THE EFFECTS OF LEUCINE, ARGININE, AND LYSINE ON ANABOLIC PATHWAYS IN HUMAN MYOBLASTS

by

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A THESIS

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Hans C. Dreyer

Total knee arthroplasty (TKA) procedures are projected to increase nearly seven-fold to 3.4 million surgeries performed annually in the U.S. by 2030. Although the surgery is effective at alleviating osteoarthritic pain, it causes long-term muscle wasting that inhibits functional mobility. A proof-of-principle report by our group showed that essential amino acid (EAA) supplementation was successful in mitigating muscle atrophy after surgery. To better understand the mechanisms of EAAs at the cellular level, we isolated human myoblasts and modeled the cellular responses to anabolic stimuli experimentally under controlled cell culture methods using young healthy isolated myoblasts as control. **Methods:** Cells from the biopsies were seeded in well plates and myogenic identity was confirmed via immunocytochemistry (ICC). After plating, cells were starved prior to stimulation in either untreated, LRK or LRK+I. Cells were lysed and phosphorylation status of targets of the mTORC1 signaling cascade were analyzed by Western Blot. **Results:** Preliminary results demonstrate that isolating myoblasts from older patients is more difficult than young controls. In the young, incubation with LRK only produced a significant effect on the phosphorylation

of Akt at 15 minutes. Incubation with LRK + I showed a greater effect, with the phosphorylation status of Akt and Gsk3β being significantly greater than the control at 15 minutes, and the phosphorylation of Akt and rpS6 were significantly greater than the control at 30 minutes. **Conclusions:** Additional research is needed to refine our isolation methods and to better understand the mechanism(s) through which amino acids attenuate the atrophy of TKA patients. If successful, recovery strategies can be refined to improve functional mobility following surgery and enhance long-term quality of life for these older individuals.

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Introduction

Total knee arthroplasty (TKA), or total knee replacement, is the surgical remediation of the knee joint in order to eliminate chronic knee pain, restore physical integrity and function to the damaged joint. Total knee arthroplasty is becoming increasingly popular with over 700,000 TKAs performed in the United States in 2012. A conservative estimate of the number of primary TKA procedures expects to see a 143% increase in incidence rates by 2050 or 1.5 million cases.[1] While other estimates project that there will be 3.48 million primary TKA procedures by 2030.[2] Total knee arthroplasty is the most commonly performed surgical remediation for patients with knee osteoarthritis. Despite the success of TKA in eliminating chronic knee pain due to osteoarthritis, muscle atrophy following TKA occurs at a rate of 1% per day for the first 2 weeks after surgery.[3] Further complicating the situation, the older adult demographic undergoing TKA are also experiencing sarcopenia, or age-associated muscle loss, in addition to post-operative muscle atrophy. It is estimated that 5-13% of elderly people aged 60-70 years and 11-50% of those aged 80 or above are affected by sarcopenia.[4] TKA exacerbates sarcopenia and accelerates total muscle loss in patients after surgery. This atrophy also impedes the ability of TKA patients to return to baseline levels of strength and function after surgery. Unfortunately, this atrophy that patients experience is likely permanent due to the age-associated anabolic resistance, leading to a reduced likelihood to regain muscle mass and strength during recovery.[5]

Therefore, there is a present and increasing demand for improving post-operative recovery for TKA patients in order to attenuate the muscle atrophy experienced during rehabilitation. In a 2013 study, Dreyer *et al*. found that twice daily

ingestion of 20 grams of EAAs between meals for 1 week before TKA and 2 weeks-post helped reduce the degree of post-operative muscle atrophy and associated weakness in patients undergoing total knee arthroplasty.[3] In a follow-up trial, published in 2018, we replicated these findings, and showing that bilateral muscle atrophy was significantly less in patients on EAAs vs. placebo.[6]

Determining the signaling pathways controlling skeletal muscle metabolism in response to essential amino acid supplementation will allow us to better understand, and target these areas and better inform clinical practice. Skeletal muscle anabolism and catabolism are the result of complex interactions between gene transcription, translation and protein breakdown.[7] Studies performed in C2C12 store bought mouse myoblasts have found that leucine is uniquely able to stimulate mTOR and downstream 4EBP1 phosphorylation to enhance anabolism within the cell.[8] A separate study, also performed in C2C12 mouse myoblasts found that arginine, a semi-essential amino acid, also activated protein anabolism through the phosphorylation of mTORC1 and downstream effectors.[9] However, these studies are limited due to their use of an immortalized mouse myoblast cell line as opposed to myoblasts isolated from humans.

Studies in young adults have found that ingestion of essential amino acids and carbohydrate mixture both alone and following resistance exercise resulted in activation of the anabolic mTOR pathway.[10,11] In older adults, phosphorylation of mTOR increased less in older adults in response to ingestion of 10 g of essential amino acids, compared to young.[12] However, the cellular response to essential amino acids in young and older human cells has not been elucidated further. Therefore, the primary purpose of this study is to examine how leucine, arginine and lysine (LRK) stimulation affects

cellular signaling pathways in young and older adults. This Honors Thesis aims to assess the anabolic cellular response to LRK, and LRK and insulin combined in human myoblasts in healthy, young individuals compared to older TKA patients to better understand potential underlying differences in anabolic signaling. This knowledge could then be used to design additional experiments to help eliminate, or reduce, the anabolic resistance at the cellular level in hopes of redesigning new clinical trials in older TKA patients.

Background

Osteoarthritis and Total Knee Arthroplasty

Chronic knee osteoarthritis is a painful joint disease where the articular cartilage and its underlying bone have progressively worn away, resulting in pain and stiffness during movement. Between 1997 and 2011, the hospitalization rate for osteoarthritis grew 160% in the United States.[13] Osteoarthritis of the knee is the most prevalent disease in the U.S., and affects over 60% of adults 65 years and older.[14]

Total knee arthroplasty (TKA) is the most commonly performed surgical remediation for patients with knee osteoarthritis. In 2005, there were approximately 500,000 TKAs performed in the United States, at a cost of over $11 billion.[15] Projections estimate that the demand for primary TKAs is projected to increase 673% to 3.4 million surgeries performed annually in the U.S.[2] While TKA remedies pain due to osteoarthritis, most patients experience significant loss of strength and muscle volume after surgery.[3,16] Two recently completed clinical trials show that essential amino acid (EAA) supplementation attenuates muscle atrophy and accelerates the return of functional mobility.[3] However, the cellular mechanisms that cause muscle atrophy, and the ability to attenuate muscle atrophy or preserve existing muscle tissue after major surgeries remains unknown.

Muscle Loss in Older Adults

Sarcopenia, the involuntary loss of muscle mass and muscle function that occurs with age, has extensive consequences on older adults.[17] Sarcopenia is the age-associated progressive loss of muscle mass and function, which causes disability, loss of

independence, and increases the risk for falls.[18] Loss of muscle with age begins after the age of 35 with a loss of 3-8% muscle mass per decade. After the age of 60, this muscle loss rate accelerates and a reduction in voluntary strength also begins to occur.[5] The loss of muscle mass in older adults is difficult to recover. Progressive sarcopenia can ultimately lead to frailty, and an increased likelihood of falls, may eventually culminate in reduced quality of life, and in some cases institutionalization.[19] The pathological changes that occur with the loss of metabolically active skeletal muscle can contribute to loss of function, as well as being associated with increased insulin resistance, fatigue, falls, and mortality.[17] Declines in muscle mass from sarcopenia drives the association with various metabolic disorders, including: obesity, insulin resistance, diabetes, dyslipidemia, and hypertension.[20] The population attributable risk in older men due to sarcopenia was 85.6% and in older women was 26.0%, meaning that if sarcopenia were completely eliminated, 85.6% of the disability cases in older men and 26.0% of the disability cases in older women would be eliminated.[21] It is estimated that in the year 2000 the United States spent $18.5 billion on healthcare directly attributable to sarcopenia.[21]

The cause of sarcopenia is unknown, but it is thought to be multifactorial, ranging from whole body physical activity to cellular and morphological changes that gradually lead to overall muscle tissue changes.[17] These changes include specific loss of Type II muscle fibers, with a portion of those being re-innervated by adjacent Type I nerves.[5] Type II muscle fibers are capable of producing four times more power than Type I fibers, which may explain the reduced power production of older adults.[5] Diminished excitation-contraction coupling and declines in satellite cell activation and

proliferation are also thought to contribute to sarcopenia.[5] While many factors may influence the incidence of sarcopenia, it results from a fundamental imbalance between muscle protein synthesis and breakdown that results in muscle fiber atrophy. The muscle protein anabolic response to amino acids and/or protein ingestion between older and young adults does not differ greatly when ample amounts of protein/amino acids are consumed either alone of when accompanied by physical activity.[22] However, older adults have a blunted anabolic response to the same dose of EAA compared to young when the amount is at or below 10 grams of EAA.[12] In order to determine the mechanism(s) of sarcopenia in older adults, careful studies of their behaviors and metabolism in comparison to younger adults must be performed. Determining the cellular signaling pathways controlling skeletal muscle metabolism in response to both anabolic and catabolic stimulus in young and older cells will allow us to better understand, and target, molecular pathways that control metabolism in order to better preserve muscle after common orthopedic surgeries in older adults.

Essential amino acids and anabolic signaling

Skeletal muscles are regulated by multiple processes that incorporate many physical signals, such as diet and exercise, into an anabolic response.[7] The regulation of these signals occurs on both a cellular and organismal level.[7] Amino acids are needed in all cells in order serve as a substrate for constructing new proteins within the cell, acting as both a substrate for polypeptide synthesis and directing the activation of regulatory mechanisms of mRNA translation.[8] Essential amino acids are a subset of amino acids that must be obtained through dietary sources because they cannot be synthesized within the human body. Branched-chained amino acids are a subset of EAAs that

include leucine, isoleucine and valine, which make up approximately one-third of skeletal muscle protein.[8] Amino acids are necessary to stimulate protein synthesis through the mTOR signaling pathway in skeletal muscle.[23] In particular, leucine is a known anabolic stimulator of the mTOR signaling pathway. Further, recent evidence indicates that lysine and arginine may also stimulate anabolism through mTOR.[24] However, the exact mechanism behind amino acid activation of protein synthesis is not fully understood.

Previous Findings of EAA Supplementation in TKA patients

The potential benefits of essential amino acid supplementation to promote an increase in muscle protein synthesis and/or reduce muscle protein catabolism have been studied for decades. More recently, using EAAs to promote muscle anabolism and reduce muscle catabolism in clinical setting have increased. Notably, successful treatment of total knee arthroplasty (TKA) patients with EAAs, as a twice daily supplement for one week prior to surgery and two weeks following,[3] or for an extended six weeks after surgery,[6] results in an attenuation of muscle atrophy. After surgery subjects on placebo demonstrated a 14%-15% loss of quadriceps and hamstring/adductor muscle volume two weeks after surgery.[3] Those who received the EAA supplement, however, reduced muscle atrophy 2- to 4.6-fold compared to the placebo group at six weeks after surgery.[3]

While EAA supplementation did not eliminate the muscle loss experienced by these subjects, the patients who received the EAA supplement also experienced greater functional mobility after surgery.[3] Older adults having TKA may benefit from EAA supplementation because it helps to reduce muscle atrophy, but this is not the case for

all patients and, moreover, identifying the critical components that can preserve muscle are needed. In our recent clinical trials, subjects had to ingest a large amount of the EAAs (20g) twice a day, which could be limiting in its application because of the volume, taste, and duration participants take the supplement (1 week before and 6 weeks after surgery). Therefore, identifying the cellular mechanisms of the EAA supplement that maximally stimulate pathways governing anabolism and catabolism will greatly enhance applicability and, potentially, efficacy. Knowledge of the specific cellular response to leucine, lysine, and arginine can provide insight into altering the treatment into a more easily managed dose, hopefully resulting in easier distribution and appeal to patients. In 2013, we published a proof-of-principle report[3] showing that EAAs significantly reduced bilateral quadriceps and hamstrings muscle atrophy and accelerated the return of functional mobility vs. placebo in older adults after TKA. In a follow-up trial[6], published in 2018, we replicated these findings, and showed that bilateral muscle atrophy was significantly less in patients on EAAs vs. placebo.

The mTOR Pathway

The mechanistic target of rapamycin (mTOR) pathway integrates nutrient and growth signals within the cell. mTOR is composed of two protein complexes known as mTORC1 and mTORC2; mTORC1 is associated with cell growth, while mTORC2 promotes cell proliferation and survival.[23] In order to stimulate cell growth, mTORC1 relies on its downstream effectors to promote anabolism. These effectors include: 4E-binding protein 1 (4EBP1), and ribosomal protein S6 (rpS6). The phosphorylation status of these effectors indicates their activation status within the cell.

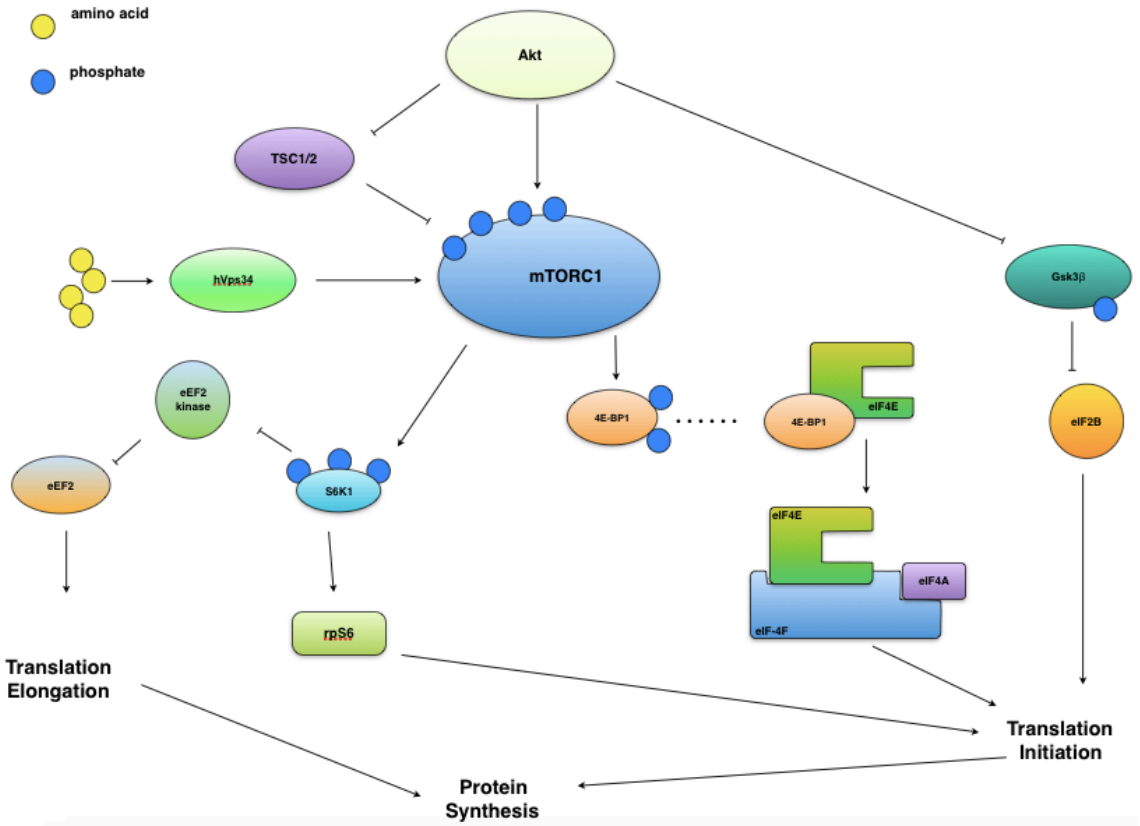

Figure 1. A simplified mTOR signaling cascade

4EBP1 is a repressor protein that regulates cap-dependent translation via promoting eukaryotic initiation factor 4F (eIF4F) assembly so that translation that can occur. Phosphorylation of 4EBP1 indicates that it is no longer able to inhibit translation.[25] Another downstream target of mTORC1 is S6K1, which is activated by amino acids and can be further activated by insulin.[26] S6K1 has many targets that it phosphorylates, including rpS6. When rpS6 is phosphorylated, this indicates that it is activated and will promote protein translation initiation and ribosome formation.[7]

Akt is an upstream effector of mTORC1. Akt is responsive to both insulin and muscle contraction.[7] Phosphorylation of Akt indicates it is activated, and it has been found that ingestion of leucine results in phosphorylation of Akt.[27] When activated, Akt phosphorylates mTORC1, resulting in its activation. Akt also phosphorylates glycogen

synthase kinase 3β (Gsk3β), which results in its inactivation.[28] When active, Gsk3β phosphorylates, and inactivates eukaryotic initiation factor 2B (eIF2B). eIF2B is a guanine nucleotide exchange factor and is necessary for eukaryotic inittation.[28,29] As such, Akt inhibits Gsk3β and allows for translation initiation to proceed (inhibiting an inhibitor).

Skeletal muscle anabolism and catabolism are results of complex interaction between gene transcription, translation, and protein breakdown.[7] Many factors influence the pathways that govern the resulting synthesis or breakdown of muscle protein. While these components influence specific regulatory mechanisms of the pathway on a cellular level, they can also be stimulated by resistance type exercise. Muscle protein synthesis is upregulated by the ingestion EAAs and carbohydrates particularly following an acute bout of resistance exercise.[7] However, essential amino acid supplementation with only modest amounts of carbohydrate produces the same maximal protein anabolic response.[30] It is clear that mTORC1 has an essential role in EAA induced signaling because when mTORC1 activity is inhibited by rapamycin, EAA induced protein synthesis is blunted in rats.[31] It is also known that the primary factor for mTOR signaling and peak stimulation of muscle protein synthesis in rats is leucine.[32] Further, recent evidence indicates that lysine and arginine may also stimulate the synthesis of ribosomal proteins.[24] The greatest effect on skeletal muscle anabolism occurs with ingestion of EAAs and exercise, however, the specific mechanisms of these within the cellular pathways remain unclear.

Limited research has been conducted on human subjects and acute and direct effects of essential amino acid supplementation. An in vivo study of young adults found

that ingestion of EAAs resulted in expression of several amino acid sensing, transport and mTOR regulatory genes.[33] This research helps to support the in vitro work that further links EAAs to specific parts of the mTOR pathway.

Studies in mouse myoblasts

Studies on the effect of essential amino acid supplementation in C2C12 mouse myoblasts have found that EAAs activate anabolic signaling. By exposing C2C12 myoblasts to each EAA, it was determined that leucine is uniquely able to stimulate mTOR and 4EBP1 phosphorylation to enhance anabolism within the cell.[8] Further, this stimulation of mTORC1 was independent of Akt, which is a potential upstream regulator of mTORC1.[8] While another study found that the other EAAs on their own do not stimulate the phosphorylation of these important proteins, finding that leucine by itself plays a significant effect on the activation of these pathways provides further incentive to investigate in human cells. Another study also using C2C12 mouse myoblasts found that arginine, a semi-essential amino acid, activated protein synthesis through the phosphorylation of mTORC1, and its downstream targets, independent of Akt.[9] These specific protein markers help to elucidate the mechanism of amino acid regulation of the cell. This is important because mTORC1 is known as a master regulator of protein synthesis, and incorporates signals from nutrients, growth factors, energy status and stress.[34] These studies are useful but are limited because they were conducted in a immortalized mouse myoblast cell line as opposed to myoblasts isolated from humans.

Studies in young adults

In young adults, essential amino acids and carbohydrate ingestion following resistance exercise enhanced mTOR signaling and muscle protein synthesis.[10] After a bout of resistance exercise mTOR phosphorylation increased twofold, while ingesting a EAA and carbohydrate mixture one hour after resistance exercise activated the mTOR pathway fivefold.[10] In a study of young males who had a muscle biopsy before and after ingesting a solution of EAAs and carbohydrates it was found that mTOR signaling to key regulators of translation initiation, seen by increased phosphorylation of S6K1 and 4E-BP1, and translation elongation, through the decreased phosphorylation, and activation, of eEF2, stimulated muscle protein synthesis.[11] After an infusion of the branch chain amino acids (BCAA) valine, leucine and isoleucine, it was found that both eIF4E-BP1 and p70s6k were phosphorylated, resulting in an increased translation initiation of skeletal muscle proteins.[35] This is notable that the changes in BCAA concentrations within the physiological range are sufficient to cause an effect on the phosphorylation of eIF4E-BP1 and p70s6k.[35]

Studies in older adults

In older adults, muscle protein synthesis is delayed following resistance exercise and essential amino acid ingestion compared with young.[36] While protein synthesis in the young was elevated 1-3 hours after exercise and ingestion of EAAs, protein synthesis in the old was only upregulated at 3 hours post, to a similar extent as seen in the young subjects.[36] Phosphorylation of mTOR increased less in older adults in response to ingestion of 10 g of essential amino acids, compared to young.[12] In the young subjects mTOR and p70s6k phosphorylation increased by 5.2-8.1-fold, while in

the older subjects mTOR and p70s6k only increased by 2.7-3.5-fold.[12] This is likely due to the anabolic resistance experienced by older adults, and suggests that the nutrient signal is not transduced as well by old muscle as the young muscle.[12] While older subjects were still able to stimulate protein synthesis, it took a larger amount of EAAs to reach the same strength of signaling as the young.

Following an acute bout of high-intensity resistance exercise, muscle protein synthesis and signaling through mTOR were upregulated in young subjects.[37] Muscle proteins synthesis was upregulated in younger subjects at 3 hours, 6 hours, and 24 hours after exercise.[37] However, there was a depressed response in both mTOR signaling and muscle protein synthesis in the old subjects.[37] Notably, the lack of signaling response in Akt, mTOR, S6K1, and 4EBP1 in the old suggests that the lack of mTOR activation is the cause of blunted muscle protein synthesis response.[37]

Methods

Ethics approval

This study was approved by the University of Oregon (UO) institutional review board for the primary cell study in young subjects, IRB number 08012014.002 and for the older subjects as part of our TKA study, IRB number 12272013.024. This study was conducted in accordance with the Declaration of Helsinki. All subjects gave informed written consent prior to study participation. This study was funded by a grant from the National Institute on Aging, NIH R01 AG0464012.

Subjects

For this study, we used cells collected from 5 young (19-23 years old) subjects, and 40% of the subjects were females. Older adult subjects (50-80 years old) were also being recruited, and biopsy tissue has been collected, but we were not as successful at isolation of satellite cells from older subjects.

Muscle biopsy

Human primary muscles cells were collected from a muscle biopsy from percutaneous *vastus lateralis* muscle. Subjects underwent local anesthesia [1% Lidocaine HCl (10 mg/mL, Hospira Inc., Lake Forest, IL, approximately 10 cc)] before the incision was made. A 0.6 cm incision was made, allowing a 5 mm Bergstrom biopsy needle, with applied suction to be inserted into the muscle. 150-300 mg of tissue was obtained in 1-2 passes. The biopsy was performed using standard surgical sterility procedures. Excised muscle biopsies were blotted to remove blood and dissected away from any adipose tissue. Tissue for cell isolation was immediately placed into cold

growth medium, transferred to a biological safety cabinet (BSC) and diced into small 'pepper' sized pieces before being explanted onto 12-well collagen coated plates. All tissue explants are grown in PromoCell skeletal muscle growth medium at 37°C at 5% CO_2.

Cell Culture

Cells from the muscle biopsies grown to 80% confluency in 12 well plates. Cells were then detached with Accutase®, a gentle enzyme mixture, and satellite cells were identified using a CD56 antigen. Satellite cells were positively selected for using anti-CD56 magnetic antibodies and separated using magnetic activated cell separation (MACS). MACS separates cells by using antibodies coated with magnetic nanoparticles that cause the CD56+ cells to attach to the magnetic nanoparticles. This allows the CD56+ cells to be separated from the non-satellite cells. The detached cells were spun in a centrifuge at 600g for 4 minutes, with the cell pellet being collected and the supernatant being discarded. The cells were then treated with anti-CD56 magnetic antibodies in MACS buffer (3% BSA, EDTA) for 15 minutes at 4°C. The cells were then centrifuged at 300g for 10 minutes. The cells were then washed through a magnetic column to elude the unbound fraction. The column was then separated from the magnet and the cells attached were flushed and spun to obtain a CD56+ cell pellet. These cells were then seeded in t12 (12 mm^2) flasks and grown to 80% confluency in PromoCell human skeletal muscle growth medium. Cells were then detached with trypsin and EDTA. Trypsin also non-selectively cleaves surface proteins, allowing for removal of

CD56 beads. The cells were then seeded into experimental 4 well plates with 20,000 cells per well for future analysis.

Immunocytochemistry

Population typing is performed by immunocytochemistry (ICC). Cells were immune-tagged with Desmin^{+}, a sarcomeric structural protein that is muscle-specific, and TE-7, a marker of fibroblasts. Cells that were positive for Desmin^{+}, and negative for TE-7 were determined to be myogenic. Experiments were performed on populations 80% myogenic or above. When experimental plates were seeded, one extra well for each data set was seeded in a separate 4 well plate, at the same density, for ICC analysis of cell population. Cells were seeded overnight, washed twice in PBS and fixed in 4% paraformaldehyde (PFA) for 10 minutes followed by 0.5% triton-X100 for 15 minutes and blocked in 3% BSA for 30 minutes at room temperature. Primary antibody was then added in 3% BSA in PBS and incubated overnight at 4°C. Cells were then washed three times in PBS and incubated in anti-Rb488 Alexa/ anti-Ms555 Alexa (Invitrogen) for 1 hour at room temperature. Cells were then washed three times in PBS and DAPI with anti-fade was added. Images were analyzed with ImageJ software, taken with a Zeiss inverted microscope with Zeiss software.

Protein stimulation

Previous work in our lab has shown a need to starve cells for 1h prior to amino acid (AA) stimulation to silence residual signaling from pre-stimulation growth medium. A custom amino acid free basal medium was produced by PromoCell for all stimulation experiments. Myoblasts will be washed twice in 1X phosphate-buffered

saline (PBS) and incubated in standard incubation conditions (37°C at 5% CO2) for 1 hour in serum and amino acid free medium. AA stimulations contain LRK, leucine:arginine:lysine, 2:1:1, 5mM:2.5mM:2.5mM in otherwise amino acid free and serum free medium. The cells were also stimulated with leucine:arginine:lysine, 2:1:1, and 1.72μM insulin in amino acid free and serum free media. Cells were stimulated for 15 minutes and 30 minutes and then immediately placed on ice and lysed in RIPA buffer with protease and phosphatase inhibitors (100x Halt, ThermoFisher Sci.). Lysed cells were then removed using a cell scraper. Lysates were then sonicated twice for 20 seconds and spun 14,000 x g for 15 minutes at 4°C. Supernatant was collected, and protein concentration measured using a fluorometer (Qubit, Invitrogen).

Antibodies

The primary antibodies used were p-Akt Serine 473 (#9271), p-Gsk3β Serine 9 (#9315), p-rpS6 Serine 235 and Serine 236 (#4858), p-4EBP1 was detected at Threonine 37 and Threonine 46 (#2855), and housekeeping GAPDH (#2118) from Cell Signaling (Beverly, MA). The secondary HRP antibodies were from Abcam (Cambridge, MA). ECL+ Anti-Rabbit IgG, horseradish peroxidase from donkey and mouse were purchased from GE Healthcare.

Western Blots

Western blotting is an analytical technique that measures the levels of specific proteins in a sample. Western blot analysis was performed on myoblast lysates collected at 15 and 30 minute time points. Anabolic arms of the mTORC1 cascade were explored, specifically as a Akt, Gsk3B, rps6 and 4EBP1. GAPDH was used as a loading control

because it is constitutively expressed in cells at high levels. Cell lysates in 4x Laemmli buffer added were loaded into 18 lane Any kD gels (Bio-Rad, Hercules, CA), and run through gel electrophoresis at 200V for 43 minutes. The protein was then transferred onto a nitrocellulose membrane and moved into a 4˚C fridge with ice block and stir bar. The proteins on the nitrocellulose membrane were then blocked for 1 hour at room temperature in 3% Bovine serum albumin (BSA) and incubated in primary antibodies overnight at 4˚C in 3% BSA with constant agitation. The membrane was incubated overnight with the primary antibodies in order to detect the phosphorylation status of each protein. The next day the membranes were washed an incubated with their respective secondary antibodies 1 hour at room temperature. The membranes were imaged using ECS substrate (BioRad) and the bands shown were analyzed with Quantity One software.

The results acquired from the Western Blots were made relative to GAPDH. However, the data in Figure 2., representing the effect of growth media compared to serum starved, was not made relative to GAPDH due to the difficulty of obtaining full saturation of the bands. The custom amino acid free media was the basal Skeletal Muscle Cell Media manufactured by PromoCell to not contain any amino acids. The cells incubated in the custom serum and amino acid media were used as a control. Then, the phosphorylated band densities representing protein concentrations were made relative to the equivalent total protein blots. This ratio of phosphorylated protein to total protein was then used to analyze the change in protein activation due to LRK and LRK + I stimulation. Results were then compared to the control using a paired t-test. Significance was set to $P^{*}<0.05$.

Results

Subject Characteristics

This study included 5 young subjects whose cells were grown and split into different flasks. The subjects were mostly female and had a mean age of 20 as shown in Table 1. Three older adults were recruited as subjects, but fibroblasts in the old cell cultures proliferated much more than the satellite cells. This made it impossible to reach 80% myogenic confluency needed to conduct the experiments.

Table 1. Subject Characteristics

	young	old
% female	40%	66%
Age *(yr)*	20 ± 0.56	58.7 ± 1.3

Values shown are means ± SE; n=5 for young; n=3 for old

Growth Media v. Serum Starved

The effect of custom serum and amino acid-free media was examined by incubating the myoblasts in the custom media for 1 hour. The phosphorylation status of Akt, Gsk3β, rpS6 and 4EBP1 were then analyzed by Western blot. The phosphorylation status of these key proteins were also measured following the cell's incubation in growth media. This serves as our positive control to show that cell signaling was diminished following serum starvation. As shown in Figure 2., the serum and amino acid starvation decreased the activation of Akt, Gsk3β, rpS6 and 4EBP1 as shown

through the decreased level of phosphorylation. Akt ($p=0.301$), Gsk3β ($p=0.456$), and rpS6 ($p=0.0111$) were all significantly different, demonstrating that the change in media did result in a silencing of the cell signaling. This provides a baseline control for cell signaling with no nutrients.

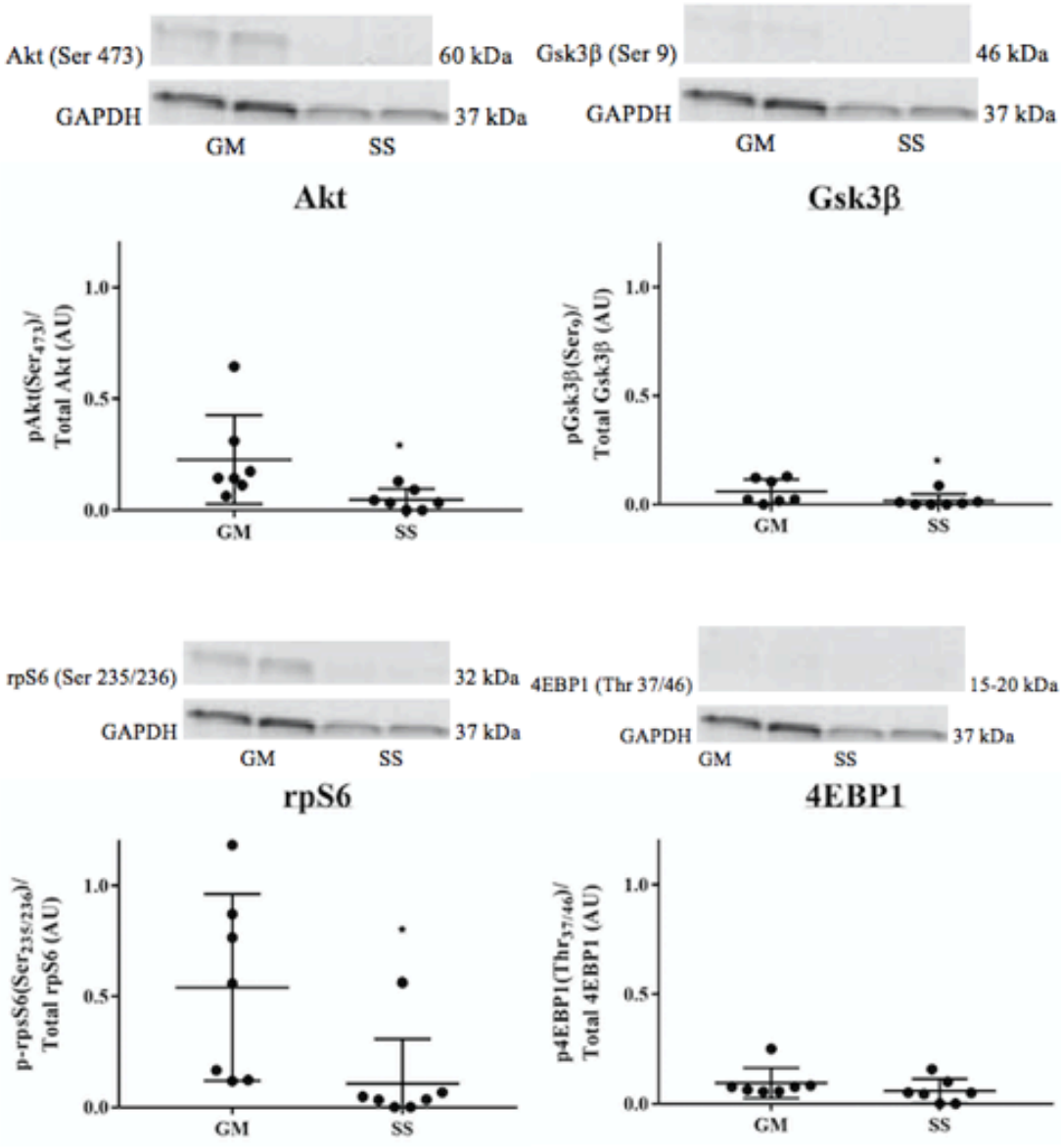

Figure 2. The effect of serum and amino acid free media on the phosphorylation of Akt, Gsk3β, rpS6 and 4EBP1

Amino Acid incubation

The effect of exposure to Leucine:Arginine:Lysine (LRK) and LRK and insulin (LRK + I) were examined at 15 and 30 minute time points. Following the cell's starvation from amino acids and serum, the cells were incubated with LRK and LRK + I

for 15 minutes. As shown in Figure 3., the phosphorylation of Akt was significantly higher after incubation with LRK compared to the control ($p=0.0084$). However, none of the other proteins' phosphorylation status were significantly different than the control. After incubation with LRK + I the phosphorylation of Akt ($p=0.0016$), Gsk3β ($p=0.0363$) were significantly higher than the control. The other data sets were not significantly greater than the control.

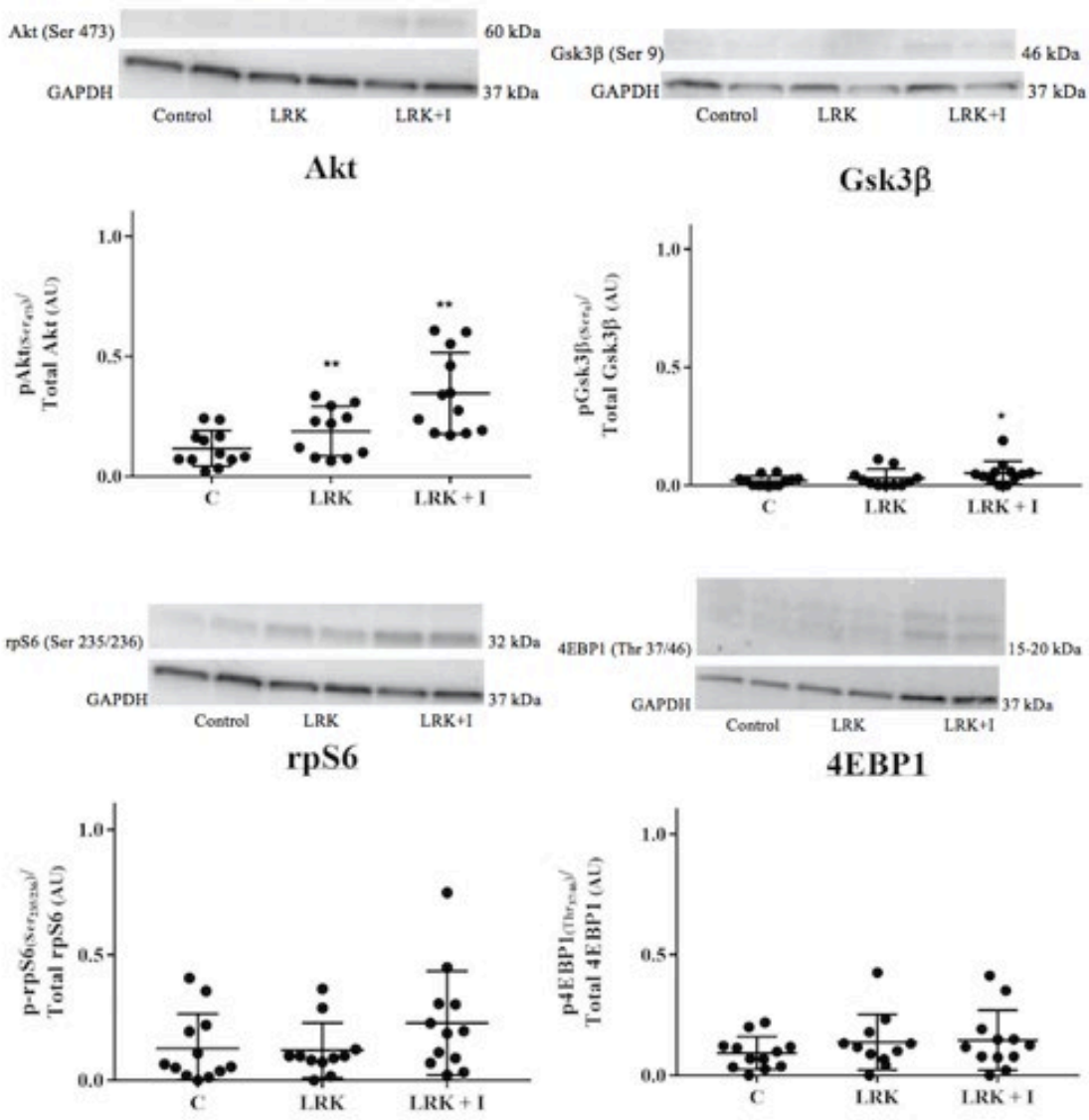

Figure 3. The effect of exposure to LRK and LRK+I on the phosphorylation of Akt, Gsk3β, rpS6 and 4EBP1 for 15 minutes

The myoblasts were also incubated with LRK and LRK + I for 30 minutes to allow greater exposure. As shown in Figure 4., the phosphorylation of Akt ($p= 0.0345$) and rpS6 ($p= 0.0018$) were significantly higher than the control when incubated with LRK + I for 30 minutes.

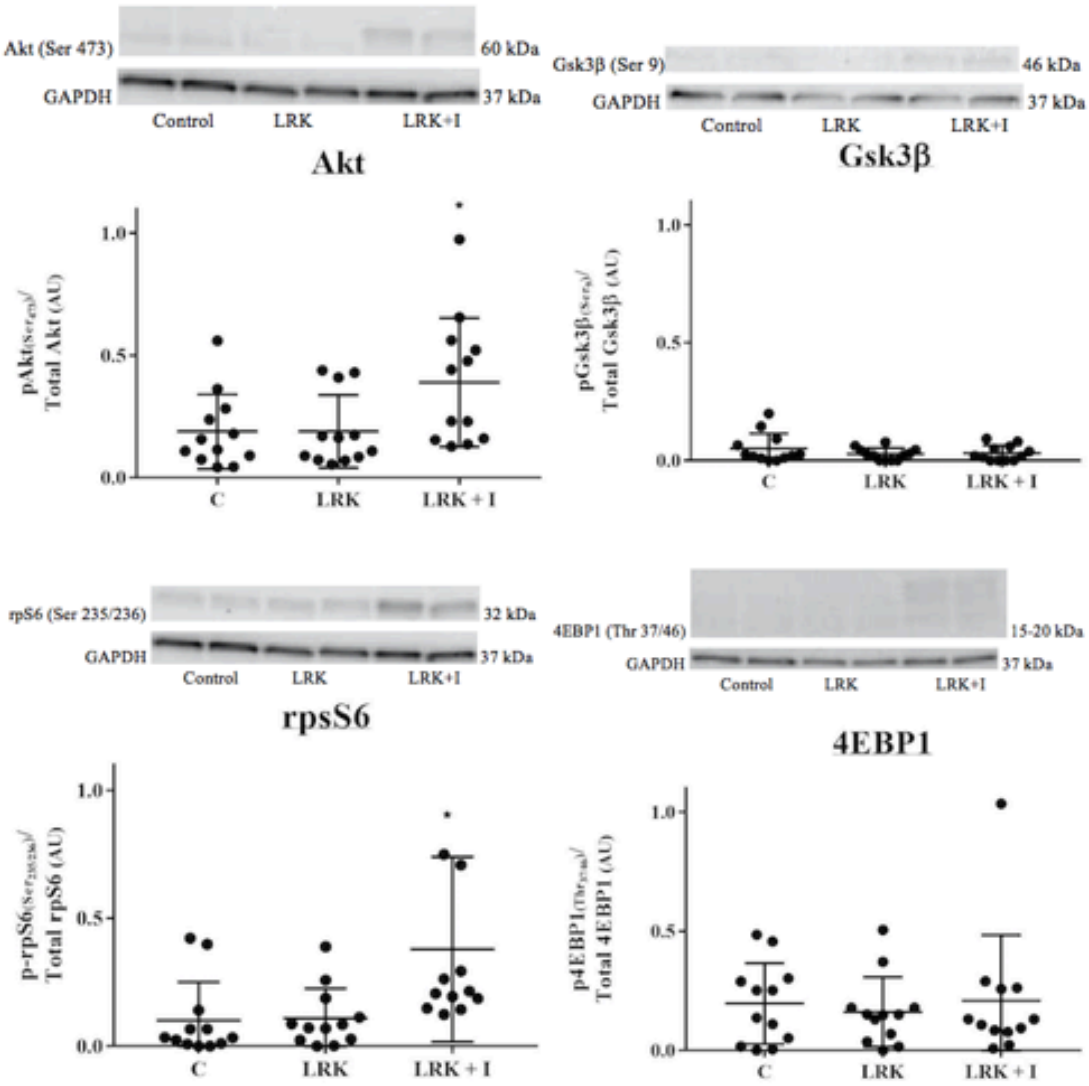

Figure 4. The effect of exposure to LRK and LRK+I on the phosphorylation of Akt, Gsk3β, rpS6 and 4EBP1 for 30 minutes

Discussion

The purpose of this study was to determine the effect of acute amino acid stimulation on anabolic mTORC1 signaling in human myoblasts. We found that use of a custom amino acid and serum free media resulted in a decrease in protein phosphorylation, showing that cell signaling was decreased when compared to cells incubated in growth media.

Incubation with LRK only showed a significant effect on the phosphorylation of Akt for 15 minutes. All other incubation with LRK did not produce any data significantly different than the control. Incubation with LRK + I showed a greater effect, with the phosphorylation status of Akt and Gsk3β being significantly greater than the control after 15 minutes. After 30 minutes incubation with LRK + I, the phosphorylation of Akt and rpS6 were significantly greater than the control. This suggests that while LRK alone may not be potent enough to activate all these proteins, the addition of insulin results in greater activation in these experimental conditions.

These data suggest that there is a difference in the response of myoblasts and myotubes to amino acid stimulation. Myoblasts are the precursors to myotubes, and while myoblasts are differentiated from satellite cells and can no longer proliferate, they do not have the developed structure of myotubes. Myoblasts are mainly trying to align with other myoblasts in order to form myotubes. Myotubes are multinucleated, and their structure includes larger proteins, including actin and myosin, which may result in a greater response for protein synthesis than myoblasts. These data do suggest a proof of principle that myoblasts can be isolated and stimulated with amino acids and insulin to create a cellular signaling response.

However, the clinical significance of these experiments is still unknown due to our lack of success at isolating myoblasts from older adults. The selected satellite cells were overwhelmed by the proliferation of fibroblast in most cell cultures. However, we were able to isolate myoblasts from one subject and run western blots, furthering our proof of principle that myoblasts can be isolated and stimulated with amino acids and insulin to create a cellular signaling response. However, with only data from one subject, results are inconclusive.

Limitations

This study was limited by the unexpected difficulty in isolating myoblasts from older biopsy tissue. The fibroblasts in these cell cultures proliferated much more than the satellite cells. This made it impossible for them to reach the 80% confluency needed to perform the experiment. This limits the clinical applications of this study for now, as we cannot compare the responses of the old to young.

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