in 3 ml of 20 or 100 mM cycloleucine (Sigma Chemical Co., St. Louis MO) in 0.84% NaCl plus 5 mM cycloheximide (Boehringer Mannheim, Indianapolis, IN.) (see results). To prevent leakage of intracellular phenylalanine, cycloleucine which is transported on the L-system in muscle (Maroni et al., 1987) was added to the wash. Cycloheximide was added to inhibit protein synthesis (Fulks et al., 1975). Intracellular free and total specific activities were measured (Garlick et al., 1980) with modification in volumes used as described below. After excision (total specific activity) or washing in cycloleucine (intracellular), frozen muscles were homogenized in 1.5 ml of ice cold 2% w/v perchloric acid (PCA) then centrifuged for 5 minutes at 2800 x g followed by 10,000 x g for 20 min. The protein pellet was washed twice in 1 ml of ice cold 2% PCA and the supernatants pooled. A 0.75 ml aliquot of the intracellular fraction was added to 5 ml aqueous scintillation counting fluid (ACS, Arlington Heights, IL) for radioactive counting.

**Results**

**Intramuscular Injection Technique**

Intramuscular injection of 3H phenylalanine was used to facilitate the measurement of in situ synthesis of separate sarcoplasmic and myofibrillar proteins (described in Chapter 3). Since there was concern that radioactive phenylalanine injected into the belly of the muscle might not diffuse rapidly to
the far ends, the distribution of radioactivity between the middle (approximately 50%) and the two ends of the muscle was determined. Protein analysis showed that 48% of the protein was contained in the middle portion, but 67% of the radioactivity was present indicating inadequate distribution of radioactivity to the ends of the muscle (Table 1). Consequently, for all IM measurements of protein synthesis, only the middle protein of the muscle was used.

Intracellular phenylalanine specific activity was analyzed in order to assess protein synthesis rates using the IM injection technique. To do so, it was necessary to rid the muscle of its high level of interstitial radioactive phenylalanine. As described in Methods, this was accomplished by washing muscles in cycloleucine. Concentrations of 20 mM or 100 mM cycloleucine were compared. Both concentrations were equally effective in trapping the phenylalanine within the cell but the higher concentration interfered with the 8-phenethylamine assay. Therefore, 20 mM cycloleucine was used as part of the wash solution to measure in situ rates of protein synthesis. Two washes with this solution were deemed sufficient since the second wash contained barely detectable amounts of radiolabeled phenylalanine.

The use of the flooding dose by IM injection provided the opportunity to compare the relative specific activities for intracellular and aminoacyl-tRNA. The values were not compared in the same muscles because of processing procedures. Instead, the specific activities were compared as a fraction of the total (intracellular and extracellular) specific activity of each group of muscles. As shown in Table 2, the aminoacyl-tRNA specific activity was 24% of the total specific activity. When intracellular specific activity was determined by washing muscles in 20 mM cycloleucine, the intracellular specific activity was 50% of the
total specific activity indicating that there was not equilibration between the amino acid pools. After normalizing the aminoacyl tRNA specific activity to the total specific activity, it was shown that the tRNA specific activity was approximately 48% of the intracellular value (0.24/0.50). Therefore, this correction factor was used in subsequent measurements of protein synthesis following IM injection to more closely approximate the fractional rate of synthesis.
Table 1. Percent distribution of $^3$H phenylalanine after intramuscular injection.

<table>
<thead>
<tr>
<th>Muscle Section</th>
<th>Protein</th>
<th>DPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ends</td>
<td>51</td>
<td>33</td>
</tr>
<tr>
<td>Mid</td>
<td>48</td>
<td>67</td>
</tr>
</tbody>
</table>

Data represent average percentages from soleus muscles of 6 animals.
Table 2. Comparision of total, intracellular and tRNA specific activities after intramuscular injection of $^3$H phenylalanine.

<table>
<thead>
<tr>
<th></th>
<th>tRNA</th>
<th>Intracellular</th>
<th>Total</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1</td>
<td>$2.48 \pm 0.25$</td>
<td>------</td>
<td>$10.29 \pm 0.83$</td>
<td>4.2</td>
</tr>
<tr>
<td>Expt 2</td>
<td>------</td>
<td>$1.48 \pm 0.09$</td>
<td>$2.94 \pm 0.28$</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Intracellular/tRNA 2.1

Data are mean ± SEM. Expt 1 involved measurement of tRNA and total specific activities. Expt 2 represents the measurement of intracellular specific activity from muscles which were washed in 20 mM cycloleucine as described in Methods.
Discussion

Relationship Between tRNA, Total and Intracellular Specific Activities

To measure in situ rates of protein synthesis in the myofibrillar and sarcoplasmic protein fractions an intramuscular injection procedure using a flooding dose of $^3$H phenylalanine was developed. Earlier studies which have measured in vivo rates of myofibrillar protein synthesis have used tRNA specific activity to assess rates of protein synthesis. Arguably, it is more correct to use the tRNA specific activity because it serves as the immediate precursor pool for protein synthesis (Thomason et al., 1989). However, several problems hinder this measurement in all experiments. First, there is limited labeling of the tRNA pool. This potentially introduces large errors in measurement of specific activities. Although, when utilizing IM rather than IP injection, the extent of protein labeling was increased, even this method produced low labeling of phenylalanyl-tRNA. Secondly, to obtain a sufficient amount of aminoacyl-tRNA (the dansylation yield is generally only about 15-20%) requires an unreasonably large number of animals in light of the extensive time course which was to be conducted. In addition, for the purposes of this dissertation, it is the qualitative pattern of changes in protein synthesis which is more critical than the absolute rates of synthesis. Furthermore, as it would not be possible to measure aminoacyl-tRNA specific activity for the time course of changes which occur from muscle unweighting, this correction at least provided a better estimate. The development of this technique for measuring in situ protein synthesis allowed the measurement of myofibrillar and sarcoplasmic proteins in unweighted soleus muscle. This method facilitated labeling of the sarcoplasmic proteins without drastically increasing the number of animals used.
In conclusion, intramuscular injection of $^3$H phenylalanine into the belly of the soleus muscles may be used to evaluate protein synthesis rates. However, due to inadequate distribution of the radioactivity, the mid section of the muscle must be used. In addition, a correction factor of $0.48$ must be applied to the intracellular specific activity to better estimate protein synthesis.
CHAPTER THREE

TIME COURSE OF THE RESPONSE OF IN SITU MYOFIBRILLAR AND SARCOPLASMIC PROTEIN METABOLISM TO UNWEIGHTING ATROPHY

Abstract

The time course of changes in myofibrillar and sarcoplasmic protein metabolism following one to four days of unweighting was investigated. Protein synthesis was measured by intramuscular injection of a flooding dose of $^3$H phenylalanine. Myofibrillar protein loss, which showed only a small decrease after two days of suspension, was greatest during day three, then diminished thereafter. In contrast, the sarcoplasmic protein pool showed no change in protein content during four days of unweighting. Beginning at 24 hours, protein synthesis rates decreased in parallel for myofibrillar and sarcoplasmic proteins (-50%). Myofibrillar protein degradation increased steadily during the first three days of unweighting thereby partially accounting for the loss of protein. However, myofibrillar degradation decreased below control thereafter. This explains the onset of slower loss in this protein pool after day three. Sarcoplasmic protein degradation increased slightly during the first two days of unweighting followed by a sharp decline. This decline in sarcoplasmic protein degradation coincided with the sparing of sarcoplasmic proteins during unweighting atrophy. These data suggest that 1) the acute loss of protein during unweighting is a consequence of decreased synthesis and increased degradation of myofibrillar proteins and 2) sarcoplasmic proteins are spared due to their
slower degradation.
Introduction

Unweighting of the soleus muscle from hindlimb suspension or exposure to microgravity causes muscle atrophy and a significant loss of protein (Jaspers and Tischler, 1984; Musacchia et al., 1983). In vivo and in vitro evaluations of protein metabolism in unweighted soleus have shown that atrophy is apparently due to a combination of decreased total protein synthesis and increased degradation (Goldspink et al., 1986; Jaspers et al., 1988; Jaspers and Tischler, 1984). However, these studies evaluated the role of total protein metabolism at three and six days after unloading thereby providing information concerning protein metabolism at a single time point. A six day time course of changes in total protein metabolism revealed that protein synthesis declined steadily while degradation increased over six days of unweighting (Satarug, 1987). During days five and six, synthesis and degradation did not undergo further changes. This study provided information concerning the time course of changes in total protein metabolism during unweighting. However, other studies suggested that myofibrillar proteins are preferentially lost during unweighting while sarcoplasmic proteins may be spared (Jaspers and Tischler, 1986; Jaspers and Tischler, 1984; Thomason et al., 1987a).

The purpose of this study was to evaluate the contributions of myofibrillar and sarcoplasmic protein metabolism to the change in total protein metabolism over four days of unweighting. This study was restricted to four days since there are no apparent changes during days five and six (Satarug, 1987). This investigation was accomplished by analyzing daily muscle protein content and in situ fractional rates of protein synthesis which were then used to calculate
degradation of the protein pools.

Methods

Treatment of Animals

Female Sprague Dawley rats (105-135 g) were provided with food and water ad libitum and maintained under standard housing conditions. Animals were killed by cervical dislocation. Body weights of animals were taken prior to tail casting and at the time of killing. Animals were tail-casted and suspended in a head-down position for one to four days (Jaspers and Tischler, 1984). Tail casts consisted of Hexcelite orthopedic tape (Kirschner Medical, Timonium, MD) and medical grade elastomer (Factor II, Lakeside, AZ).

Protein Metabolism

Myofibrillar and Sarcoplasmic Protein Content

Because of the additional processing required for separating the myofibrillar and sarcoplasmic protein pool, it was difficult to assess the protein content of these fractions in the same muscles used for analyzing protein synthesis. Therefore, separate groups of animals were used. Separation of myofibrillar and sarcoplasmic proteins was based on a published method (Perry, 1974). After excision, muscles were immediately weighed then homogenized in 3 ml of ice cold 10 mM Tris-HCl buffer, pH 7.4 containing 5 mM cycloheximide (Boehringer-Mannheim, Indianapolis, IN). Cycloheximide was added because in future experiments it would be used to terminate protein synthesis after muscle excision (Fulks et al., 1975). Muscle homogenates were
centrifuged at 2800 x g for 5 minutes and then at 10,000 x g for 20 minutes at 4° C. The myofibrillar protein pellet was washed three times with 1 ml of the Tris-HCl buffer and the supernatant solutions, which contained the sarcoplasmic protein fraction, were pooled (6 ml final volume). Sarcoplasmic proteins were precipitated by addition of 1 ml of ice-cold 100% w/v TCA to the pooled supernatant solutions followed by centrifugation as described above. Both protein pellets were washed with 2 ml of ethanol:ether (1:1 v/v) then solubilized in 2 ml of 1 N NaOH. Protein content was determined using the Lowry method with bovine serum albumin as the standard (Lowry et al., 1951).

Estimation of Protein Accretion or Loss

The fractional rates of growth (protein accretion) or atrophy (protein loss) were based on the change in protein content for each period of unweighting or weight-bearing. For each muscle, the percent change in protein was calculated as the difference between the measured value at the end of each period and the estimated value at the start of each period (initiation of suspension). Initial protein content for weight-bearing animals was estimated using regression analysis of soleus protein content at time zero versus body weight (Fig. 2, myofibrillar; and Fig. 3, sarcoplasmic). Therefore, initial body weights were used to estimate protein content on day zero. The fractional growth rate (percent/day) for each successive 24 hour period was calculated as the difference in the percent change for the appropriate periods of time. For example, the growth rate for day two, which began at 24 hours and ended at 48 hours after suspension, was calculated as the difference in the percent change between 48 hours and 24 hours of unweighting. Protein values were normalized to the
average initial body weight of all animals in an experiment, and are presented as the percent change in protein content which is corrected for body weight differences.

Estimation of Protein Synthesis and Degradation

Fractional rates (percent/day) of in situ protein synthesis were assessed by using a flooding dose of L-[side chain-\(^{3}\)H] phenylalanine (ICN, Costa Mesa, Ca). The fractional rate of degradation for suspended animals was calculated as the difference between the fractional rate of synthesis and the fractional rate of protein accretion for a 24 hour period. The final data for fractional growth (or atrophy), synthesis, and degradation are approximations for each 24 hour period.

Fractional Rates of Protein Synthesis

In situ fractional protein synthesis rates were measured by IM injection of phenylalanine as described in Chapter Two. Fifteen minutes after injection, muscles were excised and washed twice for 10 minutes in 3 ml of 20 mM cycloleucine in 0.85% NaCl and 5 mM cycloheximide in 0.85% NaCl. Specific activities of the bound (myofibrillar and sarcoplasmic) and free intracellular fractions were measured (Garlick et al., 1980) with modification limited to volumes used. Muscles were immediately frozen in liquid nitrogen then homogenized in 2 ml of ice-cold 10 mM tris buffer pH 7.4 and 5 mM cycloheximide. Homogenized muscles were centrifuged for 5 minutes at 2800 x g then at 10,000 x g for 20 minutes. After removal of the supernatant, the myofibrillar protein pellet was washed four times with 10 mM Tris buffer, and the supernatants pooled. Sarcoplasmic proteins were precipitated by addition of
1 ml of 8% TCA to the pooled supernatants. This pellet was washed once with 2 ml of 2% PCA and centrifuged for 5 minutes at 2800 x g and then 15 minutes at 10,000 x g. To 2 ml supernatant was added 1 ml saturated tripotassium citrate for neutralization. To remove potassium perchlorate, supernatants were centrifuged for 15 minutes at 2800 x g then processed for β-phenethylamine extraction, as described below. An additional 0.5 ml was removed for scintillation counting in Ecolume. Protein fractions were hydrolyzed in 6N HCl for 24 hours at 110° C, evaporated to dryness, then resuspended in 2 ml of 0.5 M sodium citrate, pH 6.3. Determination of 3H phenylalanine specific activity for the supernatants and pellets involved the enzymatic conversion of phenylalanine to β-phenethylamine. A 1 ml aliquot of hydrolyzed protein or supernatant was incubated for 12 hours at 50° C in 0.5 ml of 1-tyrosine decarboxylase suspended in 0.5 M sodium citrate (pH 6.3, 1.4 units/ml for hydrolysate and 0.7 units/ml for supernatant). After conversion, 1 ml of 3 N NaOH was added to each sample and β-phenethylamine was extracted with 10 ml chloroform : heptane (1:3 v/v). After separation, 4 ml of 0.01 M H2SO4 and 5 ml of chloroform were added to the organic layer and the aqueous phase was collected.

A 1 ml aliquot of extracted supernatant or 0.02 ml aliquot of extracted hydrolysate was fluorometrically assayed for β-phenethylamine using 20 mM β-phenethylamine (Sigma Chemical Co.) in 0.01 M H2SO4 as the standard (2 to 20 umol). Samples and standards were incubated in a dimly lit room with 0.5 ml of 2 mM leucylalanine, 1ml of 5 mM ninhydrin and 2.5 ml of 1 M potassium phosphate, pH 8.0. After incubation at 60° C for 60 minutes, samples were cooled in ice for 15 minutes then warmed to room temperature for 2 minutes. Fluorescence was measured at 495 nM (emission), 390 nM (excitation) on a
Perkin-Elmer LS-5B fluorescent spectrophotometer.

Fractional rates of protein synthesis were calculated as \( \frac{S_b}{S_i \times 0.48 \times t} \) where \( S_b \) is the protein bound specific activity, \( S_i \) is the intracellular specific activity, 0.48 is the correction factor for aminoacyl-tRNA specific activity (see Chapter Two) and \( t \) is the time in days between injection and muscle excision.

Statistical Analysis

Fractional rates of protein degradation for each 24 hour period were based on calculations from average measured values of protein content and protein synthesis at the beginning and end of the 24 hour period. Because of this, standard errors of measurement can not be determined and the data are analyzed qualitatively. Data for daily changes in protein content and fractional rates of protein synthesis were analyzed using one way analysis of variance. Data are presented as mean ± SEM.

Results

Body weight and Muscle Mass

Initial body weights of weight bearing and hindlimb suspended animals were similar so that changes in protein content over 4 days of unweighting could be estimated. Initial body weights for weight bearing animals in each experiment ranged from 115 ± 2 g to 121 ± 1 g while initial body weights in suspended animals ranged from 120 ± 2 g to 122 ± 1 g (Table 3).

Normalized muscle mass in weight bearing animals increased (P<0.05) from 38.3 mg/100g body weight on day zero to 42.1 mg/100g body weight by day three of weight bearing (Table 4). In contrast, unweighted soleus muscle
showed a relative loss of mass declining by 23% over four days of unweighting (P<0.05). This loss was significant by day two of unweighting and it continued to decline (P<0.05) through day three.

Protein Content

The relative contributions of myofibrillar and sarcoplasmic proteins to the loss of total protein during unweighting atrophy were assessed. Myofibrillar protein content remained constant in weight bearing animals, but significantly decreased by 19% over four days of unweighting (Table 5). Myofibrillar protein content was significantly lower by three days of unweighting (P<0.05). In addition, between day two and day three there was a significant decrease in myofibrillar protein content of unweighted soleus (P<0.05).

In contrast to the loss of myofibrillar proteins, there was no change in sarcoplasmic protein content during the four day unweighting period (Table 6). Myofibrillar and sarcoplasmic protein concentration (mg protein/mg muscle mass) increased slightly over four days of unweighting (Table 7, myofibrillar; Table 8, sarcoplasmic). Although myofibrillar protein concentration increased, the ratio of myofibrillar to sarcoplasmic proteins declined by 18% over four days of unweighting (Table 9). This decrease indicates a greater loss of myofibrillar proteins relative to sarcoplasmic proteins during unweighting, particularly between days two and three. The myofibrillar to sarcoplasmic protein ratio was constant in weight bearing muscle.
Table 3. Initial body weights of control and hindlimb suspended animals.

<table>
<thead>
<tr>
<th>Duration of study (days)</th>
<th>Initial Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight bearing</td>
</tr>
<tr>
<td>0</td>
<td>120 ± 1</td>
</tr>
<tr>
<td>1</td>
<td>115 ± 2</td>
</tr>
<tr>
<td>2</td>
<td>118 ± 2</td>
</tr>
<tr>
<td>3</td>
<td>122 ± 1</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are mean ± SEM for 10 to 35 animals. ND represents not determined in this experiment.
Table 4. Normalized mass of weight bearing and unweighted soleus muscle.

<table>
<thead>
<tr>
<th>Duration of study (days)</th>
<th>Muscle Weight (mg/100 g body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight bearing</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

Data are mean ± SEM for 10 to 35 animals. Muscle mass is normalized to average final body weights at the end of each period. ND represents not determined in this experiment. * P<0.05 versus day 0; ‡ P<0.05 versus unweighted day two.
Table 5. Myofibrillar protein content in weight bearing and unweighted soleus muscle.

<table>
<thead>
<tr>
<th>Duration of study (days)</th>
<th>Myofibrillar Protein Content (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight bearing</td>
</tr>
<tr>
<td>0</td>
<td>3.62 ± .05</td>
</tr>
<tr>
<td>1</td>
<td>3.69 ± .09</td>
</tr>
<tr>
<td>2</td>
<td>3.74 ± .07</td>
</tr>
<tr>
<td>3</td>
<td>4.01 ± .04</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are mean ± SEM for 10 to 35 animals. ND represents not determined in this experiment. * P<0.05 versus control; ‡ P<0.05 versus two days of unweighting.
Table 6. Sarcoplasmic protein content in weight bearing and unweighted soleus muscle.

<table>
<thead>
<tr>
<th>Duration of study (days)</th>
<th>Sarcoplasmic Protein Content (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Weight bearing</strong></td>
</tr>
<tr>
<td>0</td>
<td>1.92 ± .02</td>
</tr>
<tr>
<td>1</td>
<td>1.94 ± .04</td>
</tr>
<tr>
<td>2</td>
<td>2.01 ± .03</td>
</tr>
<tr>
<td>3</td>
<td>2.12 ± .03</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are mean ± SEM for 10 to 35 animals. ND represents not determined in this experiment. * P<0.05 versus day zero; ‡ P<0.05 versus two days of unweighting.
Table 7. Myofibrillar protein concentration in weight bearing and unweighted soleus muscle.

<table>
<thead>
<tr>
<th>Duration of Study (days)</th>
<th>Myofibrillar Protein (mg protein/mg muscle mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Weight bearing</strong></td>
</tr>
<tr>
<td>0</td>
<td>7.81 ± .07</td>
</tr>
<tr>
<td>1</td>
<td>7.92 ± .04</td>
</tr>
<tr>
<td>2</td>
<td>7.64 ± .18</td>
</tr>
<tr>
<td>3</td>
<td>7.91 ± .35</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are mean ± SEM for 10 to 35 animals. ND represents not determined in this experiment. * P<0.05 versus day zero.
Table 8. Sarcoplasmic protein concentration in control and unweighted soleus muscle.

<table>
<thead>
<tr>
<th>Duration of Study (days)</th>
<th>Sarcoplasmic Protein (mg protein/mg muscle mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight bearing</td>
</tr>
<tr>
<td>0</td>
<td>4.23 ± .03</td>
</tr>
<tr>
<td>1</td>
<td>4.29 ± .03</td>
</tr>
<tr>
<td>2</td>
<td>4.11 ± .06</td>
</tr>
<tr>
<td>3</td>
<td>4.07 ± .16</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are mean ± SEM for 10 to 35 animals. ND represents not determined in this experiment. * P<0.05 versus day zero.
Table 9. Ratio of myofibrillar to sarcoplasmic proteins in weight bearing and unweighted soleus muscle.

<table>
<thead>
<tr>
<th>Duration of Study (days)</th>
<th>Ratio (myofibrillar/sarcoplasmic)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight bearing</td>
</tr>
<tr>
<td>0</td>
<td>1.86 ± .04</td>
</tr>
<tr>
<td>1</td>
<td>1.88 ± .06</td>
</tr>
<tr>
<td>2</td>
<td>1.85 ± .05</td>
</tr>
<tr>
<td>3</td>
<td>1.90 ± .04</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are mean ± SEM for 10 to 35 animals. ND represents not determined in this experiment. * P<0.05 versus control; ‡ P<0.05 versus two days of unweighting.
Rates of Change of Myofibrillar and Sarcoplasmic Proteins

Protein content on day zero of hindlimb suspension was estimated for each muscle by using linear regression analysis of initial body weight and protein content of separate groups of animals. A regression equation was obtained by measuring initial body weight and protein content (see methods). The regression lines and equations for the myofibrillar and sarcoplasmic proteins are shown in figure 2 and 3 respectively. Using these data, the cumulative changes in protein content for one to four days of unweighting period could be calculated. Daily changes were then estimated as described in Methods.

Protein accumulation in weight bearing animals averaged 4.4 ± 0.4 percent per day for myofibrillar proteins and 4.9 ± 0.5 percent per day for sarcoplasmic proteins. The time course of cumulative changes in protein content for myofibrillar and sarcoplasmic proteins following one to four days of unweighting is shown in Table 10. Following the first day of unweighting there was neither accretion nor loss of myofibrillar proteins (P<0.05). By the end of day two of unweighting there was a small but significant loss of myofibrillar proteins. After three days of unweighting there was a considerable cumulative loss (-16.5%) of myofibrillar protein, as well as a significant amount of muscle atrophy (Table 3). These data indicate that myofibrillar protein turnover in juvenile animals is most responsive between two and three days unweighting.

In contrast to the pattern of loss observed for myofibrillar proteins, the sarcoplasmic protein pool showed no significant change at any time during four days of unweighting (Table 10). Therefore, during the time in which significant muscle atrophy and myofibrillar protein loss occurs, sarcoplasmic proteins show no response.
Fig. 2. Linear regression of myofibrillar protein content and body weight. Data represent 53 weight-bearing animals. The regression equation myofibrillar protein = (.048 x body weight) - 2.15 \( r^2 = .81 \)
Fig. 3. Linear regression of sarcoplastic protein content and body weight. Data represent 53 animals. The regression equation was sarcoplastic protein = (.013 x body weight) + .32 \( r^2 = .70 \)
Table 10. Effect of unweighting on cumulative changes in myofibrillar and sarcoplasmic protein content.

<table>
<thead>
<tr>
<th>Duration of Unweighting (days)</th>
<th>Cumulative Change in Protein Content (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Myofibrillar</td>
</tr>
<tr>
<td>1</td>
<td>-0.3 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>-5.3 ± 0.4 *‡</td>
</tr>
<tr>
<td>3</td>
<td>-16.5 ± 1.0 *‡</td>
</tr>
<tr>
<td>4</td>
<td>-19.0 ± 1.3 *‡</td>
</tr>
</tbody>
</table>

Data are means ± SEM for muscles from 10 to 15 animals for each time point. *P < .05 versus one day of unweighting; †P < 0.05 versus two days of unweighting.
Myofibrillar and Sarcoplasmic Protein Synthesis and Breakdown

In situ synthesis of myofibrillar proteins was measured to evaluate the contribution of this protein pools to the loss of total protein during soleus unweighting. To do this, fractional synthesis rates were measured following IM injection of a flooding dose of $^3$H phenylalanine in weight bearing animals and in animals subjected to 1 to 4 days of suspension. Measurements were taken at 24 hour intervals and fractional synthesis rates were determined. The synthesis data together with the data obtained from daily protein content changes were used to calculate degradation.

Myofibrillar protein synthesis decreased (-16%) within the first 24 hours of unweighting (Table 11). Synthesis declined further (-24%, P<0.05) by 48 hours of unweighting. After three days there was a 40% slower rate of myofibrillar protein synthesis while after day four it had declined by 50%. Although synthesis declined steadily over the four day period, the greatest decline occurred between days two and three (P<0.05).

Average changes in myofibrillar protein growth, synthesis and degradation are shown in Table 12. These values represent daily averages and estimations of degradation and therefore statistical analysis could not be performed. By calculating the difference in protein loss between days two and three of unweighting, the data show an average loss during the third day of 11% compared to only 5% during the second day (Table 12). During day four, the extent of protein loss declined markedly to 2.5%. This slowing of protein loss must be due to a slowing of protein breakdown since protein synthesis remained low. A comparison of daily protein changes and calculated fractional rates of protein breakdown illustrate this conclusion (Table 12). Myofibrillar protein
degradation increased slightly after one day of unweighting followed by a sharp increase during days two and three. Thereafter, protein degradation declined dramatically. The increase in breakdown over the first three days of unweighting accounts partially for the loss of protein content observed over the three day period. The slowing of protein breakdown after three days of unweighting may also account for the slower rate of loss in protein content after day three. Therefore, qualitatively, myofibrillar protein breakdown increases during the time when the loss of these proteins is greatest.

Sarcoplasmic Protein Synthesis and Breakdown

Sarcoplasmic protein synthesis and degradation rates were assessed to evaluate the apparent sparing of these proteins from loss during unweighting atrophy (Table 11). The rate of sarcoplasmic protein synthesis declined significantly by 28% after two days of unweighting (Table 11) even though these proteins were spared (Table 7). Similar to the myofibrillar proteins, sarcoplasmic protein synthesis tended to decrease by about -13% within 24 hours of unweighting. Synthesis continued to decline steadily to 52% of control after four days of unweighting.

Daily changes in sarcoplasmic protein growth, rates of synthesis and degradation are shown in Table 13. The values represent daily averages and are an estimation of degradation. In contrast to the loss of myofibrillar proteins, sarcoplasmic proteins showed no net change protein over the four days. Despite the evident sparing of these proteins, the rate of synthesis of these proteins steadily declined (Table 11). Accordingly, there may have been a slight decrease in protein content through day two of unweighting. In contrast, during days three and four, sarcoplasmic proteins showed a tendency to accumulate. Because
changes in sarcoplasmic protein synthesis did not parallel protein accumulation during days three and four, only a slowing of protein degradation could explain the sparing of these proteins. As shown in Table 13, sarcoplasmic protein breakdown increased slightly over the first two days of unweighting, then sharply declined during days three and four. This pattern of declining rates of degradation during days three and four may account for the sparing of sarcoplasmic proteins during this time since rates of synthesis remained low.
Table 11. Effects of soleus unweighting on rates of in situ myofibrillar and sarcoplasmic protein synthesis.

<table>
<thead>
<tr>
<th>Duration of Unweighting (days)</th>
<th>Protein Synthesis (% Fractional rate/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Myofibrillar</td>
</tr>
<tr>
<td>0</td>
<td>20.7 ± 0.8</td>
</tr>
<tr>
<td>1</td>
<td>18.2 ± 0.9</td>
</tr>
<tr>
<td>2</td>
<td>16.3 ± 0.4 *</td>
</tr>
<tr>
<td>3</td>
<td>13.1 ± 0.3 *†‡</td>
</tr>
<tr>
<td>4</td>
<td>10.8 ± 0.3 *†‡</td>
</tr>
</tbody>
</table>

Data are means ± SEM for 10 to 15 animals. Fractional rates of protein synthesis were determined by i.m. injection as described in Methods. * P<0.05 versus control; † P<0.05 versus one day unweighting; ‡ P< 0.05 versus two days of unweighting.
Table 12. Effects of unweighting on average daily rates of growth, synthesis and degradation of myofibrillar proteins.

<table>
<thead>
<tr>
<th>Day of Unweighting (days)</th>
<th>Growth (%/day)</th>
<th>Synthesis (%/day)</th>
<th>Degradation (%/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>3.3</td>
<td>20.7</td>
<td>17.4</td>
</tr>
<tr>
<td>Day 1</td>
<td>-0.3</td>
<td>19.5</td>
<td>19.8</td>
</tr>
<tr>
<td>Day 2</td>
<td>-5.0</td>
<td>17.8</td>
<td>22.8</td>
</tr>
<tr>
<td>Day 3</td>
<td>-11.2</td>
<td>14.7</td>
<td>25.9</td>
</tr>
<tr>
<td>Day 4</td>
<td>-2.5</td>
<td>12.0</td>
<td>14.5</td>
</tr>
</tbody>
</table>

Data represent average values of growth, synthesis and degradation of successive 24 hour periods. Day zero represents the 24 hour period prior to hindlimb suspension. Calculations are based on data in Table 10 (growth) and, Table 11 (synthesis) as described in Methods. Degradation was calculated as synthesis minus growth.
Table 13. Effects of unweighting on average daily rates of growth, synthesis and degradation of sarcoplasmic proteins.

<table>
<thead>
<tr>
<th>Duration of Unweighting (days)</th>
<th>Growth (%/day)</th>
<th>Synthesis (%/day)</th>
<th>Degradation (%/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>3.3</td>
<td>19.7</td>
<td>16.4</td>
</tr>
<tr>
<td>Day 1</td>
<td>1.8</td>
<td>18.8</td>
<td>17.0</td>
</tr>
<tr>
<td>Day 2</td>
<td>-3.3</td>
<td>16.4</td>
<td>19.7</td>
</tr>
<tr>
<td>Day 3</td>
<td>2.7</td>
<td>13.5</td>
<td>10.8</td>
</tr>
<tr>
<td>Day 4</td>
<td>0.5</td>
<td>11.2</td>
<td>10.7</td>
</tr>
</tbody>
</table>

Data represent average values of growth, synthesis and degradation of successive 24 hour periods. Calculation were based on data in Table 10 (growth) and Table 11 (synthesis) as described in Methods. Degradation was calculated as synthesis minus growth.
Discussion

A daily time course of alterations in myofibrillar and sarcoplasmic protein metabolism was evaluated to gain further understanding of the contributions of protein synthesis and degradation to unweighting atrophy in developing muscle. Although a previous time course of changes in protein metabolism had been assessed (Satarug, 1987), it involved only the measurements of total mixed protein metabolism.

Myofibrillar and Sarcoplasmic Protein Loss

As shown in Fig. 4, total mixed protein loss increased through day three of unweighting (Satarug, 1987). This loss parallels the loss of myofibrillar proteins observed in this study. Following the first day of unweighting there was no change in either total or myofibrillar proteins. By the end of 48 hours, both total and myofibrillar proteins showed significant loss. Between 48 and 72 hours of unweighting, there was a sharp decline in both protein fractions. After 72 hours, the extent of protein loss declined for both fractions. In contrast, sarcoplasmic proteins were spared over the four day period. Therefore, protein loss in unweighting atrophy can be attributed to an increased loss of myofibrillar proteins.

Loss of myofibrillar proteins from unweighting has also been shown in adult animals, the onset of loss occurs between four days and eight weeks (Desplanches et al., 1990a; Thomason et al., 1987b; Tsika et al., 1987). This contrasts the acute loss of protein observed in juvenile animals in this study. In previous studies using young animals, the loss of myofibrillar proteins was acute during six days of unweighting (Jaspers et al., 1985; Jaspers et al., 1988).
Similarly, there was no change in sarcoplasmic protein content while the ratio of myofibrillar to sarcoplasmic proteins declined. In addition to the direct evidence described in this study, indirect evidence of a preferential loss of myofibrillar proteins has been obtained from studies which assessed insulin and B-adrenergic receptors (Henriksen et al., 1986; Kirby and Tischler, 1990; Kirby et al., 1992). As described later in this discussion, these studies showed an increase in the density of these receptors which was most likely due to losses of myofibrillar proteins as the soleus atrophies.

The increased loss of total and myofibrillar proteins observed during day three occurs at the same time the soleus muscle shows an increased response to in vitro insulin stimulated glucose uptake (Henriksen and Tischler, 1988a), protein synthesis, and protein breakdown (Tischler et al., 1990). Therefore, it is possible that an increased response of protein breakdown to insulin could slow muscle atrophy at this time. In addition, an increase in the responsiveness of unweighted soleus to circulating catecholamines could contribute to the decreased loss of myofibrillar proteins after day three of unweighting by slowing degradation. This possibility was demonstrated by recent studies using clenbuterol, a selective $\beta_2$ antagonist whose anabolic effect on skeletal muscle may involve slowing of myofibrillar degradation (Lowell et al., 1986b). In addition, enhanced $\beta$-adrenergic activity has been demonstrated in unweighted soleus muscle (Kirby and Tischler, 1990; Kirby et al., 1992). One possible explanation for the attenuation of myofibrillar protein loss after three days of unweighting in developing muscle is that factors which promote muscle growth provide some protection against prolonged muscle loss in the juvenile animals.
Myofibrillar and Sarcoplasmic Protein Synthesis

As previously shown, total protein synthesis declines over four days of unweighting (Satarug, 1987). Therefore, contributions of myofibrillar and sarcoplasmic synthesis to the loss of total proteins was assessed. Myofibrillar protein synthesis declined in parallel with the total proteins over the four day period (Fig. 4). However, total mixed proteins showed a sharper decline the first two days of unweighting. In addition the magnitude of these changes differed because total proteins declined by 65% while myofibrillar proteins declined by 50%. Sarcoplasmic proteins also showed a parallel decline in synthesis, declining to 50% of control by day four.

Myofibrillar and Sarcoplasmic Protein Degradation

Total, myofibrillar and sarcoplasmic protein synthesis declined in parallel, while the patterns of protein loss differed between the myofibrillar and sarcoplasmic proteins. Therefore, the changes in protein content must be due to alterations in degradation. Similar to total proteins (Satarug, 1987), myofibrillar proteins showed an increase in estimated protein degradation over the first three days of unweighting (Fig. 5). However, protein degradation declined dramatically after day three. This decline in degradation accounts for the decreased rate of loss of both total (2.6% day four versus 11.2 % day three) and myofibrillar protein (2.5% day 4 versus 13.2 % day three). Therefore, an increase in protein degradation over three days accounts in part for the greater loss of proteins during this time. In addition, a slowing of myofibrillar degradation during day four partially accounts for the slowing of protein loss. In contrast to total and myofibrillar protein breakdown, sarcoplasmic protein degradation
showed only a modest increase over the first two days of unweighting (Fig. 5). This was followed by a sharp decrease during days three and four. Therefore, a decline in degradation must account for the sparing of sarcoplasmic proteins during unweighting. These results indicate that the loss of protein in unweighting atrophy is due to losses of myofibrillar proteins through increased rates of protein breakdown and decreased rates of synthesis. It is also evident that sarcoplasmic proteins are spared due to a decline in degradation. In addition, because the pattern of breakdown was different between the two protein fractions, these data suggest that there is independent regulation of these pools.

Sparing of Sarcoplasmic Proteins; Relationship to Carbohydrate Metabolism

Previous studies from this laboratory have demonstrated that insulin and β-adrenergic receptors are spared during unweighting atrophy (Henriksen et al., 1986; Kirby et al., 1992; Kirby and Tischler, 1990). Since membrane proteins make up a portion of the sarcoplasmic protein pool, it would follow that the proteins in this pool would be spared. Indeed, a major finding of this study was the sparing of sarcoplasmic proteins over the four days of unweighting. The sparing of these proteins was not due to increased rates of synthesis since synthesis rates declined in parallel with the myofibrillar proteins over the four day period. This conclusion may be demonstrated by regression analysis. Linear regression analysis of fractional rates of protein synthesis versus time showed similar slopes for both myofibrillar (-2.67 percent/day) and sarcoplasmic (-2.65 percent/day, $r^2=.99$) protein pools. This shows a linear parallel decline in synthesis of both pools. Within the first two days of unweighting there was a
significant decline in myofibrillar protein synthesis. In contrast, rates of myofibrillar protein synthesis decline exponentially in adult animals. This decline is rapid as synthesis rates fell by 50% within two days of unweighting. In addition, rates of total protein synthesis decline more rapidly within two days.

Several findings from previous studies also support the idea of an apparent sparing of sarcoplasmic proteins in unweighting atrophy. As previously stated, increased responses of insulin in unweighted muscle have been demonstrated in vitro and in situ. This was due to an increase in the density of receptors as the total number of receptors did not change when muscle size diminished. In addition, β-adrenergic responses are altered after three and six days of unweighting (Kirby and Tischler, 1990). This was demonstrated by the ability of isoproterenol, a β-antagonist to decrease glycogen formation, increase its breakdown and enhance the production of cAMP (Kirby et al., 1992; Kirby and Tischler, 1990). Instead of a loss of β-adrenergic receptors, there was increased binding capacity relative to muscle size in unweighted muscle. Therefore these findings were similar to those of the insulin receptor. Taken together these results demonstrate the preferential sparing of the sarcoplasmic proteins in unweighted muscle due to a decline in breakdown.

In addition to direct measurement of sarcoplasmic protein metabolism, previous studies have evaluated the role of degradative systems in protein loss from unweighting (Tischler et al., 1990). These studies indicated that proteolysis in unweighted muscle is not due to activation of the lysosomal degradation system. Instead, it appears to involve cytoplasmic proteolysis. In addition, it may involve calcium activated thiol proteases. As membrane proteins are primarily degraded in the lysosome, it may be inferred from results
of this and other studies that myofibrillar proteins are degraded via a cytosolic mechanism. Although some published data support this idea (Lowell et al., 1986a), other evidence suggests that myofibrillar proteins may be degraded in the lysosome (Gerard and Schneider, 1979). In addition, although it is generally accepted that sarcoplasmic proteins are degraded in the lysosome (Creek and Sly, 1983; Ilare, 1988; Libby and Goldberg, 1981) exercise induced protein degradation leads to the loss of sarcoplasmic proteins apparently via a non-lysosomal mechanism (Kasperek and Snider, 1989). Therefore, controversy exists concerning pathways of degradation under various physiological perturbations.
Fig. 4 Total, myofibrillar and sarcoplasmic protein accumulation and synthesis during four days of unweighting. Data for total proteins are from Satarug, 1987. Myofibrillar and sarcoplasmic protein data are from Tables 13 and 14, respectively. Data are averages of each 24 hr period.
Fig. 5 Total, myofibrillar and sarcoplasmic protein accumulation and breakdown during four days of unweighting. Data for total proteins are from Satarug, 1987. Myofibrillar and sarcoplasmic protein data are from Tables 13 and 14, respectively. Data are averages of each 24 hr period.
CHAPTER FOUR
EFFECTS OF SPACE FLIGHT AND EARTH-BASED UNWEIGHTING ON SKELETAL MUSCLE OF YOUNG RATS

Abstract

The purpose of this experiment was to assess the effects of weightlessness on skeletal muscle of juvenile rats and to compare these effects to hindlimb suspension. Measurements included mass and protein content of the soleus (SOL), plantaris (PLN), gastrocnemius (GAS), tibialis anterior (TA) and extensor digitorum longus (EDL) muscles. Insulin stimulated glucose transport in the SOL and EDL was also assessed. These parameters were measured in juvenile female rats exposed to weightlessness, asynchronous controls and hindlimb suspended animals. Food intake among the three groups was similar and consistent with preflight data. However, flight animals gained a greater percentage of body mass. The SOL of both flight and suspended animals showed marked atrophy, while the PLN and GAS showed reduced growth. The EDL and TA grew normally. In vitro insulin stimulated glucose uptake in the SOL was increased in flight (2.5 fold) and suspended (2.7 fold) compared to control animals (1.6 fold). The EDL showed similar responses to insulin in all three groups studied. For comparison, in situ insulin and IGF-1 stimulated 2-deoxyglucose uptake was assessed in SOL of weight bearing and suspended animals. 2-deoxyglucose uptake was 78% and 80% greater than control after 3 days suspension and 283% and 447% greater than control after 6 days of suspension for insulin and IGF-1, respectively. Together these results demonstrate that tail cast suspension may be used as a ground based model to
study the effects of spaceflight on skeletal muscle of juvenile rats.
**Introduction**

Significant muscle wasting has been observed in both humans (Thornton and Rumme, 1974) and animals exposed to weightlessness (Ilyina-Kakueva et al., 1976; Martin et al., 1988). The hindlimb suspension model has been successfully used to mimic the effects of weightlessness on muscle atrophy in adult rats (Flynn and Max, 1985; Michel et al., 1989). Muscle atrophy has been shown to be present after one week of spaceflight and progressively continues for 2-3 weeks (Baranski et al., 1979; Ilyina-Kakueva et al., 1976; Ohira et al., 1992; Riley et al., 1992). As previously discussed (Chapter 1), hindlimb suspension is used to mimic the effects of spaceflight and results in muscle atrophy of the weight bearing muscles of rats. Suspension induces atrophy of the soleus and to a lesser extent the plantaris (Jaspers and Tischler, 1984; Steffen and Mussachia, 1986). The extensor digitorum longus and tibialis anterior do not appear to be affected by suspension (Jaspers and Tischler, 1984). Adult rats exposed to microgravity show similar responses in soleus mass and protein content (Desplanches et al., 1990b; Martin et al., 1988; Martin, 1988; Musacchia et al., 1983; Nicholson et al., 1984; Steffen and Mussachia, 1986), cross sectional area (Desplanches et al., 1990a; Holy and Mounier, 1991; Ilyina-Kakueva et al., 1976; Miu et al., 1990) and sarcomere volume (Baranski et al., 1979) compared to hindlimb suspended animals. Therefore, hindlimb suspension may be used as a ground based model to study the effects of weightlessness on skeletal muscle of adult animals. Although findings between adult animals exposed to weightlessness or hindlimb suspension are similar, there have been no studies for comparison of juvenile animals. Therefore the purpose of this investigation was to assess muscle mass, protein content, and insulin stimulated glucose
uptake in juvenile animals exposed to microgravity and to compare these findings to normal and tail cast suspended animals. In addition, the physiological significance of increased insulin response was assessed by in situ intramuscular injection.

Methods

Preflight Assessment of Food Bar Intake
Treatment of Animals

Female rats (newly weaned, 30-40 g Sasco, Sprague Dawley, Omaha, NE) were divided into two groups (10 per group), provided with food and water ad libitum and maintained under standard housing conditions (5 animals per cage). Ten animals were fed standard food pellets while the remaining animals were provided with food bars.

Diet

Food bars were prepared by the American Institute of Baking (Manhattan, KS) from Teklad TD 88179 powdered diet (Teklad, Madison, WI) by reconstituting to 26 ± 1% with water and forming into bars. After preparation, food bars were sorbate treated and radiation sterilized (Radiation Sterilizers Inc., Tustin, CA.) then kept refrigerated until used. The food bar diet provides 2.74 Kcal/g derived from 17% protein, 3.8% fat and 43.0% carbohydrate. This compares to the Teklad 45 standard rodent pellet which provides 3.23 Kcal/g derived from 25% protein, 4.3% fat, and 46.2% carbohydrates. The greater caloric
content per gram in the standard pellet diet is from the much lower moisture content.

Food Bar Consumption

Daily food bar consumption was measured in normal (days 1-6) and hindlimb suspended animals (days 7-11). Consumption of the standard rodent pellet diet was not measured in this study but can be compared to previous findings (Jaspers and Tischler, 1984). Food waste was minimal, thereby allowing easy measurement of food consumption. Food bar handling was similar to procedures used for the flight study with the exception of the method of feeding. In flight, food bars were fixed to removable panels attached to the sides of the animal enclosure module (AEM) whereas, in this study food bars were placed on the floor of the cage.

Weight bearing bar-fed and pellet-fed animals were housed in four cages (five animals per cage). Hindlimb suspended animals (Jaspers and Tischler, 1984) were housed in individual cages.

Body Mass, Muscle Mass, and Muscle Protein Content

Body mass was measured daily in normal animals (days 1-6) and on day 5 of suspension. On day 6 of the experiment, five randomly selected animals from each diet group underwent tail cast suspension. The remaining animals were killed by cervical dislocation for determination of muscle mass and protein content. The soleus, plantaris and extensor digitorum longus muscles were excised and weighed, and then solubilized in 4 ml of 1N NaOH. After 5 days of hindlimb suspension, muscles were excised, weighed and solubilized in 4 ml 1N
NaOH. Muscle protein content was determined using the biuret assay (Gornall et al., 1949) with bovine serum albumin (Sigma Chemicals, St. Louis, MO) as the standard.

**Effect of Spaceflight on Muscle Metabolism**

**Treatment of Flight Animals**

Weanling female Sprague Dawley rats were shipped along with lactating rats (Sasco, St. Louis, MO) to Kennedy Space Center six days prior to launch. Eight rats (26 days of age) were randomly selected for space flight on September 11, 1991 at 2200 eastern daylight time (EDT) and were placed in the animal enclosure model (AEM) in the mid-deck of the shuttle Discovery 19 hours prior to launch. The AEM was equipped with an 1800 ml spring loaded water system. Food bars were fixed to removable panels on the inside walls of the AEM allowing for ad libitum consumption of food. The AEM was also equipped with a temperature sensor to record in flight changes in mid-deck temperature. A second group of eight animals with the same average body weight served as prelaunch controls. These animals were used to estimate initial body and muscle mass of flight animals at the time of launch. Within two hours after launch, these animals were killed, weighed and the soleus, plantaris, gastrocnemius, extensor digitorum longus and tibialis anterior muscles excised. Discovery was launched at 1910 hours EDT on September 12, 1991 from the Kennedy Space Center. Discovery landed at 338 hours EDT on September 18, 1991 at NASA's Dryden Flight Research Facility (DFRF) at Edwards Air Force Base. The mission duration was 5.4 days. After delivery to the research
laboratory, animals were killed by cervical dislocation. This process began at approximately two hours (at 545 hours) after landing and ended at 700 hours EDT. In order of removal, the soleus, tibialis anterior, extensor digitorum longus, plantaris and gastrocnemius muscles were excised, trimmed of connective tissue and weighed. The soleus and extensor digitorum longus were placed in incubation media to measure insulin-stimulated glucose transport (see 2-deoxyglucose uptake) and then frozen in liquid nitrogen. The extensor digitorum longus, plantaris and gastrocnemius were immediately frozen in liquid nitrogen. Muscles were placed on dry ice and transported from the DFRF to the University of Arizona for analysis.

Treatment of Control and Hindlimb Suspended Animals

Asynchronous ground control experiments were conducted at the University of Arizona seven weeks after the flight experiment. Female Sprague Dawley rats, 25 days of age were housed in a separate room in the Arizona Health Sciences Center Animal Care Facility. Ambient room temperature was adjusted (23° to 28° C) to mimic variations recorded in the shuttle mid-deck during flight. Animals were provided with food bars and water ad libitum. For AEM controls, food bars were fixed to the side panels of the AEM. Food bars were placed on the cage floor for hindlimb suspended animals. Eight ground control animals were housed in an AEM for 5.4 days plus 19 hours to replicate flight duration and preflight holding time. Another group of eight 26 day old rats were tail cast hindlimb suspended. Tail casted animals were suspended for 5.4 days and then were allowed to bear weight for two hours to replicate flight conditions. In addition, body and muscle mass and protein content were assessed
in a separate groups of animals from the same shipment at the start of the experiment in order to calculate percent changes in muscle and body weight.

Measurement of Protein Content in Flight and Asynchronous Control Animals

The soleus, extensor digitorum longus and portions of the plantaris, gastrocnemius and tibialis anterior (20-35 mg) were weighed and solubilized in 0.6 ml of 0.5 N NaOH. Ten ul of solubilized muscle was diluted into 2 ml of 1 N NaOH for analyses of protein content. Protein content was analyzed according to the Lowry method (Lowry et al., 1951).

Estimation of Changes in Protein Content

Changes in protein content were estimated for each muscle based on the protein content of muscles from animals which were killed at the beginning of each experiment. This was done by assessing protein content to body weight ratios at the outset of each experiment. Since average body weights were similar between groups, this ratio could be used to calculate percent change in protein content for each muscle.

In Vitro Measurement of 2-deoxyglucose Uptake

Glucose uptake was measured in the soleus and extensor digitorum longus according to previously described methods (Henriksen et al., 1986) After excision, muscles were rinsed in Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) which had been equilibrated with 95% O₂:5% CO₂. After rinsing, muscles were blotted, weighed, then placed in 3 ml KRB with 5 mM pyruvate and 1% BSA contained in a 25 ml flask. The flask was stoppered and the gas phase
equilibrated with 95% O₂; 5% CO₂. Muscles were incubated in a 37°C shaker bath for 75 minutes. Muscles were then transferred to a new flask containing KRB with 1% BSA, 2-(2,3 H)-deoxyglucose (1 mM, 300 uCi/mmol, ICN, Irvine CA) and [carboxy-14C] inulin (20 nCi/ml, ICN, Irvine CA) in the absence or presence of 1 munit/ml insulin. The flask was stoppered, re-equilibrated with the gas mixture then incubated for 15 minutes in a shaker bath. Incubations were terminated by blotting followed immediately freezing the muscles in liquid nitrogen. Frozen muscles were solubilized in 1 N NaOH as described above. Ten ul of diluted sample was used for protein analysis. 5.0 ml Ecolume (ICN, Irvine, CA) was added to the remaining solution for radioactive counting in the 3H and 14C channels. Extracellular space was determined from 14C radioactivity and the specific activity of the incubation media. 2-Deoxyglucose uptake was calculated as extracellular 3H activity minus total 3H activity.

In Situ Measurement of 2-Deoxyglucose Uptake

In situ insulin stimulated glucose uptake was measured in soleus muscle of normal and three or six day hindlimb suspended animals. Soleus muscles were injected as described previously for measurement of protein synthesis (Chapter 2). Injection volumes were 4 ul/100 g body weight for controls, 3.2 ul/100g body weight for 3 day suspended and 2.6 ul/100 g body weight for 6 day suspended animals. The injection solution for the right leg consisted of 20 mM [1,2,3H] 2 - deoxyglucose (300 uCi/mmol), 0.6 uCi/ml (14 C) mannitol and 0.1% BSA in 0.9% NaCl. The injection solution for the left leg contained in addition, 133 nM insulin or 200 nM IGF-1 to stimulate glucose uptake. Twenty minutes after injection, the middle two-thirds of each muscle was excised,
blotted, frozen by using clamps frozen in liquid nitrogen, and weighed. Samples were solubilized in 0.6 ml of 0.5 N NaOH. Then 5 ml of Ecolume was added for radioactive counting in the $^3$H and $^{14}$C channels. Glucose uptake was calculated as described for in vitro measurement.

Statistical Analysis

Data are presented as mean ± SEM. Calculated variables include normalized muscle mass (mg muscle/100g body mass) and normalized protein content (mg protein/100g body mass). Two way analysis of variance and Student's t-tests were used to test differences in body mass and normalized protein content between bar-fed and pellet-fed animals. Food bar data for normal animals reflects average food consumption from two cages (five animals per cage) whereas food consumption data from suspended animals represents the mean ± SEM of five animals.

Control, flight and hindlimb suspended animals were compared using one way analysis of variance (ANOVA). Data are presented as means ± SEM

Results

Dietary Intake of Food Bars and its Effects on Growth (preflight assessment)

To assess the effectiveness of a space food bar diet on body weight gain and muscle growth it was necessary to conduct a preflight experiment. Average daily food bar consumption by normal (days 1-6) and hind limb suspended (days 7-11) animals are shown in Fig. 6. In normal animals, intake increased from 10.5 g/animal during day 1 to 12.0 g/animal during day 6. When normalized to body mass these results showed an intake of 24g/100g body mass during day
one. Thereafter, the average daily food consumption was 18.2 g/100 g body mass to the end of day six. The greater relative food intake during the first 24 hours is likely a reflection of their recovery from decreased dietary intake during shipment to the animal facility. During five days of suspension, consumption increased from 13.2 ± 1.4 g/day/animal to 19.1 ± 1.4 g/day/animal. The normalized intake was 16 g food/100 g body mass during the first day of suspension and 20 g food/100 g body mass during the last day of suspension. Normalized food intake could not be calculated for the intervening period because it was not possible to measure animal body mass during suspension.

Body mass was similar between diet groups in normal animals on all days of the weight-bearing period (p > 0.05) (Fig. 7). Mean body mass increased from 43.1 ± 1.3 to 73.7 ± 1.8 for pellet-fed animals. In addition, comparable gains in body mass were observed in bar-fed (5.3 g/day) and pellet-fed animals (5.1 g/day). These results agree with those of Jaspers and Tischler (1984) who showed an average gain of 5.6 g/day in pellet-fed animals. Since body mass gained by normal food bar-fed and pellet-fed animals was similar, the food bar diet appears to provide for adequate growth under normal conditions.
Fig 6. Average daily food bar consumption by normal and hind limb suspended animals. Data from days 1-6 (normal animals) represent average intake for two cages of five animals each. Hindlimb suspended animal (days 7-11) data are the mean ± SEM of five animals housed individually. Day zero is the time the diet began.
Fig. 7. The effect of diet on body mass in weight-bearing animals. Data are mean ± SEM for 10 pellet-fed (open bar) and 10 bar-fed animals (filled bar). SEM ranged between ± 1.6 - 2.9 and are not visible due to the small values. Day zero is the time the diets began.
In agreement with the results in normal animals, body mass following suspension was similar between the two diet groups. Mean body mass after five days of suspension was $97.9 \pm 4.8$ g for bar-fed animals and $92.6 \pm 3.4$ g for pellet-fed animals. No difference was found in the initial or final body mass between the diet groups ($P>0.05$). In addition, over five days of suspension similar gain in body mass of $20.3 \pm 3.1$ g (bar-fed) and $17.3 \pm 1.6$ g (pellet-fed) were found ($P>0.05$).

Mass of the five muscles studied was measured after five days of diet treatment and after five days of suspension with diet treatment. Muscle mass per 100 g body mass for the soleus, plantaris and tibialis anterior muscles was comparable for both suspended food bar-fed and pellet-fed animals (Table 14).

No differences were found between normalized muscle protein content of food bar-fed versus pellet-fed animals for the soleus, plantaris, extensor digitorum longus, and tibialis anterior ($P>0.05$) (Fig. 8). The gastrocnemius showed a decrease in protein content between diets for suspended animals. However, the interaction between diet and suspension did not reveal a difference. Therefore, the observed difference is most likely not related to diet. These data indicate that a food bar diet does not alter the muscle response to hindlimb unweighting.
Table 14. Normalized muscle mass in weight-bearing and unweighted muscle.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>BAR FED Weight-bearing</th>
<th>BAR FED Unweighted</th>
<th>PELLET FED Weight-bearing</th>
<th>PELLET FED Unweighted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus</td>
<td>37.8 ± 0.9</td>
<td>26.0 ± 1.3*</td>
<td>37.5 ± 0.5</td>
<td>29.4 ± 1.4*</td>
</tr>
<tr>
<td>Plantaris</td>
<td>70.2 ± 2.8</td>
<td>69.9 ± 1.2</td>
<td>64.6 ± 1.7</td>
<td>67.7 ± 1.7</td>
</tr>
<tr>
<td>EDL</td>
<td>42.1 ± 0.6</td>
<td>47.1 ± 1.4</td>
<td>38.4 ± 1.5</td>
<td>40.2 ± 0.7</td>
</tr>
<tr>
<td>GAS</td>
<td>345 ± 13</td>
<td>363 ± 7</td>
<td>321 ± 14</td>
<td>330 ± 14</td>
</tr>
<tr>
<td>TA</td>
<td>166 ± 6</td>
<td>185 ± 6</td>
<td>138 ± 10</td>
<td>166 ± 3</td>
</tr>
</tbody>
</table>

Values are mean ± SEM in mg per 100 g body mass for 5 animals per group.
EDL = extensor digitorum longus; GAS = gastrocnemius; TA = tibialis anterior.
* P<0.05.
Fig. 8. Normalized protein content in soleus, plantaris, extensor digitorum longus (EDL), gastrocnemius and tibialis anterior of bar fed normal (open bar), bar fed suspended (cross hatched bar), pellet-fed normal (filled bar) and pellet-fed suspended (stippled bar) animals. Data are mean ± SEM for five animals per treatment. SEM are not visible in some cases due to the small values.
Effects of Spaceflight
Food Bar, Water Intake and Growth in Flight and Asynchronous Ground Control Animals

Food bar consumption by flight and AEM asynchronous controls averaged 19.4 g/100g body weight for controls, 20.3g/100g body weight for flight and 19.4 g/100g body weight for suspended animals. Because animals were not housed individually, statistical comparison between groups of animals could not be performed.

Water consumption was 12.5 ml/rat/day for flight animals and 12.1 ml/rat/day for AEM asynchronous controls. Water intake was not measured in suspended animals since they were not provided with spring loaded water bottles.

Initial and final body weights for flight and asynchronous ground control animals are shown in Table 15. Asynchronous ground controls were selected to have smaller initial body weights so that muscles would not become to large for incubation studies. Final body weights of flight animals were significantly greater (P<0.01) than ground based controls. Weight gain for flight animals was 9.3%/day compared to 5.1%/day for suspended animals.

Muscle Mass and Protein Content

Mass of the five muscles studied are shown in Table 16. The soleus, of flight animals were significantly smaller than controls (P<0.05). The tibialis anterior and extensor digitorum longus of flight animals were larger than controls, while the plantaris showed no change. The soleus of suspended animals, differed significantly from control (P<0.05), while the remaining
muscles showed no difference. This finding is due to the smaller body weights of control animals and the fact that body weights between groups were different (Table 15). To account for differing body weights, muscle masses were normalized to the extensor digitorum longus (Fig. 9). This muscle was used for normalization since it shows the least responsiveness to suspension (Jaspers and Tischler, 1984) or spaceflight (Martin et al., 1988). Compared to control animals, the soleus of flight animals showed the greatest reduction in mass relative to the extensor digitorum longus (35%) followed by the plantaris (21%) and the gastrocnemius (13%) ($P<0.05$). In contrast, flight and suspended animals showed similar ratios for the soleus, plantaris and gastrocnemius. The tibialis anterior : extensor digitorum longus ratio was similar between the three groups of animals ($P>0.05$). When normalized to body mass, the soleus of flight animals showed a 38% loss in mass while the soleus of suspended animals showed a 33% loss (Table 17). The plantaris of flight and suspended animals showed losses of 24% and 12%, respectively while the gastrocnemius showed losses of 16% (flight) and 9% (suspended). The tibialis anterior and extensor digitorum longus did not lose mass.
Table 15. Body mass and food bar intake in control, flight and suspended animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial</th>
<th>Final</th>
<th>% gain; (*per day)</th>
<th>Food intake (g/100g BW/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>52 ± 1</td>
<td>77 ± 1</td>
<td>48 ± 2 (7.0 ± 0.3)</td>
<td>19.4</td>
</tr>
<tr>
<td>Flight</td>
<td>62 ± 1*</td>
<td>94 ± 2*</td>
<td>51 ± 2 (9.3 ± 0.3)*</td>
<td>20.3</td>
</tr>
<tr>
<td>Suspended</td>
<td>57 ± 1*</td>
<td>73 ± 1</td>
<td>27 ± 2 (5.1 ± 0.4)*</td>
<td>19.4</td>
</tr>
</tbody>
</table>

Data are Mean ± SEM for control, flight and suspended animals (n=8 per group).

* P<0.01 versus control by one way ANOVA with Bonferroni correction.
Table 16. Effects of spaceflight and suspension on soleus (SOL), plantaris, (PLN) gastrocnemius (GAS), tibialis anterior (TA) muscles mass.

<table>
<thead>
<tr>
<th>Group</th>
<th>SOL</th>
<th>PLN</th>
<th>GAS</th>
<th>TA</th>
<th>EDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mass (mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>30.9 ± 0.7</td>
<td>66 ± 4</td>
<td>307 ± 9</td>
<td>129 ± 3</td>
<td>35.8 ± 0.7</td>
</tr>
<tr>
<td>Flight</td>
<td>23.4 ± 0.8 * †</td>
<td>61 ± 3</td>
<td>313 ± 11 †</td>
<td>158 ± 6* †</td>
<td>41.7 ± 1.1 *</td>
</tr>
<tr>
<td>Suspended</td>
<td>19.1 ± 0.6 * †</td>
<td>56 ± 1</td>
<td>279 ± 4 †</td>
<td>133 ± 2 †</td>
<td>35.4 ± 0.5 †</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. *P < 0.05 versus control; †P<0.05 flight versus suspended by one way ANOVA.
Fig 9. Effect of spaceflight and suspension on relative muscle mass. Values are mean ± SEM for the ratios of extensor digitorum longus to the soleus or plantaris (A) or gastrocnemius or tibialis (B) in control (open bar), flight (filled bar) or suspended (cross-hatched bar) animals. *P<0.05 versus control by one way ANOVA with Bonferroni correction.
Table 17. Effects of spaceflight and suspension on soleus (SOL), plantaris, (PLN) gastrocnemius (GAS), and tibialis anterior (TA) and extensor digitorum longus (EDL) normalized muscle mass.

<table>
<thead>
<tr>
<th>Group</th>
<th>SOL</th>
<th>PLN</th>
<th>GAS</th>
<th>TA</th>
<th>EDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40±1</td>
<td>85±5</td>
<td>397±14</td>
<td>167±4</td>
<td>46±2</td>
</tr>
<tr>
<td>Flight</td>
<td>25±1 *</td>
<td>65±3*</td>
<td>333±12*</td>
<td>167±5</td>
<td>44±1</td>
</tr>
<tr>
<td>Suspended</td>
<td>29±1* †</td>
<td>63±4*</td>
<td>319±11*</td>
<td>176±7</td>
<td>47±1</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. *P < 0.05 versus control; †P < 0.05 versus flight by one way ANOVA.
Changes in protein content over the 5.4 days of weightlessness or tail cast suspension were estimated by measuring initial body weight and protein content in separate groups of animals which had the same mean body weight of flight or suspended animals. Initial protein content could then be estimated using the proportion of muscle protein to body weight. The percent change in soleus protein content was 44% for control, -20% for flight, and -23% for suspended animals (P<0.05) (Fig. 10). The plantaris and gastrocnemius showed changes in protein content of 42% (control), 31% (flight) and 20% (suspended). The gastrocnemius data (45% control, 31% flight, 20% suspended) were similar to the plantaris. In contrast, the tibialis anterior and extensor digitorum longus did not show loss of protein or reduced growth. The percent changes in protein content of these muscles were similar to changes in body weight gains of flight (51%) and suspended (27%) animals.

Protein concentration of the muscles studied are shown in Table 18. The soleus of flight animals showed a significantly lower concentration than control animals (P<0.05), while the soleus of suspended animals did not show this response. The extensor digitorum longus, plantaris, gastrocnemius and tibialis anterior did not show significant differences in protein concentration in either flight or suspended animals.
Fig. 10. Effect of spaceflight on calculated changes in protein content. Values are mean ± SEM for the percent change in protein content from control (open bar), flight (filled bar) or suspended (cross-hatched) animals. Percent change was calculated as described in Methods.
Table 18. Protein concentration of the soleus (SOL), plantaris, (PLN), gastrocnemius (GAS), tibialis anterior (TA), and extensor digitorum longus (EDL) muscles of control, flight and suspended animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>SOL</th>
<th>PLN</th>
<th>GAS</th>
<th>TA</th>
<th>EDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.9 ± 0.4</td>
<td>13.0 ± 0.4</td>
<td>13.0 ± 0.4</td>
<td>12.5 ± 0.5</td>
<td>13.7 ± 0.5</td>
</tr>
<tr>
<td>Flight</td>
<td>11.8 ± 0.4*</td>
<td>13.8 ± 0.4</td>
<td>14.1 ± 0.5</td>
<td>12.8 ± 0.4</td>
<td>13.6 ± 0.4</td>
</tr>
<tr>
<td>Suspended</td>
<td>13.1 ± 0.6</td>
<td>13.0 ± 0.4</td>
<td>12.9 ± 0.3</td>
<td>12.4 ± 0.5</td>
<td>12.8 ± 0.4</td>
</tr>
</tbody>
</table>

Data are means ± SEM. *P<0.05 versus control.
Insulin Stimulated Glucose Uptake

Enhanced insulin stimulated glucose uptake has previously been demonstrated in vitro in unweighted soleus muscle (Henriksen and Tischler, 1988; Henriksen, et al., 1986). This finding is unique to unweighting as neither denervation nor immobilization show this response (Burant, et al., 1984; Nicholson, et al., 1984). The uptake of 2-deoxyglucose in the presence and absence of insulin was measured in soleus and extensor digitorum longus muscles of control, flight, and suspended animals (Table 19). Soleus 2-deoxyglucose uptake in the absence of insulin was similar amongst all groups (P>0.05). There was a 1.6 fold effect of insulin in control soleus, while in flight and suspended soleus the effect was 2.5 and 2.7 fold, respectively. This amounts to a 51% increase in insulin stimulated 2-deoxyglucose uptake for flight animals and a 71% increase for suspended animals. The extensor digitorum longus did not show an enhanced response of insulin stimulated glucose uptake in flight or suspended animals. Surprisingly there was a significantly lower uptake of 2-deoxyglucose in both the presence and absence of insulin when compared to control (Table 19).

In situ insulin or IGF-1 stimulated 2-deoxyglucose uptake was evaluated in order to assess the increased response under more physiological conditions. Soleus of weight bearing and suspended animals were injected as described in Methods. There was no increase in 2-deoxyglucose uptake in unweighted soleus in the absence of insulin or IGF-1 (Fig. 11). In the presence of insulin, control muscles showed a 157% increase in the rate of 2-deoxyglucose uptake while IGF-1 stimulated uptake by 200%. Following three days of unweighting, insulin stimulated glucose uptake was 78% greater than in control and IGF-1 stimulated
uptake was 80% greater than control. After six days of unweighting, the rates of insulin or IGF-1 stimulated uptake were 283% and 447% greater than control, respectively (P<0.01).
Table 19. Effect of spaceflight or hindlimb suspension on insulin stimulated 2-deoxyglucose uptake in soleus and extensor digitorum longus muscles.

<table>
<thead>
<tr>
<th>Group</th>
<th>Minus Insulin</th>
<th>Plus Insulin</th>
<th>Fold Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Soleus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.2 ± 0.2</td>
<td>6.5 ± 0.2 *</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Flight</td>
<td>4.0 ± 0.3</td>
<td>9.8 ± 0.6 * †</td>
<td>2.5 ± 0.3 †</td>
</tr>
<tr>
<td>Suspended</td>
<td>4.1 ± 0.3</td>
<td>11.1 ± 0.6 * †</td>
<td>2.7 ± 0.1 †</td>
</tr>
<tr>
<td><strong>Extensor Digitorum Longus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.6 ± 0.1</td>
<td>3.9 ± 0.1</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Flight</td>
<td>2.0 ± 0.1 †</td>
<td>3.1 ± 0.1 †</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Suspended</td>
<td>2.3 ± 0.1</td>
<td>3.6 ± 0.2</td>
<td>1.6 ± 0.1</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. 2-deoxyglucose uptake was measured in the absence or presence of 1 munit/ml insulin. * P<0.05 versus no insulin; Students t test; † P<0.05 versus control group by ANOVA.
Fig. 11. Effect of unweighting on in situ insulin and IGF-1 stimulated 2-Deoxyglucose uptake. Data are mean ± SEM of 5 muscles per group. Muscles were injected without (open bars) or with (filled bar) insulin as described in Methods. * P < 0.001 vs. control plus hormone; †P<0.001 vs. 3 day suspended plus hormone.
Discussion

Preflight Assessment of Body Weight and Food Intake

Although food bar consumption increased throughout the study, when normalized to body mass, it remained relatively constant. Food intake during the first 24 hours after suspension decreased slightly. This is most likely due to mild stress effects of suspension. Environmental adaptation likely accounts for the large increase in food consumption during the next twenty-four hours. The fact that food bar intake was greater in suspended, as compared to normal animals, could be attributed to their smaller size. Food bar consumption data from preflight assessment were compared to pellet intake reported in a previous study (Jaspers and Tischler, 1984). After accounting for differences in moisture content (26% bar vs. 9.6% pellet), dry food intake was comparable between the bar-fed (14.1 g/100g body mass) and pellet-fed (12.8 g/10g body mass) normal animals. Dry food intake by bar-fed (13.4 g/100 g body mass and pellet-fed (13.1 g/100 g body mass) suspended animals was also similar.

Food bar consumption was measured recently by the NASA PSE-01 experiment flown on STS-41, and in ground based (AEM) controls. These data were obtained from values for total food consumption over five days, and the average of the animals' initial and final body mass. Average daily food intake was 14.6 g/100g body mass in flight animals and 15.6 g/100g body mass in AEM controls (personal communication from L.H. Ostrach, NASA/ARC). Using the same approach for calculation, the data for days two through four (normal animals) and days six through eleven (suspended animals) yielded values of 18.0 g/100g body mass/day and 19.5 g/100 g body mass/day, respectively. Though these values are somewhat higher than those reported by NASA, there are a
number of variables which can affect these comparison including the source (Harlan vs. Sasco), sex (male vs. female) and size (130-150 average vs. 65-85 g average) of the animals, as well as the cage environment (AEM vs. standard housing). Even after taking into account these variables, there was only 15% apparent difference between the control values in these two studies. These data demonstrate that food bars provide for adequate growth during suspension.

Body Weight and Food Intake in Flight Animals

Although food consumption was not different between flight, control and suspended animals, flight animals gained more body weight than asynchronous ground controls. This difference in weight gain possibly be due to a decrease in energy expenditure during flight. Differences in body weight may also be associated with stress effects due to cage restriction in ground based AEM controls. Mild stress effects may also have led to slower weight gain in suspended animals. This is supported by previous data in which suspended animals grew slower than weight bearing controls during the first two days of suspension (Jaspers et al., 1985). In addition, AEM animals may have been due to adaptation to a smaller living space.

Muscle Mass and 2-Deoxyglucose Uptake

Because flight animals had a greater final body weight than controls or suspended animals, muscle mass was normalized to the extensor digitorum longus. The soleus showed the greatest decline in mass when normalized to the extensor digitorum longus with the plantaris and gastrocnemius showing less of a decline. In contrast, when normalized to the extensor digitorum longus, the
tibialis anterior showed no change after flight or suspension. These findings are similar to those of previous ground based studies (Holy and Mounier, 1991; Ilyina-Kakueva et al., 1976; Miu et al., 1990). In addition, the decrease in soleus muscle mass and protein content is similar to previous spaceflight findings (Martin, 1988; Martin et al., 1988; Thomason and Booth, 1990). Prior to this PARE-01 experiment, the youngest animals flown had already reached adulthood (50 days old; Martin, 1988). Even so, the response of the soleus was similar to findings of this study. Similar to the finding in juvenile animals, adult animals from the SL-3 or COSMOS experiments (Miu et al., 1990) showed a greater response of the soleus than of the other weight bearing muscle. Therefore, the results of this investigation are similar to previous studies, and indicate that hindlimb suspension mimics the effects of space flight on mass and protein content of hindlimb muscles.

Enhanced insulin stimulated glucose transport activity of both flight and suspended animals provides further evidence that tail cast suspension mimics muscle responses to spaceflight in juvenile rats. This conclusion is supported by the finding that the soleus, but not EDL, shows increased insulin response in both suspended and flight animals. The lack of a differential response in the EDL demonstrates that the alteration in 2-deoxyglucose uptake was not due to systemic changes. The finding that the EDL showed decreased 2-deoxyglucose uptake in both the presence and absence of insulin for flight compared to control animals has not been replicated, and no explanation is apparent. In addition to the in vitro finding, increased insulin response was observed in situ thereby demonstrating physiological significance. The differing amount of stimulation in vivo compared to in vitro may be due to the differing methodologies used or
due the differing insulin concentrations.

Both increased insulin binding capacity and increased levels of GLUT-4 protein can account for the increase in insulin stimulated glucose uptake in flight and suspended soleus. The increase in binding capacity occurs due to sparing of insulin receptors; thus the number of membrane receptors does not change even though the muscle atrophies (Henriksen et al., 1986). In addition, GLUT-4 proteins levels are increased in three day unweighted soleus muscle. The increase in the GLUT-4 proteins occurs concurrently with the increase in insulin stimulated glucose transport, thereby providing indirect evidence that increased GLUT-4 protein levels account for these findings (Henriksen et al., 1991).

In addition to insulin, IGF-1 increased glucose transport in situ. The effects of IGF-1 have also been observed in vitro (Henriksen et al., 1992). Since IGF-1 utilizes its own receptor system (Dohm et al., 1990), it is possible that increases in both insulin and IGF-1 receptor binding produce the effects observed in unweighting. Vanadate and phospholipase C, (PLC) which are insulin mimickers, have also been shown in vitro to enhance glucose uptake in unweighted muscle (Henriksen et al., 1992). PLC enhanced glucose uptake was evident after three days of unweighting while vanadate enhanced uptake after six days of unweighting. Vanadate is insulin dependent, but its site of action is distal from the insulin receptor whereas PLC uses an insulin dependent mechanism. Together these results provide further evidence that alterations in glucose uptake in unweighted muscle may include induction of post receptor mechanisms which act along with insulin to enhance glucose uptake.

Taken together, these results demonstrate that hindlimb suspension may be used as a ground based model to mimic the effects of weightlessness on
hindlimb muscles of juvenile rats. This is demonstrated by similar changes in mass, protein content and insulin stimulated glucose transport activity in flight and suspended animals. In addition, enhanced glucose uptake may be due to alterations in both insulin and IGF receptors.
CHAPTER FIVE
SKELETAL MUSCLE MASS, PROTEIN CONTENT AND SYNTHESIS
AFTER VOLUNTARY WHEEL RUNNING AND UNWEIGHTING

Abstract

The purpose of this investigation was to evaluate the effects of training or unweighting on mass, protein content and in vivo protein synthesis. These measurements were assessed in the soleus of normal (N), voluntary wheel run (WR), hindlimb suspended (HS) and wheel run followed by seven days hindlimb suspension (WR-HS) animals. After one week of running, soleus mass and protein content increased significantly (P<0.05). Protein synthesis rates increased after two weeks of running (P<0.05). After three weeks of running, there were maximum increases of 30% for soleus protein content and muscle weight and 41% for protein synthesis. After seven days of unweighting, the soleus showed significant losses in mass and protein content (P<0.05). Compared to controls, HS and WR-HS animals showed reduction in muscle mass, protein content and synthesis. However, WR-HS animals showed higher values for these parameters compared to HS animal. These data show that 1) wheel training provides a rapid and non invasive method to promote muscle hypertrophy, 2) protein synthesis rates are reduced by suspension, even if animals are trained, and 3) protein content and synthesis rates are higher in soleus of suspended animals compared to suspended animals when training precedes unweighting.
Introduction

Unweighting by tail cast suspension or exposure to microgravity results in skeletal muscle atrophy, primarily of the soleus and to a lesser extent the plantaris (Jaspers and Tischler, 1984; Musacchia et al., 1983; Thomason and Booth, 1990). Soleus atrophy is due to a decrease in protein synthesis and an increase in degradation (Goldspink et al., 1986; Jaspers et al., 1988; Loughna et al., 1986; Thomason et al., 1989). Since significant and rapid muscle atrophy occurs in humans and animals during spaceflight, it is imperative that countermeasures to prevent muscle atrophy be developed. There are several proposed countermeasures to prevent musculoskeletal deconditioning which occurs from spaceflight. These protocols include electrical stimulation (Duvoisin et al., 1989), artificial gravity (Burton, 1989) and exercise training (Convertino, 1990). Although relatively unsuccessful, exercise regimens have been used by astronauts and cosmonauts in an attempt to prevent the detrimental effects on muscle mass (Kozlovskaya et al., 1981; Thorton and Rumme, 1974). Efforts to prevent atrophy in animals involve using methods which stimulate muscle hypertrophy such as high load and treadmill training. In hindlimb suspended animals, periodic high load exercise training has been shown to attenuate the effects of unloading on the soleus muscle (Herbert et al., 1988). In addition, treadmill training consisting of 40 minutes per day during the suspension period provides some protection, but does not prevent atrophy (Hauschka et al., 1988). Treadmill training after suspension appears to aid in faster muscle recovery as muscle weight and fiber cross sectional area are greater in trained versus sedentary muscle (Kasper et al., 1990) thereby offering some potential benefit. In addition, ground support and uphill running during long-term suspension (4 to
8 weeks) spares both myofibril and myosin protein when performed during the atrophic phase (Thomason et al., 1987a). Although some evidence exists that training during or after suspension may be beneficial in preventing atrophy, evidence remains limited and the effects of prior training have not been assessed.

Several models have been developed to promote muscle hypertrophy in animals. These models include enforced treadmill training (Holloszy and Booth, 1976) and voluntary wheel running (Lambert and Noakes, 1990; Rodnick et al., 1992) Animals which run voluntarily on an exercise wheel show similar metabolic responses as treadmill trained animals (Bagby et al., 1986; Mondon et al., 1980; Mondon et al., 1985; Reaven and Reaven, 1981; Rodnick et al., 1989) In addition, voluntary wheel running does not require adverse stimuli such as electric shock to induce running, and allows for adequate food consumption and body weight gain (Mondon et al., 1980; Reaven and Reaven, 1981). Because voluntary wheel running selectively increases soleus muscle mass, it may be used to assess the effects of exercise on muscle dynamics (Mondon et al., 1980). Because of this specificity, it also provides a model to assess the role of exercise in prevention of soleus muscle atrophy from unweighting, as this muscle shows the greatest atrophic response to unweighting. The mechanisms which cause muscle hypertrophy from voluntary wheel running have not yet been established. To date, only one study has assessed the effect of wheel running on protein metabolism. This study found that in response to insulin, protein degradation is elevated after wheel training (Rodnick et al., 1990). The time course of increasing muscle mass and protein content has not yet been evaluated and the role of in vivo protein synthesis in muscle hypertrophy from wheel training has not been measured. Therefore, the purpose of this study was to
evaluate the time course of changes, the role of protein synthesis in muscle hypertrophy of wheel trained rats, and to determine if wheel training prior to suspension would provide protection against atrophy of the soleus muscle.

Methods

Treatment of Animals.

The effects of training and unweighting on soleus and plantaris muscle mass, protein content and total mixed protein synthesis were assessed using female Sprague Dawley rats (Sasco, Omaha, NE) which were divided into four groups. These groups were designated as; normal weight bearing for up to four weeks (N), training by voluntary wheel running for one to four weeks (WR), normal weight bearing for two or three weeks followed by hindlimb suspension for seven days (HS), and training by voluntary wheel running for two or three weeks followed by tail cast suspension for seven days (WR-HS). During the training periods, WR and WR-HS animals were placed in individual cages equipped with a running wheel (1.13 m in circumference; Lafayette Instruments, West Lafayette, IN) so that animals could run voluntarily. A mechanical counter was used to record the number of revolutions run per day in order to measure running activity. To be included in the study, animals must have run 3, 5, 6, and 7 Km/day for one through four weeks of training respectively. Body weights were measured biweekly and running activity was recorded daily. Food and water were provided ad libitum in a housing unit separate from the running wheel. Animals had continuous access to food, water and the running wheel. Normal (N) animals were housed in standard cages with four animals per cage. HS and WR-HS animals were tail casted using Hexcelite orthopedic tape.
(Kirschner Medical, Timonium, MD) and medical grade elastomer (Factor II, Lakeside, AZ). During casting, animals were tranquilized by forelimb injection of 10 μl/100g body weight Innovar-vet (4 ug Sublimaze, 200 ug Inasapine; Pittman-Moore, Washington Crossing, NJ). Tail casted animals were suspended in a head down position for seven days as previously described (Jaspers and Tischler, 1984) and housed in individual cages. At the initiation of each experiment, animal body weights averaged between 95-100 g. Since two and three week HS and WR-HS animals failed to gain significant weight during suspension, these groups were weight matched to the two or three week N and WR animals, respectively. All animals were housed under standard housing conditions (12 hr light/dark cycle) and killed by cervical dislocation. All procedures were approved by the University of Arizona Animal Care and Use Committee.

Protein Content and Total Mixed Protein Synthesis.

Animals were fasted from 7:00 am on the morning of the experiment until the time of the experiment (between 12:00 PM and 1:00 PM). Animals were anesthetized by intraperitoneal (IP) injection of pentobarbital sodium (50 mg/100 Kg body weight; Abbott Laboratories, Chicago, IL). Fractional rates of protein synthesis were measured based on published methods (Garlick et al., 1980; Jepson et al., 1986) with modification limited to volumes used. Animals were injected IP with 300 μmol and 40 μCi of L-[sidechain]-3H phenylalanine (ICN, Costa Mesa, CA) per 100 g body weight administered in 2 ml of 1% saline solution. Fifteen minutes later, the soleus from both hindlimbs were excised, frozen in clamps which had been cooled in liquid nitrogen, and then weighed.
The left muscle was homogenized in 40 ul of ice-cold 20 mM HEPES buffer, pH 7.4, containing 1 mM EDTA and 250 mM sucrose. The protein content in the homogenate was determined using the bicinchoninic acid method with fatty acid free BSA as the standard (Smith et al., 1985). The right muscle was used for measurement of protein synthesis.

For protein synthesis analysis, frozen muscles were homogenized in 2.5 ml of ice-cold 2% perchloric acid (PCA) then centrifuged for 15 min at 2800 x g. The supernatant solution was saved as the acid-soluble fraction and processed as for myofibrillar and sarcoplasmic proteins for determination of specific activity (Chapter 3). The protein pellet was washed twice in 5.0 ml PCA then hydrolyzed and further processed as described for myofibrillar and sarcoplasmic proteins. The fractional rate of synthesis was calculated as \( \frac{S_b}{0.9 \cdot S_i \cdot t} \). Where \( S_b \) is the specific activity of the bound protein, \( S_i \) is the intracellular specific activity and \( t \) is the time of incorporation in days (15 minutes), and 0.9 is a correction factor for the delay of blood \( ^3 \)H phenylalanine in reaching equilibrium.

Statistical Analysis.

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using analysis of variance (ANOVA) with Bonferroni correction.

Results

Running Activity and Body Weight Gain.

Final body weights of N and WR animals in each group were similar (P>0.05) (Table 19). Running distances increased by two weeks and were maintained thereafter. Running distances were similar between WR and WR-
HS groups at two or three weeks (Table 20).

Effect of Training on Muscle Mass, Protein Content and Synthesis.

After one week of training, soleus normalized muscle mass (mg/100 g body weight) was greater (+19%; P<0.05) in WR compared to age-matched N animals (Fig. 12A). Soleus protein content of WR was 25% (P< 0.05) greater than N animals after one week of running (Fig. 12B). The percent difference in protein content between N and WR was 23% at two weeks and 30% after three weeks of training (see Fig. 14). Muscle mass and protein content showed maximal increases by week three (+30%, P<0.05) and no further increases were observed thereafter.

The average fractional rates of soleus protein synthesis ranged from 16.7 %/day to 18.0 %/day in normal animals (Fig. 13). Soleus protein synthesis was similar in one week WR compared to age-matched N animals after one week of training (P>0.05). However, synthesis was 21% greater in two week WR compared to age-matched N animals after two weeks of training (P<0.05). Further increases in synthesis were observed after three (41% greater than N animals) and four weeks (59% greater than N animals) of training (P < 0.05). The largest increment in synthesis was observed between two and three weeks of training, while between weeks three and four there was a relatively small change. The percent differences in protein content and synthesis between N and WR are summarized in Fig. 14.

Effect of Unweighting on Muscle Mass, Protein Content and Protein Synthesis in Normal Animals
Soleus muscle mass and protein content were significantly less in HS compared to N animals (P<0.05; Fig. 12A, B). Compared to weight-matched normal animals, the soleus of two week HS animals had 32% less mass and 45% less protein (P<0.05). In the one week older group, muscle mass was 30% less and protein content was 32% less than weight-matched N animals. The percent difference between N and HS protein contents after three weeks is summarized in Fig. 15.

Protein synthesis rates were 57% (two weeks) and 55% (three weeks) slower in HS animals compared to weight-matched N animals (Fig. 13). The percent difference between N and HS protein synthesis after three weeks is summarized in Fig. 14.

Effects of Unweighting on Muscle Mass, Protein Content and Protein Synthesis of Previously Trained Animals

To assess the effects of suspension on hypertrophied soleus, animals were wheel trained for two or three weeks and then suspended for seven days. Normalized soleus muscle mass was lower (p<0.05) in WR-HS compared to WR animals at either two (-35%) or three weeks (-45%) (Fig. 12A). In addition, protein content was also lower in WR-HS animals after two (-34%) or three (-36%) weeks (Fig. 12B). The difference in soleus protein content of WR and WR-HS animals are summarized in Fig. 15.

Soleus protein synthesis was 49% higher in two week WR versus two week WR-HS animals (20.9%/day trained versus 10.2%/day; trained-suspended (Fig. 13.). After three weeks of training, synthesis was 54% higher in WR compared to WR-HS animals (25.4%/day trained versus 13.3%/day trained-
suspended). The difference in synthesis between weight-matched WR and WR-HS animals are summarized in Fig. 14.

Unweighting produced significantly lower protein content and protein synthesis rates in both the N and WR animals. However, WR-HS animals had significantly more protein and higher rates of synthesis than HS animals (P<0.05). The percent differences in protein content between HS and WR-HS groups was 48% (two week) and 22% (three week) and the percent difference in synthesis was 36% at two weeks and 64% at three weeks (Fig. 14).
Table 20  Body Weights and Running Distances of normal (N,) wheel run (WR), hindlimb suspended (HS) and wheel run- suspended (WR-HS) animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight (g)</th>
<th>Distance Run (Km/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 week N</td>
<td>142 ± 5</td>
<td></td>
</tr>
<tr>
<td>1 week WR</td>
<td>138 ± 2</td>
<td>4.8 ± 0.5</td>
</tr>
<tr>
<td>2 week N</td>
<td>155 ± 7</td>
<td></td>
</tr>
<tr>
<td>2 week HS</td>
<td>151 ± 5</td>
<td></td>
</tr>
<tr>
<td>2 week WR</td>
<td>153 ± 5</td>
<td>15.3 ± 1.3</td>
</tr>
<tr>
<td>2 week HS-WR</td>
<td>157 ± 6</td>
<td>14.1 ± 1.3</td>
</tr>
<tr>
<td>3 week N</td>
<td>178 ± 6</td>
<td></td>
</tr>
<tr>
<td>3 week HS</td>
<td>182 ± 2</td>
<td></td>
</tr>
<tr>
<td>3 week WR</td>
<td>186 ± 4</td>
<td>16.0 ± 1.2</td>
</tr>
<tr>
<td>3 week WR-HS</td>
<td>177 ± 5</td>
<td>16.0 ± 1.3</td>
</tr>
<tr>
<td>4 week N</td>
<td>198 ± 5</td>
<td></td>
</tr>
<tr>
<td>4 week WR</td>
<td>199 ± 4</td>
<td>15.8 ± 0.9</td>
</tr>
</tbody>
</table>

Values are means ± SEM for 5-12 animals per group and represent body weights at the end of the respective experimental periods and running distances at the end of the respective training periods.
Fig. 12. Effect of training, unweighting and training followed by unweighting on soleus muscle mass (A), protein content (B) of N (filled bar) WR (slashed bar), HS (open bar) and WR-HS (stippled bar) animals. Values are mean ± SEM of 5-12 animals per group. Muscle mass and protein content are normalized to body weight. Protein synthesis was measured as described in Methods and calculated as fractional rates (%/day). * P< 0.05 vs. normal animals; † P<0.05 vs. trained animals by ANOVA with Bonferroni correction.
Fig. 13. Effect of training, unweighting and training followed by unweighting on protein synthesis of N (filled) WR (slashed), HS (open) and WR-HS (stippled) animals. Values are mean ± SEM of 5 - 12 animals per group. Muscle mass and protein content are normalized to body weight. Protein synthesis was measured as described in Methods and calculated as fractional rates (%/day). * P< 0.05 vs. normal animals; †P<0.05 vs. trained animals by ANOVA with Bonferroni correction.
Fig. 14 Percent difference in protein content and synthesis due to training or training followed by unweighting. Values are percent differences in soleus protein content or synthesis in two week trained versus N (filled), two week WR-HS versus HS (cross-hatched), three week WR versus N (open) and three week WR-HS versus HS (stippled). Percentages were calculated from data shown in Fig.. 12 A, B and Fig. 13.
Fig. 15. Effects of unweighting or unweighting following 3 weeks training on protein content and synthesis. Data are percent differences in protein content (open bar) or protein synthesis (filled bar) from N versus HS or WR versus WR-HS animals. Values are calculated from data shown in Fig. 12 A, B and Fig. 13.
Discussion

Voluntary wheel running for one to four weeks was used to induce hypertrophy of the soleus muscle, while unweighting by hindlimb suspension was used to cause muscle atrophy. The combination of training and unweighting was then used to evaluate the effectiveness of training prior to suspension on the prevention of muscle atrophy.

Effect of Training on Muscle Mass, Protein Content and Synthesis

Similar to findings of previous investigations (Rodnick et al., 1992) voluntary wheel running increased muscle mass of the soleus. The increase in mass was evident by the first week of training and continued through three weeks. Wheel training for a longer period of time did not produce further increases in soleus mass. Soleus hypertrophy was accompanied by increases in protein content which also plateaued by three weeks. Synthesis rates did not increase until after two weeks of training, then continued until the fourth week. The increase in muscle mass and protein content may be partially attributed to increasing rates of protein synthesis. Although synthesis rates were elevated by wheel training, other exercise regimens have been shown to either increase, decrease or remain the same depending on the type, duration and intensity of the training (Clark and Mitch, 1983; Dohm et al., 1980; Dohm et al., 1987; Kasper et al., 1990). In addition, although protein synthesis has not been previously measured in voluntary running animals, protein breakdown is enhanced in response to insulin in perfused hindlimbs (Rodnick et al., 1990).
Effect of Unweighting on Muscle mass, Protein Content, and Protein Synthesis in Normal Animals

In normal animals, unweighting of the soleus muscle for seven days produced significant atrophy. The amount of loss was slightly greater than has previously been observed after three days of unweighting (Chapter Three) and similar to the loss obtained after six days of unweighting (Satarug, 1987). The loss of muscle mass was associated with a significant loss of muscle protein. The loss in protein is similar to the loss observed in previous studies after seven days of unweighting (Babij and Booth, 1988; Steffen and Musacchia, 1984). In agreement with the loss of mass and protein content, fractional synthesis rates declined to 7.5%/day (two week) and 8.1%/day (three week) after seven days of unweighting. This decline at least partially accounts for the loss of protein during this time. These rates are comparable to the rates of 5.6% /day measured after six days of unweighting (Satarug, 1987). Comparisons of protein synthesis with other studies are difficult due to age related differences.

Effects of Unweighting on Muscle Mass, Protein Content and Protein Synthesis of Previously Trained Animals

A significant finding of this study was that previous training did not prevent atrophy of the soleus. This is demonstrated by the finding that WR-HS animals showed 35% less mass and 34% less protein at two weeks compared to WR animals. These differences were maintained at three weeks indicating that even longer periods of training does not prevent atrophy. In addition, soleus protein synthesis of WR-HS was lower at both two and three weeks compared to
WR animals. Therefore hindlimb suspension lowers protein synthesis whether or not animals are trained. This difference in synthesis was also maintained after three weeks of training. Therefore, the loss in mass and protein must be at least partially due to lower synthesis rates. In addition, three weeks of training led to a smaller difference in protein of suspended animals, despite a greater difference in synthesis. Therefore, relative to two week muscle, the three week trained muscle must have a more rapid rate of degradation. Taken together, these results provide evidence that training prior to hindlimb suspension does not prevent atrophy of the soleus. Although wheel running did not prevent soleus atrophy, protein content and synthesis were higher in soleus of WR-HS compared to HS animals after both two or three weeks. These findings are similar to previous findings that intermittent high load exercise during unweighting does not prevent atrophy, but can attenuate muscle loss (Herbert et al., 1988). Other studies have demonstrated that daily short bouts of grid climbing (Herbert et al., 1988) as well as long duration treadmill training (Graham et al., 1989; Thomason et al., 1987a), may also aid in the loss of muscle function during unweighting. Daily short duration weight support during suspension also ameliorates but does not prevent soleus atrophy (Hauschka et al., 1988). These studies provided information concerning the ability of exercise performed during or post suspension to prevent atrophy, and are in agreement that atrophy can not be prevented, but is attenuated using these models. These findings are similar to this investigation in that atrophy could not be prevented by wheel running prior to suspension, but that mass, protein content and synthesis may be maintained at higher values in animals which are trained prior to suspension. Therefore, although the atrophic response is not prevented by
wheel running, training may allow for faster recovery from suspension.
CHAPTER SIX

SUMMARY: PROTEIN AND CARBOHYDRATE METABOLISM; RESPONSE TO UNWEIGHTING ATROPHY

These investigations evaluated the alterations in protein and carbohydrate metabolism which occur from unweighting or exposure to microgravity. This was accomplished by 1) evaluating the time course of changes in protein synthesis and degradation of myofibrillar and sarcoplasmic proteins in unweighted soleus, 2) evaluating the effects of weightlessness on muscle mass, protein content, and insulin stimulated glucose uptake, and 3) assessing the role of exercise prior to unweighting on muscle mass, protein content and protein synthesis in unweighted soleus muscle. The results of these studies are summarized below.

Tail cast suspension leads to significant atrophy of the soleus muscle which occurs by day two of unweighting and continues through day four. Soleus atrophy is accompanied by a loss of myofibrillar proteins. A significant loss of myofibrillar proteins occurs by day two of unweighting and is greatest during day three. After day three the loss of this protein diminishes. The loss of myofibrillar proteins coincides with the time during which the greatest amount of atrophy occurs. In contrast to myofibrillar proteins, sarcoplasmic proteins are spared during unweighting. The loss of myofibrillar and sparing of sarcoplasmic proteins is accompanied by a parallel slowing of protein synthesis in both pools. Synthesis rates begin to slow during the first twenty four hours of unweighting and continue through day four. Although synthesis patterns are similar between
the two protein pools, degradation rates differ. In the myofibrillar pool, protein breakdown increases steadily during the first three days of unweighting then during the fourth day, protein breakdown declines dramatically. This decline in protein degradation accounts for the slower rate of myofibrillar protein loss which occurs after day three. In contrast, the pattern for breakdown of sarcoplasmic proteins shows a modest increase over the first two days of unweighting followed by a sharp decrease through day four. This pattern accounts for the sparing of these proteins during this time. It is apparent from these studies that the loss of total protein during unweighting is due to increased loss of myofibrillar proteins from increased degradation and decreased synthesis. Sarcoplasmic proteins are spared due to a decline in degradation.

Although the effects of spaceflight on skeletal muscle are documented in adult rats, the effects on muscle of juvenile rats had not been evaluated. Therefore, juvenile rats were exposed to weightlessness in order to validate tail cast suspension as a ground based model to mimic the effects of weightlessness on juvenile muscle. Similar to hindlimb suspension, spaceflight produces significant atrophy of the soleus, and reduced growth of the plantaris and gastrocnemius. In addition, the tibialis anterior and extensor digitorum longus are unaffected by microgravity. As with hindlimb suspension, exposure to microgravity significantly enhances in vitro insulin stimulated glucose uptake in soleus muscle. In contrast, glucose uptake is not enhanced in the extensor digitorum longus of either flight or suspended animals. This finding demonstrates that the enhanced insulin response is not systemic but is due to the effects of unweighting. In addition to in vitro findings, insulin stimulated glucose uptake is also enhanced in situ in unweighted soleus. This finding
provides evidence of an enhanced insulin response under more physiological conditions. In addition to insulin, IGF-1 also enhanced 2-deoxyglucose uptake in situ, suggesting that IGF-1 and insulin act in combination to produce enhanced glucose transport into unweighted muscle. These various studies indicate that hindlimb suspension appears to mimic the effects of microgravity on the hindlimb muscles of juvenile rats. Therefore, it is a suitable model for ground based studies. In addition, the increase in glucose uptake may be due to alterations in intracellular signaling which affects both insulin and IGF-1.

A main objective of this study was to gain further understanding of the relationship between hormone regulation of carbohydrate metabolism and protein turnover of unweighted muscle. Previous studies from this laboratory have indirectly demonstrated such a relationship. These studies evaluated the insulin and β adrenergic receptor systems. Both insulin and β adrenergic receptors show increased binding capacity per milligram of muscle without demonstrating changes in total receptor number. Together these studies provide evidence for an increase in hormone sensitivity in unweighted muscle and show that membrane proteins are spared in unweighting atrophy. Further studies using in vivo and in vitro lysosomotropic agents to study proteolytic mechanisms in unweighted muscle provided evidence that the sparing of membrane proteins is due to suppression of lysosomal degradation while increased structural protein degradation occurs from cytosolic breakdown (Tischler et al., 1990). The results of this investigation provide further direct evidence that sarcoplasmic (including membrane) proteins are spared during unweighting while myofibrillar proteins are lost. The relationship between hormone regulation and protein metabolism is further demonstrated by the
finding that muscle mass and myofibrillar proteins are lost concurrently with increased insulin and \( \beta \) adrenergic binding capacity. A decline in muscle mass, subsequent preferential loss of myofibrillar proteins and sparing of sarcoplasmic proteins leads to a relative increase in hormone binding capacity and therefore results in an increase in hormone sensitivity. Thus, the link between hormonal regulation of metabolism and protein turnover in unweighting is described by increased degradation of myofibrillar proteins and sparing of sarcoplasmic protein which leads to an enhanced hormone response both in vitro and in situ.

Although many studies have demonstrated a significant loss of muscle from unweighting or after exposure to microgravity, there are currently no methods which prevent atrophy. Some exercise countermeasures have attenuated the loss of mass and protein due to unweighting. To date, no model has been successful in preventing atrophy. In this study, the effects of exercise training prior to suspension on muscle atrophy were investigated. Training by voluntary wheel running proved to be an effective model to induce hypertrophy of the soleus muscle. However, two or three weeks of training followed by hindlimb suspension slowed but did not prevent atrophy of the soleus. Although training did not prevent atrophy, muscle mass and protein content were greater in soleus which had been trained prior to suspension. Therefore, voluntary exercise training prior to suspension provides some protection against the loss of muscle from unweighting.

The project presented here focused on the effects of unweighting on protein and carbohydrate metabolism. These studies contributed to the current knowledge of alterations in muscle metabolism from unweighting, but also raised questions concerning the mechanism of degradation, increased insulin
sensitivity, as well as the potential role of exercise in the prevention of atrophy. Further studies must be conducted to evaluate the role of synthesis and degradation induced from physiological perturbations such as denervation, or other muscle wasting disorders such as muscular dystrophy and cancer. In addition, the role of proteases in the degradation of myofibrillar proteins may be further delineated to identify various pathways of degradation. Varying exercise protocols such as training during suspension may be tested. In addition, other insulin like factors may be used to gain further information concerning the increase in insulin sensitivity. In conjunction with exercise, proteolytic inhibitors may be tested for possible prevention of muscle atrophy from unweighting.

In conclusion, tail cast suspension is a unique model which may be used to study the effects of weightlessness on muscle metabolism. Changes in both protein and carbohydrate metabolism from unweighting may be studied using this model, and preventative measures to prevent atrophy may be further studied using varying combinations of exercise training and tail cast suspension.
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