THE EFFECTS OF RESISTANCE EXERCISE ON IN VIVO CUMULATIVE SKELETAL MUSCLE PROTEIN SYNTHESIS

A Dissertation

by

HEATH G. GASIER

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

DOCTOR OF PHILOSOPHY

May 2009

Major Subject: Kinesiology

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Approved by:

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	James D. Fluckey
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	Stephen B. Smith
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ABSTRACT

The Effects of Resistance Exercise on In Vivo Cumulative Skeletal Muscle Protein Synthesis. (May 2009)

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An acute bout of resistance exercise (RE) and dietary protein consumption stimulate muscle protein synthesis (MPS). This anabolic effect is believed to be attenuated with resistance exercise training (RET), however, the mechanism for this 'plateau' is unknown. In addition, the ideal timing for protein consumption to optimize MPS is not well characterized. The *central hypothesis* of this research is that RE stimulates cumulative (measured over 24-36 h) MPS in rats and humans.

Study one determined whether an acute bout of RE in rats enhances MPS when assessed with the traditional flooding dose (~ 25 min) and ^2H_2O (4 and 24 h measurements); thus a comparison of the two methodologies was made. An acute session of RE did not result in an elevation in MPS when quantified by either the flooding dose or ^2H_2O over 4 and 24 h (methods compared qualitatively). Therefore, an acute bout of RE in rats does not appear to be anabolic and adaptation resulting from multiple bouts is likely necessary.

Study two determined if RET in rats results in attenuation in MPS (plateau

effect) 16 h following the final RE session (peak anabolic window) and if it is due to an increase in 4E-BP1 (a key regulator of mRNA translation initiation) activity; or if the timing in anabolism changes, which could be detected with a cumulative assessment (^2H_2O). MPS at 16 h was unchanged following RE training. Consistent with this finding, there were no differences in 4E-BP1 activity. Conversely, cumulative MPS was significantly increased with RET, suggesting a temporal shift in anabolism.

Study three determined if dietary protein consumed immediately following RE augments cumulative (24 h) MPS in young adult human males when energy and macronutrients are controlled. RE and post-RE protein had no effect on mixed MPS; however, myofibrillar MPS was significantly increased with RE suggesting specific changes within a heterogeneous protein pool.

Collectively, these are the first studies to assess changes in cumulative MPS with RE in rats and humans. The long term goals of this research are to understand muscle protein anabolism in ‘free-living’ mammals and the mechanisms that regulate this process.

ACKNOWLEDGEMENTS

I would like to thank my committee chairs, Dr. Steven Riechman and Dr. James Fluckey, and my committee members, Dr. Christopher Woodman and Dr. Stephen Smith, for their guidance and support throughout the course of this research. In addition, I would also like to extend my gratitude to Dr. Stephen Previs for his support with stable isotopic methodologies and to Dr. Alex Buentello for assisting me with amino acid analysis (HPLC).

Special thanks to all of the financial contributors: The National Institutes of Health (AG01025), Mouse Metabolic Phenotyping Center, Sydney and J.L. Huffines Institute for Sports Medicine and Human Performance, Gatorade Sports Science Institute, U.S. Poultry and Egg Association, Office of Graduate Studies and Department of Health and Kinesiology. I have learned that research cannot be pursued without monetary support.

Thanks to my colleague and friend Michael Wiggs for being next to me during much of this research; I couldn't have completed all of this work without your assistance. Thanks to all of the students who contributed to this research and subjects who were willing to participate in the human study. I would also like to thank my close friends Joshua and Sibyl Swift for always being there, particularly during difficult times. Additionally, thank you Julie Burney for your friendship and love, which allowed me to continue on at times when I didn't think that I could; I will always be indebted to you.

Also, I would like to extend my gratitude to my uncles Michael A. Morich

(Ph.D., Electrical Engineer-Philips Medical Systems) and John R. Shainoff (Ph.D., Retired Biophysicist-Cleveland Clinic) for being role models, for your kindness and for providing words of wisdom, no matter how insignificant it may have seemed. If I can obtain a fraction of the success that you both have worked so hard to achieve, I will consider my scientific career to be extraordinary.

Thank you Aunt Harriet and Uncle Ray, had it not been for your love and support, I would never been able to have earned my M.S. degree from Case Western Reserve University, thus pursue a doctorate here at Texas A&M. I will never forget your willingness to take me in as your own and support me during the difficult times.

Finally, thanks to my mother for showing me the strength that a human can possess and always believing in me, even when no one else did. I would never have achieved this, or anything else in my life, if you were not my mother. Thank you!

NOMENCLATURE

Ala (A)	Alanine
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
AMPK	5’ AMP activated protein kinase
A site	Aminoacylated-tRNA site
ATP	Adenosine triphosphate
CRT	Chronic resistance exercise training
DNA	Deoxyribonucleic acid
eEF	Eukaryotic elongation factors
eIF	Eukaryotic initiation factors
eRF	Eukaryotic release factors
ERK	Extracellular-signal regulated kinase
E site	Ejection site
FSR	Fractional rates of protein synthesis
Gly (G)	Glycine
Glu (Q)	Glutamine
GTP	Guanosine triphosphate
^2H_2O	Deuterium oxide
^2H	Deuterated
Met (M)	Methionine

mRNA	Messenger RNA
MPS	Muscle protein synthesis
OA	Oxaloacetate
PEP-CK	Phosphoenolpyruvate carboxykinase
Phe (F)	Phenylalanine
PI3K	Phosphatidylinositol 3-kinase
P site	Peptidyl-tRNA site
Pyr	Pyruvate
Ras	GTPase
RRF	Ribosomal recycling factor
rRNA	Ribosomal RNA
RNA	Ribonucleic acid
RPS	Rates of protein synthesis
RE	Resistance exercise
Ser (S)	Serine
Thr (T)	Threonine
tRNA	Transfer RNA
1RM	One repetition maximum

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CHAPTER I

INTRODUCTION

The field of skeletal muscle biology has evolved over the past 50 years, from defining structure and function to identifying a central role in the pathogenesis of many debilitating and chronic diseases (AIDS, cancer, sarcopenia, starvation, trauma and sepsis and several metabolic disorders). Much of the interest in skeletal muscle research can be attributed to its overall contribution to structure (40-45% of body mass with ~ 60% being protein) and function (primary reservoir for amino acids and glycogen, and thus energy transduction) (27, 134, 135). As a result of its structure and function, skeletal muscle accounts for ~ 30% of whole-body protein metabolism under fasting conditions, 35% with feeding and up to 45% following resistance exercise (RE) with the ingestion of mixed meals (135). Due to the increases with feeding and resistance exercise, designing experiments to include these perturbations can further our understanding of non-steady state protein anabolism which may ultimately result in identification of the defect(s) that regulate skeletal muscle metabolism and growth.

There is little known of the effects of a single bout of RE on stimulating muscle protein synthesis (MPS) in rats as the majority of the investigations define 'acute' as 2-4 bouts of RE (23, 50-52, 54-57, 84, 85). Conversely, it is well documented that a single bout of RE in humans increases MPS from 1-48 h (15, 35, 37, 41, 108). The accepted theory behind muscle hypertrophy resulting from resistance exercise training is that the

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This dissertation follows the style of *American Journal of Physiology*.

anabolic effects are cumulative; however, when making direct comparisons to acute resistance exercise, chronic resistance exercise training appears to result in an attenuated MPS response (50, 77, 107, 109). Therefore, it would seem likely that a rate limiting control step(s) within the myocyte signal transduction pathway exists, which ultimately serves as a regulator of muscle growth. Of the most likely candidates is an inhibitory protein, eukaryotic initiation factor (eIF) 4E-BP1, which functions downstream of a key muscle growth regulator, the mammalian target of rapamycin (mTOR) protein kinase (5, 21-23, 112, 115). Upon activation by mechanical strain, growth factors and nutrients, mTOR phosphorylates 4E-BP1 allowing liberation of eIF4E to bind the 5' cap structure of a transcript to promote the initiation of mRNA translation (22, 28, 65, 95, 112, 123). Therefore, increased expression and/or hypophosphorylation of 4E-BP1 may occur with chronic resistance exercise training, serving as a break on protein synthesis, thus resulting in a plateau in skeletal muscle growth.

As mentioned, the provision of macronutrients stimulates MPS. Of the three macronutrients (carbohydrates, protein, fat) protein (amino acids) appears to be the most potent stimulator of protein synthesis. The combination of resistance exercise and nutrients yield an interactive benefit in stimulating anabolism with a concomitant decrease in skeletal muscle breakdown (40, 80, 114, 127, 128). Thus, providing adequate nutrition, protein specifically, with resistance exercise would appear to maximize one's growth potential. Although the amount of protein to be consumed with resistance exercise has been suggested to be 1.3 to 1.8 $g \cdot kg$ body $mass^{-1} \cdot d^{-1}$ (1, 68, 103, 106, 107), the precise timing of protein ingestion with resistance exercise is unknown when

controlling for energy and macronutrient composition. Therefore, more research in the area of protein consumption (variable time of ingestion) with resistance exercise is warranted to determine the optimal dietary intervention for optimizing MPS and thus, muscle growth.

Labeled amino acids (tracer) have traditionally been used to measure tissue specific (direct incorporation approach) protein synthesis (146, 152); however, the results can often times vary based on the type of tracer employed in the study. In addition, the necessity for controlling experimental conditions (post-absorptive and stress free) limits the ability to investigate mammals in free-living environments. Moreover, it is known that a wide variety of conditions can alter muscle protein anabolism (e.g. feeding, fasting, sleep, stress and activity). To circumvent these issues, an alternative tracer (deuterium oxide - ^2H_2O) has been purported to provide accurate measurements of tissue specific protein synthesis over short and prolonged periods of time non-invasively (8, 30, 44, 111, 151). To date, no investigation has examined in vivo cumulative skeletal MPS with ^2H_2O to include known perturbations (food ingestion and resistance exercise) in the assessment.

The overall objectives of this research were to determine if acute resistance exercise in rats is anabolic, if chronic resistance exercise in rats leads to an inhibition in MPS which is explainable by over-expression of 4E-BP1, and to establish whether the manipulation of the diet (timing of protein ingestion) following resistance exercise in humans influences muscle protein synthesis. Moreover, the *central hypothesis* of this research is that RE stimulates cumulative (over 24-36 h) MPS in rats and humans. In

order to conduct these experiments, a validation study between ^2H_2O (alternative, allows long-term measurements) and flooding dose (traditional, limited to short-term measurements) was necessary.

The findings from this research may change our current understanding of what occurs with resistance exercise and muscle protein synthesis. Furthermore, these data will assist muscle biologists in experimental design to encompass every day normal physiology/biochemistry which provides an overall picture of muscle anabolism. In addition, these results will be foundational for which comparisons can be made to with investigations in debilitating and metabolic disease states.

CHAPTER II

BACKGROUND

Protein Synthesis Overview

Protein synthesis (translation) is the process by which genetic information contained in transcripts (messenger ribonucleic acid, mRNA) is used to produce sequences of amino acids, or proteins (136). An adult male (~ 70 kg) contains roughly 12 kg of protein (7 kg in skeletal muscle) and 220 g of free amino acids (120 g within skeletal muscle cytoplasm and 5 g in the circulation) (134). The synthesis of proteins is among the most energetically costly biological processes as 4 mol of adenosine triphosphate (ATP) or equivalent (1 mol ATP releases 20 kcal) are required per mole of amino acid incorporated into protein (145). The process by which translation occurs is a coordinated series of events that, similar to deoxyribonucleic acid (DNA) replication and transcription, can be divided into three sequential stages; initiation, elongation and termination (136). In order to carry out each of these steps, there are four key components required: mRNA (80% of RNA), transfer RNA (tRNA,15% of RNA), ribosomal RNA (rRNA, 5% of RNA) and aminoacyl tRNA synthetases (136). The remainder of this section will focus on each of the three translational steps.

Translation initiation is the primary rate–limiting step in mRNA translation (116) and is controlled by many proteins that are activated and/or inhibited by extracellular signals. The process can be divided into three stages: i) the binding of initiator methionyl-tRNA (met-tRNA$_i$) to the 40S ribosomal subunit to form the 43S preinitiation

complex, ii) the formation of the 48S preinitiation complex by binding of the mRNA to the 43S preinitiation complex and iii) the binding of the 60S ribosomal subunit to the 48S preinitiation complex to yield the functional 80S initiation complex (78) (Fig. 1, page 7). The first stage occurs by the formation of a ternary complex which consists of eIF2 (GTPase), GTP (guanosine triphosphate) and met-tRNA$_i$ and attachment on the P site (binding site for the peptidyl-tRNA) of the 40S subunit with the assistance of eIF5B-GTP (GTPase) (22, 78, 91, 136). In addition, eIF1A (inhibits early hydrolysis of the ternary complex) and eIF3 (inhibits reassociation with the 60S subunit) bind to the aminoacylated-tRNA site (A site) and the ejection site (E site) respectively (91, 136). Prior to the formation of the 48S preinitiation complex, a three-subunit protein, eIF4F (comprised of eIF4E, eIF4G and eIF4A) must recognize and bind to the 5' cap (7-methylguanosine-triphosphate, m^{7}pppN, where N is any nucleotide) of the mRNA (91, 95, 123, 136). More descriptively, eIF4E binds directly to the 5' cap structure, eIF4G is a scaffold and eIF4A is an ATP-dependent RNA helicase that unwinds secondary structures (hairpins) once activated by eIF4B (an RNA binding protein) (91, 95, 123). Next, eIF4F, eIF4B and the transcript can bind to the 43S preinitiation complex via an eIF4G and eIF3 interaction (91). Prior to the final stage (80S initiation complex formation) the 48S preinitiation complex scans the mRNA (5' → 3') in an ATP-dependent manner for the start codon (AUG) (136). Correct base-pairing (codon on mRNA and anti-codon on tRNA) triggers the release of eIF2 (GTP hydrolysis), eIF3 and eIF4B allowing the 60S subunit to associate with the 48S complex. (136). The formation of the 80S complex stimulates eIF5B-GTP hydrolysis resulting in the release of eI5B,

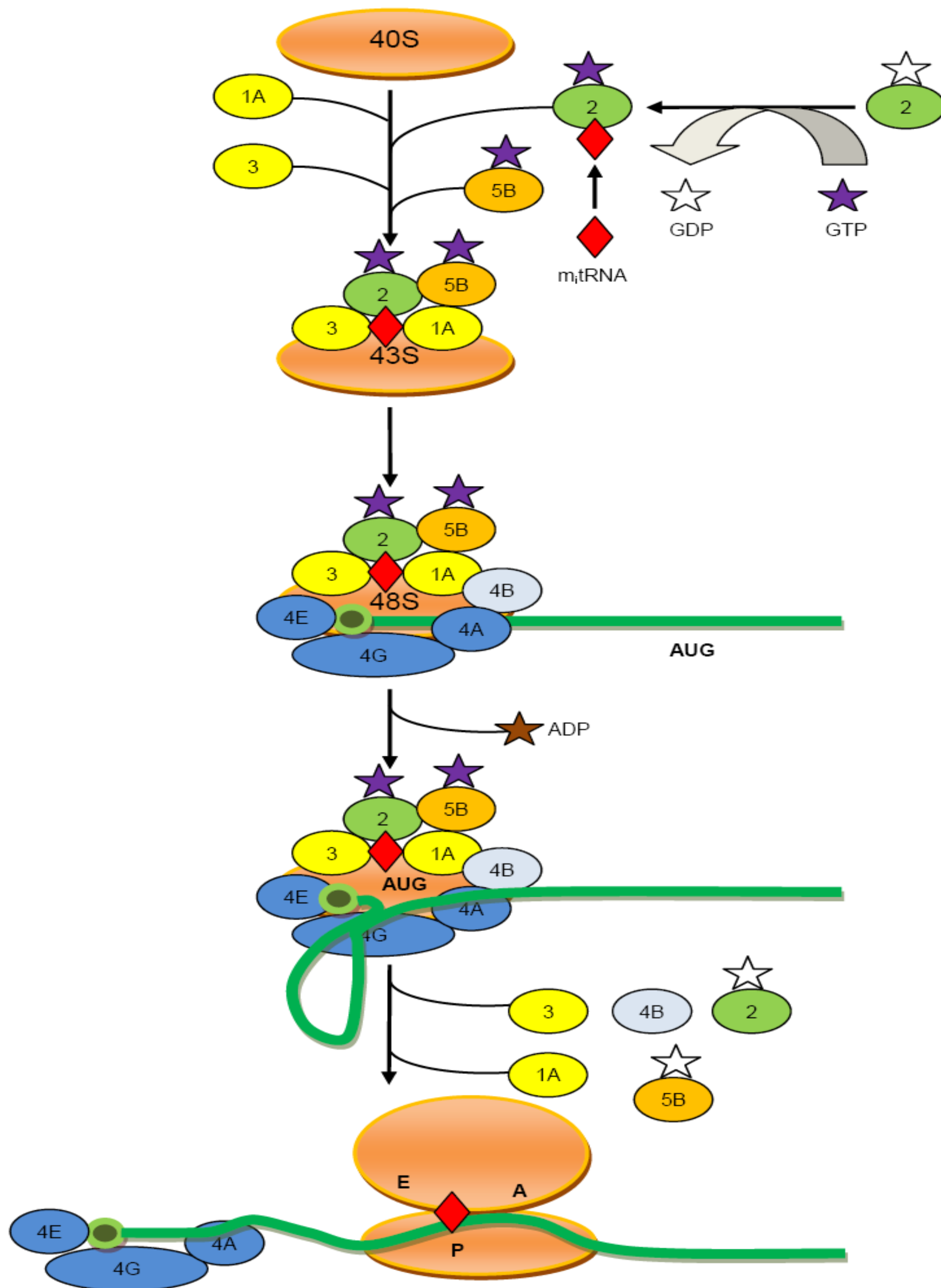


Fig. 1. Translation initiation. The figure illustrates the three stages (see text for detailed description) of translation initiation. Stage 1, the formation of the 43 preinitiation complex (initiator methionyl-tRNA binds to 40S); stage 2, the formation of the 48 preinitiation complex (mRNA with eIF4F binds to 43S) and stage 3, the formation of functional 80S ribosome (60S binds to 48S).

eIF1A and the correct placement of met-$tRNA_i$, triggering translation initiation (136). In order for another round of initiation to occur, the GDP (guanosine diphosphate) bound to eIF2 must be exchanged with GTP, which occurs via eIF2B (guanine nucleotide exchanger) (78).

Translation elongation occurs once the met-$tRNA_i$ is placed into the P site of the assembled 80S ribosome and can be explained by three sequential steps. Prior to step one and the addition of amino acids to the peptide chain, the tRNA is charged via adenylylation with the correct amino acid and the transfer of the adenylylated amino acid to the tRNA (136). Step one consists of the aminoacyl-tRNA moving into the A site of the ribosome with the assistance of eukaryotic elongation factor (eEF) 1A-GTP (112). Upon GTP hydrolysis, eEF1A-GDP is released and the aminoacyl-tRNA is moved into position of the peptide in the P site (136) with the resulting eIF1A-GDP getting recharged by the guanine nucleotide exchange factor, eEF1B (112). The second step is the formation of the peptide bond between the existing peptidyl-tRNA in the P site and the aminoacyl-tRNA in the A site (where the growing peptide is shifted to) and is catalyzed by peptidyl transferase (136). Thirdly, the peptidyl-tRNA is translocated from the A site to the P site with the assistance of eEF2-GTP (GTP hydrolysis) (112). In addition, the ribosome moves by one codon relative to the mRNA with the previous peptidyl-tRNA moving into the E site.

The final stage of translation, termination, can be explained by two sequential steps, followed by the dissociation of the 80S ribosome. First, eukaryotic release factor (eRF) 1 recognizes one of three stop codons on the mRNA (UAG, UGA or UAA) by a

peptide anticodon (136). Additionally, eRF1 possesses a glycine, glycine and glutamine (GGQ) motif that is located in close proximity to the 3' end of the peptidyl-tRNA, which results in peptide hydrolysis (136). Step two involves the binding of eRF3-GDP to the eRF1 within the intact ribosome that still has two deacylated tRNAs bound to the P and E sites. The interaction between eRF3-GDP and eRF1 results in GTP exchange with eRF3-GDP and subsequent hydrolysis resulting in the dissociation of eRF1 and eRF3 from the ribosome (136). Now that the peptide has been synthesized, the ribosome must release from the mRNA so that another round of translation can occur. Although not completely understood, it is believed that this is accomplished by a ribosomal recycling factor (RRF), eEF2-GTP and eIF3. More specifically, the RRF binds to the A site (mimicking a tRNA) and recruits eEF2-GTP to the ribosome, followed by hydrolysis which stimulates the release of the uncharged tRNAs from the P and E sites (136). Finally, eIF3 is recruited into the E site of the 40S subunit causing release of eEF2-GDP, RRF the 60S subunit and mRNA (136). The machinery is now readily available to undergo another cycle of mRNA translation.

The process of protein synthesis occurs with near flawless precision (no more than 1 in 1000 amino acids are erroneously incorporated to the growing peptide (136)) upon initiation. The process may be regulated in each phase of translation; however, since translation initiation is the rate limiting phase of protein synthesis, studies focusing on control and regulation of protein metabolism at this phase are of interest. In order for translation to occur, stimuli such as dietary factors, hormones and mechanical strain must transduce signals from the external milieu to the site of initiation. The following

sections will describe the effects of nutrition, resistance exercise and their interactive role in modulating muscle protein synthesis, followed by the regulation (signal transduction) of protein synthesis with special emphasize placed on the control of cap dependent translation initiation (primary process by which eukaryotes translate mRNA) by 4E-BP1.

Response of Muscle Protein Synthesis to Feeding

As previously mentioned, skeletal muscle is the major reservoir for free amino acids and therefore, ingestion of protein or amino acids serve to maintain the preexisting muscle protein pool. The process by which this occurs can be explained from the point of mastication. More specifically, upon ingestion of protein, the stomach pH (HCl release) stimulates the release of pepsin from parietal cells which cleaves the proteins into smaller peptides that then pass into the small intestine (73). The peptides become further hydrolyzed into amino acids and di-and tri-peptides by pancreatic enzymes (trypsin, chymotrypsins, elastase and carboxypeptidases), followed by uptake into mucosal cells (active transport) where the small peptides undergo final hydrolysis into amino acids (73). Next, the absorbed amino acids are either metabolized within the enterocyte or enter the portal circulation and carried to the liver where a portion are used and the remainder pass through the systemic circulation to be utilized by the periphery (73). Upon entering a tissue bed (skeletal muscle) the amino acids can either pass directly into the venous circulation, or be transported into the muscle via amino acid transporters where they can charge tRNA and be incorporated into protein and/or

undergo metabolism (Fig. 2, page 11) (14, 135). If the availability of amino acids from the extracellular pool become limited (20), muscle protein degradation may ensue, as well as de novo synthesis. The fate of the latter is either recycling for muscle preservation and/or release into the venous circulation to be transported to the kidney and liver (gluconeogenesis) or other tissues in need of amino acids for protein production. Therefore, the maintenance of skeletal muscle is a balance between synthesis and breakdown and feeding stimulates synthesis and depresses breakdown (129).

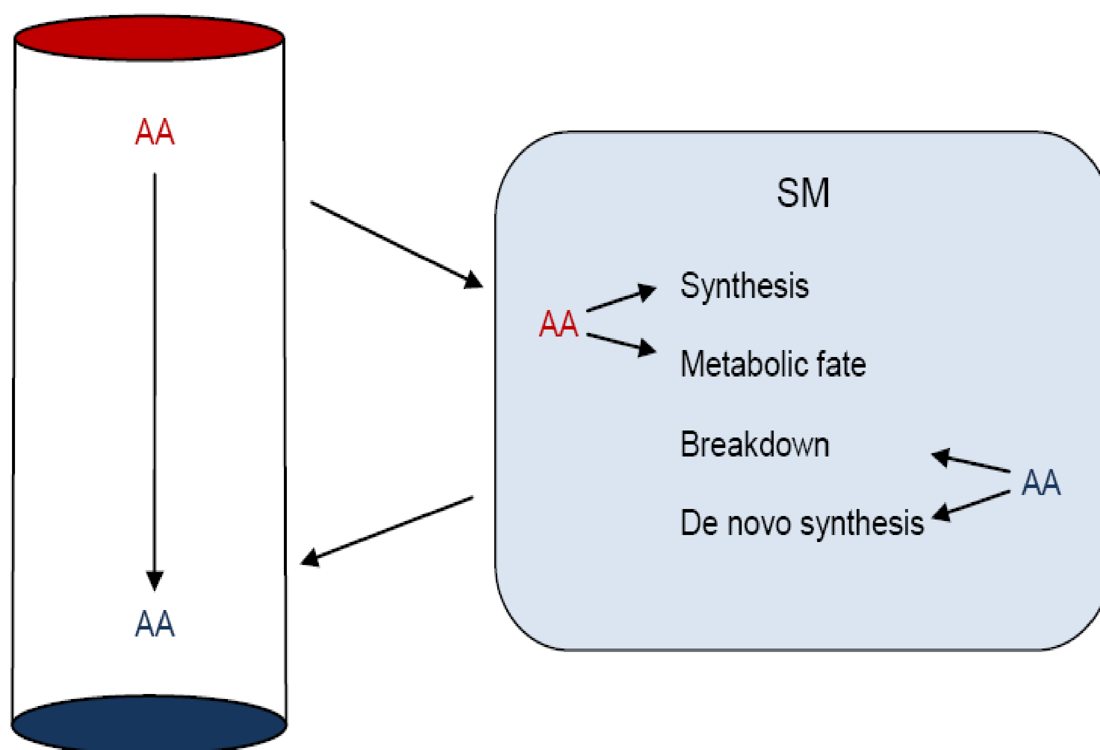


Fig. 2. General model of muscle protein metabolism. Systemic amino acids (AA) can enter skeletal muscle (SM) via amino acid transporters and charge tRNA for protein synthesis and/or undergo metabolism (disappearance). Intracellular amino acids from protein breakdown and de novo synthesis can enter the venous circulation along with amino acids that continue directly from artery to vein without intracellular uptake (appearance), or can be recycled for protein synthesis.

Much of the research over the past 25 + years has focused on the provision of amino acids to stimulate muscle protein synthesis in rats (61, 76), pigs (137) and humans (11, 12, 20, 58, 64, 96, 97, 99, 100). The reasoning for this experimental rationale is threefold. First off, although glucose and fat are the primary energy substrates under resting and exercising conditions, they do not appear to directly stimulate protein synthesis in the absence of amino acids (122). However, they may become instrumental when the energy status becomes low (increased AMP:ATP and 5'AMP-activated protein kinase, AMPK), which occurs during starvation and exercise; or through the actions of hormones, such as an increase in insulin secretion resulting from increased glucose (129). Secondly, even though mixed meals stimulate muscle protein synthesis (60, 151), amino acids (essential/indispensible) alone do not require digestion and are rapidly absorbed, creating hyperaminoacidemia in the systemic circulation which appears to modulate muscle protein synthesis (20). The mechanism by which this occurs has been suggested to be via mass action (increased intracellular availability of amino acids charge available tRNA), signal transduction of translation initiation or the presence of a membrane-bound sensor that transmits a signal to initiate translation and is independent of amino acid transporters (20, 129). To date, the evidence is mounting in support of signal transduction control, primarily through leucine in stimulating mTOR; however, it is premature to discount mass action and the presence of a membrane bound sensor due to the necessity for amino acid transport. Thirdly, the measurement of muscle protein synthesis is difficult to accurately assess under postprandial conditions due to the perturbed state of the precursor pool (plasma or

intracellular), which results in dilution of the tracer and the potential for false quantification of fractional synthesis rates of protein synthesis (FSR). Therefore, the experimental conditions generally require fasting conditions (overnight fast) and if meals are to be provided during the tracer infusion, caution is warranted, as liquid-only meals or parenteral nutrition with a precise amount of tracer must be added. Thus, at least in the post-absorptive state, nutrient ingestion (primarily indispensible amino acids) stimulates muscle protein synthesis.

Response of Muscle Protein Synthesis to Resistance Exercise

Studies examining the effects of an acute bout of resistance exercise in the post-absorptive state have reported increases in mixed (15, 41, 50, 52, 57, 77, 84, 108, 109), myofibrillar (77, 94) and sarcoplasmic (94) muscle protein synthesis in animals and humans from 1-72 h following the session; as well as a concomitant increase in breakdown (41). The increases may be attributable to the accelerated amino acid transport that occurs in coordination with increased blood flow (15, 16, 153). However, it is worth mentioning that Roy et al. (117) (resistance exercise in humans) and Fluckey et al. (54) (electrical stimulation in rats) did not observe a stimulatory effect following a single bout. The discrepancies have been proposed to be a result of the training status in humans (129) and either an excess in the stimulus provided and/or the necessity for adaptation to occur in rats. In support of this, Roy et al. (117) recruited subjects who had been participating in a regular resistance exercise training program and Farrell et al. (50, 52), Fluckey et al. (57) and Kubica et al. (84) described “acute” as being 4 progressive

bouts of resistance exercise (weighted Velcro vest protocol). One could argue that 4 sessions of resistance exercise provides more than just a “brief” stimulus to the muscle and an adaptation is actually occurring. To further strengthen this argument, Fluckey et al. (57) reported increases in total RNA content (translational capacity), which would only be expected to be observed after successive bouts of muscle overload versus acute tension (generally explainable by enhanced translational efficiency, rate of synthesis relative to RNA) (69). Thus, as a whole, the data support the notion that acute resistance exercise in the post-absorptive state increases muscle protein synthesis in humans (unknown in rats) and what has traditionally been referred to as ‘acute’ in rats may actually be an early trained state. Finally, although muscle protein synthesis is increased, muscle protein degradation is also increased and the overall balance remains negative (15, 108).

Less is known of chronic resistance exercise training and protein balance (post-absorption); likely due to the degree of complexity and cost that a regular, supervised training program adds to the design. The belief is that the anabolic effects that occur with acute resistance exercise become cumulative over time and are responsible for muscle hypertrophy. However, if this were the case than the growth potential for mammals would essentially be unlimited. Thus, it could be that there is a plateau effect with skeletal muscle growth resulting from resistance exercise training.

The results of the studies that have been reported are mixed, which can likely be explained by methodological differences. For instance, Yarasheski et al. (150) reported a 57% and 155% increase in FSR in young and aged subjects respectively, following two

weeks of daily resistance exercise training; however, the researchers compared a basal sample (prior to training) to the post-training sample (3 h following the final exercise bout). Therefore, question arises as to whether it was truly the training or the acute effect of exercise. To answer this question, Phillips et al. (109) compared resistance exercise trained (≥ 5 yr) to untrained young adults following an acute bout of unilateral leg exercise. They (109) observed an exercise effect in stimulating muscle protein synthesis (within 4 h following the session) above resting values; however, no differences between the trained (~ 0.067% $\cdot$ h^{-1}) and untrained (~ 0.080% $\cdot$ h^{-1}) subjects. Additionally, breakdown was increased by ~ 37% in the untrained following exercise, but did not change in the trained group, thus the net balance of protein accretion was in favor of the trained individuals. However, the subjects did not actually participate in a supervised resistance training program and were, instead, recruited by self reporting their training history. To alleviate this methodological limitation that may exist with a self-reported training history, others have conducted supervised resistance exercise training from 8-12 weeks in rats (50) and humans (77, 138). The findings are suggestive that the acute elevations in muscle protein synthesis no longer persist following a resistance exercise training program in the mixed muscle homogenate at a time when an acute increase is observed (50, 77); however, the myofibrillar fraction is either increased (77) or decreased (138). The discrepancy in the myofibrillar FSR can be attributed to the methodological differences (i.e. tracer used, time of measurement, length and type of training program implemented). Therefore, more research examining the effects of chronic resistance exercise and muscle protein synthesis is needed to further elucidate

whether or not a plateau does in fact exist, or if there is just a shift in the timing by which the process of translation is carried out.

Interactive Effects of Nutrition and Resistance Exercise

Since feeding stimulates muscle protein synthesis and undergoing an acute bout of resistance exercise increases synthesis and breakdown (14, 93, 129, 144), consuming nutrients with resistance exercise would seem to yield a synergistic benefit (effect greater than the sum) on skeletal muscle anabolism. Although the research in this area supports the notion that nutrient ingestion (amino acids specifically) in conjunction with resistance training are required for an optimal anabolic response, the data are not conclusive. Three questions have yet to be convincingly answered: First, what should the composition of the meal be and what time should the nutrients be ingested; second, does resistance exercise training alter the response in the fed state and third, should the measurements be made following an overnight fast with only the addition of the selected nutrients during isotopic infusions? This section will describe the research that has been conducted in this area (in the context of protein kinetic assessment) and illuminate some of the potential limitations of these studies.

Studies examining the effect of feeding prior to resistance exercise are scarce, which may be due to the need for a post-absorptive state with traditional tracer infusion protocols. The studies that have been conducted are conflicting, as Tipton et al. (128) observed increased amino acid uptake, phenylalanine disappearance (indirect indicator of MPS) and protein balance when an essential amino acid (6 g) + carbohydrate (35 g

sucrose) beverage was provided just prior to versus following resistance exercise (2 h) following an over-night fast. However, when the same group compared pre and post-resistance exercise (immediately before and 1 h after) whey protein (20 g) consumption on the same parameters, no differences were observed (126). The authors (126) concluded that the differences were because amino acids require little time for absorption as compared to whey protein; however, they did not make direct comparisons between amino acids and whey protein rendering this conclusion speculative. Moreover, the subjects were assessed following an over-night fast over ~ 5 h, thus providing any nutrients either before or after would serve to augment the increase in protein synthesis from resistance exercise (no control was used). To further clarify whether pre-resistance exercise nutrient ingestion is anabolic, Fujita et al. (59) compared an essential amino acid (20 g) + carbohydrate (27 g) supplement to an over-night fasted group, followed by an acute bout of resistance exercise. Unlike the previous investigations, Fujita et al. (59) directly measured muscle protein synthesis and observed no differences 2 h following exercise when providing amino acids + carbohydrate prior to resistance exercise. As a consequence of the above mentioned studies, it remains inconclusive whether providing nutrients prior to resistance exercise following an over-night fast is synergistically anabolic, at least in the short time-frame following the exercise session (1-5 h).

Similar to the concerns with maintenance of steady state isotopic enrichments in pre-resistance exercise feeding, little work has been reported with feeding during resistance exercise on muscle metabolism. Of the three published reports, Chesley et al. (35) was the first to address this question by assessing protein synthesis in

the biceps brachii 4 and 24 h following resistance in humans with mixed meal supplements (Ensure Supplement, ~ 50% of the subjects reported mean daily intake) during the tracer infusion. They (35) observed a 50% increase in FSR 4 h and 109% increase 24 h following exercise when comparing the exercised to the non-exercised arm in the subjects. However, it is not clear whether the subjects were assessed in a fed or fasted state and whether tracer was added to the supplements to prevent isotopic dilution, limiting the study's interpretative value.

Miller et al. (94), using a unilateral leg protocol, assessed myofibrillar and sarcoplasmic protein synthesis with mixed meal supplements (1.4 x basal metabolic rate) provided during the exercise and tracer infusion protocol. The investigators (94) reported significant elevations in both muscle fractions from 6-72 h following an acute bout of resistance exercise. Although this group suggested that this occurred in a fed state, the protocol was initiated following an overnight fast, thus one could argue that this truly wasn't a postprandial assessment. More recently, Beelen et al. (9) examined the response of protein (casein, 21 g) + carbohydrate (glucose + maltodextrin, $0.15 \text{ g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) supplemented during 2 h of whole body resistance exercise under postprandial conditions. Thus, that may represent the first study (11) to assess protein balance and muscle protein synthesis in the fed state (early evening) versus following an overnight fast, enhancing the practicality of the design. However, since no tracer was added to the supplements, the precursor enrichment (plasma) likely became diluted and yielded a false elevation in FSR. If, in fact, the investigators (9) used the protein-free enrichment as the precursor, no differences between carbohydrate and protein + carbohydrate would

have been observed. Therefore, although this design is promising for future studies in the area of nutrition and resistance exercise, the question as to whether providing nutrients during resistance exercise enhances anabolism remains inconclusive.

In contrast to 'pre' and 'during' resistance exercise designs, a substantial amount of experiments have been conducted following resistance exercise with nutrient manipulation. In regards to carbohydrate alone, providing 1 $g \cdot kg^{-1}$ of glucose (117) or 100 g of maltodextrin (25) following resistance exercise (assessment made following an over-night fast) did not result in stimulation of tracer disappearance (glucose) or muscle protein synthesis (maltodextrin). However, 3-Methylhistidine excretion and urinary urea nitrogen (indicators of protein breakdown) were decreased with glucose administration (117). Moreover, there was a decrease in tracer appearance and thus, an improvement in overall net protein balance (25) with carbohydrate administration. In both of these studies (25, 117), insulin was increased significantly with carbohydrate, which has been suggested to play a permissive role in stimulating muscle protein synthesis, as well as an instrumental role in inhibition of muscle protein breakdown (36, 66, 104). When examining the role of either a complete (16) or essential (127) amino acid mixture under fasting condition, increases in muscle protein synthesis were reported (~ 150% over non-hyperaminoacidemia) in the former and net protein balance significantly improved in the latter, suggesting that only indispensible amino acids are required. Furthermore, when combining carbohydrate with indispensible amino acids (6–45 g), mixed muscle protein synthesis increased from 1-6 h (40, 42, 114) and myofibrillar and sarcoplasmic FSR increased from 6-24 h (37) post-resistance exercise. Additionally, net protein balance

significantly improved with the combination of the amino acid + carbohydrate supplement (40, 114). However, it is worth mentioning that this nutrient augmented response was delayed in the aged (70 y) 6 h versus immediately following exercise (42). Taken together, these results suggest that hyperaminoacidemia with a concomitant increase in muscle blood flow, as well as the inhibitory action of insulin on breakdown, augment muscle anabolism following resistance exercise.

Although the post-resistance exercise increase in muscle protein synthesis and/or muscle protein balance reported with either carbohydrate and/or amino acids (indispensible) is an appealing approach to optimize muscle anabolism, one must consider that humans consume mixed meals on a daily basis and not single macronutrients. Koopman et al. (83) compared the effects of post-resistance exercise consumption of either carbohydrate (glucose + maltodextrin, ~ 122 g), carbohydrate + whey protein hydrolysate (~ 82 g whey) or carbohydrate + whey protein + leucine (+ 41 g leucine) on muscle and whole body protein balance (fasted assessment). Their findings were suggestive of a stimulatory effect on muscle protein synthesis (6 h following exercise) with both carbohydrate + whey and carbohydrate + whey + leucine; and an overall improvement in whole body protein balance, although the differences in the latter finding were minimal. In addition, Koopman et al. (82) (using a similar protocol as above (83) with the exception that there was no carbohydrate + protein group) reported elevations in both young and aged subjects, supporting the notion that there is not an impairment in protein synthesis stimulation in the elderly. More recently, the same group (80) (using a similar design) observed no added benefit on muscle protein synthesis and

whole body protein balance with either low (0.15 g · kg^{-1} · h^{-1}) or high (0.6 g · kg^{-1} · h^{-1}) carbohydrate supplemented with casein protein (hydrolysate) in the 6 h following resistance exercise. The discrepancy between the two studies can likely be attributed to the sufficient rise in insulin (~ 17 μU/ml) with casein protein in the latter study (80). To determine the effects of food on muscle protein synthesis following resistance exercise, Wilkinson et al. (141) and Elliot et al. (46) examined whether milk consumption (fasted conditions) following resistance exercise stimulates muscle anabolism. Both observed an anabolic effect, such that the Wilkinson et al. (141) reported a significant increase in synthesis rates and muscle protein balance and Elliot et al. (46) observed an increase in muscle protein balance. These results suggest, at least under post-absorptive conditions, that intact proteins with carbohydrate, which can be found in an inexpensive food item similar to milk, may be advantageous in augmenting muscle protein synthesis following resistance exercise. In addition, the optimal amount of indispensible amino acids and intact protein that have been used appears to be between 6-15 g and ~ 20 g respectively.

There are limited data on the response of chronic resistance exercise to feeding. The results that have been reported vary due to the differences in methodological approaches, making it difficult to draw solid conclusions. In addition, during training, typical approaches to these studies are that subjects consume their own meals daily and the researchers gain insight by periodic diet records, which can fail in precision as subjects may underreport by as much as 10-40% (73). The first study to examine the effects of 12 weeks of whole body resistance exercise training (5 days per week) with ingestion of meals during the tracer infusion (no tracer added) was performed by

Yarasheski et al. (149). They (149) found a 38% increase in muscle protein synthesis ~ 20 h following the last training session compared to pre-training resting values, as well as an increase in strength and muscle mass. To overcome the limitation of this comparison, Phillips et al. (107) assessed muscle protein synthesis in the 6 h following an acute bout of unilateral resistance exercise (contralateral leg served as control) prior to and following 8 weeks (6 days per week) of whole body resistance exercise training. In addition, a casein (10 g) + glucose (75 g) supplement was provided following each session and a mixed supplement was provided during tracer infusion (no tracer was added). The findings were an increase in resting muscle protein synthesis, but an attenuation in the post-resistance exercise values (no change from basal levels) (107). This may be explained by the training protocol (whole body) versus acute bout of unilateral resistance exercise used prior to and after training for protein turnover measurement. Thus the same group (124) performed a similar study, however, only trained one leg for 8 weeks to maintain consistency with the unilateral leg protocol for protein turnover assessment. In addition, they (124) made the measurements with mixed meals (tracer added) 4 h and 28 h following an acute bout of resistance exercise. The data revealed that exercise training increased muscle protein synthesis by ~ 162% following acute exercise in the trained leg compared to acute exercise prior to training; however, at 28 h the values returned to normal in the trained leg. In addition, acute resistance exercise in the untrained leg following exercise training increased muscle protein synthesis by 108% (4 h) and 70% (28 h) post-exercise. Although humans typically undergo whole body resistance exercise training programs, the unilateral leg

protocol is likely ideal for determining the response of chronic exercise to nutrients as a consequence of the protein kinetic assessment protocol being similar. Recently, Wilkinson et al. (140), using a similar unilateral leg protocol, observed an increase in resting and post-acute resistance exercise myofibrillar protein synthesis following 10 weeks of unilateral leg exercise training; however, mitochondrial FSR was decreased following the acute bout of resistance exercise with training. Therefore, the fraction of muscle protein being synthesized may be masked when mixed muscle homogenates are assessed (common), although one must wonder if 4 h is sufficient to assess a detectable amount of enrichment in the myofibrillar fraction due to the slower turnover properties. Finally, although nutrients were provided during all of the tracer infusion protocols, all measurements were initiated following an over-night fast.

Should the assessment of muscle protein turnover be made under post-absorptive conditions? Recently (as mentioned above) Beelen et al. (9) examined protein kinetics with resistance exercise at the end of the day that included regular feeding, thus measurements were made in the postprandial state. In addition, they added nutrients during the tracer infusion protocol to determine whether additional protein (above what humans consume during daily feeding) augments acute exercised induced muscle protein synthesis. The authors made the following statement to justify their design: "Studies that have reported on the benefits of protein ingestion on postexercise recovery generally investigate subjects in the overnight fasted state. Under these conditions, it might be speculated that endogenous amino acid availability from the gut and/or the intramuscular free amino acid pool might be limiting. Such postabsorptive

conditions differ substantially from normal everyday practice in which recreational sports activities are generally performed in the evening in a fed state. Thus far, no data are available on the effect of protein and carbohydrate coingestion on muscle protein synthesis during resistance-type exercise under normal, fed conditions (9)." More than a decade has past with differing study designs, but a common theme in all is assessments are made in a post-absorptive state. Thus, the improvement in muscle protein balance with nutrient supplementation may really only represent a return to baseline following 10 h of fasting with an acute bout of exercise. If we are to ultimately determine whether nutrients (protein specifically) augments the anabolic process, then muscle biologists should assess muscle protein anabolism over extended periods of time to include activities of daily living, feeding, exercise, stress and sleep. The major limitation in doing so is the influence feeding has on steady state tracer enrichments and amino acid recycling when using labeled amino acids; however, the use of an alternative stable isotope (^2H_2O) eliminates these concerns (will be discussed in the ^2H_2O section below).

In summary, providing protein or amino acids with carbohydrate in combination with resistance exercise may serve to optimize anabolism, although the optimal time to ingest these nutrients has yet to be elucidated. The degree of anabolism varies with reports in fasted-state human muscle FSR ranging from ~ 0.02-0.06% $\cdot$ h^{-1} under resting conditions, ~ 0.04% $\cdot$ h^{-1} during exercise and between ~ 0.04-0.12% $\cdot$ h^{-1} in the hours following resistance exercise. In the fed state, muscle FSR range from ~ 0.04-0.07% $\cdot$ h^{-1} under resting conditions, ~ 0.04-0.07% $\cdot$ h^{-1} during and ~ 0.05-0.14% in the hours following resistance exercise. The variability in the values can be attributed to the

different designs employed to investigate resistance exercise under fed and fasted conditions; such as the isotope used, the precursor surrogate used (plasma, intracellular protein-free or aminoacyl tRNA), the feeding regimen (amino acids, protein, with or without carbohydrate and lipid), the training state and the fraction of muscle protein assessed (mixed, myofibrillar, sarcoplasmic and mitochondrial).

As opposed to human studies, muscle protein synthesis assessed in animals is commonly determined by the amount of amino acid (nmol) incorporated into a known amount of tissue per unit of time, accounting for the protein pool versus just the enrichments of labeling. The values in the mixed gastrocnemius (commonly assessed) range from ~40-100 nmol in non-exercised animals to ~ 40-200+ nmol in those that have undergone resistance exercise. Similar to humans, these values can vary depending upon the muscle type and the different training protocols. Thus, when making comparisons between investigations, one must use caution and base results qualitatively versus quantitatively and in the context by which the study was conducted. Finally, prior to making conclusive statements in regards to the influence that feeding has in augmenting exercised induced elevations in muscle protein synthesis; more research is warranted with designs that allow for the inclusion of normal daily routines.

Control of Protein Synthesis by 4E-BP1

The independent and interactive anabolic effects of nutrients and exercise are mediated through changes in signal transduction. The signaling networks connect the external environment to that of the intracellular milieu leading to the initiation of mRNA

translation, which is the rate limiting step in mRNA translation (116). The process is highly complex and coordinated, involving several proteins that serve to activate and/or inactivate one another based on the presented stimuli. The end result is activation and acceleration in the rates of protein synthesis, and thus, hypertrophy. Although a great deal of research in the area of skeletal muscle signal transduction control with nutrition and mechanical strain has been conducted, the comprehensive picture remains to be fully elucidated. One of the primary reasons for this is the number of pathways and cross-talk that are involved in initiating or inhibiting translation. To date, there appears to be three distinct signaling cascades that directly or indirectly mediate the rate limiting step in mRNA translation initiation, the binding of eukaryotic initiation factor (eIF) eIF4E to the mRNA 5'-cap structure and eIF4G (Fig. 3, page 27) (67, 86, 91, 95, 123, 136). The three include *i)* the phosphatidylinositol 3-kinase (PI3K)-mTOR, which is activated through growth factors (insulin and insulin like growth factor-1, IGF-1); *ii)* the Ras (GTPase)-extracellular signal-regulated kinase (ERK), which is activated by growth factors (insulin and insulin like growth factor-1, IGF-1) and mechanical strain; and *iii)* the direct activation of protein kinase B (PKB)-mTOR via nutrients (amino acids) and mechanical strain (21, 30, 32, 38, 42, 55, 69-71, 92, 112, 118, 142). Figure 3 (page 27) provides a general depiction and brief overview of these signal transduction pathways and how they may act in regulating mRNA translation initiation via activation of the mammalian target of rapamycin (mTOR) with subsequent phosphorylation of eIF4E- binding proteins (4E-BP1, a repressor of eIF4E). Thus, 4E-BP1 may regulate muscle protein synthesis and ultimately growth with resistance exercise training. This section will describe the control

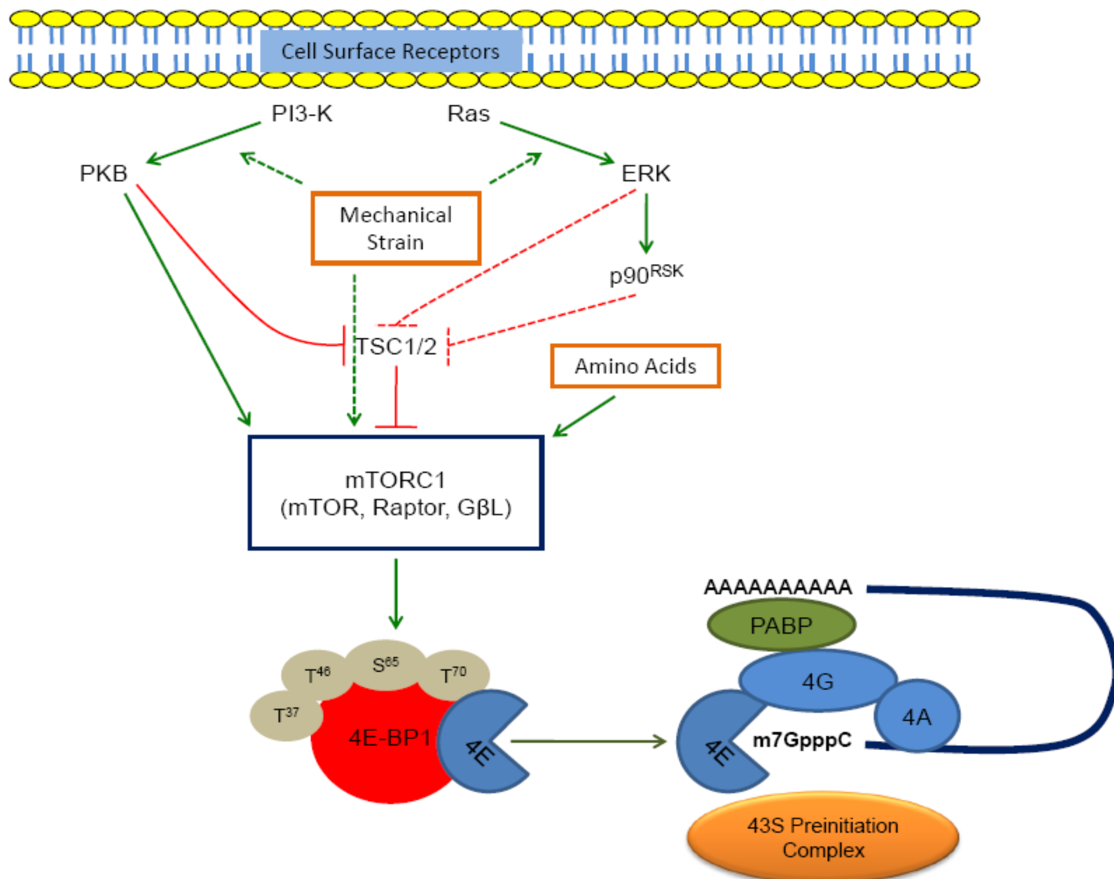


Fig. 3. Regulation of mRNA translation initiation through 4E-BP1. Signaling pathways that have been shown to be activated by hormones via tyrosine receptor kinase, mechanical strain from resistance exercise or by amino acids. Hormonal receptor binding and mechanical strain can activate both the phosphatidylinositol 3-kinase (PI3-K) and protein kinase B, and the Ras (GTPase) and extracellular signal-regulated kinase (ERK) pathways. The former acts by either direct phosphorylation of the mammalian target of rapamycin complex 1 (mTORC1) or by phosphorylation of the tuberous sclerosis complex (TSC1/2, GTPase-activating protein). The latter may serve to activate mTORC1 by ERK and/or $p90^{RSK}$ (90 kDa ribosomal protein S6 kinase) phosphorylation of TSC1/2. Phosphorylation of TSC1/2 inhibits its activity and allows mTORC1 to become activated. In addition, amino acids and possibly mechanical strain can directly stimulate mTORC1 activity. The consequences of mTORC1 phosphorylation and activation is the phosphorylation of 4E-BP1 (Thr^{37}, Thr^{46}, Ser^{65} and Thr^{70}), thus allowing eIF4E to form the eIF4F (eIF4E, eIF4G and eIF4A) complex with the mRNA and bind the remainder of the translational apparatus so that mRNA translation can ensue. Green arrows, activating steps; Blocked lines, inhibitory steps in the pathway. Dotted lines, incompletely defined mechanism. mTOR, mammalian target of rapamycin; raptor, regulatory associated protein of rapamycin; GβL, G-protein β-subunit like protein; PABP, poly A binding protein.

of protein synthesis by 4E-BP1 with special emphasis placed on the effects of RE.

As previously mentioned, eukaryotic mRNA are modified at their 5'-ends with a 7-methylguanosine-containing cap (m7GpppN) that facilitates the binding of the ribosomal 40S to the mRNA through the interaction with eIF4F (composed of three subunits; eIF4E, eIF4G and eIF4A) (13, 67, 86, 91, 95, 123, 136) (Fig 1, page 7). The binding of eIF4E to the cap and to eIF4G is necessary for translation of transcripts that contain a significant quantity of secondary structures in their 5'-untranslated regions (86). Only when the eIF4F complex is formed can the helicase activity of eIF4A (in coordination with eIF4B) unwind secondary structures (86). Thus, the availability of eIF4E (25 kDa) allows for efficient translation of highly structured messages. Although not fully understood, it is believed that there is more eIF4A and eIF4G relative to eIF4E suggesting that eIF4E limits the amount of eIF4F present within a cell, serving as a master switch for translation initiation (86, 130). This master switch is, however, regulated by a family of small translational repressor proteins, PHAS (phosphorylated heat- and acid-stable) or more commonly, 4E-BPs (86, 101, 123). The binding of eIF4E by 4E-BPs inhibits the formation of the eIF4F complex as both 4E-BPs and eIF4G interact with eIF4E through a similar binding motif (YXXXXLΦ, where X is any amino acid and Φ is a hydrophobic residue) (65). More simplistically, eIF4E can continue to bind the 5' cap structure of the mRNA when bound to 4E-BP1, it just cannot bind to eIF4G (translation initiation cannot start) until released from 4E-BP1 control.

In 1981, Denton et al. (39) reported insulin-stimulated phosphorylation of a ~ 22 kDa protein (4E-BP1). In 1994, Hu et al. (72) determined, through purification

techniques, that the protein was ~ 12 kDa. Additionally, this group and others (90, 101) obtained the amino acid sequence for 4E-BP (1 and 2) in the rat, mouse and human. Later, 4E-BP3 was identified (110); thus in mammals, the 4E-BP family consists of 4E-BP1, 4E-BP2 and 4E-BP3; however, 4E-BP1 and 4E-BP2 are the predominant forms expressed in tissues involved in glucose and lipid homeostasis, including skeletal muscle, adipose tissue, the pancreas and liver (87). Furthermore, it was determined that 4E-BP1 was the form that controls cell growth (mTOR mediated) as rapamycin administration has been reported to reduce 4E-BP1 phosphorylation, thus preventing eIF4E to bind with eIF4G inhibiting cap-dependent translation and ultimately cell size (4, 13, 28, 53, 67). mTOR control was confirmed by Azpiazu et al. (4) who, in addition to incubating muscles in rapamycin, also incubated with the mitogen activated protein kinase kinase (MEK; ERK pathway, see Fig. 3 on page 27 for description) inhibitor, PD 098059 and insulin. They (4) observed an increase in phosphorylation of 4E-BP1 with insulin; however, this was abolished with rapamycin, but not PD 098059, suggesting mTOR as being the primary regulator of 4E-BP1. As a result of these findings, it would seem plausible that the anabolic effects of resistance exercise are mediated through 4E-BP1 control.

Due to 4E-BP1 being a relatively newly discovered protein involved in the control of protein synthesis, it was not until 2000 that Farrell et al. (52) were the first to examine the effects of acute resistance exercise on 4E-BP1 in rats. They (52) found that 16 h following resistance exercise (point at which muscle protein synthesis is significantly increased) there were no changes in total 4E-BP1 or phosphorylated (γ

form), or in the association of 4E-BP1 with eIF4E or eIF4E with eIF4G, suggesting that the timing of assessment may be of concern. To determine if timing was a concern, the same group (23) extracted muscle from 5-60 min following acute resistance exercise (same paradigm) and reported significant increases in 4E-BP1 phosphorylation (γ form) and an association of eIF4E with eIF4G at 5 and 10 min following exercise, suggesting that the control of translation initiation is an immediate response following acute resistance exercise. In further support of this was a recent investigation conducted by Thomson et al. (125) who observed a significant increase in 4E-BP1 phosphorylation (γ form) immediately, 20 min and 40 min following an acute bout of high intensity electrical simulation in rats. Therefore, translational control appears to occur within minutes following resistance exercise, increasing protein synthesis; however, the time by which this becomes significant in terms of measurement (synthesis rates) may take hours.

One limitation in the above mentioned investigations is the means by which phosphorylation state of 4E-BP1 was determined. 4E-BP1 resolves into distinct electrophoretic forms (α, β and γ, Fig. 4, page 31) (23, 86). However, there are 4 key phosphorylation sites (Thr-37, Thr-46, Ser-65 and Thr-70) on 4E-BP1 (48, 49) and although it is assumed that they are within the γ form (at least some) (86), it is not completely descriptive of whether 4E-BP1 is fully phosphorylated and thus, inhibited or not. 4E-BP1 can remain bound to eIF4E with mTOR phosphorylation on Thr-37 and Thr-46 (65). In addition, Tomoo et al. (130) reported that single Ser-65 phosphorylation was inadequate for releasing 4E-BP1 from eIF4E. Taken together, phosphorylation of

4E-BP1 on Thr-37 and Thr-46 serves as a priming event for subsequent phosphorylation on carboxy terminal sties (Ser-65 and Thr-70), which appear to be required for full release of eIF4E from 4E-BP1 (65, 112). In support of this, Kubica et al. (84) examined the effects of acute resistance exercise in rats on muscle protein synthesis and translation initiation factors with specific phosphorylation sites on 4E-BP1 measured. This group's (84) findings were an increase in the γ form, increased phosphorylation on Thr-37 and Thr-46, but not Thr-70 16 h following resistance exercise (protein synthesis was significantly increased). In addition, although the data appeared to support a reduction in the association of 4E-BP1 with eIF4E in the resistance exercise animals, they were not statistically different from the control. Thus, examination of Thr-70 phosphorylation within 4E-BP1 (in addition to associations) may be more descriptive of its activity.

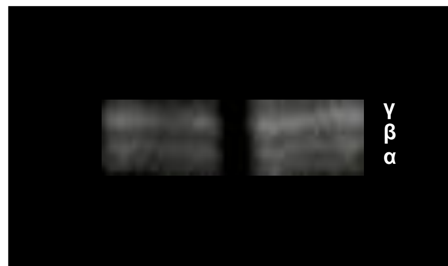


Fig. 4. Expression of 4E-BP1 proteins in the soleus muscle from ~ 7 mo Sprague Dawley rats. The electrophoretic separation of the α, β and γ forms from a 10% discontinuous polyacrylamide gel. The γ form represents the hyperphosphorylated form; however, specific phosphorylated sites (Thr-37, Thr-46, Ser-65 and Thr-70) cannot be determined and thus, whether or not there was complete separation from eIF4E. Therefore, mobility shift assays may not be completely useful without measurement with anti-antibodies specific to the phosphorylated residue of interest.

Although there are data on the effects of acute resistance exercise on 4E-BP1 control of mRNA translation, there is only one study examining the effects of chronic

resistance exercise training on 4E-BP1 (88). That group (88) observed no changes in 4E-BP1 phosphorylation (Thr-37 and Thr-46) 48-72 h following 8 weeks of resistance exercise training in humans, which significantly increased strength and thigh cross-sectional area. Muscle protein synthesis was not measured so it is difficult to determine if 4E-BP1 activity correlated with changes in synthesis rates.

Based on the reported research from rats (23, 52, 84, 125) and humans (38, 40-42, 143), there is an increase in 4E-BP1 within minutes (23, 125), a decrease in 4E-BP1 phosphorylation (Thr-37 and Thr-46) during or immediately after (23, 38, 40, 41, 125, 143) and an increase in 4E-BP1 phosphorylation (Thr-37 and Thr-46) hours (38, 42, 84, 143) following acute resistance exercise. However, due to the paucity of data that exists on 4E-BP1 activity and chronic resistance exercise training, little can be concluded. More research in this area is warranted due to the changes in muscle protein synthesis that occur with acute (increased) and chronic (attenuated response) resistance exercise. It may be that the changes in phosphorylation of 4E-BP1 that are observed with acute resistance exercise support the elevations in protein synthesis and that the attenuated responses that occur with chronic resistance exercise training are associated with an increase in 4E-BP1 expression and decreased phosphorylation.

Approaches for Assessing Muscle Protein Synthesis

There have been four approaches developed over the years to assess protein metabolism: i) nitrogen balance (intake – loss) (89), ii) arteriovenous differences to determine amino acid balance (6), iii) turnover of proteins by labeling and monitoring its

decay (75) and iv) direct incorporation using precursor-product labeling (152); however, the direct incorporation method is preferred for determining muscle protein synthesis due to its precision in measurement. One drawback of the precursor-product labeling method is that it only provides data on the changes in synthesis and is not descriptive of breakdown (protein balance is the difference in synthesis and breakdown). As a result of this, the addition of the arteriovenous balance technique across the area that the muscle is sampled from can yield a breakdown rate by determining the enrichments in the artery, vein and muscle (14, 15, 146). However, this approach has gained a great deal of criticism over the years due to the potential for amino acid recycling (i.e. if a protein is broken down and the amino acid is reincorporated back into a protein, less will be released into the venous circulation and a falsely low breakdown rate will be determined) and the contribution of turnover from other muscles in the vicinity of sampling. Thus, few studies exist which have attempted to assess muscle protein breakdown. Overwhelmingly, studies examining protein metabolism report changes in muscle fractional synthesis rates (FSR, the fraction of the total protein-bound pool that is being synthesized), which is often expressed per unit of time and assumes that FSR and the pool size are constant (146).

In order to measure protein synthesis using direct incorporation (precursor-product labeling), a tracer (labeled amino acid) and tracee (same amino acid but unlabeled) are administered. The tracers that are commonly used to measure muscle protein synthesis include radionuclides (atoms with an unstable nucleus that emit radiation), such as [^{14}C]leucine and [^{3}H]phenylalanine; and stable isotopes (atoms with

an additional neutron, thus altered mass), such as [^{13}C] leucine, or [^{13}C], [^{15}N], or [^{2}H]phenylalanine (146). Although both leucine and phenylalanine have recently been shown to yield similar results (119), phenylalanine is generally preferred since it is not oxidized in skeletal muscle (134). The tracer and tracee can be delivered to the organism as either a continuous infusion (constant infusion over time of measurement), a primed-continuous infusion (bolus, followed by constant infusion over time of measurement) or as a flooding dose (supraphysiological bolus over a few seconds-minutes) (146); although, due to the time necessary to reach a plateau in isotopic enrichment of the precursor (plasma, intracellular or aminoacyl-tRNA) and thus, product (protein-bound), researchers commonly use the primed-continuous or flooding dose protocols.

Flooding Dose

One of the major limitations that has plagued researchers who study tissue protein synthesis is assessment of the true precursor pool, the aminoacyl-tRNA (137). However, due to the rapid turnover and difficulty in measuring aminoacyl-tRNA, a surrogate marker (plasma or intracellular) is normally used (146). Several studies (7, 33, 36, 137) have compared the plasma, intracellular (protein-free) and aminoacyl-tRNA when using the primed-continuous infusion protocol and have reported different results. For instance, Watt et al. (137) reported higher plasma leucine and leucyl-tRNA then protein-free leucine values in rat gastrocnemius muscle. However, Baumann et al. (7) observed higher protein free leucine then leucyl-tRNA, but very similar values when using phenylalanine as the tracer in swine semimembranosus and semitendinosus

muscle. Caso et al. (33) also reported similar protein-free phenylalanine and phenyl-tRNA in the bicep femoris muscle of dogs. More recently, however, Chow et al. (36) reported similar plasma and protein-free leucine with leucyl-tRNA in human vastus lateralis muscle; thus there are apparent discrepancies as to what precursor surrogate should be used. Although, it should be noted that the three investigations that used [^{13}C]leucine (7, 36, 137) as the tracer, the venous α-ketoisocaproic acid (KIC) values corresponded most closely with the leucyl-tRNA (leucine undergoes transamination, and thus, oxidation (134)) and should be used as the precursor surrogate when [^{13}C]leucine is used as the tracer. Taken together, variable synthesis rates can be determined when using different tracers and different surrogates (plasma *vs.* protein-free) as the precursor pool.

To circumvent this concern/issue, the flooding dose was developed and presented by Garlick et al. (63) in 1980. The flooding dose consists of injecting a large bolus of tracer (typically [^{3}H]phenylalanine in animals and [^{2}H]phenylalanine in humans) along with the same unlabeled amino acid used over seconds in rats (63) to ~ 10 minutes in dogs (33) and humans (34). The amount of the labeled and unlabeled amino acid are several-fold higher than the endogenous free pool of the amino acid used (e.g. phenylalanine); thus, the tissue uptake becomes saturated in a shorter period of time (~ 10, 45 and 90 min in rats, dogs and humans respectively) (33, 34, 63). Furthermore, due to short assessment times, there is little concern for dilution of the specific activity as a consequence of muscle protein breakdown. In addition, the short time of measurement only warrants one biopsy *vs.* two or more, which are generally needed with the primed-continuous infusion protocols to insure that the protein-bound label incorporation

exceeds background. The result is that there is no question as to the precursor enrichment as the plasma, protein-free and aminoacyl-tRNA are very similar due to saturation (33, 63).

Although the flooding methodology eliminates some of the major issues that exist with primed-continuous protocol; there is one concern that the supraphysiological bolus of amino acid that is given could influence the rate of protein synthesis. Garlick et al. (63), in conjunction with the flooding dose experiment, provided two different tracers ([^{3}H]lysine and [^{14}C]threonine) with either 0.9% NaCl or 150 mM of unlabelled phenylalanine (amount provided with flooding dose experiments in rats) and reported no differences with either tracer between the saline and phenylalanine bolus. Furthermore, Garlick et al. (62) did a follow up study in 1989 in humans using [^{13}C]leucine as the tracer and observed similar plasma leucine, plasma α-KIC and protein-free leucine in the vastus lateralis with synthesis values ~ 2.0% per day (within range that has been reported). However, Jahoor et al. (74) reported that the flooding dose resulted in significantly higher rates in protein synthesis than the primed-constant infusion in the hindlimb muscle of dogs and that providing a bolus of leucine renders the method invalid as a result of increasing the precursor specific activity and decreasing tracer incorporation into protein if a tracer other than leucine is used. Thus it would appear leucine, a potent stimulator of protein synthesis, may not be the best choice of tracer when using the flooding dose protocol.

To determine if other indispensable amino acids stimulate human muscle protein synthesis with the flooding dose, Smith et al. (120) provided a primed-continuous

infusion of [^{13}C]leucine for 6 h, at which time, a flooding dose of either phenylalanine, threonine (both indispensible), arginine, [^{13}C]serine or [^{13}C]glycine were provided. Their findings (120) were that both phenylalanine and threonine stimulated protein synthesis, whereas, the others did not. However, these individuals were measured in the fasted state and the provision of indispensible amino acids would likely serve to be anabolic. Therefore, Caso et al. (34) measured fed state muscle protein synthesis with both flooding dose and primed-continuous infusion with [$^{2}H_{5}$]phenylalanine in humans and observed no differences between either of them when the protein-free compartment (vastus lateralis) was used for calculation of FSR with the primed-continuous infusion. Thus, the potential for the flooding dose to stimulate muscle protein synthesis does exist; however, this concern can be minimized by avoiding prolonged fasting conditions.

The concerns that generally exist with the primed-continuous infusion (length of time for precursor and product enrichments, time of infusions and risk of amino acid recycling, to name a few) can be eliminated with the flooding dose. Although there have been discrepant findings from flooding dose experiments in dogs and humans, rat experiments have been consistent and this may be associated to the time needed to flood all compartments (~ 10 min in rats *vs.* ~ 20-90 min in dogs and humans respectively), as well as the tracer used and the fed/fasted state. Therefore, it can be concluded that flooding with phenylalanine in rats does not stimulate muscle protein synthesis and is a reliable and valid method for determining synthesis rates. In addition, when using the flooding dose in humans, phenylalanine should be used as the tracer and the infusion should be started in an early post-absorptive state or with the provision of nutrients

during the experiment.

^2H_2O

One of the major limitations with using labeled amino acids as tracers is the time by which assessment of muscle protein synthesis can be made. Primed-continuous infusion protocols typically run for ~ 360 minutes and flooding dose infusions run from seconds to ~ 10 minutes in larger mammals. Although these provide reliable and valid results under those specific conditions of measurement, they do not yield data over long periods of time (days and even weeks). Muscle FSR (expressed as a % per day) values are believed to be ~ 2.0% in humans (145) and anywhere from 3-20+ % in rats (64) (variability is likely due to rapid growth patterns and short life span). However, these values come from extrapolating values that were obtained with short isotopic infusions over a 24 h day and rarely account for feeding. Thus, the data that exist today on skeletal muscle protein synthesis rates are specific to the precise methods and exclusive to the conditions of measure. In addition, although feeding in close proximity to the start of a flooding dose or shortly thereafter is recommended when using an essential amino acid as the tracer, large quantities of nutrient provision are likely inappropriate as this may decrease the likelihood that label will accumulate into the protein. With primed-continuous infusions, feeding prior to the infusion of the tracer is generally not recommended due to the potential for dilution of the precursor pool (more unlabeled amino acid will be present from exogenous sources and decrease the specific activity). Additionally, when nutrients are provided during the tracer protocol, additional tracer

should be added to the nutrients to avoid the dilution. However, even if care is taken by adding more tracer, selecting the correct precursor surrogate becomes questionable (both plasma and protein-free should be measured). Another limitation to tracer experiments is the invasiveness of the procedures and cost, both of which limit the number of investigations that are conducted on muscle protein synthesis.

An alternative approach by which these limitations can be avoided for the study of protein synthesis is the use of ^2H_2O; first presented by Hans Ussing (Copenhagen University) in 1941 (131). Dr. Ussing determined that by providing ^2H_2O to mice and rats, one could determine protein renewal by the incorporation of ^2H into protein; including liver, kidney , skeletal muscle and specific proteins such as myosin (131). He believed that if ^2H_2O was provided to an animal and the concentration was maintained then the ^2H would accumulate into protein over time approaching a constant value that could be determined by their synthesis rates (131). More specifically, Dr. Ussing thought that this occurred by the transfer of ^2H with the H of amino acids through transamination reactions and similar processes, which would then be incorporated into protein (131). Additionally, he suggested that measurement of this process would be easiest if the exchange in ^2H with amino acids occurred very rapidly so that their ^2H content would not change throughout the incorporation process as long as a consistent ^2H_2O concentration was maintained (131). Due to the difficulty in obtaining ^2H_2O during that time, follow up investigations were not possible (131). However, in 1961 Oshima and Tamiya (98) were able to confirm Ussing's hypothesis that ^2H exchange with the H of amino acids (alanine) occurs through transamination (Fig. 5, page 40). Furthermore,

alanine labeling occurs rapidly through transamination, and although other amino acids (leucine, glycine and glutamate) undergo this process as well, it is the number of hydrogen's and the simplicity in the structure of alanine that makes it easily quantifiable.

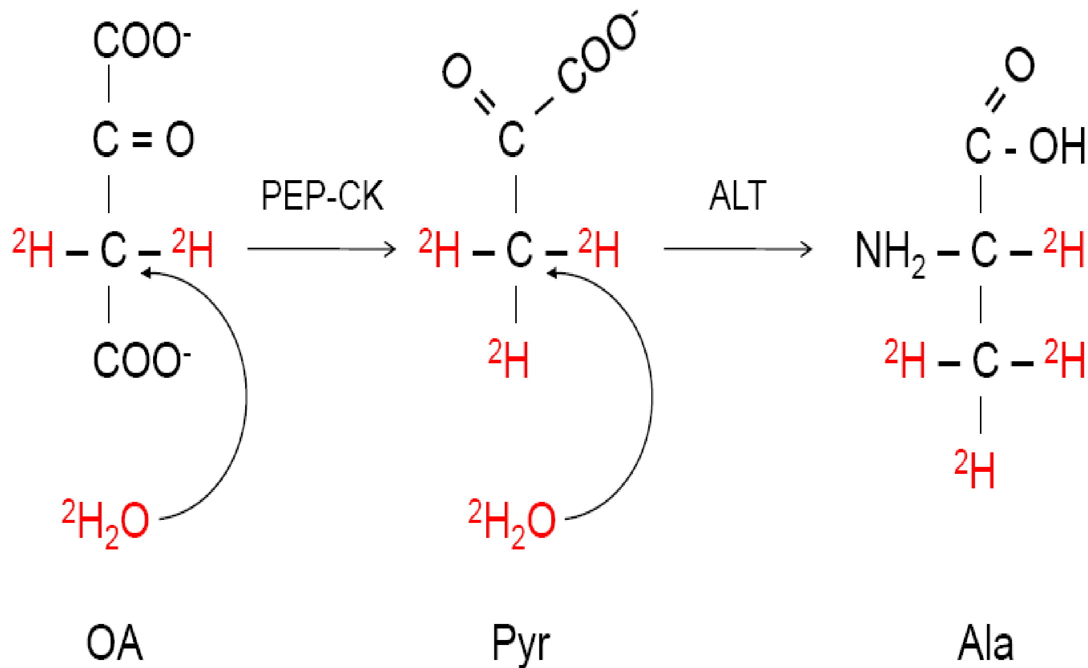


Fig. 5. ^{2}H labeling of alanine using ^2H_2O. Administration of ^2H_2O results in rapid equilibration with body H_2O (~ 20 min), which can then label Ala (alanine) through intermediary metabolism and become incorporated into a protein. Dissimilar to labeled amino acids which must gain entry into the cell via transporters, the ^{2}H labeling of Ala occurs intracellularly. OA, oxaloacetate; PEP-CK, phosphoenolpyruvate carboxykinase; Pyr, Pyruvate; ALT, alanine aminotransferase.

Although it was determined that ^2H_2O could be used for measuring muscle protein synthesis (131) and the process by which this occurred was understood (98), it still wasn't clear whether feeding (potential disruption of the alanine pool) influenced the labeling, and thus the ability to assess synthesis rates. If this were the case, then the use of ^2H_2O would be limited to post-absorptive experiments. In 2004, however, Dufner

et al. (44) provided rats with ^2H_2O (bolus intraperitoneal injection) and determined that the percent of ^2H_2O in body water was identical with the percent of ^{2}H-labeled alanine and the time by which this was achieved was ~ 20 minutes. Additionally, the same group (44) provided a large bolus of unlabeled alanine after 90 minutes and observed similar ^{2}H-labeled alanine and ^2H_2O in body water within ~ 30 minutes, supporting the notion that feeding will not alter the precursor pool (i.e. ^2H_2O). However, this experiment was conducted on plasma and not within a specific protein, site of transamination of alanine; thus it could not be concluded at this time if the plasma labeling was equal to the labeling of the protein-free compartment. Thus, additional experiments (44, 111) by the same group compared protein-free ^{2}H labeling of alanine in rat liver tissue (44) and human albumin (111) with that of plasma ^{2}H labeling of alanine and ^2H_2O and observed very similar values. In addition to the benefits of intracellular ^{2}H labeling of alanine and the lack of an alteration in labeling gradients with feeding, ^2H_2O can also be used safely (102) for prolonged periods of time (111) at a relatively low cost and noninvasively to assess protein synthesis. However, to date, no studies have been published the area of exercise and muscle protein synthesis using ^2H_2O as a tracer. The benefit of conducting these experiments would be that cumulative differences in muscle protein synthesis are assessed in real-time environments (no laboratory or controlled environment required). In addition, the measurements could be made over extended periods of time to provide an actual daily rate *vs.* an extrapolated one.

CHAPTER III

A COMPARISON OF ^2H_2O AND PHENYLALANINE FLOODING DOSE TO INVESTIGATE MUSCLE PROTEIN SYNTHESIS WITH ACUTE EXERCISE IN RATS

Introduction

Tracers have been used to study whole-body and tissue-specific protein synthesis under various conditions (i.e. post-absorptive, post-prandial, disease and exercise) for more than 35 years. Questions are addressed using either “stochastic methods” (which measure the dilution/excretion of a carbon- or a nitrogen-labeled amino acid) or using “direct incorporation methods” (which compare the labeling of a protein-bound amino acid against the labeling of the amino acid precursor). Direct incorporation methods (i.e. precursor:product labeling ratios) are generally accepted as yielding reliable measurements of protein synthesis in specific proteins. Since skeletal muscle accounts for ~ 60% of total body protein (134) and plays a pivotal role in health and the pathology of many chronic diseases, direct examination of this protein pool is of great interest to researchers.

The use of continuous infusion with either [^{13}C]leucine, or [^{13}C], [^{15}N], or [^{2}H] phenylalanine is common, but requires adequate time to achieve a steady state in precursor enrichment (146) and substantial incorporation into newly made protein. In addition, uncertainty regarding the measurement of the true precursor pool (which is assumed to be the tRNA-bound amino acid) exists. However, since the tRNA-bound

pool of amino acids is relatively small and is expected to have a high turnover, investigators have proposed various surrogates (intracellular or plasma), e.g. α-ketoisocaproate (KIC) labeling as a marker of intracellular leucine labeling.

An alternative to the continuous infusion is the flooding dose. The principle is that by providing a large bolus that contains a mixture of labeled and unlabeled amino acids over a short period of time (seconds to minutes), there will be a saturation of the uptake of the amino acid by a tissue(s) and therein minimize labeling gradients between plasma and tRNA-bound amino acids, thus making amino acid labeling in the plasma pool appropriate to use as the precursor (33, 63). An advantage of the flooding dose method is that it allows measurements over short periods of time (10 min in animals and 90 min in humans). However, the primary concern regarding the flooding dose centers on the physiological perturbation that occurs; therefore, one must consider whether the increase in the pool size of the tracee alters the metabolism under investigation. In support of this, Jahoor et al. (74) and Smith et al. (120) have observed increases in muscle protein synthesis when using the flooding dose (with essential amino acids) in dogs and humans, respectively; however, Garlick et al. (63) did not find a stimulatory effect when flooding with phenylalanine in rats. Although the differences may be explained by the species used, the possibility that supraphysiological concentrations of essential amino acids stimulate muscle protein synthesis warrants consideration.

These methods are faced with major challenges when the aim is to study protein synthesis under perturbed conditions. For example, nutrient ingestion can dilute the tracer and therein influence assumptions regarding precursor labeling. The use of $^{2}H_2O$

(a "primed-infusion method") minimizes precursor labeling gradients since cells are labeled from the inside via transaminase reactions (8, 30, 43, 98, 111) (*Fig. 6, page 45*). Since cells readily produce alanine, its labeling should reflect that of body water under many conditions, especially since alanine undergoes rapid turnover, even in the fed state. Thus, one can compare the precursor labeling (body water) with either the ^{2}H labeling of the α-hydrogen or total ^{2}H labeling of protein-bound alanine divided by "n", the number of incorporated deuteriums, which we have found to be 3.7 (a factor that is consistent with isotope effects on enzyme catalyzed reactions and is supported by other recent data) (8, 44, 111). The use of ^2H_2O has practical advantages in that studies do not require a post-absorptive state and can assess protein synthesis over short and long periods of time.

Belloto et al. (10) recently demonstrated similar apoB100-VLDL protein synthetic rates with ^2H_2O and a primed-infusion of leucine, further validating the reliability of using labeled water. However, to our knowledge, no studies exist comparing the flooding dose and ^2H_2O methodologies on muscle protein synthesis. We aimed to determine if ^2H_2O and flooding with L-[2,3,4,5,6-^{3}H]phenylalanine would yield comparable results. The experiments examined two physiological scenarios (i.e. basal *vs*. an acute bout of resistance exercise) and considered measurements over different periods of time.

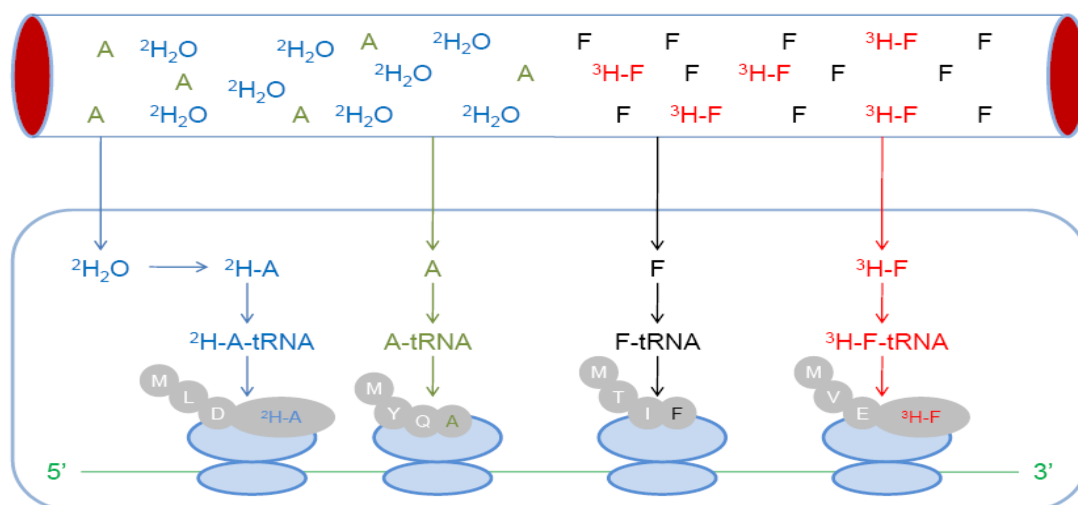


Fig. 6. Depiction of mRNA translation elongation with two different isotopes (^2H_2O and L-[2,3,4,5,6-^{3}H]F). The flooding dose works on the assumption that providing a large bolus of labeled (^{3}H-F) and unlabeled (F) over seconds-minutes will result in similar specific activities (SA) between the plasma, intracellular and ultimately the aminoacyl-tRNA. The ^2H_2O method works on the assumption that ^{2}H rapidly equilibrates with the body water which leads to the intracellular ^{2}H labeling of alanine via transamination reactions; therein bypassing the requirement for rapid transmembrane amino acid transport.

Materials and Methods

Chemicals and Supplies

Unless specified, all chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA). ^2H_2O (99.9 atom percent excess) and L-[*ring*-$^{13}C_6$]phenylalanine (F) (99% enriched) were purchased from Cambridge Isotopes (Andover, MA). MTBSTFA + 1% TBDMCS was purchased from Pierce (Rockford, IL). L-[2,3,4,5,6-^{3}H]F was purchased from GE Healthcare Life Science-Amersham (Buckinghamshire, England). Gas chromatography-mass spectrometry supplies were purchased from Agilent Technologies (Wilmington, DE) and Alltech (Deerfield, IL). Kinase buffer (10X) was purchased from Cell Signaling Technologies (Danvers, MA).

Animals and Operant Conditioning

Twenty-four male Sprague-Dawley rats (5 mo old) were individually housed in a climate controlled small animal facility with a 12 hour light (start at 10:00 pm) and dark cycle (start at 10:00 am). Rats were fed a standard feed ad libitum and had free access to water. The rats were assigned by body mass into one of four groups: 4 h control (CON 4h, n = 6), 4 h resistance exercise (RE 4h, n = 6), 24 h control (CON 24h, n = 6) or 24 h resistance exercise (RE 24h, n = 6). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Texas A&M University.

The RE animals were operantly conditioned to depress an illuminated bar located high on a Plexiglas exercise cage (56). Negative reinforcement via an electrical foot shock (<3 mA, 60Hz) was used to train the rats to perform the desired movements. After the animals were conditioned (4 sessions), a non-weighted Velcro vest was strapped over the scapulae and the rats were required to touch the illuminating bar 50 times for an additional two sessions. The CON animals were free from physical activity and remained single-housed over the course of the study.

Experimental Protocol

On the day of the study, half of the animals (24 h CON and 24 h RE) received 20 µl per g of body mass, via an intraperitoneal injection (IP), of 99.9% ^2H_2O and allowed free access to drinking water enriched to 4.0% ^2H_2O (Fig. 7, page 47). In addition, half of the animals (24 h RE and 4 h RE) performed a progressive bout of RE. The protocol consisted of the RE animals performing 0 g (10 repetitions), 80 g (15 repetitions), 110 g (12 repetitions), 180 g (10 repetitions), 230 g (8 repetitions) and 80 g (15 repetitions).

Ten out of the 12 RE animals completed this protocol, however, two failed to complete the 8 repetitions with 230 g, and therefore, we increased the repetitions of the final set at 80 g to equal the total volume that the other rats completed. Twelve hours following the RE session, the remaining animals (4 h CON and 4 h RE) received the same quantity of ^2H_2O and allowed free access to drinking water enriched to 4.0% ^2H_2O. In addition, the food was withdrawn at this time.

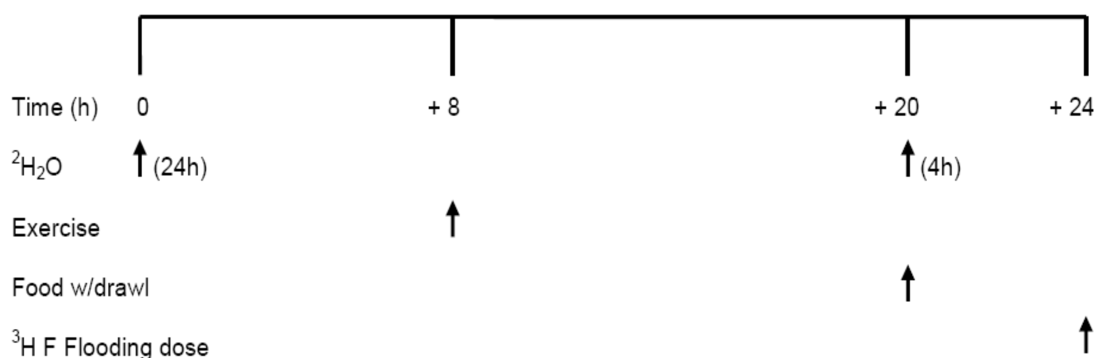


Fig. 7. Schematic display of study design used to measure muscle protein synthesis with ^2H_2O and flooding dose of phenylalanine. At time 0, an intraperitoneal bolus of ^2H_2O was provided to the rats (*n = 12)* and 4% ^2H_2O drinking water was provided ad libitum. The acute bout of resistance exercise (*n = 12)* was completed at time + 8 h. At time + 20 h, the other group of rats (*n = 12)* were provided an intraperitoneal bolus of 99.9% ^2H_2O and 4% ^2H_2O ad libitum drinking water. At + 24 h, all animals (*n = 24)* were anesthetized, cannulated and administered a F flooding dose, as well as muscle harvest to assess fractional rates of protein synthesis (FSR).

Four hours later, all rats were anesthetized with Ketamine (40-55 mg/kg) and Medetomidine (0.4 mg/kg) via an IP injection. The left carotid artery and right jugular vein were cannulated with sterile, heparinized polythene tubing (PE-50). While sedated a flooding dose of L-[2,3,4,5,6-^{3}H]F (0.5 mCi/rat) in unlabeled F (150 mM, 1ml · 100 g body mass^{-1}) was injected into the venous catheter over a 10- to 15-s period (63). Three arterial blood samples (1 ml) were taken from the carotid cannula during a 12 min period

(2, 6 and 12 min) for the determination of F specific radioactivity. After arterial sampling, the mixed gastrocnemius (M. Gastroc), plantaris (Plant) and soleus (Sol) muscles were excised, immediately frozen to the temperature of liquid nitrogen between aluminum blocks, and stored at -80ºC until analyzed.

Flooding Dose Sample Preparation and Analyses

Tissue and plasma specific radioactivity were determined using a modified version of the protocols outlined by Garlick et al. (63) and Vary et al. (133) (see Appendix A for detailed description). A known amount of muscle (~ 0.150-0.200 g) was pulverized under liquid nitrogen followed by homogenization in 2.0 ml of 10% trichloroacetic acid (TCA) and centrifuged at 3,800 rpm for 15 min at 4ºC. The supernatant (SPNT) was discarded and the protein precipitate was washed four additional times in 2.0 ml of 10% TCA, then freed from lipid with 2.0 ml of acetone, washed with 2.0 ml of distilled H_2O and dissolved in 2.0 ml 0.25 N NaOH. A portion of the protein was used to quantify protein content using the bicinchoninic acid (BCA) method (121). To determine tissue specific radioactivity, 0.5 ml of the protein solution was measured by liquid-scintillation counting with appropriate corrections made for quench. Protein-bound F was determined by re-precipitating the protein from the 0.25 N NaOH solution with 1.0 ml of 10% TCA and centrifugation at 3,800 rpm for 15 min at 4ºC, followed by 2 additional wash steps. The precipitate was then hydrolyzed for 24 h at 110ºC with the addition of 0.1 ml of 10 μmol/ml of [ring-$^{13}C_6$]phenylalanine to serve as an internal standard (14). Blood samples (2, 6 and 12 min) were centrifuged at 3,800 rpm for 30 min at 4ºC followed by removal of 0.4 ml of plasma and the addition of 0.4

ml of 10% TCA and 0.4 ml of 30-60 nmol/ml [ring-$^{13}C_6$]phenylalanine to quantify the total F in the plasma samples. To determine plasma radioactivity, 0.05 ml was measured by liquid-scintillation counting with appropriate corrections made for quench.

In order to quantify both plasma and tissue phenylalanine, 0.05 ml was dried under a stream of N_2, followed by the addition of a 1:1 solution of acetonitrile:*N*-methyl-*N*-*tert*-(t-butyldimethylsilyl)trifluoracetamine ± *tert*-butyl dimethylchlorosilane (MTBSTFA + 1% TBDMCS); modified from Calder et al. (31). After reacting at room temperature for 30 min, the samples were analyzed using an Agilent 5975C-MSD equipped with an Agilent 7890 GC system, and a HP-5ms capillary column (30 m × 0.25 mm × 0.25 µm). The following temperature program was used: 160ºC initial and hold for 5 min, increase by 15°C/min to 270ºC and hold for 8 min. The sample was injected at a spit ratio of 40:1 with a helium flow of 30 mL/min. Phenylalanine eluted at ~ 12 min. The mass spectrometer was operated in electron impact mode (70eV). Selective ion monitoring of mass-to-charge ratios (*m/z*) 234 (M) and 240 (M + 6) was conducted using a dwell time of 30 ms per ion.

^2H_2O Preparation and Analysis

Labeling (^{2}H) of body water was assessed by exchange with acetone described by Yang et al. (148) (see Appendix B for detailed description). The reaction occurred with 20 µL of a sample/standard, 2 µL of 10N NaOH and 4 µL of a 5% (vol/vol) solution of acetone in acetonitrile for 24 h. Acetone was removed by the addition of 0.6 ml of chloroform and 0.5 g Na_2SO_4. The samples were mixed and 0.1 ml of the chloroform was transferred to a GC-MS vial. The samples were analyzed using an

Agilent 5975C-MSD equipped with an Agilent 7890 GC system, and a HP-5ms capillary column (30 m × 0.25 mm × 0.25 μm). The following temperature program was used: 60ºC initial, increase by 20°C/min to 100ºC, increase by 50ºC/min to 220ºC, and hold for 1 min. The sample was injected at a spit ratio of 40:1 with a helium flow of 1 mL/min. Acetone eluted at ~ 1.7 min. The mass spectrometer was operated in electron impact mode (70eV). Selective ion monitoring of mass-to-charge ratios (*m/z*) 58 (M) and 59 (M + 1) was conducted using a dwell time of 10 ms per ion.

Labeling (^{2}H) of protein-bound alanine was measured as previously described by Dufner et al. (44) (see Appendix B for detailed description). Briefly, ~ 0.030 g of M. Gastroc, Plant and Sol were homogenized on ice in 0.3 ml of a 10% (wt/vol) TCA and centrifuged at 3,750 rpm at 4°C for 15 min. The SPNT was discarded and the protein pellet was washed 3 additional times with TCA. The protein pellet was then dissolved in 6 N HCl (0.1 mL/0.010 g tissue) and reacted at 100ºC for 18 h. In order to determine if there was a difference in mixed muscle versus myofibrillar, ~ 0.060 g of M. Gastroc was homogenized on ice in 400 μL of a 1 X kinase buffer (25 mM Tris-HCl, 5 mM ß-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) with 200 μM ATP and 0.01% Triton. Homogenates were centrifuged at 14,000 rpm at 4ºC for 30 min. After centrifugation, the SPNT containing the cytosolic and membrane portion was discarded and the precipitate (representative of the myofibrillar fraction) was prepared the same as the mixed muscle. An aliquot (0.1 ml) of the hydrolysate was dried under a stream of N_2 for ~ 1 h. A 3:2:1 ratio (0.1 mL) of “Methyl-8” reagent (Pierce, Rockford, IL), methanol and acetonitrile was added to the residue to determine the ^{2}H labeling of

alanine on its methyl-8 derivative. All samples were analyzed using an Agilent 5973N-MSD equipped with an Agilent 6890 GC system, and a DB17-MS capillary column (30 m × 0.25 mm × 0.25 µm) was used in all analyses. The following temperature program was used: 90ºC initial, hold for 5 min, increase by 5°C/min to 130ºC, increase by 40ºC/min to 240ºC, and hold for 5 min. The sample was injected at a spit ratio of 20:1 with a helium flow of 1 mL/min. Alanine eluted at ~12 min. The mass spectrometer was operated in electron impact mode. Selective ion monitoring of mass-to-charge ratios (*m/z*) 99 (M) and 100 (M + 1) was conducted using a dwell time of 10 ms per ion.

Calculations (See Appendix D for detailed description)

Rates of protein synthesis (RPS) were calculated for the F flooding dose using the equation described by Vary et al. (133):

$$(DPM \cdot g\ wet\ wt^{-1}) \cdot (S_{\bar{A}})^{-1} \cdot (t)^{-1}\ (eq\ 1)$$

where DPM (disintegrations per minute) · g wet wt^{-1} is the quantity of L-[2,3,4,5,6-^{3}H]F incorporated into the available pool (mg protein per g of tissue), $S_{\bar{A}}$ is the mean specific activity in plasma (DPM · nmol F^{-1}) over 12 minutes of flooding and *t* is incorporation time in hours. Fractional rates of protein synthesis (FSR) with F were calculated using the equation described by Garlick et al. (63):

$$S_P \cdot (S_{\bar{A}}\ x\ t)^{-1}\ x\ 100\ (eq\ 2)$$

where S_P is the specific radioactivity of protein (DPM · nmol F^{-1}) (63). When ^2H_2O was provided, FSR was calculated using the equation:

$$E_A \cdot [E_{BW}\ x\ 3.7\ x\ time\ (h)]^{-1}\ x\ 100\ (eq\ 3)$$

where E_A represents amount of protein-bound ^{2}H Ala (MPE, mole percent excess), E_{BW}

is the quantity of ^2H_2O in body water (MPE) and 3.7 represents the exchange of ^2H between body water and alanine (44, 111, 146). The assumption is that ^2H-labeled body water equilibrates with free alanine more rapidly than ^2H-labeled alanine incorporates into newly made protein and that protein synthesis is linear over time.

Statistical Analysis

Analyses were completed using SigmaStat version 3.5 and values are expressed as means ± SE. A one-way ANOVA was used to compare differences between the exercise and control groups, the different approaches (4 h ^2H_2O vs. 24 h ^2H_2O and flooding), F specific radioactivities, mg protein relative to tissue and plasma ^2H_2O labeling. A two-way ANOVA was used to compare differences between the myofibrillar and mixed muscle (activity x muscle fraction), as well for ^2H-labeled alanine (activity x time of administration). If a test of normality or equal variance failed, a ranks test followed by a Dunn's Test was conducted. A Fisher LSD *Post hoc* test was used when a main effect was observed. We accepted an analysis as statistically significant if $P < 0.05$.

Results

Exercise vs. Control Assessed with F Flooding Dose

The specific activities (SA) of the plasma and muscle ($DPM \cdot nmol\ F^{-1}$) are shown in Tables 1 and 2 (pages 53 and 54). In all cases we obtained sufficient counts to allow for the calculation of protein synthesis. RE did not affect protein synthesis in any muscle type (Fig. 8, page 55; main panel FSR and inset RPS). As a result of this finding, we collapsed the groups to compare muscle type differences (data not shown) and observed

higher RPS in the soleus (89%, slow oxidative (3)) compared to the plantaris and mixed gastrocnemius ($P < 0.001$); which is consistent with previous findings (50, 57, 132) regardless of whether plasma or the intracellular compartment was used as the precursor pool in the calculations. In addition, FSR was significantly higher in the soleus vs. the plantaris and mixed gastrocnemius muscles ($P < 0.05$). The nmol of F and DPM relative to the pool size (mg protein · g wet weight^{-1}) did not differ statistically between the mixed gastrocnemius (122 ± 6 and 36 ± 5) plantaris (131 ± 9 and 41 ± 4) and soleus (109 ± 7 and 56 ± 7); however, given that total F was similar for all muscles, but more DPM relative to the pool size in the soleus, it suggests that muscle composed of predominately slow muscle have greater RPS and FSR values.

Table 1. *Plasma phenylalanine specific activity (F SA) and ^{2}H-labeling of body water*

Group	n	F SA	^2H_2O at 1h	^2H_2O at 24h
CON 4h	6	592.68 ± 47.55	NA	2.47 ± 0.12
RE 4h	6	575.76 ± 23.67	NA	2.80 ± 0.08
CON 24h	6	NA	2.63 ± 0.02	2.71 ± 0.16
RE 24h	6	NA	2.65 ± 0.13	2.69 ± 0.05

Values are means ± S.E. F SA, specific activity of plasma (DPM · nmol phenylalanine^{-1}); Plasma ^2H_2O expressed as MPE, mole percent excess.

Table 2. *Tissue phenylalanine SA and ^{2}H-labeling of protein-bound alanine*

Group	n	F SA Tissue	^{2}H-Labeling Tissue
		Mixed Gastrocnemius	
CON 4h	6	0.22 ± 0.06	0.37 ± 0.04
RE 4h	6	0.36 ± 0.02	0.50 ± 0.06
CON 24h	6	- -	0.72 ± 0.04 ‡
RE 24h	6	- -	0.71 ± 0.05 †
		Mixed Gastrocnemius Myofibrillar	
CON 24h	6	- -	0.69 ± 0.04
RE 24h	6	- -	0.65 ± 0.04
		Plantaris	
CON 4h	6	0.24 ± 0.05	0.43 ± 0.10
RE 4h	5	0.36 ± 0.02	0.35 ± 0.04
CON 24h	5	- -	0.74 ± 0.06 †
RE 24h	6	- -	0.71 ± 0.06 ‡
		Soleus	
CON 4h	5	0.44 ± 0.10	0.38 ± 0.07
RE 4h	6	0.49 ± 0.05	0.41 ± 0.05
CON 24h	6	- -	0.94 ± 0.10 ‡
RE 24h	6	- -	0.86 ± 0.04 ‡

Values are means ± S.E. F SA, specific activity of tissue ([DPM · nmol phenylalanine^{-1}]); ^{2}H labeling of protein-bound alanine expressed as MPE, mole percent excess. †Significantly different in 24 h from 4 h in same group (P<0.01); ‡Significantly different in 24 h from 4 h in same group (P<0.001).

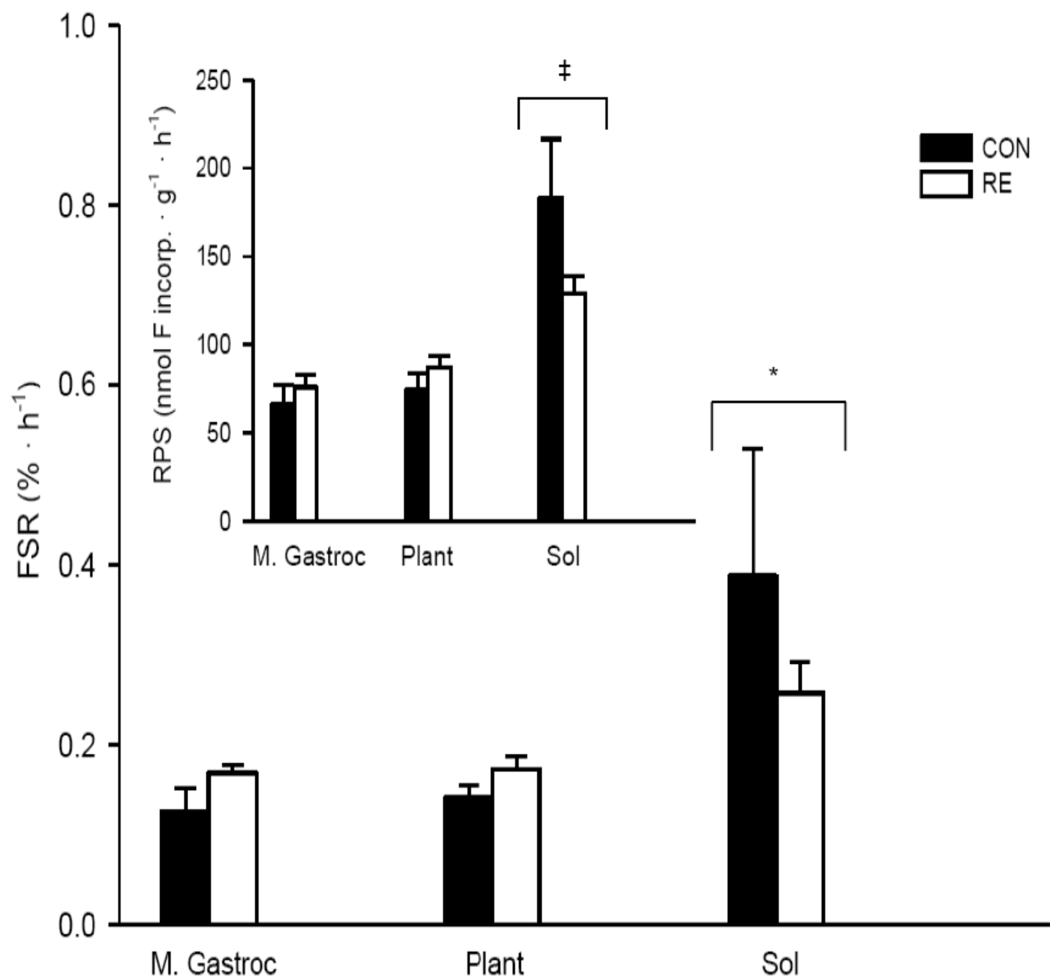
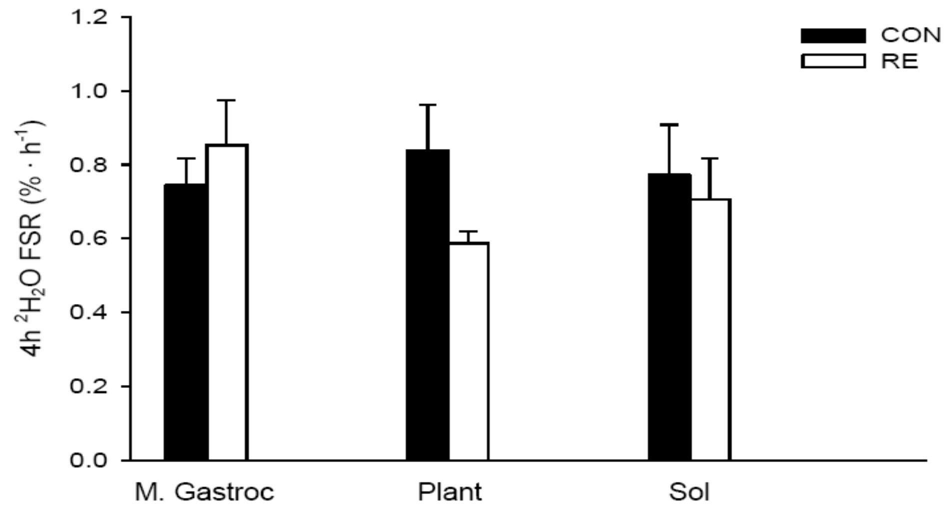


Fig. 8. Fractional rates of protein synthesis (FSR, main panel) and rates of protein synthesis (RPS, inset) assessed with L-[2,3,4,5,6-^{3}H]F in the mixed gastrocnemius (M. Gastroc), plantaris (Plant) and soleus (Sol) 16 h following the resistance exercise (RE) session or control (CON). Data are expressed as % · h^{-1} (nmol F incorp. · g^{-1} · h^{-1}, inset) and presented as means ± SE; *(n = 5-6 per group)*. The groups were collapsed to determine muscle type differences. * = significant differences between the Sol and the M. Gastroc and Plant for FSR ($P < 0.05$); ‡ = significant differences between the Sol and the M. Gastroc and Plant for RPS ($P < 0.001$); *(n = 11-12 per muscle)*.

Exercise vs. Control Assessed with $^{2}H_{2}O$

Tables 1 and 2 (pages 53 and 54) demonstrate that the labeling of body water remained virtually constant over the 24 h period and show incorporation of protein-bound ^{2}H Ala into the various muscles. Consistent with the F flooding dose, the $^{2}H_{2}O$ method did not detect any effect(s) of RE on protein synthesis in the 4h $^{2}H_{2}O$ (Fig. 9A, page 57) or the 24h $^{2}H_{2}O$ rats (Fig. 9B, page 57). In addition, similar to the F flooding dose higher FSR in the soleus muscle, we observed a significant increased in the 24h $^{2}H_{2}O$ rats soleus compared to the mixed gastrocnemius and plantaris (data not shown) when the CON and RE groups are collapsed ($P < 0.01$). It is commonly accepted that mixed muscle protein synthesis should be reflective of myofibrillar proteins (which comprise > 70% of the total protein pool (105)). However, myofibrillar proteins turnover at a much slower rate relative to other proteins in the mixed pool, which may limit one's ability to detect alterations in protein synthesis using methods over short periods. Thus, we directly examined the gastrocnemius myofibrillar fraction in the animals that received $^{2}H_{2}O$ over 24 h in order to determine whether or not protein synthesis in the myofibrillar fraction was reflective of the total mixed pool. Interestingly, FSR in the mixed muscle or myofibrillar proteins fractions did not differ ($P > 0.05$) in the 24h $^{2}H_{2}O$ CON (0.27±0.01 vs. 0.26±0.01 respectively), nor was it affected by RE (0.29±0.02 vs. 0.26±0.01, respectively).

A



B

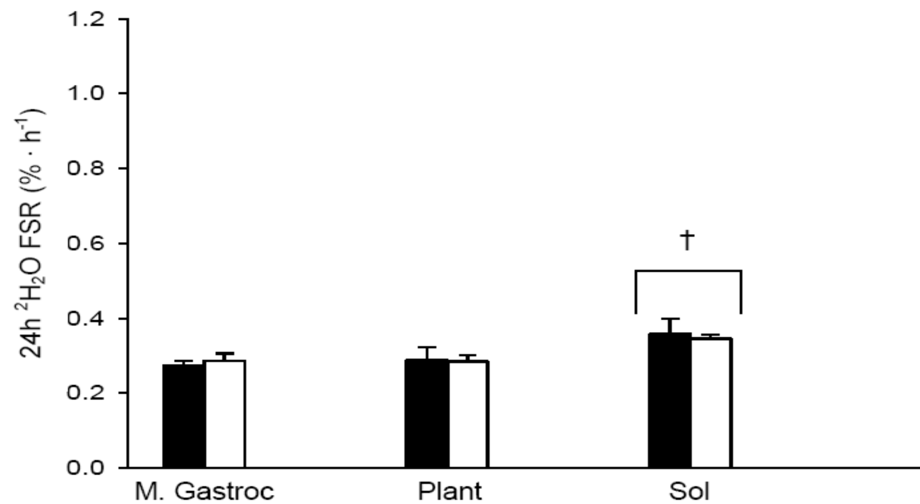


Fig. 9. Fractional rates of protein synthesis (FSR) assessed with ^2H_2O in animals over 4 h (*A*) and 24 h (*B*) in the mixed gastrocnemius (M. Gastroc), plantaris (Plant) and soleus (Sol). Data are expressed as % · h^{-1} and presented as means ± SE; *(n = 5-6 per group)*. The groups were collapsed to determine muscle type differences. † = significant differences between the Sol and the M. Gastroc and Plant in the 24 h *(B)* animals ($P < 0.01$); *(n = 11-12 per muscle)*.

F Flooding vs. 4h ^2H_2O and 24h ^2H_2O

In addition to making qualitative comparisons, we also wanted to make direct comparisons (quantitatively) between ^2H_2O (different time of assessment) and flooding dose expressed % · h^{-1} (Fig. 10, page 59). As a result of not detecting an exercise effect, we collapsed the groups and observed significant differences between 4h ^2H_2O approach and F flooding and 24h ^2H_2O in the mixed gastrocnemius, plantaris and soleus ($P < 0.001$); and between the 24h ^2H_2O and F flooding in the mixed gastrocnemius and plantaris ($P < 0.05$). Note that a greater percentage of protein was synthesized in the 24h ^2H_2O (7.2 ± 0.3, 7.4 ± 0.5 and 9.1 ± 0.6) vs. 4h ^2H_2O (4.4 ± 0.4, 4.0 ± 0.5 and 4.0 ± 0.5) and F flooding (0.05 ± 0.01, 0.05 ± 0.01 and 0.09 ± 0.01) in the mixed gastrocnemius, plantaris and soleus respectively ($P < 0.001$). These observations underscore the importance of tissue heterogeneity (i.e. the complexity of the total protein pool) and its affect(s) when calculating synthesis rates.

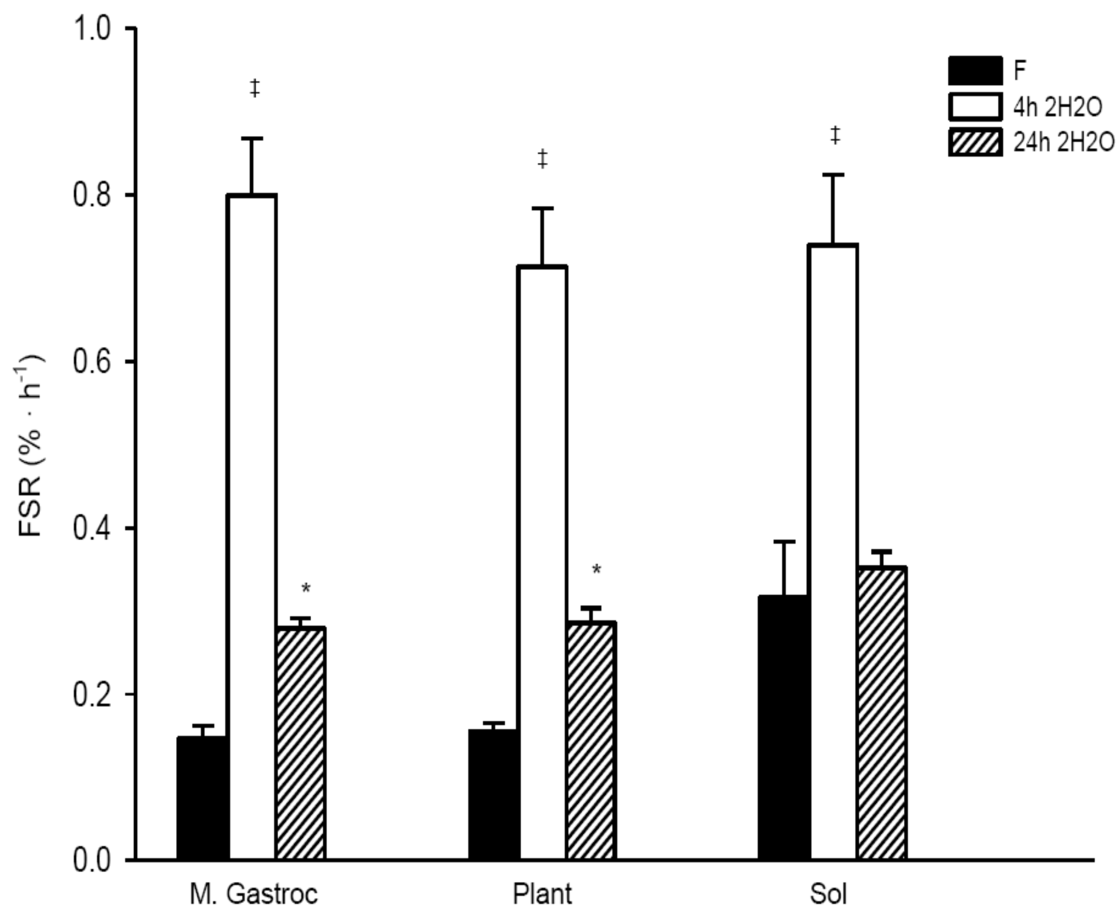


Fig. 10. Fractional rates of protein synthesis (FSR) assessed with F (flooding dose) and ^2H_2O (4 and 24 h) in the mixed gastrocnemius (M. Gastroc), plantaris (Plant) and soleus (Sol). Data are expressed as % · h^{-1} and presented as means ± SE; ‡ = significant difference between 4h ^2H_2O vs. F and 24h ^2H_2O for M. Gastroc, Plant and Sol ($P < 0.001$); * = significant difference between 24h ^2H_2O and F in the M. Gastroc and Plant; *(n = 11-12 per group)*.

Discussion

The main purpose of the present work was to determine if ^2H_2O provided comparable results as the traditional flooding dose (using L-[2,3,4,5,6-^{3}H]F as the tracer) with an acute bout of high intensity resistance exercise. We chose to include an acute bout of high intensity resistance exercise due to its anabolic nature and role in prevention and treatment of many diseases. Additionally, we wanted to measure protein synthesis rates over different time points with ^2H_2O (4 h and 24 h) to ascertain whether extrapolating short-term synthesis rates ($\% \cdot h^{-1}$) over a day provides similar values to the measured (% newly synthesized protein). We report three novel findings from our study; *i)* ^2H_2O provides comparable results to the flooding dose when studying sedentary and exercising conditions, *ii)* a single bout of high intensity resistance exercise without prior training does not appear to be anabolic in rats, and *iii)* extrapolating values that are measured over a short interval does not accurately reflect what might occur over an entire day.

The ability to quantify protein synthesis during non steady-state metabolism has presented a challenge to investigators since it can be difficult to assess amino acid precursor labeling, particularly as temporal changes or perturbations in biochemical flux (such as what occurs with feeding or exercise) disrupt steady state metabolism. The flooding dose method offers some advantages, in that measurements of protein synthesis can be made with infusion periods of ~ 10 to 90 min (33, 34, 63, 74, 120) by imposing a saturation of the precursor pool. Furthermore, flooding dose methodologies may be preferred in short term intervention studies where rapid physiological responses are

expected. However, flooding dose can become problematic when designing experiments since vascular cannulation has traditionally been required and measurements of protein flux over longer periods is not practical. The use of ^2H_2O for assessing protein synthesis can bypass some of the traditional tracer limitations; such as measurement of the true precursor pool, the need for post-absorptive conditions prior to initiation of the tracer, the necessity of a general clinical research center and laboratory confinement (if using humans) and skilled personnel for cannulation. In addition, measurements can be made over relatively short (~ 4 h) and long intervals (days or perhaps weeks), therein permitting acute and cumulative studies of protein synthesis *in vivo* (10, 30, 44, 111, 151). Note that ^2H_2O results a rapid equilibration (~ 20 min) between the hydrogen labeling in body water and those stably bound to free alanine (10, 44, 111), thus the labeling of plasma water seems to provide a reliable surrogate of the precursor labeling over a range of experimental conditions making the use of ^2H_2O a suitable tracer in the free living environment.

In the current studies we were unable to detect any stimulatory effect(s) of a single bout of resistance exercise on protein synthesis. Particular attention was focused on 16 h post-resistance exercise, since we and others have demonstrated that this time corresponds to the peak 'anabolic window' (time of elevated rates of protein synthesis) (23, 51, 52, 56, 57) after 2 or 4 bouts of exercise without prior training. Likewise, the cumulative measurements over a 24-hour period made using ^2H_2O did not reveal any stimulatory effect of resistance exercise on protein synthesis. These observations raise important questions regarding alterations of muscle protein synthesis after a single bout

of resistance exercise in animals that are consuming food, *ad libitum*. For example, resistance exercise exerts both anabolic and catabolic stimuli, whereas feeding simultaneously stimulates protein synthesis and inhibits protein breakdown (103, 106, 115, 127, 129). One might expect that while resistance exercise may lead to elevated protein turnover, the integrated effect of exercise and food consumption over a 24 h period should lead to elevated labeling of proteins in skeletal muscle. Our data suggest that a single bout of high intensity resistance exercise, without prior training in rats, may not be followed by a net anabolic effect. It is possible that our findings are a result of either the intense nature of the activity or that more time is necessary to observe the metabolic adaptations necessary to support the anabolic response to exercise.

One could argue that our resistance exercise regimen was too intense to observe anabolic responses to exercise, as opposed to our prior work (56, 57) and work by others (50-52, 84) which used lower intensities. Consistent with that possibility, we previously demonstrated that rates of muscle protein synthesis are blunted following a single bout of high intensity, eccentric exercise; however, a second bout of exercise of the same magnitude one week later demonstrated elevated rates of synthesis in a fiber-specific manner (54). Therefore, while we cannot rule out that the higher intensity of the resistance exercise protocol in the present study potentially blunted post-exercise anabolic responses, it is more likely that a single bout without prior training leads to adaptations in skeletal muscle that are necessary before anabolic responses can be observed with subsequent bouts.

We investigated muscle protein synthesis using two methodologies to account for

possible differences in the time of assessment. Reported fractional rates of mixed muscle protein synthesis typically range from ~ 0.05% per h post-absorptive to ~ 0.10% per h following feeding and RE in human vastus lateralis muscle (34, 41) and 0.2-0.9% per h under fasting-feeding conditions respectively in lower-limb rat muscle (63, 64, 133, 151). Clearly, it is difficult to extrapolate measurements of protein synthesis made over minutes to a few hours and draw conclusions regarding the total amount of newly made protein over the course of an entire day. In support of this, had we extrapolated the 4h ^2H_2O h and F flooding dose over 24, we would have over-estimated the % of newly made protein with the 4h ^2H_2O by 165% in the mixed gastrocnemius, 131% in the plantaris and 96% in the soleus ($P<0.001$) as well as underestimated FSR with the F flooding dose by 105% in the mixed gastrocnemius and 98% in the plantaris ($P<0.01$). However, when looking at the effect of time on the incorporation of label, it is clear that there is greater incorporation of ^2H-labeled alanine into muscle protein after 24 h vs. 4 h exposure to the isotope and therefore, as expected more protein was synthesized over 24 h (*Tables 1 and 2, pages 53 and 54*).

In addition to technical differences between the tracer methods, our seemingly discrepant differences in quantitative FSR extrapolated over time, particularly using the two deuterium oxide techniques, may be partly explained on the basis of food consumption. For example, although food was removed from all of the rats 4 hours prior to our assessment of muscle protein synthesis, it is possible that the rats consumed food up until the time of removal, allowing for labeling in the 4h group to be initiated in the postprandial state, ultimately enhancing FSR. While this happened in the other groups as

well, incorporation of label only occurred either minutes before the assessment of muscle protein synthesis 4 hours following food removal (F flooding), or over the entire 24 h period (24h ^2H_2O). Thus, the impact of food consumption during this period of time would be minimized in the former (postabsorptive) and diluted with respect to the 24 h labeling period of the latter. Figure 11 (*page 64*) shows the consequences of sampling at different time intervals, such that if one were to measure FSR over 3 different periods of time, it is possible to obtain measurements that do not reflect the true values during each period. This simple example emphasizes the importance (or consequences) of understanding the metabolic state of the tissue (fed, fasted, pre or post-exercise, etc) during assessments of muscle protein synthesis.

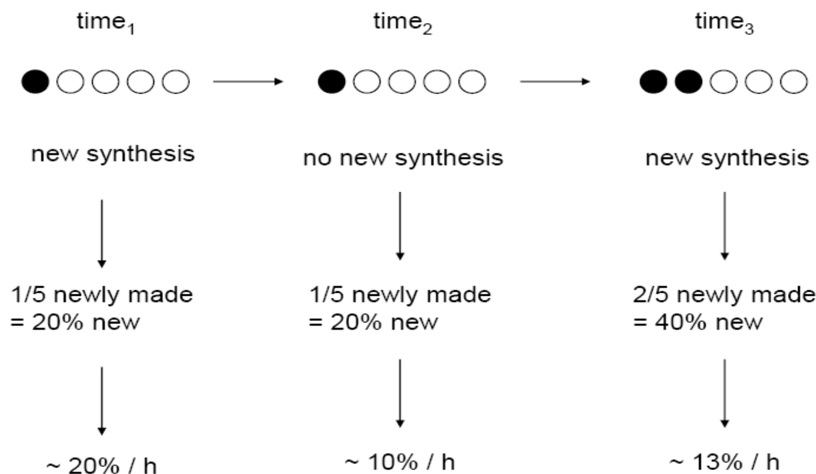


Fig. 11. Proposed scheme of measuring non-steady state protein synthesis in a homogeneous (purified protein) pool at different time points. Assuming that the time interval is the same (e.g. 1 h), if 1/5th of the available pool is newly synthesized protein, the percent per hour expression would be 20%. If in the next hour no new synthesis occurs, then 1/5th of the pool would be newly synthesized protein, however, the percent per hour expression would now only be 10%. If in the third hour of measurement another 1/5 of the pool is synthesized (2/5th total), the percent per hour expression would be ~13%, however, this fractional synthesis rate (FSR) does not represent the values obtained over either of the other intervals.

In summary, the present study demonstrates that a single bout of high intensity resistance exercise without prior training in rats does not stimulate muscle protein synthesis (mixed or myofibrillar) and that the phenylalanine flooding dose and $^{2}H_2O$ provide qualitatively similar results. Moreover, we conclude that one should consider the impact of extrapolating (regardless of methodology) *vs*. measuring FSR over different periods. Finally, $^{2}H_2O$ is an attractive stable isotope method for measuring protein synthesis due to the ease of administration and relative low cost, and most importantly because it can be effectively used during a variety of physiological perturbations.

CHAPTER IV

THE ASSESSMENT OF MUSCLE PROTEIN SYNTHESIS FOLLOWING CHRONIC RESISTANCE EXERCISE: A PLATEAU EFFECT?

Introduction

Resistance exercise training improves skeletal muscle function and in most, muscle mass. The observed muscle hypertrophy occurs as a result of an increase in muscle protein synthesis to a greater extent than muscle protein breakdown. This process has been well documented following acute resistance exercise in animals (50, 51, 55, 57, 84) and humans (15, 35, 108, 109) and it is believed that these anabolic effects are cumulative and responsible for growth over time. However, when compared to acute resistance exercise, chronic resistance exercise training appears to result in an attenuated muscle protein synthesis response (50, 77, 107, 109). The reasoning for these observations could be due to an adaptation occurring within muscle serving to blunt growth beyond a mammalian organism's potential, which can only be surpassed with altered stimuli; or the timing of muscle protein synthesis has changed.

The primary rate-limiting step in mRNA translation is initiation (116) and the rate limiting step in translation initiation is the binding of eukaryotic initiation factor 4E (eIF4E) to the transcript 5' cap structure, m^7GpppN, where N is any nucleotide (22, 28, 65, 95, 112, 123). A repressor binding protein (4E-BP1/PHAS1) modulates the activity of eIF4E by sharing the same binding site as the scaffolding protein eIF4G; however, this does not inhibit the cap interaction. The regulation of 4E-BP1 is via protein kinase B

(PKB) and the mammalian target of rapamycin (mTOR) protein kinase, which when activated by external stimuli (mechanical strain, growth factors and nutrients) result in phosphorylation of 4E-BP1 on Thr-37/46 (65). This phosphorylation serves as a priming event, which is required for subsequent phosphorylation of carboxy-terminal sites such as Thr-70 resulting in dissociation of eIF4E from 4E-BP1, thus allowing eIF4E to complex with eIF4G. Kubica et al. (84), using an exercise paradigm similar to that of the present study, demonstrated elevated mTOR-dependent (rapamycin-sensitive) 4E-BP1 phosphorylation 16 h after resistance exercise at the onset of training. Thus, 4E-BP1may be a suitable marker for mTOR activation with chronic resistance exercise training.

Although it is not completely understood whether or not cumulative hyper-phosphorylation of 4E-BP1 is necessary for elevated rates of synthesis after acute resistance exercise, we felt that a reduced capacity to phosphorylate 4E-BP1 may limit the capacity of skeletal muscle to elevate rates of protein synthesis. Therefore, the purpose of this study was to determine if rates of protein synthesis were augmented in response to an acute exercise bout after 5 weeks of resistance exercise training. Our hypothesis was that muscle protein synthesis after exercise training would be similar to control levels, and this would be, in part, due to attenuated phosphorylation of 4E-BP1.

Materials and Methods

Chemicals and Supplies

Unless specified, all chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA). $^{2}H_2O$ (99.9 atom percent excess) and was purchased from

(Cambridge Isotopes, Andover, MA). L-[2,3,4,5,6-^{3}H]phenylalanine (F) was purchased from GE Healthcare Life Science-Amersham (Buckinghamshire, England). Gas chromatography-mass spectrometry (GC-MS) supplies were purchased from Agilent Technologies (Wilmington, DE) and Alltech (Deerfield, IL). Antibodies, rabbit (polyclonal) anti-eIF4E (9742), anti-phospho-4E-BP1-Thr^{70} (9455), anti-4E-BP1 (9452), anti-rabbit IgG, HRP (7074) and kinase buffer (10X; 9802) were purchased from Cell Signaling Technologies (Danvers, MA).

Animals and Operant Conditioning

Forty-two male Sprague-Dawley rats (5-6 month old) were individually housed in a climate controlled small animal facility with a 12 hour light and dark cycle. Rats were fed ad libitum an experimental diet (Purina Test Diet, 5001) that was comprised of 24% protein, 12% fat, 54% carbohydrate, 7% ash, 5% fiber, and vitamins. Cholesterol content was manipulated so that half of the animals received 180 ppm and the other half received 1800 ppm to determine if cholesterol influenced protein synthesis and growth. At this time there is inadequate data to support an interactive effect of cholesterol and exercise on anabolism, therefore, the data was pooled and the rats were randomized by body mass to resistance exercise (RE, n = 16), exercise control (EC, n = 15) or sedentary control (CC, n = 11) groups. Additional work (studies) is required to accurately assess if cholesterol is a potent mediator of skeletal muscle hypertrophy. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Texas A&M University.

The RE and EC animals were operantly conditioned to depress an illuminated

bar located high on a Plexiglas exercise cage (56). Negative reinforcement via a brief electrical foot shock (1 mA, 60Hz) was used to train the rats to perform the desired movements. After the animals were conditioned (4 sessions), a non-weighted Velcro vest was strapped over the scapulae and the rats were required to touch the illuminating bar 50 times for two additional sessions. The CC animals were free from physical activity and remained single-housed over the course of the study.

Experimental Protocol

The RE and EC rats performed 15 training sessions (three sessions per week interspersed by at least 48 hours of rest) over a 5-wk period, modified from Westerlind et al. (139) and Farrell et al. (50). The RE group completed a progressive resistance training program with a starting weight of 80 g for 50 repetitions on session one and increasing to 410 g for 16 repetitions on session 15. The total volume was decreased (increased resistance and decreased repetitions) by 5% per week for a total of 25% over the 5-wk training period. The EC rats completed the same protocol following the resistance training sessions, performing the same number of repetitions and receiving the same number of electrical foot shocks, without resistance, to elicit a similar stress response experienced by the RE group (adrenal mass did not differ among groups, data not shown).

Prior to and after 5-weeks of training, all rats were anesthetized with Ketamine (40-55 mg/kg) and Medetomidine (0.4 mg/kg) and dual x-ray absorptiometry (DEXA, GE Lunar Prodigy small animal program) scans were completed to assess changes in body composition.

Twenty hours prior to the final exercise bout (Fig. 12, page 70), all animals received an intraperitoneal injection (IP) of 1.5 ml of ^2H_2O and allowed free access to drinking water enriched to 4.0% ^2H_2O. Food was withdrawn 4 h prior to the animals being anesthetized as previously described, and the left carotid artery and right jugular vein were cannulated with sterile, heparinized polythene tubing (PE-50). Rats remained sedated after the insertion of catheters and a flooding dose of tritiated L-[2,3,4,5,6-^{3}H]F (~1 mCi/rat) in unlabeled F (150 mM, 1ml · 100 g body mass^{-1}) was injected into the venous catheter over a 10- to 15-s period (63). Following injection, arterial blood samples (1 ml) were taken from the carotid cannula during a 12 min period (2, 6 and 12 min) for the determination of F specific radioactivity. After arterial sampling; the fast glycolytic fiber plantaris (Plant) and the slow oxidative fiber soleus (Sol) (3) were excised, wet weights recorded and immediately frozen between aluminum blocks and cooled in liquid nitrogen for later analysis.

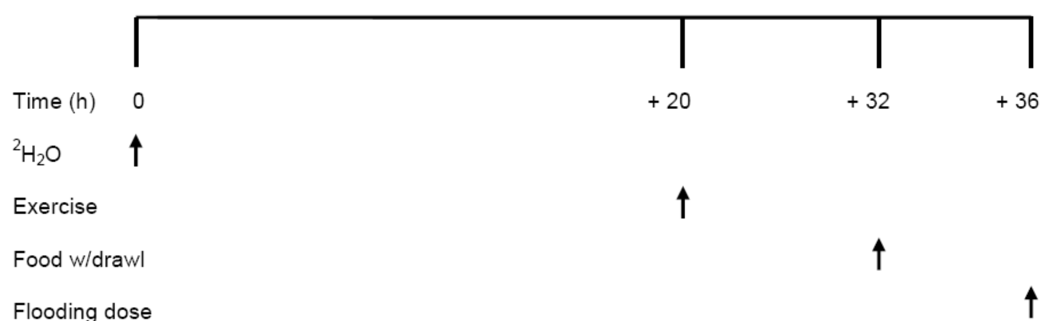


Fig. 12. Schematic display of study design used to measure the final 36 h following 5-weeks of resistance exercise training. The first 14 resistance exercise sessions were conducted over 5 weeks and the final session was held on wk-6. At time 0, 1.5 ml of ^2H_2O was provided (IP injection) to each rat and 4.0% ^2H_2O was added to the drinking water. The final resistance exercise session was completed at time + 20 h and at time + 36 h the animals were cannulated and underwent flooding dose methodology, followed by muscle harvest to assess muscle protein synthesis.

Rates of Protein Synthesis (RPS)

Approximately 0.150 g of muscle tissue was used to estimate the rate of incorporation of radioactive F into of the Plant and Sol mixed muscle proteins described by Vary et al. (133) (see Appendix A for detailed description). Briefly, the samples were pulverized under liquid nitrogen, homogenized in 2.0 ml of 10% trichloroacetic acid (TCA) and centrifuged at 3,800 rpm for 15 min at 4ºC. The supernatant (SPNT) was discarded and the protein precipitate was washed four additional times in 2.0 ml of 10% TCA, then freed from lipid with 2.0 ml of acetone, washed with 2.0 ml of distilled H_2O and dissolved in 2.0 ml 0.25 N NaOH. A portion of the protein was used to quantify protein content using the bicinchoninic acid (BCA) method (121). Radioactivity was determined in both tissue and plasma samples (0.5 ml and 0.05 ml, respectively) via liquid-scintillation counting using appropriate correction for quench. The total concentration of plasma F was determined using high-performance liquid chromatography (HPLC) with a fluorescence detector after 1.5 mol/l $HClO_4$ was added to deproteinize the sample according to Buentello and Gatlin (29). Rates of protein synthesis are reported as nmol F incorporated · g wet weight of muscle^{-1} · h^{-1} (133).

Fractional Protein Synthesis Rates (FSR)

^{2}H Labeling of body water was assessed by exchange with acetone as described by Yang et al. (148) (see Appendix B for detailed description). We administered (i.p.) much less ^2H_2O (1.5 ml) than has been described previously (8, 10, 30, 44, 151), but were able to achieve adequate ^{2}H labeling of body water ($\geq$ 2.0%) by the addition of 4.0% ^2H_2O to the drinking water. The reasoning for higher body water ^2H_2O in the CC

and EC animals could be due to the stressor of the final resistance exercise bout on the RE group, preventing them from drinking as much water. The reaction occurred with 20 µl of a sample (or standard), 2 µl of 10N NaOH and 4 µl of a 5% (vol/vol) solution of acetone in acetonitrile for 24 h. Acetone was removed by the addition of 600 µl of chloroform and 0.5 g Na_2SO_4. The samples were mixed and 100 µl of the chloroform was transferred to a GC-MS vial. The sample was analyzed using an Agilent 5973N-MSD equipped with an Agilent 6890 GC system, and a DB17-MS capillary column (30 m × 0.25 mm × 0.25 µm). The following temperature program was used: 60ºC initial, increase by 20°C/min to 100ºC, increase by 50ºC/min to 220ºC, and hold for 1 min. The sample was injected at a spit ratio of 40:1 with a helium flow of 1 mL/min. Acetone eluted at ~ 1.7 min. The mass spectrometer was operated in electron impact mode (70eV). Selective ion monitoring of mass-to-charge ratios (*m/z*) 58 (M) and 59 (M + 1) was conducted using a dwell time of 10 ms per ion.

^2H Labeling of protein-bound alanine was measured as previously described by Dufner et al. (44) (see Appendix B for detailed description). We chose to measure FSR in the plantaris and soleus due to the growth in the plantaris and the difference in fiber types (fast *vs.* slow). Briefly, ~ 0.030 g of Plant and Sol was homogenized on ice in 0.3 ml of a 10% (wt/vol) TCA and centrifuged at 3,800 rpm at 4°C for 15 min. The SPNT was discarded and the protein pellet was washed 3 additional times with 10% TCA prior to dissolving in 6 N HCl (0.1 ml per 0.030 g tissue) and reacting at 100ºC for 18 h. An aliquot (0.100 ml) of the hydrolysate was freeze dried for 24 h. A 3:2:1 ratio (0.1 mL) of "Methyl-8" reagent (Pierce, Rockford, IL), methanol and acetonitrile was added to the

residue to determine the ^{2}H labeling of alanine on its methyl-8 derivative. All samples were analyzed using an Agilent 5973N-MSD equipped with an Agilent 6890 GC system, and a DB17-MS capillary column (30 m × 0.25 mm × 0.25 µm) was used in all analyses. The following temperature program was used: 90ºC initial, hold for 5 min, increase by 5°C/min to 130ºC, increase by 40ºC/min to 240ºC, and hold for 5 min. The sample was injected at a spit ratio of 20:1 with a helium flow of 1 mL/min. Alanine eluted at ~ 12 min. The mass spectrometer was operated in electron impact mode. Selective ion monitoring of mass-to-charge ratios (*m/z*) 99 (M) and 100 (M + 1) was conducted using a dwell time of 10 ms per ion.

We calculated fractional synthetic rate of mixed muscle proteins (FSR) by measuring the incorporation of ^{2}H alanine into protein (E_A) and using the precursor-product model (see Appendix D for detailed description):

$$FSR = E_A/(E_{BW} \cdot 3.7 \cdot t) \text{ (eq 1)}$$

where E_A represents amount of protein-bound ^{2}H Ala (%), E_{BW} is the quantity of $^{2}H_2O$ in body water (%), 3.7 represents the exchange of ^{2}H between body water and alanine (44, 111, 146) and *t* is time of label exposure (36 h). Additionally, an absolute rate of protein synthesis (ABS) was calculated using the product of FSR and protein concentration per g of wet weight (146, 147):

$$APS = FSR/\ (mg\ protein \cdot g\ wet\ wt^{-1}) \text{ (eq 2)}$$

Quantification of 4E-BP1, Phospho-4E-BP1 and 4E-BP1/eIF4E Association

To determine eIF4E-BP1, phospho-eIF4E-BP1 (Thr 70) and eIF4E, 0.050 g of Plant was homogenized on ice in 400 µl of a 1 X kinase buffer (25 mM Tris-HCl, 5 mM

ß-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) with 200 µM ATP, 0.01% Triton and protease inhibitors (Sigma-Aldrich, St. Louis, MO) added. Homogenates were centrifuged at 14,000 rpm at 4ºC for 30 min. After centrifugation, the supernatant containing the cytosolic and membrane portion was extracted, protein content in the SPNT was assayed using the bicinchoninic acid (BCA) method (121), and 20 µg was added to a 10% discontinuous polyacrylamide gel. After electrophoresis, gels were transferred to a PVDF membrane using a semidry method (Immobilon-P; Millipore) and immunoblotted with the specific antibodies of interest mentioned above. Samples of mixed cardiac muscle from these animals were prepared identical and an equal quantity of protein was added to each gel to serve as a control. To determine if 4E-BP1 was bound to eIF4E, eIF4E was precipitated out of 50 µg of protein with anit-eIF4E, Super Block (Fisher Scientific, Pittsburgh, PA) and Protein A (Sigma-Aldrich, St. Louis, MO); modified from Kimball et al. (79). An equal amount (30 µl) was then added to a 10% discontinuous polyacrylamide gel as above, followed by electrophoresis, gel transfer and then immunoblotted with anti-4E-BP1.

Statistical Analysis

All data analysis was completed using SigmaStat version 3.5 and values are expressed as means ± S.E.M. Mean weekly body mass change comparisons were performed using a two way repeated measure analysis of variance (ANOVA) with the effects being physical activity and time. All other comparisons were made using a one way ANOVA with the effect being physical activity. *Post hoc* testing was performed using the Fisher LSD method when a main effect was observed. If a test of normality or

equal variance failed, then ANOVA on ranks followed by a Fisher LSD *post hoc* comparison test was performed. We accepted an analysis as statistically significant if $P < 0.05$.

Results

Effectiveness of the RET Protocol

There were no differences in pre-weights between RE (402 ± 2.5 g), EC (415 ± 2.6 g) or CC (407 ± 3.0 g) groups (Fig. 13, page 76), however, there were significant differences in the main effect interaction (physical activity x time) between RE vs. EC and CC from week 3 to the end of the study ($P < 0.001$), such that the RE group gained less wt (2.1%) than the EC (9.3%) and CC (9.1%) groups. Total body fat percentage and total lean tissue were measured pre-and post training with DEXA (Fig. 14, page 77). There were significant differences in body fat percentages (Fig. 14A, page 77) between RE (-6.6 ± 5.6%) and EC (+6.0 ± 5.4%) vs. CC (+44.9 ± 5.8%) animals following the training protocol ($P < 0.001$); but no differences in lean tissue accretion (Fig. 14B, page 77) among the groups. These data demonstrated a training effect in resistance exercised rats, as well as the efficacy of resistance exercise in the control of fat mass.

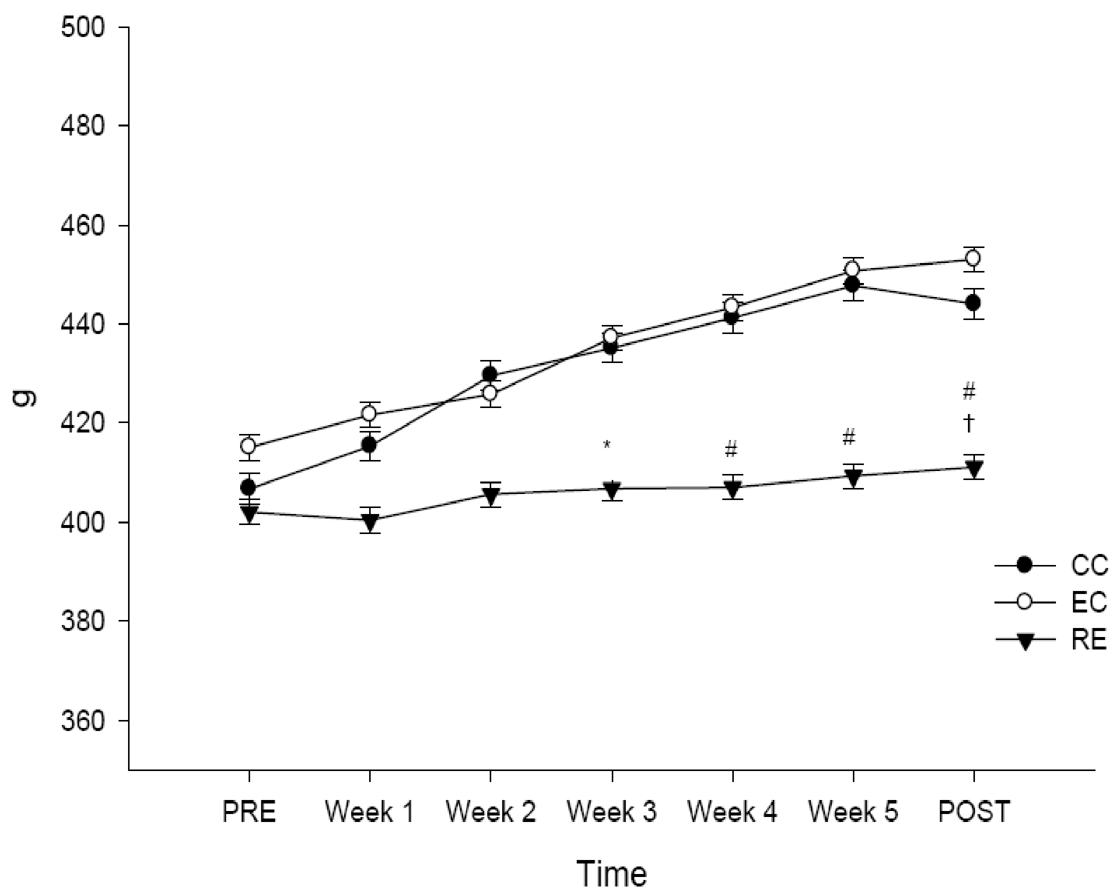


Fig. 13. Mean weekly weight gain in cage control (CC), exercise control (EC) and resistance exercised (RE) rats over 5 weeks. * = significant difference ($P < 0.05$) in RE vs. EC and CC rats. # = significant difference ($p < 0.01$) in RE vs. EC and CC rats. † = significant difference ($P < 0.001$) in RE vs. EC rats. Data are presented as means ± SE; *(n = 11-16 per group)*.

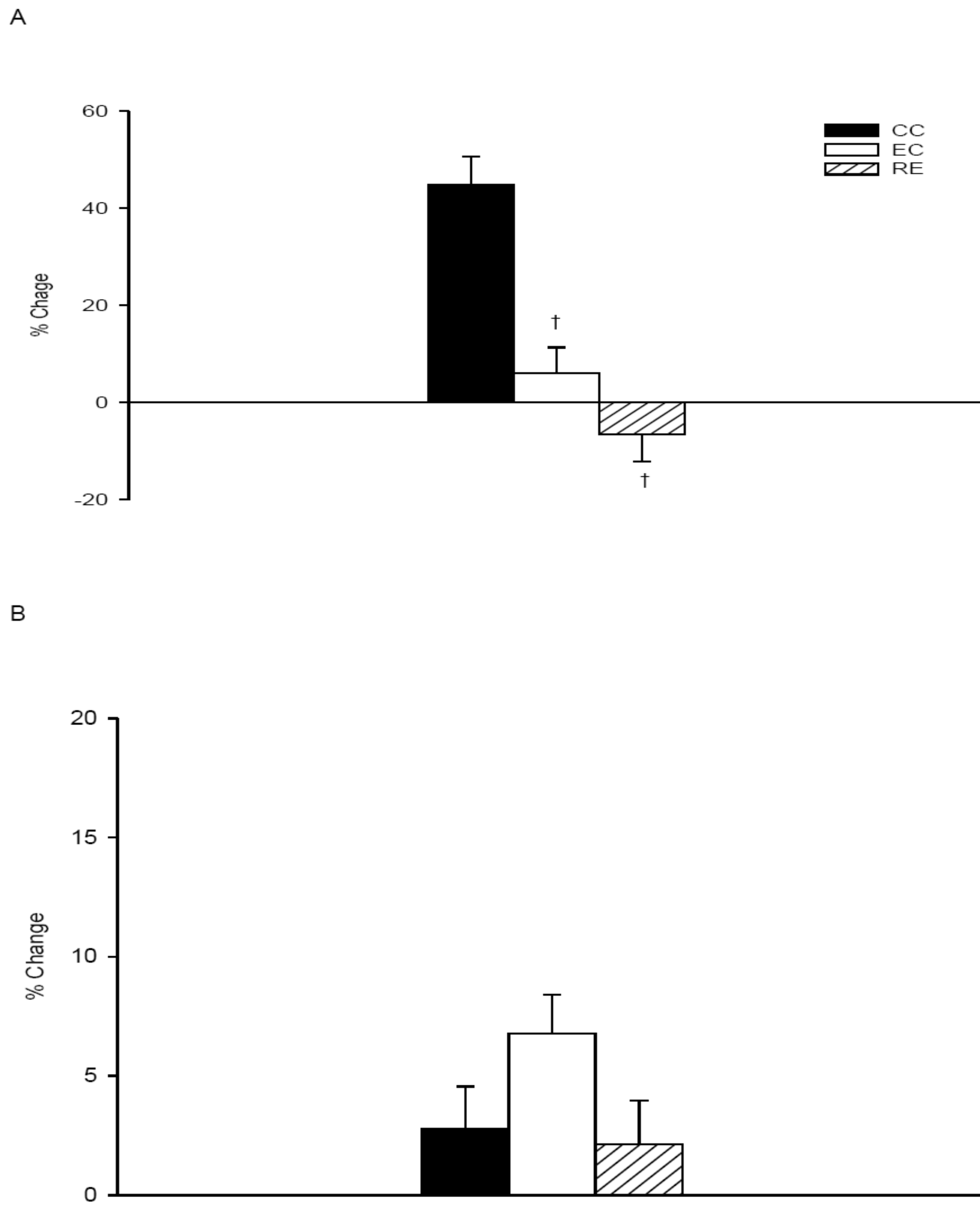


Fig. 14. Total body fat percentage *(A)* and total lean tissue *(B)* assessed (DEXA) before and after 5 weeks of RE training. Cage control (CC), exercise control (EC) and resistance exercised (RE) rats. † = significant difference ($P < 0.001$) in RE and EC vs. CC rats. Data are expressed as % change (pre and post) and presented as means ± SE; *(n = 11-16 per group)*.

Anabolic Response

Plant wet weights were significantly greater in the RE vs. EC (5.7%, $P < 0.05$) and RE vs. CC (8.2%, $P < 0.01$) animals (Fig. 15, page 78), whereas, no differences among groups for soleus muscle mass were observed. In spite of an increased growth response in the Plant, rates of muscle protein synthesis (assessed in a subset of animals) did not differ among RE, EC or CC groups in the Plant and Sol muscles (Fig. 16, page 79).

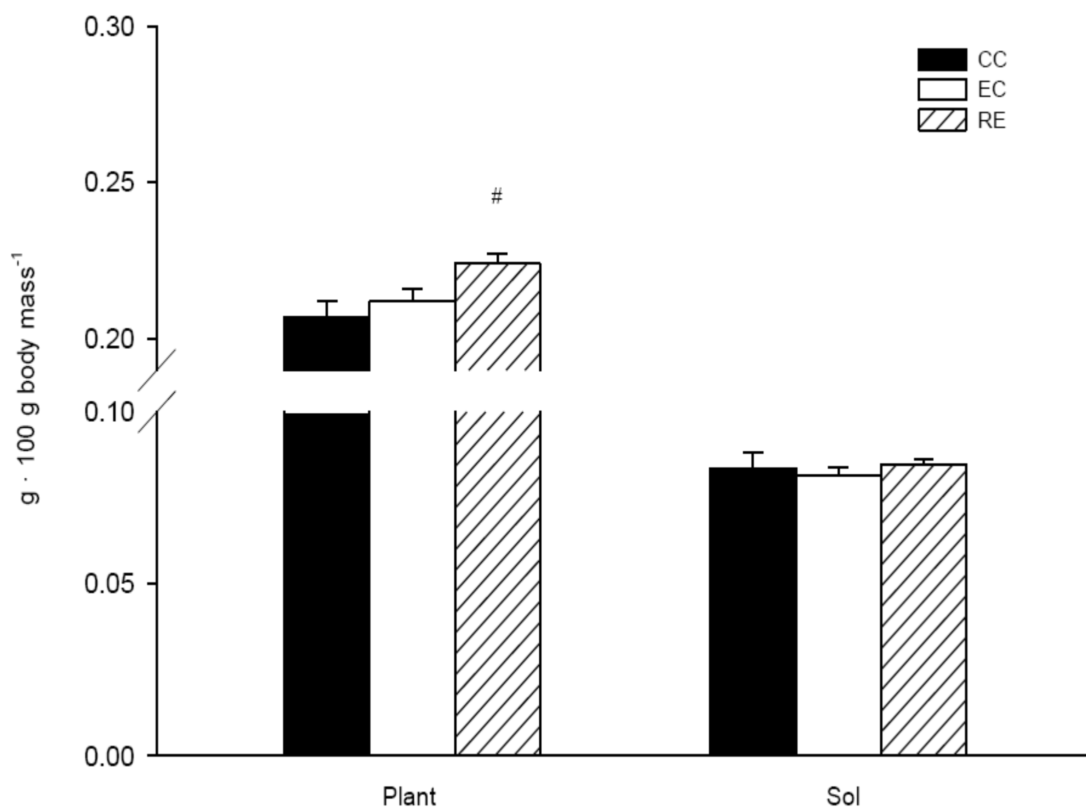


Fig. 15. Wet weight of plantaris (Plant) and soleus (Sol) muscles following 5 weeks of RE training. Cage control (CC), exercise control (EC) and resistance exercised (RE) rats. * = significant difference ($P < 0.05$) in RE vs. EC rats. # = significant difference ($P < 0.01$) in RE vs. CC rats. Data are presented as means ± SE; *(n = 8-15 per group)*.

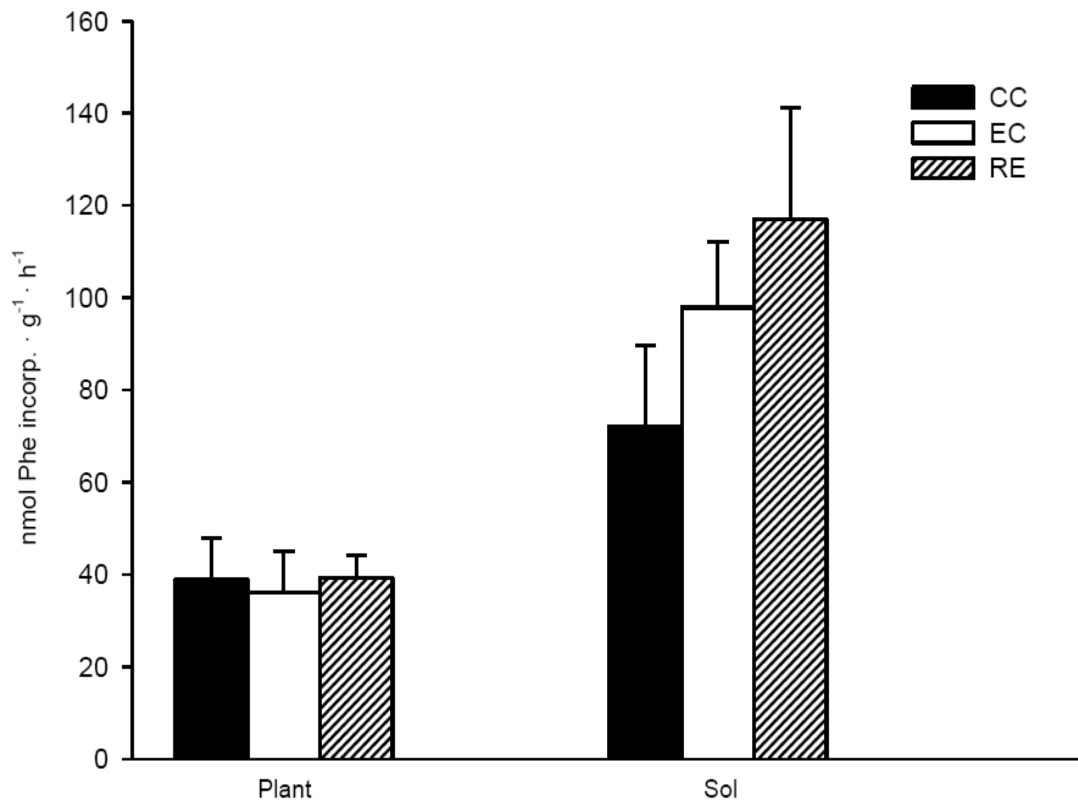


Fig. 16. Rates of mixed muscle protein synthesis (RPS) for plantaris (Plant) and soleus (Sol) assessed 16 h following the final bout of RE after 5 weeks of RE training. Cage control (CC), exercise control (EC) and resistance exercised (RE) rats. Data are presented as means ± SE; *(n = 5 for CC and EC, n = 9 for RE).*

4E-BP1, Phospho-4E-BP1 and 4E-BP1/eIF4E Association

In order to determine if similarities in muscle protein synthesis among groups were due to attenuated mTOR downstream signaling, we determined the total expression of 4E-BP1, the phosphorylation status of 4E-BP1 (Thr 70) and the bound 4E-BP1 to eIF4E in the Plant relative to control (heart standard) (Fig. 17, page 80). There were no differences between the RE, EC or CC groups in the expression of 4E-BP1 (Fig. 17A, page 80), phosphorylation of 4E-BP1 (Fig. 17B, page 80) or the 4E-BP1 and eIF4E complex (Fig. 17C, page 80).

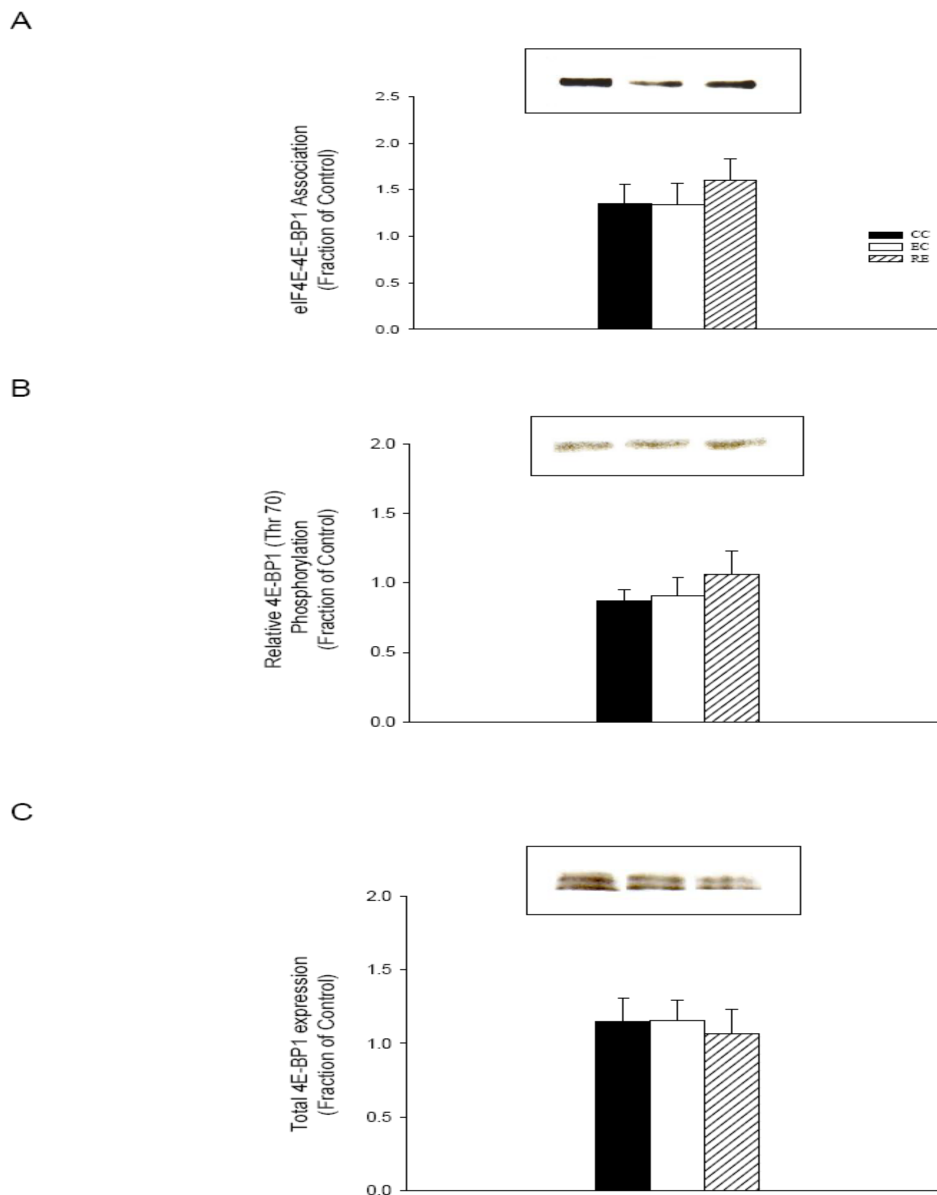


Fig. 17. Downstream targets of mTOR assessed 16 h following the final bout of RE after 5 weeks of RE training in the plantaris. Control, heart standards. Western blot analysis and graphical summaries of mean densitometry values are depicted. *(A)* eIF4E-4E-BP1 complex in cage control (CC) *(lane 1)*, exercise control (EC) *(lane 2)* and resistance exercised (RE) *(lane 3)*. *(B)* 4E-BP1 phosphorylation in cage control (CC) *(lane 1)*, exercise control (EC) *(lane 2)* and resistance exercised (RE) *(lane 3)*. *(C)* Total 4E-BP1 in cage control (CC) *(lane 1)*, exercise control (EC) *(lane 2)* and resistance exercised (RE) *(lane 3)*. Data are presented as means ± SE; *(n = 7-10 per group)*.

Assessment of Protein Synthesis with ^2H_2O

The IP injection with1.5 ml of ^2H_2O and the 4.0% ^2H_2O added to the drinking water yielded at least 2.0% plasma ^2H_2O, with the CC and EC animals obtaining higher enrichment (Table 3, page 81). The only difference in protein labeling (^{2}H-Ala) was in the Plant; with the RE group possessing much higher labeling (Table 3, page 81). The fractional synthesis rates (Fig. 18A, page 82) in the RE group were 142% higher than EC and 210% greater than the CC animals ($P < 0.001$). Due to the time of the labeling with ^2H_2O (36 h) and the hypertrophy of the plantaris, we calculated absolute protein synthesis rates (mg of protein synthesized per gram of tissue) to account for any changes that may have occurred in the protein pool (Fig. 18B, page 82). The trend was similar with RE yielding a 139% greater absolute rate of protein synthesis than EC and 250% greater than CC ($P < 0.001$). No differences were observed for either fractional or absolute synthesis rates in the Sol muscle.

Table 3. *^{2}H-labeling of body water and skeletal muscle proteins*

Group	n	Plasma (MPE)	Plantaris (MPE)	Soleus (MPE)
CC	7-8	2.92 ± 0.18	1.16 ± 0.12	1.00 ± 0.12
EC	9-10	2.31 ± 0.11#	1.16 ± 0.10	1.08 ± 0.11
RE	9-10	2.00 ± 0.06*†	2.53 ± 0.29†	0.89 ± 0.12

Values are means ± SE. MPE, mole percent excess. Cage control, CC; exercise control, EC and resistance exercise, RE. * Significantly different (P<0.05) from EC group; $^\#$ significantly different (P<0.01) from CC group; † significantly different (P<0.001) from CC group in plasma and from CC and EC groups in the plantaris.

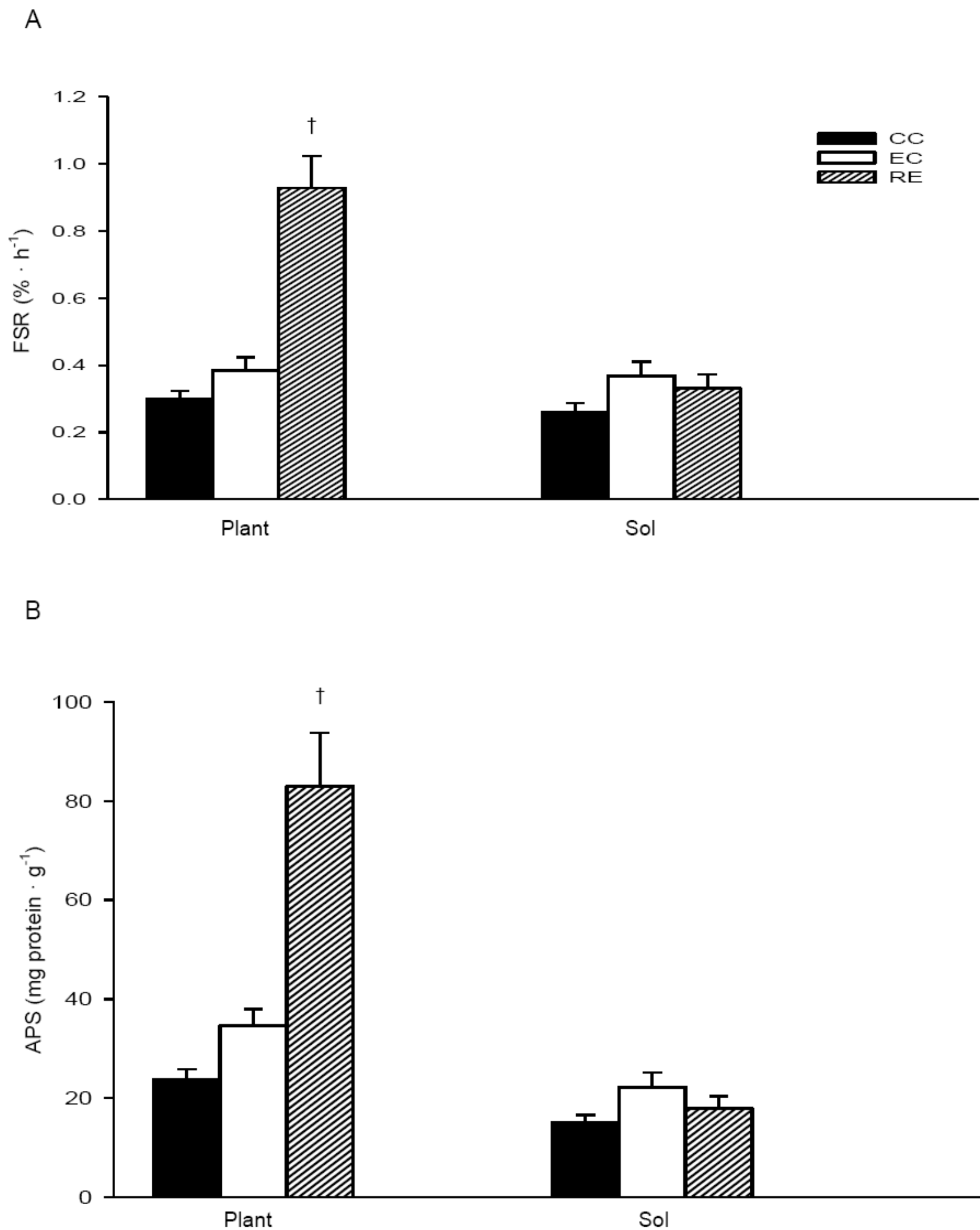


Fig. 18. Mixed muscle protein fractional synthetic rate (FSR) and absolute rates of protein synthesis (ASR) assessed over 36 h following 5 weeks of RE training in the plantaris of cage control (CC), exercise control (EC) and resistance exercised (RE) rats; and soleus of CC, EC and RE muscles. † = significant difference ($P < 0.001$) in RE vs. EC and CC rats. Data are presented as means ± SE; *(n = 7-10 per group)*.

Discussion

Part of the significance of the present study is that it reports the absence of an acute anabolic response to resistance exercise training when rates of protein synthesis are examined in the pre-training anabolic window. Protein synthesis was even normalized in the plantaris, which hypertrophied with resistance exercise training. Consistent with our measurements in the traditional anabolic window, both the phosphorylation on 4E-BP1 (Thr 70) and the fraction of 4E-BP1 bound to eIF4E was similar in resistance exercise training and sedentary controls. Taken together, these results appear to indicate that chronic resistance exercise training, even that leading to the accumulation of muscle mass, may accomplish muscle hypertrophy with no sustained elevations of muscle protein synthesis. However, when the cumulative anabolic response was assessed over a 36 h period, the FSR with training was significantly greater than in the non-resistance trained muscle (at least in hypertrophied muscle), suggesting that evaluations of muscle protein synthesis in the traditional anabolic window prior to training may not accurately reflect the integrative anabolic potential that exercise training provides.

The lack of an apparent anabolic effect of exercise with training is not novel. Farrell et al. (50) observed the lack of an anabolic response (rates of muscle protein synthesis) in selected hindlimb muscles following 8 wk of resistance exercise using a similar model in rats. In humans, Phillips et al. (109) observed an attenuation in mixed muscle FSR within 4 h following an acute bout of resistance exercise in individuals who had been undergoing resistance exercise training for more than 5 years (self-reported).

Additionally, Kim et al. (77) reported a decrease in mixed muscle FSR and no change in myofibrillar FSR when comparing a trained vs. untrained leg 12 h following an 8-week unilateral resistance exercise leg protocol. In the case of the human studies, it should be noted that both studies examined FSR in the fasted state, which may have influenced the lack of an anabolic response with training. However, another study by Phillips et al. (107), which examined the effects of an 8 wk resistance exercise training program on mixed muscle FSR in the fed state found that prolonged resistance exercise training may reduce the anabolic stimulus of resistance exercise. Thus the use of periodic assessments of muscle protein synthesis typically demonstrates a training-associated loss of the anabolic potential with chronic resistance exercise training, even when hypertrophy occurs.

Consistent with our observations regarding rates of muscle protein synthesis, we found that phosphorylation of 4E-BP1 (Thr70), as well as 4E-BP1/eIF4E association was similar among groups. The lack of response in plantaris muscle, which underwent hypertrophy, may be an important contributor to the aforementioned 'anabolic plateau' with resistance exercise training. Interestingly, we initially hypothesized that this plateau may be due to an over expression of 4E-BP1, which would serve to inhibit muscle protein synthesis even with adequate mTOR activation. However, since over expression of 4E-BP1 was not observed in the present study, we may alternatively conclude that the lack of hyperphosphorylation of this important anabolic suppressor, at least 16 h post exercise, may be due to attenuated mTOR activation after chronic resistance exercise training. More studies are necessary to elucidate this possibility.

Another possibility is that the anabolic response to exercise is still intact, but our ability to assess the effect of exercise in the traditional 'anabolic window' may be diminished. To explore this possibility, we assessed FSR over the final 36 h period of resistance exercise training with $^{2}H_2O$ and found a significant, cumulative, anabolic response in plantaris muscle. To our knowledge, this study is the first to utilize this methodological approach in skeletal muscle with exercise. The use of $^{2}H_2O$ allows for ^{2}H to label body H_2O and alanine, which may then be incorporated into proteins, including skeletal muscle, allowing measurements of protein synthesis in either an acute mode or during chronic exposure to the isotope (44, 111, 151). We chose to provide $^{2}H_2O$ over the final 36 h to allow for a *'cumulative'* assessment of FSR, thus allowing for food, sleep and exercise to influence the outcome (normal physiology), and ultimately, rule out the possibility that we may have missed the 'anabolic window' with traditional flooding dose methodology. In addition, because the time of measurement was over 36 h, we determined the absolute rate of protein synthesis to account for any changes that may occur within the plantaris or soleus protein pool and observed a similar pattern as the FSR changes. Our data clearly show that FSR, when assessed for extended periods of time, are greater than 2-fold in plantaris muscle of resistance exercise trained rats, a finding not observed using flooding dose techniques.

In support of a shift in the anabolic window comes from a recently published investigation by Tang et al. (124), whereby an 8-week unilateral resistance exercise leg protocol was followed, similar to Phillips et al. (107); the difference between those studies (107, 124) was that the relative work of the former load was changed following

training to represent the strength gains. They (124) reported significant increases in muscle protein synthesis in the trained leg 4 h post-exercise, but attenuation at 28 h. Thus, identification of this altered window becomes problematic when choosing which time points to examine. This issue can be resolved by utilizing ^2H_2O, which can be used for prolonged periods of time.

In contrast to the plantaris muscle, the cumulative assessment of FSR in soleus muscle demonstrated no differences among groups, which was consistent with our findings using flooding dose techniques. Interestingly, unlike plantaris muscle, the soleus did not hypertrophy in response to chronic resistance exercise training, and therefore may not be responsive to this training paradigm. At the onset of training, the present work demonstrates that the soleus muscle is highly active during the exercise movements (unpublished observations) and responds with elevated rates of synthesis after exercise. However, it is possible that this highly active, postural/locomotor muscle is not responsive to chronic, voluntary training in intact hindlimbs.

It is not clear why these muscle fibers responded so differently with exercise training; however, it is possible that anabolic responses to training may also depend on fiber composition, as plantaris is predominately composed of faster fibers (93%) and soleus is composed of slower fibers (89%) (3). Irrespective of fiber composition, findings of the present experiment reveal that cumulative absolute protein synthesis and FSR are almost identical, supporting the notion that skeletal muscle protein turnover is low, regardless of fiber composition. From the present work, it cannot be inferred if the enhanced cumulative anabolic response in plantaris muscle is due to a temporally

distinct anabolic window at another time-point(s), or a subtle, but persistent elevation of protein synthesis for greater periods of time. Therefore, more studies are necessary to explore these possibilities.

In summary, this study highlights the importance of assessing total, cumulative anabolic responses to interpret anabolic potential of skeletal muscle with exercise training. When using shorter-term flooding dose procedures, there were no observable differences in mixed muscle rates of protein synthesis of exercised *vs.* sedentary muscle controls, 16 h following the final exercise session. This finding is consistent with the notion that chronic resistance exercise training leads to normalized muscle protein synthesis, even in hypertrophic muscle. However, when cumulative anabolic responses were assessed on hypertrophied muscle fibers over an extended period of time, the present work demonstrated that exercised muscle fibers display anabolic responses more than two-fold higher than those of the control muscles. Results from the present work do not support a ‘plateau’ effect following chronic resistance exercise training, and further suggest that protein anabolism is still greater with training, although the precise mechanisms cannot be explained at this time.

CHAPTER V

IMMEDIATE POST-RESISTANCE EXERCISE PROTEIN CONSUMPTION DOES NOT AUGMENT HUMAN CUMULATIVE MIXED MUSCLE PROTEIN SYNTHESIS

Introduction

There is little disagreement among muscle biologists that skeletal muscle protein synthesis must be, in some order magnitude, greater than protein breakdown for muscle growth to occur. Known stimuli of increasing protein synthesis are nutrient ingestion, physiological secretion of hormones/steroids and an adequate amount of overload produced from resistance exercise. Feeding alone increases human muscle protein synthesis and it is believed that the extracellular amino acid concentrations supplied by the diet mediate this response (20, 34, 58). Additionally, upon ingestion of nutrients, a pulsatory secretion of hormones (insulin) supports this process, mainly by inhibiting protein breakdown (33, 36, 66). Resistance exercise, in the post-absorptive state, has been reported to increase mixed muscle (15, 41, 50, 52, 57, 77, 84, 108, 109), myofibrillar (77, 94) and sarcoplasmic (94) protein synthesis in animals and humans from 1-72 h following the session; as well as increasing breakdown (41). The combined effect of resistance exercise and nutrients provide an interactive benefit in stimulating anabolism and attenuating catabolism in skeletal muscle (17, 40, 80, 114, 127, 128). The ideal timing for nutrient ingestion with resistance exercise training is, however, not well defined.

Long-term (12 weeks) studies by Esmarck et al. (47) and Andrews et al. (2) support the notion that protein consumption in the early period following resistance exercise is paramount for optimal muscle growth to occur in seniors. However, Rankin et al. (113) did not observe differences between post-exercise protein or carbohydrate consumption in 18-25 year old men who underwent 10-weeks of resistance exercise training. None of the investigations measured protein turnover; therefore, it is difficult to conclude whether post-exercise protein consumption truly influences the growth process.

There have been several acute resistance exercise investigations that directly measured muscle protein metabolism with protein and/or carbohydrate before (59, 117, 126, 128), during (9, 18) or after (25, 40, 80, 114, 126, 127) with mixed results. The discrepancy in the results can be attributed to differences in the methodological approach, such as the stable isotope ([^{13}C] leucine, or ([^{13}C], [^{15}N], [^{2}H]) phenylalanine) and the precursor pool used for calculation of muscle fractional rates of protein synthesis (FSR); as well as the intensity of the resistance exercise bout and the amount of nutrients provided at varying time points. In addition, the subjects are typically studied following an over-night fast due to the influence that feeding has on precursor enrichment (label dilution). This raises questions as to the applicability of the findings as humans eat, perform activities of daily living and sleep during a 24 h cycle.

Cumulative assessments (over 24 h) of whole body protein synthesis have been made by using oral [1-13C]lysine (45) or intravenous [1-13C]leucine (24); however, no studies have examined skeletal muscle over 24 h. Thus, it is assumed that a measurement obtained over minutes to 6 h can be extrapolated to yield daily rates, which in human

skeletal muscle is believed to be 1.5-2% per day (62). However, we recently discovered that this is not the case when measuring skeletal muscle FSR in rats over 24 h with ^2H_2O compared to extrapolated values obtained with either flooding dose of L-[2,3,4,5,6-^{3}H]phenylalanine over 12 min or a 4 h ^2H_2O measurement (calculated values can under and/or over estimate FSR). The use of ^2H_2O allows for protein synthesis measurements to be made over short or extended periods of time. In addition, since ^2H_2O is not influenced by feeding, exercise, stress and sleep (normal biological processes); one can study perturbations that are typically difficult to account for with traditional tracer methodologies, thus providing a cumulative, real life picture.

We, therefore, sought out to determine the effects of high intensity resistance exercise on cumulative FSR and whether the timing of post-exercise protein consumption (1 or 3 h) influences this response when energy and macronutrients are controlled. Our hypothesis was that FSR would be increased with an acute bout of high intensity exercise and that immediate post-exercise protein consumption would augment this anabolic response. We chose to examine both the mixed muscle and myofibrillar fraction to determine if differences in protein specific FSR responses occur over 24 h.

Materials and Methods

Chemicals and Supplies

Unless specified, all chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA). ^2H_2O (99.9 atom percent excess) was purchased from Cambridge Isotopes (Andover, MA). Gas chromatography-mass spectrometry supplies

were purchased from Agilent Technologies (Wilmington, DE) and Alltech (Deerfield, IL). Kinase buffer (10X) was purchased from Cell Signaling Technologies (Danvers, MA).

Subjects

Twelve healthy, recreationally active young males (Table 4) volunteered to participate in an acute bout of high intensity resistance exercise. All participants were

Table 4. *Subjects' characteristics*

	PRO (*n=6*)	CON (*n=6*)
Age, yrs	22.33 ± 0.67	20.83 ± 0.31
Ht, cm	179.00 ± 2.91	181.93 ± 1.91
Wt, kg	81.14 ± 5.64	82.70 ± 3.57
BMI, kg/m^2	25.13 ± 1.23	25.00 ± 1.32
Lean tissue, kg	65.75 ± 3.45	64.31 ± 0.82
Body fat, %	15.35 ± 2.24	18.42 ± 3.58
1RM LP, kg	244.09 ±16.55	201.29 ±23.52
1RM LP/lean tissue	3.74 ± 0.25	3.21 ± 0.35
1RM KE, kg	99.24 ± 8.34	96.21 ± 6.83
1RM KE/lean tissue	1.52 ± 0.11	1.50 ± 0.11

Values are expressed means ± SE. PRO, immediate post-exercise protein; CON, carbohydrate control; BMI, body mass index; 1RM, single repetition maximum (LP, leg press; KE, knee extension). No significant difference in any characteristics.

informed of study purpose, procedures and risks associated with participation prior to providing informed consent. Subjects were randomized by lean tissue, in a double blinded manner, to either immediate post-exercise protein ingestion (PRO, n = 6) or an isocaloric carbohydrate control (CON, n = 6). Subjects completed a medical screening questionnaire to insure that they were healthy and free of chronic disease. Exclusion from participation included the following; a) collegiate athletes, b) regular resistance training in the previous 6 months, and c) consuming ergogenic aids. The Institutional

Review Board of Texas A&M University approved all experimental procedures.

Diet and Post-Exercise Supplementation

All subjects received 1 h of nutrition education by a registered dietitian (author) and were required to record 72 h (2 week days and 1 weekend day) of normal nutrient intake; as well as 24 h prior to the day of the study. Diets were analyzed for macronutrient composition using NutriBase 7.0 Clinical (Phoenix, AZ). The 72 h mean energy and macronutrient intake were 12,035 ± 988 kJ, 47 ± 2% carbohydrate, 20 ± 2% protein (1.7 ± 0.2 g protein $\cdot kg^{-1}$ body mass) and 33 ± 2% lipid.

In order to control diet for 24 h (study day), subjects were provided all meals in the form of liquid supplements (Boost Plus and Boost High Protein; Novartis Medical Nutrition, Fremont, IN). Each participant consumed 4 Boost Plus and 2 Boost High Protein during the 24 h period (Fig. 19, page 94) to provide a total of 8033 kJ with 56% being supplied by carbohydrates, 15% from protein (1.1 ± 0.04 g protein $\cdot$ kg^{-1}body mass) and 29% from lipid. Additionally, subjects received a beverage immediately following resistance exercise and 3 h after. The PRO group received the protein beverage immediately following resistance exercise and the placebo 3 h later, whereas, the CON group consumed the placebo immediately after exercise and the protein beverage 3 h later. The volume of beverage supplied was based on the amount of protein relative to body mass (0.3 g protein $\cdot kg^{-1}$) in both groups. This amount was chosen to provide an adequate precursor substrate for protein synthesis/anabolism and has been used previously (2, 81). The protein containing beverage (Gatorade Nutrition Shake; Chicago, IL) supplied 1959 ± 51 kJ, 0.8 g carbohydrate $\cdot kg^{-1}$, 0.3 g protein $\cdot kg^{-1}$ (milk

isolate, whey and casein) and 0.1 g fat $\cdot kg^{-1}$; whereas the placebo (85% Waximaize Fruit Punch; Innovative Delivery Systems, Oviedo, FL and 15% Peanut Oil; commercially available) provided 1974 ± 52 kJ, 1.2 g carbohydrate $\cdot kg^{-1}$, 0.0 g protein $\cdot kg^{-1}$ and 0.1 g fat $\cdot kg^{-1}$.

In total, subjects consumed 12,217 ± 128 kJ, 56% carbohydrate, 15% protein (1.4 ± 0.2 g protein $\cdot kg^{-1}$ body mass) and 29% lipid during the study day. Energy (kJ) and macronutrient composition (g) did not differ between the subjects 72 h, 24 h and study day intake; however, composition as a percentage was different ($p < 0.05$). We chose to increase the percentage of carbohydrates and reduce the percentage of protein and lipid to remain consistent with the dietary guidelines for Americans and athletes (1, 73).

Acute Resistance Exercise

Subjects performed an acute bout of dynamic resistance exercise (85 % of 1 repetition maximum (1RM) until exhaustion) of leg-press (5 sets) and leg-extension (5 sets) (Keiser, Fresno, CA) using one leg with the contralateral leg serving as a control. Subjects were allowed 2 min rest between sets and 5 min between the two exercises. A familiarization period and a 1 RM test were completed > 7 days prior to the study day, with at least 48 h separation between the two. Participants were instructed to maintain regular physical activity, but to refrain from any resistance activity 7 days prior to the day of the study.

Body Water Enrichment of ^2H_2O

All study participants reported to The Human Countermeasures Laboratory at 0800 (Fig. 19, page 94) the morning of the study and consumed the first of four 75 mL

boluses of 70% ^2H_2O. This amount was chosen based on previous human investigations (30, 111) with the goal of obtaining ~ 0.04 atom percent excess of ^2H labeling of body water. EDTA serum was collected at 4 time points over 24 h for measurements of body water ^2H enrichment. No adverse affects occurred in any of the subjects.

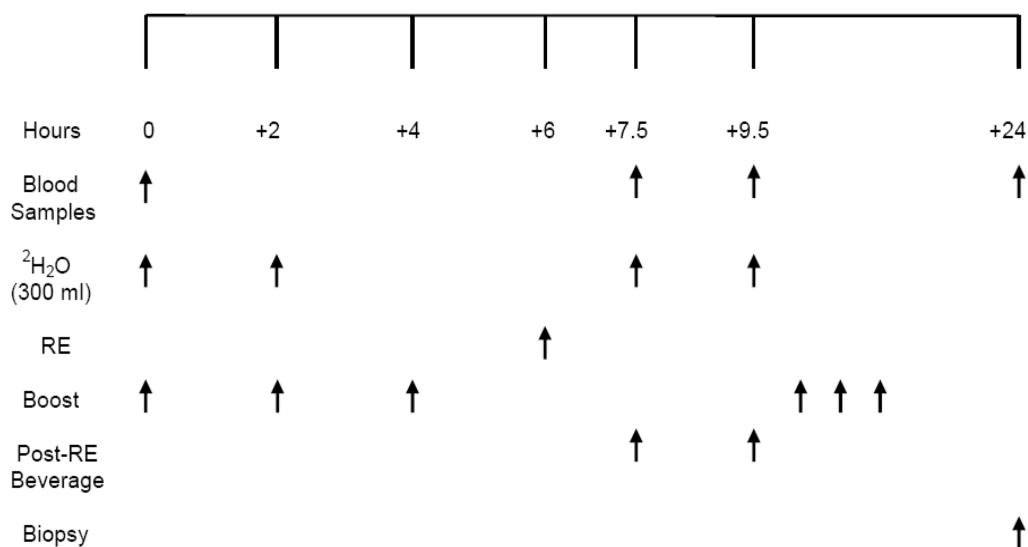


Fig. 19. Study Design. Blood and muscle sampling, ^2H_2O and beverage consumption are indicated by the arrows. The design of the study was identical for the immediate post-exercise protein (PRO, *n = 6*) and carbohydrate control (CON, *n = 6*) groups except for the timing of protein supplement ingestion. See methods for detailed description of the study design.

Plasma Sample Analysis

Blood samples were collected in EDTA containing tubes and centrifuged at 2,500 rpm at 4ºC for 20 min. Aliquots of plasma were immediately stored at -80 ºC. Glucose and lactate were measured with an YSI 2300 STAT Plus glucose and lactate analyzer (YSI Life Sciences, Yellow Springs, OH). Insulin was determined by an ELISA

kit (Alpha Diagnostic International, San Antonio, TX) with absorbance measurement completed on a DTX 880 Multimode Detetector (Beckman Coulter, Inc., Fullerton, CA). Plasma amino acids (isoleucine, leucine and valine) were determined by using high-performance liquid chromatography (HPLC) as described by Buentello and Gatlin (29) (see Appendix C for detailed description). Briefly, samples were deproteinized on ice with 1.5 mol/l $HClO_4$ (1:1 vol), followed by neutralization with 2 mol/l K_2CO_3 (2:1 vol) and lipid removal with diethyl ether (1:3 vol). Upon filtering of samples (0.22-µm polycarbonate syringe filters), 1.0 ml of sample underwent precolumn derivatization with opthaldialdehyde (Sigma-Aldrich, St. Louis, MO).

The ^{2}H labeling of body water was determined by acetone exchange (148) (see Appendix B for detailed description). The reaction occurred with 0.02 ml of sample, 2 µL of 10N NaOH and 4 µL of a 5% (vol/vol) solution of acetone in acetonitrile for 24 h. Acetone was removed by the addition of 0.6 ml of chloroform and 0.5 g Na_2SO_4. The samples were mixed and 0.1 ml of the chloroform was transferred to a GC-MS vial. The samples were analyzed using an Agilent 5975C-MSD equipped with an Agilent 7890 GC system, and a HP-5ms capillary column (30 m × 0.25 mm × 0.25 µm). The following temperature program was used: 60ºC initial, increase by 20°C/min to 100ºC, increase by 50ºC/min to 220ºC, and hold for 1 min. The sample was injected at a spit ratio of 40:1 with a helium flow of 1 mL/min. Acetone eluted at ~ 1.7 min. The mass spectrometer was operated in electron impact mode (70eV). Selective ion monitoring of mass-to-charge ratios (*m/z*) 58 (M) and 59 (M + 1) was conducted using a dwell time of 10 ms per ion.

Muscle Sample Analysis

The following morning, 16 h after the acute bout of resistance exercise, subjects returned to laboratory at 0800 (+ 24 h) for muscle biopsies. Samples were obtained from both the exercised and control leg (vastus lateralis) with a 5 mm Bergström biopsy needle, under sterile procedure and local anesthesia (1% lidocaine). Fat, connective tissue and blood were removed from the samples, frozen in liquid N_2 and stored at -80°C until further analysis.

The ^{2}H labeling of protein-bound alanine was measured as previously described by Dufner et al. (44) (see Appendix B for detailed description). Briefly, ~ 0.030 g of muscle was homogenized on ice in 0.3 ml of a 10% (wt/vol) TCA and centrifuged at 3,750 rpm at 4°C for 15 min. This was repeated 3 additional times prior to dissolving the protein pellet in 6 N HCl (0.1 mL/0.030 g tissue) and reacting at 100ºC for 18 h. In order to determine if there was a difference in mixed muscle versus myofibrillar, ~ 0.040 g of muscle was homogenized on ice in 0.4 mL of a 1 X kinase buffer (25 mM Tris-HCl, 5 mM ß-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) with 200 µM ATP and 0.01% Triton. Homogenates were centrifuged at 14,000 rpm at 4ºC for 30 min. After centrifugation, the supernatant containing the cytosolic and membrane portion was discarded and the precipitate representing the myofibrillar fraction was prepared similar to the mixed muscle. An aliquot (0.100 ml) of the hydrolysate was freeze dried for 24 h. A 3:2:1 ratio (0.1 mL) of “Methyl-8” reagent (Pierce, Rockford, IL), methanol and acetonitrile was added to the residue to determine the ^{2}H-labeling of alanine on its methyl-8 derivative. All samples were analyzed using an Agilent 5973N-

MSD equipped with an Agilent 6890 GC system, and a DB17-MS capillary column (30 m × 0.25 mm × 0.25 μm) was used in all analyses. The following temperature program was used: 90ºC initial, hold for 5 min, increase by 5°C/min to 130ºC, increase by 40ºC/min to 240ºC, and hold for 5 min. The sample was injected at a spit ratio of 5:1 with a helium flow of 1 mL/min. Alanine eluted at ~12 min. The mass spectrometer was operated in electron impact mode. Selective ion monitoring of mass-to-charge ratios (*m/z*) 99 (M) and 100 (M + 1) was conducted using a dwell time of 10 ms per ion.

We calculated fractional synthetic rates of mixed muscle proteins (FSR) by measuring the incorporation of ^2H alanine into protein (E_A) and using the precursor-product model (see Appendix D for detailed description):

$$FSR = E_A/(E_{BW} \cdot 3.7 \cdot t) \ (eq\ 1)$$

where E_A represents amount of protein-bound ^2H Ala (%), E_{BW} is the quantity of ^2H_2O in body water (%), 3.7 represents the exchange of ^2H between body water and alanine (44, 111, 146) and t is time of label exposure (36 h).

Statistics

All data analysis was completed using SigmaStat version 3.5 and values are expressed as means ± SE. Comparisons for glucose, lactate, insulin and amino acids were made using a two-factor repeated measures ANOVA with the main effects being diet and time. A one-factor repeated measure ANOVA for ^2H labeling of plasma was used. A one-factor ANOVA was used to compare differences in ^2H labeling of tissue alanine and the effect of exercise in the myofibrillar fraction. A two-factor ANOVA (diet and activity) was used to compare differences in mixed muscle FSR. *Post hoc*

testing was performed using the Fisher LSD method when a main effect was observed. If a test of normality or equal variance failed, a Kruskal-Wallis test was conducted, and the Fisher LSD *Post hoc* test was used when a main effect was observed. We accepted an analysis as statistically significant if $P < 0.05$.

Results

Exercise Session

All subjects completed 5 sets of leg-press and 5 sets of knee-extension at 85% of their 1RM. There was no difference in the leg-press resistance used between the PRO (202 ± 10 kg) and CON (171 ± 20 kg) groups, nor was there any differences between the groups in the number of repetitions performed over the course of the 5 sets, PRO (11 ± 2) vs. CON (13 ± 2). However, the CON group's powers output (854 ± 71 W) was significantly greater over the 5 sets of leg-press than the PRO group (730 ± 77 W) and thus, work ($P < 0.01$). In addition, there were no difference in the knee-extension resistance used (84 ± 7 vs. 79 ± 6 kg), the repetitions over 5 sets (2 ± 0.3 vs. 2 ± 0.2) or power output (136 ± 8 vs. 148 ± 5 W) between the PRO and CON groups respectively.

Plasma Glucose, Lactate and Insulin

Plasma glucose concentrations remained constant over the course of the 24 h period (Table 5, page 99) and did not differ at any time point between groups. In contrast, plasma lactate tended to increase (~ 2 fold over basal values, time 0 h) 1 h following the acute bout of resistance exercise in both groups and remained elevated at 3 h following exercise in the PRO group ($P < 0.05$); whereas lactate decreased at 3 h post-

exercise, but increased ~ 3 fold over pre-study concentrations 16 h after exercise in the CON group ($P < 0.05$) and was significantly different between treatments at this time ($P < 0.05$). Plasma insulin concentrations also increased 1 h following resistance exercise in both the CON ($P < 0.05$) and PRO ($P < 0.01$) groups vs. basal levels, but returned to normal concentrations the remainder of the time points. Additionally, there were no significant differences observed between treatments ($P > 0.05$).

Table 5. *Plasma glucose, lactate and insulin concentrations*

	0	1 h Post-Ex	3 h Post-Ex	16 h Post-Ex
		Glucose (mM)		
CON	4.9 ± 0.3	4.7 ± 0.3	5.0 ± 0.2	5.5 ± 0.5
PRO	4.8 ± 0.3	4.1 ± 0.2	5.0 ± 0.3	5.0 ± 0.2
		Lactate (mM)		
CON	1.0 ± 0.1	2.2 ± 0.8	1.0 ± 0.1	4.2 ± 1.3$^{\#}$
PRO	0.9 ± 0.1	2.7 ± 0.6	2.8 ± 0.9$^{\#}$	1.9 ± 0.6*
		Insulin (µU/mL)		
CON	7.7 ± 2.1	18.5 ± 5.0$^{\#}$	15.3 ± 3.5	9.0 ± 2.4
PRO	8.1 ± 0.7	50.0 ±13.8†	7.8 ± 1.9	8.3 ± 2.1

Immediate post-exercise protein (PRO, *n = 4-6*) and carbohydrate control (CON, *n = 6*) measured prior to the start of the study (0h), 1h post-exercise (+7.5h), 3h post-exercise (9.5h) and 16h post-exercise (+24h). Measurements are expressed means ± SE. $^{\#}P<0.05$ vs. time 0 within same group; $^{\dagger}P<0.01$ vs. time 0 within same group. $^{*}P<0.05$ vs. CON at same time point.

Plasma Amino Acids

The branch chain amino acid concentrations over time are shown in Figure 20 (page 101). Plasma isoleucine, leucine and valine all increased 1 h after resistance exercise over pre-exercise values in the PRO group ($P < 0.001$) and were significantly higher over the CON group ($P < 0.001$). In addition, plasma isoleucine and leucine responses were lower in the CON group 3 h following exercise vs. basal values ($P < 0.01$) and treatment differences persisted at this time point with greater concentrations in the PRO group for isoleucine ($P < 0.01$), leucine and valine ($P < 0.001$). The amino acids returned to basal values 24-hours from the start of the study (16 h following resistance exercise) ($P > 0.05$). Although not shown, we observed a similar pattern for the majority of other amino acids, however, only chose to show the branched chain amino acids due to their proposed role in stimulating muscle protein synthesis (19).

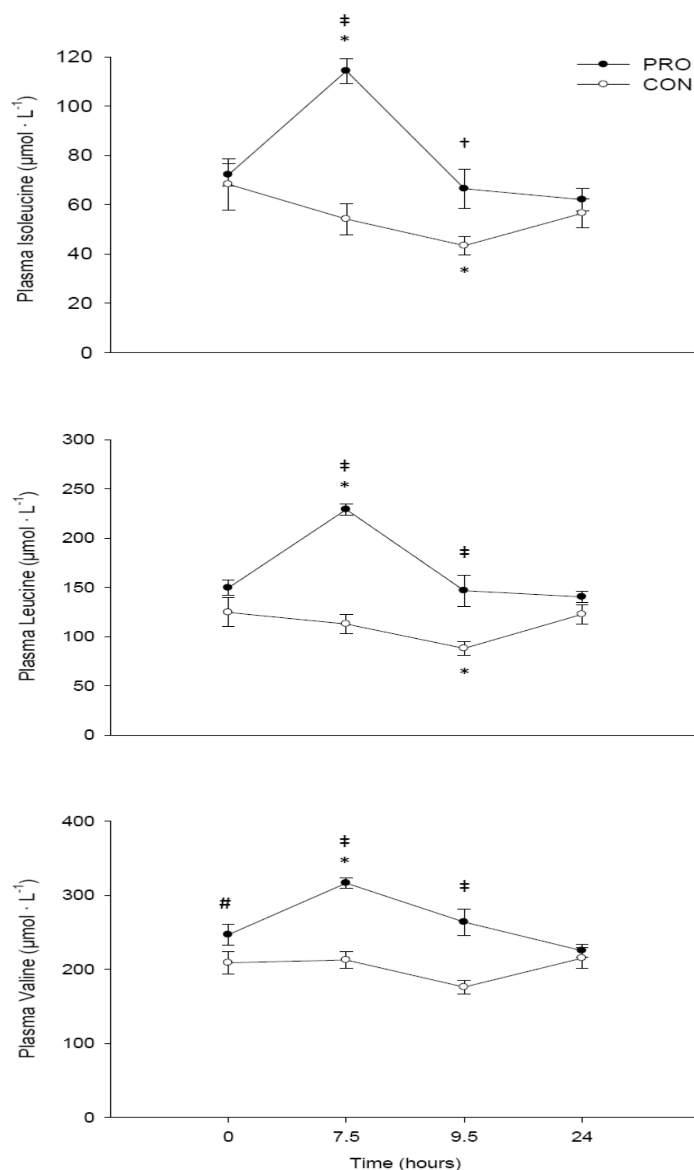


Fig. 20. Plasma isoleucine, leucine and valine concentrations in immediate post-exercise protein (PRO, *n = 6*) and carbohydrate control (CON, *n = 6*) measured prior to the start of the study (0h), 1h post-exercise (+7.5h), 3h post-exercise (9.5h) and 16h post-exercise (+24h). Values are means ± SE. Data were analyzed with repeated-measures ANOVA (treatment x time). *Plasma isoleucine*: $^{*}P < 0.001$ vs. 0h in PRO group and $P < 0.01$ in CON group; $^{\dagger}P < 0.01$ vs. CON at same respective time point; $^{\ddagger}P < 0.001$ vs. CON at same respective time point. *Plasma leucine*: $^{*}P < 0.001$ vs. 0h in PRO group and $P < 0.01$ in CON group; $^{\ddagger}P < 0.001$ vs. CON at same respective time point. *Plasma valine*: $^{*}P < 0.001$ vs. 0h in PRO; $^{\#}P < 0.05$ vs. CON at same respective time point; $^{\ddagger}P < 0.001$ vs. CON at same respective time point.

Muscle Protein Synthesis

Body water ^2H enrichment reached ~ 0.4 % excess (Table 6, page 103) over the course of the study and the ^2H labeling of alanine did not differ between the exercised (Ex) and non-exercised (NoEx) legs. Both plasma and muscle ^2H enrichments were adequate for FSR calculations. No differences in FSR ($\% \cdot h^{-1}$) were observed between the PRO and CON groups, or between the Ex and NoEx legs (Fig. 21, page 104) in mixed muscle when assessed 16 h following the resistance exercise session (measured over 24 h). To determine whether there were differences in specific proteins being synthesized due to the length of FSR assessment, we examined the myofibrillar fraction in a subset of the muscle samples (insufficient amount of tissue to compare the main effect of protein) and observed a 20% increase in FSR in the Ex leg vs. the NoEx leg ($P < 0.05$), (Fig. 22, page 105). Thus, assessment of the myofibrillar fraction may be a preferred indicator of an anabolic response resulting from exercise in skeletal muscle.

Table 6. *^{2}H-labeling of body water and skeletal muscle proteins*

	Plasma (MPE)				Mixed SM (MPE)	
Subject	Pre	7.5 h	9.5 h	24 h	Ex Leg	NoEx Leg
1	0	0.264	0.392	0.374	0.183	0.304
2	-0.025	0.203	0.321	0.369	0.184	0.231
3	0.030	0.188	0.303	0.386	0.235	0.169
4	-0.009	0.198	0.341	0.368	0.152	0.153
5	0.038	0.178	0.322	0.370	0.152	0.166
6	0.026	0.212	0.310	0.362	0.177	0.316
7	-0.007	0.199	0.318	0.376	0.259	0.178
8	-0.015	0.145	0.229	0.340	0.085	0.244
9	0.034	0.317	0.343	0.425	0.192	0.203
10	0.056	0.233	0.306	0.356	0.179	0.178
11	0.007	0.236	0.475	0.439	0.289	0.103
12	0.023	0.151	0.231	0.341	0.254	0.298
Mean	*0.013*	*0.210**	*0.324**	*0.376**	*0.195*	*0.212*
SEM	*0.007*	*0.014*	*0.019*	*0.009*	*0.016*	*0.019*

Plasma ^{2}H labeling measured prior to the start of the study (0h), 1h post-exercise (+7.5h), 3h post-exercise (9.5h) and 16h post-exercise (+24h) in the plasma and ^{2}H labeling of protein-bound alanine measured 16h post-exercise (+24h) in the exercised leg (Ex) and non-exercised leg (NoEx). MPE, mole percent excess. *Plasma ^{2}H labeling was significantly higher at 7.5, 9.5 and 24 h than pre (time 0) values, $P < 0.001$.

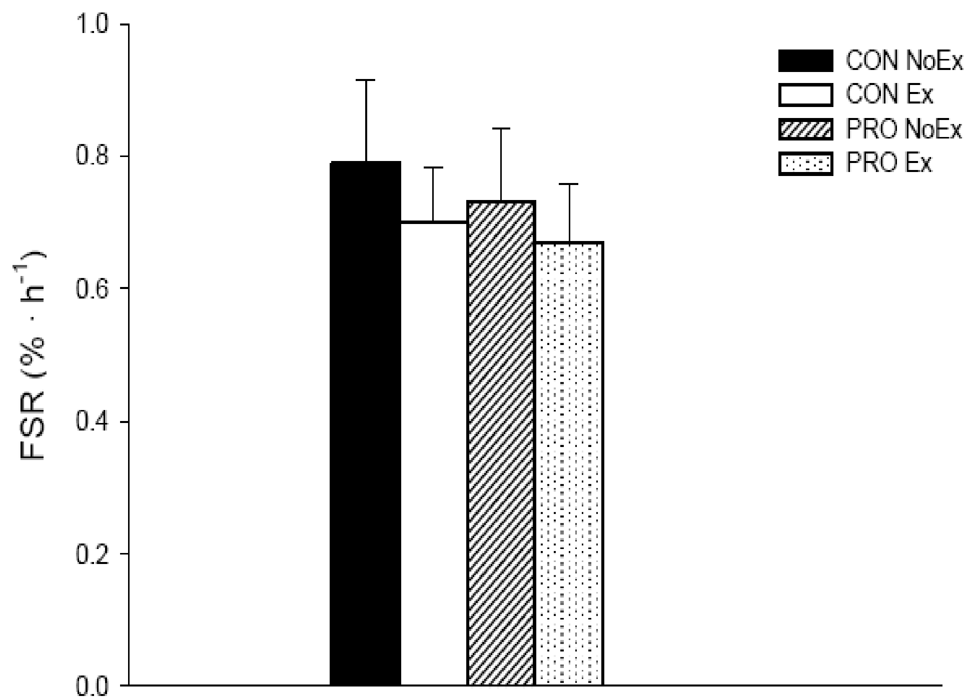


Fig. 21. Mixed muscle protein fractional synthesis rates (FSR) assessed over 24 h in the carbohydrate non-exercised leg (CON NoEx, *n = 6*), carbohydrate trained leg (CON Ex, *n* = 6), immediate post-exercise protein non-exercised leg (PRO NoEx, *n = 6*) and immediate post-exercise protein exercised leg (PRO Ex, *n = 6)* groups. Data are presented as means ± SE.

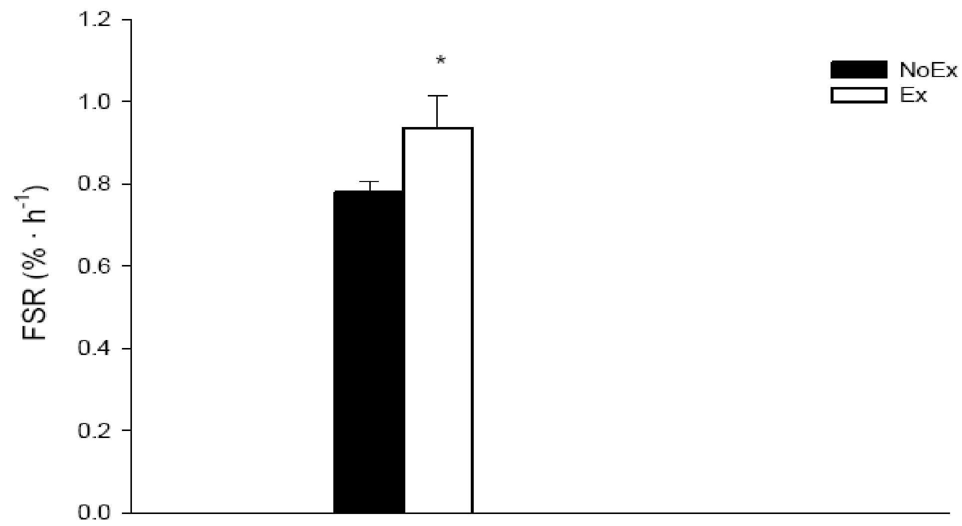


Fig. 22. Myofibrillar protein fractional synthesis rates (FSR) assessed over 24 h in the non-exercised leg (NoEx, n = *8*) and the exercised leg (Ex, n = *4*). Data are presented as means ± SE. *$P < 0.05$.

Discussion

To our knowledge, this is the first study to examine human skeletal muscle protein synthesis over 24 h to include feeding, resistance exercise, post-resistance exercise protein supplementation, hormonal stimulation/inhibition (biological patterns) and sleep; providing a cumulative assessment. Contrary to our hypotheses, we did not observe an exercise effect in stimulating mixed muscle FSR, nor did we find an augmentation in synthesis rates with the provision of a protein (0.3 g · kg body mass^{-1}) containing beverage immediately following a bout of high intensity resistance exercise when total energy and macronutrients were controlled over a 24 h time frame in 20-24 year old males. However, we did observe an exercise effect on myofibrillar protein FSR. We explain our findings by *i)* the potential for there being differences in protein pool turnover rates and *ii)* the dietary control over the experimental protocol, such that the timing may not be as critical as believed when sufficient energy and protein are provided throughout the day.

The fact that we observed an exercise effect in the myofibrillar fraction (*Fig. 22, page 105*) but not in the mixed muscle (*Fig. 21, page 104*) is intriguing. Skeletal muscle is a mixture of proteins with very different renewal rates (131). However, mixed muscle protein has traditionally been measured to determine FSR as it represents an average of all muscle proteins. Myofibrillar protein (primarily actin and myosin filaments) constitutes the majority of the total muscle protein pool, > 70% (105), thus any changes in this fraction should (in theory) be observed with mixed muscle measurement. There are a limited number of human investigations that examine the synthesis of specific

fractions of muscle protein (myofibrillar, mitochondrial and sarcoplasmic). Studies by Cuthbertson et al. (37) and Miller (94) examined myofibrillar and sarcoplasmic FSR at 3, 24 and 6 and 72 h, respectively, following an acute bout of resistance exercise (unilateral-leg kicking in the former and dynamic shortening or lengthening in the latter). They reported similar changes in both fractions; however, Miller (94) observed elevations in myofibrillar FSR at 72 h, but not sarcoplasmic. More recently, Wilkinson et al. (140) reported differences in mitochondrial and myofibrillar FSR following endurance and resistance exercise training (unilateral leg protocol). Although these studies (37, 94, 140) support the notion that different proteins within a heterogeneous pool turnover at dissimilar rates, it is not obvious as to why changes in the largest fraction (myofibrillar) were not apparent in the mixed muscle. Furthermore, had we not examined the myofibrillar fraction, we would have concluded that an acute bout of high intensity resistance exercise does not stimulate FSR when cumulative assessments are made.

Our assessment of muscle protein synthesis was made over 24 h to include feeding, normal activities of daily living, an acute bout of dynamic high intensity resistance exercise, controlled feeding with adequate energy and macronutrients to support anabolism in this population, and sleep. We feel that our experimental design made it possible to observe increases in myofibrillar FSR with no changes in the total protein pool (*Fig. 23, page 108*). We provided 4 boluses of ^2H_2O with the first being at 0800, thus ^{2}H-labeled alanine would start to accumulate and be accelerated with the meals supplemented prior to the exercise protocol on both the Ex and NoEx legs. Upon

completion of the high intensity acute bout of unilateral-leg resistance exercise, there was possibly a decrease in the rate of ^{2}H-labeled alanine incorporation due to the intensity (85% of 1RM until exhaustion, not fixed number of repetitions typically used) in the Ex leg compared to a constant rate in the NoEx leg. However, in the hours following with the provision of nutrients (protein) and rest, the rate of the ^{2}H labeling of alanine in tissue was accelerated with the total accumulation of ^{2}H-labeled alanine in the mixed muscle being similar over the 24 h time frame. Furthermore, the accelerated rate of ^{2}H labeling of alanine in the myofibrillar fraction of the Ex leg, compared to similar changes in all fractions in the NoEx leg could explain the discrepancy between the mixed and myofibrillar protein fractions. Thus, our findings support the concept that resistance exercise increases FSR, however, when a cumulative assessment is made, the observed changes are within the fraction of muscle protein that is responsible for hypertrophy that occurs over time; myofibrillar protein.

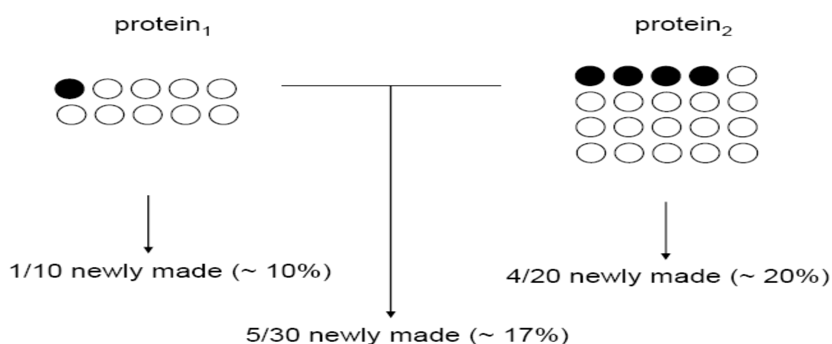


Fig. 23. Proposed scheme of measuring protein synthesis in a heterogeneous pool (mixed muscle). The consequences of assessing a mixed pool are that specific proteins with variable masses can have different synthesis rates. For example, if protein 1 synthesizes 10% (1/10) of its structure and protein 2 makes 20% (4/20) over the same period of time, the percentage of newly made protein (17%, 5/30) does not reflect the average of each (15%, 30/2). Thus, mixed muscle assessment may not always be reflective of individual proteins (e.g. myofibrillar).

The fact that we did not find an effect of immediate post-exercise protein supplementation on mixed muscle FSR is not a novel finding, however, the context in which measurements were made is unique. More descriptively, many observe an augmentation in FSR with the consumption of protein and/or indispensible amino acids with or without carbohydrate following an acute bout of resistance exercise (15, 16, 37, 40, 80, 82, 114, 127). However, in order to draw conclusions as to whether the timing following the early hours following resistance exercise is truly paramount, than a comparison to a different time point of ingestion should be included in the experimental design. In support of this, Rasmussen et al. (114) compared the provision of an essential amino acid-carbohydrate supplement either 1 h or 3 h following an acute bout of resistance exercise and reported elevations in FSR at both time points that were dependent upon the time of ingestion of the nutrients. Therefore, as a result of providing the protein immediately following (PRO) or 3 h after (CON) exercise, the timing was insignificant. Additionally, in this study, the participants started the acute bout of exercise ~ 1.75 h after the last meal and, therefore, consumed 4,017 kJ, 43 g protein (0.5 ± 0.02 g $\cdot$kg^{-1}), 123 g carbohydrate and 34 g of lipid. This provided ~ 34% of energy, 63% of protein, 35% of carbohydrate and 33% of lipid daily requirements for a moderately active 20-24 year old male (73). Thus, it would appear that subjects were, at least, in protein balance (although we did not directly measure this) prior to exercising and any additional protein could potentiate the protein synthetic response. In support of this, the PRO group displayed elevations in branched chain amino acids (BCAAs) up to 3 h (*Fig. 20, page 101*) following the exercise session. Although it is believed that

indispensible amino acids, primarily leucine, promote anabolic signaling and muscle protein synthesis (26, 40, 127, 144), we chose to provide mixed beverages (protein was in the form of milk isolate) as people consume mixed meals, not just amino acid solutions. In addition, insulin also increased 1 h following exercise (*Table 5, page 99*), but the elevations were no different between groups ($P < 0.09$). Although the biochemical conditions appeared superior for the PRO group, at 24 h (16 h following resistance exercise) the BCAAs and insulin returned to pre-study values, the point at which muscle biopsies were extracted for measurement. It is possible that if we had measured FSR over the immediate time following exercise (up to 3 h) only, we would have observed elevations in the PRO group over the CON group. In contrast, had our measurements been made from 3-6 h post-exercise, we may have observed increases in the CON group over the PRO group; however, we wanted to examine '*cumulative'* muscle protein synthesis to encompass perturbations (sleep, exercise, activities of daily living and rest) that occur daily in humans. Based on our observations, controlling the composition of diet over an extended period of time, negates the timing effect that post-resistance exercise protein consumption has on augmenting muscle protein synthesis. However, we acknowledge that a limitation of this study was the inability to measure the main effect of the timing of protein on myofibrillar FSR, and thus an interaction (insufficient sample size). Therefore, we cannot discount the possibility that immediate post-exercise protein consumption was the reasoning for our observed increases in the Ex groups myofibrillar protein synthesis. Thus, more work in this area focusing on specific protein pools and proteins should be conducted.

Another finding that deserves mentioning is the calculated FSR values ($\% \cdot h^{-1}$), which were ~ 5-10 fold higher than what is typically reported with feeding/exercise human investigations (~ 0.08 - 0.16%). We explain our results by our methodological approach for assessing muscle FSR and the limitations associated with tracer amino acids. First, ^2H_2O allows for long-term FSR assessment; however, our value does not make any description of protein degradation, thus although we are reporting an FSR of ~ $0.8\% \cdot h^{-1}$, it is possible that breakdown occurred at a comparable rate. Second, in regards to tracer amino acid protocols, measurements are generally made (at least begin) in the post-absorptive state (following an overnight fast) with primed-continuous infusion protocols to insure that basal precursor and tissue enrichments are accurately determined. One concern with such designs becomes what physiological state is actually being examined as humans typically ingest nutrients upon awakening, which alters the hormonal state from catabolic to anabolic. If food is provided during the protocol, additional tracer should be added to insure that appropriate enrichment of the precursor is maintained to avoid a dilution effect. Another concern is the precursor pool that is used to calculate FSR. Due to the difficulty in obtaining the true precursor (aminoacyl-tRNA), surrogates such as the intracellular (e.g. [^{2}H]phenylalanine) or plasma (e.g. [^{13}C]leucine, plasma α-KIC) are generally used. The concern with using plasma sampling is the site of collection relative to the tissue that is being collected, such that even if a femoral vein is used, consideration must be given to the influence of other muscle fibers and skin in the region. The use of ^2H_2O bypasses these limitations in that ^{2}H labeling of alanine occurs intracellularly via transamination reactions (30, 98) versus

the requirement of transmembrane transport with tracer amino acids. In addition to our methodological approach and short-comings of labeled amino acid study designs, one must consider that a calculated FSR is a ‘percentage’ of the existing protein pool (assumed to be constant) synthesized over a period of time (146), however, does not describe changes in the absolute pool size (protein concentration). Thus, FSR comparisons between studies should be viewed ‘qualitatively’.

In conclusion, we report that an acute bout of high intensity resistance exercise stimulates cumulative myofibrillar protein synthesis in spite of detecting no change in mixed muscle. Additionally, the provision of protein immediately following high intensity resistance exercise does not appear to be advantageous than providing it 3 h later when energy and macronutrients are controlled. Future studies in the area of nutrition/exercise should focus more on specific protein fractions versus mixed muscle alone. Additionally, more long-term measurements of muscle protein synthesis are needed to fully comprehend real-time protein renewal; which can be accomplished with the use of $^{2}H_2O$ with relative ease.

CHAPTER VI

CONCLUSIONS

These experiments were carried out to determine the effects of acute (rats and humans) and chronic resistance exercise (rats) on cumulative muscle protein synthesis. In order to conduct these studies, a validation study comparing ^2H_2O with the flooding dose was carried out. Five novel findings resulted from this reported research. First, ^2H_2O provides qualitatively similar muscle protein synthesis results as the flooding dose when studying perturbations, such as exercise and feeding. Secondly, a single bout of resistance exercise does not appear to stimulate muscle protein synthesis in rats as it does in humans. This may either be due to the intensity or the necessity for subsequent sessions to promote skeletal muscle adaptation. Thirdly, chronic resistance exercise continues to be anabolic; however, the time by which this can be observed changes. Fourthly, immediate post-resistance exercise protein consumption does not augment human mixed muscle protein synthesis when a cumulative assessment (over 24 h) is made. Fifthly, an acute bout of resistance exercise stimulates human cumulative myofibrillar muscle protein synthesis. The fact that mixed muscle was apparently not increased further supports the notion that proteins within a heterogeneous pool undergo different turnover rates. Thus, measurement of specific proteins *vs.* the total pool may be more descriptive of what is actually occurring within a tissue of interest.

It was my (as well as my mentors and colleagues) intention to advance our understanding of the stimulatory effect of resistance exercise on skeletal muscle protein

anabolism. More specifically, we report that acute resistance exercise may not be anabolic in rats, there is a temporal shift in the timing of muscle protein synthesis following resistance exercise training in rats, immediate post-resistance exercise protein consumption does not augment human mixed muscle protein synthesis and there can be differences in specific proteins within the total pool (myofibrillar *vs.* mixed muscle). Future studies should be carried out to assess changes in cumulative muscle protein synthesis in specific disease states, such as debilitating and metabolic pathologies. Additionally, more emphasis should be placed on the assessment of specific proteins *vs.* a mixed sample. The data from this research could potentially serve as the foundation by which future comparisons in the area of muscle protein anabolism are made.

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APPENDIX A

MUSCLE PROTEIN SYNTHESIS PROCESSING (ASSESSED WITH L-[2,3,4,5,6-^{3}H]PHENYLALANINE)

I. Plasma Specific Radioactivity (HPLC-used for "The Assessment of Muscle Protein Synthesis Following Chronic Resistance Exercise: A Plateau Effect?").

Required Items	Catalog Number
1.5 ml microcentrifuge tubes	Fisher, 02-681-284
2.0 ml microcentrifuge tubes	Fisher, 02-681-291
0.45 µm pore size membrane filter	Fisher, R04SP04700
0.22 µm pore size membrane filter	Fisher, R02SP01300
Amino acids:	
L-Aspartic acid ($C_4H_7NO_4$)	Sigma, A4534
L-Asparagine ($C_4H_8N_2O_3$)	Sigma, A8824
Taurine ($C_2H_7NO_3S$)	Sigma, T0625
L-Phenylalanine ($C_9H_{11}NO_2$)	Sigma, P8324
L-Ornithine ($C_5H_{12}N_2O_2$)	Sigma, O2375
Borate ($Na_2B_4O_7$)	Sigma, B9876
Brij-35 $(C_2H_4O)_nC_{12}H_{26}O$	Sigma, B4184
Ethanolamine ($NH_2(CH_2)_2OH$)	Sigma, E0135
Hydrochloric acid (12N HCl)	EMD, HX0603-3
Methanol (CH_3OH-HPLC grade)	Fisher, MXO 475-1
2-Mercaptoethanol (C_2H_6OS)	Sigma, 3148
Perchloric acid ($HClO_4$)	J.T. Baker, 9652-02
Phthaldialdehyde ($C_8H_6O_2$)	Sigma, P0657
Potassium carbonate (K_2CO_3)	Sigma, P5833
Shell vials for Waters autosampler	Fisher, 03-391-23
Sodium acetate ($C_2H_3O_2Na$)	Sigma, S7670
Tetrahydrofuran (C_4H_8O)	Sigma, 34865
Trichloroacetic acid (TCA-Cl_3CCOOH)	EMD, TX1045-5
Tuberculin slip tip (1ml)	BD, PK 100309602
Water (H_2O-HPLC grade)	Fisher, W5-4

Procedure:

A. Plasma Phenylalanine Preparation:

1. Centrifuge 1.0 ml of 2, 6 and 12 minute blood samples at 3,800 rpm at

2. 4ºC for 20 min.

3. Extract plasma with 1.0 ml pipette and place into 1.5 ml microcentrifuge tubes, freeze at -80ºC.

4. Weigh two 1.5 ml microcentrifuge tubes for each sample and record weights. Label tubes "A" and "B", document.

5. In tube A, place 0.25 ml plasma and 0.25 ml 10% TCA (Cl_3CCOOH).

6. Reweigh tube A, document weight.

7. Centrifuge at 3,800 rpm at 4ºC for 5 min.

8. Transfer supernatant with 1.0 ml pipette into tube B.

9. Reweigh tubes A and B, record weights.

10. Add 0.3 ml of 0.5 M Borate ($Na_2B_4O_7$), which will yield a pH of 9.0 in each sample.

11. Reweigh tube B and record weights.

12. Extract 0.05 ml of sample with 0.1 ml pipette and place into 1.5 ml microcentrifuge tubes.

13. To 0.05 ml sample, add 0.45 ml HPLC grade H_2O, cap and vortex.

14. Remove 0.2 ml of sample, place into 1.5 ml microcentrifuge tubes and add 0.2 ml 1.5M perchloric acid ($HClO_4$); cap and vortex.

15. Add 0.1 ml 2.0 M potassium carbonate (K_2CO_3), cap and vortex.

16. Centrifuge at 6,000 rpm at room temperature for 12 min.

17. Remove 0.2 ml of supernatant and place in 2.0 ml microcentrifuge tubes. Add 1800 ml HPLC grade H_2O, cap and vortex.

18. Extract 1.0 ml of sample with tuberculin slip tip syringe and filter 1.0 ml of sample through a 0.22 μm membrane filter into 1.0 ml HPLC vials and place into refrigerator without caps overnight.

19. Inject 1:1 (0.1 ml) of sample and OPA into Waters HPLC (phenylalanine elutes at 40 min).

B. High Performance Liquid Chromatography (HPLC):

1. System:

    a. HPLC Waters solvent delivery system (2 Waters 501 pumps, a 717 Waters Autosampler, and Automated Gradient Controller [curve 6 chosen] Waters 470 Scanning Fluorescence Detector [attenuation 350 nm, gain 450 m]).

    b. Column, Suelcosil LC-18 (58985) 15 cm x 4.6 mm, 3 micron beads.

2. Gradient Solvent (A):

    a. Add 0.8 L of HPLC grade H_2O into 1.0 L flask.

    b. Add 13.65 g sodium acetate ($C_2H_3O_2Na$), 0.048 ml 6N HCl, 5.0 ml tetrahydrofuran (C_4H_8O) and 0.09 ml methanol (CH_3OH-HPLC grade).

    c. Filter through 0.45 μm membrane filter into 1.0 L flask with side arm and q.s. to 1.0 L.

3. Solvent B:

    a. Filter 1.0 L methanol (CH_3OH-HPLC grade) through 0.45 μm membrane filter.

4. Phthaldialdehyde ($C_8H_6O_2$) (OPA) Reagent:

    a. Add 0.05 g OPA to sterile 20.0 ml amber bottle.

    b. Add 1.25 ml methanol (CH_3OH-HPLC grade), 11.20 ml sodium borate buffer, 0.05 ml 2-Mercaptoethanol (C_2H_6OS) and 0.4 ml Brij-35 $(C_2H_4O)_nC_{12}H_{26}O$, cap and store in the refrigerator.

        i. Sodium borate buffer:

            1. Mix 15.22 g Borate ($Na_2B_4O_7$) in 1.0 L HPLC grade H_2O; pH 9.2.

5. HPLC Settings and Flow Rate: Linear gradient (1500 psi).

Time (min)	Flow (ml/min)	% Solvent A	%Solvent B	Curve
0	1.1	86	14	6
20	1.1	86	14	6
24	1.1	70	30	6
26	1.1	65	35	6
28	1.1	53	47	6
34	1.1	50	50	6
38	1.1	30	70	6
40	1.1	0	100	6
42	1.1	0	100	6
48.1	1.1	86	14	6
48.5	1.1	86	14	6

C. Linear Standard Curve Preparation:

1. Stock Solution (250 nmol/ml):

a. To 0.6 ml of HPLC grade H_2O, add the following:

M.W. x M x Vol (L)

L-Aspartic acid ($C_4H_7NO_4$), M.W. = 133.10 (0.0020 g)
L-Asparagine ($C_4H_8N_2O_3$), M.W. = 132.12 (0.0020 g)
Taurine ($C_2H_7NO_3S$), M.W. = 125.15 (0.0019 g)
L-Phenylalanine ($C_9H_{11}NO_2$), M.W. = 165.19 (0.0024 g)
L-Ornithine ($C_5H_{12}N_2O_2$), M.W. = 168.62 (0.0025 g)

b. Add 0.6 ml ethanolamine ($NH_2(CH_2)_2OH$) and 4.8 ml HPLC grade H_2O to the above; total volume 6.0 ml.

nmol/ml	ml of stock solution	ml HPLC H_2O	total ml	ratio
250.00	1.0	0	1.0	1:0
125.00	0.5	0.5	1.0	1:1
62.50	0.5	1.5	2.0	1:3
41.68	0.2	1.0	1.2	1:5
35.71	0.1667	1.0	1.1667	1:6
22.73	0.1	1.0	1.1	1:10
15.63	0.1	1.5	1.6	1:15
11.91	0.1	2.0	2.1	1:20
9.62	0.05	1.25	1.3	1:25
4.90	0.039	1.96	1.99	1:50

c. Add 0.05 ml of each standard to 1.5 ml microcentrifuge tubes and add 0.45 ml HPLC grade H_2O, cap and vortex.

d. Remove 0.2 ml of sample, place into 1.5 ml microcentrifuge tubes and add 0.2 ml 1.5M perchloric acid ($HClO_4$); cap and vortex.

e. Add 0.1 ml 2.0M potassium carbonate (K_2CO_3), cap and vortex.

f. Centrifuge at 6,000 rpm at room temperature for 12 min.

g. Remove 0.2 ml of supernatant and place in 2.0 ml microcentrifuge tubes. Add 1800 ml HPLC grade H_2O, cap and vortex.

h. Extract 1.0 ml of sample with tuberculin slip tip syringe and filter 1.0 ml of sample through a 0.22 µm membrane filter into 1.0 ml HPLC vials and place into refrigerator without caps overnight.

i. Inject 1:1 (0.1 ml) sample and (0.1 ml) OPA into Waters HPLC (phenylalanine elutes at 40 min).

II. Plasma Phenylalanine (GC-MS used for "A Comparison of ^2H_2O and Phenylalanine Flooding Dose Methodologies to Investigate Muscle Protein Synthesis in Rats").

Required Items	Catalog Number
1.5 ml microcentrifuge tubes	Fisher, 02-681-284
2.0 ml microcentrifuge tubes	Fisher, 02-681-291
Acetonitrile (CH_3CN)	Fisher, A998-4
GC-MS glass vials	Agilent, 5182-0714
GC-MS vial inserts	Agilent, 5183-2085
GC-MS vial screw top caps	Agilent, 5185-5820
L-Phenylalanine ($C_9H_{11}NO_2$)	Sigma, P2126
L-[*ring*-$^{13}C_6$]phenylalanine ($F\text{-}C_3{}^{13}C_6H_{11}NO_2$)	Cambridge Isotopes, CLM-1055
MTBSTFA + 1% TBDMCS	Pierce, 48927
Trichloroacetic acid (TCA-Cl_3CCOOH)	EMD, TX1045-5
Water (H_2O-HPLC grade)	Fisher, W5-4

Procedure:

A. Plasma Phenylalanine Preparation:

1. Centrifuge 1.0 ml of 2, 6 and 12 minute blood samples at 3,800 rpm at 4ºC for 20 min.

2. Extract plasma with 1.0 ml pipette and place into 1.5 ml microcentrifuge tubes, freeze at -80ºC.

3. Extract 0.3 ml of sample with 1.0 ml pipette and place into 1.5 ml microcentrifuge tubes.

4. Add 0.3 ml of 10% TCA (Cl_3CCOOH) and 0.3 ml of 0.03 nmol/ml L-[*ring*-$^{13}C_6$]phenylalanine ($F\text{-}C_3{}^{13}C_6H_{11}NO_2$) to each sample, cap and vortex.

5. Centrifuge at 3,000 rpm at 4ºC for 15 min.

6. Remove 0.05 ml of the supernatant with 0.1 ml pipette and add to 1.5 ml microcentrifuge tubes; leave tops open.

7. Dry under stream of N_2 for 1h.

8. Add 0.05 ml of acetonitrile (CH_3CN) and 0.05 ml of MTBSTFA + 1% TBDMCS (1:1, vol/vol); cap and vortex.

9. Allow to sit at room temperature for 20 min (capped), vortex and add 0.1 ml of solution to the GC-MS vial inserts (in GC-MS vials) and cap.

10. Inject sample into GC-MS at 1 µl/min; selective ion monitoring *(m/z)* 234 and 240.

B. Gas Chromatography Mass Spectrometry (GC-MS):

1. System:

    a. Agilent 5975C-MSD equipped with an Agilent 7890 GC system.

    b. Column, HP-5ms capillary column (30 m × 0.25 mm × 0.25 µm).

2. Settings:

    a. The following temperature program was used: 160ºC initial and hold for 5 min, increase by 15°C/min to 270ºC and hold for 8 min. The sample was injected at a spit ratio of 40:1 with a helium flow of 30 mL/min. The mass spectrometer was operated in electron impact

mode (70eV).

b. Selective ion monitoring of mass-to-charge ratios (*m/z*) 234 (M) and 240 (M + 6) was conducted using a dwell time of 30 ms per ion. Phenylalanine eluted at ~ 12 min.

C. Linear Standard Curve Preparation:

1. Stock Solution of Phenylalanine (F) (10 µmol/ml):

a. To 100 ml of HPLC grade H_2O, add the following:

M.W. x M x Vol (L)

L-Phenylalanine ($C_9H_{11}NO_2$), M.W. = 165.19 (0.165 g)

2. Stock Solution of L-[*ring*-$^{13}C_6$]phenylalanine (0.06 µmol/ml):

a. To 100 ml of HPLC grade H_2O, add the following:

L-[*ring*-$^{13}C_6$]phenylalanine ($F\text{-}C_3{}^{13}C_6H_{11}NO_2$), M.W. = 171.15 (0.0103 g)

3. Preparation and Measurement:

µmol/ml	ml of stock F	ml HPLC H_2O	total ml	ratio
10.0	1.0	0	1.0	1:0
7.5	0.750	0.250	1.0	3:1
5.0	0.500	0.500	1.0	1:1
2.5	0.250	0.750	1.0	1:3
1.225	0.1225	0.8775	1.0	1:7
0.625	0.0625	0.9375	1.0	1:15
0.313	0.0313	0.9687	1.0	1:30
0	0	1.0	1.0	0:1

a. Add 0.3 ml of each standard (above), 0.3 ml of 10% TCA (Cl_3CCOOH) and 0.3 ml of 0.06 µmol/ml L-[*ring*-$^{13}C_6$]phenylalanine ($F\text{-}C_3{}^{13}C_6H_{11}NO_2$) to 1.5 ml microcentrifuge tubes, cap and vortex.

b. Remove 0.05 ml of the standard with 0.1 ml pipette and add to 1.5 ml microcentrifuge tubes; leave tops open.

c. Dry under stream of N_2 for 1h.

d. Add 0.05 ml of acetonitrile (CH_3CN) and 0.05 ml of MTBSTFA +

1% TBDMCS (1:1, vol/vol); cap and vortex.

e. Allow to sit at room temperature for 20 min (capped), vortex and add 0.1 ml of solution to the GC-MS vial inserts (in GC-MS vials) and cap.

f. Inject sample into GC-MS at 1 µl/min; selective ion monitoring *(m/z)* 234 and 240.

III. Plasma L-[2,3,4,5,6-^{3}H]Phenylalanine Preparation:

Required Items	Catalog Number
1.5 ml microcentrifuge tubes	Fisher, 02-681-284
6.0 ml scintillation vials	Fisher, 592928
Complete counting cocktail	Research Products, Inc., 111167
Trichloroacetic acid (TCA-Cl_3CCOOH)	EMD, TX1045-5

Procedure:

1. Centrifuge 1.0 ml of 2, 6 and 12 minute blood samples at 3,800 rpm at 4ºC for 20 min.

2. Extract plasma with 1.0 ml pipette and place into 1.5 ml Microcentrifuge tubes, freeze at -80ºC.

3. Extract 0.3 ml of sample with 1.0 ml pipette and place into 1.5 ml microcentrifuge tubes.

4. Add 0.3 ml of 10% TCA (Cl_3CCOOH) to each sample, cap and vortex.

5. Centrifuge at 3,000 rpm at 4ºC for 15 min.

6. Remove 0.05 ml of the supernatant with 0.1 ml pipette and add to 6.0 ml scintillation vials.

7. Add 0.5 ml of ultrapure dH_2O to each sample.

8. Add 5.0 ml of complete counting cocktail, cap and vortex.

9. Place in a scintillation counter (Beckman Coulter LS 6500 Multi-Purpose Scintillation Counter) and allow to settle overnight.

10. Count ^{3}H DPM (disintegrations per minute), each sample for 1h. Make corrections for quench (subtract background from each sample).

IV. Tissue L-[2,3,4,5,6-^{3}H]Phenylalanine and Total Phenylalanine:

Required Items	Catalog Number
1.5ml microcentrifuge tubes	Fisher, 02-681-284
2.0ml microcentrifuge tubes	Fisher, 02-681-291
2.0ml screwtop tubes	United Labs., UP 2031
6.0ml scintillation vials	Fisher, 592928
Acetone (CH_3COCH_3)	EMD, AX0120-8
Bicinchoninic acid	Sigma, B9643
Copper (II) sulfate	Sigma, C2284
Culture test tubes (17x100mm)	Fisher, 14-956-1J
GC-MS glass vials	Agilent, 5182-0714
GC-MS vial inserts	Agilent, 5183-2085
GC-MS vial screw top caps	Agilent, 5185-5820
Hydrochloric acid (12N HCl)	EMD, 258148
Kinase buffer (10X)	Cell Signaling, 9802
L-Phenylalanine ($C_9H_{11}NO_2$)	Sigma, P2126
L-[*ring*-$^{13}C_6$]phenylalanine ($F\text{-}C_3{}^{13}C_6H_{11}NO_2$)	Cambridge Isotopes, CLM-1055
MTBSTFA + 1% TBDMCS	Pierce, 48927
Protein Standards (BSA)	Sigma, P09M-5AMP
Sodium hydroxide (NaOH)	Fisher, 5612-3
Trichloroacetic acid (TCA-Cl_3CCOOH)	EMD, TX1045-5
Water (H_2O-HPLC grade)	Fisher, W5-4

Procedure:

A. Tissue L-[2,3,4,5,6-^{3}H]Phenylalanine:
Note: All steps must be done on ice.

1. Weigh out between 0.1-0.2 g of powdered skeletal muscle in culture test tubes (17x100mm), record weights.

2. Add 2.0 ml of 10% TCA (Cl_3CCOOH), and homogenize using a polytron homogenizer at full speed. Take 0.025 ml of homogenate and place into 2.0 ml microcentrifuge tubes for BCA protein assay (refrigerate – do not freeze). Ideally, large orifice tips should be used

for transfer only on this step.

a. The 0.025 mL aliquot should be diluted with Kinase buffer (1X), 1:6 (i.e., add 0.125 ml of 1X Kinase buffer to vial and vortex). Note: May use Pilches buffer in place of 1X Kinase buffer.

3. Centrifuge homogenate in super-speed at 3,750 rpm (using SM-24 rotor) for 15 minutes at 4^oC.
4. Aspirate or carefully decant supernatant, and to the pellet add 2.0 ml 10% TCA and vortex to resuspend.
5. Centrifuge suspension in super-speed at 3,750 rpm (using SM-24 rotor) for 15 minutes at 4^oC.
6. Aspirate or carefully decant supernatant, and to the pellet add 2.0 ml 10% TCA and vortex to resuspend.
7. Centrifuge suspension in super-speed at 3750 rpm (using SM-24 rotor) for 15 minutes at 4^oC.
8. Aspirate or carefully decant supernatant, and to the pellet add 2.0 ml 10% TCA and vortex to resuspend.
9. Centrifuge suspension in super-speed at 3800 rpm (using SM-24 rotor) for 3 minutes at 4^oC.
10. Aspirate or carefully decant supernatant, and to the pellet add 2.0 ml ice-cold HPLC grade acetone (CH_3Cl_3) and vortex to resuspend.
11. Centrifuge suspension in super-speed at 10,000 rpm (using SM-24 rotor) for 5 minutes at 4^oC.
12. Aspirate or carefully decant supernatant, and to the pellet add 2.0 ml ice-cold water (ultrapure grade) and vortex to resuspend.
13. Centrifuge suspension in super-speed at 10,000 rpm (using SM-24 rotor) for 5 minutes at 4^oC.
14. Aspirate or carefully decant supernatant, and dissolve pellet in 2.0 ml of 0.25 N NaOH.
15. Incubate tubes (capped/sealed) at 60^oC for 30 minutes or until protein is soluablized. Vortex often. If needed, use polytron homogenizer to break-up pellet.
16. Remove 0.5 ml of protein solution and add to 6.0 ml scintillation vials containing 5.0 ml of complete counting cocktail and 0.5 ml ultrapure

water. Shake vigorously and allow settling overnight before counting.

17. Take 0.025 ml of protein solution for post-assay protein analysis. Take remaining portion and place in 2.0 ml microcentrifuge tubes and freeze at -80°C.

    a. Dilute protein solution with 0.075 ml (1:4) of 1X Kinase buffer and run in triplicate (aliquot 0.010 ml per well). It is acceptable to conduct both pre and post protein assays at the same time. Must have standards on each plate. Label tubes numerically with alphabetic designation "B" for post.

    b. Add 0.2 ml of BCA-Copper (II) sulfate (50:1, vol/vol) with repeat pipetter.

    c. Used a Beckman Coulter DTX 880 Multimode Detector (absorbance @ 595).

B. Tissue Total Phenylalanine:

1. Remove 1.0 ml of the NaOH protein solution (step 17 tissue L-[2,3,4,5,6-^{3}H]Phenylalanine preparation) and place in 2.0 ml microcentrifuge tubes.

2. Add 1.0 ml of 6 N HCl to sample, cap and vortex.

3. Place 1.0 ml of solution into 2.0 ml screwtop tubes.

4. Add 0.1 ml of a 10 µmol/ml to sample (1 µmol/ml with 1.0 ml of sample).

5. Incubate at 110ºC for 24h.

6. Remove 0.05 ml of the sample with 0.1 ml pipette and add to 1.5 ml microcentrifuge tubes; leave tops open.

7. Dry under stream of N_2 for 1h.

8. Add 0.05 ml of acetonitrile (CH_3CN) and 0.05 ml of MTBSTFA + 1% TBDMCS (1:1, vol/vol); cap and vortex.

9. Allow to sit at room temperature for 20 min (capped), vortex and add 0.1 ml of solution to the GC-MS vial inserts (in GC-MS vials) and cap.

10. Inject sample into GC-MS at 1 µl/min; selective ion monitoring *(m/z)* 234 and 240.

C. Linear Standard Curve Preparation:

1. Stock Solution of Phenylalanine (F) (10 µmol/ml):

   a. To 100 ml of HPLC grade H_2O, add the following:

   M.W. x M x Vol (L)

   L-Phenylalanine ($C_9H_{11}NO_2$), M.W. = 165.19 (0.165 g)

2. Stock Solution of L-[*ring*-$^{13}C_6$]phenylalanine (10 µmol/ml):

   a. To 100 ml of HPLC grade H_2O, add the following:

   L-[*ring*-$^{13}C_6$]phenylalanine (F-$C_3{}^{13}C_6H_{11}NO_2$), M.W. = 171.15 (0.171 g)

3. Preparation and Measurement:

µmol/ml	ml of stock F	ml HPLC H_2O	total ml	ratio
10.0	1.0	0	1.0	1:0
7.5	0.750	0.250	1.0	3:1
5.0	0.500	0.500	1.0	1:1
2.5	0.250	0.750	1.0	1:3
1.225	0.1225	0.8775	1.0	1:7
0.625	0.0625	0.9375	1.0	1:15
0.314	0.0313	0.9687	1.0	1:30
0	0	1.0	1.0	0:1

   a. Add 1.0 ml of each standard (above) and 1.0 ml of 6 N HCl.

   b. Take 1.0 ml of above sample and add 0.1 ml 10.0 µmol/ml L-[*ring*-$^{13}C_6$]phenylalanine (F-$C_3{}^{13}C_6H_{11}NO_2$) to 1.5 ml microcentrifuge tubes, cap and vortex.

   c. Remove 0.05 ml of each standard with 0.1 ml pipette and add to 1.5 ml microcentrifuge tubes; leave tops open.

   d. Dry under stream of N_2 for 1h.

   e. Add 0.05 ml of acetonitrile (CH_3CN) and 0.05 ml of MTBSTFA + 1% TBDMCS (1:1, vol/vol); cap and vortex.

   f. Allow to sit at room temperature for 20 min (capped), vortex and

add 0.1 ml of solution to the GC-MS vial inserts (in GC-MS vials) and cap.

g. Inject sample into GC-MS at 1 µl/min; selective ion monitoring *(m/z)* 234 and 240

APPENDIX B

MUSCLE PROTEIN SYNTHESIS PROCESSING (ASSESSED WITH ^2H_2O)

I. ^2H Labeling of Body Water.

Required Items	Catalog Number
1.5ml microcentrifuge tubes	Fisher, 02-681-284
2.0ml microcentrifuge tubes	Fisher, 02-681-291
^2H_2O	Cambridge Isotopes, DLM2259-1
Acetone (CH_3COCH_3)	EMD, AX0120-8
Acetonitrile (CH_3CN)	Fisher, A998-4
GC-MS glass vials	Agilent, 5182-0714
GC-MS vial inserts	Agilent, 5183-2085
GC-MS vial screw top caps	Agilent, 5185-5820
Chloroform (CH_3Cl_3)	Fisher, C298
Sodium hydroxide (NaOH)	Fisher, 5612-3
Sodium Sulfate (Na_2SO_4)	Fisher, S414
Trichloroacetic acid (TCA-Cl_3CCOOH)	EMD, TX1045-5

Procedure:

A. Preparation of Plasma Samples:

1. Centrifuge 1 ml of 2, 6 and 12 minute blood samples at 3,800 rpm at 4ºC for 20 min.

2. Extract plasma with 1.0 ml pipette and place into 1.5 ml microcentrifuge tubes, freeze at -80ºC.

3. Place 0.02 ml of plasma into 2.0 ml microcentrifuge tubes.

4. Add 2.0 µl of 10N NaOH to samples.

5. Add 4.0 µl of a 5% (vol/vol) acetone (CH_3Cl_3) in acetonitrile (CH_3CN), cap and allow settling for a minimum of 24 h.

6. Add ~0.5 g sodium sulfate (Na_2SO_4) to samples.

7. Add 0.6 ml of chloroform (CH_3Cl_3) to each sample, cap and vortex vigorously.

8. Inject sample into GC-MS at 1 µl/min; selective ion monitoring *(m/z)* 58 and 59.

B. Gas Chromatography Mass Spectrometry (GC-MS):

1. System:

a. Agilent 5975C-MSD equipped with an Agilent 7890 GC system.

b. Column, HP-5ms capillary column (30 m × 0.25 mm × 0.25 µm).

2. Settings:

a. The following temperature program was used: 60ºC initial, increase by 20°C/min to 100ºC, increase by 50ºC/min to 220ºC, and hold for 1 min. The sample was injected at a spit ratio of 40:1 with a helium flow of 1 mL/min. The mass spectrometer was operated in electron impact mode (70eV).

b. Selective ion monitoring of mass-to-charge ratios (*m/z*) 58 (M) and 59 (M + 1) was conducted using a dwell time of 10 ms per ion. ^{2}H acetone elutes at ~ 1.7 min.

C. Linear Standard Curve Preparation

1. Stock Solution of $^{2}H_2O$:

a. 5% $^{2}H_2O$ (5.0 ml + 95 ml of ultrapure dH_2O)

% labeled	ml of stock	ml of ultrapure dH_2O	total ml
5.0	1.0	0	1.0
2.5	0.5	0.5	1.0
1.25	0.25	0.75	1.0
1.0	0.20	0.8	1.0
0.5	0.10	0.9	1.0
0.25	0.05	0.95	1.0
0.125	0.025	0.975	1.0
0	0	1.0	1.0

b. Place 0.02 ml of each standard into 2.0 ml microcentrifuge tubes.

c. Add 2.0 µl of 10N NaOH to samples.

d. Add 4.0 µl of a 5% (vol/vol) acetone (CH_3Cl_3) in acetonitrile (CH_3CN), cap and allow settling for a minimum of 24 h.

e. Add ~0.5 g sodium sulfate (Na_2SO_4) to samples.

f. Add 0.6 ml of chloroform (CH_3Cl_3) to each sample, cap and vortex vigorously.

g. Inject sample into GC-MS at 1 µl/min; selective ion monitoring *(m/z)* 58 and 59.

II. ^{2}H Labeling of Protein-Bound Alanine.

Required Items	Catalog Number
2.0ml microcentrifuge tubes	Fisher, 02-681-291
2.0ml screwtop tubes	United Labs., UP 2031
Acetonitrile (CH_3CN)	Fisher, A998-4
ATP ($C_{10}H_6N_5O_{13}P_3$)	Sigma, A9187
GC-MS glass vials	Agilent, 5182-0714
GC-MS vial inserts	Agilent, 5183-2085
GC-MS vial screw top caps	Agilent, 5185-5820
Hydrochloric acid (12N HCl)	EMD, 258148
Kinase buffer (10X)	Cell Signaling, 9802
L-Alanine ($C_3H_7NO_2$)	Sigma, A7627
L-Aalanine-2-d ($CH_3CD(NH_2)CO_2H$)	Isotec, 485861
Methanol (CH_3OH-HPLC grade)	Fisher, MXO 475-1
Methyl-8 reagent	Pierce, 49350
Triton X-100 (t-Oct-C_6H_4-$(OCH_2CH_2)_xOH$, x= 9-10)	Sigma, T8532
Trichloroacetic acid (TCA-Cl_3CCOOH)	EMD, TX1045-5

Procedure:

A. Preparation of Muscle Samples:

1. Weight out ~ 0.03 g of muscle (0.06 g if assessing myofibrillar fraction as it makes up 50% of the mixed muscle homogenate).

2. Place into 2.0 ml microcentrifuge tubes and add 0.03 ml of 10% TCA (Cl_3CCOOH) on ice.

a. If want to separate myofibrillar from mixed muscle, place ~0.06 g of mixed muscle into a 10:1, vol:wt ratio of Kinase buffer (1X) with 200 μM ATP ($C_{10}H_6N_5O_{13}P_3$) and 0.01% Triton; keep on ice.

b. Homogenize using a polytron homogenizer at full speed and allow settling on ice for a minimum of 1 h.

c. Centrifuge at 14,000 rpm for 30 min at 4ºC. Decant supernatant and add 0.3 ml of 10% TCA on ice. The remainder of the steps are the same as the mixed muscle homogenate.

3. Homogenize using a polytron at full speed, centrifuge at 3,800 rpm, decant supernatant and add 0.3 ml of 10%TCA on ice.

4. Repeat step 3 three additional times.

6. Add 0.3 ml of 6 N HCl, vortex and place into 2.0 ml screwtop tubes.

7. Incubate at 100ºC for 24 h.

8. Take 0.1 ml of hydrolysate and place into GC-MS glass vials and dry under a low stream of N_2 (can place in heating block at heat at 100ºC with top off for 1 h). Freeze the remainder of the hydrolysate.

9. Add a 3:2:1 solution of Methyl-8, methanol (CH_3OH) and acetonitrile (CH_3CN); cap and vortex vigorously.

10. Place sample into GC-MS vial inserts and place into the GC-MS glass vials and cap.

11. Inject sample into GC-MS at 1 μl/min; selective ion monitoring *(m/z)* 99 and 100.

12. NOTE: You may need to dilute you samples by 5, 10 or even 20.

B. Gas Chromatography Mass Spectrometry (GC-MS):

1. System:

a. Agilent 5973N-MSD equipped with an Agilent 6890 GC system.

b. DB17-MS capillary column (30 m × 0.25 mm × 0.25 μm).

2. Settings:

   a. The following temperature program was used: 90ºC initial, hold for 5 min, increase by 5°C/min to 130ºC, increase by 40ºC/min to 240ºC, and hold for 5 min. The sample was injected at a spit ratio of 20:1 (5:1 for human tissue) with a helium flow of 1 ml/min. The mass spectrometer was operated in electron impact mode (70eV).

   c. Selective ion monitoring of mass-to-charge ratios (*m/z*) 99 (M) and 100 (M + 1) was conducted using a dwell time of 10 ms per ion. ^2H alanine elutes at ~ 12 min (9 min on our system).

C. Linear Standard Curve Preparation

1. Stock Solution of L-alanine ($C_3H_7NO_2$):

   a. Place 10 mg L-alanine into 1.0 ml of ultrapure dH_2O (Solution A).

2. Stock Solution of L-alanine-2-d ($CH_3CD(NH_2)CO_2H$):

   a. Place 1 mg of L-alanine-2-d into 1.0 ml of ultrapure dH_2O (Solution B).

% labeled	Sol A (ml)	Sol B (ml)	H_2O (ml)	total (ml)
2.0	1.0	0.2	0.8	2.0
1.0	1.0	0.1	0.9	2.0
0.75	1.0	0.075	0.925	2.0
0.5	1.0	0.050	0.950	2.0
0.25	1.0	0.025	0.975	2.0
0	1.0	0	1.0	2.0

   b. Take 0.1 ml of each standard and place into GC-MS glass vials and dry under a low stream of N_2 (can place in heating block at heat at 100ºC with top off for 1 h). Freeze the remainder of the hydrolysate.

   c. Add a 3:2:1 solution of Methyl-8, methanol (CH_3OH) and acetonitrile (CH_3CN); cap and vortex vigorously.

   d. Place sample into GC-MS vial inserts and place into the GC-MS glass vials and cap.

e. Inject sample into GC-MS at 1 µl/min; selective ion monitoring *(m/z)* 99 and 100.

APPENDIX C

PLASMA AMINO ACID ANALYSIS

I. Plasma Amino Acid (HPLC)

Required Items	Catalog Number
1.5 ml microcentrifuge tubes	Fisher, 02-681-284
2.0 ml microcentrifuge tubes	Fisher, 02-681-291
0.45 µm pore size membrane filter	Fisher, R04SP04700
0.22 µm pore size membrane filter	Fisher, R02SP01300
Amino acid standards	Sigma, AAS18
Amino acids:	
B-Alanine ($C_3H_7NO_2$)	Sigma, A7752
L-Asparagine ($C_4H_8N_2O_3$)	Sigma, A8824
L-Citrulline ($C_6H_{13}N_3O_3$)	Sigma, C7629
L-Glutamine ($C_5H_{10}N_2O_3$)	Sigma, G3126
Taurine ($C_2H_7NO_3S$)	Sigma, T0625
L-Tryptophan ($C_{11}H_{12}N_2O_2$)	Sigma, T8659
L-Ornithine ($C_5H_{12}N_2O_2$)	Sigma, O2375
Benzoic acid (C_6H_5COOH)	Sigma, 109479
Borate ($Na_2B_4O_7$)	Sigma, B9876
Brij-35 $(C_2H_4O)_nC_{12}H_{26}O$	Sigma, B4184
Diethyl Ether ($C_4H_{10}O$)	EMD, EX0190-8
Ethanolamine ($NH_2(CH_2)_2OH$)	Sigma, E0135
Methanol (CH_3OH-HPLC grade)	Fisher, MXO 475-1
2-Mercaptoethanol (C_2H_6OS)	Sigma, 3148
Perchloric acid ($HClO_4$)	J.T. Baker, 9652-02
Phthaldialdehyde ($C_8H_6O_2$)	Sigma, P0657
Potassium carbonate (K_2CO_3)	Sigma, P5833
Shell vials for Waters autosampler	Fisher, 03-391-23
Sodium acetate ($C_2H_3O_2Na$)	Sigma, S7670
Tetrahydrofuran (C_4H_8O)	Sigma, 34865
Tuberculin slip tip (1ml)	BD, PK 100309602
Water (H_2O-HPLC grade)	Fisher, W5-4

Procedure:

A. Plasma Amino Acid Preparation:

1. Centrifuge 1.0 ml of 2, 6 and 12 minute blood samples at 3,800 rpm at 4ºC for 20 min.

2. Extract plasma with 1.0 ml pipette and place into 1.5 ml microcentrifuge tubes, freeze at -80ºC.

3. To 0.15 ml sample, add 0.15 ml HPLC grade H_2O, cap and vortex.

4. Remove 0.2 ml of sample, place into 1.5 ml microcentrifuge tubes and add 0.2 ml 1.5 M perchloric acid ($HClO_4$); cap and vortex.

5. Add 0.1 ml 2.0 M potassium carbonate (K_2CO_3), cap and vortex, then decap and allow settling for 5 min at room temp.

6. Centrifuge at 6,000 rpm at room temperature for 12 min.

7. Remove 0.3 ml of supernatant and place in 1.5 ml microcentrifuge tubes. Add 0.9 ml of diethyl ether ($C_4H_{10}O$), cap and vortex.

8. Aspirate top layer (lipid, will see separation with a film).

9. Extract 0.2 ml of bottom layer and add 0.1 ml of 1.2% benzoic acid (C_6H_5COOH) and 1.5 ml of HPLC grade H_2O in 2.0 ml microcentrifuge tubes; cap and vortex.

11. Remove sample with tuberculin slip tip syringe and filter 1.0 ml of sample through a 0.22 µm membrane filter into 1.0 ml HPLC vials and place into refrigerator without caps overnight.

12. Inject 1:1 (0.1 ml) of sample and OPA into Waters HPLC.

B. High Performance Liquid Chromatography (HPLC):

1. System:

   a. HPLC Waters solvent delivery system (2 Waters 501 pumps, a 717 Waters Autosampler, and Automated Gradient Controller [curve 6 chosen] Waters 470 Scanning Fluorescence Detector [attenuation 350 nm, gain 450 m]).

   b. Column, Suelcosil LC-18 (58985) 15 cm x 4.6 mm, 3 micron beads.

2. Gradient Solvent (A):

   a. Add 0.8 L of HPLC grade H_2O into 1.0 L flask.

b. Add 13.65 g sodium acetate ($C_2H_3O_2Na$), 0.048 ml 6 N HCl, 5.0 ml tetrahydrofuran (C_4H_8O) and 0.09 ml methanol (CH_3OH-HPLC grade).
c. Filter through 0.45 μm membrane filter into 1.0 L flask with side arm and q.s. to 1.0 L.

3. Solvent B:

a. Filter 1.0 L methanol (CH_3OH-HPLC grade) through 0.45 μm membrane filter.

4. Phthaldialdehyde ($C_8H_6O_2$) (OPA) Reagent:

a. Add 0.05 g OPA to sterile 20.0 ml amber bottle.

b. Add 1.25 ml methanol (CH_3OH-HPLC grade), 11.20 ml sodium borate buffer, 0.05 ml 2-Mercaptoethanol (C_2H_6OS) and 0.4 ml Brij-35 $(C_2H_4O)_nC_{12}H_{26}O$, cap and store in the refrigerator.

i. Sodium borate buffer:

1. Mix 15.22 g Borate ($Na_2B_4O_7$) in 1.0 L HPLC grade H_2O; pH 9.2.

5. HPLC Settings and Flow Rate: Linear gradient (1500 psi).

Time (min)	Flow (ml/min)	% Solvent A	%Solvent B	Curve
0	1.1	86	14	6
20	1.1	86	14	6
24	1.1	70	30	6
26	1.1	65	35	6
28	1.1	53	47	6
34	1.1	50	50	6
38	1.1	30	70	6
40	1.1	0	100	6
42	1.1	0	100	6
42.1	1.1	86	14	6
48.5	1.1	86	14	6

C. Linear Standard Curve Preparation:

1. Stock Solution (250 nmol/ml):

a. To 200 ml of HPLC grade H_2O, add the following (GATCOTA):

M.W. x M x Vol (L)

B-Alanine ($C_3H_7NO_2$), M.W. = 87.09 (0.0435 g)
L-Asparagine ($C_4H_8N_2O_3$), M.W. = 132.12 (0.066 g)
L-Citrulline ($C_6H_{13}N_3O_3$), M.W. = 175.2 (.0876 g)
L-Glutamine ($C_5H_{10}N_2O_3$), M.W. = 146.1 (0.073 g)
Taurine ($C_2H_7NO_3S$), M.W. = 125.15 (0.0625 g)
L-Tryptophan ($C_{11}H_{12}N_2O_2$), M.W. = 204.2 (0.102 g)
L-Ornithine ($C_5H_{12}N_2O_2$), M.W. = 168.62 (0.0843 g)

b. Add 0.3 ml of amino acid standard, 0.3 ml of GATCOTA, 0.3 ml ethanolamine ($NH_2(CH_2)_2OH$ and 2.1 ml of HPLC grade H_2O; total volume = 3.0 ml.

nmol/ml	ml of stock solution	ml HPLC H_2O	total ml	ratio
250.00	1.0	0	1.0	1:0
125.00	0.5	0.5	1.0	1:1
62.50	0.5	1.5	2.0	1:3
41.68	0.2	1.0	1.2	1:5
35.71	0.1667	1.0	1.1667	1:6
22.73	0.1	1.0	1.1	1:10
15.63	0.1	1.5	1.6	1:15
11.91	0.1	2.0	2.1	1:20
9.62	0.05	1.25	1.3	1:25
4.90	0.039	1.96	1.999	1:50

c. Add 0.15 ml of each standard to a 1.5 ml microcentrifuge tubes and add 0.15 ml HPLC grade H_2O, cap and vortex.

d. Remove 0.2 ml of sample, place into 1.5 ml microcentrifuge tubes and add 0.2 ml 1.5 M perchloric acid ($HClO_4$); cap and vortex.

e. Add 0.1 ml 2.0 M potassium carbonate (K_2CO_3), cap and vortex, than decap and allow settling for 5 min at room temp.

f. Centrifuge at 6,000 rpm at room temperature for 12 min.

g. Remove 0.3 ml of supernatant and place in 1.5 ml microcentrifuge tubes. Add 0.9 ml of diethyl ether ($C_4H_{10}O$), cap and vortex.

h. Aspirate top layer (lipid, will see separation with a film).

i. Extract 0.2 ml of bottom layer and add 0.1 ml of 1.2% benzoic acid (C_6H_5COOH) and 1.5 ml of HPLC grade H_2O in 2.0 ml microcentrifuge tubes; cap and vortex.

j. Remove sample with tuberculin slip tip syringe and filter 1.0 ml of sample through a 0.22 µm membrane filter into 1.0 ml HPLC vials and place into refrigerator without caps overnight.

k. Inject 1:1 (0.1 ml) of sample and OPA into Waters HPLC.

APPENDIX D

CALCULATIONS

Assessment With L-[2,3,4,5,6-^{3}H]Phenylalanine

I. Determination of Plasma and Tissue Specific Radioactivity (SA):

A. Plasma SA with HPLC: $(DPM \cdot ml^{-1}) \cdot (F \cdot ml^{-1})^{-1}$

1. $(DPM \cdot ml^{-1})$ = DPM x 40

2. $(F\ nmol \cdot ml^{-1}) = [(F) \times B3] \cdot [(B2 - B1) \times ((A2 - A1) - (A3 - A1))]^{-1}$

a. (F) = value of F in ml obtained from HPLC.

b. A1 = weight of tube A empty.

c. A2 = weight of tube A with 0.25 ml of plasma and 0.25 ml 10% TCA (Cl_3CCOOH).

d. A3 = weight of tube A with precipitate only (supernatant removed).

e. B1 = weight of tube B empty.

f. B2 = weight of tube B with supernatant.

g. B3 = weight of tube B with supernatant and 0.3 ml of 0.5M Borate ($Na_2B_4O_7$).

B. Plasma SA with GC-MS: $(DPM \cdot ml^{-1}) \cdot (F \cdot ml^{-1})^{-1}$

1. $(DPM \cdot ml^{-1})$ = DPM x 40

2. $(F\ nmol \cdot ml^{-1}) = (C \times 60) \cdot 0.3^{-1}$

a. C = Corrected ratio of 234/240 which is obtained by taking the

ratio, subtracting the intercept (measured standard 234/240 ratios) and dividing by the slopes (expected 234/240 ratios obtained by taking standard values and dividing by the quantity of the internal standard (nmol/ml).

b. 60 = internal standard quantity ($nmol \cdot ml^{-1}$).

c. 0.3 = quantity of sample used (ml).

C. Tissue SA with GC-MS: $(DPM \cdot ml^{-1}) \cdot (F \cdot ml^{-1})^{-1}$

1. $(DPM \cdot ml^{-1}) = (DPM \cdot 0.5^{-1}) \times 2$

   a. 2 = volume that was originally used (2.0 ml).

2. $(F\ nmol \cdot ml^{-1}) = ((C \times 1000) \cdot 1.0^{-1}) \times 2$

   a. C = Corrected ratio of 234/240 which is obtained by taking the ratio, subtracting the intercept (measured standard 234/240 ratios) and dividing by the slopes (expected 234/240 ratios obtained by taking standard values and dividing by the quantity of the internal standard (nmol/ml).

   b. 1000 = internal standard quantity ($nmol \cdot ml^{-1}$).

   c. 1.0 = quantity of sample used (ml).

   d. 2 = volume that was originally used (2.0 ml).

II. Calculation of Fractional Rates of Protein Synthesis (FSR): $\% \cdot h^{-1}$

A. FSR: $S_P \cdot (S_{\bar{A}} \times t)^{-1} \times 100$ (expressed as $\% \cdot h^{-1}$)

1. S_P = Specific radioactivity of tissue.

   a. $(DPM \cdot ml^{-1}) \cdot (F \cdot ml^{-1})^{-1}$

2. $S_{\bar{A}}$ = Mean specific radioactivity (2, 6, 12 min bloods) of plasma.

   a. $(DPM \cdot ml^{-1}) \cdot (F \cdot ml^{-1})^{-1}$

3. t = time from start of flooding to placement of tissue into liquid N_2 (h).

4. 100 = conversion to a % (expressed as % · h^{-1}).

III. Calculation of Rates of Protein Synthesis (RPS): nmol F · g^{-1} · h^{-1}

A. RPS: (DPM · protein $pool^{-1}$) · $(S_{\bar{A}})^{-1}$ · $(t)^{-1}$

1. DPM = [(DPM · 0.5^{-1}) · post-$protein^{-1}$] x [(2 x post-protein) · g $tissue^{-1}$)]

a. (DPM · 0.5^{-1}) = DPM is 0.5ml of NaOH.

b. post protein = protein content in NaOH (mg · ml^{-1}).

c. (2 x post-protein) = quantity of protein (mg · ml^{-1}) in 2.0ml (original vol.).

2. $S_{\bar{A}}$ = Mean specific radioactivity (2, 6, 12 min bloods) of plasma.

b. (DPM · ml^{-1}) · (F · $ml^{-1})^{-1}$

3. t = time from start of flooding to placement of tissue into liquid N_2 (h).

Assessment with ^2H_2O

I. Determination of Plasma and Tissue Enrichment:

A. Plasma Enrichment: (59) · $(58)^{-1}$

1. *(m/z)* 59 = M+1 labeling of acetone (CH_3COCH_3). You could potentially have up to 6 ^2H (6H in acetone), M+6, however, do not need to measure the (m/z) 64.

2. *(m/z)* 58 = M, meaning you have 0 labeling of acetone.

. Tissue Enrichment: (100) · $(99)^{-1}$

1. *(m/z)* 100 = M+1 labeling of Methyl-8 fragment of alanine

($C_3H_7NO_2$).

2. *(m/z)* 99 = M, meaning you have 0 labeling Methyl-8 fragment of alanine.

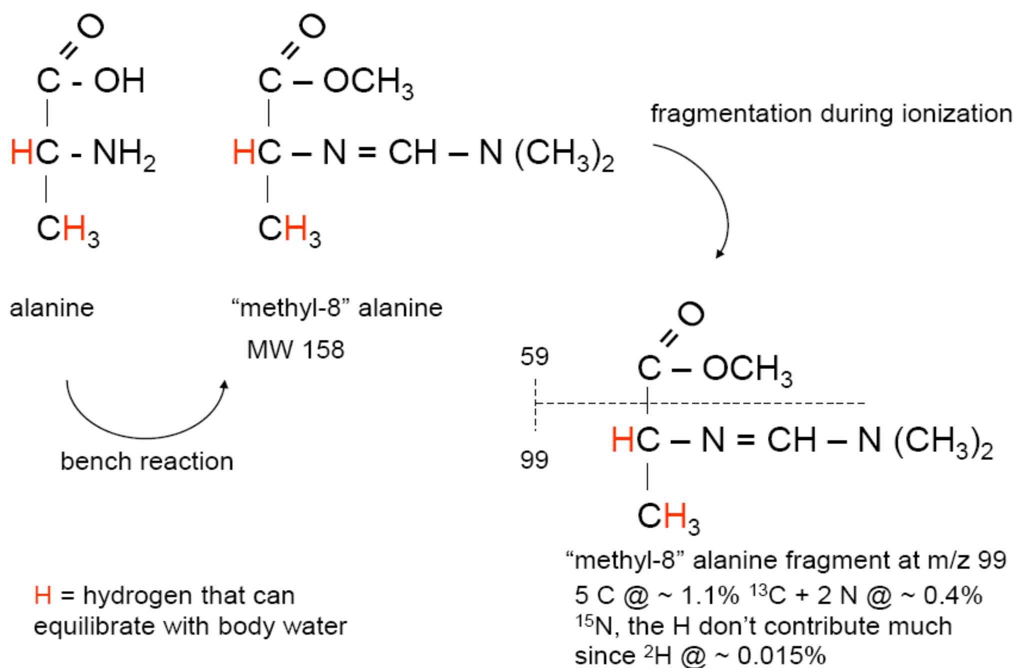
II. Calculation of Fractional Rates of Protein Synthesis (FSR):

A. FSR: ^{2}H-labeling of Ala in protein · [^{2}H-labeling body water x 3.7 x time (h)]$^{-1}$

1. Multiplying X 100 will convert to a % (% · h^{-1})

2. 3.7 represents exchange of ^{2}H between body water and alanine.

3. The assumption is that ^{2}H labeled body water equilibrates with free alanine more rapidly than ^{2}H labeled alanine incorporates into newly made protein and that proteins synthesis is linear over time.



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