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Amino Acid Sensing in Muscle: Role of the lysosome and the effect of protein supplementation on improving amino acid sensitivity in aging muscle

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Amino Acid Sensing in Muscle: Role of the lysosome and the effect of protein supplementation on improving amino acid sensitivity in aging muscle

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Dedication

Dedicated to all the people along the way who made my dissertation possible during this long journey.

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Amino Acid Sensing in Muscle: Role of the lysosome and the effect of protein supplementation on improving amino acid sensitivity in aging muscle

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Protein ingestion is a well-studied stimulus for increasing skeletal muscle protein synthesis. Only recently has the mechanism for this phenomenon been elucidated at the cellular level. To this point, most of the mechanistic research into this process has been conducted in human embryonic kidney cells. Therefore, it is necessary to determine if this cellular mechanism is conserved in muscle. If so, it may be possible to manipulate this mechanism in order to enhance skeletal muscle protein turnover, that is the balance between protein synthesis and protein breakdown. Potentially a protein supplement that could exploit this mechanism to promote protein anabolism through either increasing protein synthesis or decreasing protein breakdown would result in improved skeletal muscle health. Reduced strength and muscle mass are predictors of early mortality. This highlights the importance of developing more effective methods to increase muscle mass and strength. Reduced muscle mass and weakness increases the risk for falls in older adults. A fall can lead to a loss of independence and placement in a care facility. Excessive muscle wasting and weakness is also considered a key risk factor for survival with cancer and other diseases. A better understanding of the biology of muscle wasting is needed to develop evidence-based rehabilitation protocols for improving muscle function for a variety of clinical conditions associated with muscle loss.

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CHAPTER 1

Introduction

TRANSLATIONAL RESEARCH

There is a new emphasis in biomedical research for taking what is discovered at the bench and using that knowledge to improve human health. This new focus has been coined "translational research". The overall goal of translational research is to integrate basic science research into clinical practice in order to benefit the public at large. [1]

Basic science is typically considered science for the pursuit of knowledge and without clearly defined benefits for human health. In 1945, the National Science Foundation defined basic research as, "Basic research is performed without thought of practical ends. It results in general knowledge and an understanding of nature and its laws. This general knowledge provides the means of answering a large number of important practical problems, though it may not give a complete specific answer to any one of them. The function of applied research is to provide such complete answers." [2] Since that time, clinical research has also been defined by the National Institutes of Health as any research conducted on humans whether through clinical trial or large epidemiological data-set collection. [3]

This new focus on translational research is the attempt to bridge this research gap between basic science, clinical research and ultimately the implementation of new health care strategies for the general public. Bridging this gap would translate discoveries at the bench into tangible benefits for the public at large. Therefore it is in the best interest of those in the scientific community to relay the importance of translational research to those

in the basic science field so that even research at the most basic level can be guided by the tenet of ensuring that all new knowledge have some translatable benefit to human health.

Translational research from bench to beside is the major focus of this research paradigm but is not the only research method for improving patient outcomes. The reverse direction, bedside to bench, has taken hold as a new research focus and has been coined "reverse translational research". [4] Phenomena witnessed in the clinic may sometimes have no explanation. A drug or other intervention may result in an outcome for the patient for which there is no account found in the scientific literature. This new outcome provides the impetus for a new area of scientific experimentation. Researchers can take these outcomes seen in the clinic and form hypotheses for the origin of these results which can then be tested at the bench. This allows for the entire experimental milieu to be at the researcher's disposal as these drugs or other interventions can be verified in an animal model or even in cell culture. In this case, results in humans can be tested in cells as opposed to information gained from cell experiments being applied to humans.

Translational research, either starting at the bench or at the bedside, is a new method of funneling current research towards improving patient-centered outcomes. This shift in research methodology makes it necessary to focus not only on one individual research model but instead to use all models to ultimately improve the public health. One area of health that would greatly benefit from translational research is health disability.

DISABILITY AND AGING

According to the Department of Health and Human Services, the budget for Medicare spending for the year 2015 is 605.9 billion dollars. In addition, the average number of enrollees per year has tripled over the past ten years. [5,6] As the number of enrolled individuals increases and costs continue to climb for treating older citizens, it is becoming even more imperative that we discern the reason behind the increases in medical

care needs and costs in this country. One major factor for the increased burden on the healthcare system is disability. [7] As the population continues to enjoy a longer life-expectancy, it is the goal of modern medicine to ensure a later onset or absolute avoidance of disability altogether to ensure a high quality of life. Unfortunately this is often not the case. Approximately 56 million Americans are currently living with a disability. Those with disability are at greater risk for falls, frailty, loss of independence and ultimately residence in a care facility. [7]

The concept of the compression of morbidity was conceived in 1980. This theory posits that advances in medical care delay the onset of disability. As a result, people are able to enjoy improved quality of living while performing typical daily tasks of living until a later age. When disability is reached, it is at the very end of the lifespan. Therefore the individual does not suffer from the consequences of disability for very long. The opposite hypothesis states that modern medicine allows people to survive longer with disabilities that were once considered fatal. As a result, a longer life expectancy results in living longer with disability. This means reduced activities of daily living as well as overall quality of life for all of those additional years of the lifespan. [8] While neither hypothesis has been shown to be conclusive, what is not in doubt is the need to create strategies to fight disability in order to improve quality of life.

Many chronic diseases such as diabetes, heart disease and cancer can lead to disability. [9] As such preventing these diseases may prevent the onset of disability. Doctors often prescribe smoking cessation to reduce the risk of lung cancer or a change in diet to lessen the likelihood of developing diabetes. The one major risk factor that is often overlooked much more so than these behavioral or dietary modifications is age. Getting older is the greatest risk factor for developing a host of chronic diseases that can lead to disability. The mortality rate for a host of diseases including: respiratory disease, kidney disease, cardiovascular disease and diabetes increases tenfold between the ages of fifty and seventy and another tenfold from seventy to over eighty-five. [10]

Since aging is a major risk factor for disability, and disability can lead to falls and frailty, determining the root cause of these outcomes is necessary in order to treat them. Outcome measures such as frailty have a direct link with loss of muscle mass. [11] Therefore loss of muscle mass throughout the lifespan can be detrimental to overall health. Events of the lifespan during which muscle is lost are now categorized as “catabolic crises”. [12] These events typically coincide with an injury or illness that requires significant time in bed. In healthy older adults, ten days of bedrest has been shown to lead to the loss of 0.95kg of leg lean mass. [13] While one kilogram of lost leg lean mass may not be detrimental to overall health and function, repeated bouts of bedrest-induced atrophy may result in muscle loss too great to overcome. According to the catabolic crisis model, an individual may undergo an injury or illness in their fifties. During this time, that individual may lose one to two kilos of lean mass. Following recovery, the individual resumes normal activities but does not restore all lean tissue lost during bedrest. A decade later, another illness requires an extended stay in bed. Again the individual loses one to two kilos of lean mass. As these bouts continue over a thirty-year span, the individual continues to lose lean mass during each catabolic crisis with recovery never reaching one hundred percent. Therefore, after each episode, the person retains less muscle as compared to before bedrest. With each cumulative loss of lean mass, ultimately the person reaches full body lean mass levels typically seen in individuals much older. The end result of these catabolic crises is frailty at an early age. [12]

SARCOPENIA

In addition to the muscle mass lost from various adverse catabolic events, advancing age can negatively impact whole body lean mass reservoirs as well. Reduced muscle mass from aging, known as sarcopenia, diminishes physical function over time. This is due to the fact that not only is muscle mass reduced as we age, but muscle quality

as well. [14] Essentially older individuals have less muscle, and the muscle they do retain does not function as well as the muscle in their younger counterparts. Older muscle is typically more fibrotic and has a higher lipid content resulting in less power and force production. [15]

Since older individuals have less muscle overall and the muscle has reduced function, they cannot endure any additional muscle atrophy. Thus catabolic crises in combination with sarcopenia provide a dangerous formula for maintaining health and independence while avoiding falls and frailty. Therefore the best method to avoid frailty is to maintain enough functional muscle mass throughout the lifespan so as to avoid falling into disability following the natural course of muscle loss due to sarcopenia. [16] Two methods for maintaining lean mass are amino acid/protein ingestion and resistance exercise.

PROTEIN TURNOVER

Protein turnover is a necessary process for maintaining the health of many tissues of the body. Over time, tissues of the body breakdown and must be reconstituted. The replacement of these lost proteins by the synthesis of new proteins encompasses whole body protein turnover. Within this whole body protein turnover, muscle undergoes a constant remodeling to ensure suitable muscle health over the course of the lifespan. [17]

All tissues undergo remodeling. Skeletal muscle experiences turnover of approximately 2% each day. Muscle turnover is a function of synthesis, the amount of new protein being created and incorporated into muscle, and breakdown, the amount of protein within muscle broken down into its constituent amino acids. The balance between synthesis and breakdown is referred to as protein net balance. Anabolism is the state when synthesis exceeds breakdown. Conversely when the rate of breakdown exceeds synthesis, this is a catabolic state. At rest protein net balance is approximately -0.05% per hour. [17]

While in the catabolic state, muscle is broken down in order to release amino acids into the blood stream. Since muscle is the largest pool of amino acids in the body, muscle serves to replenish the other tissues of the body during the fasted state. These amino acids are either taken up by other tissues of the body or converted into energy in the liver. The body may shift into the anabolic state following certain stimuli such as amino acid ingestion or resistance exercise. [17]

NUTRITIONAL INTERVENTIONS FOR ACTIVING PROTEIN SYNTHESIS

Preserving lean mass requires activation of the muscle to shift from a catabolic state to an anabolic state. The ingestion of amino acids has been shown to provide the necessary stimulation to induce muscle protein synthesis in both younger and older subjects. [18-20] Similarly protein ingestion alone has the same effect on protein anabolism as amino acid ingestion. [21] Therefore either nutritional intervention, protein or its constituent amino acids, can transition the muscle from catabolism to anabolism and thus potentially help to maintain lean tissue over the long-term. [22]

Supplementing with amino acids or protein is not as simple as taking a dose from a bottle right off the shelf. The dose and timing of supplement ingestion is critical for receiving maximum benefit from protein/amino acid consumption. The current recommended dietary allowance is a minimum of 0.8g of protein per kilogram of overall bodyweight. This protein intake level has been somewhat controversial as many studies have demonstrated benefits for muscle health and function only at higher intake levels. [23,24] One specific variable often overlooked is timing of protein ingestion. According to the National Health and Nutrition Examination Survey (NHANES), protein ingestion at dinner is approximately triple that of protein intake at breakfast. [25] This typical skewed diet in American adults provides for adequate intake at dinner but falls short of the overall protein needed to shift protein turnover from catabolism to anabolism and maximally

stimulate protein synthesis during other meals throughout the day. Instead of consuming the bulk of the protein found in the diet at one meal, spreading the protein out equally between all three meals was found to be more beneficial for maintaining an anabolic state with elevated protein synthesis as compared to the more traditional skewed diet. Therefore it would appear that a strategy of 30g of protein three times per day is favorable over 90g total protein with 60g consumed during the evening meal. [26]

RESISTANCE EXERCISE FOR ACTIVATING PROTEIN SYNTHESIS

Stimulation of muscle through a single bout of resistance exercise has been determined to adequately stimulate skeletal muscle protein synthesis in humans up to 72h post exercise. [27,28] Unlike the boost from nutritional interventions which benefit both young and old alike, the large increases in muscle protein synthesis from resistance exercise alone are only seen in younger individuals. Older individuals do not appear to reap the same benefits from resistance exercise while in the fasted state. [29,30] Yet it would be folly to negate the benefits in improved lean mass gains witnessed in older adults undergoing a resistance exercise protocol. [31] As mentioned earlier, reductions in lean mass associated with aging or in conjunction with catabolic crises can increase the likelihood of falls and frailty. Therefore any intervention that can maintain lean mass in older adults must be considered. Since exercise alone does not significantly stimulate muscle protein synthesis in older adults, it is necessary to combine interventions for the greatest possible benefit to muscle health. Combining both exercise and nutritional supplementation holds the key to shifting older individuals into an anabolic state to attenuate age-related muscle loss.

EXERCISE AND NUTRITION

The strategy of incorporating amino acid or protein supplementation with resistance exercise has been thoroughly tested over the decades. What has been realized is that amino acid/protein supplementation can provide an additive benefit not seen with exercise alone in stimulating muscle protein synthesis and the accumulation of muscle size and strength. This holds true for people of all ages. Traditionally the investigation for the benefits of supplementation and resistance exercise was done in an acute setting, that is, typically over a single day. [27-29] These types of studies allowed for the measurement of changes in muscle protein synthesis and breakdown and thus overall protein turnover. While many different exercise modalities exist, these studies often engaged subjects in high-intensity exercise requiring maximal exertion. The purpose of these studies is to maximally stimulate the targeted muscle to encourage growth. These exercises were often unilateral or bilateral leg exercises performed on the leg extension machine to engage the four muscles comprising the quadriceps group (vastus lateralis, vastus medialis, vastus intermedius and rectus femoris). Other modalities might include squats to engage leg extensors or biceps curls to engage arm flexors. These studies were limited in their ability to measure changes over a few hours. The provision of nutritional supplementation into these studies, either amino acids or intact protein beverages, provides an opportunity to test both protein timing and dose in these subjects. Again, the outcome measures tested, such as muscle protein synthesis or muscle cell signaling, can only be considered over a brief period. [31-33]

Over the past few years many laboratories have tested the results of acute studies with more chronic intervention studies. The benefit of longer-term studies, ranging from as little as three to as many as twenty-four weeks or longer, allows for the researchers to correlate acute changes in muscle protein synthesis with changes in lean body mass, power and strength. Ultimately the goal is to improve these outcome measures to preserve physical function and activities of daily living. These studies employ either whole-body

exercise training or more limited muscle-specific training protocols. [34-36] An overall examination of the literature does not provide a consensus for the benefit of protein supplementation when combined with exercise training as resistance exercise alone elicits improvements in muscle mass and strength in both older and younger individuals. [37] Therefore further research is necessary to tease out the impact of protein supplementation within these exercise studies.

ANABOLIC RESISTANCE

If chronic resistance exercise results in lean mass accumulation and muscle function, an incongruity must be present in order to explain the ever increasing numbers of frail or disabled individuals within the population. The population-wide health consequences outlined at the beginning of this chapter illustrate the public need to combat sarcopenia as well as catabolic crises. One clear answer is supplementation and exercise. The one factor not discussed previously is the detrimental effect of aging on the ability of older individuals to benefit from these specific interventions.

Resistance exercise and protein supplementation can maximally stimulate the muscle to grow in younger individuals, both individually and together. This is not the case for older individuals. Older individuals are now thought to suffer from anabolic resistance. Anabolic resistance is the reduced sensitivity for aging muscle to respond to known muscle-building interventions.[38] Resistance exercise alone does not maximally stimulate muscle protein synthesis in older individuals. [28] Similarly protein or amino acid supplementation alone does not always result in maximal stimulation of muscle protein synthesis. [39,40] Recent evidence supports the notion that older individuals must ingest a minimum amount of protein much higher than seen with younger individuals to enjoy the same benefits. [41] That minimum of 20g of protein provides the necessary stimulation to significantly increase protein synthesis in older subjects. [42] Combining

both interventions together appears to be the best method for overcoming anabolic resistance. [43]

Muscle protein accumulation is a function of changes in both synthesis and breakdown. [17] Resistance exercise alone or in conjunction with supplementation stimulates muscle protein breakdown. [44,45] In order to facilitate net accrual of skeletal muscle protein following exercise and feeding, it is necessary to promote muscle protein synthesis such that increases in synthesis may offset concomitant increases in breakdown.

mTOR Signaling Pathway

This review has focused on the interventions of resistance exercise and protein/amino acid supplementation to combat the negative consequences of muscle loss typically seen in older individuals. As mentioned earlier, resistance exercise alone does not stimulate muscle protein synthesis in older adults. Either a protein dose (>20g) or protein intake following resistance exercise is required to activate muscle protein synthesis. [25] This activation of protein synthesis takes place at the cellular level and is controlled by the mammalian (mechanistic) target of rapamycin, (mTOR). While other signaling pathways contribute to the regulation of muscle protein synthesis, the mTOR pathway has been shown to be the key regulatory pathway governing protein synthesis. [46]

The mTOR protein is 289 kilodaltons and is comprised of two distinct complexes known as mTORC1 and mTORC2. mTORC2 contains Rictor, G protein β-subunit-like protein (GβL), mammalian stress-activated protein kinase interacting protein 1 (mSIN1) and DEPTOR. mTORC2 is not sensitive to rapamycin and is not thought to play a role in protein synthesis. mTORC1 contains the mTOR protein and is joined by Raptor, PRAS40 as well as DEPTOR. mTORC1 is rapamycin sensitive and is important in regulating protein synthesis through hormonal, nutritional and contractile inputs. These inputs have been

shown to activate the mTOR complex and initiate a signaling cascade in order to activate protein synthesis rapidly following anabolic stimulation. [46,47]

Hormones such as insulin along with certain growth factors can activate mTOR signaling via the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Akt activation disassociates tuberous sclerosis complex 2 (TSC2) from TSC1. When this dimer is disrupted, TSC2 is unable to hydrolyze Ras-homolog enriched in brain allowing for Rheb to remain in its GTP-bound state and act on mTORC1. [48] Similarly amino acids can activate mTOR kinase activity and its downstream signaling cascade. For an overview of amino acid activation of mTOR, see the amino acid sensing section below.

Regulation of protein translation at the level of the ribosome is comprised of three phases, initiation, elongation and termination. mTORC1 plays a role in both initiation and elongation. For translation initiation, eukaryotic initiation factors (eIFs) recruit mRNA to the small ribosomal subunit 40s. Additionally, eIFs enlist the methionyl-tRNA to recognize the start codon on the mRNA to begin translation. The eIF4E protein is known to bind the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). 4E-BP1 serves as a translation inhibitor through its binding of eIF4E. Following mTORC1 activation, its downstream target 4E-BP1 is phosphorylated and thus disassociates from eIF4E. This disassociation allows for eIF4E to bind to other eIFs and begin translation initiation. [49]

mTOR can influence translation elongation by phosphorylating its downstream target ribosomal protein S6 kinase beta-1 (S6K1). S6K1 acts on eukaryotic elongation factor 2 (eEF2) to prevent binding of eEF2 with the ribosome. This allows the ribosome to continue with translation elongation. During this phase of translation, the ribosome moves along the mRNA at three nucleotides or one codon so the peptidyl-tRNA can transfer from the ribosomal A site to the ribosomal P site. [50]

AMINO ACID SENSING

For amino acid activation of mTOR to occur, the amino acids must enter the muscle from the blood stream. Recent discoveries in amino acid transport have identified factors involved with transporting amino acids through the cell membrane and into the muscle cell. Leucine is the most highly-studied of the amino acids as it has been shown to be the strongest stimulator of muscle protein synthesis. [51] The amino acid transporter SNAT2 is responsible for transporting glutamine into the cell. Glutamine transport is necessary for leucine transport due to the anti-porter nature of LAT1, another transporter. Leucine is ferried into the cell as glutamine is removed from the cell. [51] The requirement for glutamine and leucine transport by SNAT2 and LAT1 was demonstrated in studies that inhibited SNAT2 and LAT1 expression respectively by using cellular acidosis for SNAT2 and L-y-glutamyl-p-nitroanilide for LAT1. These transport inhibition experiments showed a decrease in intracellular leucine levels. [52, 53]

Prior to 2008, the mechanism for leucine activation of protein synthesis through the mTOR signaling cascade was unknown. Since that time, much of the mechanism has been elucidated in human embryonic kidney cells with the discovery of the role of the lysosome in amino acid activation of mTOR signaling. The lysosome is a cellular organelle with a more acidic pH (~5) as compared to other organelles within the cell. This acidity provides a conduit for the cellular destruction of waste products. The lysosome is an integral part of cellular autophagy. During amino acid sensing, the mTOR complex translocates to the lysosome to induce activation of the mTOR signaling cascade. [54]

Following the introduction of leucine into the cell via LAT1, the amino acid moves into the lysosome. The mechanism for leucine transport into the lysosome has yet to be determined. The incorporation of leucine into the lysosome activates the lysosome to initiate a binding with mTORC1. Following lysosomal activation, the Vacuolar ATPase on the surface of the lysosomal membrane transmits the amino acid sensing signal to the Rag

GTPases. The V-ATPase triggers the binding of the Ragulator proteins with mTORC1. [55] The Ragulator complex provides a scaffolding on the lysosome for the Rag proteins A/B in a GTP-bound state as well as Rag proteins C/D in a GDP-bound state. The amino acid sensing leucyl-tRNA synthetase binds to the Rag A/B proteins. The leucyl-tRNA synthetase acts as a GTPase activating protein that triggers the binding of these Rag proteins with the mTORC1 protein Raptor. [56] This colocalization of the lysosome with mTORC1 is possible only when amino acids are present within the cell. During periods of low amino acid availability, the GATOR1 protein acts as a negative regulator of this amino acid sensing pathway. GATOR1 consists of three proteins: DEPDC5, Nprl2, and Nprl3. GATOR1 serves as a GAP for the Rag A/B proteins. The GATOR2 complex consists of five proteins: WDR24, WDR59, Mios, Sec13, and Seh1L. During high amino acid availability, GATOR2 serves as a positive regulator of this pathway by inhibiting GATOR1 through a coupling with GATOR1 although its specific function has not been clearly identified. It is currently believed that Sestrin2 prevents the association of GATOR1 and GATOR2 and thus allows GATOR1 to act upon the RAG proteins. [57,58] Following lysosome/mTORC1 binding, mTORC1 kinase activity is activated through the Ras homolog enriched in brain (Rheb) protein. Rheb binds to the catalytic domain of the mTOR protein to initiate the mTOR signaling cascade. mTOR pathway substrates become phosphorylated after linking with the mTORC1 protein Raptor. [59]

SUMMARY

Translational research provides the framework for exploring the mechanism responsible for diseases or conditions. In some cases, this means making discoveries at the bench which can be used in the clinic to treat patients. In other instances, observations made in the clinic can be used to determine specific causes of conditions in the laboratory.

As the prevalence of disability continues to increase, the use of these techniques will allow researchers to better understand both the causes and consequences of disability.

One factor leading to disability is sarcopenia. At this point, age-related muscle loss is inevitable. Current research would suggest that nutritional and exercise interventions may hold the key to delaying the onset of sarcopenia. Unfortunately older individuals are less sensitive to these interventions and suffer from anabolic resistance. Older individuals need to consume more protein/amino acids than their younger counterparts when not paired with resistance exercise to shift from a catabolic to an anabolic state. Since older individuals typically consume less protein, it is important to make sure that the feeding dose and timing is providing the most benefit possible. [60] As a result, the study outlined in chapter 2 sought to determine the benefit of a blended protein source versus a single protein source for improving muscle protein turnover in older men following a bout of resistance exercise. The study discussed in chapter 3 tested the mechanism of amino acid sensing in muscle, both at the cellular and human level. These two studies taken together will advance our knowledge of how protein supplementation improves muscle protein turnover at the mechanistic level so that we may ultimately manipulate this mechanism in order to improve muscle health at the human level.

Chapter 2

Effect of Protein Blend Ingestion on Muscle Turnover in Aging

INTRODUCTION

As we age, reduced strength and muscle mass, sarcopenia, are predictors of early mortality [59]. With the aging of the baby boomer generation, the prevalence of sarcopenia will only increase. A sarcopenic population will only add to the ever-increasing health care costs as older individuals will require enhanced care from weakness, bedrest and loss of independence. Additionally reduced muscle mass and weakness increases the risk for falls in older adults [60]. A fall can lead to hospitalization and placement in a care facility [13, 14].

Amino acid/protein supplementation and resistance exercise are well-studied interventions for maximizing muscle protein synthesis in adults of all ages [32, 62-67]. Unfortunately these stimuli do not enhance muscle protein synthesis in the elderly as robustly as in younger individuals. This phenomenon has been classified as anabolic resistance [38, 39, 68]. While the mechanism for anabolic resistance is unknown, evidence suggests that a dose threshold must be breached for protein supplementation to maximally

stimulate muscle protein synthesis in older individuals [16,40]. Current research suggests that fewer than 20g of protein is insufficient for maximal stimulation of protein synthesis [36,39]. Therefore any supplement provided to older individuals should contain 20g of protein at a minimum [41]. Muscle protein breakdown is less well-studied, especially in older individuals. FBR increases following resistance exercise alone or when combined with feeding [21, 44, 45]. Therefore maximizing protein synthesis is needed to counteract these increases in protein breakdown in order to shift into an anabolic state and reach a positive net balance.

Adequate protein intake is only one potential aspect for using protein supplementation as a tool for overcoming anabolic resistance and improving muscle protein turnover. The protein source is also important as protein sources contain different amino acid compositions. [69,70] In addition, certain proteins have differing digestion rates [71]. Since activation of skeletal muscle protein synthesis is contingent on the amino acids being taken up by the muscle from the blood, the overall length of elevated blood amino acids or aminoacidemia is crucial [72].

The most well-studied protein supplement, whey protein, contains high levels of branched-chain amino acids compared to other protein sources. It is especially high in leucine content, which has been demonstrated as an amino acid responsible for activating protein synthesis [73]. Whey protein is a fast-digesting protein and thus results in a rapid spike in blood amino acid levels [74]. The other milk protein, casein, has a slower digestion profile than whey. As a result, casein prolongs elevated aminoacidemia longer than whey protein. While the increase in blood amino acids following casein ingestion does not reach the magnitude seen with whey ingestion, protein synthesis is still activated post exercise [75, 76]. Milk is not the only protein source for post exercise supplementation. The plant protein soy is also capable of stimulating muscle protein synthesis [16,77]. In addition, soy contains many anti-oxidants and is a good alternative source of protein for those on a vegetarian diet [78]. Since these three proteins, whey, casein and soy all have different

amino acid profiles as well as digestion rates, a blended protein supplement would theoretically provide the benefits of all three proteins [79].

A prior study using this blended protein supplement in young adults demonstrated that the protein blend was able to prolong amino acid net balance across the leg for up to two hours post ingestion as compared to only 20min for the whey alone group [80]. In addition, the protein blend was able to increase muscle protein synthesis for 4h post ingestion as compared to 2h for the whey alone group [79]. This prolonged anabolic stimulus may hold the key for improving skeletal muscle turnover and attenuating muscle atrophy in older populations.

As a result of this prior research, we conducted a double-blind randomized controlled clinical trial in men ages 55-75yrs. This research study compared the effects of whey protein (WP) to a protein blend (PB) ingested 1h after a bout of high intensity resistance exercise. The outcomes measures for this study: blood and muscle amino acid concentrations, mTOR signaling as well as muscle protein synthesis and breakdown measures will allow us to demonstrate a beneficial change in muscle protein turnover in older men with the protein blend that may provide a new strategy for attenuating the debilitating consequences of sarcopenia.

METHODS

SCREENING OF PARTICIPANTS

We recruited twenty healthy, older men 55-75 years of age for this double-blind, randomized clinical trial. Participant characteristics are shown in Table 1. The participants were recruited through flyers, newspaper advertisements, and word of mouth. Participants were required to be healthy, only recreationally active (no high-intensity resistance exercise regimen), non-smoking and not currently using any protein supplements. Participants were screened on two separate days at the Institute for Translational Sciences-

Clinical Research Center (ITS-CRC) at the University of Texas Medical Branch. Screening one included: laboratory tests (complete blood count with differential, liver and kidney function tests, coagulation profile, fasting blood glucose, hepatitis B and C screening, HIV test, thyroid stimulating hormone, lipid profile, urinalysis, and drug screening), clinical history with physical exam, and one repetition maximum (1RM) testing. The second screening included: a dual-energy X-ray absorptiometry (DEXA) scan (Hologic QDR 4500W, Bedford, MA) for measuring lean and fat mass, an additional 1 RM test as well as a cardiac stress test. 1RM testing was performed on a leg extension machine (Cybex-VR2, Medway, MA, USA). The repetition maximum was considered the average of the heaviest weight lifted from each of the two sessions. All participants provided written informed consent before enrollment in the study. The study was approved by the Institutional Review Board of the University of Texas Medical Branch, and is in compliance with the Declaration of Helsinki as revised in 1983.

STUDY DESIGN

The infusion protocol for this study was identical for both groups (Illustration 1). Enrolled participants checked into the ITS-CRC at ~600 h on the day of the study. Participants were instructed to refrain from exercise for at least 48h prior to admission. Participants were fasted for ~10h before beginning the infusion but were provided water ad libitum. The participants were randomized to ingest a 30 gram protein blend (N=9 PB) or whey protein beverage (N=10 WP) at 1h post high-intensity leg resistance exercise.

EXPERIMENTAL PROTOCOL

On the morning of the experiment, an 18 Gauge polyethylene catheter was inserted into the antecubital vein in order to begin the primed, constant infusion (~10h) of L-[ring-

$^{13}C_6$] phenylalanine and L-[^{15}N] phenylalanine (Sigma-Aldrich, St. Louis, MO, USA). A background blood sample was taken prior to commencement of the isotope infusion. The priming dose for the labeled phenylalanine was 2 μmol·kg^{-1} and the infusion rate was 0.05 μmol·kg^{-1}·min^{-1}. A retrograde catheter was inserted into a hand vein on the contralateral arm so that arterialized blood may be taken for sampling when heated. Muscle biopsies were performed on the lateral aspect of the vastus lateralis for the determination of resting mixed muscle FSR at 2h and 4h following infusion initiation. All biopsies were taken with a 5mm Bergström biopsy needle under sterile procedure and local anesthesia (1% lidocaine). After the second biopsy, the participants were moved to a leg extension machine (Cybex-VR2, Medway, MA, USA) for high-intensity resistance exercise consisting of eight sets of ten repetitions. The first 3 sets were below 70% 1RM to allow the subject to warm up and reach the goal of 70% without risk of injury or early-onset fatigue. Sets four through eight were performed at ~ 70% 1RM. The subjects were given three min rest between sets. Three additional muscle biopsies were taken 1, 3 and 5h after the completion of exercise (0, 2, 4h post supplement ingestion). The nutritional supplements were ingested immediately following the 1hr post exercise biopsy. Two biopsies were performed from each of the first two incisions with one biopsy taken from the final incision. Multiple sampling from the same area was limited by separating the incisions by ~7 cm. Biopsies taken from the same incision were angled ~5 cm from the previous one. This method has been utilized in both our lab [28,81] and as well as others. [82,83] Muscle tissue was immediately blotted, frozen in liquid nitrogen and stored at -80oC until analysis. Blood samples were collected during the resting (0, 120, 180, 185, 195, 205, 215, 225, 240 min) and post-ingestion (-60, 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 185, 195, 205, 215, 225, 240 min) time periods (Illustration 1) for the determination of blood L-[ring-$^{13}C_6$] phenylalanine enrichment (see below) and select amino acid concentrations. The infusion study concluded with the fifth muscle biopsy at which time the participants were fed a standard meal.

PROTEIN BEVERAGE INTERVENTION

The protein beverages (PB or WP) were consumed one hour following exercise. The beverages were dissolved in 300 mL of water and enriched (8%) with L-[ring-$^{13}C_6$] and L-[^{15}N] phenylalanine to maintain isotopic steady state in arterialized blood. The PB consisted of 30 g total protein (providing 2.78g leucine) composed of 50% protein from sodium caseinate, 25% protein from whey protein isolate and 25% protein from soy protein isolate. The WP consisted of 30g of protein (providing ~3.26g leucine). 30g was chosen as the leucine content of both supplements has been shown to be sufficient to stimulate skeletal muscle protein synthesis in older subjects [16].

FREE BLOOD AMINO ACID CONCENTRATION, PLASMA GLUCOSE, AND LACTATE

Concentrations of phenylalanine and the branch-chained amino acids (leucine, isoleucine, and valine) were measured in deproteinized whole blood using gas chromatography-mass spectrometry (GCMS) as previously described using an internal standard solution. [84, 85] An automated glucose and lactate analyzer (YSI, Yellow Springs, OH) was used to measure plasma glucose and lactate on the day of the study at time points (min) 0, 120, 240, 250, 256, 262, 268, 278, 318, 358, 378, 468.

MEASUREMENT OF LEAN MASS

Lean mass was estimated using a DEXA scan (Hologic QDR 4500W). The coefficient of variation for repeated measures of lean tissue is <1%.

CALCULATION OF MUSCLE PROTEIN SYNTHESIS

Muscle proteins and intracellular free amino acids were extracted from biopsy samples as previously described. [81] GCMS (GCMS, 6890 Plus CG, 5973N MSD, 7683 autosampler, Agilent Technologies, Palo Alto, CA) Bound muscle and intracellular free concentrations were calculated with the internal standard method using tracer enrichments for L-[ring-$^{13}C_6$] phenylalanine, L-[^{15}N] phenylalanine and appropriate internal standards (d_3 Leucine, ^{13}C Isoleucine, ^{13}C Valine, ^{15}N Phenylalanine). Measurements were determined as previously described. [40] Mixed-muscle protein-bound phenylalanine enrichment was analyzed by GCMS after protein hydrolysis and amino acid extraction, [86] using the external standard curve approach. [87] The fractional synthesis rate (FSR) of mixed muscle proteins was calculated from the incorporation rate of L-[ring-$^{13}C_6$]Phenylalanine into the mixed muscle proteins, and the free-tissue phenylalanine enrichment:

$$FSR = (\Delta Ep/t)/[(EM(1) + EM(2))/2] \bullet 60 \bullet 100$$

where $\Delta EP/t$ is the slope of the straight line that fits the protein-bound phenylalanine enrichment across two sequential biopsies, t is the time interval encompassing the two biopsies, EM(1), and EM(2) are the mean phenylalanine enrichments (tracer/tracee) in the free muscle pool in two biopsies. The results are presented as $\%.h^{-1}$. Phenylalanine is used because it is an essential amino acid that is not oxidized in the muscle tissue.

CALCULATION OF MUSCLE PROTEIN BREAKDOWN

Muscle protein fractional breakdown rate (FBR) was measured with phenylalanine tracers using the precursor-product method. [85] The method requires measurement of intracellular free phenylalanine enrichment at steady-state and after 1h of tracer decay. Frequent arterialized blood sampling during that 1h period is necessary for tracking the decay of blood enrichment via dilution by endogenous tracee released from muscle during protein breakdown. To measure FBR at baseline, the L-[ring-$^{13}C_6$]Phenylalanine enrichment at 4 hours was used as the plateau enrichment and L-[^{15}N] Phenylalanine enrichment at 4 hours was used for the 1 h decay enrichment. FBR was calculated using the formula:

$$FBR = \frac{\Delta E_M}{\left[p\int E_A(t)dt - (1+p)\int E_M(t)dt\right]\cdot\left(\frac{Q_M}{T}\right)}$$

WESTERN BLOT ANALYSIS

Muscle tissue samples were immediately quick-frozen in liquid nitrogen following the biopsy, and kept in liquid nitrogen until analyzed. Phosphorylation as of mTOR, 4E-BP1, S6K1, and S6 was measured using Western blot techniques as previously described [81]. Samples from both groups were included on the same blot. 50μg of protein from each sample was loaded in duplicate onto a 7.5% or 15% polyacrylamide gel (Criterion; Bio-Rad) and subjected to electrophoresis at 150 V for 70 min. Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) that were then blocked in 2.5% non-fat dried milk. Membranes were incubated with a primary antibody overnight at 4°C. The following rabbit polyclonal primary antibodies (Cell Signaling, Beverley, MA) were used: mTOR (Ser2448), S6K1 (Thr389), 4EBP1 (Thr37/46), and ribosomal protein S6 (Ser240/244). Blots were incubated with secondary antibody (Amersham Bioscience) washed, and then a chemiluminescent solution (ECL

plus; Amersham BioSciences, Piscataway, NJ, USA) was administered. Optical density measurements were then obtained with a digital imager (Bio-Rad) so that a densitometric analysis (Quantity One software, version 4.5.2; Bio-Rad) could be performed. All data is expressed relative to the internal control (rat phosphorylation standard).

STATISTICAL ANALYSIS

All values are expressed as Mean ± SEM. Data were transformed using the Box-Cox set of transformations to stabilize the variance and make the data approximately normally distributed. To test differences between groups, the data were modeled using an ANCOVA model with resting/baseline values as a covariate. The testing of differences was thus accomplished through a t-test of the parameter indicating the difference between groups. Comparisons with resting values were based on testing contrasts across time using a mixed model with subject as a random intercept term. Significance was set at $p < 0.05$. All calculations were done in R. [88]

RESULTS

SUBJECT CHARACTERISTICS

Table 1 Participant characteristics

	N	Age, *years*	BMI, kg/m^2	Fat *%*	Lean Mass, *kg*	1RM
Whey	10	69.29 ± 2.1*	26.5 ± 0.5	31.5 ± 2.2	53.9 ± 1.2	175.0 ± 8.5
Protein Blend	9	62.22 ± 1.5*	25.1 ± 1.1	27.3 ± 1.5	53.1 ± 3.1	178.6 ± 15.1

Data are mean ± SEM. 1RM, One-repetition maximum. *difference in age between treatment groups, p=.038.

Descriptive characteristics for all subjects are shown in Table 1. The participants had similar lean mass, percent body fat and one repetition maximum (1RM) values. There was a difference in ages between the groups with WP statistically older than the PB ($p<0.05$).

GLUCOSE AND LACTATE

Table 2 Plasma lactate and glucose concentrations in older adults at rest, during the exercise period and post-exercise recovery period following ingestion of the protein blend (PB) or whey protein (WP) 1 h after completion of resistance exercise

			Time post-ingestion (min)					
	Rest	**Exercise**	**0**	**20**	**60**	**100**	**150**	**240**
Lactate				*mmol/L*				
Whey	1.05 ± 0.10	4.36 ± 0.50*	7.05 ± 0.64*	1.44 ± 0.13*	1.20 ± .06	0.99 ± 0.06	0.84 ± 0.08	0.85 ± 0.06
Protein Blend	1.07 ± 0.08	4.48 ± 0.49*	7.28 ± 0.67*	2.02 ± 0.36*	1.57 ± 0.15*	1.58 ± 0.28	1.02 ± 0.13	0.94 ± 0.13
Glucose				*mmol/L*				
Whey	102.7 ± 4.6	101.1 ± 4.1	107.6 ± 4.1	98.5 ± 2.1	99.1 ± 1.9	97.4 ± 2.5	97.2 ± 2.2	90.8 ± 2.7*
Protein Blend	103.8 ± 3.8	102.2 ± 4.6	113.5 ± 7.4	102.0 ± 4.5	103.6 ± 3.3	100.8 ± 3.5	98.7 ± 3.9	89.8 ± 3.9*

Data are mean ± SEM. * different from pre, P<.05.

Glucose measurements were reduced from baseline ($p<0.05$) at 240min for both groups. Lactate measurements were elevated from baseline ($p<0.05$) until 20min post exercise for Whey. Protein Blend measurements were elevated from baseline ($p<0.05$) until 60min post exercise. (Table 2)

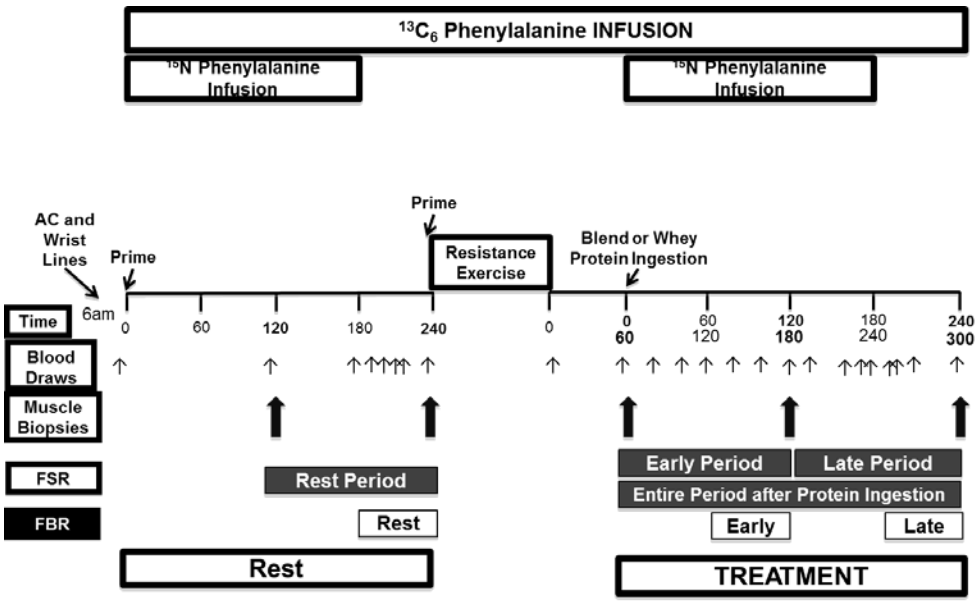


Illustration 1 Schematic of randomized, double-blinded experimental protocol. Participants ingested either the PB or WP 1 h following the completion of 8 sets of knee extension RE. The small arrows represent blood draws whereas the large arrows represent biopsies. FSR, fractional synthesis.

BLOOD AND MUSCLE AMINO ACID CONCENTRATIONS

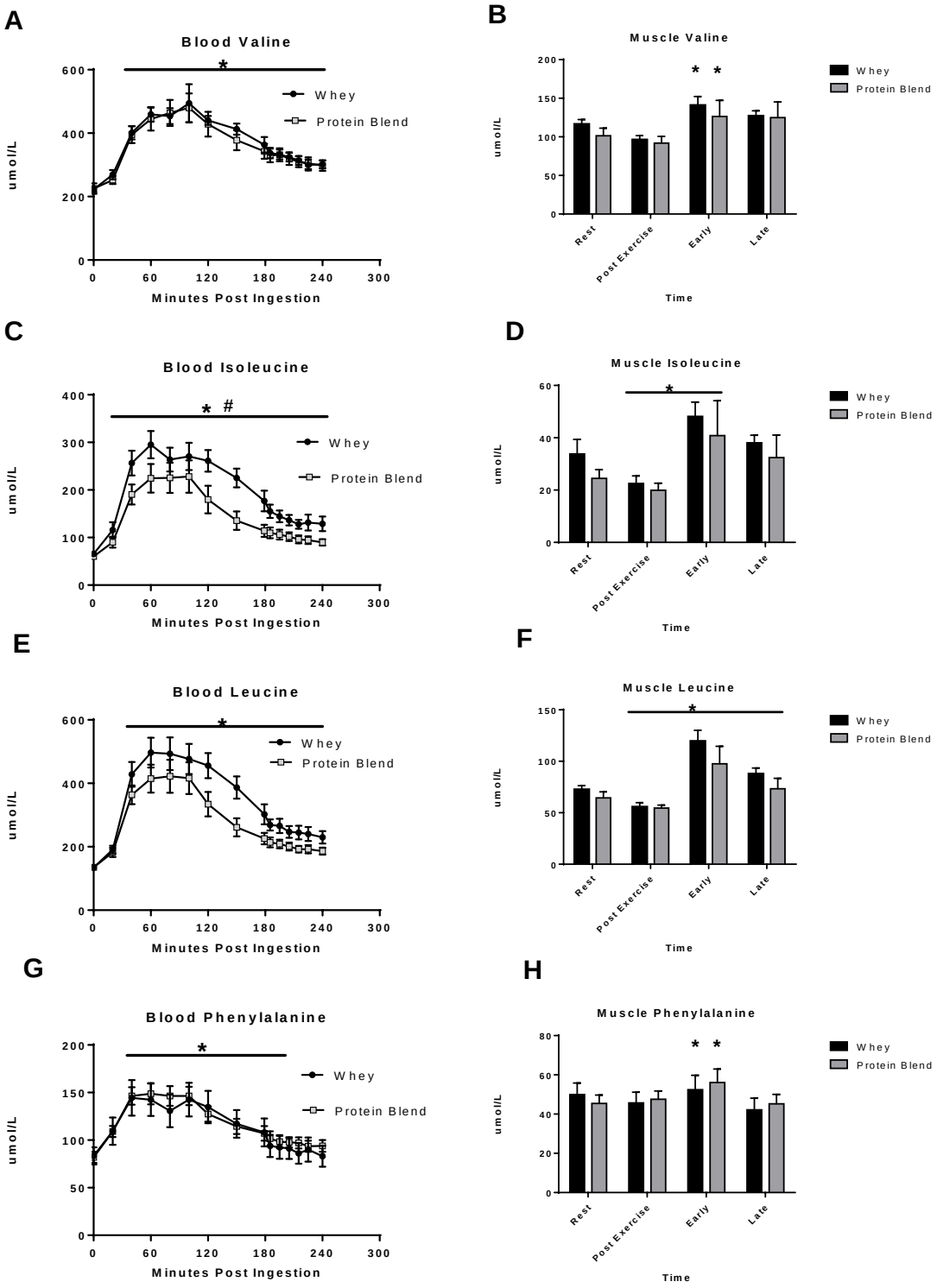


Figure 1

Changes from rest in blood Valine (A), muscle Valine (B), blood Isoleucine (C), muscle Isoleucine (D), blood Leucine (E), muscle Leucine (F), blood Phenylalanine (G), and muscle Phenylalanine (H) in older adults during the post exercise recovery period following ingestion of whey or protein blend 1hr after a bout of resistance exercise. Data are mean ± SEM. *different from resting values, $p<0.05$. #difference between treatment groups, $p<0.05$.

Blood concentrations for valine (Figure 1A) were elevated from rest ($p<0.05$) for both treatment groups for the entire treatment period post ingestion. Valine intracellular muscle concentrations were elevated in both groups for the early period compared to rest, $p<0.05$.(Figure 1B). Isoleucine concentrations in the blood were elevated from rest ($p<0.05$) for both treatment groups for the entire treatment period post ingestion with a significant increase at every time point for Whey over Protein Blend, $p<0.05$. (Figure 1C) Both groups were significantly reduced 1hr post exercise for muscle isoleucine concentrations compared to baseline but were significantly increased 2hr post ingestion. $p<0.05$. (Figure 1D) Blood concentrations for leucine were elevated from rest ($p<0.05$) for both treatment groups for the entire treatment period post ingestion with no difference between groups. (Figure 1E) Leucine intracellular muscle concentrations were reduced compared to baseline in both groups 1hr post resistance exercise bout but were significantly elevated for both the early and late periods compared to rest, $p<0.05$.(Figure 1F). Blood phenylalanine concentrations were elevated in both groups up to 205 minutes post ingestion with no difference between groups, $p<0.05$. (Figure 1G) Phenylalanine intracellular muscle concentrations were elevated in both groups for the early period compared to rest, $p<0.05$. (Figure 1H).

MUSCLE MTORC1 SIGNALING

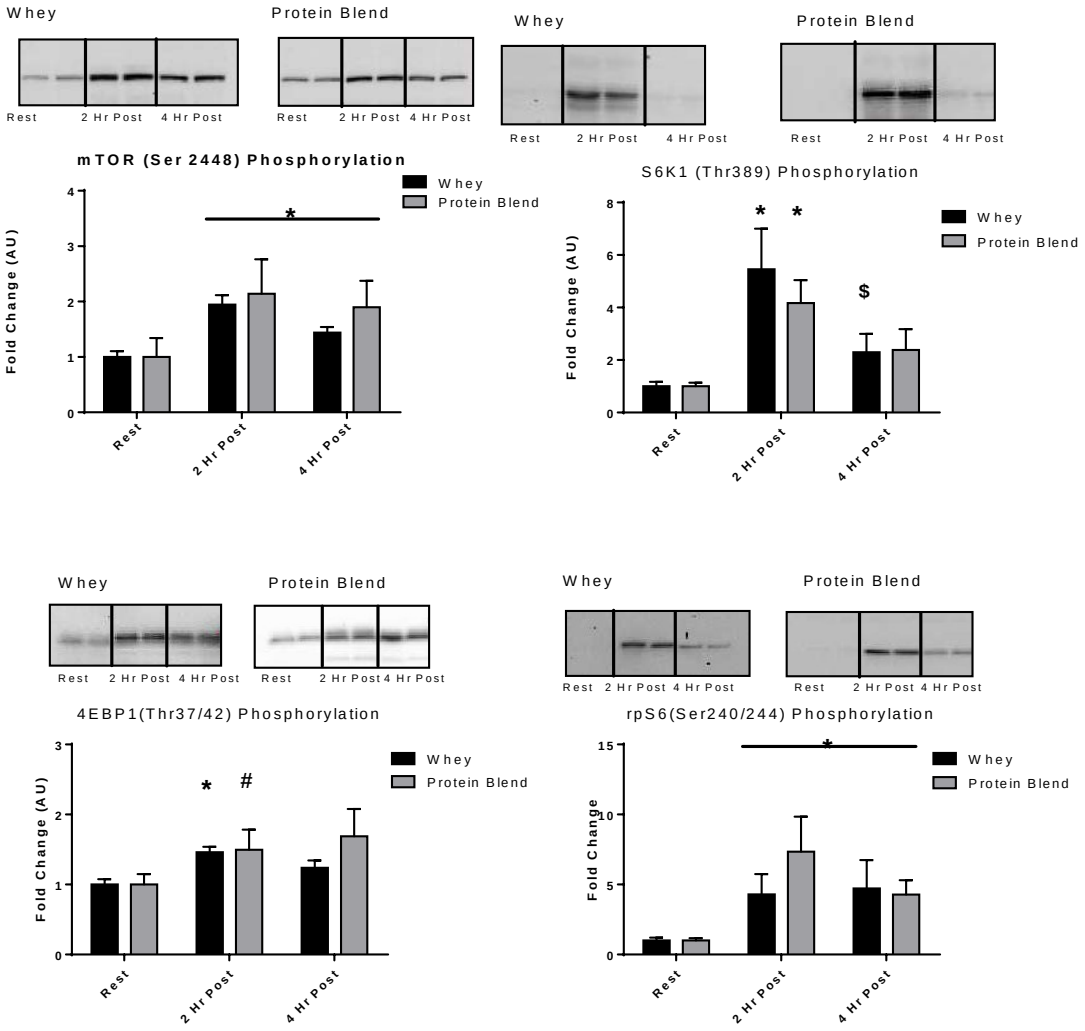


Figure 2 Western blot analyses of mTORC1 (A) and mTOR pathway-related proteins: S6K1 (B), 4EBP1 (C) and rpS6 (D), in older adults during the treatment period following a bout of resistance exercise. Data are mean ± SEM. * different from Rest, $p<0.05$. # trend difference from Rest, $p=.059$. $ different from 2 Hr Post, $P<.05$. & trend difference from 2 Hr Post, $p=.088$.

The phosphorylation status of mTORC1 (Ser2448) was significantly increased ($p < 0.05$) at both 2hr and 4hr post ingestion in both groups compared to baseline. (Figure 2A) S6K1 (Thr389) phosphorylation status was elevated at 2hr post ingestion for both groups. At 4hr post ingestion, the Whey group was significantly reduced ($p<0.05$) from the 2hr time point but was not different than baseline. The Protein Blend group was not significantly lower at 4hr compared to 2hr (trend of $p=0.088$). (Figure 2B) 4E-BP1 (Thr37/42) only showed significant elevation at 2hr ($p<0.05$) in Whey although Protein Blend showed a trend of $p=0.059$. (Figure 2C) Lastly the phosphorylation status of rpS6 (Ser240/244) was significantly increased ($p < 0.05$) at both 2hr and 4hr post ingestion in both groups compared to baseline. (Figure 2D)

FRACTIONAL SYNTHETIC RATE

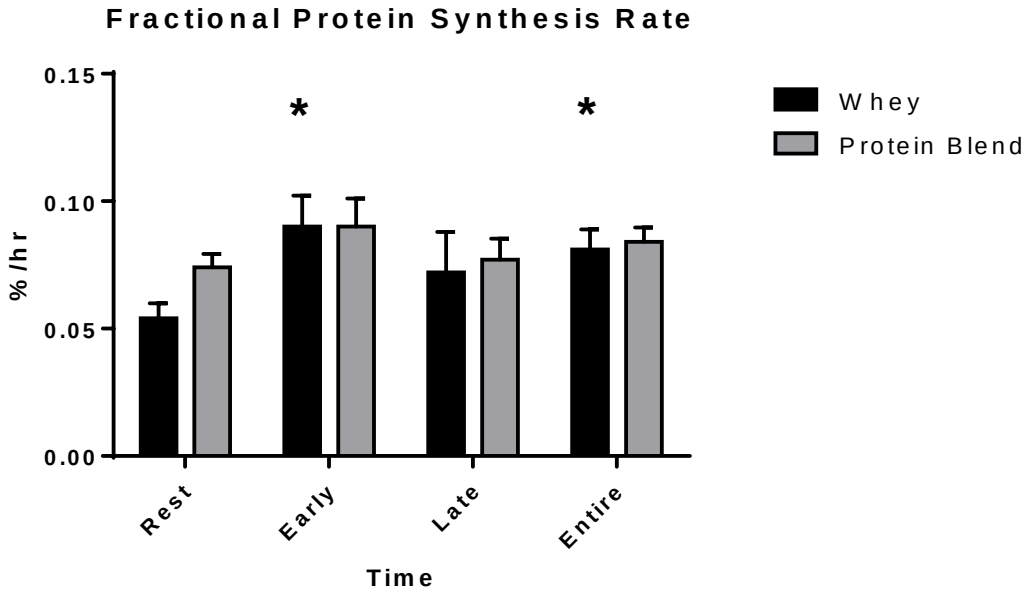


Figure 3 FSR (vastus lateralis) of older adults at rest and during the treatment period following an acute bout of resistance exercise and ingestion of whey or protein blend 1hr

post exercise. Early is 0-2hr post ingestion, late is 2-4hr post ingestion with entire being 0-4hr post ingestion. Data are mean ± SEM. * different from rest for Whey, $p<0.05$. FSR, fractional synthesis rate.

There was no difference between groups for muscle protein synthesis at any time point. The post-exercise FSR was elevated from resting values for Whey only in the Early Period as well as Entire, $p< 0.05$). (Figure 3)

FRACTIONAL BREAKDOWN RATE

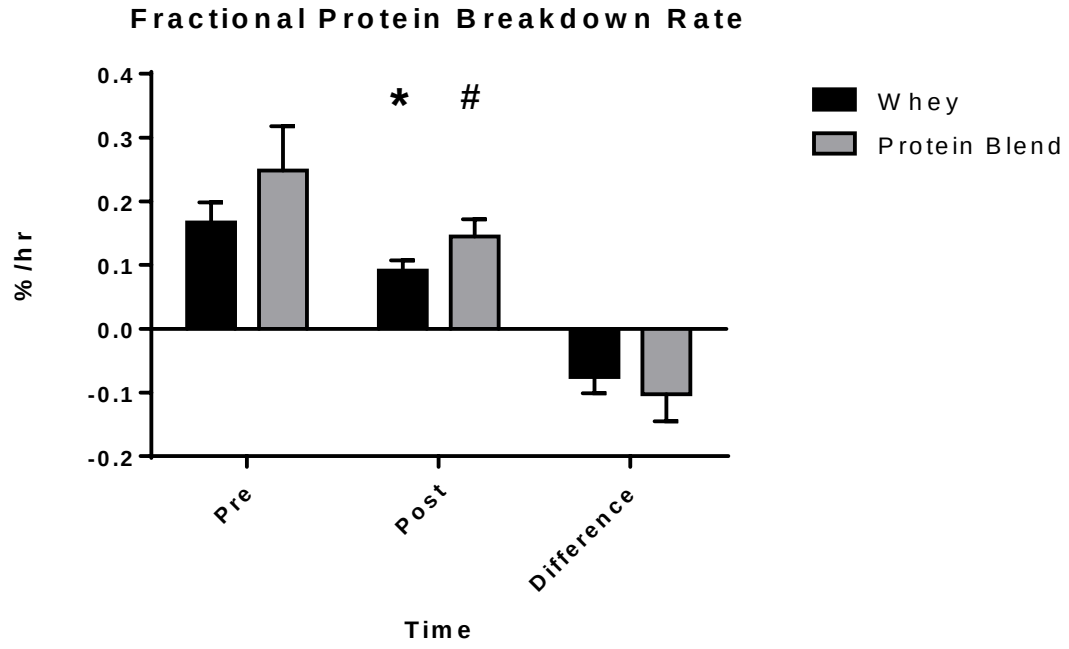


Figure 4 FBR (vastus lateralis) of older adults at rest and during the final 1hr of the treatment period following an acute bout of resistance exercise and ingestion of whey or protein blend 1hr post exercise. Pre is the hour prior to biopsy two. Post is the hour prior

to biopsy 5. Data are mean ± SEM. * different from pre for Whey, $p<0.05$. # trend difference from pre for Protein Blend, $p= 0.077$. FBR, fractional breakdown rate.

There was no difference between groups for muscle protein breakdown at any time point. Whey showed a significant reduction in breakdown during the final hour of the treatment period as compared to the baseline breakdown measure, ($p< 0.05$). Protein Blend showed a trend of $p=0.077$. (Figure 4)

DISCUSSION

The stimulation of muscle protein turnover provided by resistance exercise and/or protein supplementation to overcome anabolic resistance in older populations has recently become a major focus in the field of sarcopenia. Feeding alone can be effective for increasing muscle protein synthesis in older populations with the proper dosage. Cuthbertson at al. [39] showed a significant increase in FSR up to 3.5hr post ingestion. For this study, they utilized a dose-response protocol to test the threshold necessary for maximally stimulating protein synthesis using essential amino acids. They found that the response in older subjects did not match those of their younger counterparts. Still mixed muscle FSR was significantly increased using 10-20g. Similarly Kastansos et al. [16] saw a marked increase in FSR in older subjects following ingestion of a 6.7 EAA beverage containing 2.8g of leucine. This was the equivalent leucine concentration of a 25-30g protein supplement. Moore et al. [41] demonstrated a greater need for protein intake for stimulating myofibrillar protein synthesis in older subjects compared to young in a retrospective study with older subjects requiring a protein intake of 0.40g/kg body mass per meal with young only requiring 0.24g/kg body mass.

Unlike feeding, resistance exercise alone does not promote a robust increase in skeletal muscle protein synthesis in older adults. Using high- intensity resistance exercise, Fry et al. [28] were unable to induce an increase in FSR in older adults, as was shown in

younger adults, following 8 sets of 10 leg extensions at 70% 1RM. Kumar et al. [29] saw a similar disparity between young and old subjects following resistance exercise alone and concomitant increases in FSR.

All studies examining muscle protein turnover thus far have used either crystalline amino acids or a single protein source as a supplement in older individuals. This study has differentiated itself by the novel application of a protein supplement comprised of three separate proteins in the specific ratio of 25% whey, 25% soy and 50% casein. Each protein supplement is considered high quality as it contains all of the essential amino acids and is readily digestible. Yet, it would appear that leucine concentration in the supplement may be a key factor for promoting anabolic signaling and skeletal muscle protein synthesis [16, 67,73]. In older adults, Katsanos [16] et al. showed that 2.8g of leucine was necessary for increasing FSR while a 1.7g leucine dose was insufficient even when the total EAA beverage remained at 6.7g. Bukhari et al. [89] demonstrated significant elevations in muscle protein synthesis in older women using a 3g EAA beverage containing 1.2g leucine in combination with resistance exercise. The Katsanos study [16], along with Moore et al listed above [41], offers further evidence that body mass may play an integral role in protein/leucine dosage in older individuals. The mean lean body mass for subjects in the Bukhari study was only 40.5kg as compared to ~53.5kg for the Katsanos study. This weight discrepancy may explain the increases in muscle protein synthesis following ingestion of 1.2g leucine in one study not seen with 2.8g in the other study.

Whey protein contains high levels of leucine as compared to other protein sources [69]. The two groups in this study, WP and PB were not matched for leucine content. The WP group received 3.26g of leucine while the PB received 2.78g. Even though the two groups did not ingest equal amounts of leucine, according to the work of Katsanos et al [16], both groups received enough leucine to exceed the minimum threshold for shifting protein turnover into an anabolic state. Blood leucine concentrations remained elevated 240min post supplement ingestion with no difference between groups. Both groups also

had similar peak leucine concentrations between 450 and 500 umol/L at 60min post protein ingestion. Similarly both groups showed a similar increase in muscle leucine concentrations 2h post ingestion. Therefore leucine availability was comparable for both PB and WP.

The sustained availability of amino acids in the blood past 2hr for both groups may provide the explanation for increased anabolic signaling through the mTOR pathway 2-4h post ingestion of the supplement. This aminoacademia is vital for stimulating anabolic signaling in older individuals. Fry et al. [28] showed no increase in mTOR signaling or muscle protein synthesis following resistance exercise alone in older adults. We found that both mTOR and its downstream target, rpS6, were phosphorylated for the entire 4h treatment period. In addition, S6K1 was elevated for both groups for the first 2h post supplementation. 4EBP1 only showed significance in the Whey group at 2h, but the Blend group showed a trend towards significance with a p value of 0.059.

While both aminoacidemia and mTOR signaling were similarly elevated between groups, only WP showed a statistically significant increase in FSR during the Early period ($p<0.05$). As there was no difference between groups, muscle protein synthesis was similar for both the Early and Late periods for both WP and PB. PB had a higher baseline value and therefore did not show a statistically significant increase during the Early period. A large variance within the baseline measures for PB prevented the increase in FSR during the Early period to show statistical significance. Still considering the similarities between groups in amino acid concentrations, cell signaling and overall FSR levels during the Early, Late and Entire periods, it is possible to speculate that the Protein Blend group would have matched the Whey group during the Early period if not for a few unexplained high values during the baseline period.

Protein turnover is not only governed by changes in muscle protein synthesis. The other side of the net balance equation, muscle protein breakdown, may serve an important role in controlling muscle accretion or atrophy [29]. While protein breakdown has not been

measured as often as protein synthesis, some studies have thus far have shown an increase in FBR following exercise but then an attenuation following feeding in younger adults [63, 64]. The study of protein breakdown in older adults is not as comprehensive. One study showed no change in FBR among older adults following resistance exercise alone between baseline and 24hr post exercise. [44]

The design for this study allowed for the measurement of protein breakdown both during the rest period as well as the treatment period of the study as muscle protein breakdown was measured both at the 1-2hr mark during the rest period as well as 3-4hr post supplement ingestion. Protein supplementation resulted in an attenuation of the increase in FBR that is commonly observed following resistance exercise.

This particular study population included only men. While sex differences are not apparent in younger populations for anabolic signaling and FSR follow RE, we cannot make any inferences on the potential sex differences in older populations when combining RE with a protein blend [90]. In addition, the subjects did not spend the previous night at the hospital. Therefore it was not possible to control diet, activity or fasting 12h prior to commencement of the infusion.

The results of this study demonstrate the benefit of a protein supplementation, whether whey alone or a blend of proteins, for improving protein turnover in older men following resistance exercise. The effectiveness of a blended protein supplement must be further tested longitudinally in conjunction with a chronic training program to better identify the overall health benefits of this supplement for attenuating muscle loss in older populations.

Chapter 3

Amino Acid Sensing in Skeletal Muscle

INTRODUCTION

Various anabolic stimuli activate muscle growth through the stimulation of the mammalian target of rapamycin complex 1 (mTORC1). Stimulation of mTORC1 results in the initiation of a signaling cascade that promotes the enhancement of protein initiation and translation. Thus protein synthesis during anabolism is a direct consequence of activation of the mTORC1 kinase. [46]

Phosphorylation of mTORC1 via growth factors and insulin has been well-studied. [91,92] What has not been determined is the mechanism of mTORC1 activation following ingestion of amino acids. Ingestion of protein or amino acids has been shown to result in increases in skeletal muscle protein synthesis in animal and human models.[93-97] These studies are all descriptive by nature. While it is clear that amino acid ingestion is an anabolic stimulus for activating protein synthesis, the potential mechanism behind this effect was unknown until recently.

Amino acid introduction into the cell shifts the environment within the cell from a catabolic state to an anabolic state. During catabolism, mTORC1 is dormant. The inactive state of mTORC1 coincides with an upregulation of autophagy through the lysosomal degradation pathway. [98] This increase in autophagy provides substrates that may be

converted into energy during periods of low energy availability. Conversely, during the anabolic state, autophagy is suppressed. Instead of breaking down protein, the lysosome becomes an integral component in the creation of new proteins. [99]

Recent work has identified some of the players involved in amino acid sensing at the cellular level. During periods of low energy availability when amino acids are not present within the cell, amino acid-induced mTORC1 signaling is repressed. During this time, the Rag proteins are in a GDP-bound state and thus prevent localization of mTORC1 to the lysosome. [100, 101]

The rise of amino acids within the cell activates the amino acid sensing machinery upon introduction of the amino acids into the lysosome. The newly discovered arginine transporter SLC38A9 may be the first amino acid transporter identified that signals availability of amino acids to the lysosome. This allows for the Rag proteins to switch from a GDP-bound state to a GTP- bound state. The Rag A/C heterodimer is now active and recruits the mTORC1 complex to bind to the lysosome. This colocalization of mTORC1 and the lysosome initiates the mTOR signaling cascade. [102, 103]

The mTORC1 complex protein Raptor physically binds to the lysosome when the Rag proteins are in the GTP-bound state. This allows for the interaction of Raptor and Rheb on the surface of the lysosome. Once mTORC1 has become active, it initiates a signaling cascade through two downstream targets, eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and ribosomal kinase S6 kinase 1 (S6K1). 4E-BP1 serves as a translational inhibitor of the eukaryotic initiation factors (eIFs). When phosphorylated, 4E-BP1 disassociates from eIF4E allowing for the binding of eIF4E with eIF4G which promotes translation initiation. [1] Ribosomal protein S6 (rpS6) is located on the 40s ribosomal subunit. Phosphorylation of rpS6 by S6K1 leads to increased translation elongation via eukaryotic elongation factor 2 (eEF2) phosphorylation. [104] In this manner, mTOR signaling is able to increase protein synthesis when in the presence of amino acids.

Amino acid supplementation has been shown to activate mTOR signaling as well as significantly increase skeletal muscle protein synthesis in humans. [105] Since this newly discovered mechanism of amino acid sensing was studied only in human embryonic kidney cells (HEK), we proposed to determine if this mechanism is conserved in skeletal muscle. We hypothesized that chloroquine treatment would inhibit mTOR signaling activation following leucine administration in muscle cells. [106] Therefore we tested activation and subsequent inhibition of mTOR signaling in C2C12 mouse myoblasts using the amino acid leucine as well as the lysosomotropic agent chloroquine. We followed this study with a randomized controlled clinical trial in adults aged 18-40 years of age. These subjects were given a 10g EAA cocktail previously shown to stimulate muscle protein synthesis. [107] Half of the subjects were given chloroquine prior to ingestion of the EAA beverage. The outcome measures of mTOR signaling, protein synthesis as well as protein breakdown should provide evidence for the need of the lysosome for promoting mTOR signaling and protein synthesis from amino acid stimulation. For this study, we hypothesized that the mTOR signaling as well as protein synthesis activation seen in previous studies using a 10g EAA cocktail would be disrupted in the group given 750mg of chloroquine prior to beverage ingestion.

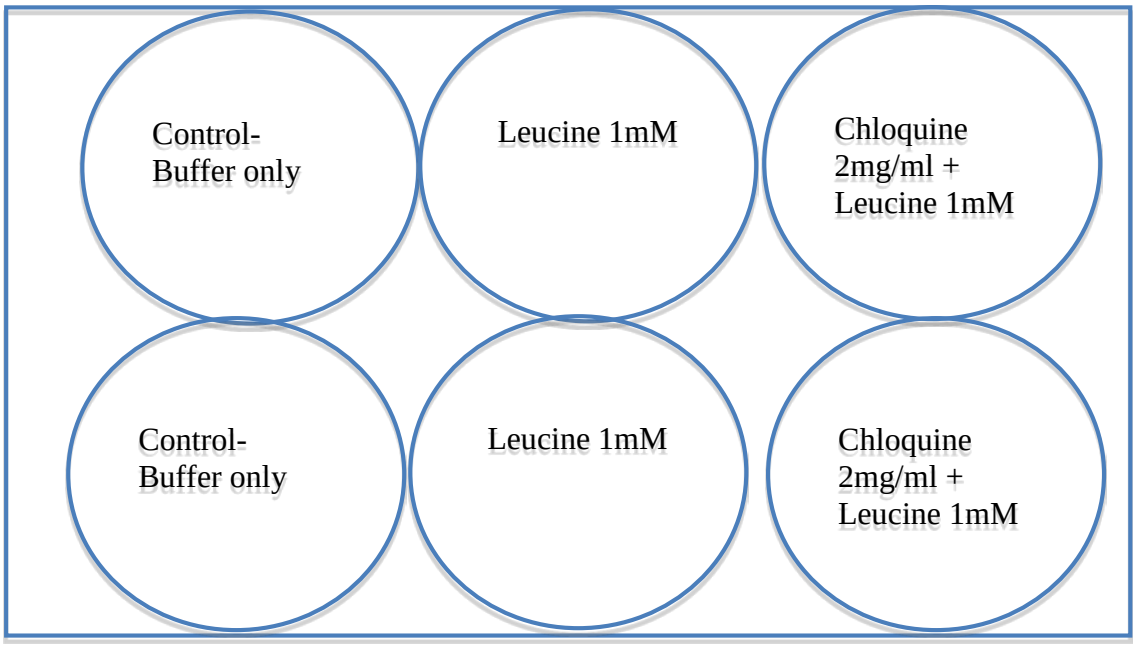
METHODS

CELL CULTURE

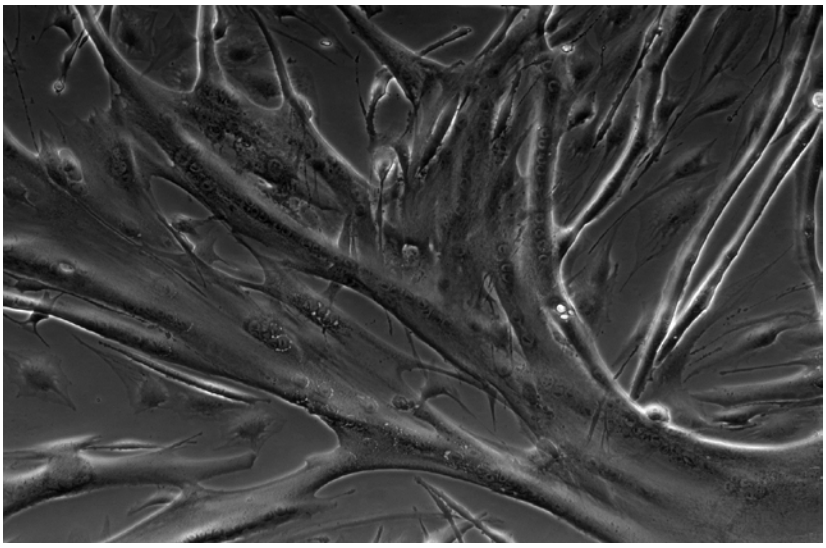
Murine C2C12 myoblasts were obtained from American Type Culture Collection and cultured on 0.1% gelatin-(Sigma-Aldrich, St. Louis, MO) coated tissue 6-well cultureware plates in growth media (high-glucose Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 50 U of penicillin/mL, 50 μg of streptomycin/mL; Invitrogen, Carlsbad, CA). The cells were incubated in an atmosphere

of 5% CO2/95% air at 37°C. At ~90% confluency, differentiation medium (low-glucose Dulbecco's modified Eagle medium supplemented with 2% horse serum, 50 U of penicillin/mL, 50 µg of streptomycin/mL; Invitrogen, Carlsbad, CA) was added to cultures for approximately 5 days to allow for formation of multinucleated myotubes.

Illustration 2: 6 Well Cell Culture Plate



CELL IMAGES



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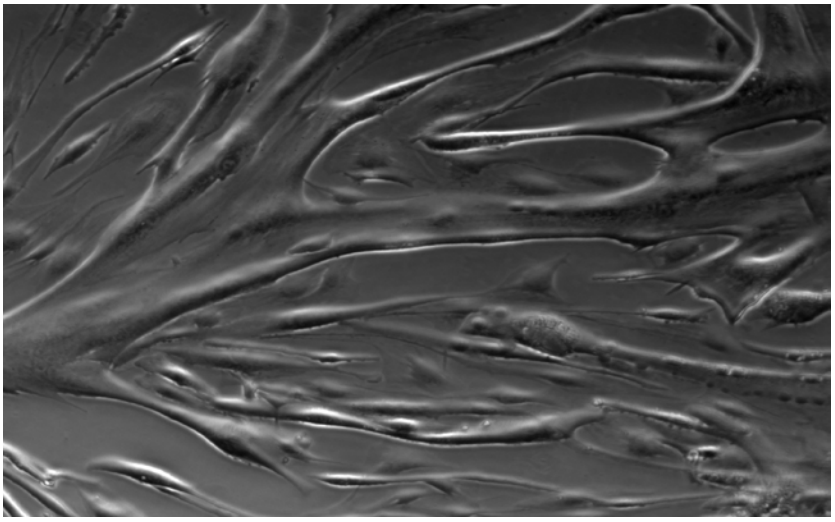
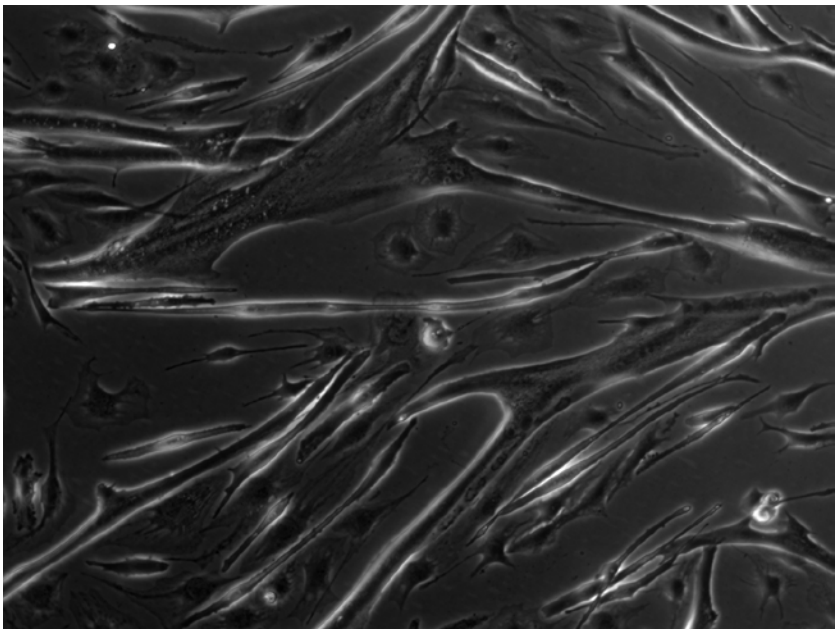


Leucine only



Leucine plus Chloroquine

EXPERIMENTAL DESIGN

At the outset of the experiment, myotubes were nutrient starved for 8 hrs in HEPES-buffered saline (HBS, 20 mmol/L HEPES/Na, 140 mmol/L NaCl, 2.5 mmol/L MgSO4, 5 mmol/L KCl, and 1 mmol/L CaCl2; pH 7.4; Sigma-Aldrich). For each plate, two wells were designated “control” and allowed to be starved for an additional 70min for a total of 9hr and 10min. Two more wells were designated “leucine only”. These wells were also starved for a total of 9 hrs and then administered 1mM leucine for ten minutes. Lastly the final two wells were designated “chloroquine plus leucine”. These wells were nutrient starved for 8 hrs and then administered 2mg/ml chloroquine for 60 min followed by 1mM leucine treatment for ten minutes. The choice of 1mM leucine was done to mirror postprandial leucine concentrations in the blood. The 2mg/ml concentration of chloroquine was selected after many trials that were conducted with concentrations ranging from .4mg/ml to 4mg/ml. 2mg/ml appeared to be the lowest concentration that effectively

downregulated leucine-induced activation of mTOR and S6K1 phosphorylation. All wells were washed with PBS between treatment administrations. This specific protocol is the result of many months of pilot testing in order to properly optimize these experimental conditions. The starvation conditions were chosen so as to reduce basal metabolic activity for all samples.

Following treatments, myotubes were rinsed with PBS and each well scraped in ice-cold extraction buffer (50 mmol/L Tris-HCl, 250 mmol/L mannitol, 50 mmol/L NaF, 5 mmol/L Na pyrophosphate, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 1 mmol/L DTT, 1 mmol/L benzamidine, 0.1 mmol/L PMSF, 5 μg/mL soybean trypsin inhibitor, pH 7.4). Samples were frozen in liquid nitrogen.

For the protein concentration assay, the samples were thawed and vortexed three times and later sonicated for 15 sec. Protein concentrations were calculated using the Bradford Protein Assay (Smartspec Plus, Bio-Rad, Hercules, CA).

WESTERN BLOT ANALYSIS

For Western blot analysis, cell lysates were diluted (1:1) in a 2× sample buffer mixture (125 mmol/L Tris, pH 6.8, 25% glycerol, 2.5% SDS, 2.5% β-mercaptoethanol, and 0.002% bromophenol blue) and then boiled for 3 min at 100°C. Equal amounts of total protein (12ug) were loaded into each lane and the samples were separated by electrophoresis at 150 V for 60 min on a 7.5% or 15% polyacrylamide gel (Criterion, Bio-Rad). All samples were loaded in duplicate with a loading control and molecular weight ladder (Precision Plus, Bio-Rad).

Following electrophoresis, the protein was transferred to a polyvinylidene difluoride membrane (Bio-rad) at 50 V for 60 min. Blots were blocked in 1% bovine serum albumin for 1h and then incubated with primary antibody overnight at 4°C. The following morning, secondary antibody was added for 1hr at room temperature. Blots were incubated

in a chemiluminescent solution (ECL plus, Amersham BioSciences, Piscataway, NJ) for 5min and optical density measurements quantified using a digital imager (ChemiDoc, Bio-Rad) and densitometric analysis was performed using Quantity One 4.5.2 software (Bio-Rad). Membranes were stripped using Restore Western Blot Stripping buffer (Pierce Biotechnology, Rockford, IL). Phosphorylation values were normalized to the loading control.

AMINO ACID SENSING IN HUMANS

SCREENING OF PARTICIPANTS

We recruited fourteen healthy, men and women 18-40 years of age for this double-blind, randomized clinical trial. Participant characteristics are shown in Table 4. The participants were recruited through flyers, newspaper advertisements, and word of mouth. Participants were required to be generally healthy with no tobacco usage or protein/amino acid supplement usage. Participants received a screening at the Institute for Translational Sciences-Clinical Research Center (ITS-CRC) at the University of Texas Medical Branch. The screening included: laboratory tests (complete blood count with differential, liver and kidney function tests, coagulation profile, fasting blood glucose, hepatitis B and C screening, HIV test, thyroid stimulating hormone, lipid profile, urinalysis, and drug screening), clinical history with physical exam, and a dual-energy X-ray absorptiometry (DXA) scan (Hologic QDR 4500W, Bedford, MA) for measuring lean and fat mass All participants provided written informed consent before enrollment in the study. The study was approved by the Institutional Review Board of the University of Texas Medical Branch, and is in compliance with the Declaration of Helsinki as revised in 1983.

STUDY DESIGN

The infusion protocol for this study was identical for both groups (Illustration 3). Enrolled participants checked into the ITS-CRC at ~1800h the night before the study. Participants refrained from exercise for at least 48h prior to admission. Subjects were fed a standardized dinner (10 kcal/kg of body weight; 60% carbohydrate, 20% fat,, and 20% protein) and a 2100h snack (5 kcal/kg of body weight; 60% carbohydrate, 20% fat,, and 20% protein), and asked to sleep in the UTMB CRC. After 2300h, they were allowed only water and the 10 gram essential amino acid beverage until the end of the trial. The participants were randomized to control (N=7 CON) or chloroquine (N=7 CHQ). Control subjects received nothing. Chloroquine subjects received a 250mg dose at 2000h the night before the study and 500mg the next morning following commencement of the stable isotope tracers consistent with a previous study. [108]

EXPERIMENTAL PROTOCOL

On the morning of the experiment, an 18G polyethylene catheter was inserted into the antecubital vein in order to begin the primed, constant infusion (~10h) of L-[ring-$^{13}C_6$] phenylalanine and L-[^{15}N] phenylalanine (Sigma-Aldrich, St. Louis, MO, USA). A background blood sample was taken prior to commencement of the isotope infusion. The priming dose for the labeled phenylalanine was 2 $\mu mol \cdot kg^{-1}$ and the infusion rate was 0.05 $\mu mol \cdot kg^{-1} \cdot min^{-1}$. A retrograde catheter was inserted into a hand vein on the contralateral arm so that arterialized blood may be taken for sampling. Muscle biopsies were performed on the lateral aspect of the vastus lateralis for the determination of resting mixed muscle FSR at 2h and 4.5h following infusion initiation. All biopsies were taken with a 5mm Bergström biopsy needle under sterile procedure and local anesthesia (1% lidocaine). The EAA beverage was consumed following the second biopsy with biopsies three and four performed 60m and 120m post ingestion respectively. Two biopsies were performed from

each of the two incisions. Multiple sampling from the same area was limited by separating the incisions by ~7 cm. Biopsies taken from the same incision were angled ~5 cm from the previous one. This method has been utilized in both our lab [28,81] and as well as others. [82,83] Muscle tissue was immediately blotted, frozen in liquid nitrogen and stored at -80^{o}C until analysis. Blood samples were collected during the resting (0, 89, 95, 105, 115, 125, 135, 150 min) and post-ingestion (0, 15, 30, 45, 60, 75, 90, 105, 120min) time periods for the determination of blood L-[ring-$^{13}C_6$] phenylalanine enrichment (see below) and amino acid concentrations. The infusion study concluded with the fourth muscle biopsy at which time the participants were fed a standard meal.

ESSENTIAL AMINO ACID INTERVENTION

The EAA beverage was consumed following biopsy two. The amino acids were dissolved in 300 mL of Fresca® and enriched (8%) with L-[ring-$^{13}C_6$] and L-[^{15}N] phenylalanine to maintain isotopic steady state in arterialized blood. The composition of the beverage is shown in (Table 3).

Table 3: EAA Beverage

	% of total	Desired amt (g)
Histidine	11	1.1000
Isoleucine	10	1.0000
Leucine	18	1.8500
Lysine	16	1.5500
Methionine	3	0.3000
Phenylalanine	16	1.5500
Threonine	14	1.4500
Valine	12	1.2000

FREE BLOOD AMINO ACID CONCENTRATION, PLASMA GLUCOSE, AND LACTATE

Concentrations of phenylalanine and leucine were measured in deproteinized whole blood using Gas chromatography-mass spectrometry (GCMS) as previously described

using an internal standard solution. [84, 85] An automated glucose and lactate analyzer (YSI, Yellow Springs, OH) was used to measure plasma glucose and lactate on the day of the study.

MEASUREMENT OF LEAN MASS

Muscle mass was estimated using a DEXA scan (Hologic QDR 4500W). The coefficient of variation for repeated measures of lean tissue is <1%.

MUSCLE PROTEIN TURNOVER

Enrichments of free L-[ring-$^{13}C_6$]Phenylalanine, and L-[^{15}N]Phenylalanine in blood and tissue fluid were measured by GCMS after addition of appropriate internal standards and precipitation of blood and tissue proteins with sulfosalycilic acid, extraction with cation exchange chromatography, and tert-butyldimethylsilyl derivatization (t-BDMS). Correction for skewed isotopomer distribution and overlapping spectra were performed as previously described. [85] The incorporation of L-[ring-$^{13}C_6$]Phenylalanine in the mixed muscle proteins was measured after protein extraction and hydrolysis, amino acid extraction with cation exchange chromatography, t-BDMS derivatization, and GCMS analysis.

CALCULATION OF MUSCLE PROTEIN SYNTHESIS

Muscle proteins and intracellular free amino acids were extracted from biopsy samples as previously described. [86] GCMS (GCMS, 6890 Plus CG, 5973N MSD, 7683 autosampler, Agilent Technologies, Palo Alto, CA) bound muscle and intracellular free concentrations were calculated with the internal standard method using tracer enrichments

for L-[ring-$^{13}C_6$] phenylalanine, L-[^{15}N] phenylalanine and appropriate internal standards. Measurements were determined as previously described. [85] Mixed-muscle protein-bound phenylalanine enrichment was analyzed by GCMS after protein hydrolysis and amino acid extraction, [86] using the external standard curve approach. [87] The fractional synthesis rate (FSR) of mixed muscle proteins was calculated from the incorporation rate of L-[ring-$^{13}C_6$]Phenylalanine into the mixed muscle proteins, and the free-tissue phenylalanine enrichment:

where $\Delta EP/t$ is the slope of the straight line that fits the protein-bound phenylalanine enrichment across two sequential biopsies, t is the time interval encompassing the two biopsies, EM(1), and EM(2) are the phenylalanine enrichments (tracer/tracee) in the free muscle pool in the two biopsies. The results are presented as $\%.h^{-1}$. Phenylalanine is used because it is an essential amino acid that is not oxidized in the muscle tissue. Thus, phenylalanine utilization in the muscle is an index of muscle protein synthesis seen in the following equation:

$$FSR = (\Delta Ep/t)/[(EM(1) + EM(2))/2] \bullet 60 \bullet 100.$$

CALCULATION OF MUSCLE PROTEIN BREAKDOWN

Muscle protein fractional breakdown rate (FBR) was measured with phenylalanine tracers using the precursor-product method. [85] The method requires measurement of intracellular free phenylalanine enrichment at steady-state and after 1h of tracer decay. Frequent arterialized blood sampling during that 1h period is necessary for tracking the decay of blood enrichment. To measure FBR at baseline, the L-[ring-$^{13}C_6$]Phenylalanine enrichment at 4 hours was used as the plateau enrichment and L-[^{15}N] Phenylalanine enrichment at 4 hours was used for the 1 h decay enrichment. FBR was calculated using the formula:

$$FBR = \frac{\Delta E_M}{\left[p\int E_A(t)dt - (1+p)\int E_M(t)dt\right] \cdot \left(\frac{Q_M}{T}\right)}$$

WESTERN BLOT ANALYSIS

Muscle tissue samples (150-300 mg) were immediately quick-frozen in liquid nitrogen following the biopsy, and kept in liquid nitrogen until analyzed. Phosphorylation of mTOR, 4E-BP1, p70S6K1, and rpS6 was measured using Western blot techniques as previously described [85]. 50μg of protein from each sample was loaded in duplicate onto a 7.5% or 15% polyacrylamide gel (Criterion; Bio-Rad) and subjected to electrophoresis at 150 V for 70 min. Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) that were then blocked in 5% non-fat dried milk. Membranes were incubated with a primary antibody overnight at 4°C. The following rabbit polyclonal primary antibodies (Cell Signaling, Beverley, MA) were used: mTOR (Ser2448), S6K1 (Thr389), 4EBP1 (Thr37/46), and ribosomal protein S6 (Ser240/244). Blots were incubated with secondary antibody (Amersham Bioscience) washed, and then a chemiluminescent solution (ECL plus; Amersham BioSciences, Piscataway, NJ, USA) was administered. Optical density measurements were then obtained with a digital imager (Bio-Rad) so that a densitometric analysis (Quantity One software, version 4.5.2; Bio-Rad) could be performed. Following detection of the phosphorylated protein, blots were stripped of primary and secondary antibodies and then re-probed for other proteins. All data is expressed relative to the internal control.

WHOLE BODY PROTEOLYSIS

Whole body proteolysis was measured by dividing the L-[ring-$^{13}C_6$] phenylalanine tracer infusion rate by the L-[ring-$^{13}C_6$] phenylalanine enrichment (tracer to tracee ratio) at each given time point.

STATISTICAL ANALYSIS

All values are expressed as Mean ± SEM. Data were transformed using the Box-Cox set of transformations to stabilize the variance and make the data approximately normally distributed. To test differences between groups, the data were modeled using an ANCOVA model with resting/baseline values as a covariate. The testing of differences was thus accomplished through a t-test of the parameter indicating the difference between groups. Comparisons with resting values were based on testing contrasts across time using a mixed model with subject as a random intercept term. All baseline comparisons were done using two-group t-tests. Fold changes were tested against baseline using a one-sample t-test. Significance was set at $p < 0.05$. All calculations were done in R. [88]

RESULTS

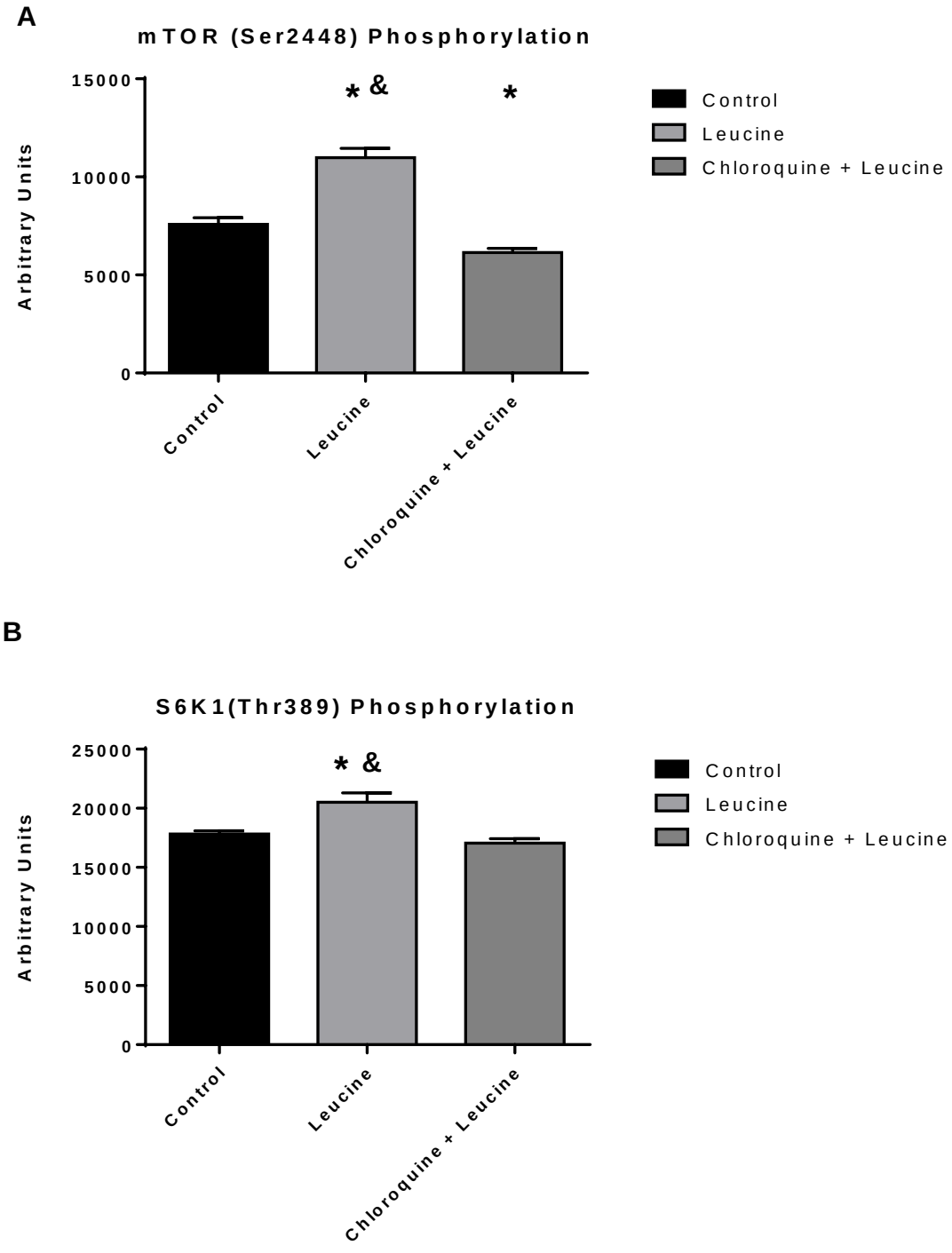

A
mTOR (Ser2448) Phosphorylation
Arbitrary Units
15000
10000
5000
0
* &
*
Control
Leucine
Chloroquine + Leucine
B
S6K1(Thr389) Phosphorylation
Arbitrary Units
25000
20000
15000
10000
5000
0
* &
Control
Leucine
Chloroquine + Leucine

Figure 5 Western blot analyses of mTORC1 (A) and mTOR pathway-related protein S6K1 (B). Data are mean ± SEM. N=8 for both treatment groups. * different from control, P<.05. & different from chloroquine +leucine.

The phosphorylation status of mTORC1 (Ser 2448) was significantly increased with 1mM leucine administration ($p < 0.05$) compared to baseline and chloroquine + leucine. (Figure 5A) The phosphorylation status of S6K1 (Thr389) was significantly increased with 1mM leucine administration ($p < 0.05$) compared to baseline and chloroquine + leucine. (Figure 5B)

SUBJECT CHARACTERISTICS

Table 4 Participant characteristics

	N	Gender	Age, *years*	BMI, *kg/m²*	Fat *%*	Lean Mass, *kg*
Control	7	5M, 2F	24.9 ± 1.5	22.0 ± 0.8	21.3 ± 2.1	47.4 ± 2.9
Chloroquine	7	5M, 2F	26.9 ± 1.9	23.0 ± 1.0	22.0 ± 2.2	50.3 ± 4.1

Data are mean ± SEM. 1RM,

Descriptive characteristics for all subjects are shown in Table 4. The participants had similar lean mass, percent body fat and BMI.

GLUCOSE AND LACTATE

Table 5 Plasma lactate and glucose concentrations at baseline and following ingestion of the EAA beverage for Control and Chloroquine

		Time post-ingestion (min)						
	Baseline	**0**	**15**	**30**	**45**	**60**	**90**	**120**
Lactate				*mmol/L*				
Control	1.08 ± 0.21	1.24± 0.24	1.02 ± 0.17	0.90 ± 0.12	0.92± . 12	0.85 ± 0.15	0.72 ± 0.09*	0.79 ± 0.10
Chloroquine	1.06 ± 0.10	1.04± 0.10	0.82 ± 0.04*	0.89 ± 0.06*	0.93 ± 0.06	0.95 ± 0.09	0.70 ± 0.02*	0.78 ± 0.08*
Glucose				*mmol/L*				
Control	95.8 ± 3.6	99.0± 2.7	98.5 ± 2.1	89.7 ± 3.2*	89.0 ± 3.4*	90.2 ± 3.7	90.0 ± 2.5	90.0 ± 2.8*
Chloroquine	91.8 ± 3.0	88.6± 2.1	85.3 ± 2.6	90.8 ± 1.8	85.7 ± 1.9	85.9 ± 1.8*	86.1 ± 2.4*	87.0 ± 2.6*

Data are mean ± SEM. * different from pre, P<.05.

Lactate measurements were reduced from baseline ($p<0.05$) at 15min, 30min, 90min and 120min for Chloroquine only. Glucose measurements were reduced from baseline ($p<0.05$) at 30min, 45min and 120min for Control. Chloroquine had reductions from rest ($p<0.05$) at 60min, 90min and 120min. (Table 5)

STUDY SCHEMATIC

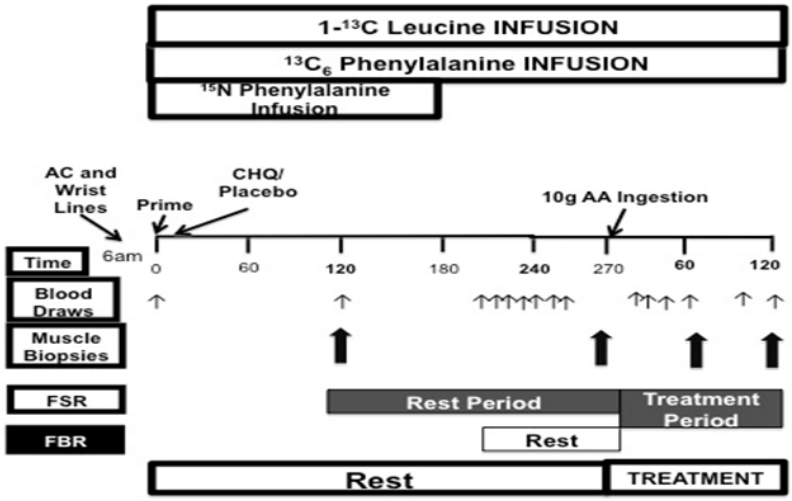


Illustration 3 Schematic of randomized, double-blinded experimental protocol. Participants ingested 10g EAA following biopsy two. The small arrows represent blood draws whereas the large arrows represent biopsies. FSR, fractional synthesis rate. FBR, fractional breakdown rate.

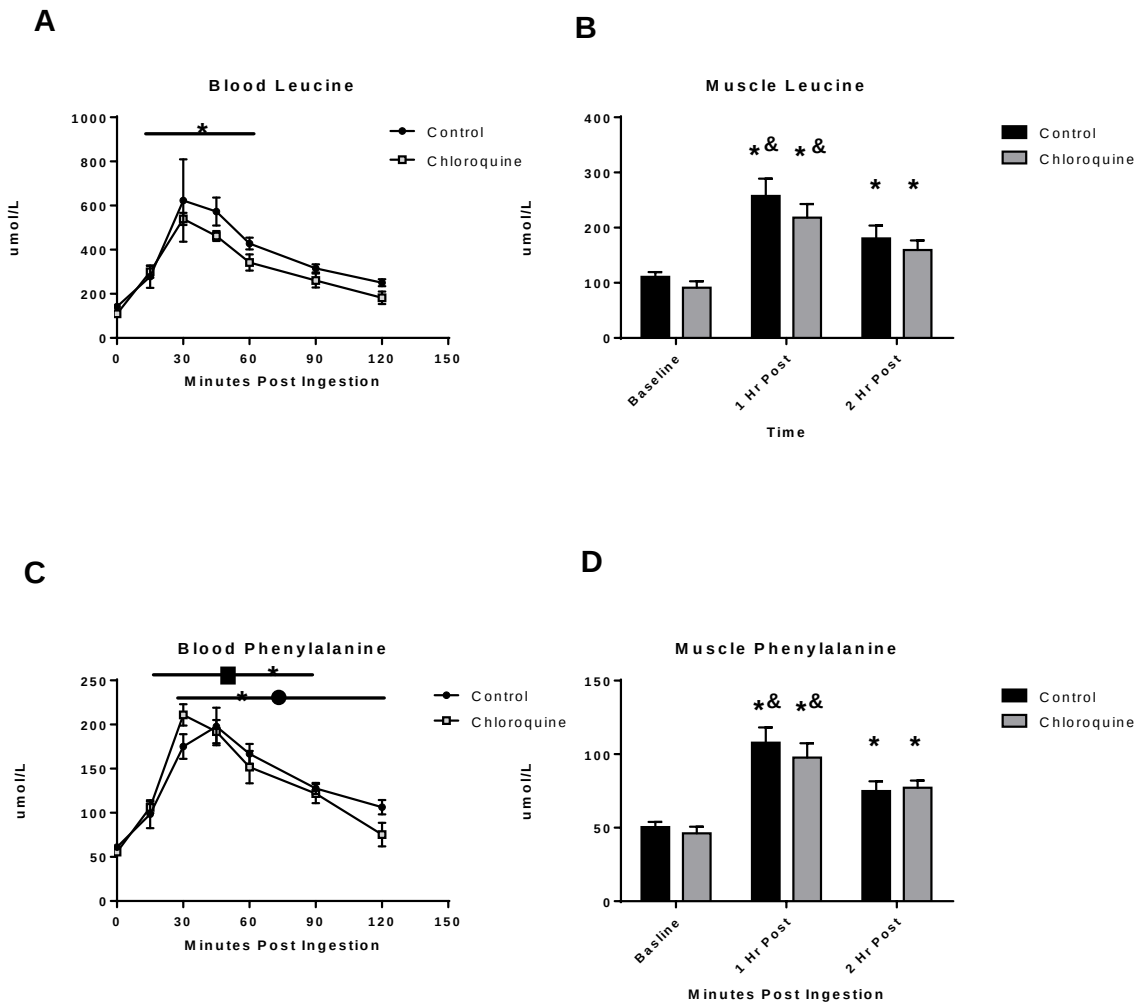


Figure 6

Changes from rest in blood Leucine (A), muscle Leucine (B), blood Phenylalanine (C), and muscle Phenylalanine (D) at baseline and following ingestion of a 10g EAA beverage.

Data are mean ± SEM. N=7 for both treatment groups. *different from resting values, $p<0.05$. & difference between 1hr post and 2hr post, $p<0.05$.

Blood concentrations for leucine (Figure 6A) were elevated from baseline ($p<0.05$) for both treatment groups for 60min post ingestion. Leucine intracellular muscle concentrations were elevated in both groups at 1hr and at 2hr post ingestion compared to baseline, $p<0.05$. 1hr post ingestion was significantly different than 2hr post ingestion for both groups (Figure 6B). Phenylalanine concentrations in the blood were elevated from baseline ($p<0.05$) for Control from 30min to 120min post ingestion. Chloroquine was elevated from rest ($p<0.05$) from 15min to 90min post ingestion. (Figure 6C) Phenylalanine intracellular muscle concentrations were elevated in both groups at 1hr and at 2hr post ingestion compared to baseline, $p<0.05$. 1hr post ingestion was significantly different than 2hr post ingestion for both groups. (Figure 6D)

MUSCLE MTORC1 SIGNALING

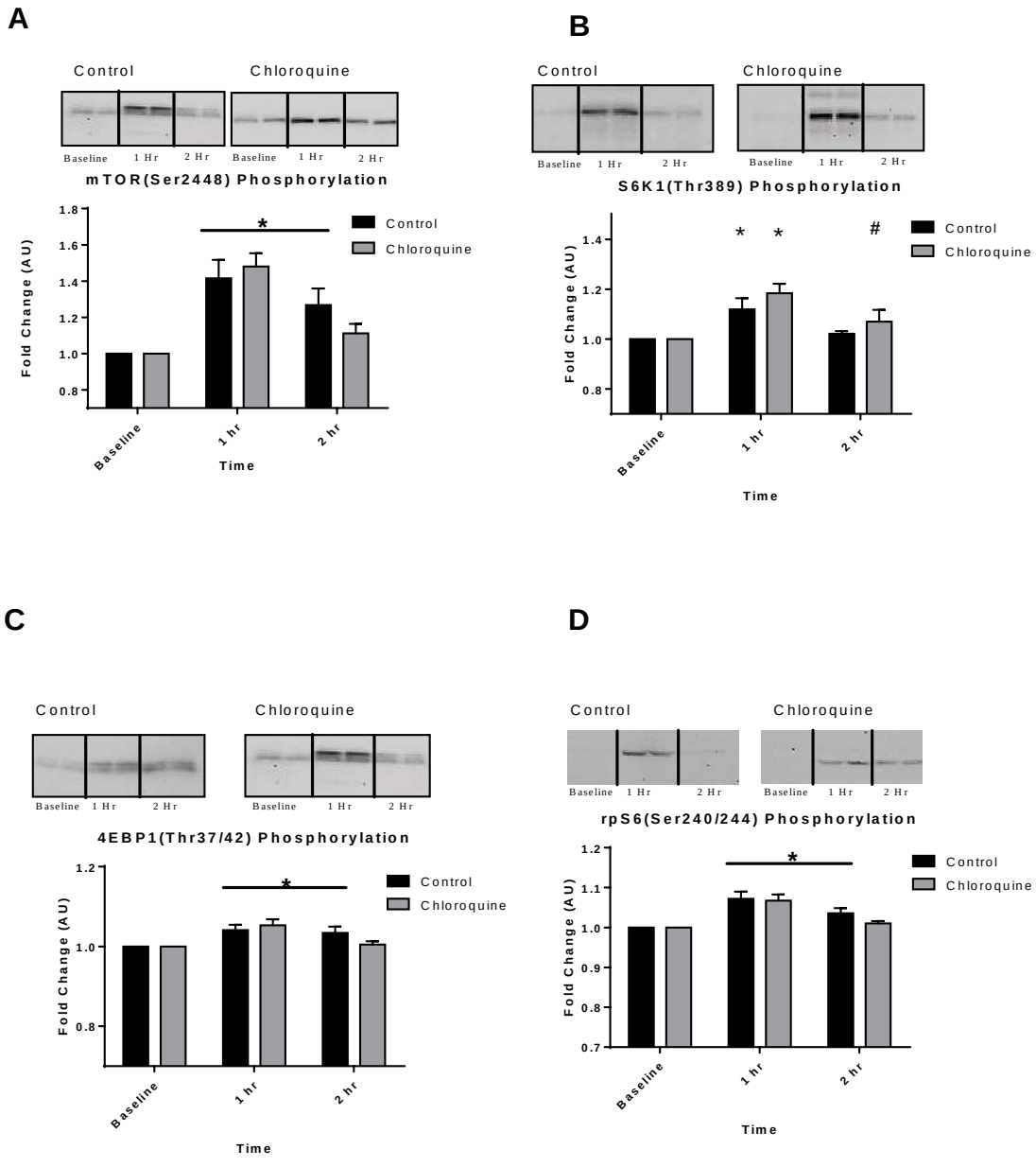


Figure 7 Western blot analyses of mTORC1 (A) and mTOR pathway-related proteins: S6K1 (B), 4EBP1 (C) and rpS6 (D), at baseline and following a 10g EAA beverage. Data are mean ± SEM. N=7 for both treatment groups. * different from pre, P<.05. # trend difference from pre, P=.07.

The phosphorylation status of mTORC1 (Ser 2448) was significantly increased ($p < 0.05$) at 1hr post ingestion in both groups compared to baseline. Control was significantly elevated at 2hr compared to baseline. (Figure 7A) S6K1 (Thr389) phosphorylation status was elevated at 1hr post ingestion for both groups. At 2hr post ingestion, Chloroquine showed a trend of $p=0.07$ compared to baseline. (Figure 7B) 4E-BP1 (Thr37/42) was significantly increased ($p < 0.05$) at 1hr post ingestion in both groups compared to baseline. Control was significantly elevated at 2hr compared to baseline. (Figure 7C) Lastly the phosphorylation status of rpS6 (Ser240/244) was significantly increased ($p < 0.05$) at 1hr post ingestion in both groups compared to baseline. Control was significantly elevated at 2hr compared to baseline. (Figure 7D)

FRACTIONAL SYNTHETIC RATE

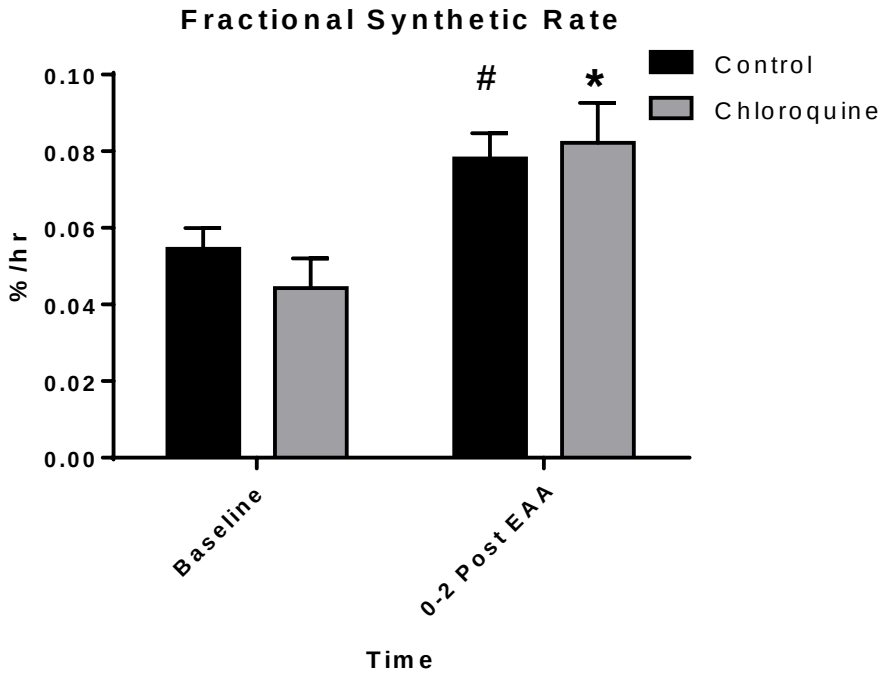


Figure 8 FSR (vastus lateralis) at baseline and for the two hour period post ingestion of the 10g EAA beverage. Data are mean ± SEM. N=7 for both treatment groups. * different from rest, $p<0.05$. # trend difference from pre, $p=.06$. FSR, fractional synthesis rate.

There was no difference between groups for muscle protein synthesis at any time point. The post 10g EAA beverage FSR was elevated from resting values for Chloroquine, $p< 0.05$, with Control showing a trend of $p=0.06$. (Figure 8)

FRACTIONAL BREAKDOWN RATE

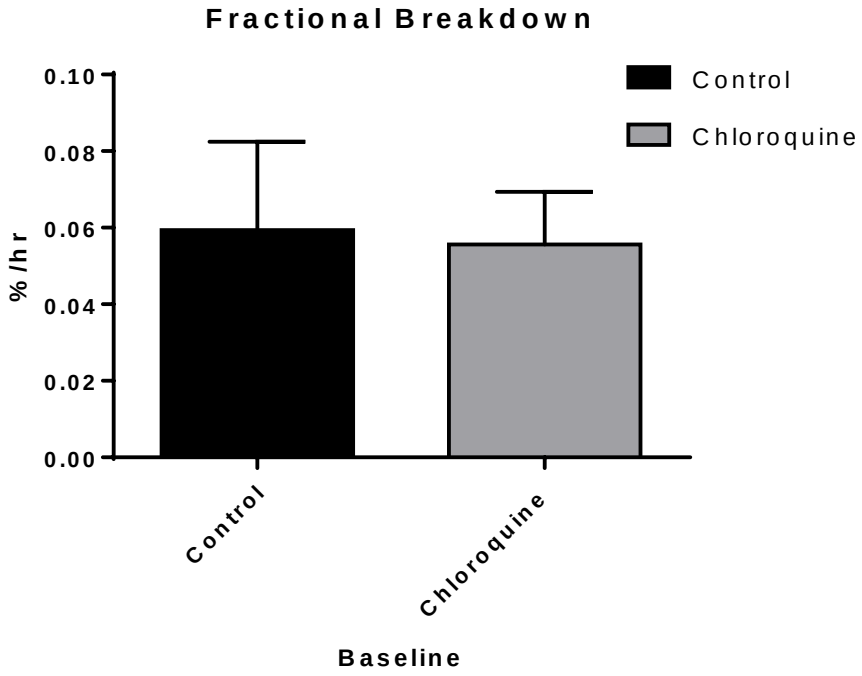


Figure 9 FBR (vastus lateralis) for the 1hr period prior to ingestion of the 10g EAA beverage. Data are mean ± SEM. N=7for both treatment groups. FBR, fractional breakdown rate.

There was no difference between groups for FBR following an overnight fast prior to the EAA intervention. (Figure 9)

WHOLE BODY PROTEOLYSIS

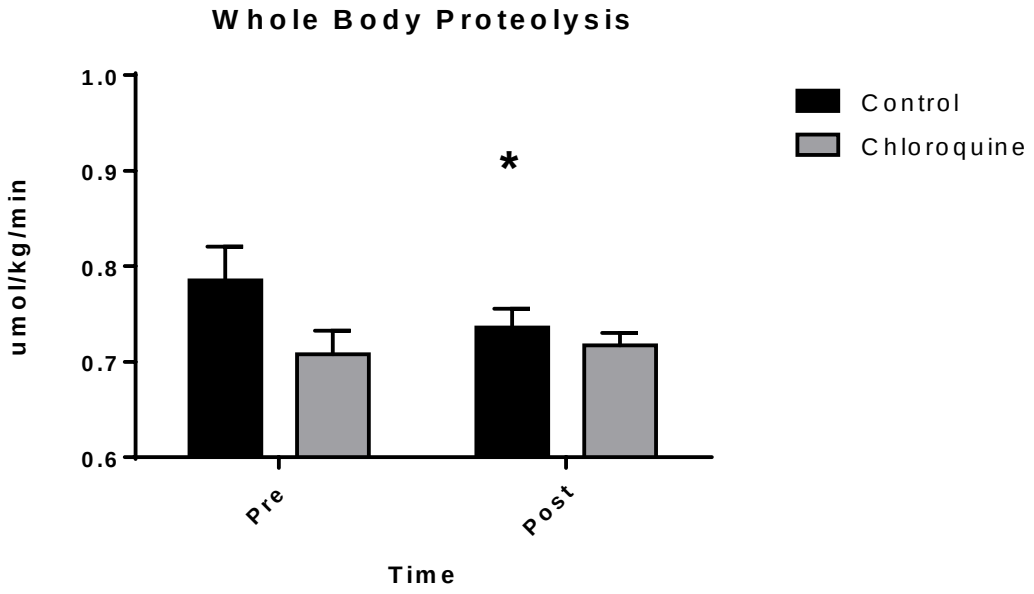


Figure 10 Whole body proteolysis at baseline and for the two hour period post ingestion of the 10g EAA beverage. Data are mean ± SEM. N=7 for both treatment groups. * different from pre, $p<0.05$.

There was no difference between groups at either time point. The post 10g EAA beverage proteolysis measure was significantly decreased from baseline for Control, $p<0.05$. (Figure 10)

DISCUSSION

The benefits of amino acid supplementation have been investigated for many decades going as far back as the 1940s. [107] Since that time, it has become understood that amino acids provide a boost for protein synthesis and net balance. [109] Yet the

mechanism of amino acid activation of protein synthesis remained undiscovered during that time. Only since 2008 has the role of amino acids in protein anabolism been ascertained at a preliminary level.

Sancak et al. 2008 provided the first evidence for amino acid activation of protein synthesis by uncovering the interaction of the mTOR complex with the Rag A/B and Rag C/D proteins. Mutating the Rag protein complex so that it remained in a constitutively active GTP-bound state, the researchers were able to demonstrate a lack of sensitivity to amino acid deprivation by the mTOR signaling pathway. This counteracted the findings that under typical low amino acid conditions, the Rag proteins are in their GDP-bound state and thus inactive. This inactive state negates mTOR activity. Conversely, Rag proteins that were mutated so as not to transition to the GTP-bound state under high amino acid conditions failed to activate mTOR signaling. This research provided the first step towards discovering how mTOR signaling and ultimately protein synthesis is activated by amino acids. [54]

The subsequent paper from the same group, Sancak et al 2010, detailed two additional contributors to the amino acid sensing mechanism. Those two contributors were the lysosome and a complex named Ragulator. The lysosome is known for cellular autophagy. In this case, the Ragulator protein complex resides on the surface of the lysosome. In the presence of amino acids, Ragulator recruits the Rag proteins in their GTP-bound state to the lysosomal membrane in order to dock with the mTOR complex and initiate mTOR signaling. [57] A year later, Zoncu et al 2011 revealed an additional component of amino acid sensing, the vacuolar H(+)-adenosine triphosphatase ATPase (v-ATPase). The v-ATPase was shown to provide a critical interaction with the scaffolding protein Ragulator during amino acid activation of mTOR signaling. [56]

Other players in the amino acid sensing mechanism have since been identified. GATOR1 is an inhibitor of this pathway as it acts on the GTP-bound Rag proteins. GATOR2 works to inhibit GATOR1 in the presence of amino acids. Lastly the Sestrins are

a family of proteins that interact with GATOR2 and are necessary for the colocalization of the lysosome and mTOR. [110]

As all of the research described above was conducted in kidney cells, this current study sought to test the conservation of this amino acid sensing mechanism in muscle. Settembre et al. 2012 demonstrated reduced mTOR signaling with the inhibition of downstream target S6K1 using the drug chloroquine. [106] Chloroquine is a lysosomotropic agent that raises the internal pH of the lysosome. This change in pH causes lysosomal dysfunction and inhibition of lysosomal protein degradation. [110]

For this study, chloroquine was similarly used to impair mTOR signaling in muscle. C2C12 mouse myoblast cells showed a significant increase in both mTOR and S6K1 phosphorylation following ten minutes of 1mM leucine administration. This increase was not seen in cells provided 2mg of chloroquine for 1hr prior to leucine administration. Therefore it would appear that leucine-stimulated activation of mTOR signaling is hampered in muscle as it is in kidney or liver cells in the presence of chloroquine. While it is not possible to determine if lysosomal disruption is the sole cause of this diminished signaling, it does provide evidence that is comparable to that seen with the study conducted by Settembre et al. mentioned above. [106]

To test the role of the lysosome in amino acid sensing in human muscle, we conducted a human trial utilizing a 10g EAA cocktail that has been shown to enhance mTOR signaling and muscle protein synthesis during previous studies. [105,112] One group was provided 750mg of chloroquine (250mg the night before and 500mg the morning of the study) while the other group was provided nothing to serve as the control. Both groups showed similar results consistent with younger subjects ingesting a 10g EAA cocktail. [105]

Amino acid concentrations were elevated in the blood up to 60 minutes post ingestion for leucine in both groups while phenylalanine elevation from baseline was delayed by 15min in the control group. Muscle amino acid concentrations were the same

for both groups with increases at 1hr post ingestion compared to baseline. Both groups showed increases in mTOR signaling phosphorylation at 1hr post ingestion as compared to baseline for all 4 proteins tested. Control also maintained increased phosphorylation of mTOR, 4EBP1 and rpS6 while Chloroquine showed a trend ($p=0.07$) increase at 2hr post ingestion for S6K1.

For muscle protein synthesis, Chloroquine was significantly elevated during the 2hr post ingestion period while the increase for Control did not reach statistical significance with a p value of 0.06. There was no difference between groups during the baseline muscle protein breakdown period. Control did show a significant decrease following amino acid ingestion for whole body proteolysis. Chloroquine did not show any change following consumption of the supplement.

These results show that there was no effect of the chloroquine on any of the muscle specific parameters that were tested for this study. Both mTOR signaling as well as muscle protein synthesis were not diminished in response to 10g of EAAs for Chloroquine. Therefore it is not possible to confirm the validity of amino acid sensing through the lysosome in humans from this study. Yet the whole body proteolysis may provide some supporting evidence for this mechanism. De feo et al 1994 showed that a 750mg dose of chloroquine is sufficient to reduce whole body proteolysis in humans. They concluded that the reduction in proteolysis by approximately 11% was caused by lysosomal disruption at the whole body level. [108] For this study, while there was no difference between groups at the baseline level, the absolute value for Chloroquine was less than the control group (.71 v .79 umol/kg/min) and may not have reached significance due to a lack of power. Yet the difference of approximately 11% between groups was consistent with the earlier study. [108] Therefore it is possible that proteolysis was impaired in Chloroquine as compared to Control prior to amino acid ingestion.

Amino acids have been shown to reduce both whole body as well as muscle protein breakdown. [113,114] A decrease in whole body proteolysis was confirmed in this study

with the reduction seen in Control following amino acid ingestion. Whole body proteolysis was lower at baseline for Chloroquine than Control (not statistically lower though) and did not change following amino acid administration since the lysosomes were disrupted and could not become engaged in the amino acid sensing mechanism. Control saw a drop in proteolysis since the lysosomes were not impaired and thus shifted from autophagy to amino acid sensing. Therefore we are able to speculate that the chloroquine did in fact disrupt lysosomal function at the whole body level, just not at the level of the muscle.

Chloroquine is known to accumulate in certain tissues of the body, specifically the liver, spleen, kidney and lung. These organs were found to have chloroquine concentrations 200-500 times that found in the blood. [115] Therefore it is possible that the dosage for this study was insufficient to reach the levels necessary to interrupt lysosomal function within skeletal muscle. That would explain the disparity between the results seen at the whole body level versus at the muscle specific level.

The two muscle studies, one in cells and one in humans, sought to test the mechanism of amino acid sensing originally found in kidney cells. The mTOR pathway phosphorylation data from the *in vitro* experiment potentially demonstrates the necessity of a functional lysosome for amino acid sensing in muscle. While the human data did not mirror these findings, the whole body proteolysis data does shed some light on the possibility of this mechanism being present systemically with the dose provided in our study. Therefore further research must be performed before a definitive picture of amino acid sensing and the role of the lysosome in muscle can be realized.

Chapter 4

Conclusions

The aging of the population has become a critical issue of late. With life expectancies increasing due to improvements in modern medicine, the population in this country continues to get older. Now as the baby boomer generation transitions into retirement, the consequences of aging are becoming more pronounced and need to be dealt with so as to avoid the high cost that we as a society will have to pay for our older citizens.

If being healthy and active is the ultimate goal as we advance in age, we must maintain the very thing responsible for our locomotion. That thing is skeletal muscle. Skeletal muscle is the tissue of the body that provides the necessary force required for movement. In fact, the area of study that focuses on muscle, kinesiology, is actually the study of movement. A base amount of skeletal muscle is critical for maintaining normal daily function. When individuals dip below that basic threshold, disability is often quick to follow. With reduced muscle tissue or lean mass, the incidence of falls and frailty are more likely. The consequence of a fall can be catastrophic when the end result is a long stay in a hospital bed. Considering the rapid loss of lean mass during bedrest, a short hospital stay following a fall for someone with reduced lean mass can be truly detrimental for recovery and ultimately result in death. In the very least, that person could easily transition into a

frail state. Frailty is the culmination of loss of muscle over the adult lifespan that pushes people out of their homes and into assisted-living facilities.

If the general aim of aging research is to keep people at home and out of the nursing home, we as a scientific field must understand the cause for this muscle loss in order to stop it. This age-related muscle loss, known as sarcopenia, has become a major focus of the muscle field although the etiology of sarcopenia is still unknown. What is known is that the morphology of older muscle is not the same as younger muscle. There lies a point when a shift in muscle quality occurs, and it is this worsening in muscle quality that results in detriments in overall muscle health. Muscle quality is typically defined as the ability of muscle to generate force. Younger muscle is capable of generating more force per muscle fiber or muscle fiber cross sectional area than older muscle. This is due to the infiltration of fat and fibrosis within older muscle. Thus older muscle does not generate force at the same level as it once did. [116]

Since older muscle is smaller and of worse quality than younger muscle, it is imperative that we understand the mechanisms for these impairments in order to create interventions to reverse these unhealthy trends. At this point, research points to two interventions that have provided results for improving muscle health in older individuals. Those two interventions are protein supplementation and resistance exercise. Both interventions provide the most robust anabolic stimulus, when used in combination, for activating the machinery necessary to build muscle. Countless studies have demonstrated the benefits of resistance training for people of all ages, either acutely or in a chronic setting. Recently work has been published showing that resistance training aids in the fight against sarcopenia and even frailty. Older individuals show improvements in strength, force production and maintaining lean mass when on a resistance exercise regimen. This even holds true for older people with certain disabilities. [117] The old notion of keeping people in bed or not pushing them too hard following injury or disability has become

antiquated. Instead we now realize that a high intensity resistance exercise program provides the greatest benefits for muscle health outcomes.

While the benefits of resistance exercise in this population are clear, there are two drawbacks to relying solely on resistance exercise as the intervention to attenuate muscle loss and frailty in the elderly. First off is the inability of resistance exercise alone to maximally stimulate muscle protein synthesis. A single bout of resistance exercise in younger individuals is adequate for activing muscle protein synthesis for over 24h. [26] This is not so in the elderly. [28] This becomes an issue since people are typically in a catabolic state of protein breakdown. It is only through a stimulation of anabolic signaling that they transition to an anabolic state. This interplay of synthesis and breakdown that governs protein turnover must be shifted to anabolism for the net balance to become positive. During positive net balance, protein accrual can exceed loss. Older individuals do not reach a positive net balance from a bout of resistance exercise. They require more stimulation than exercise alone.

A second issue with resistance exercise in the elderly is that many elderly, especially those struggling with a disability, cannot endure a high resistance exercise protocol. Most studies that have tested resistance exercise in the elderly only recruit "healthy" older subjects. These subjects typically do not have a disability, do not take medications, and are generally somewhat active in their daily life. They do not accurately represent the older population as a whole. Older subjects who cannot lift 70% of their one repetition maximum are out of luck for seeing all of these positive results. Lucky a new training protocol has been introduced over the past few years that may hold the key for those people unable to lift substantial weight due to physical limitations. Blood flow restriction (BFR) exercise may provide similar benefits to traditional resistance exercise while using only a fraction of the weight. Under blow flow restriction, the individual performs the exercise using only 20% one repetition maximum as opposed to the more traditional 70% 1RM. This weight reduction could allow for younger people recovering

from injury or older people with physical limitations to still use a resistance exercise protocol without further physical risk. BFR uses blood pressure cuffs to restrict blood flow both during exercise sets as well as during the between set rest period. Recent studies have shown similar increases in mTOR signaling with activation of mTOR as well as downstream target S6K1 between BFR and traditional high intensity exercise. Similarly the muscle protein synthetic response is comparable between exercise modalities. [118,119] This makes BFR a potential option for stimulating muscle growth in older individuals who cannot tolerate more traditional exercise.

The second intervention for shifting protein turnover from the catabolic state to the anabolic state is protein supplementation. Protein supplementation alone or jointly with resistance exercise provides enough stimulus to maximally stimulate protein synthesis in older adults. Therefore adequate protein intake is key in the elderly. As with resistance exercise, there are a few caveats worth considering when touting the merits of increased protein ingestion for muscle health. It has been well-established that older individuals require more protein at any given meal than their younger counterparts. This protein intake threshold can prove to be a mitigating step as most people do not spread their protein out throughout the day. While we now know that an equal distribution of 30g of protein at each meal is the best strategy for maintaining anabolism through the day, most people eat inadequate amounts of protein at both breakfast and lunch. Thus they only reach their minimum protein synthesis stimulating threshold at dinner. The excess protein consumed at dinner does not provide any further benefit since excess protein is oxidized by the body. [120] Therefore a change in diet practice would be required to receive maximum protein intake benefit on stimulating muscle growth. A second issue with increased protein ingestion in the elderly is that elderly individuals eat less in general including less protein. The elderly sometimes go through what is known as “anorexia of aging.” This anorexia of aging has many causes both physical and psychological. From a physical standpoint, appetite suppression and difficulty digesting can plague those with certain diseases, ulcers

or bowel obstructions. In addition, medications for many diseases can result in loss of appetite. It is not only physical issues alone that account for this phenomenon. The psychological distress caused by disease itself could cause appetite suppression. Loss of independence, moving to a nursing home or extended hospital stay can all contribute to reduced food intake. [121] Therefore just prescribing an increase in protein intake as part of the regular diet may not suffice for improving muscle anabolism in this population.

In light of this current evidence, it is critical to combine exercise and nutrition for older individuals to overcome their anabolic resistance and maintain proper muscle health. The results of the study in Chapter 2 suggest that combining both interventions with a minimum amount of protein is key for maximally stimulating protein synthesis. Both groups showed similar shifts in both amino acid concentration profiles as well as anabolic signaling. Only one group, Whey, showed a statistically significant improvement in FSR since Protein Blend had an unusually high baseline protein synthesis rate. Still it would appear that the nature of the supplement is not critical for improved anabolism. What is critical is the dose. These results complement the other similar studies outlined in Chapter 2. The issue with studies of this nature is that these were all healthy male subjects lacking comorbidities while being studied acutely in the fasted state. Since many people of this age range suffer from certain conditions or are taking medications, the groups typically chosen for these studies do not accurately reflect the population at large. Therefore it is difficult to extrapolate the results of this study for the entire elderly population. Also most people do not fast for up to 18hrs at a time. Still it is another step forward in trying to determine the efficacy of protein supplementation combined with resistance exercise in older individuals. Also it is impossible to do more than speculate whether improvements in FSR, say from 0.06% per hour to 1.0% per hour during an acute study have any real clinically beneficial effects over the long-term. That is why it is necessary to complete more chronic studies to determine if short-term acute improvements seen in the hospital provide true long-term benefits for the population in question. Recently published long-term studies have provided

some evidence that chronic resistance exercise is helpful for post illness or injury rehabilitation as well as maintaining activities of daily living. The benefits of protein supplementation is less clear. Yet it would seem that protein supplementation has a place in the treatment of the elderly for those unable to consume the minimum protein threshold to activate muscle protein synthesis. Protein supplementation is an inexpensive tool for this population that could provide a simple solution in hospitals, nursing homes or in peoples' homes to reduce the risk for frailty and detrimental muscle loss.

All of the results provided in Chapter 2 are descriptive in nature. This is a common issue for studies of this kind. Both groups saw increases in anabolic cell signaling as a result of both exercise and protein supplementation. One group saw a statistically significance increase in protein synthesis. The other group failed to show a statistical increase due to a high basal synthesis rate. This data is a good representation of studies of this type that report increases or decreases in certain measures following the intervention. These descriptive studies are normal for human research as there are strict limits for what types of treatments or tests can be performed on human subjects. Safety is always paramount and comes first for all clinical trials. The science comes second. As a result, it is very difficult to perform mechanistic studies in human test subjects. Still it is possible to discover mechanistic explanations for the descriptive data collected during clinic trials. Employing reverse translational methodology can provide a clue as to the underlying cause of the phenomena witnessed in the clinic.

It is well-accepted that exercise and protein or amino acid supplementation activates anabolic signaling and the protein synthesis machinery muscle for young and old alike. In order to understand the mechanism behind this anabolism, we employed reverse translational thinking in opting to experiment within mouse muscle myoblast cells. Going from humans to cell culture provided an opportunity to specifically test amino acid sensing in a model that allows for considerable physiology modifications not possible in humans. The work of the Sabatini lab over the years, outlined in both Chapters 1 and 3, provided a

working model for amino acid sensing in kidney cells. The project in Chapter 3 sought to determine if this mechanism was conserved in muscle. The cell culture experiments revealed a disruption in mTOR signaling activation from the drug chloroquine prior to leucine exposure. Since lysosomal function was not directly tested in these experiments, it is only possible to speculate that lysosomal disruption was the direct cause of the inhibition in mTOR signaling. Yet is was proof of concept in that a lysosomotropic agent could negatively impact amino acid sensing in skeletal muscle. This finding aligned itself well with the work done in kidney cells. Also working in cells allowed for the freedom to choose dosages of leucine and chloroquine not possible in humans. The liberty to hone in on ideal concentrations with in vitro experiments ultimately leads to better results. While it is true that a dish of cells is not comparable to a human being, the ability to test all matter of conditions and treatments to determine the proposed mechanism has tremendous value. In this case, there was positive evidence for the role of the lysosome in amino acid sensing in muscle. As a result, the second part of the project outlined in Chapter 3 involved testing this mechanism in humans.

Taking results seen in a cell culture plate and translating those results into a human trial can be challenging. A collection of cells is a very minimal system. There is no interplay between organ systems of the body. In addition, the environment is tightly controlled. While clinical trials seek to minimize variability by controlling the environment, tremendous variability still exists within a human research study. This variability can potentially explain the discrepancies in results between the bench and the clinic. The muscle specific outcomes for this study were very similar between groups. Both groups saw comparable increases in both amino acid concentrations as well as anabolic signaling. The chloroquine group showed a significant increase in protein synthesis following ingestion of a 10g EAA cocktail. The control group had a similar increase, but a statistically non-significant p value of 0.06. This EAA cocktail has been shown to significantly increase all of these outcome measures in young fasted subjects. Therefore this study was

consistent with previous studies and allows for the conclusion that the chloroquine did not have an effect on amino acid sensing within muscle.

The other result of the study, that whole body proteolysis was reduced following amino acid ingestion only for the control group does allow for some conclusions to be made outside of the muscle. Amino acid supplementation does reduce protein breakdown at both the muscle as well as whole body level. Whole body proteolysis was confirmed in this study. The lack of change for the chloroquine group makes it possible to speculate that the lysosomes were disrupted in other tissues of the body. Unfortunately anabolic signaling and protein synthesis were not measured directly in any tissues of the body other than muscle. Therefore it is impossible to know if the potential lysosomal disruption caused a deficit in anabolic response. One problem with a study such as this as compared to cell culture is that the dosage had to be kept under a certain amount to remain safe for use with humans. It was not possible to continuously increase the dosage until we saw a change within the muscle. The incongruity between studies is what makes translational research so difficult. Often times, the discoveries at the bench stay at the bench. Translating the findings into a human model is often not obvious with the restrictions imposed within human research. Yet there is value in testing these systems in humans. After all, the ultimate goal is to improve human health, not cell or rodent health. Therefore all hypotheses need to be confirmed in the human.

While amino acid sensing cannot be determined at this point in humans, this study does provide a part of the framework that will allow for further testing in the future. With a better understanding of amino acid sensing within muscle, it may be possible to exploit this mechanism to aid in muscle anabolism and attenuation of muscle loss. For the elderly population especially, activating this mechanism without outside stimulation would circumvent many of the issues we currently are facing. Anabolic resistance would no longer pose a problem if mTOR signaling and protein synthesis could be activated synthetically. Also an inability to tolerate the consumption of adequate protein would no

longer pose a problem. Perhaps as our understanding improves with improvements in technology, some type of manipulation of this mechanism could provide the much sought after solution to the issues of sarcopenia and frailty. If so, this would allow for people to stay in the home, remain active and compress their disability until later in the lifespan.

Glossary

1RM – One repetition maximum
4E-BP1 – Eukaryotic initiation factor 4E binding protein 1
AC line- Antecubital line
AIDs – Acquired immune deficiency syndrome
AMT- Amount
ANCOVA- Analysis of covariance
ANOVA – Analysis of variance
Akt – Protein kinase B
AU – Arbitrary units
BFR – Blood flow restriction
BMI – Body mass index
CHQ- Chloroquine
CON – Control
DEXA – Dual-energy X-ray absorptiometry
DTT – Dithiolthreitol
EAA- Essential amino acids
ECG – Electrocardiogram
eEF2 – Eukaryotic elongation factor 2
eIF4E – Eukaryotic initiation factor 4E
eIFS- Eukaryotic initiation factors
EM – Phenylalanine enrichments in the free intracellular pool
Ep – Increment in protein-bound phenylalanine enrichment
FBR- Fractional breakdown rate
FSR – Fractional synthetic rate
GAP- GTPase-activating protein
GCMS – Gas chromatography mass spectrometry
GβL – G protein β-subunit-like protein
GH – Growth hormone
GDP-Guanosine diphosphate
GTP- Guanosine triphosphate
GTPase- Guanosine triphosphatases
HEK- Human embryonic kidney cells
HIV – Human immunodeficiency virus
Hr- Hour
IC- Intracellular
IGF-1 – Insulin-like growth factor 1
IgG – Immunoglobulin G

ITS-CRC – Institute for Translational Sciences Clinical Research Center
LAT1- L-type amino acid transporter 1
Min- Minutes
mSIN1- Mammalian stress-activated protein kinase interacting protein 1
mTOR- mammalian (mechanistic) target of rapamycin
mTORC1 – Mammalian target of rapamycin complex 1
mTORC2 – Mammalian target of rapamycin complex 2
NFDM – Non-fat dry milk
NHANES- National Health and Nutrition Examination Survey
PI3K- Phosphatidylinositol 3-kinase
PB- Protein blend
PBS- Phosphate buffered saline
PMSF – Phenlymethylsulfonyl fluoride
PRAS40 – Proline-rich Akt substrate-40
PVDF – Polyvinylidene fluoride
Rag- Recombination-activing gene
Raptor – Regulatory associated protein of mTOR
RE- Resistance Exercise
Rheb – Ras-homologue enriched in brain
Rictor – Rapamycin-insensitive companion of mTOR
RPM – Revolutions per minute
rpS6 – Ribosomal protein S6
S6K1 – Ribosomal protein S6 kinase beta-1
SBTI – Soybean tripson inhibitor
SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM- Standard error of the mean
Ser – Serine
SNAT2- System A transporter 2
t – Time
t-BDMS- tert-butyldimethylsilyl
TBS – Tris buffered saline
Thr – Threonine
TSC1- Tuberous sclerosis complex 1
TSC2 – Tuberous sclerosis complex 2
Tyr – Tyrosine
v-ATPase- Vacuolar H(+)-adenosine triphosphatase ATPase
WP- Whey protein

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Vita

Michael Steven Borack was born on June 21, 1978 in Livingston, New Jersey. He completed his Bachelor of Arts degree from the University of Wisconsin-Madison in 2000 with a focus on Political Science. He later enrolled in the Master's program within the school of Kinesiology at the University of Michigan in 2007. During his time at Michigan, Michael worked in a laboratory with a focus on the role of genetics in the exercise response. Specifically, the lab investigated the role of cardiovascular fitness on disease prevention. Michael worked in a second laboratory that focused on pediatric obesity and exercise in the human model. In 2011, Michael came to the University of Texas Medical Branch in Galveston, TX in order to pursue a PhD. During his time at UTMB, Michael has mentored a high school student through the Bench Tutorials program at UTMB in conjunction with Ball High School, as well as two medical Students through the mSTAR program. Additionally, Michael has received several scholarships and awards while at UTMB including the Charles F Otis Endowed Award for Clinical Research.

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Publications

Manuscripts

Dickinson JM, Gundermann DM, Walker DK, Reidy PT, Borack MS, Drummond MJ, Arora M, Volpi E, Rasmussen BB. Leucine-enriched amino acid ingestion after resistance exercise prolongs myofibrillar protein synthesis and amino acid transporter expression in older men. J Nutr. 2014 Nov; 144(11):1694-702.

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Borack MS, Reidy PT, Husaini SH., Markofski MM., Deer RR, Richison AB, Lambert BS, Cope MB, Mukherjea R, Jennings K, Volpi E, Rasmussen BB. Protein Blend and Muscle Turnover in Aging.

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