# REGULATION OF LEUCYL-TRNA SYNTHETASE AND RAGB EXPRESSION IN HUMAN SKELETAL MUSCLE BY ESSENTIAL AMINO ACIDS

by

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# STATEMENT OF THESIS APPROVAL

The following faculty members served as the supervisory committee chair and members for the thesis of Matthew Brian Carlin.

Dates at right indicate the members' approval of the thesis.

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## ABSTRACT

Essential amino acids (EAA) are a potent stimulator of mammalian target of rapamycin complex 1 (mTORC1) signaling and muscle protein synthesis. However, the molecular regulators upstream of mTORC1 signaling that are stimulated by EAA are not well described in human skeletal muscle. Leucyl tRNA synthetase (LRS) and RagB have recently been shown to be important in mTORC1 activation, but their role in human skeletal muscle following EAA ingestion is unknown. The purpose of this study was to determine changes in protein expression and the association of the LRS- and RagB-mTORC1 complexes following acute EAA ingestion in healthy human skeletal muscle. Muscle biopsies (*vastus lateralis*) were obtained from 13 young adults (7M, 6F, 22.9 ± 0.9y, 21.7 ± 0.9 BMI) in the fasted state (basal) and 1 and 3 h after EAA (13g; 2.4g Leu) ingestion. Co-immunoprecipitation and western blotting were used to determine LRS- and RagB-mTORC1 protein complexes and LRS and RagB protein expression, respectively. We report that EAA ingestion did not alter LRS- or RagB-mTORC1 association or LRS protein expression ($P > 0.05$) contrary to the robust increase in mTOR phosphorylation ($P < 0.05$). However, we found that EAA increased RagB protein abundance following EAA ingestion ($P < 0.05$). We conclude that LRS- and RagB-mTORC1 complexes are not altered 1 and 3 h following EAA ingestion in healthy young adult skeletal muscle. However, the transient increase in RagB protein expression after EAA ingestion may be an important mechanism to promote protein anabolism.

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# INTRODUCTION

Net protein balance (NB) is regulated by the overall difference between muscle protein synthesis (MPS) and muscle protein breakdown (MPB). When MPB exceeds MPS in a given day, a negative nitrogen balance ensues. However, when MPS exceeds MPB, a positive nitrogen balance occurs allowing for hypertrophic adaptations and maintenance of muscle tissue. While both MPS and MPB are affected by external and internal stimuli, such as essential amino acid (EAA) ingestion or insulin release (1, 2), MPS appears to be the more dynamic variable in a healthy population. In a recent study, Glynn et al. showed that 10g EAA significantly increased MPS but did not affect MPB in young skeletal muscle (3).

The mammalian target of rapamycin (mTOR), a 289 kDa protein kinase, has been established as a central regulator of MPS and cell size through mRNA translational control (4, 5). mTOR interacts with several other proteins to form at least two main active protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 consists of the proteins mTOR, regulatory associated protein of mTOR, DEP-domain-containing mTOR-interacting protein (Deptor), proline-rich Akt substrate-40, and β-subunit-like protein GβL (6). The majority of research has focused on mTORC1 because of its sensitivity to rapamycin and its fundamental role in stimulating human MPS following a variety of anabolic stimuli such as hormones (i.e., insulin) (2, 7), contraction (8), and amino acids (9–11). mTORC1 activation appears to

be a prerequisite for the acute stimulation of MPS in humans in response to EAAs as described by Dickinson and colleagues (11). In this crossover study, phosphorylation of S6K1, a downstream effector of mTORC1, and MPS significantly increased in skeletal muscle of young adults at 1 hour following EAA ingestion. However, the normal EAA-induced increase in S6K1 phosphorylation and MPS was attenuated when these subjects consumed rapamycin (16mg) two hours before 10g EAA ingestion (11).

A wealth of information is known about the downstream signaling events of the mTOR pathway and subsequent stimulation of MPS in response to EAA. On the other hand, upstream nutrient sensors leading to mTORC1 activation are currently less described in human skeletal muscle. Several amino acid sensing molecules, including leucyl-tRNA synthetase (LRS) and RagB, have been investigated to describe how amino acids influence mTORC1 activation (12, 13).

The four Rag GTPases (Rags A, B, C, and D) facilitate the binding of Rheb to mTORC1 by coordinating the translocation of mTORC1 to the lysosomal membrane (13). Rag B has been shown to be most active in the GTP-bound state (13, 14). In cells overexpressing Rag $B^{GTP}$, mTORC1 translocates to the lysosomal surface regardless of amino acid deprivation, unlike cells expressing wild type Rag B. This indicates that the GTP loading of Rag B is a rate limiting step to promote mTORC1 translocation. Additionally, in Rag $B^{GTP}$ knockdown cells, mTOR translocation does not occur (13). Thus, the amino acid-sensitive GTP loading of Rag B appears to be vital to stimulating lysosomal translocation of mTORC1.

Amino-acyl tRNA synthetases have a key role in the first steps of protein synthesis by binding an amino acid with a corresponding tRNA (15). LRS has recently

been identified in both the yeast cell and mammalian cell as an “amino acid sensing” protein. During low amino acid concentration conditions, LRS, along with mTOR and Raptor, are found mainly in the cytosol and the endoplasm. Upon addition of amino acids, LRS, mTOR, and Raptor translocate to the lysosomal membrane, where the Rags are bound. Knockdown of LRS reveals an inability of mTOR and Raptor to translocate to the cell membrane, indicating that it has a pivotal role in coordinating translocation along with Ragulator. Moreover, knockdown of LRS reduced S6K1 phosphorylation, while knockdown of isoleucyl tRNA synthetase, methionyl-tRNA synthetase, or valyl-tRNA synthetase did not (12).

Upon translocation to the lysosome, LRS bound with leucine interacted directly with RagD, functioning as a GTPase activating protein to facilitate the transition from RagD-GTP to RagD-GDP, in turn activating the mTORC1 signaling pathway. Together these data suggest that LRS has a unique role compared to the other branched-chain amino acyl tRNA synthetases and is critical for mTORC1 activation.

While LRS and Rag proteins have been shown to be necessary to increase mTORC1 activation in response to amino acids in animal and cell models, there is no current literature describing this relationship in human skeletal muscle. Therefore, the aim of this study is to investigate the acute changes in mTORC1-associated LRS and RagB protein expression in response to a bolus of EAA’s in adult human skeletal muscle.

## METHODS

### Screening of Participants

A total of 13 healthy male and female young participants (age range: 18–28) were recruited through poster advertisements on the University of Utah campus and in the surrounding Salt Lake City community. Participant characteristics are listed in Table 1. The subjects were recreationally active, but were not engaged in any regular exercise training program as defined by two or more exercise training sessions of moderate to high intensity aerobic or resistance exercise per week. Exclusion criteria included, but were not limited to heart, lung, blood, vascular, liver, kidney, infectious, oncologic, and neurological diseases. All subjects gave their written informed consent before participating in the study, which was approved by the Institutional Review Board of the University of Utah.

**Table 1.**
Subject characteristics and body composition of healthy, young adults

| Characteristic | Measure |
|---|---|
| Age | 22.9 ± 0.88 |
| Height | 1.7 ± 0.02 |
| Weight | 65.9 ± 3.78 |
| BMI | 21.7 ± 0.91 |

Values are means ± SE; $n = 13$

## Acute Amino Acid Ingestion Study

After a standardized evening meal (30% fat, 15% protein, 55% carbohydrate) and an overnight fast, subjects arrived at 0600 by transportation to the Center for Clinical and Translational Sciences at the University of Utah. The subjects were allowed to rest in their hospital room for 2 hours after which a muscle biopsy was taken from the *vastus lateralis*. Immediately after the biopsy, subjects ingested ~13g of crystalline EAA (Glanbia Nutritionals) mixed in a 400 ml flavored low calorie, noncaffeinated beverage. Additional muscle biopsies were taken 1 and 3 h after EAA ingestion. The composition of EAA mixture was the following: histidine (1.6g), isoleucine (1.0g), leucine (2.5g), lysine (3.1g), methionine (0.8g), phenylalanine (1.2g), threonine (1.2g), and valine (1.5g). The proportion of EAA, in particular leucine, has been shown to maximally stimulate MPS in young adults (9, 16, 17).

## Muscle Biopsy Procedure

Muscle biopsies were sampled from the *vastus lateralis* of the right leg using aseptic technique, local anesthesia (1% lidocaine), and a 5 mm Bergström biopsy needle with manual suction (18). The first muscle biopsy (baseline) was taken from a single incision. The second and third biopsies were taken from a separate incision, ~7 cm proximal from the first and inserted at an angle to separate the two sampling sites (biopsies 2 and 3) by at least 5 cm. All muscle tissue was immediately blotted and dissected of visible nonmuscle tissue, flash-frozen in liquid nitrogen, and stored at -80°C for later analysis.

Co-Immunoprecipitation and Western Blotting

Details to the co-immunoprecipitation for mTORC1 have been reported previously (19). Frozen muscle was homogenized in CHAPS buffer [40 mM HEPES (pH 7.5), 120 mM β-glycerolphosphate, 40 mM NaF, 1.5 mM sodium orthovanadate, 0.3% CHAPS, 0.1 mM PMSF, 1 mM benzamadine, and 1 mM DTT], and the homogenate was rotated for 20 min at 4°C and then centrifuged at 10,000 rpm for 5 min at 4°C. Protein concentration was determined by Bradford Assay (BioRad). 700 μg of protein was aliquoted and diluted 3x in CHAPS buffer without inhibitors. Six microliters of mTOR antibody (Cell Signaling; Catalog #2972) was added to each sample then rotated overnight at 4°C. The mTOR protein-antibody complex was isolated by adding BioMag goat anti-rabbit IgG (Qiagen, Valencia, CA) bead slurry then rotated for 1 h at 4°C. Just before using the BioMag beads, the beads were washed twice with CHAPS buffer without protease inhibitors, collected using a centrifuge tube magnetic stand (Qiagen) after each wash, then resuspended with one-quarter of the volume of CHAPS buffer with 0.1% nonfat dry milk (NFDM). 500-μl of the bead+buffer mixture was added to the sample then rotated for 1 h at 4°C.

Following incubation the bead-antibody-protein complex was isolated and collected using a magnetic stand (Qiagen), washed twice with CHAPS buffer without protease inhibitors and once in CHAPS buffer containing 150 mM of NaCl and 50 mM of HEPES. The bead-antibody-protein complex was eluted with 60μl of 2X sample buffer (125mM Tris, pH 6.8, 25% glycerol, 2.5% SDS, 2.5% β-mercaptoethanol and 0.002% bromophenol blue), then boiled for 5 min at 100°C. An immunoprecipitated sample (25 μl) was loaded on a 7.5 or 15% Tris-HCL polyacrylamide gel (Criterion; BioRad,

Hercules, CA), depending on the protein target size of interest, then separated with SDS-PAGE for 1 hour at 150V.

Each gel contained 6 samples in duplicate from two subjects (baseline, 1 and 3 h post EAA) and a molecular weight ladder. An internal control (mouse quadriceps homogenate) was loaded in duplicate on each gel for band normalization and comparison across blots. Protein was transferred (50V, 1 hour) to a polyvinylidene diflouride membrane then blocked for 45 min at room temperature with 2% NFDM in Tris-buffered saline in 0.1% Tween-20 (TBST) on a rocker. Membranes were cut and incubated overnight separately in anti-leucyl-tRNA synthetase rabbit polyclonal antibody (1:1000; abcam, Cambridge, MA; Catalog #ab31534) and RagB rabbit polyclonal antibody (1:2000; Cell Signaling Technology, Inc., Danvers, MA; Catalog #8150) diluted in 2% NFDM. The next morning, blots were rocked in rabbit secondary antibody (1:6000; sc2799; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature then washed 3x (5 min) with TBST. Chemiluminescence reagent (ECL Plus, GE Healthcare) was applied to each blot then incubated for 5 min at room temperature. Optical density measurements were obtained with a digital imager (ChemiDoc XRS+, BioRad). Membranes were stripped (Restore Western Blot Stripping Buffer; Pierce Biotechnlogy, CA) of primary and secondary antibodies then reprobed for total mTOR (1:1000; Cell Signaling Technology, Inc., Danvers, MA; Catalog #2972) on a separate day. Densitometric analysis was performed using Lab version 4.1 software (BioRad). Co-immunoprecipitation data were normalized to the internal control, and replicate samples were averaged. Co-immunoprecipitation data were reported as target protein/total mTOR. No signal was present when using an IgG control antibody (1:1000; Cell

Signaling Technology, Inc., Danvers, MA; Catalog #2729) for precipitation.

## Total Protein Expression

To determine total protein abundance of LRS and RagB in skeletal muscle biopsy samples, frozen muscle samples were homogenized (1:9, w/v) in a buffer containing 50 mM Tris-HCl, 250 mM mannitol, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, pH 7.4, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF and 5 μg $ml^{-1}$ soybean trypsin inhibitor (SBTI). DTT, benzamidine, PMSF, and SBTI were added to the buffer immediately prior to use. Supernatant was collected after centrifugation at 10,000 rpm for 10 min at 4°C. Protein concentration was determined by Bradford Assay (BioRad) and later boiled at 95°C for 3 minutes. Forty micrograms of protein were loaded on a Tris-HCL polyacrylamide gel (Criterion; BioRad, Hercules, CA) then separated with SDS-PAGE for 1 h at 150V.

Protein was transferred (50V, 1 h) to a polyvinylidene diflouride membrane then blocked for 45 min at room temperature with 2% NFDM in Tris-buffered saline in 0.1% Tween-20 (TBST) on a rocker. Membranes were cut and incubated overnight individually in anti-leucyl-tRNA synthetase rabbit polyclonal antibody (1:1000; abcam, Cambridge, MA; Catalog #ab31534), RagB rabbit polyclonal antibody (1:2000; Cell Signaling Technology, Inc., Danvers, MA; Catalog #8150), and phospho-mTOR (Ser2481) rabbit polyclonal antibody (1:1000; Cell Signaling Technology, Inc., Danvers, MA; Catalog #2974) diluted in 2% NFDM. The next morning, blots were rocked in rabbit secondary antibody (1:6000; sc2799; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature then washed 3x (5 min) with TBST. Chemiluminescence

reagent (ECL Plus, GE Healthcare) was applied to each blot then incubated for 5 min at room temperature. Optical density measurement was obtained with a digital imager (ChemiDoc XRS+, BioRad). Membranes were stripped (Restore Western Blot Stripping Buffer; Pierce Biotechnlogy, CA) of primary and secondary antibodies then reprobed for total mTOR (1:1000; Cell Signaling Technology, Inc., Danvers, MA; Catalog #2972) on a separate day. Densitometric analysis was performed using Lab version 4.1 software (BioRad). Whole muscle homogenate data were normalized to the internal control, and replicate samples were averaged and reported as fold change from basal. Phosphorylated mTOR (Ser2481) was reported as p-mTOR/total mTOR (fold change).

## Statistical Analysis

A 1-way repeated measures ANOVA was used to analyze differences across time (basal and 1 and 3 h after EAA ingestion). *Post hoc* tests (Bonferroni) were conducted to assess specific interactions. Significance was set at $P < 0.05$. All values are presented as mean ± SE. All analyses were performed with SigmaPlot (Version 12.0).

## RESULTS

LRS-mTORC1 association was not different from basal at 1 h (1.11 ± 0.12-fold; $P = 0.28$ vs. basal) or 3 h post-EAA ingestion (1.10 ± 0.10-fold; $P = 0.36$ vs. basal) (Fig. 1A). Similarly, RagB-mTORC1 association was not different from basal at 1 h (1.02 ± 0.09-fold; $P = 0.62$ vs. basal) or 3 h post-EAA ingestion (1.04 ± 0.16-fold; $P = 0.67$ vs. basal) (Fig. 1B).

mTOR phosphorylation at Ser2481 (relative to total mTOR) increased at 1 h (1.29 ± 0.12-fold; $P = 0.03$ vs. basal) and returned to baseline 3 h (1.01 ± 0.12-fold; $P = 0.95$ vs. basal) following EAA ingestion (Fig. 2A). LRS total protein expression was not different from basal at either 1 h (1.09 ± 0.08-fold; $P = 0.32$) or 3 h (1.08 ± 0.10-fold; $P = 0.34$) following EAA ingestion (Fig. 2B). RagB total protein abundance was not different from basal at 1 h (1.70 ± 0.17-fold; $P = 0.10$ vs. basal) following EAA ingestion but increased at 3 h (1.95 ± 0.42-fold; $P = 0.03$ vs. basal) following EAA ingestion (Fig. 2C). Figure 3 contains representative western blot images for proteins analyzed.

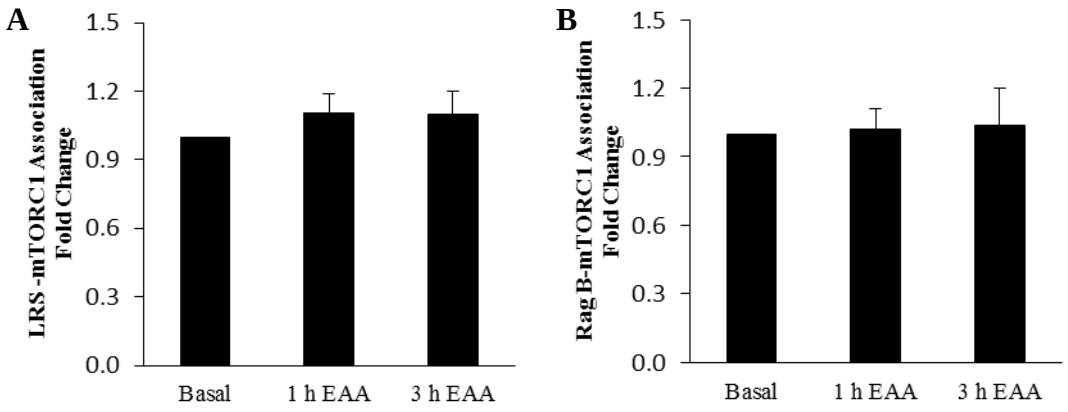


**Figure 1.**

LRS- and RagB-mTORC1 Co-Immunoprecipitation in Human Skeletal Muscle at Basal and 1 and 3 h after EAA Ingestion. A) LRS-mTORC1 Association Fold Change. B) RagB-mTORC1 Association Fold Change.

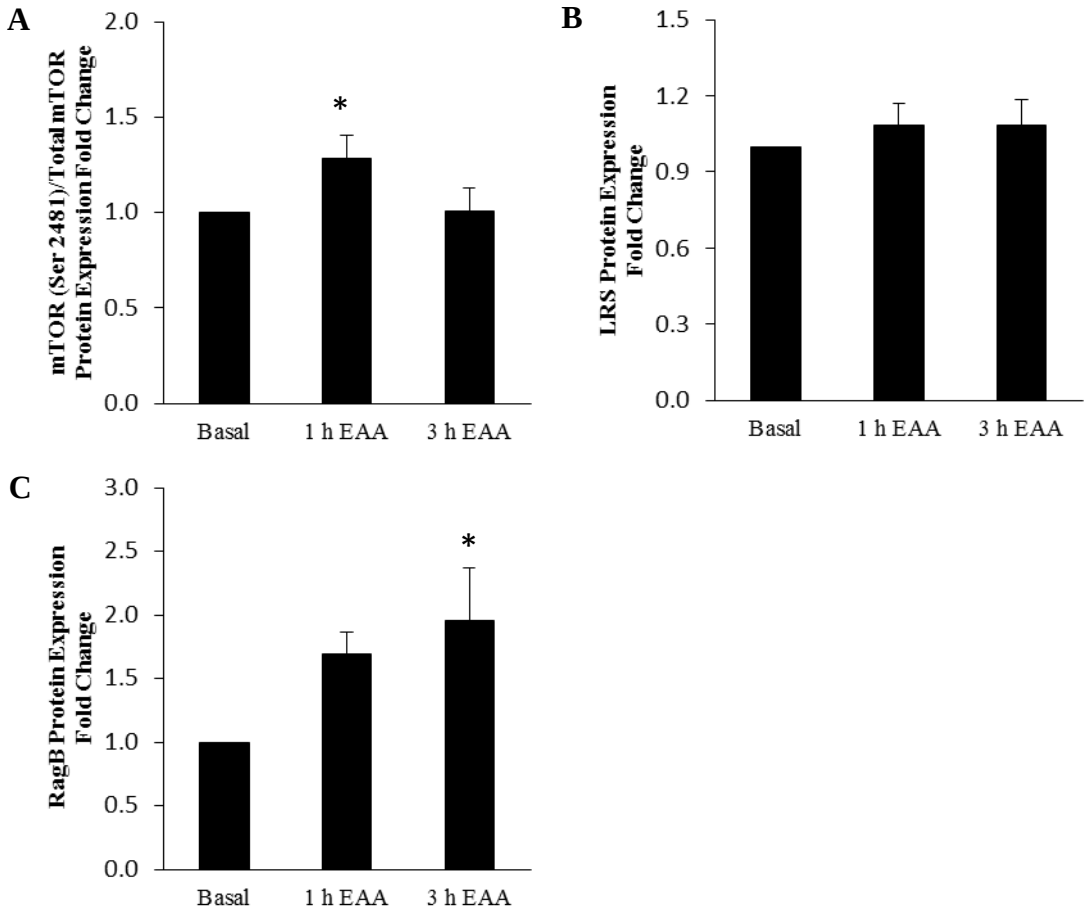


**Figure 2.**
mTOR (Ser2481), LRS, and RagB Total Protein Expression in Human Skeletal Muscle at Basal and 1 and 3 h after EAA Ingestion. A) mTOR (Ser 2481)/Total mTOR Protein Expression Fold Change. B) LRS Protein Expression Fold Change. C) RagB Protein Expression Fold Change.

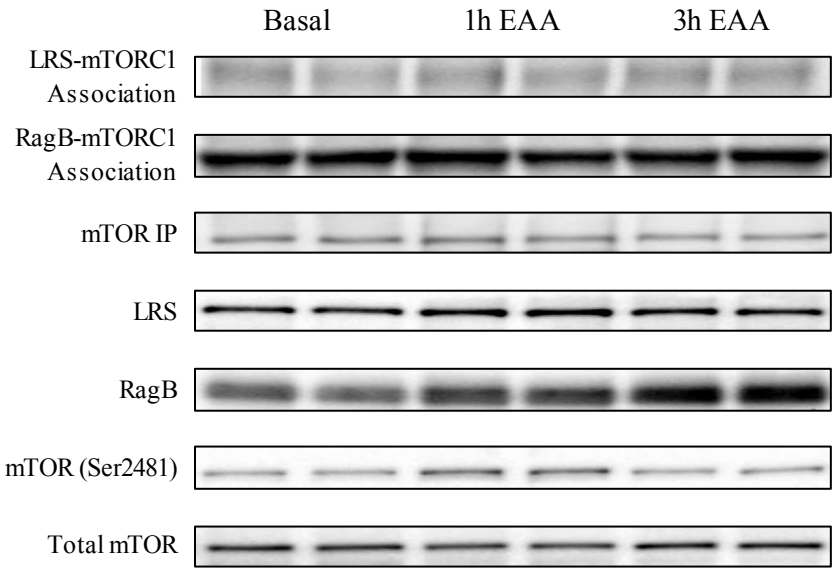


**Figure 3.**
Representative western blot images for proteins analyzed.

## DISCUSSION

We originally hypothesized that LRS- and RagB-mTORC1 association in human skeletal muscle would increase acutely following an ingested bolus of EAA: a dose previously known to maximally stimulate muscle protein synthesis. However, contrary to our hypothesis, we did not detect a change in LRS- and RagB-mTORC1 association from basal at either 1 or 3 h following EAA ingestion in young healthy human skeletal muscle. This occurred despite an increase in mTOR phosphorylation in whole muscle homogenates following EAA ingestion. Alternatively, skeletal muscle RagB total protein abundance increased at 1 h following EAA ingestion. Although we observed a robust anabolic response to EAA ingestion in human skeletal muscle (e.g., mTOR phosphorylation), LRS- and RagB-mTORC1 association does not occur 1 or 3 h following EAA ingestion in healthy human muscle. The increase in RagB protein abundance following EAA ingestion may be an important mechanism to increase the protein anabolic response to EAAs and/or increase sensitivity to a subsequent anabolic stimulus (e.g., exercise).

Recent cell studies have shown that a critical step in amino acid-induced mTORC1 activation is the binding of LRS and RagB to mTORC1 at the lysosome (12, 13). However in the current study, LRS- and RagB-mTORC1 association was not altered following EAA ingestion, despite a robust anabolic environment as evidenced by increased mTOR phosphorylation. The EAA dose used in the current study (13g EAA;

2.4g Leu) has been identified to elicit a maximal MPS response (10g EAA; 1.8g Leu) in young adult skeletal muscle (16, 17). Moreover, the time points we chose were in accordance with maximal amino acid-induced MPS and mTORC1 activity (3, 10, 20–22). Our findings are consistent with those of Suryawan and colleagues, who found no change in RagB-Raptor association following a 2 h amino acid infusion in neonatal pig skeletal muscle (23). While we initially hypothesized that LRS- and RagB-mTORC1 association might occur earlier than 2 h in human muscles (i.e., 1 h following EAA ingestion), this timepoint may still be too late to detect the formation of an LRS or RagB-mTORC1 complex. This notion is not unreasonable since an increase in plasma EAA concentrations are detected in as little as 30 min after whey protein (22), and 4E-BP1 phosphorylation (downstream of mTORC1 activation) has been detected as early as 45 min after EAA+CHO ingestion (24).

The lack of RagB-mTORC1 complex formation observed in this study may be due to a transient association/dissociation with mTORC1 that occurs in an alternative time sequence than hypothesized in the current study. Prior cell studies have shown RagB to be a central regulator of mTORC1 activity by promoting mTORC1 translocation from the cytosol to the lysosome (13, 25). However, Sancak and colleagues determined that Rag function was no longer needed for the activation of mTORC1 when mTORC1 was localized at the lysosomal surface (25). These data suggest that once RagB has initiated mTORC1 translocation to the lysosome, RagB could possibly dissociate from the mTORC1 protein complex even in the presence of subsequent mTORC1 activation and muscle protein synthesis.

Based on our LRS-mTORC1 data, we propose that the binding of LRS to the

mTOR complex, much like RagB, may be transient and dissociates after initiating mTORC1 translocation to the lysosome. In a cell model, LRS translocates to the lysosome and forms a complex with mTORC1, activating the mTORC1 pathway (12). The basic functions of LRS are to "charge" leucyl-tRNA with a leucine molecule and to "proofread" misacetylated $tRNA^{Leu}$ molecules, ensuring the correct amino acid is placed on the tRNA (26). More recently, LRS has been proposed to be necessary in mTORC1 activation by translocating to the lysosome, forming a complex with mTORC1, and facilitating the transition of $RagD^{GTP}$ to more active $RagD^{GDP}$. The conformation of Rag $D^{GDP}$ and RagB $^{GTP}$ has been shown to promote the highest activation of mTORC1 translocation and subsequent activation (12). Once LRS facilitates the transition of $RagD^{GTP}$ to $RagD^{GDP}$, LRS may dissociate from mTORC1, although this has yet to be confirmed. This data fits a model in which LRS translocates to the lysosomal membrane upon amino acid stimulation, acts as a GTPase-activating protein towards $RagD^{GTP}$, and dissociates from the complex. Like RagB-mTORC1 association, presumably LRS-mTORC1 association may be very transient (<1 h) to EAA ingestion despite a continual mTOR activation and protein synthesis response.

Although we were unable to detect changes in LRS- or RagB- association to the mTORC1 complex, RagB protein abundance was shown to increase 3 h following EAA ingestion in human skeletal muscle. We are unsure of what is responsible for the nearly 100% increase in RagB protein abundance within hours of EAA ingestion. However, it is not unreasonable to suspect parallel changes in RagB mRNA abundance contributing to this protein response since we have previously observed acute increases in both mRNA and protein abundance of other nutrient sensors following EAA ingestion in humans (27).

The increase in RagB protein abundance 3 h following EAA ingestion may not be related to the acute anabolic response to EAA ingestion since mTOR phosphorylation returns back to baseline at this timepoint. Rather, an increase in RagB protein may be useful to amplify a subsequent anabolic stimulus such as resistance exercise as has been observed previously (28–30). The abundance of LRS needed for translocation of mTORC1 might already be at an optimal level such that additional LRS protein is unnecessary to serve as an additional signal for mTORC1 translocation and activation.

In conclusion, RagB- and LRS-mTORC1 association was not altered in the early hours following EAA ingestion in human skeletal muscle. While this differs from previous cell data, complex formation of RagB and LRS is likely transient and may occur outside our sampling time points (e.g., <1 h EAA ingestion). An increase in RagB protein abundance may serve to amplify the signal for mTORC1 translocation and further activation of postprandial human muscle protein synthesis. A more elaborate time course of LRS- and RagB- association following EAA ingestion is warranted in humans.

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