Skeletal Muscle Protein Metabolism and Molecular Responses to Endurance Exercise and Nutritional Interventions

by

Shanon Loree Casperson

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Dedication

Dedicated to my husband and son whom I love with everything I have. And to my mom and dad who gave me the foundation from which to grow.

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First I would like to thank all the study volunteers, without their selflessness these studies would not have been possible. Second I would like to thank each of my committee members, Melinda Sheffield-Moore, Douglas Paddon-Jones, Bill Durham, Elena Volpi, and Michele Keske, for your time and mentorship during my graduate studies. Each of you has helped me grow as a scientist and for this I will be indebted to you. I would also like to thank all the nurses and staff of the ITS-CRC for all their support and expertise in helping me conduct these studies. And finally I would like to thank my friends and colleagues for all your love and support in getting me through all those tough times.

SKELETAL MUSCLE PROTEIN METABOLISM AND MOLECULAR RESPONSES TO ENDURANCE EXERCISE AND NUTRITIONAL INTERVENTIONS

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Shanon Loree Casperson, Ph.D.

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Supervisor: Melinda Sheffield-Moore

It has been suggested that the gradual loss of muscle mass observed with healthy aging is the result of a resistance of aged muscle tissue to normally potent anabolic stimuli. Recent evidence indicates an age-related anabolic resistance in skeletal muscle tissue to exogenous amino acids and to a wide range of resistance exercise intensities. In addition, aging is associated with adaptations within the vascular system that may adversely affect blood flow, thereby potentially impacting amino acid delivery, exchange and utilization. Endurance-type exercises have been shown to elicit significant increases in muscle blood flow and stimulate muscle protein synthesis; however, it is not known whether age-related anabolic resistance occurs in response to endurance exercise. These studies were designed and implemented to determine whether endurance exercise and amino acid supplementation independently and synergistically contribute to the stimulation of skeletal muscle protein synthesis. Stable isotopic techniques (i.e. arteriovenous balance measurements and tracer incorporation), macro- (indocyanine green dye dilution and Doppler) and microvascular (contrast-enhanced ultrasound and microdialysis) techniques, immunoblotting, and hormone assays were used to examine skeletal muscle metabolism, leg blood flow and skeletal muscle perfusion, and intracellular signaling events associated with translational control of muscle protein accretion. The principal findings are that acute endurance exercise increases amino acid exchange stimulating muscle protein synthesis without an age-related resistance to the anabolic effects of exogenous amino acids during exercise. This is contradictory to the situation following endurance-type exercise; when older adults exhibit anabolic resistance to exogenous amino acids. It is important to note that this age-related anabolic resistance did not manifest into an overall decrement in net protein balance. The molecular events underlying these changes are marked by increases in the activation of proteins involved in muscle hypertrophy signaling, and decreases in muscle atrophy signaling. Lastly, daily leucine supplementation enhances the anabolic stimulus of a mixed nutrient meal in older adults. Taken together, these results suggest that endurance exercise and amino acids, specifically leucine, are effective interventional strategies that may be used to stimulate muscle protein anabolism in older adults to ameliorate or prevent muscle loss.

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CHAPTER 1

INTRODUCTION[1]

As the elucidation of skeletal muscle biology continues, the importance of muscle tissue as a major metabolic organ has become clear. Unfortunately, a seemingly inevitable negative aspect of aging is the subtle but continuous loss of skeletal muscle tissue, termed sarcopenia. Sarcopenia occurs in persons who are otherwise free of disease, and it does not discriminate based on gender, ethnicity or wealth [1]. Over the past several years our understanding of the physiological and pathophysiological influences that modulate age-associated skeletal muscle loss has shifted from a theory of dysregulation of basal muscle protein metabolism to include the concept of "anabolic resistance." The term "anabolic resistance" was coined by Cuthbertson *et al.* [2] and has come to mean the age-related inability of anabolic stimuli to induce an "appropriate" anabolic response in skeletal muscle tissue. Specifically, in healthy young muscle tissue, anabolic stimuli significantly increase skeletal muscle protein synthesis; however, aged muscle tissue exhibits a diminished synthetic responsiveness to some anabolic stimuli. Thus, age-related anabolic resistance is thought to be the principal contributor to the decline in skeletal muscle mass with aging [3]. As such, there is clearly a need to advance our knowledge concerning age-related anabolic resistance in skeletal muscle tissue in order to develop therapeutic interventions targeted at preventing, attenuating and/or reversing age-associated muscle loss.

Regardless of age, the two most potent anabolic stimuli of muscle protein synthesis are protein/essential amino acids [4] and muscle contraction [5]. However, there are several reports suggesting an impaired anabolic response to such potent stimuli in older adults. The first

[1] Excerpts from Durham WJ, Casperson SL, Dillon EL, Keske MA, Paddon-Jones D, Sanford AP, Hickner RC, Grady JJ, Sheffield-Moore M. Age-related anabolic resistance after endurance-type exercise in healthy humans. *FASEB J.* 2010 Oct; 24(10):4117-27. © Federation of American Societies for Experimental Biology, and Casperson SL, Sheffield-Moore M, Hewling S, and Paddon-Jones D. Leucine supplementation chronically improves muscle protein synthesis in older adults consuming the RDA for protein. *Clinical Nutrition;* In press, 2012. © The European Society for Clinical Nutrition and Metabolism, reproduced with permission.

demonstration of age-related anabolic resistance in muscle tissue to amino acid stimulation was a study conducted by Volpi, *et al.* in 2000 [6]. During a 3-hour oral administration of an amino acid/glucose mixture (a total of 40 g glucose and 40 g crystalline amino acids) the rate of muscle protein synthesis increased in the young but not the older subjects [6]. In a follow up study, Guillet *et al*. [7] also found reduced muscle protein synthesis rates in older subjects during a constant infusion of an amino acid/glucose mixture. Taken together, these observations suggest that the anabolic effect of insulin or amino acids on protein metabolism is impaired in older adults. However, when insulin secretion is clamped at postabsorptive values (i.e. fasting) it becomes apparent that the anabolic stimulus of amino acids *per se* is blunted in older adults [2]. Since these initial studies, age-related anabolic resistance in muscle tissue to physiological doses of amino acids has been confirmed by others [8-10].

The concept of age-related anabolic resistance in muscle tissue has recently been extended to differential activation of muscle protein synthesis in response to exercise [11-13]. Several studies have shown that the muscle protein synthetic response to a wide range of resistance exercise intensities is reduced in older adults when compared to their younger counterparts [11-14]. Drummond *et al.* [11] reported that following resistance exercise (8 sets of 10 repetitions of 2-legged extension exercises at 70% of their one-repetition maximum (1 RM)) the muscle anabolic response is delayed for up to three hours in older adults. Likewise, Kumar *et al.* [13] reported a blunted response of the exercise-stimulated rate of protein synthesis in older men in response to unilateral isotonic leg extension and flexion exercises at a range of 20 – 90% of their 1 RM. In a follow up study by Fry *et al.* [12], following 8 sets of 10 repetitions at 70% 1RM older adults demonstrated a blunted response at 3 hours postexercise, and this response persisted for at least 24 hours. Taken together, these studies suggest an age-related anabolic resistance to resistance-type exercise in muscle tissue. The mechanisms behind this apparent age-related anabolic resistance of skeletal muscle tissue to protein/amino acids and resistance exercise are not fully understood. It may be that older adults require increases in both dietary

protein and exercise volume to achieve a more robust "youthful" response. In addition, it is not known whether endurance–type exercise elicits similar anabolic resistance in aged muscle tissue. In considering these possibilities, I will first draw upon recent evidence to characterize how healthy adults respond to endurance exercise and nutritional countermeasures.

Endurance-type exercise as a countermeasure to age-related anabolic resistance

Endurance exercise and skeletal muscle protein metabolism

Although it is generally assumed that endurance-type exercise does not increase skeletal muscle mass, until recently its influence on skeletal muscle protein synthesis has been relatively unstudied. Several studies have now shown that both myofibrillar and mitochondrial protein synthesis are increased in response to endurance-type exercise [15-18]. In 1990, Carraro *et al.* [15] was the first to report that a single bout of endurance-type exercise (4 hours of treadmill walking at 40% VO_{2max}) stimulates muscle protein synthesis by ~20% above rest in young adults. In a follow up study employing the same exercise intensity but studying a more practical duration of exercise (45 minutes vs. 4 hours), Sheffield-Moore *et al.* [17] demonstrated that this relatively low-intensity endurance exercise is also capable of stimulating muscle protein synthesis in older men to the same extent as seen in the young, at least during and immediately following exercise. These observations have recently been confirmed by Mascher *et al.* [19], who found that muscle protein synthesis was elevated ~22% after a bout of moderate-intensity (1 hour at 65-70% VO_{2max}) endurance exercise in a group of young men. It is important to note that these studies showed elevated rates of muscle protein synthesis without the benefit of supplementary nutrition.

However, many individuals do not exercise in the fasting state, and there is currently considerable curiosity among researchers as well as everyday exercisers about the potential for pre- or during-exercise protein/amino acid supplements to influence anabolic responses [20-24]. When subjects are given a nutritional supplement, either concurrently or following a bout of

moderate or intense endurance exercise, muscle protein synthesis is further increased in young men, peaking 24 hours after exercise [16, 18, 25]. In fact, Miller *et al.* [16] showed elevated rates of muscle protein synthesis for up to 72 hours after a 1-hour bout of one-legged kicking at 67% of maximum workload (W_{max}). On the other hand, recent studies in physically active young men have shown that the addition of protein to a carbohydrate supplement does not further increase muscle protein synthesis rates during endurance-type exercise [22, 24]. However, these studies have conflicting results in the recovery period. Hulston *et al.* [24] observed higher rates of muscle protein synthesis, where as Beelen *et al.* [22] observed no difference when supplementary protein was administered during exercise. Two possible explanations for this discrepancy are the subject training status and the duration of the exercise session (2 hours vs. 3 hours). Specifically, in trained young male cyclist, 2 hours of continuous cycling at 55% W_{max} [22] may not have placed a high metabolic demand on the muscle tissue thus influencing the ability of protein supplementation to stimulate muscle protein synthesis. Taken together, these varied durations and intensities of endurance-type exercises have shown it capable of stimulating muscle protein synthesis; overcoming years of dogma that have claimed it incapable. In addition, provision of protein further augments the anabolic response following a bout of endurance-type exercise in healthy untrained adults.

The role of microvascular perfusion in skeletal muscle metabolism

One potential limitation to overcoming age-related anabolic resistance in muscle tissue is that a variety of age-related adaptations within the vascular system may adversely affect blood flow, thereby potentially impacting nutrient flow, exchange and utilization. Historically, the skeletal muscle vasculature has been viewed as a passive system, designed to deliver hormones and nutrients and remove waste products based upon the metabolic demand of the muscle [26]. However, since the groundbreaking studies of the 1960s and 1970s [27, 28], it has been suggested that skeletal muscle contains two different vascular flow routes, nutritive and non-nutritive, based on their effects on metabolism and nutrient exchange [26, 29-32]. Anatomically,

skeletal muscle vessels of the nutritive route are considered to be in close proximity to muscle fibers, whereas vessels of the non-nutritive route are considered to serve as a functional vascular shunt with poor nutrient exchange (based on decreased oxygen consumption) with muscle, constituting approximately 62% of total resting blood flow [27]. Therefore, the access of nutrients and hormones to muscle is controlled by both total blood flow and the relative distribution of this flow between nutritive and non-nutritive routes. Furthermore, the classic studies of Honig *et al.* [33, 34] showed that in skeletal muscle the regulation of the hemodynamic response to exercise shifts from the "active" terminal arteriole to the "passive" individual capillaries [33, 34]. As a result, emerging evidence now suggests a controlling mechanism by which the skeletal muscle vasculature, specifically microvascular perfusion, controls muscle metabolism [26, 32, 35-43].

The redistribution of nutritive and non-nutritive flow occurring during exercise is associated with increased substrate delivery, potentially influencing muscle metabolism, in particular protein accretion [32, 44, 45]. However, in the absence of this shift to nutritive flow, or if tissue permeability to substrates is low, the influence of the hemodynamic response of exercise on tissue substrate exchange will be minimal [46]. In theory, impaired endothelial function could interfere with the response of skeletal muscle to elevated plasma amino acids by reducing capillary blood flow and thus limiting transcapillary amino acid exchange with the interstitial fluid. In the young, exercise elicits increased microvascular perfusion in skeletal muscle regulating blood flow in a local and graded manner to promote nutrient exchange [32]. While it has not been established whether the skeletal muscles of older individuals respond to exercise by similarly enhancing muscle perfusion and nutrient exchange as in the young, it is one aim of this dissertation to investigate this phenomenon.

Amino acids as a countermeasure to age-related anabolic resistance

Metabolic roles of nutrients in skeletal muscle metabolism

Amino acid availability, primarily the essential amino acids, is vital in the regulation of skeletal muscle protein metabolism, suggesting that muscle protein synthesis is modulated by the availability of amino acids [4, 47-51]. Studies have shown unequivocally that increased plasma and intracellular amino acid concentrations independently stimulate muscle protein synthesis in a dose dependent manner [4, 51-55]. However, the protein/amino acid dose required to stimulate muscle protein synthesis in older adults remains controversial. Katsanos *et al.* [8] showed that after a single bolus ingestion of a small amount of amino acids (~7 g of essential amino acids) the muscle protein anabolic response was diminished in older adults compared to young. By contrast, Cuthbertson *et al*. [2] showed that younger and older adults exhibit a similar skeletal muscle anabolic response to a single bolus ingestion of less than 10 g of essential amino acids, and that age-related anabolic resistance in muscle tissue to exogenous amino acids was only apparent at doses of 10 g or more. In line with these findings, Yang *at al.* [56] demonstrated that the skeletal muscle anabolic response in older adults plateaus after ingestion of 20 g of whey protein, containing ~10 g of essential amino acids. If the results from these studies are correct then the recommendation that older adults consume ~20 g (based on the RDA of 0.8 g/kg/day for a 75 kg adult) of high-quality protein with each meal would maximally stimulate muscle protein synthesis. However, a typical 20 g serving of protein supplies only 5 – 8 g of essential amino acids. Therefore, a larger protein serving (> 25 g, supplying 10 – 15 g of essential amino acids) is needed to induce a positive net accretion in aged muscle tissue [4, 8, 9, 54, 57]. In support of this notion, Symons *et al.* [58, 59] reported that a moderate serving of intact protein (30 g total; ~12g essential amino acids) maximally stimulates muscle protein synthesis in both younger and older adults. However, when protein/amino acids are consumed in combination with carbohydrates the anabolic response is blunted [6, 7]. Although the anabolic response to a mixed nutrient meal is reduced with aging, pure amino acid supplementation can stimulate muscle protein synthesis to a

rate comparable to that of younger adults [47, 51, 58, 60]. In a recent study by Dillon *et al*. [54] the addition of 15 g/day of essential amino acids to the daily diets of older women for three months enhanced muscle protein synthesis leading to significant increases in lean body mass. These data provide evidence suggesting that aged muscle tissue retains the ability to respond to the anabolic stimulus of amino acids, albeit to a lesser extent than the muscle of young.

Leucine's role in skeletal muscle metabolism

The amino acid leucine is distinct as an effective nutrient regulator of skeletal muscle protein synthesis. [9, 57, 61-75]. For over 30 years leucine has consistently been shown to positively affect muscle protein synthesis, either alone or by enhancing the anabolic effect of amino acid mixtures [9, 11, 57, 61-72, 74, 75]. Recent studies in both animals [62, 75-77] and humans [72] have demonstrated that leucine ingestion provides an anabolic stimulus comparable to that of all essential amino acids combined without altering food intake [72, 75, 77-79]. Although there is sufficient evidence that leucine is able to stimulate muscle protein synthesis, its effectiveness to improve muscle mass remains controversial. Aged muscle tissue appears to be less responsive to physiological leucine concentrations, suggesting a leucine "threshold" that must be exceeded in aged muscle tissue for the stimulation of muscle protein synthesis [9, 76]. Several studies in both animals [75, 80] and humans [9, 65, 72] have shown that increasing the leucine content of regular mixed nutrient meals can normalize or even increase muscle protein anabolism in older adults, demonstrating that age-related anabolic resistance to exogenous amino acids in muscle tissue can be overcome with greater amounts of leucine. Specifically, Rieu *et al.* reported that leucine, when added to a mixed nutrient meal, normalized muscle protein synthesis in older animals [75]. In a follow-up study in older adults, muscle protein synthesis was acutely increased in response to a leucine supplemented meal [72]. The results of these studies indicate that the addition of leucine to regular meals acutely stimulates muscle protein synthesis in older adults. However, the impact of chronic leucine supplementation on improving age-related anabolic resistance to protein/amino acids in muscle tissue remains relatively unstudied.

Therefore, it is one aim of this dissertation to investigate the efficacy of leucine *per se* to counteract age-related anabolic resistance in muscle tissue by increasing the leucine content of each meal.

Regulatory mechanisms of muscle protein synthesis

Molecular responses to nutrition

The molecular events underlying the age-related anabolic resistance in muscle tissue to a provision of amino acids are being elucidated but are still far from being clear. The primary pathway through which amino acids are thought to regulate muscle protein synthesis is the mammalian target of rapamycin (mTOR) signaling pathway [81-83]. In mammalian cells, mTOR is found in two protein complexes: mTORC1 and mTORC2. Both complex 1 and 2 are comprised of the mTOR protein and a G protein β-subunit-like protein (GβL) [84]. Differences in the two complexes stem from the association with either raptor (mTORC1), which is rapamycin sensitive, or rictor (mTORC2), which is rapamycin-insensitive [82, 85]. Activation of mTORC1 is primarily in response to nutrients and contractile activity, therefore, suggesting it is a key regulator of muscle protein synthesis [86]. mTOR is a serine/theronine kinase that when activated promotes mRNA translation initiation and stimulation of protein synthesis promarioly through activation of two downstream targets - eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and 70-kDa ribosomal protein S6 kinase 1 ($p70^{S6K}$) [85-90]. The phosphorylation of 4E-BP1 allows the release of eIF4E which is then available to bind to eIF4G, forming the eIF4F pre-initiation complex [91]. The phosphorylation of

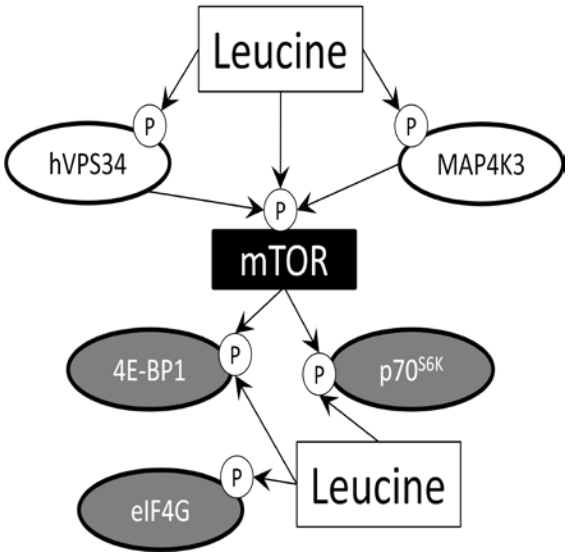


Figure 1: Leucine regulation of skeletal muscle protein synthesis.

$p70^{S6K}$ enhances cellular translational capacity and cell size, however, its physiological role remains obscure [91].

As noted above, the distinct qualities of leucine make it an effective nutrient regulator of skeletal muscle protein synthesis [9, 57, 61-75]. Although leucine is an insulin secretogogue, its stimulation of muscle protein synthesis is activated via an insulin-independent mechanisms through direct stimulation of the mTOR- signaling pathway [92]. The mechanisms through which leucine directly stimulates mTOR are yet to be elucidated but recent evidence supports a role of both the vacuolar protein sorting 34 (hVPS34) [93, 94] and mitogen-activiated protein kinase kinase kinase (MAP4K3) [95]. Independent of mTOR, leucine also enhances the association of eIF4E with eIF4G through increased stimulation of 4E-BP1 and eIF4G phosphorylation, therefore, modulating distinct steps of translation initiation and protein synthesis [62]. In addition to the elevated 4E-BP1 and eIF4G phosphorylation, oral administration of leucine increases $p70^{S6K}$ phosphorylation, particularly at Thr^{389} which is associated with increased activation of muscle protein synthesis [96]. Together, leucine amplifies intracellular signaling events associated with accelerated translational control of muscle protein synthesis. However, the extent to which the proteins involved in these intracellular signaling events are activated in response to chronic leucine supplementation has yet to be determined.

Molecular responses to exercise

Like amino acids, exercise-induced increases in muscle protein synthesis are stimulated through the phosphorylation of proteins in the mTOR-signaling pathway [85, 97, 98]. However, the mechanical signal generated during contraction initiates an intricate matrix of molecular events involved in specific signaling pathways which cross-talk to produce a complex transduction network [7, 11, 99-106]. Figure 2 shows some of the interconnecting signaling cascades that are activated in response to exercise. Following a single bout of endurance exercise, the activation of mTOR increases $p70^{S6K}$ phosphorylation for up to 90 minutes in the

fasted state [19] and up to 24 hours with the addition of supplementary nutrition [107]. These results suggest a synergetic effect of endurance exercise and nutrients to increase muscle protein synthesis. Nonetheless, exercise-induced increases in muscle protein synthesis may be accompanied by an equal or greater increase in muscle protein breakdown [17, 24].

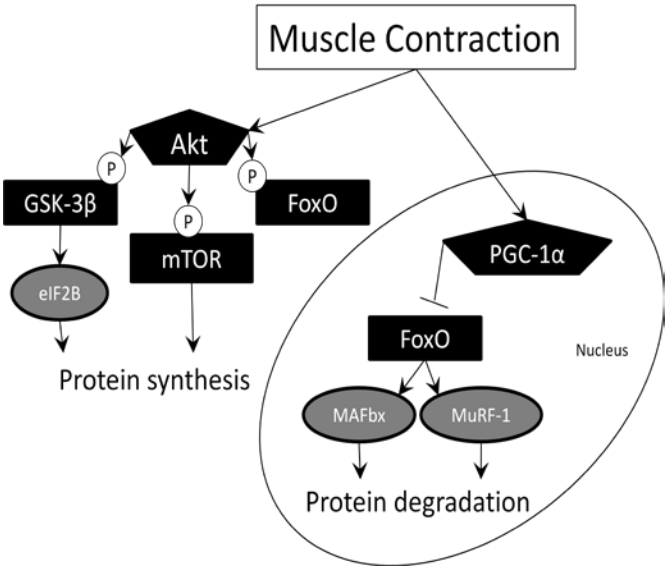


Figure 2: Molecular signaling events in response to exercise.

Skeletal muscle protein breakdown is regulated primarily through the localization of the class O of forkhead box transcription factors (FoxO) [108, 109]. Skeletal muscle contains three isoforms of the FoxO transcription factors: FoxO1 (FKHR), FoxO3 (FKHRL1), and FoxO4 (AFX) which appear to be coordinately regulated [105]. In myotubes and animal models, FoxO translocation has been shown to mediate the upregulation of the muscle-specific E3 ligases [109, 110]. The primary muscle-specific E3 ligases, which are part of the ubiquitin-proteasome pathway thought to be the primary pathway through which the bulk of all intracellular proteins are degraded, are muscle RING-finger protein-1 (MuRF-1) and muscle-specific atrogin-1 (MAFbx) [111]. The functional importance of MuRF-1 and MAFbx in skeletal muscle atrophy processes has been shown in both animals [109, 110] and humans [112]. Since the maintenance of skeletal muscle is an intricate balance between protein synthesis and breakdown, it is important to characterize the molecular events responsible for these processes in response to endurance exercise; it is one aim of this dissertation to characterize these molecular events.

Summary

The dynamic processes through which endurance exercise and amino acids regulate gain, maintenance, or loss of muscle mass is the result of intricate cellular alterations culminating in the alteration of skeletal muscle metabolism. Although aging is associated with a blunted synthetic response to anabolic stimuli, I have presented possible countermeasures to prevent, attenuate and/or reverse age-associated muscle loss. My **general hypothesis** is that the age-related anabolic resistance in muscle tissue can be attenuated with low-intensity endurance exercise and increased amino acid availability. In my first research project, I examined how aging affects the exercise-induced muscle microvascular blood flow response and the subsequent response of muscle protein synthesis in the postexercise period when a provision of amino acids was provided. My next research project focused on the during-exercise response of skeletal muscle protein metabolism to a single bout of endurance-type exercise during concomitant amino acids administration. Specifically, I examined the extent to which skeletal muscle contributes to leg protein metabolism during exercise. In my third study, I examined how the mTORC1 and FoxO signaling pathways were affected following a single bout of endurance-type exercise in combination with an amino acid infusion. In my final project, I examined how supplementing the regular daily meals of community dwelling older adults with leucine would influence the efficiency of the skeletal muscle anabolic response. These translational studies focus on interventional strategies to stimulate muscle protein anabolism in older adults to overcome age-related anabolic resistance in muscle tissue. The data presented herein significantly contributes to the growing body of knowledge in both the exercise physiology and nutrition scientific communities.

CHAPTER 2

AGE-RELATED ANABOLIC RESISTANCE AFTER ENDURANCE-TYPE EXERCISE IN HEALTHY HUMANS [2]

Introduction

Most individuals experience a gradual loss of muscle mass as they age, a process termed *sarcopenia.* When sarcopenic losses reduce skeletal muscle mass below a critical threshold, activities of daily living are compromised and disability ensues [113]. Although multiple etiologic factors have been associated with sarcopenia [114-116], the nature of sarcopenic progression is unclear. In particular, it is uncertain whether there is an early stage of resistance to anabolic stimuli that precedes frank muscle loss. Although changes in basal muscle protein metabolism have been reported to occur in some [117-121] but not all [122-127] studies of otherwise healthy adults, aberrant responses to anabolic stimuli are thought to play an important role [128]. Accordingly, evidence is accumulating that skeletal muscle of older individuals exhibits resistance to anabolic stimuli such as amino acids, insulin, and resistance exercise [129, 130].

Before circulating amino acids can be used for skeletal muscle protein synthesis, they must first leave skeletal muscle capillaries, traverse the interstitial fluid, and be transported into muscle fibers. Previous studies suggest that the transit of amino acids from the blood to the interstitial space occurs primarily via diffusion and is rate-limiting for the net uptake of circulating amino acids by muscle fibers [131-133]. One mediator of this uptake by skeletal muscle may be the relative distribution of blood flow between routes of optimal nutrient transfer (termed “nutritive” flow routes) and suboptimal nutrient transfer (termed “non-nutritive” routes)

[2] Durham WJ[1], Casperson SL[1], Dillon EL, Keske MA, Paddon-Jones D, Sanford AP, Hickner RC, Grady JJ, Sheffield-Moore M. Age-related anabolic resistance after endurance-type exercise in healthy humans. *FASEB J.* 2010 Oct; 24(10):4117-27.

[134, 135]. Thus, reduced nutritive flow is one potential contributor to the blunted anabolic responsiveness of older individuals to anabolic stimuli. However, there is evidence that other factors are likely involved as well. Previous studies suggest that the capacity for translation by ribosomes and their associated factors is reduced by aging [136, 137]. In addition, aging has been reported to decrease skeletal muscle capillarization and increase capillary basement membrane width [138], with unknown effects on transcapillary efflux of amino acids into the interstitial fluid.

To our knowledge, no previous studies have determined whether the generalized phenomenon of age-related amino acid resistance includes the period following endurance-type ("aerobic") exercise. Although such exercise stimulates skeletal muscle protein synthesis both acutely [139, 140] and chronically [141, 142] in the fasted state, it is not associated with hypertrophy [143], in contrast to resistance type exercise. Divergent responses to resistance and endurance exercise may result from differences in energetic signaling responses [144-146], including intensity-dependent adaptive responses such as mitochondrial biogenesis [147, 148]. Such responses may interfere with stimulation of muscle protein synthesis by exogenous amino acids and/or increase muscle catabolism during post-absorptive conditions [149]. Consistent with this notion, acute moderate-intensity (75% $VO_{2\ peak}$) endurance-type cycling exercise [150] blunted the anabolic response to constant amino acid administration observed in healthy young subjects at rest [151, 152]. In contrast, repetitive knee extension exercise (67% maximal work rate) for one hour did not interfere with the anabolic response to chronic amino acid administration [153], suggesting anabolic resistance induced by endurance exercise is specific to the type of exercise performed. However, whether lower-intensity endurance exercise, which provides less energetic stress and less stimulation of mitochondrial adaptive responses, blunts amino acid-induced anabolic responses is unknown. Further, whether such blunting occurs in older subjects in response to walking, the mode of endurance exercise most commonly engaged in by older individuals has not been studied.

Accordingly, in the current study we examined the response of muscle protein metabolism to endurance exercise in younger and older subjects during provision of amino acids. We also measured amino acid concentrations in the blood and interstitial fluid, determined phenylalanine transport rates in skeletal muscle, and utilized both contrast-enhanced ultrasound (CEU) and the microdialysis ethanol technique, two independent methods for assessing skeletal muscle nutritive flow [154-162]. We studied younger and older subjects who were free of disease and not taking medications in an attempt to study the effects of aging *per se* and reduce the potential independent effects of disease and pharmacological interventions on metabolic responses, as well as to complement investigations of older individuals who were less healthy or already exhibited markedly reduced muscle mass. We hypothesized that older subjects, as compared with the young, would be resistant to the anabolic effects of amino acids and that this resistance would be associated with reductions in post-exercise nutritive blood flow, interstitial amino acid concentrations, and amino acid transport from the blood into skeletal muscle. Although we did find evidence for an age-related deficit in nutritive blood flow, our data comprehensively refute our hypothesis that impaired nutritive flow limits amino acid availability in healthy older individuals, as circulating and interstitial amino acid concentrations and phenylalanine transport into skeletal muscle were all equal or higher in older individuals than in the young. Instead, our data direct attention to metabolic and oxidative stress, altered skeletal muscle membrane integrity, and hemodynamic mechanisms apart from amino acid availability as potential mediators of age-related anabolic resistance following an acute bout of endurance-type exercise.

Experimental Procedures

Subjects:

Seventeen healthy untrained men (eight older [O, 67 ± 1.6 (SE) yr] and nine younger [Y, 29.8 ± 1.7 (SE) yr]), were studied before and after a single bout of low- to moderate-intensity

endurance exercise (walking) during continuous infusion of amino acids (AA). Informed written consent, which was approved by the Institutional Review Board of the University of Texas Medical Branch, was obtained from all volunteers prior to any study-related procedures. Volunteers were screened at the University of Texas Medical Branch (UTMB) General Clinical Research Center (GCRC) to determine study eligibility. Exclusion criteria included the following: cardiac, liver, kidney, pulmonary, autoimmune or vascular disease; hypo- or hypercoagulation disorders, diabetes, cancer, obesity, anemia, infectious diseases or an allergy to iodides. Subjects taking antihypertensive or lipid-lowering medications, anabolic steroids or corticosteroids in the past 6 months were excluded, as were subjects unable to discontinue anti-inflammatory or prophylactic aspirin therapy or nutritional supplement use (for 14 days) or those engaged in regular aerobic or resistance exercise training. All older subjects had their ankle-brachial index (ABI) determined to screen for peripheral arterial disease of the legs. Subjects were instructed to continue all regular activities of daily living and maintain their usual diet during the week preceding the study.

Pre-testing:

Subjects were admitted as outpatients to the GCRC two weeks prior to conducting the metabolism study. Total body fat, leg lean and leg fat mass were determined by Dual Energy X-Ray Absorptometry (DEXA, Hologic, Inc., Natick, MA). Following the DEXA, subjects were escorted to the UTMB Heart station for determination of VO_{2peak}. VO_{2peak} was measured on a treadmill using expired gas analysis (SensorMedics, Yorba Linda, CA) during a medically supervised progressive walk/run exercise test, as previously described [163].

Experimental Protocol:

The experimental protocol is outlined in figure 4. Subjects reported to the GCRC at noon the day before the study. Subjects were fed a standardized, meat-containing mixed meal the

evening before the study and fasted from 10 PM until amino acids were initiated in the immediate preexercise (rest) period the following day. The morning of the study, polyethylene catheters were inserted into the antecubital vein of both arms for infusion of stable isotopes, amino acids, Definity microbubbles (for contrast ultrasound; Bristol-Myers Squibb, New York, NY, USA), and arterialized venous blood sampling. Polyethylene catheters were also inserted into the femoral artery and vein of one leg for arterial and venous blood sampling. Indocyanine green (ICG; Akorn, Buffalo Grove, IL) was infused into the femoral artery for measurement of leg plasma flow, as described previously [164, 165]. Baseline blood samples were drawn approximately 150 minutes prior to exercise (time = -195 minutes in Figure 4) for the analysis of background isotopic enrichment and ICG concentration. Thereafter, blood samples were taken at $t = -120$, -105, -90, and -75 min for the analysis of isotopic enrichment, ICG concentration, insulin concentration, and glucose concentration. In addition, the $t = -75$-min sample was also analyzed for amino acid concentrations (see below). Following exercise, blood samples were obtained at 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, and 180 min for measurements of isotopic enrichment, ICG concentration, insulin concentration, and glucose concentration. In addition, the 60, 120, and 180 min postexercise samples were analyzed for amino acid concentrations, as described below. A primed (2 $\mu mol \cdot kg^{-1}$) continuous infusion of l-[ring-$^{13}C_6$] phenylalanine (Phe) (0.08 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) was started (~time=−300) and continued uninterrupted until study conclusion (Figure 4). In the final 30 min of the rest period, an infusion of amino acids (Premasol 10%; Baxter, Deerfield, IL) was started (prime=0.45 $ml \cdot kg^{-1}$; IR=1.35 $ml \cdot kg^{-1} \cdot h^{-1}$) and continued throughout the remainder of the study. After a 30-min stabilization period, subjects performed treadmill exercise (walking) for 45 min at ~ 40% Vo_{2peak}. Following exercise, subjects were immediately returned to bed for the 180-min postexercise period.

Muscle biopsies (~100–200 mg) were taken 150 and 30 min before exercise (t = −195 and −75 min in Figure 4) and at 10 and 180 min postexercise from the vastus lateralis muscle, ~20 cm above the knee, as described previously [166, 167]. All tissue was snap-frozen in liquid nitrogen and kept frozen at−80°C for later analysis. At the e nd of the study, all infusions were stopped, catheters were removed, and the subjects were fed, monitored for 2 h, and discharged home with follow-up care instructions.

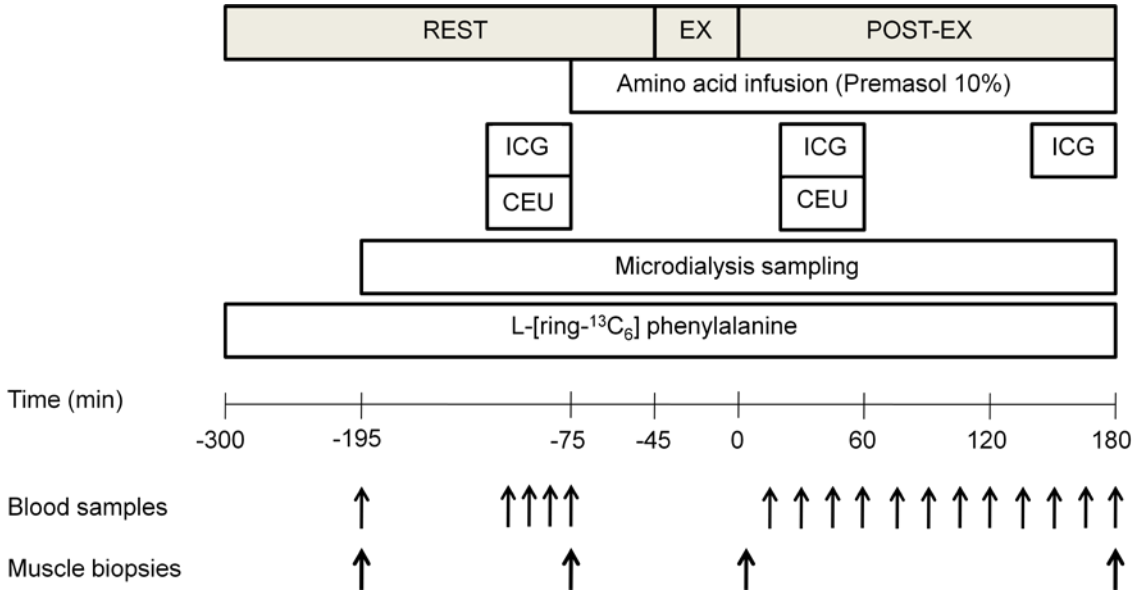


Figure 3: Study timeline. Abbreviations: ICG - indocyanine green sampling, EX – exercise at ~40% VO2peak, POST-EX - post-exercise, CEU - contrast-enhanced ultrasound.

Leg blood flow:

Leg plasma flow was determined utilizing the ICG dye-dilution technique and converted to leg blood flow using hematocrit as previously described [168, 169].

Contrast-enhanced ultrasound:

Imaging of the vastus lateralis muscle was performed in a transaxial plane ~15–20 cm above the patella over the midportion of the muscle using a P4–2 phased array transducer interfaced with the HDI-5000 ultrasound system (Philips ATL Ultrasound, Andover, MA, USA). Power Doppler imaging was performed as described by others [170-172]. In summary, an intravenous infusion (3.5 ml·min^{-1} for 8 min) of a suspension of perflutren microbubbles (Definity) was performed at $\sim t = -85$ min at rest and again at ~50 min postexercise. Contrast-enhanced ultrasound was only performed 2 times because the FDA limits the amount of Definity that can be infused per day. A mechanical index of 1.3 was used and a compression of 80%. Once the systemic microbubble concentrations reached steady-state (~2 min), background images were obtained at a frame rate of 1 s^{-1}. Intermittent imaging was then performed using an internal timer at pulsing intervals (PI) ranging from 1 to 25 s, thus allowing progressively greater replenishment of the ultrasound beam elevation between destructive pulses. Depth, focus, and gain were optimized at the beginning of each study and held constant throughout. Data were recorded on an SVHS tape and digitized for analysis using an offline system. A minimum of 3 images were acquired at each PI. The background-subtracted video intensity (VI) at each PI was measured from a region of interest (ROI) within the vastus lateralis muscle. PI *vs.* VI data were curve fitted to the function:

$$y = A\,(1-e^{-\beta t})$$

where y is the video intensity at PI t, A is the plateau video intensity (an index of microvascular blood volume, MBV), and β is the rate of microvascular re-filling (an indicator of microvascular flow velocity, MFV) [173]. The product (MBV x MFV) is a measure of microvascular blood flow (MBF).

Microdialysis:

Three CMA 60 microdialysis probes (30 mm, 20-kDa cutoff; CMA Microdialysis, Solna, Sweden) were inserted percutaneously into the vastus lateralis muscle of one leg with an 18-gauge needle following 1% lidocaine administration ~20 cm above the patella. Microdialysis probes were perfused at a rate of 5.0 μl · min^{-1} using a CMA 102 microinfusion pump (CMA Microdialysis) with a solution consisting of Na^+ (147 mEq), K^+ (4 mEq), Ca^{2+} (2.3 mM), Cl^- (156 mM), and 40 g · L^{-1} Dextran 70. EtOH (5 mM) was included in the microdialysis perfusion medium to monitor skeletal muscle nutritive blood flow in the area of the microdialysis probe.

Microdialysis probe recovery for phenylalanine and leucine was determined by the internal reference technique [174] by adding 0.108 μCi·ml^{-1} of d-[^{3}H]phenylalanine and 0.108 μCi·ml^{-1} of d-[^{14}C]leucine (Amersham Pharmacia Biotech, Piscataway, NJ, USA) to the perfusate. Probe recovery was determined by measuring disintegrations per minute (DPM) of ^{14}C Leu and ^{3}H Phe in the perfusate and dialysate, placing 10 μl in 15 ml of scintillation fluid and counting for 10 min on a LS 6500 multipurpose scintillation counter (Beckman Coulter, Fullerton, CA, USA). *In vivo* recovery was calculated using the following formula:

$$(\text{Perfusate}_{\text{dpm}} - \text{Dialysate}_{\text{dpm}})/\text{Perfusate}_{\text{dpm}}$$

As described previously [175], recoveries for phenylalanine and leucine were used for lysine and alanine, respectively. For all other interstitial amino acids, the average of the phenylalanine and leucine recoveries was applied to estimate interstitial concentrations and allow relative comparisons between young and old.

Once inserted, probes were perfused for 45 min to reach equilibrium, after which dialysate samples were collected in 30-min aliquots during the preexercise and postexercise time periods in sealed microvials (Milian, Geneva, Switzerland) that were weighed before and after dialysate collection to determine dialysate volume. The microvials for each 30-min collection

were immediately stored at 4°C until an aliquot was removed later in the day for ethanol analysis, with the remaining volume stored at −80°C.

Ethanol concentration in each perfusate and dialysate sample was measured according to the method described by Hickner *et al.* [176, 177]. Briefly, 150 μl of reagent mixture consisting of glycine-hydrazine buffer at pH 8.9 (74 μM $Na_4P_2O_7$, 22 μM glycine, and 60 μmM hydrazine) and 0.54 μM NAD^+ was added to a 96-well plate. Then, 2 μl of sample was added, followed by 20 μl of enzyme (1.7 mg alcohol dehydrogenase in 1 ml ddH_2O). Ethanol concentrations were measured in the perfusate and dialysate solutions by fluorometric-enzymatic assay (Fluoraskan II; MTX Labs Systems, Inc., Vienna, VA, USA), with the results expressed as the ethanol outflow/inflow concentration ratio:

$$[\text{ethanol}]_{\text{collected dialysate}} / [\text{ethanol}]_{\text{infused perfusion medium}} = C_{out}/C_{in}$$

where C_{out} is the concentration of the dialysate and C_{in} is the concentration of the perfusate. Sample determinations from each time period (rest, 0–60 min postexercise, 61–120 min postexercise, 121–180 min postexercise) were averaged. The ethanol outflow/inflow (O/I) ratio is inversely related to local nutritive blood flow [178].

Analytical methods:

Phenylalanine enrichments and concentrations in arterial and venous blood samples were determined after the addition of an internal standard, deproteinization with sulfosalicylic acid, extraction using cation exchange chromatography, and *tert*-butyldimethylsilyl (*t*-BDMS) derivatization using gas-chromatography mass-spectrometry (GCMS) in electron impact mode (GC HP 5890, MSD HP 5989; Hewlett Packard, Palo Alto, CA, USA) as previously described [17].

Muscle samples were weighed, and the proteins were precipitated with 800 μl of 10% perchloric acid. Tissue homogenization and centrifugation were performed twice, and the supernatant was collected. The remaining pellet was then washed 3 times with 2% perchloric

acid, once with 200 proof ethanol and ethyl ether, with centrifugation following each wash. The pellet was then dried overnight at 50°C. The following day, 3 ml of 6 N hydrochloric acid was added and then placed on a heating block at 110°C for 24 h to hydrolyze the proteins in the pellet. The enrichment of free tissue phenylalanine was determined on its *t*-BDMS derivative using GCMS [179]. The enrichment of bound tissue phenylalanine was determined on its *t*-BDMS derivative using GCMS monitoring the ions 237 and 239 and using the standard curve approach [180]

Plasma and microdialysate amino acid concentrations were determined using a Hitachi L8800 amino acid analyzer (Hitachi, Tokyo, Japan). Plasma was precipitated to remove proteins by mixing equal volumes of 7.5% SSA in 0.02 N HCl containing internal standard with plasma sample. Samples were centrifuged for 15 min at 10,000 *g*. Supernatants were filtered through a 0.22-μm filter and injected on the Hitachi L8800 for analysis using standard physiological analysis methods, buffers, and reagents as provided by the manufacturer.

For analysis of AMPK and mTOR phosphorylation by immunoblotting, ~30 mg of muscle biopsy tissue was homogenized in tissue lysis buffer [10 mM HEPES-KOH, pH 7.9; 1.5 mM $MgCl_26H_2O$; 10 mM KCl; 2 mM phenylmethylsulfonyl fluoride; 1 mM dithiothreitol; and 1 mM protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) at 1:18 w/v] followed by incubation on ice for 10 min and vortexing, and centrifugation at 16,110 *g* for 30 s. The supernatant was collected, and protein concentrations were determined *via* Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). A total of 80 μg of protein, diluted in sample buffer (NuPAGE LDS Sample Buffer; Invitrogen, Carlsbad, CA, USA) and boiled for 3 min., was loaded per lane and separated by SDS-PAGE (NuPAGE Novex 3–8% tris-acetate gels for mTOR and NuPAGE Novex Bis-Tris Gels for AMPK; Invitrogen) at a constant 150 V for 60 min. A molecular weight ladder and rodent normalization standard were also loaded on each gel. Following SDS-PAGE, proteins were transferred (100 V for 60 min) to polyvinylidene difluoride (PVDF) membranes (Hybond-P; Amersham Biosciences, Piscataway, NJ, USA) and placed in

blocking buffer for 1 h. Blots were serially washed and incubated with primary antibody overnight at 4°C with constant agitation. Primary antibodies were purchased from Cell Signaling (Beverly, MA, USA): phospho-mTOR (Ser-2448; 1:1000), phospho-AMPKα (Thr^{172}; 1:1000), total mTOR (1:1000), and total AMPKα (1:1000). The next day, the blots were rinsed and incubated with secondary antibody for 1 h at room temperature with constant agitation. Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was purchased from Amersham Biosciences (1:5000). Blots were serially washed and incubated for 5 min with enhanced chemiluminescence reagent (ECL Advanced Western blotting Detection System; Amersham Biosciences). Optical density measurements were obtained with a ChemiDoc XRS imaging system (Bio-Rad). Densitometric analysis was performed by using Quantity One 4.5.2 1-dimensional analysis software (Bio-Rad). Data are expressed as the signal from the phosphorylated kinase divided by the total protein signal for the kinase, normalized to a rodent standard that was run on each gel, in arbitrary units.

Calculations:

Skeletal muscle phenylalanine transmembrane transport and intracellular kinetics were studied using established techniques as previously described [181, 182].

To assess the efficiency of amino acid handling, we calculated the following additional characteristics: the proportion of leg delivery that was transported into the muscle (inward transport efficiency), the proportion of the intracellular rate of appearance (R_a) that was transported out of the muscle to the vein (outward transport efficiency), the proportion of intracellular R_a used for muscle protein synthesis (synthetic efficiency), the ratio of phenylalanine utilization for muscle protein synthesis to the arterial phenylalanine concentration (synthetic clearance), and the ratio of phenylalanine NB to the arterial phenylalanine concentration (anabolic clearance):

$$\textbf{Inward transport efficiency, (\%)} = 100 \times F_{M,A}/F_{in}$$

$$\textbf{Outward transport efficiency, (\%)} = 100 \times F_{V,M}/(F_{M,A} + F_{M,O})$$

$$\textbf{Synthetic efficiency, (\%)} = 100 \times F_{O,M}/(F_{M,A} + F_{M,O})$$

$$\textbf{Synthetic clearance, (mL/min)} = F_{O,M}/C_A$$

$$\textbf{Anabolic clearance, (mL/min)} = NB/C_A$$

Mixed muscle fractional synthesis rate (FSR; %/h) was calculated from the incorporation of Phe into protein, using the precursor-product model:

$$\textbf{FSR} = [(E_{P2} - E_{P1}) / (E_M \times t)] \times 60 \times 100$$

where E_{P1} and E_{P2} are the enrichments of bound Phe in the first and second muscle biopsies of a time period, t is the time interval between biopsies (min), and E_M is the mean Phe enrichment in the muscle intracellular pool [183].

Statistical Analysis:

The outcome variables were all continuous and approximately normally distributed. A repeated-measures general linear mixed model (GLMM) was used to analyze the treatment effects over time. Initial assessments for time × group interactions suggested no evidence of interaction in the model, and this term was dropped for all subsequent models. The models were fit in the MIXED procedure in SAS® (SAS Institute INC. 2004. *SAS/STAT® 9.1 User's Guide.* Cary, NC: SAS Institute Inc.). To obtain meaningful (positive) values for 3-pool model fluxes calculated using both muscle and blood enrichments (*e.g.*, $F_{M,A}$), the muscle intracellular enrichment must be lower than that of the blood. In one younger and one older subject, this condition was not met during the postexercise period, and, as a result, only F_{in}, F_{out}, and NB were included in analyses for these subjects. In addition, in another older subject, the NB 60-min postexercise was negative (and >7sd away from the mean) despite receiving an amino acid

infusion; model fluxes from this subject at this time point were excluded from statistical analysis. We used a 2-sided α level of significance of 0.05 to assess statistical significance. Correlation coefficients are presented as Supplemental Data.

Results

Subject characteristics:

Subject characteristics are presented in Table 1. Except for age and aerobic capacity ($VO2_{peak}$), subjects were well-matched, with similar heights, weights, BMI values, leg volumes, leg lean and fat mass, and body composition.

	Younger Men (n=9)	**Older Men (n=8)**
Age, yr	30 ± 2	67 ± 2
Height, cm	170 ± 1	176 ± 3
Weight, kg	78 ± 2	84 ± 4
BMI, kg/m^2	27 ± 1	27 ± 1
Body fat, %	22 ± 1	24 ± 1
Total Fat Free Mass, kg	55 ± 2	59 ± 2
Total leg lean mass (R), kg	9.6 ± 0.3	9.9 ± 0.4
Total leg fat mass (R), kg	3.0 ± 0.3	3.2 ± 0.2
Total leg volume (R), L	8.8 ± 4	8.5 ± 3
Total leg lean mass (L), kg	9.6 ± 0.3	9.7 ± 0.5
Total leg fat mass (L), kg	3.1 ± 0.3	3.2 ± 0.2
Total leg volume (L), L	8.6 ± 4	8.4 ± 3
$VO_{2peak,}$ $ml \cdot kg^{-1} \cdot min^{-1}$	52 ± 4 #	41 ± 2 #

Table 1: Characteristics of subjects. Values are means ± SE. #Significant difference between groups, P≤0.05.

Leg blood flow, microvascular blood flow, and interstitial ethanol exchange:

Total leg blood flow did not differ between young and old at any time (Figure 4A). MBF (MBV×MFV) was significantly elevated relative to rest period at 60 min postexercise in both young and old (Figure 4B; P=0.006 for time main effect). In addition, MBF was significantly higher in the young than in the older group (P=0.03). At 60 min postexercise, skeletal muscle microvascular blood volume was higher than at rest in both young and old (Figure 4C; P=0.0006 for time main effect). MFV did not differ between young and old (Figure 4D), and there was no change from baseline at 60 min postexercise. There was a marginal (P=0.06) effect of age on interstitial fluid ethanol exchange, whereas the effect of time on this variable was highly (P=0.0006) significant (Figure 4E), with means of 0.47 ± 0.02, 0.40 ± 0.02, 0.42 ± 0.02, and 0.42

± 0.02 for rest and 0–60, 61–120, and 121–180 min postexercise, respectively, in the repeated mixed model.

Amino acid concentrations:

Because older individuals have elevated first-pass splanchnic extraction of oral amino acids [60], we provided amino acids via infusion in an attempt to normalize delivery of exogenous amino acids to the circulation. Arterial concentrations of several amino acids, including leucine, were marginally higher (P=0.04, 0.08, 0.08, for leucine, phenylalanine, and essential amino acids, respectively) in the older than in the younger group (Figure 5, Appendix E). However, this was not due to an elevated overall (i.e., endogenous+exogenous) rate of appearance of amino acids in the older subjects, based on the fact that whole-body phenylalanine R_a was fairly well matched in the two groups (Figure 5G), with values in the younger slightly (but significantly; P=0.007) higher than in the older. As expected, total whole-body phenylalanine R_a increased in response to the amino acid infusion (Figure 5G, P<0.0001 for time main effect). Endogenous phenylalanine R_a was reduced in both groups in response to the amino acid infusion and exercise and was significantly lower in the older group (Figure 5H; P=0.007 and P=0.02 for age and time effects, respectively).

Interstitial concentrations of many amino acids were significantly correlated with plasma concentrations (Appendix G). In particular, plasma-interstitial fluid correlations for the branched chain amino acids and the sum of the essential amino acids were significant (P<0.0001) and moderately high (R^2=0.65–0.76). As in the plasma, several amino acids were present at higher concentrations in the interstitium of the older individuals than in the young (Figure 5D, Appendix F). Relative interstitial ammonia and urea levels were also significantly higher in the older group (Appendix F). We were not able to determine interstitial levels of all species measured in plasma. In particular, the summed interstitial summed essential amino acids do not include histidine, as we were not able to measure interstitial histidine levels in all subjects.

Phenylalanine kinetics:

Phenylalanine concentration and enrichment data are presented in Appendix H and demonstrate that subjects were in isotopic steady state during the postexercise period. Consistent with previous studies [11, 184], basal muscle protein metabolism was similar in the younger and older groups (Appendix I). Significant time effects were found for arterial inflow (F_{in}, P<0.0001,), venous outflow (F_{out}, P<0.0001), inward transport into muscle ($F_{M,A}$, P<0.0001), arteriovenous shunting ($F_{V,A}$, P<0.0001), outward transport from muscle ($F_{V,M}$, P=0.003), muscle protein breakdown ($F_{M,O}$, P=0.006), net balance (NB, P<0.0001), and muscle protein synthesis ($F_{O,M}$, P=0.02) (Appendix I). None of these variables were found to differ significantly between the young and older subjects, although there were marginal group effects for inward transport (P=0.09) and outward transport (P=0.07).

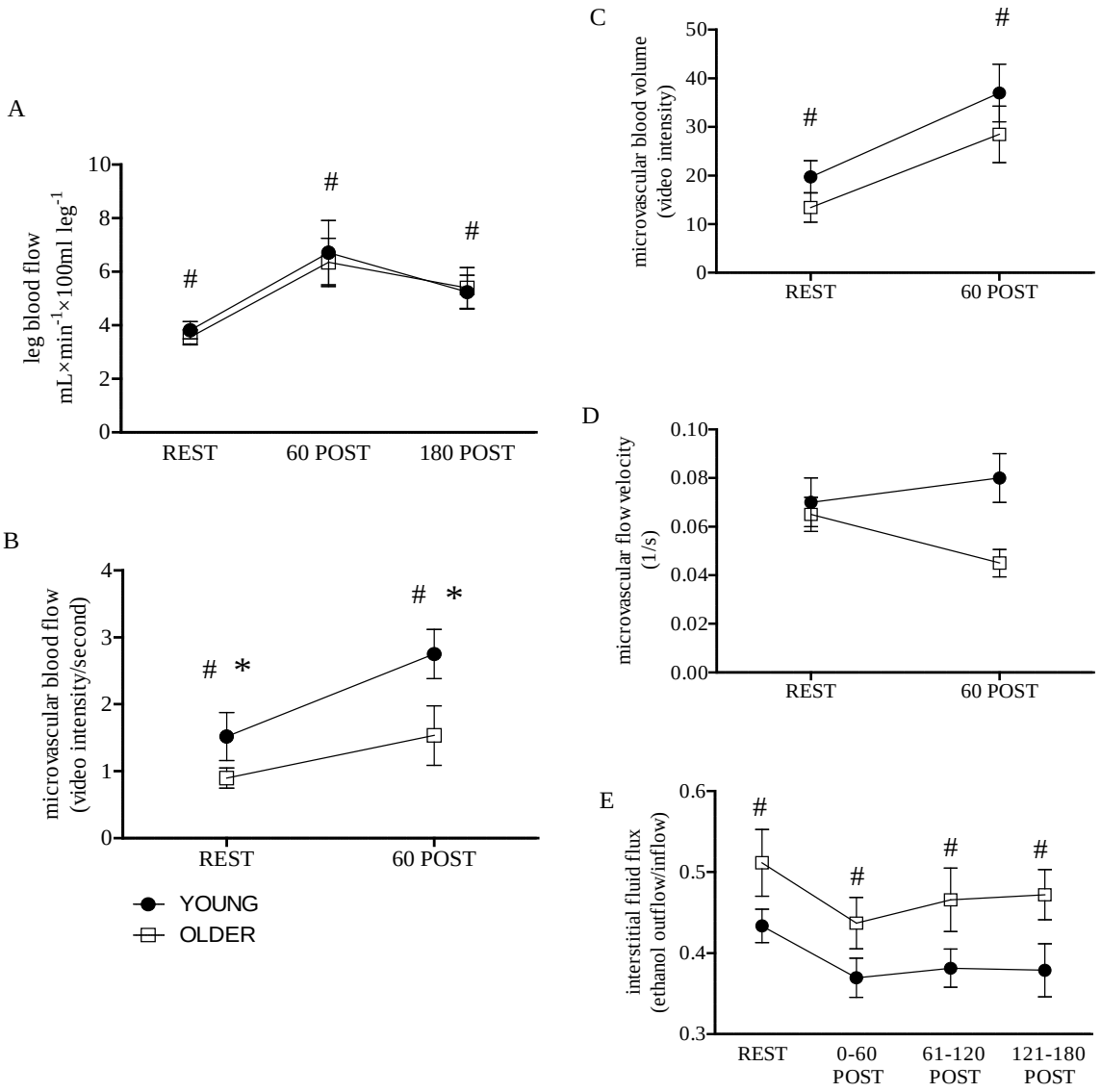


Figure 4: Total leg blood flow and skeletal muscle nutritive flow. A) Leg blood flow did not differ between young and older groups. $^{\#}P < 0.0001$ for time effect. B) Microvascular blood flow was different between young and older groups and between rest and 60-post groups. $*P = 0.03$ for young vs. older groups; $^{\#}P = 0.006$ for rest vs. 60-post groups. C) Microvascular blood volume increased postexercise but did not differ between young and older individuals. $^{\#}P = 0.0006$. D) Microvascular flow velocity did not differ between age groups or times. E) Interstitial ethanol exchange was marginally different between age groups (P=0.06), with a significant effect of time. $^{\#}P = 0.0006$ for time effect.

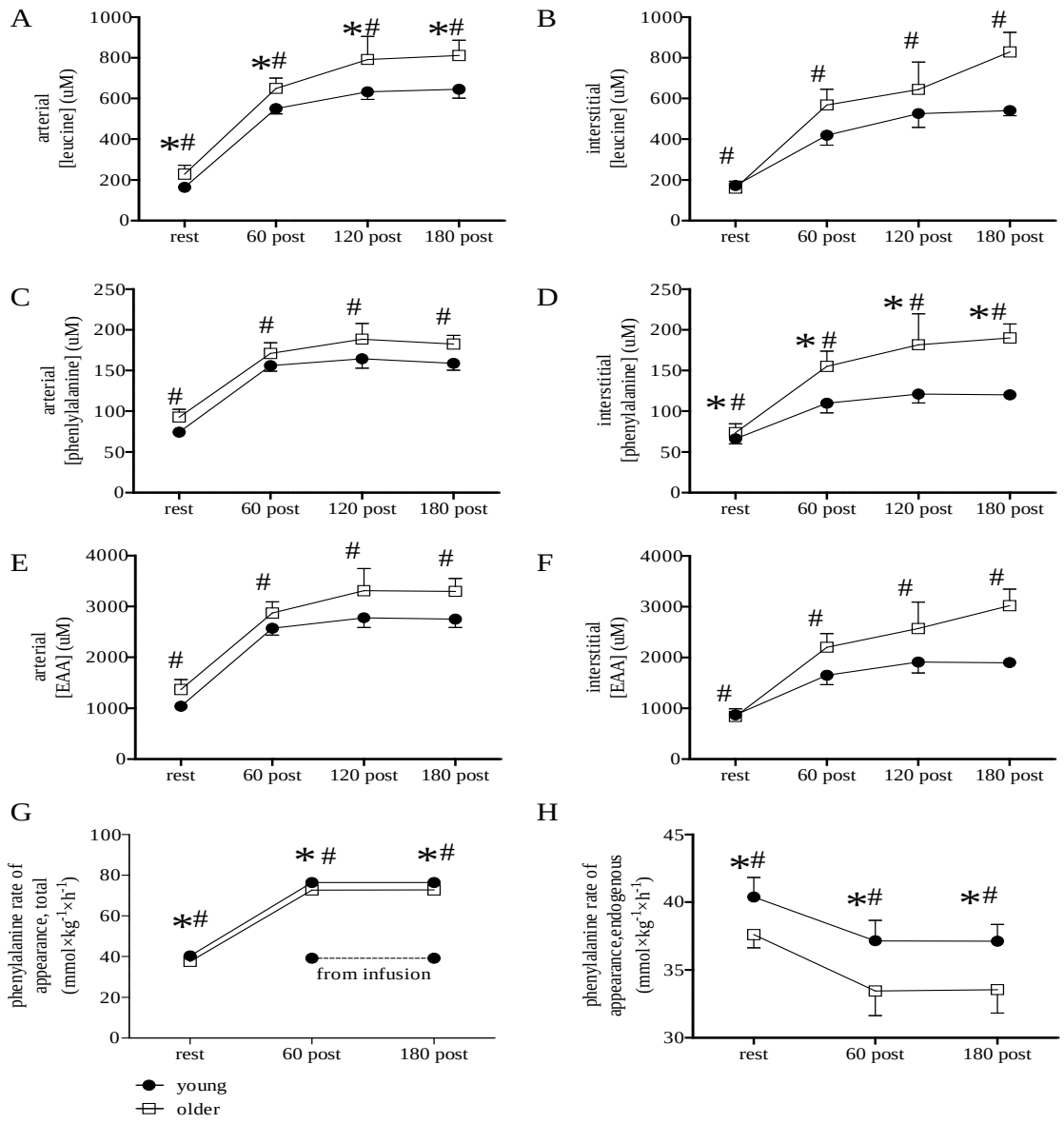


Figure 5: Amino acid availability. A–F) Both arterial (A, C, E) and interstitial (B, D, F) concentrations of leucine (A, B), phenylalanine (C, D), and the sum of the essential amino acids (EAA; E, F) increased with time; $^{\#}P < 0.0001$. Arterial leucine concentrations (A) and interstitial phenylalanine concentrations were significantly higher in older subjects; *P = 0.04. G, H) Total (endogenous+exogenous; G) and endogenous (H) rates of phenylalanine appearance in the circulation were lower in the older individuals and exhibited a significant effect of time. *P = 0.007 vs. younger group; $^{\#}P<0.0001$ (G), $^{\#}P = 0.02$ (H) for time effect. See also Results and Appendix E and Appendix F for other amino acids.

Fractional Synthesis Rate (FSR):

Mixed muscle protein FSR was significantly higher postexercise than during rest (P<0.001 for time main effect), without age-related differences (0.061±0.005%·h^{-1} in the young subjects at rest and 0.070±0.006%·h^{-1} in the older subjects at rest; 0.098±0.009%·h^{-1} in the young subjects postexercise and 0.111±0.011%·h^{-1} in the older subjects postexercise).

Serum Insulin Concentrations:

Insulin concentrations responded differently to the combined intervention of amino acid infusion and exercise in the younger and older subjects (group× time interaction P = 0.01), with older subjects exhibiting higher insulin concentrations at 60 min postexercise than the younger subjects (3.0±0.8, 3.2±1.1, and 3.6±1.1 pM at rest and at 60 and 180 min postexercise, respectively, in the young; 3.3±0.4, 7.3±1.2, 5.2±0.8 pM at rest and at 60 and 180 min postexercise, respectively, in the older subjects).

Sensitivity of muscle protein synthesis to amino acids:

Because recent evidence suggests that older individuals exhibit resistance to anabolic stimuli, in particular, amino acids, we performed a number of calculations assessing amino acid handling and anabolic sensitivity in the young and older groups. Fractional outward transport and synthetic efficiency, two reciprocally related factors, increased and decreased, respectively, with time after exercise and were significantly different between young and old, with older subjects exhibiting greater fractional outward transport and lower synthetic efficiency (Figure 6). We also calculated synthetic efficiency by dividing the FSR by the mean postexercise rate of phenylalanine appearance and obtained results similar to those obtained using the 3-pool model (Appendix A).

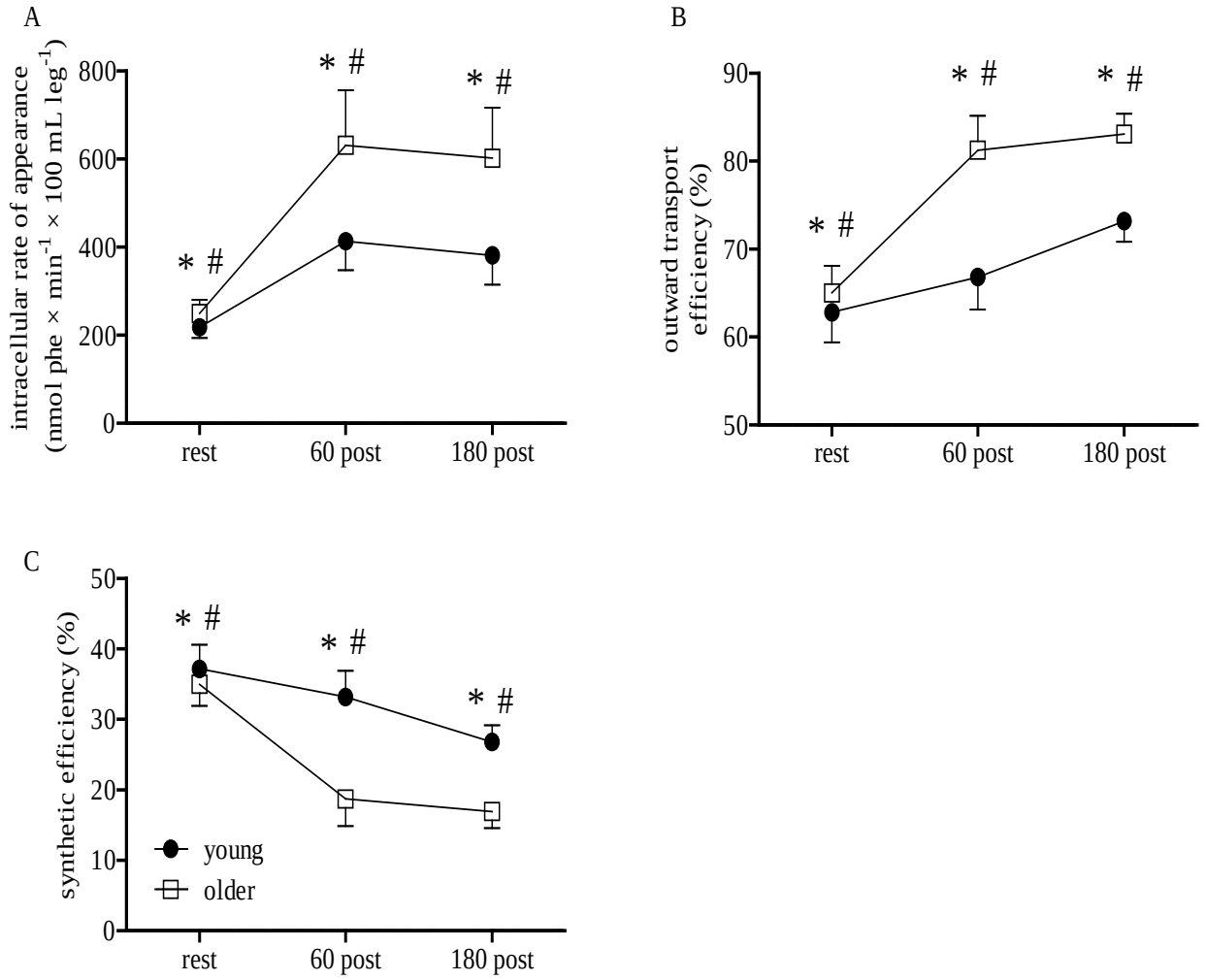


Figure 6: Skeletal muscle phenylalanine trafficking. A) Intracellular rate of appearance was significantly different between young and older subjects, with a significant effect of time. *P = 0.01 vs. younger group; #P = 0.0005 for time effect. B) Outward transport efficiency was different between young and older subjects, with a significant effect of time. *P = 0.05 vs. younger group; #P = 0.0001 for time effect. C) Synthetic efficiency was higher in young than in older subjects, with a significant effect of time. *P = 0.05 vs. younger group; #P = 0.0001 for time effect.

Relationships between amino acid concentrations and muscle anabolism:

Correlations between circulating and interstitial amino acid concentrations and protein synthesis, protein breakdown, and the net balance between synthesis and breakdown, using all time points (i.e. rest and 60 and 180 min postexercise) are presented in Appendix J and Appendix K. For the infused amino acids, the correlations between the interstitial or plasma concentrations and protein synthesis generally suggested a weak to moderate relationship, whereas the correlations with protein breakdown suggested at most a weak relationship (Appendix B Appendix J and Appendix K). In contrast, correlations between net balance and the interstitial or plasma concentrations of the infused amino acids suggested a moderate to strong association (Appendix B Appendix J and Appendix K).

Energetic and anabolic signaling response (AMPK and mTOR activation):

To address the possibility that the exercise bout represented a greater metabolic stress in the skeletal muscle of the older subjects and thereby interfered with anabolic signaling responses, we assessed skeletal muscle AMPKα phosphorylation (Thr^{172}) and mTOR (Ser^{2448}) phosphorylation (Appendix C). AMPKα phosphorylation was on average higher in the older subjects than in the young at each time point, but this difference did not reach statistical significance (P=0.12). Likewise, there were no group differences in mTOR phosphorylation; however, there was a significant effect of time (P=0.01).

Leg glucose uptake:

Leg glucose uptake in the younger group was 70.9 ± 7.3, 97.9 ± 29.2, and 73.1 ± 9.5 $\mu mol \cdot min^{-1} \cdot leg^{-1}$ at rest and at 60 and 180 min postexercise, respectively. In the older group, the leg glucose uptake was 60.4 ± 2.7, 88.2 ± 14.2, and 68.1 ± 5.6 $\mu mol \cdot min^{-1} \cdot leg^{-1}$ at rest and at 60 and 180 min postexercise, respectively. Leg glucose uptake did not differ significantly in young and older subjects and did not exhibit a significant effect of time.

Discussion

Loss of skeletal muscle mass is a common consequence of aging. Although age-related comorbidities can contribute to this loss, studies in healthy older individuals suggest that aging *per se* contributes to development of age-related resistance to anabolic stimuli [185-189]. Here, we expand on this body of literature by reporting for the first time that aging induces anabolic resistance to amino acids following low-intensity endurance exercise. As expected in a group of older subjects who are healthy and nonsarcopenic, the amino acid resistance observed in this study did not manifest as marked changes in the overall balance between muscle protein synthesis and breakdown. Rather, it was a subclinical entity, evident in a reduced sensitivity of muscle protein synthesis to stimulation by amino acids.

Previous studies have determined that the stimulation of muscle protein synthesis in response to protein or amino acid administration occurs *via* an unidentified sensing mechanism responsive to elevated extracellular concentrations and/or increased intracellular rates of appearance of essential amino acids [190-196]. In the current study, we compared anabolic sensitivity in the younger and older groups by expressing anabolic responses relative to the intracellular rate of appearance of phenylalanine and the arterial phenylalanine concentration (Figure 6, Appendix A and Appendix D). These calculations uniformly demonstrated marked amino acid resistance in skeletal muscle of the older subjects. These findings are consistent with previous studies in which the response of skeletal muscle protein synthesis to amino acids was found to be blunted in the old under nonexercised conditions [197] and demonstrate that age-related anabolic resistance extends to the period following an acute bout of endurance-type exercise.

In this study, we hypothesized that reduced "nutritive" blood flow in older *vs.* younger subjects would be associated with reduced interstitial amino acid concentrations and inward phenylalanine transport, which would thereby limit muscle protein synthesis by limiting essential amino acid availability. In theory, impaired endothelial function could interfere with the response

of skeletal muscle to elevated plasma amino acids by reducing capillary blood flow and thus limiting transcapillary amino acid exchange with the interstitial fluid. Our results comprehensively refute our hypothesis. Despite evidence of reduced nutritive blood flow by two independent measures, the older individuals had equal or greater amino acid availability than the young, based on circulating and interstitial amino acid concentrations and intracellular appearance rates of phenylalanine. Thus, age-related anabolic resistance to amino acids is apparently not mediated *via* reduced amino acid availability secondary to impaired hemodynamics. However, we cannot rule out a role for impaired microvascular flow in anabolic resistance, but by some mechanism other than amino acid availability (*e.g.,* reduced hormone/nutrient delivery [198] and/or reduced removal of cellular "waste" products such as ammonia (Supplemental Table 2)), as both capillary recruitment and microvascular blood flow were positively correlated with net balance.

The markedly higher interstitial ammonia levels (Appendix F) in skeletal muscle of the older subjects were unexpected. Skeletal muscle ammonia production can occur by several mechanisms involving deamination of AMP or amino acids [199-201], although the relative importance of these are still not certain, with the exception of very intense exercise, during which AMP deamination is generally agreed to be the most important pathway for skeletal muscle ammoniagenesis. Recent human and animal studies suggest that the susceptibility of older skeletal muscle to metabolic stress is increased, on the basis of activation of the energy sensor AMPK [202, 203]. Such findings are consistent with the notion that mitochondrial ATP-generating capacity is reduced with aging [204]. It is conceivable that this greater metabolic stress in older skeletal muscle results in significant ammonia production from AMP deamination. In the current study, skeletal muscle AMPK phosphorylation was, on average, higher in the older than in the younger subjects, but this difference did not reach statistical significance. The finding that arterial glutamine levels were significantly higher in the old than in the young, whereas interstitial concentrations were not different between the two groups, could also indicate that

activity of glutaminase, which catalyzes conversion of glutamine to ammonia and glutamate, is higher in skeletal muscles of older *vs.* younger individuals. However, the facts that glutamate, aspartate, and alanine were given in the amino acid infusion and no tracers were infused for the purpose of studying amino acid deamination prevent any strong conclusions regarding which, if any, amino acid deamination pathways were differentially stimulated in older and younger skeletal muscle.

Circulating levels of 3-methylhistidine, an accepted marker of myofibrillar protein breakdown [205], were significantly higher in the old than in the young (Appendix E). However, the fact that neither skeletal muscle protein breakdown nor whole body phenylalanine R_a were greater in the old subjects is inconsistent with greater release of 3-methylhistidine from protein breakdown of skeletal muscle or other tissues. An alternative explanation is that skeletal muscle sarcolemmal integrity is compromised with aging, possibly due to chronic suboptimal nutrition [206, 207], with the result that exchange between muscle fibers and the interstitial fluid is increased, as suggested previously [208]. The significantly greater fractional outward transport from skeletal muscle in the older subjects is consistent with such an interpretation, as our model of phenylalanine kinetics does not distinguish between transporter-mediated exchange and amino acid exchange occurring through a compromised sarcolemma. Notably, a metabolomic investigation found that 3-methylhistidine is a sensitive marker of oxidative stress [209], suggesting that age-related oxidative stress may have contributed to reduced sarcolemmal integrity in the older subjects. Likewise, cystine, the oxidized form of cysteine, was also higher in both interstitial fluid and plasma of the older subjects than in the young, confirming previous reports of higher circulating cystine levels in the elderly [210-213] and consistent with the development of redox stress with aging [214-216].

By design, we studied healthy older subjects who do not take prescription medications, are glucose tolerant, and do not participate in a regular exercise program. This allowed us to study the effects of aging *per se* in the absence of comorbidities or pharmacological effects.

However, our results, therefore, may underestimate the extent of impairment in anabolic responsiveness in older individuals who are not as healthy. Likewise, by selecting glucose-tolerant elderly subjects, we have minimized the likelihood of observing impairments in leg glucose uptake. Future studies will be important to determine the degree of impairment in less healthy populations.

Regardless of how it occurs, the existence of age-related anabolic resistance to amino acids, as found in this study, as well as in several previous ones, suggests two general, nonexclusive approaches for treating or preventing sarcopenia: increase ingestion of amino acids and/or protein by older individuals; and increase the anabolic sensitivity of older skeletal muscle to amino acids. The first approach may be viable in principle, as rates of skeletal muscle protein synthesis in older subjects have equaled those of younger subjects in some [217, 218] (as well as the present study) but not all [219] studies when large enough amounts of protein or amino acids were given. However, the diminished anabolic responsiveness of older individuals to amino acids, as well as the uncertain effects of long-term high protein/amino acid diets on deleterious signaling in tissues other than skeletal muscle [220, 221], implies that increasing anabolic sensitivity is the preferable approach. The present study suggests that interventions to reduce metabolic and redox stress and maintain sarcolemmal integrity are worthy of investigation in this regard.

CHAPTER 3

MINOR ROLE FOR SKELETAL MUSCLE IN AMINO ACID TRAFFICKING CHANGES DURING EXERCISE

Introduction

Public health organizations recommend that adults exercise approximately 2.5 hours each week for health and up to 4 hours or more for lasting weight loss [222], which equates to ~130-216 hours spent exercising each year. Because of the large total time investment, even incremental metabolic changes occurring during an exercise bout could have a large cumulative effect. In addition to the potential public health significance, the during-exercise response of skeletal muscle protein metabolism to endurance exercise is of fundamental scientific interest and has received little attention in humans [223]. Notably, it is important to determine how different and potentially opposing stimuli in the during-exercise period are integrated into a coordinated system-level metabolic response. For example, exercise-enhanced muscle perfusion will increase amino acid delivery to skeletal muscle, which might be expected to stimulate skeletal muscle protein synthesis [224-228], whereas energetic stress occurring during exercise might be expected to exert the opposite effect [11, 223, 229, 230].

The dearth of studies examining the during-exercise response of skeletal muscle protein synthesis likely reflects the methodological difficulties that make measurements during the performance of endurance exercise difficult to obtain or interpret [223]. Although arteriovenous (AV) balance measurements across a limb can be obtained during endurance exercise, and offer the advantage of good temporal resolution, they include all tissues within the limb (e.g. skin, bone, adipose tissue), making attribution of exercise-induced changes to skeletal muscle tenuous. In addition, changes in limb AV balance of an amino acid may reflect changes in the intracellular or interstitial pool size of the amino acid being monitored rather than a change in utilization for muscle protein synthesis. On the other hand, measurements of labeled amino acid incorporation

into skeletal muscle protein are unaffected by changes in pool size and are thus theoretically more robust; however, sufficient time for measurable tracer incorporation to occur is necessary, resulting in less temporal resolution than AV balance measurements. Perhaps for this reason, most studies of during-exercise responses of muscle protein metabolism have employed long-duration (2-4 hours) protocols [15, 22, 24]. Although valid for the specific type, intensity, and duration of exercise used in such studies, designs employing greater exercise duration may augment the influence of energetic stress relative to other factors such as amino acid availability and thus may not be relevant for individuals whose exercise regimens are briefer. Our group previously reported that during a single bout of shorter duration (45 minutes) low- to moderate-intensity exercise in the fasting state muscle protein synthesis was stimulated in younger and older men [17]. To my knowledge, this is the only study that has addressed the during-exercise response of muscle protein synthesis to endurance-type exercise lasting less than 2 hours.

As many individuals do not exercise in the fasting state, there is currently considerable curiosity among researchers as well as everyday exercisers about the potential for pre- or during-exercise amino acid or protein supplementation to influence anabolic responses [20-24]. Notable from both technical and practical perspectives, the reported lag phase between the initiation of amino acid administration and the response of skeletal muscle protein synthesis (using tracer incorporation techniques) is at least 30 minutes [53, 63, 231], suggesting that anabolic responses to exogenous amino acids are unlikely to be detected during shorter-duration exercise bouts, particularly if the amino acid provision is not initiated at least 30 minutes prior to exercise. Accordingly, the aims of the current study were to determine i) the during-exercise response of skeletal muscle protein metabolism to a bout of endurance-type exercise during concomitant amino acid administration initiated 30 minutes prior to exercise, ii) the extent to which skeletal muscle contributes to leg protein metabolism during perturbation with nutrition and exercise, and iii) whether this response differed in younger and older men, using a combination of AV balance and tracer incorporation techniques along with microdialysis sampling of skeletal muscle

interstitial fluid. The hypothesis was that the during-exercise response of skeletal muscle protein metabolism in older individuals would exhibit an age-related resistance to the anabolic effects of exogenous amino acids, similar to what occurs in the postexercise period following a bout of low-intensity endurance exercise [232]. Instead, the results suggest that healthy younger and older men respond in a generally similar manner, with markedly increased leg amino acid uptake and an augmentation of muscle protein synthesis, the latter in fact was marginally higher in the older group. Notably, unique findings were uncovered from the AV balance, microdialysis, and tracer incorporation data which, when viewed in total, provide a compelling deductive argument that endurance-type exercise evokes marked changes in amino acid handling in non-muscle tissues. These data are presented along with the age-related during-exercise metabolic comparisons.

Experimental Procedures

Subjects:

Seventeen healthy untrained men (eight older [O, 67 ± 1.6 (SE) yr] and nine younger [Y, 29.8 ± 1.7 (SE) yr], whose general characteristics have been previously reported (Ch. 2, p. 26), were studied before (REST) and again during a continuous infusion of amino acids at rest (REST+AA) and during treadmill walking (40% of VO_{2peak}) (EX+AA). Informed written consent, which was approved by the Institutional Review Board of the University of Texas Medical Branch (UTMB), was obtained from all volunteers prior to study-related procedures. Volunteers were screened at the UTMB Institute for Translational Sciences - Clinical Research Center (ITS-CRC) as previously described (Ch. 2, p. 15).

Pre-testing:

Two weeks prior to their study, subjects were admitted as outpatients to the ITS-CRC to determine total body lean and fat mass, as well as leg lean and fat mass via Dual Energy X-Ray

Absorptiometry (DEXA, Hologic, Inc., Natick, MA), and VO_{2peak}, as previously described (Ch. 2, p. 15).

Experimental Protocol:

Subjects reported to the ITS-CRC at noon the day before the study to control timing and nutritional intake of the evening meal and to ensure that each subject fasted from 2200. The morning of the study, polyethylene catheters were inserted into the antecubital vein of both arms for infusion of stable isotopes and amino acids, and arterialized venous blood sampling. In addition, 3.0Fr/8cm Cook polyurethane catheters were inserted into both the femoral artery (A) and vein (V) approximately 2-5cm below the inguinal ligament and advanced in the proximal direction. To prevent the possibility of the catheter slipping out during exercise, each catheter was fixed to the skin with sutures. Indocyanine green (ICG; Akorn, Buffalo Grove, IL) was infused into the femoral artery for measurement of leg plasma flow as previously described [233, 234]. Three microdialysis probes (m.w. cut-off 20,000 Da; CMA 70, CMA Microdialysis, Solna, Sweden) were inserted approximately 10-15cm above the patella into the *vastus lateralis* of the right leg for determination of nutrient flow and interstitial amino acid concentrations as previously described (Ch. 2, pp. 19-20).

Baseline blood samples were drawn for the analysis of background isotopic enrichment and for use in ICG blood flow determinations, after which a primed (2 μmol/kg) continuous infusion of L-[ring-$^{13}C_6$]-phenylalanine (Cambridge Isotope Laboratories, Andover, MA) (0.08 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) was started (time 0) and continued uninterrupted. Beginning three hours after the start of the infusion, femoral artery and vein blood samples were obtained every 15 minutes for the analysis of isotopic enrichment as well as glucose, lactate and amino acid concentrations. Plasma glucose and lactate concentrations were determined enzymatically (YSI 1500, Yellow Springs Instruments, Yellow Springs, OH, USA). In the final 30 minutes of REST, a primed continuous infusion of amino acids (prime = 0.45 ml/kg; IR = 1.35 ml/kg/hr, Premasol 10%;

Baxter, Deerfield, IL) was started. After the 30 minute amino acid stabilization period, subjects were placed on the treadmill and exercised for 45 min at ~40% VO_{2peak}.

A total of three muscle biopsies (~100-200 mg) were taken from the *vastus lateralis* of the right leg, 15-20 cm above the knee, as previously described [166, 167]. Two biopsies were taken during rest and the final biopsy was taken upon cessation of exercise. Biopsies were snap frozen in liquid nitrogen to abruptly stop all enzymatic reactions and frozen at -80˚C for later analysis.

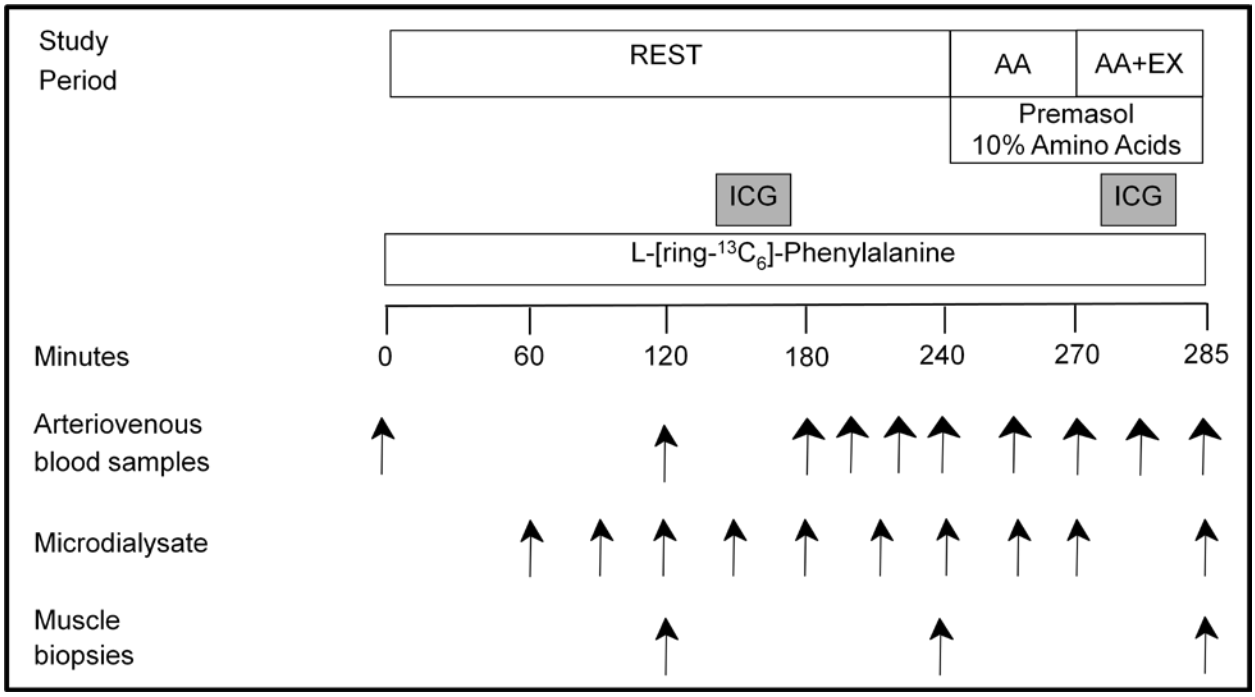


Figure 7: Experimental protocol.

Analytical methods:

Phenylalanine enrichments and concentrations in arterial and venous blood samples and muscle samples were determined as previously described [232].

Calculations:

Leg plasma flow was determined using the indocyanine green (ICG; Akorn, Buffalo Grove, IL) dye dilution technique and converted to leg blood flow (BF) using hematocrit as previously described (Ch. 2, p.18). Data are expressed as ml/min.

The microdialysis ethanol technique was used as an indicator of skeletal muscle nutritive blood flow as previously described (Ch. 2, pp. 19-22).

The rate of phenylalanine disappearance (R_d) from the artery (nmol/min) is calculated as:

$$\mathbf{R_d = ((E_A \times C_A) - (E_V \times C_V)) \times LBF / E_A}$$

where E_A and E_V are the blood phenylalanine enrichments and C_A and C_V are the blood phenylalanine concentrations in the femoral artery and vein, respectively. Individual R_d values were averaged for the REST, REST+AA and EX+AA periods to calculate an average response during each of these periods.

Because DEXA measurements of fat free soft tissue (FFST) include contributions from skeletal muscle, connective tissue, adipose tissue, and skin, lower limb skeletal muscle mass from DEXA FFST was calculated by utilizing the descriptive prediction equation developed by Shih et al. [235]:

$$\mathbf{SM = (0.692 \times FFST) - (0.017 \times age) + (0.068 \times BMI) + 0.11}$$

where FFST is the fat free soft tissue (kg) as measured using DEXA and BMI is the body mass index.

The total phenylalanine present in leg skeletal muscle protein was estimated using the following equation:

$$\mathbf{SM_{Phe} = 233 \times [SM \times (M_{Dry} / M_{Wet}) \times 4.37/4.05]}$$

where 233 is the amount (nmol/mg) of phenylalanine in dried muscle protein [233], M_{Dry} and M_{Wet} are the dry and wet weights, respectively, of the muscle tissue obtained from the biopsies, 4.37/4.05 represents the increase in skeletal muscle mass, due to fluid shifts, during exercise [236]. SM is expressed in mg.

Mixed muscle fractional synthesis rate (FSR) was calculated by measuring the incorporation of L-[ring-$^{13}C_6$]-phenylalanine into protein, using the precursor-product model:

$$\mathbf{FSR = [(E_{P2} - E_{P1}) / (E_{ic} \times \mathit{t})] \times 60 \times 100}$$

where E_{P1} and E_{P2} are the enrichments of bound L-[ring-$^{13}C_6$]-phenylalanine in two muscle biopsies, *t* is the time interval between biopsies, and E_{ic} is the L-[ring-$^{13}C_6$]-phenylalanine enrichment in the muscle intracellular precursor pool [237, 238]. For the FSR during exercise and amino acid infusion, the EX+AA intracellular enrichment was used for the precursor enrichment (as this muscle sample is likely more representative of the combined REST+AA and the EX+AA periods), whereas for the basal REST period the average intracellular enrichment from the first and second biopsies was used as the precursor enrichment. In addition, a during-exercise FSR was calculated using the assumption that muscle protein synthesis remained at basal levels during the initial 30 minutes of amino acid infusion (i.e. FSR during the REST+AA period = FSR during REST), based on the stereotypical "lag-stimulation-resolution" response of muscle protein synthesis to increased amino acid availability [53, 63, 231] and in particular the time course study of Bohe *et al.* [53] in humans showing a 30 minute lag time in the stimulation of skeletal muscle protein synthesis following initiation of amino acid infusion.

Interstitial (extracellular, EC) skeletal muscle phenylalanine concentration (C_{EC}, nmol/ml) was calculated as [232]:

$$\mathbf{C_{EC} = 100 \times ([Phe]_{dialysate} / \text{probe recovery})}$$

The concentration (C) (nmol/ml) of phenylalanine in total muscle tissue fluid was calculated as:

$$\mathbf{C= [Q_{IS} / (V_{IC} \times E_{IS})]}$$

where Q_{IS} (nmol) is the amount of internal standard added to the sample, V is the volume of muscle tissue fluid and E_{IS} is the tracer-to-tracee ratio of internal standard in total muscle water.

To account for the influence of skeletal muscle interstitial phenylalanine on the calculation of intracellular phenylalanine ($[Phe]_{IC}$), including the fluid shift in skeletal muscle during exercise, as well as to calculate leg skeletal muscle interstitial fluid volume and phenylalanine content (below), skeletal muscle intracellular and interstitial fluid volumes from Sjøgaard, *et al* [236], were used. Hence, H_2O_{IC} was calculated as:

$$\mathbf{H_2O_{IC\ REST} = [SM \times (1-(M_{Dry} / M_{Total}))] \times 90\%}$$

$$\mathbf{H_2O_{IC\ EX} = [SM \times (1-(M_{Dry} / M_{Total})) \times 4.37/4.05] \times 86\%}$$

Thus

$$\mathbf{Phe_{IC} = [(C - p_v \times C_{EC}) / (1- p_v)] \times H_2O_{IC}}$$

Where p_v is the proportion of the biopsy water volume accounted for by interstitial fluid and Phe_{IC} is the intracellular content (nmol phe/mL) of phenylalanine.

The proportion of the intracellular phenylalanine pool coming from the artery (vs. breakdown of skeletal muscle proteins) is given by the ratio E_{ic}/E_A. In order to estimate the amount of leg R_d accounted for by changes in intracellular phenylalanine concentration during the REST+AA and the EX+AA periods, conservative estimates were made such that the proportion of the total change in intracellular phenylalanine concentration occurring during each period was directly related to the duration of each period. Thus, the fraction of the intracellular phenylalanine pool (F_{ic}) coming from the artery during the combined REST+AA and EX+AA periods was calculated as:

$$\mathbf{F_{IC} = E_{IC} / (E_{A(Rest+AA)} \times 0.4) + (E_{A(EX+AA)} \times 0.6)}$$

where 0.4 and 0.6 represent the proportion of time spent in the REST+AA and EX+AA periods, respectively. Therefore, the amount (nmol phe) of arterial phenylalanine used to change the intracellular concentration was calculated as:

$$\Delta \mathbf{Phe_{IC}} = \mathbf{(([Phe]_{IC(EX+AA)} \times H_2O_{IC\ (EX+AA)}) - ([Phe]_{IC(REST)} \times H_2O_{IC\ (REST)})) \times F_{IC}}$$

Interstitial phenylalanine content was calculated using the same assumptions as for intracellular phenylalanine concentration:

$$\mathbf{H_2O_{EC(REST)} = [SM \times (1-(M_{Dry} / M_{Total}))] \times 10\%}$$

$$\mathbf{H_2O_{EC(EX+AA)} = [SM \times (1-(M_{Dry} / M_{Total})) \times 4.37/4.05] \times 14\%}$$

Thus

$$\mathbf{Phe_{EC} = C_{EC} \times H_2O_{EC}}$$

where Phe_{EC} is the interstitial (extracellular) content of phenylalanine.

The fraction (F) of the interstitial phenylalanine pool that is being supplied from the artery was calculated as:

$$\mathbf{F_{EC} = (E_{EC} - E_{IC}) / (E_A - E_{IC})}$$

where F is the fraction from the artery and E_{EC} is the average of the intracellular and venous enrichments (used here to estimate interstitial phenylalanine enrichments, based on the relationship between interstitial and blood phenylalanine enrichments from Miller et al. [238]). Hence, the amount of arterial phenylalanine contributing to the net change in the extracellular phenylalanine content was calculated as:

$$\Delta \mathbf{Phe_{EC}} = \mathbf{(([Phe]_{EC(EX+AA)} \times H_2O_{EC(EX+AA)}) - ([Phe]_{EC(REST)} \times H_2O_{EC(REST)})) \times F_{EC}}$$

The amount of plasma phenylalanine used for skeletal muscle protein synthesis (FSR_{Phe}) per minute per leg was calculated as:

$$\mathbf{FSR = ((SM_{Phe} \times FSR)/60) \times F_{IC}}$$

Note that FSR calculated here is in units of per hour, rather than % per hour (as described above). This value was divided by 60 to convert this value to the same units as leg R_d (i.e. nmol phe/min).

Statistical Analysis:

Between-group differences were tested by two-way ANOVA with repeated measures, the effects being age and time. Post hoc testing (Bonferroni) was performed when appropriate to identify pair-wise differences. Student's t-test was used to determine group difference for phenylalanine disposal. Significance was set at $P \leq 0.05$. All analyses were done with SPSS Statistics 17.0. Descriptive data are presented as means ± SEM.

Results

Leg Blood Flow:

Blood flow to the leg was significantly higher during exercise than at rest in both Y (3488 ± 316 and 375 ± 43 ml/min, respectively) and O (3440 ± 502 and 315 ± 19 ml/min, respectively).

Skeletal Muscle Nutritive Flow:

Vastus lateralis nutritive flow was significantly increased during REST+AA and EX+AA in both the Y (REST 0.4391 ± 0.0209, REST+AA 0.3931 ± 0.0265, and EX+AA 0.1541 ± 0.0218) and O (REST 0.5032 ± 0.0334, REST+AA 0.4307 ± 0.0173, and EX+AA 0.1709 ± 0.0317), based on the microdialysis ethanol O/I ratio, which is inversely proportional to nutritive flow.

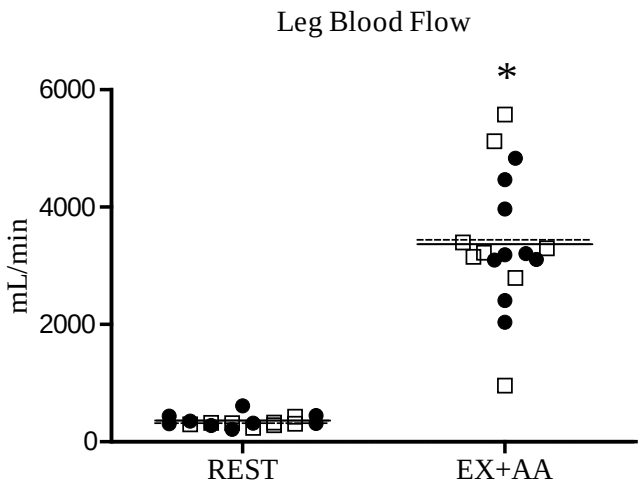


Figure 8: Total leg blood flow. Comparison of total leg blood flow in healthy younger (●) and older (□) men measured at REST and during 45 minutes of treadmill walking exercise with concomitant amino acid infusion (EX+AA). Values are expressed as ml•min^{-1}. * Significant difference from REST.

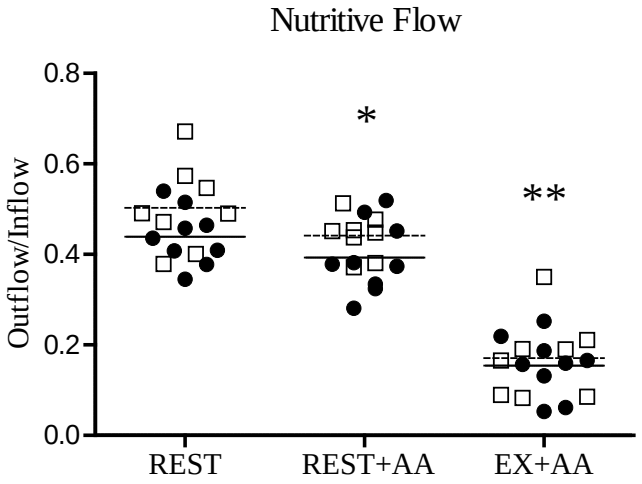


Figure 9: Local skeletal muscle nutritive flow. Comparison of skeletal muscle nutritive flow in healthy younger (●) and older (□) men measured at REST, during 30 minutes of amino acid infusion (REST+AA) and during 45 minutes of treadmill walking exercise with concomitant amino acid infusion (EX+AA). Values are expressed as ml•min^{-1}. * Significant difference from REST. * * Significant difference from REST+AA.

Leg Glucose and Lactate Concentrations:

Glucose concentrations increased in response to amino acid provision in both the arterial (Y: 4.91 ±0.10 vs. 5.28 ±0.08 mmol/L; O: 4.86 ± 0.17 vs. 5.23 ± 0.22) and venous (Y: 4.73 ± 0.10 vs. 5.03± 0.08 mmol/L; O: 4.68 ± 0.15 vs. 4.89 ± 0.18 mmol/L; $p < 0.05$) samples. However, both arterial (4.90 ± 0.14 mmol/L) and venous (4.76 ± 0.14 mmol/L) glucose concentrations returned to REST levels in the younger men during exercise; while in the older men arterial (5.11 ± 0.18 mmol/L) and venous (4.96 ± 0.17 mmol/L) glucose concentrations remained elevated ($p < 0.04$). A significant age x time interaction was observed ($P \leq 0.05$).

Arterial lactate concentrations increased in response to amino acid (Y: 0.87 ± 0.09 vs. 0.94 ± 0.08 mmol/L; O: 0.80 ± 0.07 vs. 0.90 ± 0.12 mmol/L), whereas venous lactate concentrations (Y: 0.98 ± 0.06 vs. 1.01 ± 0.06 mmol/L; O: 0.91 ± 0.06 vs. 0.90 ± 0.08 mmol/L) remained unchanged. During exercise arterial (Y: 1.32 ± 0.14 mmol/L; O: 1.17 ± 0.08 mmol/L) and venous (Y: 1.41 ± 0.17 mmol/L; O: 1.20 ± 0.11 mmol/L) lactate concentrations were increased ($p < 0.02$).

Phenylalanine Enrichments and Concentrations:

Within each time period, arterial and venous phenylalanine enrichments and concentrations were stable. No significant difference in the blood phenylalanine enrichments was observed between the groups. Arterial and venous enrichments significantly decreased from REST during the amino acid infusion ($P < 0.001$) without further changes in response to exercise. This was expected as no additional tracer was added to the amino acid infusion.

The arterial and venous phenylalanine concentrations rose significantly within 15 minutes of the start of the amino acid infusion, and remained ~200% above REST throughout the remainder of the study. Significant main effects were found for age and time ($P < 0.0001$) and for the age x time interaction ($P < 0.0001$) with AV blood phenylalanine concentrations being higher in the older men ($P < 0.001$).

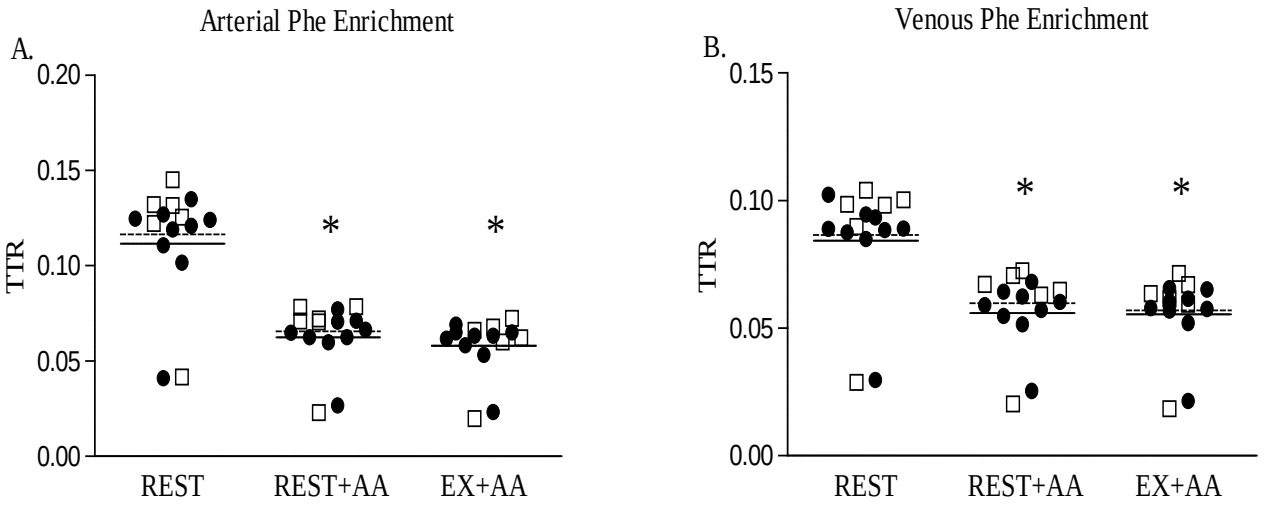


Figure 10: Plasma phenylalanine enrichments. Comparison of arterial (A) and venous (B) phenylalanine enrichments in healthy younger (●) and older (□) men measured at REST, during 30 minutes of amino acid infusion (REST+AA) and during 45 minutes of treadmill walking exercise with concomitant amino acid infusion (EX+AA). Values are expressed as nmol•leg^{-1}. * Significant difference from REST.

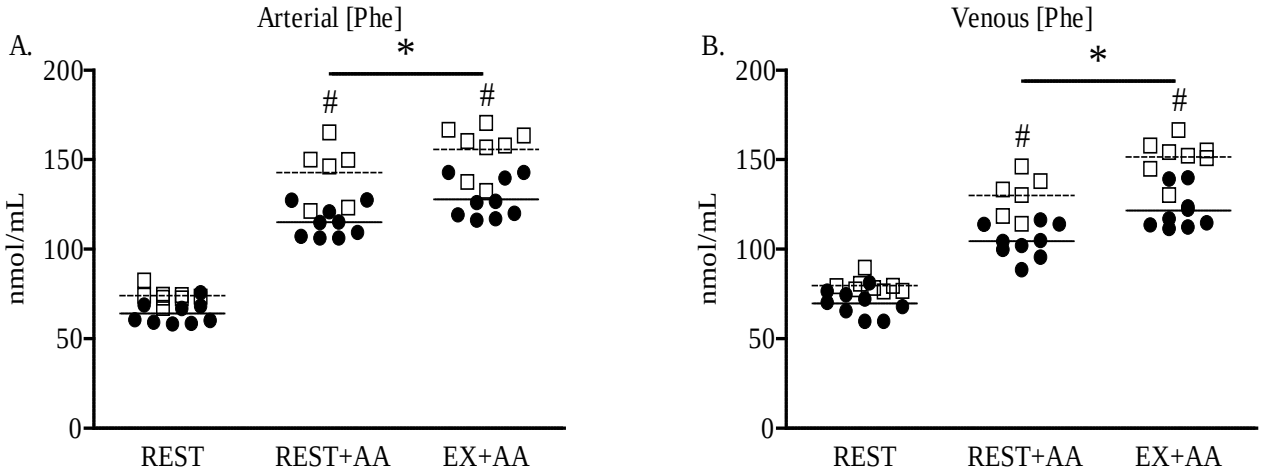


Figure 11: Plasma phenylalanine concentrations. Comparison of arterial (A) and venous (B) phenylalanine concentrations in healthy younger (●) and older (□) men measured at REST, during 30 minutes of amino acid infusion (REST+AA) and during 45 minutes of treadmill

walking exercise with concomitant amino acid infusion (EX+AA). Values are expressed as nmol•leg^{-1}. * Significant difference from REST. # Significant group difference.

Muscle free intracellular phenylalanine concentrations were greater in the older men (71 ± 6 and 110 ± 13 nmol/mL for the REST and EX+AA biopsies, respectively) in comparison to the younger men (52 ± 3 and 76 ± 4 nmol/mL for the REST and EX+AA biopsies, respectively). Significant main effects were found for age (P = 0.006) and time (P < 0.0001). Muscle free intracellular phenylalanine content in the leg increased in response to exercise and was greater in the older men in comparison to the younger men (522394 ± 90628 and 293948 ± 33041 nmol, respectively). Significant main effects were found for age (P = 0.009) and time (P < 0.02). Leg muscle interstitial phenylalanine content increased during amino acid infusion with no difference

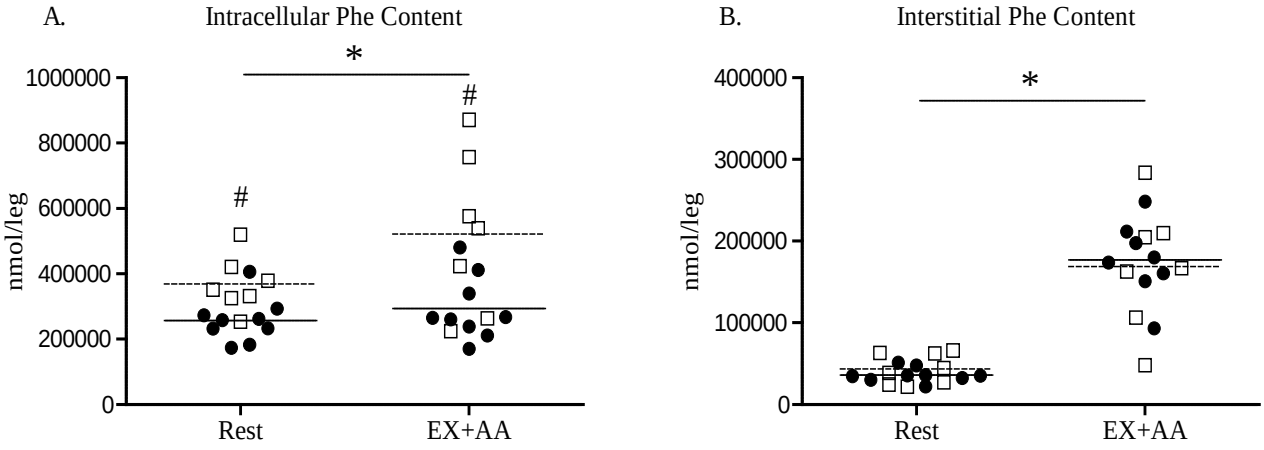


Figure 12: Skeletal muscle phenylalanine content. Comparison of muscle intracellular (A) and interstitial (B) phenylalanine content in healthy younger (●) and older (□) men measured at REST and during 45 minutes of treadmill walking exercise with concomitant amino acid infusion. Values are expressed as nmol•leg-1. * Significant difference from REST. # Significant group difference.

between the younger and older men (168924 ± 28742 and 177055 ± 16205 nmol, respectively).

Leg Phenylalanine Delivery and R_d:

The delivery of phenylalanine to the leg was not significantly different between the younger men (270 ± 38 nmol/ min) and the older men (266 ± 23 nmol/min) at REST. During the REST+AA period, phenylalanine delivery to the leg significantly increased from REST in both the younger (477 ± 64 nmol/ min) and older men (540 ± 39nmol/ min). This increase was further amplified with the addition of exercise (Y: 4785 ± 508 nmol/min and O: 5063 ± 693 nmol/min). The leg phenylalanine R_d did not differ between the younger and older men. During the amino acid period phenylalanine R_d significantly increased from REST in both the younger (4512 ± 1045 and 7597 ± 1172 nmol/ min, respectively) and older men (4131 ± 377 and 6596 ± 1640 nmol/min, respectively). This increase was further amplified with the addition of exercise (Y: 36259 ± 6732 nmol/min and O: 33190 ± 5605 nmol/ min).

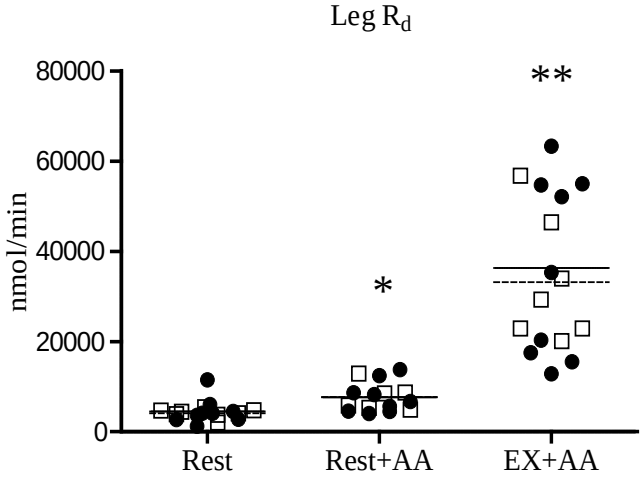


Figure 13: Leg rate of phenylalanine disappearance. Comparison of the rate of phenylalanine disappearance (Rd) in healthy younger (●) and older (□) men measured at REST, 30 minutes of amino acid infusion (REST+AA) and during 45 minutes of treadmill walking exercise with concomitant amino acid infusion (EX+AA). Values are expressed as nmol•ml^{-1}. * Significant difference from REST. ** Significant difference from REST and REST+AA.

Mixed-muscle fractional synthesis rate (FSR):

FSR increased from REST during amino acid infusion in the younger (0.061 ± 0.005% and 0.083 ± 0.012%, respectively) and older men (0.075 ± 0.010% and 0.127 ± 0.019%, respectively), using the EX+AA E_{ic} as the precursor enrichment. A significant main effect was found for time ($P < 0.0001$), with marginal effects of age and time x age interaction. Furthermore, the calculated during-exercise FSR was significantly greater in the older men (0.150 ± 0.026%) when compared to the young (0.093 ± 0.017%) ($P < 0.05$).

Phenylalanine disposal during concomitant endurance-type exercise and AA infusion:

Phenylalanine disposal was similar in the younger and older men and is presented in Table 2.

	Younger Men (n=9)	**Older Men (n=8)**
Leg Skeletal Muscle Mass (g)	6636 ± 230	6855 ± 281
Leg Phe R_d, nmol/min	23566 ± 3867	22552 ± 2671
Plasma Phe required for muscle protein synthesis, nmol/min	3379 ± 580	3956 ± 592
F_{IC}(fraction of IC Phe from artery)	0.72 ± 0.08	0.84 ± 0.03
Plasma Phe required for $\Delta[Phe]_{IC}$, nmol/min	51 ± 144	1305 ± 926
F_{ISF} (fraction of ISF Phe from artery)	0.38 ± 0.04	0.30 ± 0.04
Plasma Phe required for $\Delta[Phe]_{EC}$, nmol/min	676 ± 115	512 ± 128

Table 2: Plasma phenylalanine (Phe) disposal during moderate aerobic exercise. Values are means ± SEM. Rd – Rate of phenylalanine disappearance, IC – Intracellular concentration, EC – Extracellular concentration, ISF – Interstitial fluid.

Changes in the intracellular and interstitial fluid phenylalanine content represented a minor fate for phenylalanine taken up from the blood (less than 10% of leg R_d). Skeletal muscle phenylalanine utilization for protein synthesis also reflected a minority (~20%) of total leg phenylalanine disappearance.

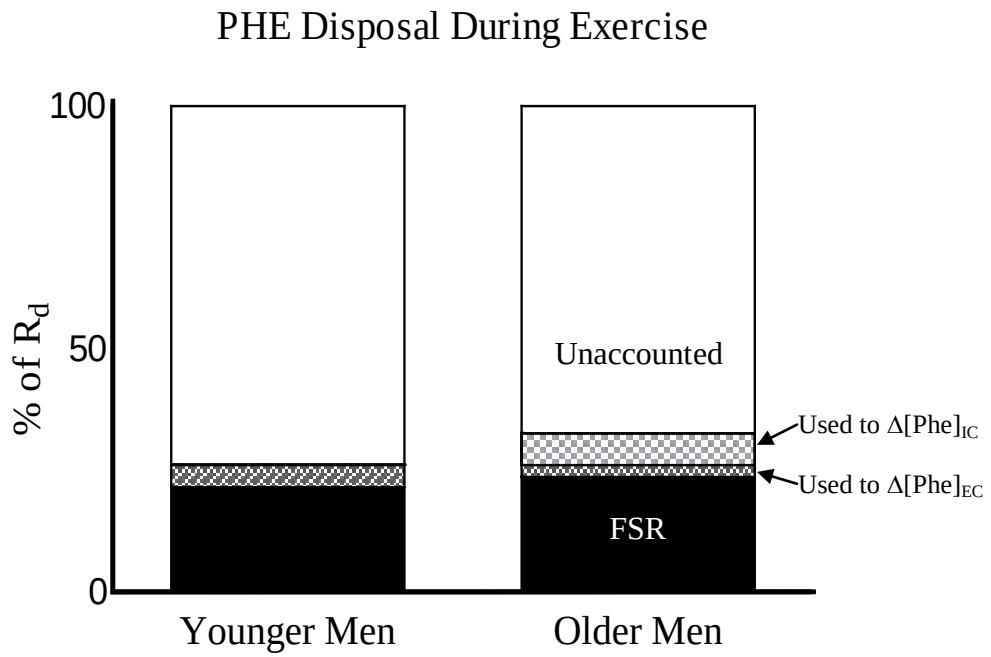


Figure 14: Plasma phenylalanine disposal during exercise. Comparison of the percentage of total phenylalanine disposal in each measured compartment during exercise in healthy younger and older men. The black section represents the percentage of phenylalanine Rd being used to achieve FSR. The hatch mark section represents the percentage of phenylalanine being used to change the concentration in the interstitial fluid. The checkered section represents the percentage of phenylalanine being used to change the concentration in the intracellular fluid. The white section represents the percentage of phenylalanine that is unaccounted for.

Discussion

The response of muscle protein metabolism during endurance-type exercise in humans has received little attention, likely due to methodological difficulties associated with its study. However, the response is of potential importance given that i) numerous physiological responses occur in the during-exercise period that, under different circumstances, have been associated with changes in muscle protein metabolism and ii) it is recommended that everyone (young and old) perform a minimum of 130 hours of this type of exercise every year, meaning that even an incremental change in response to exercise could have a large cumulative metabolic effect. The principal findings of this study were that i) endurance exercise, in the form of walking, markedly increased substrate exchange between the blood and leg tissues and ii) healthy younger and older men responded in a qualitatively similar manner, with markedly increased leg amino acid uptake and an augmentation of muscle protein synthesis; the latter in fact was marginally higher in the older group. These data thus refute the hypothesis that skeletal muscle of older individuals would exhibit an age-related resistance to the anabolic effects of exogenous amino acids during endurance exercise. This finding contrasts with the situation following endurance exercise, when older individuals exhibit both anabolic resistance to exogenous amino acids and reduced nutritive flow relative to the young [232].

Under fasting conditions using tracer incorporation methods, skeletal muscle protein synthesis was previously reported to be significantly higher than at rest during 45 minutes [17] but not 4 hours of walking exercise [15] at low to moderate intensity. Recently, muscle protein synthesis in trained young male cyclists was reported to be higher during 2 hours of cycling exercise at 55% W_{max} with concomitant supplementation of carbohydrate alone, or carbohydrate with protein, than under basal conditions [22]. A separate investigation reported, similarly, that supplemental protein did not augment skeletal muscle protein synthesis during 3 hours of cycling at the same intensity, although a comparison of during-exercise versus pre-exercise muscle protein synthesis was not performed. Although not a direct comparison, the response of FSR to

exercise in the current study, in which amino acids were infused, was similar to what has previously been observed during the same type of exercise under fasting conditions [17]. In addition, chronic endurance-type exercise training was recently reported to stimulate skeletal muscle protein synthesis but this stimulation was not further augmented by protein supplementation during training. The present study thus contributes to a small but growing body of literature suggesting that skeletal muscle protein synthesis can be stimulated during endurance-type exercise, at least during a bout of exercise lasting 2 hours or less, but that this stimulation is insensitive to exogenous amino acids. This study is also the first to report that age-related resistance to exogenous amino acids is not observed during low- to moderate-intensity endurance-type exercise. However, in light of the studies noted above suggesting that the stimulation of muscle protein synthesis during endurance-type exercise is not influenced by exogenous amino acids, it is perhaps more accurate to say that there does not appear to be an age-related resistance to the stimulation of skeletal muscle protein synthesis that occurs during endurance-type exercise. It is intriguing to note that in both this study as well as our previous study conducted under fasting conditions [17] there was a trend for skeletal muscle protein synthesis to be higher in older than in younger individuals during endurance-type exercise. If confirmed in future studies this would represent the only circumstance I am aware of in which muscle protein synthesis responds to a greater degree in older than in younger individuals.

Notably, time represents an important physiological and design variable that will need to be considered before the nature of the during-exercise response of muscle protein metabolism can be fully elucidated. For example, do the responses to different durations of endurance-type exercise, which cursory consideration suggests are divergent, actually under closer scrutiny simply represent different sampling points along a similar response timeline characterized by a transient period of stimulation of muscle protein synthesis, followed by a subsequent return to basal levels (similar to the normal “lag-stimulation-resolution” response to an increase in amino acid delivery under non-exercising conditions [53, 63, 231])? This issue was previously

considered by Bohe et al. [53], who noted that the time course of the response of skeletal muscle protein synthesis to a step-change in amino acid availability (under non-exercised conditions) meant that longer tracer incorporation times would eventually lead to an underestimation of the anabolic response to amino acids. Further, does exercise alter the normal "lag-stimulation-resolution" response of skeletal muscle to increased amino acid delivery [53]? Additional studies will be necessary to resolve these issues and improve our understanding of the regulation of skeletal muscle protein synthesis during endurance-type exercise.

In the examination of the isotopic data, novel findings were uncovered that suggest endurance-type exercise evokes marked changes in amino acid handling in non-muscle tissues regardless of age. In vivo, substrates are disseminated into physically distinct tissues (e.g. skeletal muscle, skin, bone, and adipose tissue) and compartments (e.g. plasma, interstitial fluid, and intracellular fluid). There are several fates for phenylalanine taken up from the circulation across a limb. The AV balance method of determining limb R_d estimates rates of amino acid utilization for protein synthesis and breakdown. However, the metabolic contributions from non-muscle tissues are also incorporated into this measurement and use of limb R_d as an indicator of muscle protein synthesis may lead to over- or underestimation of blood amino acid utilization for muscle protein synthesis in some circumstances, such as when blood flow distribution between tissues is altered. Therefore, a principal goal of the present study was to find out the extent to which skeletal muscle contributes to leg protein metabolism during perturbation with nutrition and exercise. By employing a combination of AV balance and tracer incorporation techniques along with microdialysis sampling of skeletal muscle interstitial fluid, the contributions of skeletal muscle phenylalanine interstitial and intracellular concentration changes and phenylalanine utilization for protein synthesis were determined. In contrast to previous estimates that 10 – 15% of leg amino acid uptake is accounted for by non-muscle tissues under fasting conditions at rest [233, 239], the present results suggest that roughly 70% of leg phenylalanine R_d is accounted for by non-muscle tissues during endurance exercise with concomitant amino

acid administration (Figure 14). Although other tissues were not sampled, these results provide a strong deductive argument that endurance exercise markedly stimulates amino acid uptake by other tissues, including skin, bone, and adipose tissue. These results, in combination with the finding that younger and older men respond in a metabolically similar fashion in the during-exercise period of endurance-type exercise, provide supportive evidence that endurance exercise is metabolically important for enhancement of substrate exchange and utilization not only in skeletal muscle, but also in other metabolically active tissues.

CHAPTER 4

SKELETAL MUSCLE INTRACELLULAR SIGNALING EVENTS IN RESPONSE TO ENDURANCE EXERCISE AND AMINO ACIDS

Introduction

Throughout the past two decades the influence of resistance exercise and amino acids, independently and synergistically, on skeletal muscle protein synthesis has been well characterized [82, 96, 97, 240-242]. However, our understanding of the skeletal muscle protein synthetic response to endurance-type exercise is only beginning to be elucidated. Recently, endurance-type exercise has been shown to stimulate muscle protein synthesis [17, 20, 22, 24], and when combined with nutritional supplementation the effect is enhanced and prolonged [16, 18, 25, 232]. Nevertheless, the influence of endurance exercise on the skeletal muscle metabolic response and the molecular signaling events involved remains poorly understood. It has been shown that the response to anabolic stimuli, such as amino acids and exercise, is controlled through numerous interconnecting signaling cascades within which the serine/threonine protein kinase Akt serves as a central regulatory node [105, 243, 244]. Activation of Akt results in phosphorylation of a wide range of proteins, including other kinases such as the mammalian target of rapamycin (mTOR) and glycogen synthase kinase-3 beta (GSK-3β), in addition to transcription factors such as the class O of forkhead box transcription factors (FoxO). Differential phosphorylation of these Akt substrates and their subcellular localization plays an important role in skeletal muscle metabolism [245-247].

The molecular mechanisms underlying muscle protein synthesis in response to exercise are primarily thought to occur through stimulation of the phosphatidylinositol-3 kinase (PI3K)/Akt/mTOR pathway [99, 100, 105, 243]. Independent of the PI3K/Akt pathway, amino acids influence muscle protein synthesis by directly affecting the activation of mTOR [82, 100]. The phosphorylation of mTOR exerts well-characterized effects on two downstream targets -

eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and 70-kDa ribosomal protein S6 kinase 1 ($p70^{S6K}$) [85, 86, 88, 90, 248]. The phosphorylation of 4E-BP1 allows eIF4E to be released and bind to eIF4G, forming the eIF4F preinitiation complex [91]. The phosphorylation of $p70^{S6K}$ and subsequent phosphorylation of the 40S ribosomal subunit protein S6 and the eukaryotic translation initiation factor 4B (eIF4B) is thought to control cell size through enhanced translation, although how it does so is unclear [91, 249].

The molecular mechanisms underlying muscle protein breakdown in response to exercise may involve alterations in components specific to the ubiquitin-proteasome pathway (UPP). In addition, amino acid supplementation appears to attenuate some of the components of the UPP [250-252]. The two most induced genes of the UPP are the muscle-specific E3 ubiquitin ligases - muscle RING-finger protein-1 (MuRF-1) and muscle-specific atrogin-1 (MAFbx) [105]. The expression of these E3 ubiquitin ligases is regulated by the cellular localization of the FoxO transcription factors [108-110]. Specifically, *in vivo* FoxO1 and FoxO3a overexpression induces skeletal muscle atrophy [109], and constitutively activated FoxO significantly lessens disuse-induced muscle fiber atrophy [253-255]. However, limited studies have investigated the UPP in response to resistance exercise [112, 256, 257], endurance-type exercise [258] or combined resistance/endurance exercise [91]. The changes in the UPP-related gene expression observed in these studies are consistent with alterations in muscle protein breakdown in response to resistance exercise [112, 259]. Still, the role of these cellular mechanisms in mediating the muscle proteolytic response to endurance-type exercise is unknown.

As such, the purpose of this study was to further characterize the affect of a single bout of low- to moderate-intensity endurance exercise (i.e. treadmill walking) in combination with amino acids on skeletal muscle hypertrophy- and atrophy-related intracellular signaling responses in healthy adults. Therefore, we hypothesized that low-intensity endurance exercise concomitant with amino acids would increase the phosphorylation of key proteins involved in the Akt and mTOR intracellular-signaling pathways leading to stimulation of muscle protein

synthesis. Likewise, components of the UPP would be decreased reflecting changes in the proteolytic environment immediately following and during recovery from endurance exercise.

Experimental Procedures

Subjects:

Seventeen healthy sedentary men (eight older [O, 67 ± 1.6 (SE) yr] and nine younger [Y, 29.8 ± 1.7 (SE) yr]), whose general characteristics have been previously reported (Ch. 2, p. 26) completed the study. Informed written consent, which was approved by the University of Texas Medical Branch (UTMB) Institutional Review Board, was obtained prior to any study-related procedures. Volunteers were screened at the UTMB Institute for Translational Sciences - Clinical Research Center (ITS-CRC) to determine study eligibility as previously described (Ch. 2, p. 15).

Pre-testing:

Prior to performing the actual protocol, VO_{2peak} of each subject was determined as previously described (Ch. 2, p. 16).

Experimental Protocol:

Subjects reported to the ITS-CRC at noon the day before the study to control timing and nutritional intake of the evening meal and to ensure that each subject fasted from 2200. The following day, a total of three muscle biopsies (~100-200 mg) were taken from the *vastus lateralis* of the right leg, 15-20 cm above the knee, as previously described (8, 29). Biopsies were taken at rest, immediately upon cessation of exercise, and after 3 hours of recovery. Biopsies were snap frozen in liquid nitrogen to abruptly stop all enzymatic reactions and stored at -80˚C until analyzed.

Western Blot Analysis:

Primary antibodies were purchased from Cell Signaling (Beverly, MA): phosphor-Akt (Ser^{473}), phospho-GSK-3β (Ser^{9}), phospho-$p70^{S6K1}$ (Thr^{389}), phospho-4E-BP1 ($Thr^{37/46}$), phospho-Fox01 (Ser^{256}), phospho-Fox03a ($Ser^{318/321}$), total GSK-3β, total $p70^{S6K1}$, total 4E-BP1, total Fox01, and total Fox03. MuRF-1 and MAFbx were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PGC-1α was purchased from EMD Chemicals (Philadelphia, PA). Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was purchased from Amersham Bioscience (Piscataway, NJ). Anti-mouse IgG horseradish peroxidase-conjugated secondary antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

For analysis of protein phosphorylation by immunoblotting, muscle tissue was weighed and homogenized in cytoplasmic extract buffer (18µl per mg of muscle tissue) containing 10 mM Hepes-KOH (pH 7.9), 1.5 mM $MgCl_2 6H_2O$, 10 mM KCl, 2 mM phenylmethylsulfonyl floride (PMSF), 1 mM dithiothreitol (DTT) and 1 mM protease inhibitor cocktail (Sigma-Aldrich Co. LLC, St Louis, MO). After homogenization, the sample was vortexed and incubated on ice for 10 minutes, after which the sample was vortexed, frozen in liquid nitrogen and allowed to thaw on ice. Once thawed, the sample was vortexed and centrifuged at 4°C, 16,110 g for 30 seconds. The supernatant (cytosolic fraction) was collected and stored at -80° C until analyzed To the remaining pellet, ice cold nuclear extract buffer (4 µl per mg of muscle tissue) containing 20 mM Hepes-KOH (pH 7.9), 1.5 mM $MgCl_2 6H_2O$, 5% glycerol, 420 mM NaCl, 0.2 mM ethylene-diamine tetraacetic acid (EDTA), 2 mM PMFS, 1 mM DTT and 1 mM protease inhibitor cocktail was added and the sample was vortexed to resuspend the pellet. The sample was incubated on ice for 30 minutes, vortexing 10 seconds every 10 minutes. The sample was then centrifuged at 4°C, 16,110 g for 4 minutes and the supernatant (nuclear fraction) was collected and protein concentrations were determined. All samples for stored at -80°C.

For the determination of protein concentrations, protein standards were prepared at concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mg/ml and the protein fractions were diluted 1:50. In 13 x 100mm test tubes, 50 µl of each standard and sample and 2.5 ml of diluted dye reagent (Protein Assay Dye Reagent Concentrate; Bio-Rad Life Science Research, Hercules, CA). The samples were incubated at room temperature for 5 minutes and measured at 595 nm absorbance (Beckman UV/Vis Spectrophotometer; Beckman Coulter, Inc., Indianapolis, IN).

A total of 80 µg of protein was loaded per lane and separated by SDS-PAGE (NuPAGE®; Invitrogen, Carlsbad, CA) at a constant 200 volts for 60 min as previously described (Ch. 2, pp. 23-24). Data are expressed as the signal from the phosphorylated divided by the total protein and normalized to a rodent standard that was run on each gel, in arbitrary units.

Statistical Analysis:

Between-group differences were tested by two-way ANOVA with repeated measures, the effects being age and time. Post hoc testing (Bonferroni) was performed when appropriate to identify pair-wise differences. As no differences were found between the younger and older men, the two treatment groups were combined and a paired Student's *t*-test was conducted to evaluate changes from the baseline measurement. Significance was set at $P \leq 0.05$. All analyses were made using SPSS (Ver. 18) and all data are expressed as means ± SEM.

Results

PGC-1α, Akt and GSK-3β phosphorylation.

PGC-1α, a marker of mitochondrial biogenesis and energy metabolism, was not influenced by low-intensity endurance exercise with provision of amino acids. Additionally, Akt (Ser^{473}) and GSK-3β (Ser^{9}) phosphorylation remained unchanged postexercise.

$P70^{S6K1}$ and 4E-BP1phosphorylation.

The phosphorylation of $p70^{S6K1}$ (Thr^{389}) and 4E-BP1 ($Thr^{37/46}$) were increased in response to low-intensity endurance exercise with provision of amino acids. Figure 15 illustrates changes in $p70^{S6K1}$ (A) and 4E-BP1 (B) phosphorylation. Directly after exercise, the level of $p70^{S6K1}$ phosphorylation was increased ($P < 0.05$), and was further increased during the 3-hour recovery period (112%; $P < 0.05$). Similarly, 4E-BP1 phosphorylation increased ($P < 0.05$) and levels of this species persisted for the full recovery period ($P < 0.001$).

FoxO1and FoxO3 phosphorylation.

Figure 16 illustrates changes in both fractions (cytosolic and nuclear) of FoxO1 and FoxO3 phosphorylation. The phosphorylation of nuclear FoxO1 (B) and FoxO3 (D) were found to be unchanged in response to low-intensity endurance-exercise with provision of amino acids. The phosphorylation of cytosolic FoxO3 (C) increased in response to low-intensity endurance exercise with provision of amino acids (29%, $P < 0.05$); while, the phosphorylation of cytosolic FoxO1 (A) remained relatively unchanged postexercise.

MuRF-1 and MAFbx total protein expression.

Directly after exercise, the total protein expression of MuRF-1 increased (28%, $P < 0.05$), however, this increase was short lived (Figure 17A). In contrast, the total protein expression of MAFbx remained relatively unchanged immediately postexercise, but was significantly reduced during the 3-hour recovery period (36%, $P < 0.001$; Figure 17B).

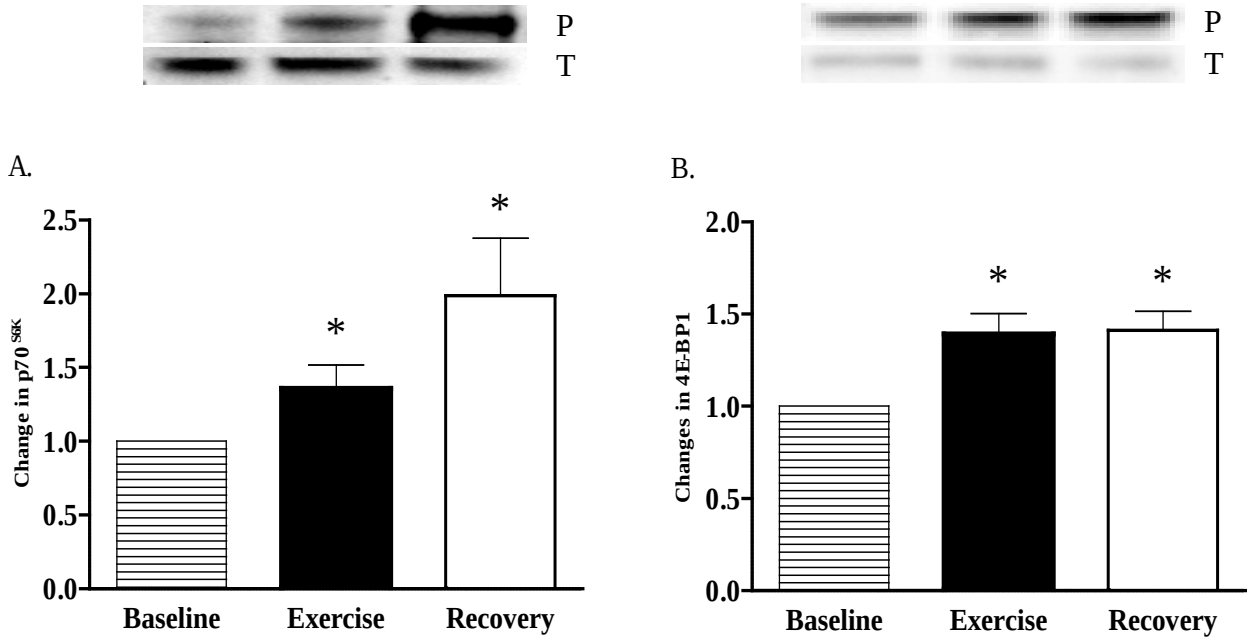


Figure 15: Changes in $p70^{S6K}$ and 4E-BP1 phosphorylation in response to endurance-type exercise. Changes in the phosphorylation status of $p70^{S6K}$ (A) and 4E-BP1 (B) immediately following (black bars) and again 3 hours (white bars) after an acute bout of low intensity endurance exercise. Data are expressed as the phosphorylated divided by the total protein signal, normalized to a rodent standard that was run on each gel, relative to baseline measurements, and are expressed as Mean ± SEM. Insert shows representative western blots for phosphorylated (P) and total (T) protein. * Significant from baseline.

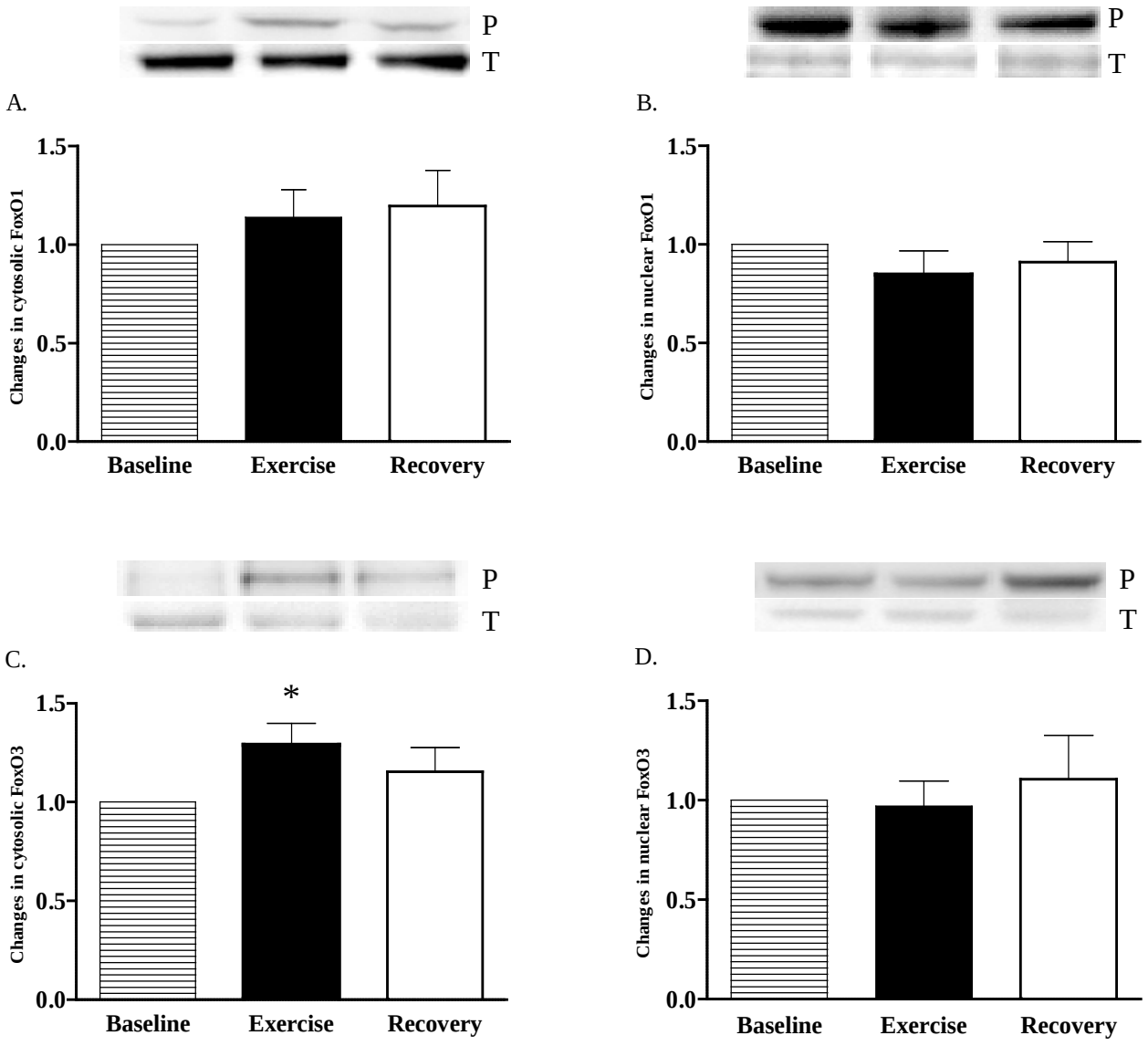


Figure 16: Changes in FoxO1 and FoxO3 cytosolic and nuclear fraction phosphorylation in response to endurance-type exercise. Changes in the phosphorylation status of FoxO1 cytosolic (A) and nuclear (B) fractions and FoxO3 cytosolic (C) and nuclear (D) fractions immediately following (black bars) and again 3 hours (white bars) after an acute bout of low intensity endurance exercise. Data are expressed as the phosphorylated divided by the total protein signal, normalized to a rodent standard that was run on each gel, relative to baseline measurements, and are expressed as Mean ± SEM. Insert shows representative western blots for phosphorylated (P) and total (T) protein. * Significant from baseline.

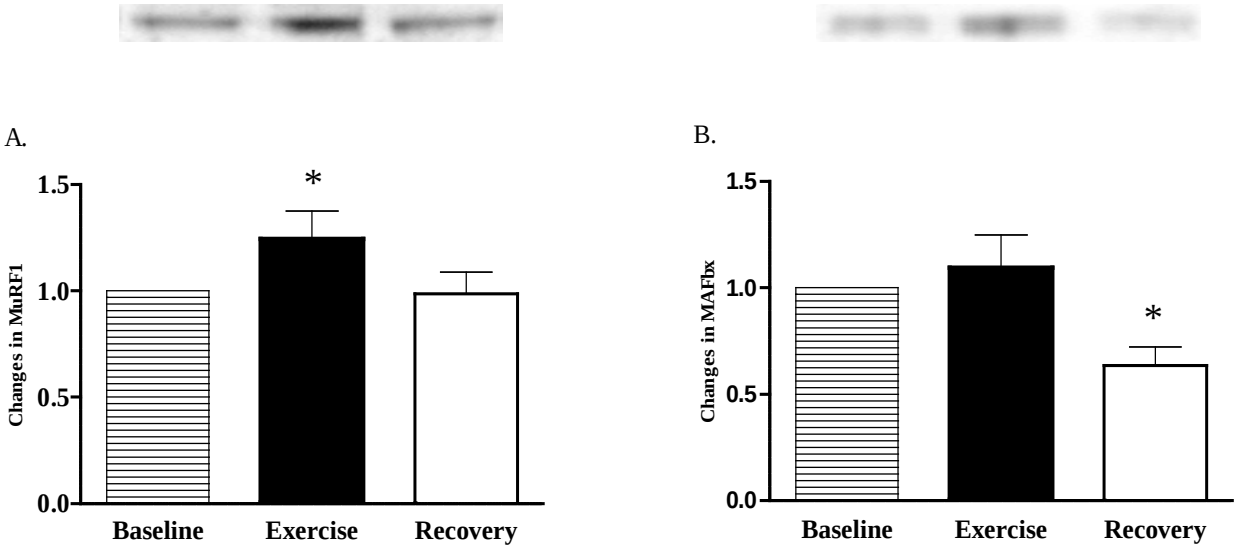


Figure 17: Changes in total protein expression of MuRF-1 and MAFbx in response to endurance-type exercise. Changes in the total protein of MuRF-1 (A) and MAFbx (B) immediately following (black bars) and again 3 hours (white bars) after an acute bout of low intensity endurance exercise. Data are expressed as the phosphorylated divided by the total protein signal, normalized to a rodent standard that was run on each gel, relative to baseline measurements, and are expressed as Mean ± SEM. Insert shows representative western blots for total protein.* Significant from baseline.

Discussion

The present study details intracellular signaling events in response to an acute bout of endurance exercise during provision of amino acids. Although endurance-type exercise is not typically thought of as a stimulator of with skeletal muscle protein synthesis, studies show that it enhances the activation of key proteins involved in the muscle synthetic response [19, 260-262]. In the current study, endurance exercise with a provision of amino acids produced significant alterations in the phosphorylation of some but not all key proteins within the synthetic signaling pathways, along with a reduction in the total protein expression of MAFbx. More importantly, this is the first study to report a decrease in the muscle specific E3 ligase after an acute bout of exercise.

Skeletal muscle protein breakdown is tightly regulated through the UPP. These findings indicate that the total protein expression of MAFbx was decreased after a 3-hour recovery period while MuRF-1 was transiently elevated during exercise before returning to baseline levels. Similar changes in the proteolytic gene expression of these E3 ligases were recently reported after a single high-volume bout of resistance exercise (four sets each of 10 repetitions at 80% of 1-RM and 15 repetitions at 65% of 1-RM) [263]. Borgenvik *et al.* [263] found that amino acid supplementation started prior to initiation of exercise and continuing for 45 minutes of recovery reduced mRNA levels of MAFbx after a 3-hour recovery period in healthy young adults. The findings of this study as well as those of Borgenvik *et al.* [263] are contradictory to the recent report by Pasiakos *et al.* [261] which showed that moderate-intensity endurance exercise (60 minutes of cycling at 60% VO_{2peak}) increases the expression of MuRF-1 and MAFbx mRNA, and that this increase persist for 3 hours postexercise [112, 261]. The reason for these discrepancies may be explained by the differences in the exercise modalities and intensities, as well as the provision of amino acids. In addition, the divergent response of MuRF-1 and MAFbx to exercise may be explained by the functional differences of these two E3 unbquitin ligases; MuRF-1 appears to degrade components of the contractile apparatus [100], whereas MAFbx

targets include MyoD, calcineurin, and eukaryotic initiation factor 3F (eIF-3F), important in mTOR-$p70^{S6k}$ signaling [100, 263]. The immediate increase in the total expression of MuRF-1 following endurance exercise may reflect increased contractile protein degradation, an effect that was short lived in the presence of amino acids. The current finding that MAFbx expression decreased after endurance exercise with provision of amino acids supports the findings of Borgenvik *et al.* [263] suggesting that MAFbx may function to attenuate breakdown of proteins involved in mTOR signaling.

Alterations in skeletal muscle metabolism in response to endurance-type exercise are relatively unknown. The current study was designed to expand upon findings from a our previous study [17] showing increases in muscle protein synthesis in response to endurance exercise in the fasted state. In agreement with this prior study [17], the rates of muscle protein synthesis reported in chapters 2 and 3 resulted in 4E-BP1 and $p70^{S6K}$ phosphorylation increasing immediately postexercise, an effect that persisted 3 hours into recovery (Figure 15). Our understanding of $p70^{S6K}$ phosphorylation in response to exercise and nutrition comes from the study of two separate activation sites: Ser^{424}/Thr^{421} and Thr^{389} [264]. As several studies have shown differential phosphorylation of $p70^{S6K}$ at Ser^{424}/Thr^{421} and Thr^{389} [18, 19, 260, 261, 265], it is important to note that it is the phosphorylation of Thr^{389} that is the chief event through which full activity is achieved [264]. For this reason, $p70^{S6K}$ (Thr^{389}) was measured in response to low-intensity endurance exercise with provision of amino acids. Although endurance-type exercise has been shown to immediately stimulate $p70^{S6K}$ (Thr^{389}) phosphorylation, there are mixed reports as to the prolonged effect [18, 19, 243, 261]. Several studies report an increase in $p70^{S6K}$ (Thr^{389}) phosphorylation by endurance exercise followed by a return to baseline measurements. In contrast, Camera *et al.* [243] showed that in physically active young men, $p70^{S6K}$ (Thr^{389}) phosphorylation continually increased throughout 60 minutes of recovery from cycling exercise (60 minutes at 70% VO_{2peak}), although this increase failed to reach significance. The significant alterations in $p70^{S6K}$ (Thr^{389}) phosphorylation observed in the current study may be explained by

the provision of amino acids, as amino acid supplementation is associated with increased $p70^{S6K}$ (Thr^{389}) phosphorylation [241, 266, 267]. Consistent with the findings of the present study, Cuthbertson *et al.* [107] reported an increase in $p70^{S6K}$ (Thr^{389}) phosphorylation at 3 hours of recovery, which was sustained for up to 24 hours, when amino acids where given for the two hours following 12 minutes of a step-up/step-down exercise protocol. Taken together, the increase in $p70^{S6K}$ (Thr^{389}) phosphorylation following low-intensity endurance exercise and the persistent phosphorylation in the subsequent 3 hours is consistent with the observation that amino acids not only provide substrate for protein synthesis but also stimulate intracellular signaling important in the regulation of translation initiation.

In conclusion, low-intensity endurance exercise with provision of amino acids not only enhances protein utilization through stimulation of muscle protein synthetic signaling events but may also serve to attenuate the proteolytic effect of exercise. As both young and older men volunteered to participate in this research project, these data provide compelling evidence that the intracellular signaling mechanisms are intact and stimulated in aged muscle, as no age-related differences were observed. In addition, these data suggest that low-intensity endurance exercise is stimulatory to skeletal muscle and provides a practical solution to maintaining skeletal muscle mass throughout the aging process. Given that the combination of amino acid supplementation and endurance-type exercise stimulated skeletal muscle protein synthesis as shown in chapters 2 and 3, the increased 4E-BP1 and $p70^{S6K}$ (Thr^{389}) activity observed here provides a plausible molecular mechanism through which these enhanced effects are governed. In addition, the localization of the FoxO transcription factors to the cytosol and the subsequent decrease in the E3 ligases in the recovery period provides further evidence that low-intensity endurance exercise is a key component to maintaining skeletal muscle mass throughout the aging process. However, linking a specific molecular signaling event to a specific muscle metabolic response is complicated as these pathways are nonlinear in their degrees of regulation and activation [91]. Furthermore, the measured proteins are only a snap shot of the molecular events taking place

during the dynamic processes of protein synthesis and breakdown. Further studies are needed to fully understand the molecular events involved in protein synthesis and breakdown in response to endurance-type exercise and nutritional supplementation.

CHAPTER 5

LEUCINE SUPPLEMENTATION CHRONICALLY IMPROVES THE ANABOLIC RESPONSE TO MEALS IN OLDER ADULTS CONSUMING THE RDA FOR PROTEIN[3]

Introduction

Aging is commonly associated with the progressive loss of skeletal muscle tissue and functional capacity. Illness and injury notwithstanding, sarcopenia is facilitated by factors secondary to the adoption of a more sedentary lifestyle and consumption of a less than optimal diet. While maintaining or increasing physical activity is clearly desirable, establishing a basic nutrition foundation focusing on adequate protein and energy consumption is necessary if other interventions are to succeed.

It is generally accepted that aging is associated with a blunted protein synthetic response to meals containing less than approximately 15–20 g of protein or the equivalent essential amino acid content [2, 6, 8]. While this deficiency may be partially or wholly overcome by simply increasing protein intake, obstacles such as total energy consumption, palatability, cost, satiety and habitual practices must be considered [268, 269]. Consequently, the practical validity of prescribed supplementation regimens must also be critically examined. For example, in controlled metabolic studies it is easy to demonstrate acute increases in muscle protein synthesis or markers of anabolism following ingestion of a protein or amino acid supplement [47, 58, 270]. However, in practice, there is a risk that acute responses will not translate into positive chronic adaptations in muscle mass or function. The reasons for a lack of success are varied, but we suspect they more often reflect compliance-related issues or consumer choice, rather than a temporally diminished physiological capacity [271, 272]. To this end, we propose that an

[3] Casperson SL, Sheffield-Moore M, Hewling S, and Paddon-Jones D. Leucine supplementation chronically improves muscle protein synthesis in older adults consuming the RDA for protein. *Clinical Nutrition,* In press, 2012.

effective supplement should produce a robust anabolic response, and be i) low-volume and easily incorporated into existing menu plans, ii) palatable, and iii) cost effective.

From a practical and mechanistic perspective, the branch chain amino acid leucine is an attractive supplement. Leucine has many well described effects on the regulatory mechanisms controlling translation initiation and muscle protein synthesis [8, 9, 62, 63, 65, 71, 76, 96, 270, 273]. Nevertheless, aging muscle appears to be less responsive to the stimulatory effects of normal/typical postprandial concentrations of leucine [76]. Several studies in both animals [75, 80] and humans [72, 78, 79] suggest that increasing the leucine content of regular mixed nutrient meals may normalize or even increase protein synthesis in older populations. Specifically, Rieu *et al*. [75], reported that following a mixed meal, muscle protein synthesis in an older rat population was blunted compared to younger rats. However, the addition of supplemental leucine to the meal restored/normalized muscle protein synthesis in the older animals [75]. In a follow-up study in older adults (70 ± 1 yr), muscle protein synthesis was acutely increased in response to a leucine supplemented meal (0.053 ± 0.009%/h vs. 0.083 ± 0.008%/h, $p < 0.05$) [72]. Of note, however, is the fact that for 4 days preceding the metabolic study, the older adults consumed a controlled diet that limited protein intake to 0.8 g/kg/day [72]. As described below, we propose that habitual protein intake is a key determinant of the efficacy of leucine/protein supplementation regimens.

While the headline results from the leucine studies mentioned above are encouraging, an important emerging caveat is that leucine supplementation is not unconditionally effective. Specifically, ingestion of supplemental leucine appears to be of little benefit to: i) younger adults [270], and/or, ii) individuals who habitually consume a protein/leucine-sufficient diet [274]. For example, in two well designed studies, no change in skeletal muscle mass or strength was reported in a cohort of healthy [274] (n=15; 71±4 yrs) or diabetic older adults [275] (n= 29; 71±1 yrs) who received 2.5 g of supplemental leucine per meal for 3-and 6 months respectively. In both cases, the average daily protein intake was approximately 1.0 g/kg body weight/day and

regular daily exercise was described as moderate. Consequently, we concur with the authors' assertion that a habitual moderate-to-high dietary protein intake may provide sufficient leucine to facilitate a maximal meal-induced protein synthetic response.

The purpose of this study was to determine whether the increase in protein synthesis following a small, low protein and carbohydrate simulated meal could be chronically increased by 2-weeks of low-volume leucine supplementation in healthy, community-dwelling older adults habitually consuming close to the recommended daily allowance (RDA) for protein. We hypothesized that leucine supplementation would improve mixed muscle protein synthesis by stimulating the mTOR signaling pathway and increasing muscle protein synthesis via a more efficient use of meal-derived amino acids.

Experimental Procedures

Subjects:

Eight healthy but sedentary older adults participated in this project. The study was approved by the Institutional Review Board of the University of Texas Medical Branch. Written informed consent was obtained prior to participation. Volunteers were screened at the University Of Texas Medical Branch (UTMB) Institute for Translational Sciences Clinical Research Center (ITS-CRC) to determine eligibility. Inclusion criteria included a habitual protein intake (*assessed via a 3-day dietary recall questionnaire)* at or near the current RDA for protein (*range*: *0.75–0.85 g protein/kg/day*). Exclusion criteria included: a history of regular physical exercise or training (>30 min, 3 times/wk) cardiac, liver, kidney or autoimmune disease; hypo- or hypercoagulation disorders; diabetes; cancer; uncontrolled hypertension, infectious disease, a BMI >30; recent history of anabolic steroid or corticosteroid use or recent participation in a weight loss diet. During the experimental period, subjects were instructed to continue all regular activities of daily living and maintain their usual diet.

Experimental Protocol:

Each subject completed two inpatient visits at the ITS-CRC (days 1 and 15) with an intervening two-week outpatient period involving leucine supplementation (4 g per meal; 3 meals per day; days 2-14). Subjects reported to the ITS-CRC at noon the day before each metabolic study at which time total body fat and lean mass were determined via Dual Energy X-Ray Absorptiometry (DEXA, Hologic, Inc., Natick, MA). On the morning of the metabolic study, following an overnight fast, polyethylene catheters were inserted into the antecubital vein of both arms for infusion of the stable isotope and arterialized venous blood sampling. Baseline blood samples were drawn for the analysis of hormones and background amino acid enrichment. A primed (2 μmol/kg), continuous infusion of L-[ring-$^{13}C_6$]-phenylalanine (Phe) (0.08 μmol·kg^{-1}·min^{-1}) was started (time 0) and continued uninterrupted until the conclusion of each metabolic study (Figure 18). Blood samples were obtained at hours 2 and 3 (*fasting)* and every 20 minutes for the hour prior to and following ingestion of the simulated meal. To simulate a mixed meal, a standardized beverage containing 7 g of essential amino acids and 10 g carbohydrates (sucrose) (Table 3) dissolved in approximately 300 mL of diet soda was ingested at hour 4. L-[ring-$^{13}C_6$] phenylalanine (0.05g) was added to maintain an isotopic steady state. The amino acid content of this meal is consistent with a 80-90 g serving of most animal proteins (e.g., beef, chicken, fish) and while the digestion/absorption kinetics of amino acids would certainly differ from protein-rich foods [58], this relatively small simulated meal has been previously shown to elicit only a modest anabolic response in older adults [8].

A total of four muscle biopsies (~100-200 mg) were taken from the mid-portion of the *vastus lateralis* muscle during each stable isotope study to capture data from the postabsorptive (*biopsy 1 & 2*) and postprandial (*biopsy 3 & 4*) periods (Figure 18). Samples were snap frozen in liquid nitrogen to abruptly stop all enzymatic reactions and frozen at -80˚C for later analysis.

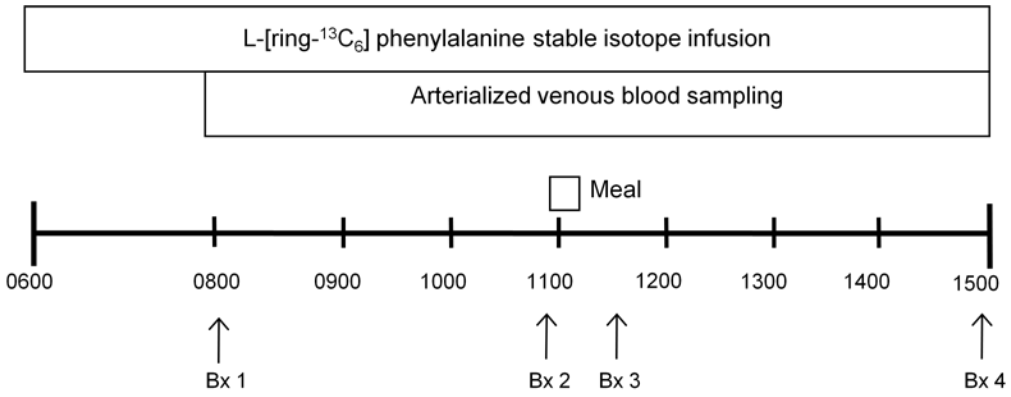


Figure 18: Stable isotope infusion protocol. Stable isotope infusion studies were performed prior to (Day 1) and following (Day 15) leucine supplementation. Arterialized blood samples were obtained at 20-min intervals during an infusion of L-[ring-$^{13}C_6$]-phenylalanine. Basal muscle biopsies from the *vastus lateralis* were obtained at 120 and 240 min. Postprandial muscle biopsies were obtained 30 and 180 min after consuming a simulated meal containing 7 g EAA and 10 g sugar.

Essential Amino Acids	**Weight (g)**
His	0.3
Ile	0.8
Leu	1.7
Lys	1.4
Met	0.3
Phe	0.5
Thr	0.9
Val	0.7
Table Sugar	10
L-[ring-13C6]-phe	0.05

Table 3: Composition of the standardized beverage used as the simulated meal on day 1 and 15.

Analytical Methods:

Dietary analysis from a 3-day diet recall period (pre-study screening) and daily diet logs (14 day supplementation period) were analyzed using Nutrition Data System for Research software version 2006, (Nutrition Coordinating Center Minneapolis, MN).

Phenylalanine enrichments in arterialized venous blood samples were determined after deproteinization with sulfosalicylic acid, extraction with cation exchange chromatography (Dowex AG 50W-8X, 100-200 mesh H+ form; BioRad Laboratories, Inc., Richmond, CA), dried under vacuum (Savant Speedvac, Thermo Fisher Scientific, Inc., Waltham, MA), and *tert*-butyldimethylsilyl (*t*-BDMS) derivatization using gas-chromatography mass-spectrometry (GCMS) in electron impact mode (GC HP 5890, MSD HP 5989, Hewlett Packard, Palo Alto, CA) [276].

Muscle samples were weighed and the proteins precipitated with 800 µl of 10% sulfosalicylic acid. Tissue homogenization and centrifugation were performed on two occasions, and the pooled supernatant was collected. Intracellular amino acids were purified using cation exchange chromatography (Dowex AG 50W-8X, 200-400 mesh H+ form; BioRad Laboratories, Inc., Richmond, CA) and *t*-BDMS derivatization using GCMS in electron impact mode [277]. The remaining pellet containing bound mixed-muscle proteins was repeatedly washed, dried at 50°C overnight and hydrolyzed in 3ml of 6N HCL at 110ºC for 24 hrs. Amino acids in the hydrolysate were extracted and derivatized, as previously described, by monitoring the ions 238 and 240 and using the external standard curve approach [278].

Calculations:

Mixed muscle fractional synthesis rate (FSR) was calculated by directly measuring the incorporation of L-[ring-13C6]-phenylalanine into muscle protein using the precursor-product model:

$$\mathbf{FSR = [(E_{BP2} - E_{BP1}) / (E_P \times t)] \times 60 \times 100}$$

where E_{BP1} and E_{BP2} are the enrichments of bound L-[ring-$^{13}C_6$]-phenylalanine in sequential muscle biopsies, *t* is the time interval between biopsies, and E_P is the mean L-[ring-$^{13}C_6$]-phenylalanine enrichment in the muscle intracellular or plasma precursor pools.

Muscle phenylalanine concentration (C) (μmol/L) in total muscle tissue fluid was determined using a chloride correction [166] and calculated as:

$$\mathbf{C = [Q_{IS} / (V \times E_{IS})]}$$

where Q_{IS} (μmol) is the amount of internal standard added to the sample, V is the volume of muscle tissue fluid and E_{IS} is the tracer-to-tracee ratio of internal standard in total muscle water.

Western Blot Analysis:

Primary antibodies were purchased from Cell Signaling (Beverly, MA): phospho- mTOR (Ser^{2449}), phosphor-p70S6K1 (Thr^{389}), phospho-4E-BP1 ($Thr^{37/46}$),total mTOR, total p70S6K1, total 4E-BP1. Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was purchased from Amersham Bioscience (Piscataway, NJ).

Biopsy samples from the postabsorptive (biopsy 1) and postprandial (biopsy 3 and 4) periods were homogenized in tissue lysis buffer, and protein concentrations were determined. A total of 80 μg of protein was loaded per lane and separated by SDS-PAGE. Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes (PVDF) (Hybond-P, Amersham Biosciences, Piscataway, NJ) and blocked for 1 h. Blots were serially washed and incubated with primary antibody overnight at 4 °C with constant agitation. The next day, the blots were washed and incubated with secondary antibody for 1 h at room temperature with constant agitation. Blots were serially washed and incubated for 5 min with enhanced chemiluminescence reagent (ECL Advanced Western Blotting Detection System, Amersham Biosciences, Piscataway, NJ). Optical density measurements were obtained with a ChemiDoc XRS imaging system (BioRad, Hercules, CA). Densitometric analysis was performed by using Quantity One 1-D analysis software (Ver 4.5.2) (BioRad, Hercules, CA). Data are expressed as

the phosphorylation divided by total protein content (in arbitrary units) normalized to a rodent standard.

Amino Acid Analysis:

Amino acids were analyzed by a solid phase extraction followed by derivatization and a liquid/liquid extraction (EZ:faast Amino Acid Analysis Kit, Phenomenex, Inc., Torrance, CA). Briefly, calibration standards were prepared at concentration of 50, 100 and 200 nmol/mL from 2 standard amino acid solutions. Standard 1 contains a mixture of AAA (α-aminoadipic acid), ABA (α-aminobutyric acid), aIle (allo-isoleucine), Ala, Asp, βAIB (β-aminoisobutyric acid), C-C, Glu, Gly, His, Hyp (4-hydroxyproline), Ile, Leu, Lys, Met, Orn, Phe, Pro, Sar, Ser, Thr, Tyr, and Val. Standard 2 contains Asn, Gln and Trp, which are unstable in acid solution. Calibration standards and serum samples were passed through a sorbent packed tip, binding the amino acids while allowing interfering compounds to flow through. The amino acids on sorbent were then extruded and quickly derivatized in an aqueous solution to facilitated concomitant migrate of the amino acids to the organic layer for additional separation from interfering compounds. The organic layer was then removed, evaporated, and re-suspended in redissolution solvent and analyzed using gas-chromatography mass-spectrometry (GCMS: GC HP 5890, MSD HP 5989, Hewlett Packard, Palo Alto, CA).

Insulin and Glucose Concentrations:

Serum insulin concentrations were measured via an IMMULITE ®2000 (Siemens Healthcare Diagnostics, Deerfield, IL, USA). Plasma glucose concentrations were measured by using an automated glucose analyzer (YSI, Yellow Springs, OH, USA). Four blood draws were taken prior to ingestion of a test meal, and these samples were averaged and reported as the basal value. All postprandial blood draws were analyzed and reported individually.

Statistical Analysis:

Each subject served as his or her own control. All values are expressed as means ± SEM. Comparisons were performed by using ANOVA with repeated measures, the effects being time and day. For post hoc testing we used Bonferroni when appropriate. Significance was set at $P \leq 0.05$. Descriptive data are presented as means ± SEM.

Results

Subject Characteristics:

Eight healthy volunteers (*5 male*, *3 female*) completed the study. Subjects characteristics were: age: 68 ± 2 yr, height: 168 ± 4 cm, weight: 76 ± 2 kg, BMI: 27 ± 2 kg/m^2, body fat: 30 ± 3%. There was no change in lean (Day 1: 47.3 ± 3.7 kg vs. Day 15: 47.0 ± 3.7 kg) or fat mass (Day1: 21.5 ± 2.6 vs. Day 15 = 22.6 ± 3.3 kg) during the 2-week intervention ($p > 0.05$). Habitual protein consumption during the study period was 0.81 ± 0.04 g protein/kg/day, or approximately 62 g protein/day. Daily energy consumption from dietary record analysis was 1840 ± 125 kcal/day. No compliance issues were noted during the study.

Plasma and Muscle Enrichments:

Plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments are depicted in figure 19. There were minor fluctuations in postprandial phenylalanine enrichment, however, no differences were observed from day 1 to day 15. Muscle phenylalanine enrichments and concentrations are also presented in Table 4. Muscle phenylalanine concentrations remained constant throughout the study with no differences from Day 1 to Day 15. Muscle free phenylalanine enrichments increased over time with no differences from Day 1 to Day 15.

Skeletal Muscle Protein Synthesis:

Two weeks of leucine supplementation increased postabsorptive muscle protein synthesis (Day 1: 0.063 ± 0.004 vs. Day 15: 0.074 ± 0.007 %/h; p = 0.004) (Figure 20A). The response to the simulated meal was also increased by leucine supplementation (Day 1: 0.075 ± 0.006 vs. Day 15: 0.10 ± 0.007 %/h; p = 0.01) (Figure 20B). A similar pattern was observed using plasma phenylalanine as the precursor (see calculations) for postabsorptive (Day 1: 0.040 ± 0.004 vs. Day 15: 0.048 ± 0.004 %/h; p=0.02) (Figure 20C) and postprandial (Day 1: 0.060 ± 0.002 vs. Day 15: 0.077 ± 0.008 %/h; p = 0. 001) muscle protein synthesis (Figure 20D).

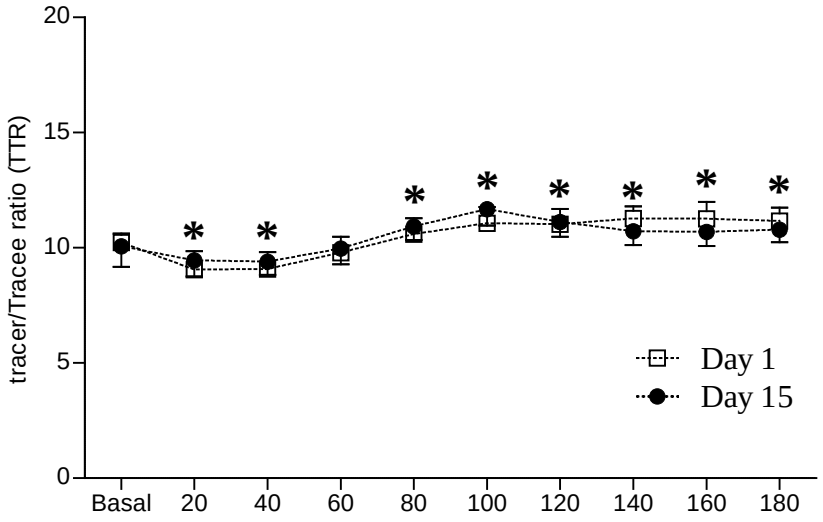


Figure 19: Plasma phenylalanine enrichments. Plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments before and after the simulated meal on Day 1 and Day 15. Values are expressed as means ± SEM. * Significant difference from postabsorptive values.

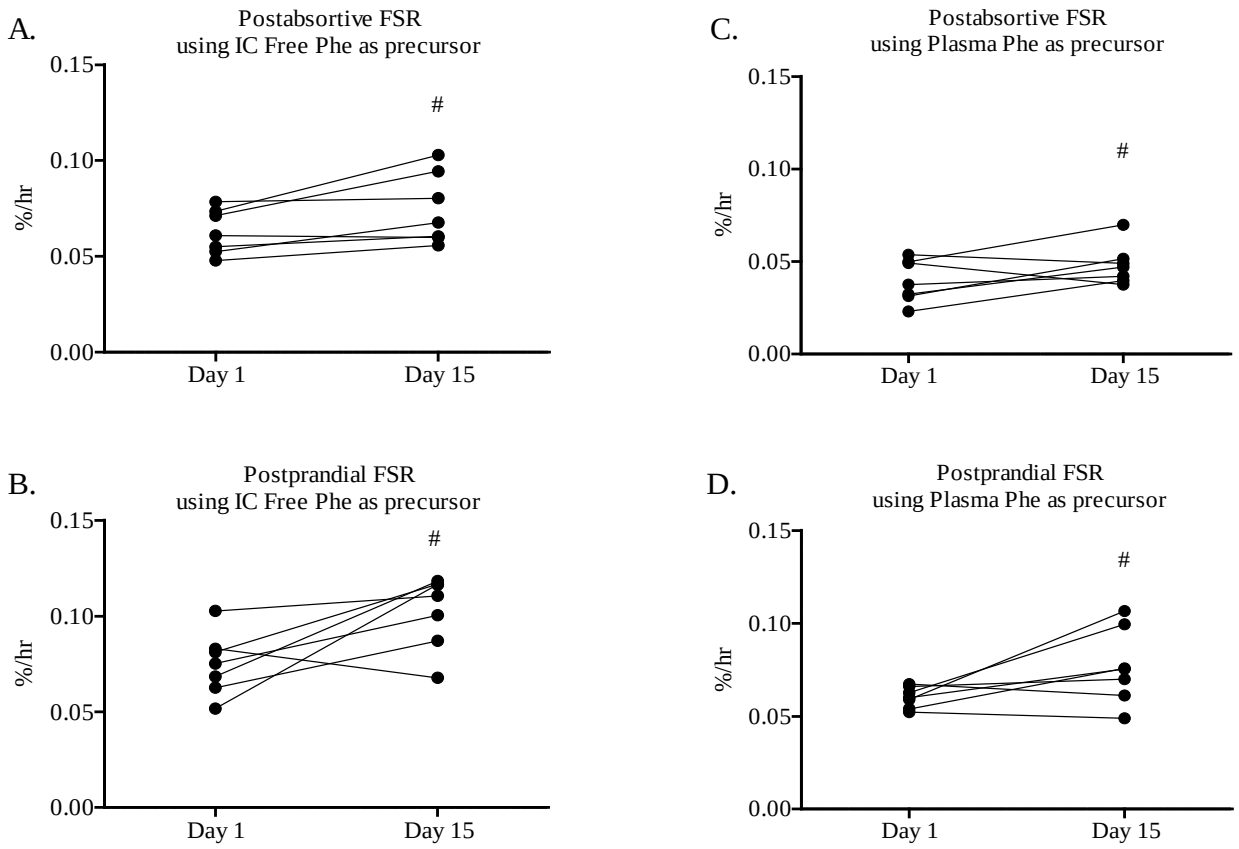


Figure 20: Fractional Synthesis Rate following 14 days of leucine supplementation. Comparison of the mixed-muscle postabsorptive (A) and postprandial (B) FSR measured on Day 1and again on Day 15 utilizing muscle free phenylalanine as the precursor pool. Comparison of the mixed-muscle postabsorptive (C) and postprandial (D) FSR measured on Day 1and again on Day 15 utilizing plasma phenylalanine as the precursor pool. Individual values are presented. Group values are expressed as means ± SEM. # Significant difference from Day 1.

	Day 1			Day 15		
	M1	M2	M4	M1	M2	M4
Muscle phe concentrations, µmol/L	103 ± 18	95 ± 13	90 ± 10	102 ± 16	92 ± 12	97 ± 13
Muscle free phe enrichments (TTR)	0.0541 ± 0.0066	0.0772 ± 0.0057 *	0.0987 ± 0.0085 *	0.0530 ± 0.0047	0.0721 ± 0.0052 *	0.0852 ± 0.0039 *
Muscle bound phe enrichment (TTR)	0.00029 ± 0.00007	0.00037 ± 0.00007 *	0.00060 ± 0.00009 *	0.00066 ± 0.00007	0.00075 ± 0.00007 *	0.0010 ± 0.00007 *
Δ muscle protein enrichment		0.00008 ± 0.00001	0.00023 ± 0.00003		0.00009 ± 0.00001	0.00025 ± 0.00002

Table 4: Muscle phenylalanine concentrations, tracer enrichments and mixed muscle fractional synthesis rate. Values are expressed as means ± S.E.M. TTR, tracer-to-tracee ratio; Muscle phenylalanine concentrations represent the phenylalanine concentration in muscle biopsies taken before (M1 and M2) and after the simulated meal (M4). Muscle phenylalanine enrichments represent the free intracellular phenylalanine enrichment in each muscle biopsy. Muscle protein enrichments represent the bound phenylalanine enrichment in each muscle biopsy. Δ muscle protein enrichment represents the change in muscle bound phenylalanine enrichment between the muscle biopsies. * Significantly different from biopsy 1 ($p < 0.05$).

Nutrient Anabolic Signaling:

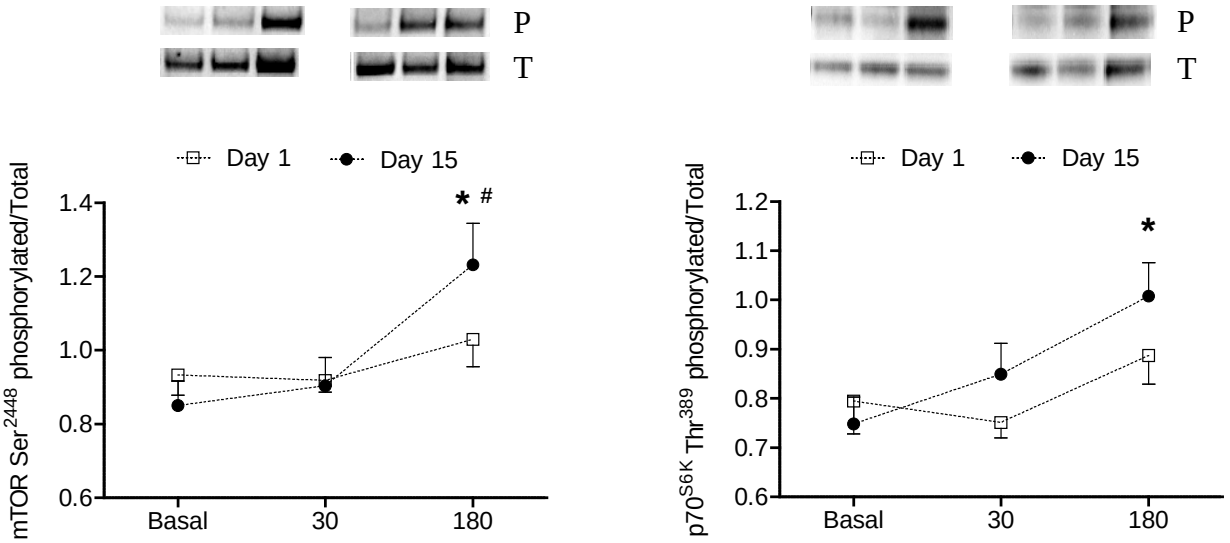
The simulated meal increased the phosphorylation of mTOR, p70S6K1, and 4E-BP1 ($p < 0.05$; Figure 21). Two weeks of leucine supplementation increased mTOR (Day 1: 1.03 ± 0.07 vs. Day 15: 1.23 ± 0.11 AU; p=0.01) and p70S6K1 (Day 1: 0.89 ± 0.06 vs. Day 15: 1.01 ± 0.07 AU) phosphorylation in response to the simulated meal; ($p < 0.05$). I also noted an increase in postabsorptive 4E-BP1 phosphorylation (Day 1: 0.30 ± 0.02 vs. Day 15: 0.37 ± 0.03 AU; p = 0.03). Total protein did not significantly change in response to the simulated meal or from Day 1 to Day 15.

Amino Acid Analysis:

Postabsorptive plasma concentrations of essential and non-essential amino acids did not change following 14 days of leucine supplementation (Table 5).

	DAY 1	DAY 15
Essential Amino Acids		
Leucine	127 ± 9	132 ± 10
Isoleucine	69 ± 7	66 ± 7
Valine	278 ± 19	250 ± 19
Histidine	87 ± 7	80 ± 5
Lysine	182 ± 10	196 ± 15
Methionine	21 ± 2	24 ± 2
Phenylalanine	71 ± 3	82 ± 6
Threonine	144 ± 14	140 ± 13
Non-essential Amino Acids		
Alanine	512 ± 51	483 ± 35
Asparagine	49 ± 5	56 ± 6
Aspartic Acid	27 ± 5	28 ± 4
Glutamic Acid	115 ± 13	119 ± 16
Glutamine	728 ± 58	645 ± 43
Glycine	336 ± 49	330 ± 44
Proline	305 ± 43	278 ± 34
Serine	115 ± 14	114 ± 12
Tyrosine	68 ± 7	76 ± 4
Tryptophan	62 ± 4	67 ± 4
Sarcosine	631 ± 63	596 ± 43
a-Aminobutyric Acid	14 ± 2	16 ± 23
b-Aminoisobutyric Acid	89 ± 0.3	89 ± 0.3
Hydroxyproline	19 ± 2	22 ± 2
a-Aminoadipic Acid	7 ± 0.4	5 ± 0.9
Ornithine	97 ± 7	96 ± 8
Cystine	51 ± 2	52 ± 3

Table 5: Mean serum amino acid concentrations before and after 14 days of leucine-supplementation



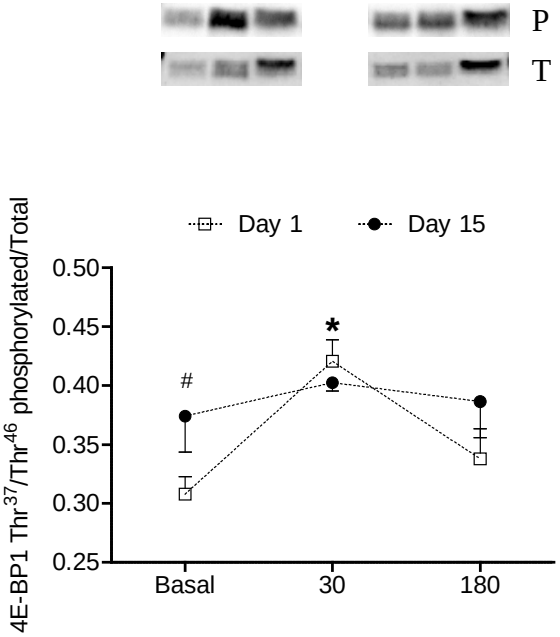


Figure 21: Effects of chronic leucine supplementation on markers of nutrient signaling. Comparisons of the phosphorylation state of mTOR (A), $p70^{S6K}$ (B), and 4E-BP1 (C) obtained on Day 1□(and Day 15 (●). Protein phosphorylation and total protein were determined before and again at 30 and 180 min after ingestion of the simulated meal. A representative blot of the phosphorylation on Day 1 and Day 15 is shown above each graph. Values are expressed as means ± SEM. * significantly different from basal and # significantly different from Day 1; $P < 0.05$.

Serum Insulin and Glucose Concentrations:

No changes in postabsorptive serum insulin concentrations following 2-weeks of leucine supplementation were noted ($P < 0.05$). However, on Day 15, subjects experienced a 33 % lower insulin response 20 minutes following the simulated meal (Day 1: 17 ± 3 pmol/L vs. Day 15: 10 ± 2 pmol/L; p=0.04), (Figure 22A). Glucose concentrations were elevated for approximately 60 minutes following the simulated meal on days 1 and 15 (p=0.02), however, no differences attributable to leucine supplementation were noted ($P < 0.05$; Figure 22B).

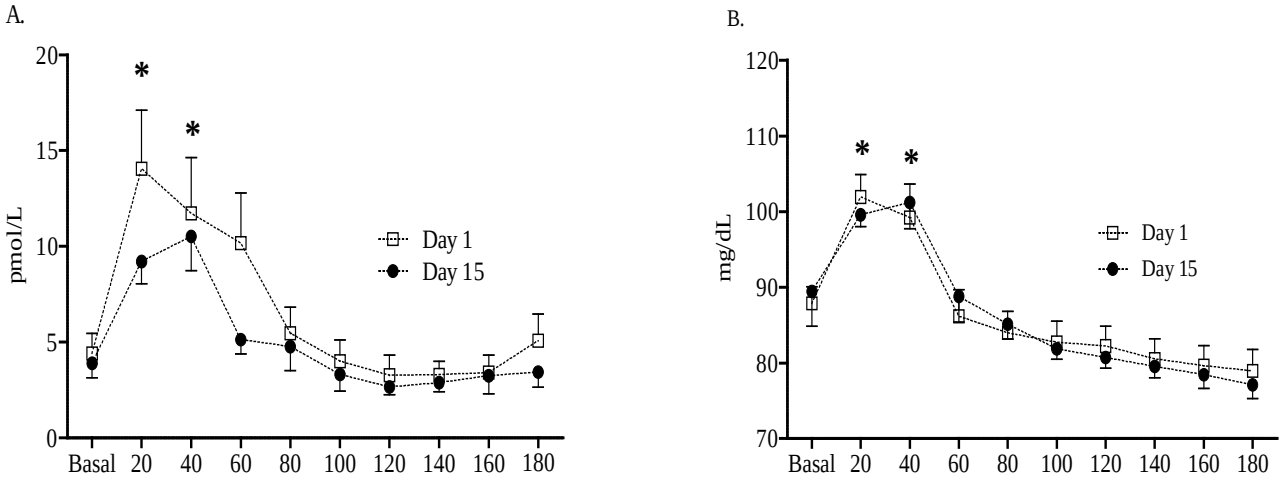


Figure 22: Plasma insulin and glucose concentrations. Plasma insulin (A) and glucose (B) concentrations obtained at Day 0 (□) and Day 14 (●) of leucine supplementation. Me asurements were made before and every 20 minutes after ingestion of the simulated meal. Values are expressed as means ± SEM. * significantly different from basal and # significantly different from Day 1; $P < 0.05$.

Discussion

Our data suggest that leucine supplementation may be an energetically efficient and practical means of chronically improving muscle protein synthesis in response to a low protein meal in older adults who habitually consume close to the RDA for protein. In the context of preventing or slowing sarcopenic muscle loss, the rationale for the use of a dietary supplement should take into account one or more of the following assumptions: i) supplementation will improve net muscle protein anabolism above that afforded by regular meals alone, ii) the supplement will not negatively influence consumption of normal daily meals and iii) use of the supplement will not be compromised or restricted by a lack of compliance, complex preparation, high cost or poor palatability. To this end, we demonstrated that supplementing regular daily meals with a relatively small amount of leucine improves both mixed-muscle protein synthesis and anabolic signaling in older adults. Further, while we did not conduct a specific assessment of practical issues such as longer duration compliance, ease of use or palatability, our low-volume approach is intuitively less likely to be burdensome than most traditional higher-volume amino acid supplement regimens.

A key finding of this study was the postabsorptive and postprandial improvement in muscle protein synthesis after 2-weeks of leucine supplementation. Speed of digestion and absorption notwithstanding, the study meal containing 7 g of essential amino acids and 10 g carbohydrates was designed with two goals in mind. First, we wanted it to mimic the amino acid and carbohydrate content of a typical small meal or snack consumed by older adults (e.g., approximately a half of a chicken breast and a half cup of cooked rice). Second, we wanted to provide a meal that would initially produce a marginal protein synthetic response; a characteristic of meals ingested by older adults at risk of, or experiencing sarcopenic muscle loss. Data from Day 1 clearly show that the simulated meal had a minimal effect on muscle protein synthesis. However, the response to the same meal after 2-weeks of leucine supplementation was quite robust, despite the fact that leucine supplementation was not consumed during the

metabolic study days (Day 1 and 15). Conceptually, this suggests that leucine supplementation is able to chronically up regulate the potential for muscle protein synthesis thereby making an otherwise insufficient or marginal quantity of protein more biologically available and efficient. Notably, we saw no differences in plasma amino acid concentrations during the metabolic studies on Day 1 and 15. In some instances, elevated plasma leucine concentrations have been associated with a reduction in the plasma or intracellular concentrations of other essential amino acids, particularly isoleucine and valine [279].

The anabolic potential of leucine has been attributed to its capacity to stimulate translation initiation both dependently and independently of the mTOR signaling pathway [71, 92]. As noted, aging has been associated with impairment of nutrient-sensing signaling pathways [76]. However, our data show that the postprandial phosphorylation of mTOR, and its downstream effector $p70^{S6K}$, was more pronounced following chronic leucine supplementation [62, 71, 92]. Further, leucine supplementation increased postabsorptive 4E-BP1 phosphorylation, suggesting that leucine supplementation effects muscle protein synthesis through increased eIF4E availability and constitutive hyperphosphorylation of 4E-BP1.

In addition to its effects on muscle anabolism, there is some evidence suggesting that leucine augments glucose-induced insulin secretion. In animal models, chronic leucine supplementation improved insulin sensitivity despite the consumption of a high-fat diet [280]. However, recently Verhoeven, *et al.* [274] reported no such benefit in healthy older individuals. Furthermore, in a group of type 2 diabetic older men no changes were observed in glycemic control with leucine supplementation [275]. Consistent with these findings, basal glucose and insulin concentrations in the present study did not change. However, we did detect a decrease in insulin concentrations immediately after the consumption of the simulated meal. This subtle improvement suggests a contribution of leucine to the enhancement of insulin-mediated glucose disposal in normal healthy aging.

While our results are encouraging, there are clearly limitations both in the design of this study and its generalizability. This was a small, non-randomized study with a limited cohort and no direct measure of muscle protein breakdown Participants were not overtly sarcopenic, but rather representative of a population at increased risk of muscle loss. Despite the lack of a traditional control group, our pre-post-test design demonstrated that leucine supplementation was more effective than the status quo. Further, there is no mechanism or previous data to suggest that an isoenergetic control could provide a comparable result. Similarly, it is possible, but unlikely that an alternate, low-volume isonitrogenous supplement (e.g., 4 g BCAA or EAA/meal) would have produced a similar result. Enthusiasm for an amino acid cocktail is further diminished by palatability, cost and compliance issues.

Given the right circumstances it seems clear that leucine supplementation can acutely increase muscle protein synthesis [9, 57, 65, 273]. Unfortunately however, there is too often disconnect between positive acute metabolic changes and longer-term improvement in outcomes that really matter (e.g. muscle mass, strength) [274]. In the current study, 2 weeks is obviously a very short period of time to observe a measurable change in muscle mass. However, the magnitude of the increase we observed in basal and postabsorptive muscle protein synthesis during this period should have mathematically increased muscle mass by approximately 4% – conservatively assuming no change in muscle protein breakdown. Moving forward, it is clear that additional steps must be taken to clarify and define groups of individuals who will, and importantly, will not benefit from longer-term leucine supplementation.

In conclusion, we have shown that leucine supplementation increases the potential for muscle protein synthesis in older adults and may make an otherwise insufficient or marginal quantity of meal-derived protein more biologically available for muscle tissue growth and repair.

CHAPTER 6

SUMMARY

These studies were designed and implemented to address age-related anabolic resistance in muscle tissue to amino acid supplementation independently and in combination with endurance-type exercise. Specifically, to assess the protein synthetic and intracellular signaling responses of aged muscle tissue during and after a single bout of endurance-type exercise (i.e. treadmill walking) with provision of amino acids, and the affect of increasing the leucine content of each meal consumed by healthy older adults. The principal findings are that: i) during endurance exercise amino acid exchange increases, stimulating muscle protein synthesis without an age-related resistance to the anabolic effects of exogenous amino acids, ii) following an acute bout of endurance exercise the anabolic effect of exogenous amino acids is reduced in aged muscle, although this does not manifest as noticeable changes in overall net protein balance, iii) the molecular events underlying these changes are marked by increases in the activation of proteins involved in muscle hypertrophy signaling, and decreases in muscle atrophy signaling, and iv) increasing the leucine content of each meal enhances the anabolic stimulus of a mixed nutrient meal in older adults. Taken together, these results provide interventional strategies to stimulate muscle protein anabolism in older adults to counter age-associated muscle loss, and provide insight into the intracellular signaling events involved in the skeletal muscle response to both endurance exercise and amino acid supplementation.

When addressing the issue of age-associated muscle loss, resistance training is typically recommended as the best countermeasure. Time and again, however, the anabolic effect of resistance exercise has been shown to be diminished in aged muscle tissue [11-14]. In addition, older adults are not as likely to begin a regimen of resistance training due to a residual cultural nonacceptance by their generation, fear of injury, monetary or transportation constraints, and a general lack of knowledge of resistance exercises. Therefore, low-intensity endurance exercise such as walking is a convenient and suitable form of exercise for older adults. The combined

stimulatory effect of endurance exercise and exogenous amino acids on microvascular perfusion, substrate exchange, and protein accretion on aged muscle tissue, as observed in the preceding studies, signify that endurance exercise is a practical countermeasure to help combat age-related anabolic resistance. These studies demonstrate that endurance-type exercise is capable of stimulating muscle protein synthesis in both younger and older adults similarly; overcoming years of dogma that have claimed it incapable. Taken together, these results suggest that relatively low-intensity endurance exercise is capable of producing a robust "youthful" anabolic response in aged muscle tissue. On the other hand, the results also confirm that aging *per se* is associated with a diminished anabolic responsiveness to physiological doses of amino acids, thereby stressing the importance of taking in sufficient high quality protein.

Establishing a basic nutrition foundation and focusing on adequate protein and energy consumption is necessary to combat age-associated muscle loss. It is important to note that simply increasing protein intake may not be sufficient, as muscle protein synthesis is not only influenced by the quantity but also by the quality of the protein. In addition, older adults appear to require a greater amount of leucine to stimulate muscle protein synthesis [72, 78, 79]. Accordingly, the addition of 4 g of leucine to each meal was utilized to enhance the anabolic response of a typical low-protein/mixed nutrient meal commonly consumed by older adults. The results from the study presented in chapter 5 suggest that older adults should focus on increasing the quantity of high-quality, leucine-rich proteins at each meal. Thus signifying that increasing the leucine content of meals is an effective nutritional countermeasure to increase anabolic sensitivity of aged muscle tissue to exogenous amino acids, facilitating a robust skeletal muscle protein synthetic response.

In order to continually develop or improve existing interventions to combat age-related muscle loss it is important to understand the intracellular signaling events involved in the skeletal muscle response. Both the nutritional and exercise interventions used in these studies stimulated muscle protein synthesis through the activation of the mTOR-signaling pathway, supporting the

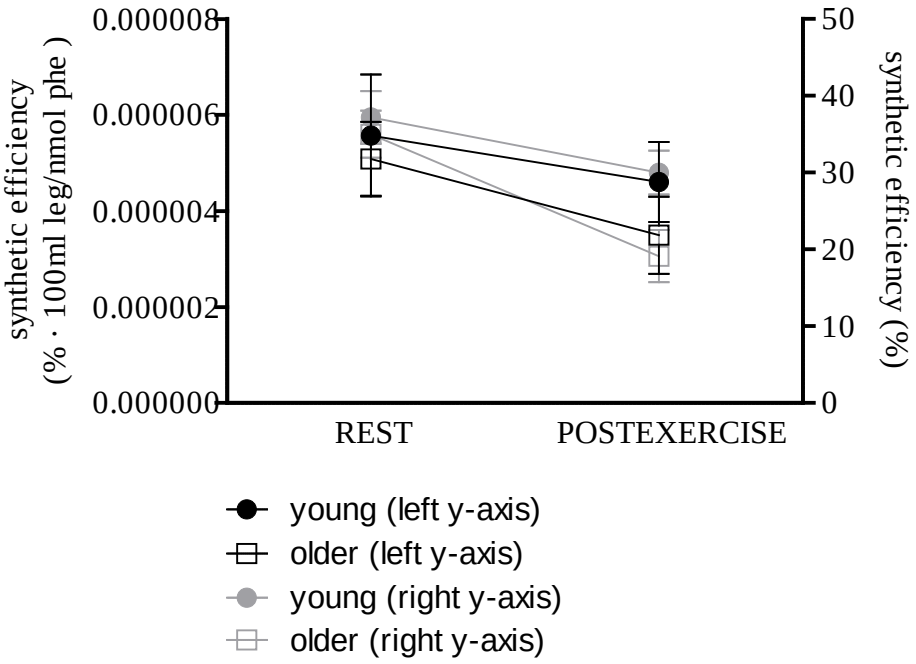
concept that it is an important and necessary molecular mechanism for the anabolic response of skeletal muscle. In addition, muscle protein breakdown is reduced following a single bout of low-intensity endurance exercise with provision of amino acids as evidenced by a reduction in the total protein expression of MAFbx. Since the maintenance of skeletal muscle requires an intricate, yet dynamic, balance between both muscle protein synthesis and muscle protein breakdown, these results further support the concept of using low-intensity endurance exercise as a countermeasure to help combat age-related anabolic resistance.

Collectively, the results from these studies provide evidence-based, practical interventions to increase the sensitivity of aged muscle tissue to the anabolic stimulus of exercise and mixed nutrient meals. While the focus of these studies was centered on age-related anabolic resistance, the practical approach of these studies also makes them applicable to many other at-risk populations (e.g. chronic illness, hospitalization, cancer, and HIV). Continued research is needed to fully understand the mechanisms involved with the loss of skeletal muscle and the intricate yet complex relationship between nutrition and exercise.

APPENDICES

Appendix A

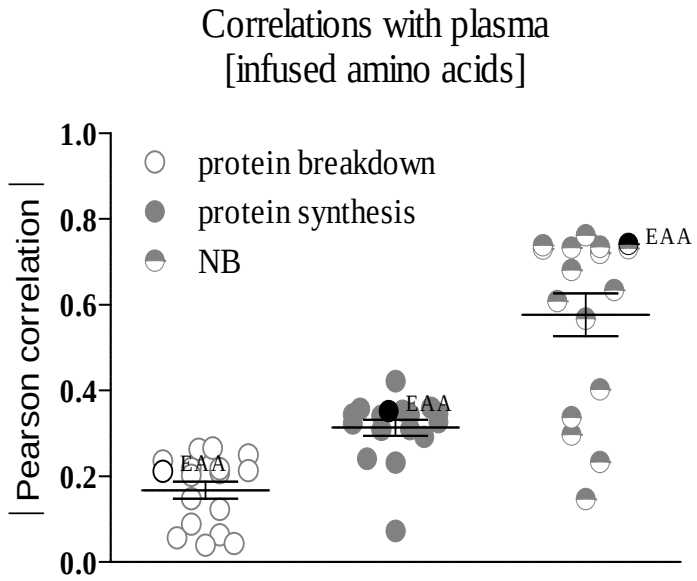
Skeletal muscle protein synthetic efficiency calculated with FSR (left y-axis) and with protein synthesis calculated using the 3-pool model described in Experimental Procedures (right y-axis). For the post-exercise determinations, means values of postexercise intracellular phenylalanine rate of appearance and postexercise protein synthesis (model-derived) were used.



Appendix B

Dot-plot of correlations between infused amino acids and protein synthesis, protein breakdown, and net balance (NB). Correlations for the sum of the infused essential amino acids are also plotted.

A



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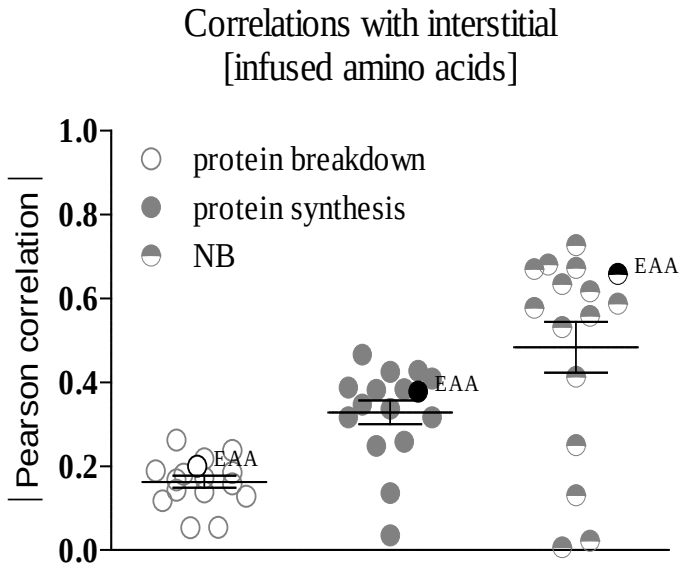
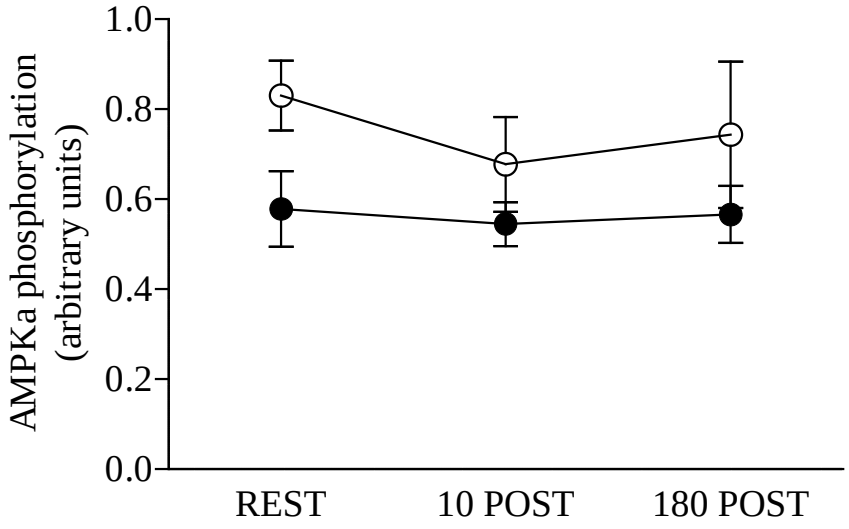


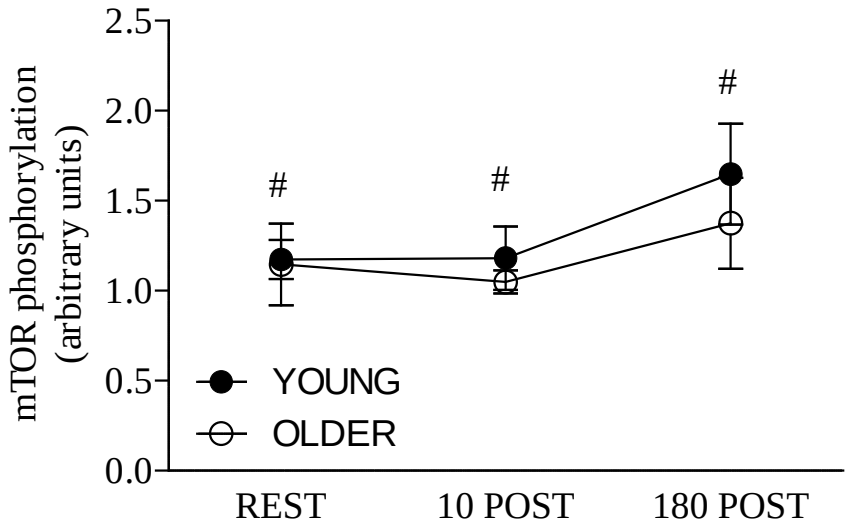
Appendix C

Phosphorylation of AMPK (A) and mTOR (B) in young and older subjects at REST and at 10 and 180 minutes post-exercise (POST). #Significant main effect of time (P=0.01)

A



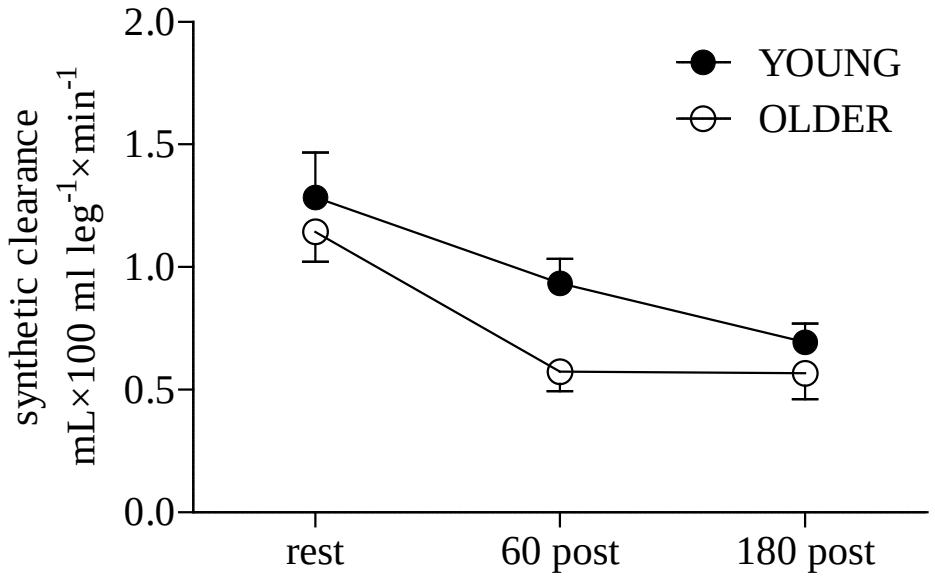
B



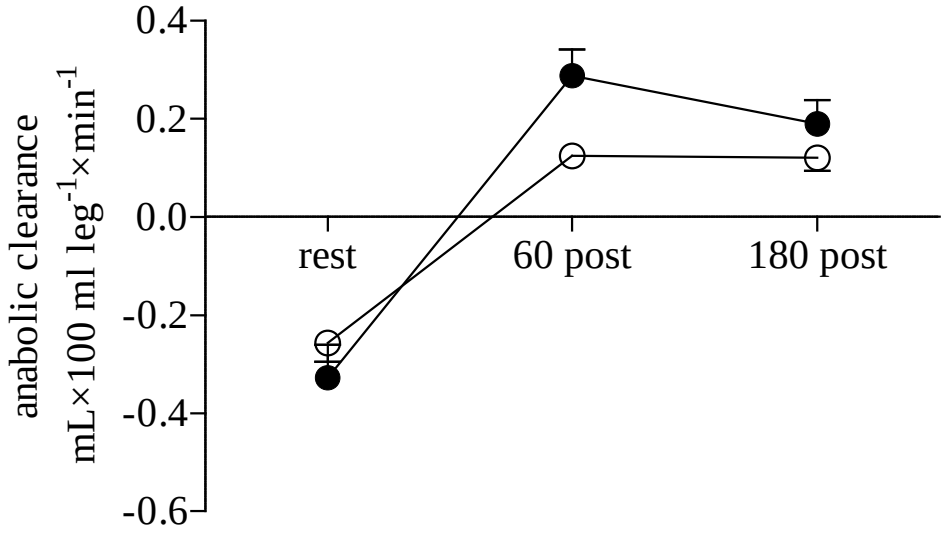
Appendix D

Anabolic clearance (A) and synthetic clearance (B) in the young and older individuals.

A



B



Appendix E

	REST						60 POST						120 POST						180 POST						P values for main effects	
	Young	sd	N	Old	sd	N	Young	sd	N	old	sd	N	young	sd	N	old	sd	N	young	sd	N	old	sd	N	age	time
Taurine	8.6	2.5	9	10.3	2.6	7	8.2	1.4	9	10.6	2.5	7	7.4	0.9	9	10.8	1.6	6	8.2	1.9	9	9.2	1.5	6	0.0107	0.5616
Phosphoserine	69.7	22.2	9	73.7	20.0	7	87.7	21.1	9	88.4	13.8	7	81.2	22.3	9	94.5	23.7	6	79.8	29.9	9	81.3	12.1	6	0.6663	0.0033
Urea	6896	3703	9	9102	4390	7	9295	3368	9	12151	5411	7	8253	3080	9	9459	2400	6	8038	2812	9	8674	1573	6	0.1403	0.0984
Aspartic Acid	7.8	2.8	9	12.7	11.5	7	32.8	5.8	9	40.1	11.2	7	29.4	9.0	9	42.0	14.7	6	28.0	12.5	9	39.0	9.5	6	0.0231	<.0001
Threonine	136.6	34.5	9	149.3	57.6	7	230.7	58.8	9	230.8	28.1	7	234.1	66.9	9	241.7	52.5	6	222.9	61.1	9	234.8	28.6	6	0.7591	<.0001
Serine	114.7	28.6	9	134.2	43.8	7	180.7	43.3	9	186.3	33.6	7	175.2	45.9	9	179.3	37.2	6	167.9	46.2	9	172.8	21.6	6	0.5484	<.0001
Asparagine	68.1	7.9	9	83.0	31.5	7	56.3	13.2	8	56.7	23.2	7	41.4	12.5	8	58.7	28.1	4	40.1	14.2	4	54.3	26.3	3	0.4894	<.0001
Glutamic Acid	161.5	44.2	9	187.9	56.6	7	208.1	33.0	9	256.3	75.3	7	215.8	54.4	9	277.3	115.8	6	196.0	42.3	9	249.7	69.9	6	0.1515	<.0001
Glutamine	798.6	68.0	9	1054.2	241.4	7	818.7	133.6	9	938.0	170.5	7	803.3	100.2	9	923.3	221.0	6	761.2	111.9	9	877.7	121.3	6	0.0115	0.2094
Glycine	266.8	28.4	9	308.6	146.5	7	327.3	48.7	9	336.4	68.6	7	316.0	50.6	9	321.4	64.1	6	294.8	56.4	9	318.2	69.6	6	0.4948	0.0854
Alanine	299.7	82.4	9	373.5	91.1	7	431.9	81.7	9	484.9	120.4	7	403.4	114.4	9	432.6	154.7	6	366.1	78.2	9	394.3	92.0	6	0.2665	<.0001
Citrulline	38.5	9.6	9	53.9	13.0	7	49.5	8.0	9	64.0	11.0	7	49.9	7.2	9	70.4	18.3	6	49.8	7.6	9	68.3	8.5	6	0.0015	<.0001
a-Aminobutyric Acid	24.7	8.1	9	27.8	4.7	7	29.5	9.6	9	30.7	6.9	7	31.9	8.6	9	33.8	10.6	6	32.5	10.6	9	35.1	8.6	6	0.5783	<.0001
Valine	213.2	19.9	9	286.3	84.1	7	532.3	66.8	9	620.8	124.3	7	615.2	95.0	9	748.2	245.2	6	629.8	93.0	9	774.0	144.3	6	0.0296	<.0001
Methionine	26.1	5.6	9	37.7	25.9	7	94.0	16.4	9	108.1	18.9	7	95.2	24.2	9	115.4	37.1	6	89.2	17.9	9	108.3	24.4	6	0.0793	<.0001
Cystine	7.0	3.6	6	13.2	5.4	7	9.6	3.1	9	20.1	6.1	7	9.8	3.3	8	21.6	7.1	6	10.0	2.5	9	19.2	5.4	6	<.0001	0.0013
Isoleucine	56.6	21.7	9	101.4	69.7	7	286.7	41.9	9	312.7	140.9	7	313.4	95.3	9	422.5	151.9	6	332.0	67.0	9	434.2	101.2	6	0.0661	<.0001
Leucine	163.4	33.4	9	229.0	115.1	7	550.0	77.2	9	649.8	133.7	7	632.7	112.6	9	791.8	278.7	6	645.4	129.6	9	811.3	183.0	6	0.0414	<.0001
Tyrosine	56.3	18.7	9	63.7	26.9	7	85.7	14.1	9	87.5	15.6	7	79.8	23.0	9	74.9	34.2	6	70.0	16.1	9	70.0	12.5	6	0.8339	<.0001
Phenylalanine	74.3	10.6	9	92.7	25.6	7	156.0	20.1	9	170.9	35.1	7	164.4	33.9	9	188.6	47.5	6	158.9	25.0	9	182.5	26.0	6	0.0796	<.0001
NH3	107.7	10.7	9	101.1	20.2	7	131.9	13.8	9	124.0	17.2	7	133.0	18.9	9	130.7	26.7	6	125.7	17.5	9	123.9	20.9	6	0.5431	<.0001
Ornithine	79.1	13.0	9	86.2	31.7	7	133.1	23.4	9	168.0	48.2	7	155.9	32.1	9	245.5	91.2	6	159.4	28.8	9	236.7	54.4	6	0.0201	<.0001
Lysine	226.8	42.0	9	307.2	96.6	7	476.3	103.0	9	525.7	89.0	7	481.2	136.4	9	556.6	179.0	6	446.0	104.2	9	516.8	95.2	6	0.1318	<.0001
1-Methyl Histidine	13.4	6.2	9	16.2	9.0	7	16.0	9.1	9	15.0	5.5	7	16.4	7.4	9	15.7	5.1	6	15.0	9.0	9	14.9	6.4	6	0.9795	0.4658
Histidine	85.2	12.2	9	101.3	36.2	7	160.3	26.1	9	165.0	29.5	7	163.0	34.5	9	172.1	52.0	6	156.3	30.3	9	165.6	26.9	6	0.4903	<.0001
3-Methyl Histidine	5.4	1.1	8	5.9	1.4	7	4.6	0.8	7	6.5	1.9	7	5.3	0.7	8	5.8	2.6	5	4.9	0.6	6	5.5	1.0	6	0.0233	0.8299
Arginine	96.3	29.2	9	168.7	116.1	7	373.5	100.1	9	415.8	111.8	7	387.6	119.3	9	399.5	150.3	6	363.6	101.5	9	395.5	62.2	6	0.2656	<.0001
EAA	1038.4	130.6	9	1368.6	510.5	7	2572.0	397.6	9	2871.4	580.8	7	2778.9	584.3	9	3311.7	1058.4	6	2750.6	498.0	9	3297.5	613.4	6	0.0755	<.0001

Appendix F

	REST						60 POST						120 POST						180 POST						P values for main effects	
	young	sd	N	old	sd	N	young	sd	N	old	sd	N	young	sd	N	old	sd	N	young	sd	N	old	sd	N	age	time
Taurine	23.6	9.6	9	26.5	14.2	8	21.7	5.3	8	30.5	7.5	6	23.9	8.5	8	31.6	12.2	5	29.5	10.1	3	30.4	8.9	5	0.2212	0.6478
Phosphoserine	117.5	73.3	9	141.1	114.3	8	118.7	53.8	8	143.6	62.4	6	86.2	45.3	8	99.5	31.3	5	77.2	32.7	3	101.3	39.5	5	0.3752	0.1826
Urea	8233.8	2563.0	8	12588.4	3825.4	6	8953.7	4664.2	8	16069.2	8034.1	6	9879.6	3398.7	8	12294.8	5619.8	5	12014.8	5138.8	3	14971.8	6152.6	5	0.0379	0.3113
Aspartic Acid	42.1	11.3	2			0	29.6	10.2	7	40.9	9.9	6	24.3	12.7	8	45.8	20.3	5	25.2	8.6	3	49.4	23.5	5	0.0135	0.4645
Threonine	126.0	46.6	9	105.6	48.0	8	177.6	67.1	8	200.6	62.7	6	182.5	66.3	8	218.0	76.5	5	168.8	22.6	3	223.9	35.7	5	0.7926	<.0001
Serine	88.3	29.0	9	96.2	44.4	8	130.1	41.8	8	184.8	54.2	6	137.4	53.4	8	170.4	83.3	5	129.2	12.3	3	185.1	50.5	5	0.1304	0.0004
Glutamine	510.6	180.9	9	582.1	338.3	8	520.3	168.5	8	691.2	209.0	6	490.2	151.6	8	618.8	253.9	5	473.3	18.7	3	624.6	131.1	5	0.3256	0.8423
Glycine	294.1	78.2	9	394.8	248.2	8	369.7	199.8	8	436.4	149.2	6	320.7	87.9	8	431.1	166.4	5	302.8	43.3	3	462.9	128.1	5	0.2393	0.6918
Alanine	410.4	122.6	9	388.3	179.7	8	500.9	169.2	8	597.2	180.0	6	457.2	124.9	8	498.4	313.4	5	465.8	76.4	3	544.4	257.3	5	0.7125	0.0242
Citrulline	57.3	37.7	8	66.7	65.1	7	53.1	34.5	8	77.9	45.2	6	26.4	19.1	8	77.6	41.9	5	25.9	21.1	3	77.3	20.1	5	0.0814	0.5675
a-Aminobutyric Acid	30.1		1	25.2	11.1	4	20.4	15.7	2	43.2	3.2	2	22.8	15.9	4	45.1	6.6	3	20.2	0.1	2	45.5	7.9	4	ND	ND
Valine	190.5	63.7	9	209.2	134.4	8	340.6	116.1	8	545.7	152.6	6	409.8	127.4	8	654.3	296.9	5	423.4	28.3	3	793.6	172.0	5	0.0192	<.0001
Methionine	27.5	7.5	9	25.2	15.7	6	65.1	19.4	8	89.3	27.6	6	75.3	20.9	8	84.7	50.5	5	73.5	3.0	3	110.0	39.6	5	0.2161	<.0001
Cystine	26.8	7.2	9	50.2	26.2	8	26.0	10.6	8	58.3	18.9	6	31.0	12.2	8	63.5	25.0	5	34.1	8.6	3	67.7	14.8	5	0.0021	0.1972
Isoleucine	45.5	13.1	9	44.7	27.2	7	169.3	59.1	8	241.3	89.3	5	208.5	73.2	8	293.1	142.0	5	200.6	5.7	3	369.4	96.4	5	0.0829	<.0001
Leucine	171.7	53.7	9	159.5	95.9	8	419.4	137.8	8	569.0	187.8	6	526.6	191.1	8	644.3	302.6	5	541.7	45.4	3	829.1	213.9	5	0.1667	<.0001
Tyrosine	67.9	15.5	9	54.0	30.3	8	63.2	16.3	8	53.3	28.5	6	64.9	14.2	8	57.7	27.9	5	66.5	7.9	3	52.0	12.7	5	0.0898	0.9526
Phenylalanine	66.1	18.3	9	73.8	30.9	8	110.0	33.9	8	155.1	46.3	6	121.2	30.6	8	181.7	84.9	5	120.0	4.4	3	190.1	38.8	5	0.0388	<.0001
NH3	291.6	187.0	9	453.5	245.2	8	136.2	123.1	8	418.2	256.7	6	157.7	111.0	7	372.8	182.8	5	11.4	6.8	2	398.9	171.2	5	0.0111	0.1576
Ornithine	45.2	15.1	9	146.2	263.2	8	65.9	25.4	8	127.6	47.1	6	89.0	30.3	8	166.3	80.3	5	88.6	14.2	3	196.5	57.6	5	0.0128	0.6447
Lysine	175.5	56.3	9	180.9	85.8	8	307.4	90.0	8	388.7	104.0	6	322.2	97.4	8	435.8	206.4	5	305.1	3.5	3	450.8	141.3	5	0.1863	<.0001
1-Methyl Histidine	110.6	57.8	6	99.3	44.2	7	134.6	77.7	5	195.8	50.7	5	114.8	90.0	3	147.2	111.9	5	146.9	57.4	3	144.5	124.3	5	0.7388	0.1984
Arginine	63.5	31.3	9	85.9	46.7	8	202.3	92.3	8	273.9	78.7	6	218.5	135.0	8	305.2	123.2	5	206.0	79.8	3	331.4	57.1	5	0.0496	<.0001
EAA (-his)	870.6	257.6	9	841.0	428.1	8	1652.6	526.5	8	2202.7	650.5	6	1911.1	610.5	8	2569.6	1167.1	5	1899.6	57.1	3	3019.0	727.6	5	0.1328	<.0001

Appendix G

	TAURINE	PHOSPHOSERINE	UREA	ASPARTATE	THREONINE	SERINE	GLUTAMINE	GLYCINE	ALANINE	VALINE	METHIONINE	CYSTINE	ISOLEUCINE	LEUCINE	TYROSINE	PHENYLALANINE	NH3	ORNITHINE	LYSINE	ONEMH	HISTIDINE	ARGININE	EAA
Pearson r	.116	.088	.085	.399	.697	.655	.430	.624	.547	.824	.783	.660	.872	.869	.063	.716	-.091	.343	.721	-.493	.858	.716	.807
P	.433	.550	.578	.021	.000	.000	.002	.000	.000	.000	.000	.000	.000	.000	.672	.000	.549	.017	.000	.003	.000	.000	.000
N	48	48	45	33	48	48	48	48	48	48	46	44	46	48	48	48	46	48	48	35	17	48	48

Appendix H

	Ea		Ev		Em		Ca		Cv		Cm	
	young	old	young	old	young	old	young	old	young	old	young	old
Rest	0.109918	0.128162	0.083916	0.097461	0.058434	0.073438	62.6	75.0	68.3	80.3	53.5	88.0
SD	0.029582	0.009065	0.022536	0.006124	0.019755	0.009820	4.5	3.4	6.3	4.3	10.1	25.2
60 Post	0.057306	0.065133	0.053447	0.060954	0.045044	0.055798	138.1	179.0	131.9	174.7	78.4	120.6
SD	0.014672	0.003629	0.013976	0.002771	0.013596	0.006840	11.4	9.4	12.0	8.3	9.3	38.1
180 Post	0.057160	0.066616	0.052624	0.061812	0.045581	0.057936	138.1	173.2	133.9	168.9	80.4	122.5
SD	0.015301	0.004414	0.014177	0.003353	0.013483	0.006089	13.7	19.6	14.8	18.7	9.1	33.5

Appendix I

	Fin		Fout		NB		Fma		Fvm		Fva		Fmo		Fom	
	young	old	young	old	young	old	young	old	young	old	young	old	young	old	young	old
REST	239.4	267.7	260.3	287.1	-20.8	-19.4	117.7	143.8	138.5	163.2	121.8	123.8	100.9	105.7	80.1	86.3
SD	60.2	65.5	62.5	73.0	12.1	8.3	52.7	55.2	56.8	62.4	44.1	42.1	34.8	31.5	32.4	26.5
60 POST	914.6	997.9	875.6	975.7	39.1	22.2	325.4	552.4	285.0	530.2	625.5	445.5	88.6	79.2	129.0	101.5
SD	433.7	259.0	428.0	258.9	18.9	7.4	161.9	292.4	158.9	289.4	298.1	307.0	30.4	35.2	39.3	32.5
180 POST	717.6	903.0	692.1	882.4	25.5	20.5	307.8	528.1	286.3	506.5	396.5	359.7	73.9	74.2	95.3	95.8
SD	228.9	251.1	223.4	246.8	17.0	12.3	167.2	257.0	161.2	248.6	121.3	251.7	22.7	29.2	27.9	39.9

Appendix J

Amino Acid		NB	PB	PS
taurine	Pearson Correlation	-.147	.264	.073
	N	40	38	38
phosphoserine	Pearson Correlation	.320	.090	.216
	N	40	38	38
urea	Pearson Correlation	.122	.319	.325
	N	40	38	38
aspartate	Pearson Correlation	.609	-.089	.358
	N	40	38	38
threonine	Pearson Correlation	.635	-.250	.241
	N	40	38	38
serine	Pearson Correlation	.568	-.064	.346
	N	40	38	38
asparagine	Pearson Correlation	-.391	.510	.106
	N	33	31	31
glutamate	Pearson Correlation	.298	.044	.232
	N	40	38	38
glutamine	Pearson Correlation	-.112	.159	.018
	N	40	38	38
glycine	Pearson Correlation	.234	.214	.310
	N	40	38	38
alanine	Pearson Correlation	.338	.057	.292
	N	40	38	38
Alpha aminobutyric acid	Pearson Correlation	.391	.202	.397
	N	40	38	38
Valine	Pearson Correlation	.732	-.236	.324
	N	40	38	38
Methionine	Pearson Correlation	.731	-.209	.353
	N	40	38	38
Cystine	Pearson Correlation	.146	.049	.128
	N	37	35	35
Isoleucine	Pearson Correlation	.734	-.218	.341
	N	40	38	38
Leucine	Pearson Correlation	.739	-.218	.344
	N	40	38	38
Tyrosine	Pearson Correlation	.403	.040	.309
	N	40	38	38
Phenylalanine	Pearson Correlation	.721	-.203	.345
	N	40	38	38
NH3	Pearson Correlation	.555	-.068	.321
	N	40	38	38
Ornithine	Pearson Correlation	.572	-.200	.240
	N	40	38	38
Lysine	Pearson Correlation	.681	-.148	.360
	N	40	38	38
oneMH	Pearson Correlation	.051	-.119	-.201
	N	40	38	38
Histidine	Pearson Correlation	.735	-.123	.422
	N	40	38	38
3-methylhistidine	Pearson Correlation	-.129	.333	.221
	N	35	33	33
Arginine	Pearson Correlation	.762	-.266	.327
	N	40	38	38
Citrulline	Pearson Correlation	.381	.020	.279
	N	40	38	38
EAA	Pearson Correlation	.742	-.212	.352
	N	40	38	38

Appendix K

Amino Acid		NB	PB	PS
IF taurine	Pearson Correlation	.131	.238	.337
	N	35	33	33
IF phosphoserine	Pearson Correlation	-.058	.289	.205
	N	35	33	33
IF urea	Pearson Correlation	.219	.036	.231
	N	32	30	30
IF aspartate	Pearson Correlation	.023	.158	.136
	N	20	18	18
IF threonine	Pearson Correlation	.578	-.263	.249
	N	35	33	33
IF serine	Pearson Correlation	.532	-.139	.317
	N	35	33	33
IF glutamine	Pearson Correlation	.093	.071	.142
	N	35	33	33
IF glycine	Pearson Correlation	.250	.054	.259
	N	35	33	33
IF alanine	Pearson Correlation	.414	.129	.466
	N	35	33	33
IF citrulline	Pearson Correlation	-.021	.362	.271
	N	33	31	31
IF valine	Pearson Correlation	.559	-.181	.317
	N	35	33	33
IF methionine	Pearson Correlation	.670	-.190	.384
	N	33	31	31
IF cystine	Pearson Correlation	.021	-.005	.015
	N	35	33	33
IF isoleucine	Pearson Correlation	.673	-.218	.382
	N	33	31	31
IF leucine	Pearson Correlation	.681	-.185	.410
	N	35	33	33
IF tyrosine	Pearson Correlation	.007	-.055	-.035
	N	35	33	33
IF phenylalanine	Pearson Correlation	.588	-.173	.347
	N	35	33	33
IF NH3	Pearson Correlation	-.245	.085	-.116
	N	34	32	32
IF ornithine	Pearson Correlation	.113	-.070	.037
	N	35	33	33
IF lysine	Pearson Correlation	.635	-.118	.425
	N	35	33	33
IF 1-MH	Pearson Correlation	.450	.079	.578
	N	29	27	27
IF histidine	Pearson Correlation	.727	-.168	.428
	N	10	8	8
IF 3MH	Pearson Correlation	-.788	.994	.632
	N	3	3	3
IF arginine	Pearson Correlation	.617	-.143	.388
	N	35	33	33
IF EAA	Pearson Correlation	.659	-.200	.378
	N	35	33	33

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VITA

Shanon L. Casperson was born on July 18, 1967 in Brooksville, FL to Kenneth and Betty Cooprider. Shanon graduated from Crystal River High School in 1985 and attended the University of Florida were she was a member of the Gator Marching Band. In 1992 Shanon joined the US Army where she studied the Russian language and was stationed in Germany where she met her husband of 15 years, Darrel. In 1998 Shanon gave birth to their son and soon after was honorably discharged. At this time Shanon began working on her nutrition degree and in 2003 was licensed as a registered dietetic technician. In 2004 Shanon graduated from the University of Houston Clear Lake receiving her B.S. in Fitness and Human Performance. Following graduation, Shanon worked as a research associate for Melinda Sheffield-Moore, Ph.D. In 2006, while maintaining her work in Dr. Moore's lab, Shanon entered the Graduate School of Biomedical Sciences at the University of Texas Medical Branch as a student in Cell Biology. Shanon has made significant contributions to the nutrition and exercise physiology scientific community, receiving several prestigious awards for scientific achievement at both the state and international levels. Shanon's achievements have been recognized with the receipt of several poster competition awards and invitations for oral presentations. In addition, Shanon has been the recipient of scholarships and travel awards to present at various professional conferences. Some of her achievements include the Peyton and Lydia Schapper Endowed Scholarship (2007 and 2010), the Energy and Macronutrient Metabolism Research Interest Section (2009 and 2010), and the prestigious Environmental & Exercise Physiology Section Predoctoral Gatorade Award (2007). In addition, Shanon was first recipient of the Gail E. Butterfield Nutrition Travel Award.

EDUCATION:

A.S., December 2002, San Jacinto College, Houston, Texas

B.S., May 2004, University of Houston Clear Lake, Houston, Texas

PUBLICATIONS:

Published Articles in Peer-Reviewed Journals (10 total, 8 published, 2 in press or review):

Sheffield-Moore, M., Paddon-Jones, D., **Casperson, S.L.**, Gilkison, C., Volpi, E., Wolf, S.E., Jiang, J. and Urban, R.J. Androgen therapy induces muscle protein anabolism in older women. *J. Clin. Endocrinol. Metab.*, 91(10), 3844-3849, 2006.

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First Author Presentations/Abstracts:

Free Weight Power Testing with Muscle Lab. Oral Presentation at the American College of Sports Medicine - Texas Chapter Annual Meeting, 2005.

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Pharmacologic vasodilation increases muscle perfusion and muscle protein anabolism similarly in elderly and young. Experimental Biology Annual Meeting, 2008.

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Anabolic and energetic signaling in human skeletal muscle in response to amino acids and endurance exercise. Experimental Biology Annual Meeting, 2010.

Metabolic Responses to Amino Acids Before and During Aerobic Exercise in Older and Younger Men. American College of Sports Medicine Annual Meeting, 2011.

Permanent address: 406 Shirleen Dr., Seabrook, TX 77586

This dissertation was typed by Shanon L. Casperson.