Mammalian Target of Rapamycin Complex 1 Activation Is Required for the Stimulation of Human Skeletal Muscle Protein Synthesis by Essential Amino Acids^{1–3}

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Abstract

The relationship between mammalian target of rapamycin complex 1 (mTORC1) signaling and muscle protein synthesis during instances of amino acid surplus in humans is based solely on correlational data. Therefore, the goal of this study was to use a mechanistic approach specifically designed to determine whether increased mTORC1 activation is requisite for the stimulation of muscle protein synthesis following L-essential amino acid (EAA) ingestion in humans. Examination of muscle protein synthesis and signaling were performed on vastus lateralis muscle biopsies obtained from 8 young individuals who were studied prior to and following ingestion of 10 g of EAA during 2 separate trials in a randomized, counterbalanced design. The trials were identical except during 1 trial, participants were administered a single oral dose of a potent mTORC1 inhibitor (rapamycin) prior to EAA ingestion. In response to EAA ingestion, an \( \text{\textasciitilde}60\% \) increase in muscle protein synthesis was observed during the control trial, concomitant with increased phosphorylation of mTOR (Ser^{2448}), ribosomal S6 kinase 1 (Thr^{389}), and eukaryotic initiation factor 4E binding protein 1 (Thr^{37/46}). In contrast, prior administration of rapamycin completely blocked the increase in muscle protein synthesis and blocked or attenuated activation of mTORC1-signaling proteins. The inhibition of muscle protein synthesis and signaling was not due to differences in either extracellular or intracellular amino acid availability, because these variables were similar between trials. These data support a fundamental role for mTORC1 activation as a key regulator of human muscle protein synthesis in response to increased EAA availability. This information will be useful in the development of evidence-based nutritional therapies targeting mTORC1 to counteract muscle wasting associated with numerous clinical conditions.  J. Nutr. 141: 856–862, 2011.

Introduction

Increased amino acid availability, primarily the essential amino acids (EAA),^{2} results in a potent and rapid elevation in the rate of human skeletal muscle protein synthesis (1–4). Consequently, the use of nutritional interventions continues to be of keen scientific interest not only to enhance the adaptive response of skeletal muscle when coupled with exercise training regimens (5–11), but also as an independent stimulus to promote a net positive muscle protein balance (4). The independent benefits of elevated amino acid levels on muscle protein metabolism may provide a useful strategy to help counteract the dramatic reduction in muscle size and function that accompany numerous clinical conditions (2,12–14), especially those that do not allow for regular exercise. However, the development of evidence-based nutritional therapies to counteract muscle wasting must rely on a better understanding of the precise cellular mechanisms by which an increase in EAA availability stimulates human skeletal muscle protein synthesis.

The mammalian target of rapamycin complex 1 (mTORC1) has become a focal point in the regulation of muscle cell size (15). An increase in the activity of mTORC1 leads to the phosphorylation of 2 key direct downstream effectors, ribo-
somal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (1,16). The activity of both S6K1 and 4E-BP1 contributes to the regulation of translation initiation, and further downstream, the activity of S6K1 also enhances translation elongation through signals that eventually lead to decreased eukaryotic elongation factor 2 (eEF2) phosphorylation (17). The mTORC1 pathway has received considerable attention as a key regulator of protein synthesis following an increase in amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cells with the immunosuppressant drug rapamycin, a potent mTORC1 inhibitor, has been shown to block the increase in S6K1 and 4E-BP1 phosphorylation in response to elevated amino acid availability. For instance, treatment of cell...
Concentrations of phenylalanine and leucine were determined in blood and muscle intracellular fluid using tracer enrichments and l-[1-\(^{13}\)C]phenylalanine and l-[5,5-\(^2\)H]leucine as internal standards for phenylalanine and leucine, respectively, as previously described (30).

**Calculation of muscle protein fractional synthesis rate.** The fractional synthesis rate (FSR) of mixed muscle protein was determined by examining the rate of l-[ring-\(^{13}\)C]phenylalanine incorporated into mixed muscle protein using the precursor product model:

\[
\text{FSR} = \frac{\Delta \text{Ep}}{\text{t}} = \frac{\text{Ep}_{\text{M}(1)} + \text{Ep}_{\text{M}(2)}}{2} 
\]

where \(\text{Ep}\) is the increment in protein-bound l-[ring-\(^{13}\)C]phenylalanine enrichment between 2 muscle biopsies, \(t\) is the time between the 2 muscle biopsies, and \(\text{Ep}_{\text{M}(1)} + \text{Ep}_{\text{M}(2)}\) are the l-[ring-\(^{13}\)C]phenylalanine enrichments in the free intracellular pool in the 2 muscle biopsies. Data are expressed as percent per hour.

**Immunoblot analysis.** Immunoblot analysis was performed as previously detailed (27). Briefly, frozen tissue was homogenized, centrifuged for 10 min at 4°C, and the supernatant collected. Total protein concentrations were determined using the Bradford assay (Smartspec Plus, Bio-Rad). The supernatant was diluted (1:1) in a 2× sample buffer mixture containing 125 mmol/L Tris, pH 6.8, 25% glycerol, 2.5% SDS, 2.5% β-mercaptoethanol, and 0.002% bromophenol blue, then boiled for 3 min at 100°C. Equal amounts of total protein (30 μg) were loaded into each lane and the samples were separated by electrophoresis (150 V for 60 min) on a 7.5 or 15% polyacrylamide gel as determined by the size of the target protein (Criterion, Bio-Rad). Each sample was loaded in duplicate and each gel contained an internal loading control and molecular weight ladder (Precision Plus, Bio-Rad). Additionally, all samples from a given experimental trial were loaded onto the same gel and each gel contained samples from both the control and rapamycin trials.

Following electrophoresis, protein was transferred to a polyvinylidene difluoride membrane (Bio-Rad) at 50 V for 60 min. Blots were then blocked for 1 h in 5% nonfat dry milk and incubated in primary antibody overnight at 4°C (see below). The next morning, blots were incubated in secondary antibody for 1 h at room temperature. Blots were then incubated in a chemiluminescent solution (ECL plus, Amersham BioSciences) for 3 min and OD measurements were obtained with a phosphorimager (Chemidoc, Bio-Rad) and densitometric analysis was performed using Quantity One 4.5.2 software (Bio-Rad). Membranes containing phospho-detected proteins were stripped of primary and secondary antibodies using Restore Western Blot Stripping buffer (Pierce Biotechnology) and were subsequently reprobed for total protein with the specific antibody of interest. Phospho and total density values were normalized to the internal loading control and the phosphotototal protein ratios were determined. Immunoblot data are expressed as phospho divided by total protein and adjusted to represent fold change from basal.

**Antibodies.** The phosho and total antibodies used for immunoblotting were purchased from Cell Signaling: phospho-mTOR (Ser\(^{2448}\); 1:250), phospho-4E-BP1 (Thr\(^{37/46}\); 1:500), phospho-4E-BP2 (Thr\(^{37/46}\); 1:1000), and phospho-mTOR (Ser\(^{2448}\); 1:5000). Total protein was detected using an antibody dilution of 1:1000. Anti-rabbit IgG HRP-conjugated secondary antibody was purchased from Amersham Bioscience (1:2000).

**Statistical analysis.** A 2-way repeated-measures ANOVA was used to test time by trial differences. A 1-way ANOVA was used to examine the time course of rapamycin in the blood; however, no detectable rapamycin was observed in any participant’s baseline blood sample (i.e., blood sample obtained prior to rapamycin administration) and therefore this time point was not included in data analysis. A Tukey’s post hoc analysis was used when necessary to determine specific differences within an ANOVA. All data were analyzed using SigmaStat v.11.0 (Systat Software). Significance for all analyses was set to \(P < 0.05\). Data are presented as mean ± SEM.

**Results**

**Time course of rapamycin in blood.** Administration of 16 mg (−0.23 mg/kg body weight \(^{-1}\)) of rapamycin significantly elevated blood rapamycin concentrations (rapamycin trial only) (Fig. 1). Relative to 30 min after rapamycin administration, concentrations of rapamycin in the blood were further elevated \((P < 0.05)\) at 1 h postadministration (corresponding to 1 h prior to EAA ingestion) and remained similarly elevated throughout the duration of the study \((P < 0.05)\).

**Blood and intracellular amino acid concentrations.** There were no differences between trials in blood or intracellular phenylalanine and leucine concentrations at any time point. Blood phenylalanine and leucine concentrations increased during both trials at 30 min after EAA ingestion and remained elevated throughout the remainder of the study \((P < 0.05)\) (Fig. 2). Intracellular phenylalanine and leucine concentrations were elevated during both trials at 1 h after EAA ingestion \((P < 0.05)\). During both trials, the intracellular phenylalanine concentration remained elevated at 2 h postingestion \((P < 0.05)\), whereas the intracellular leucine concentration was no longer elevated at 2 h after EAA ingestion (Table 1).

**Muscle protein synthesis.** The basal muscle protein synthesis rate was similar between trials \((P > 0.05)\). During the period after EAA ingestion, muscle protein synthesis rate was increased during the control trial \((P < 0.05\) vs. basal\(), whereas the muscle protein synthesis rate was unchanged from basal during the rapamycin trial \((P > 0.05)\). Muscle protein synthesis rate was higher in the control trial than in the rapamycin trial during the period after EAA ingestion \((P < 0.05)\) (Fig. 3).

**Cell signaling.** Phosphorylation of mTOR (Ser\(^{2448}\)) tended to increase at 1 h after EAA ingestion during the control trial \((P = 0.07)\) and was elevated above that of the rapamycin trial at 2 h after EAA ingestion \((P < 0.05)\) (Fig. 4). No change was observed in mTOR phosphorylation after EAA ingestion during the rapamycin trial at any time point. 4E-BP1 phosphorylation (Thr\(^{37/46}\)) increased during both trials at 1 h postingestion \((P < 0.05)\); however, the increase in 4E-BP1 phosphorylation tended to be higher during the control trial \((P = 0.06)\). Phosphorylation
of S6K1 (Thr389) increased at 1 h after EAA ingestion during the control trial (P < 0.05) with a trend for a difference between trials at 1 h after EAA (P = 0.08), whereas S6K1 phosphorylation did not increase at any time point during the rapamycin trial. Phosphorylation of eEF2 (Thr56) did not differ at any time point or between trials.

**Discussion**

The primary and novel finding from the present study is that the increase in human skeletal muscle protein synthesis rate in response to EAA ingestion is completely blocked by prior administration of the potent mTORC1 inhibitor, rapamycin. In addition, rapamycin administration blocked or attenuated the activation of key downstream components of the mTORC1 signaling pathway. Taken together, these data support a fundamental role for mTORC1 activation in the stimulation of human skeletal muscle protein synthesis in response to EAA ingestion.

**TABLE 1**

Intracellular phenylalanine and leucine concentrations under basal conditions and following ingestion of 10 g of L-EAA in young men and women

<table>
<thead>
<tr>
<th>Trial</th>
<th>Basal</th>
<th>1 h after EAA</th>
<th>2 h after EAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine, μmol L⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>45.5 ± 5.4</td>
<td>70.0 ± 5.9*</td>
<td>53.2 ± 4.4*</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>46.5 ± 9.8</td>
<td>73.8 ± 8.4*</td>
<td>54.9 ± 7.0*</td>
</tr>
<tr>
<td>Leucine, μmol L⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>115 ± 14.9</td>
<td>168 ± 17.9*</td>
<td>138 ± 13.8</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>106 ± 18.3</td>
<td>165 ± 21.0*</td>
<td>136 ± 13.4</td>
</tr>
</tbody>
</table>

1 Data are mean ± SEM, n = 8. *Different from basal, P < 0.05.

To our knowledge, this is the first investigation to use an in vivo mechanistic approach in humans designed to determine the fundamental role of mTORC1 in the regulation of skeletal muscle protein synthesis following EAA ingestion. Specifically, we administered 16 mg (~0.23 mg·kg⁻¹) of rapamycin to inhibit mTORC1 activity 2 h prior to the ingestion of a mixture of EAA. This timeframe has been shown in both the current study (Fig. 1) and previously by our laboratory (25) to coincide with peak concentrations in the blood that remained elevated throughout the duration of the current investigation. Whereas we observed a noticeable increase (~60%) in skeletal muscle protein synthesis rate following EAA ingestion during the control trial, prior administration of rapamycin completely blocked this increase in protein synthesis rate (Fig. 3). This finding is supported by previous work in animal skeletal muscle (20,22). For example, Anthony et al. (22) demonstrated that injecting 0.75 mg·kg⁻¹ of rapamycin into the tail vein of rats prevented an increase in the skeletal muscle protein synthesis rate occurring at 1 h after oral leucine administration. Furthermore, whereas Anthony et al. (22) showed that the muscle protein synthesis rate was inhibited at 1 h after leucine ingestion (determined using an ~10-min flooding dose) with prior rapamycin injection, our data show that rapamycin administration has a prolonged inhibitory effect on human skeletal muscle protein synthesis rate, such that a basal level is maintained throughout a 2-h period following EAA ingestion.

Rapamycin is a potent inhibitor of mTORC1 activity (31), and therefore to obtain mechanistic insight into the fundamental role of mTORC1 signaling we focused on key signaling proteins downstream of mTORC1 at time points known to be influenced by EAA ingestion (4). Concomitant with the inhibition of skeletal muscle protein synthesis, prior rapamycin administration blunted mTORC1 (Ser2448) and S6K1 (Thr389) phosphorylation following EAA ingestion. Furthermore, although the phosphorylation of 4E-BP1 (Thr37/46) was increased at 1 h during both trials, a larger increase was observed during the control trial (P = 0.06). The incomplete blocking of 4E-BP1 phosphorylation with prior rapamycin administration has also been observed following feeding in rodents (21); additionally, several studies in cells have highlighted that rapamycin treatment does not completely inhibit the phosphorylation of 4E-BP1 at levels that do inhibit S6K1 phosphorylation (32,33). These data suggest that rapamycin may not provide complete inhibition of mTORC1-dependent phosphorylation of 4E-BP1 or that an additional pathway may be involved in the increase in 4E-BP1 phosphorylation following increased amino acid availability. Nevertheless, the signaling data from the current investigation suggest that the dose of rapamycin used in the current study likely did inhibit mTORC1 activity, which was not overcome by an increase in EAA availability, and that the mTORC1 pathway is a key mechanism through which the increase in human skeletal muscle protein synthesis is regulated following EAA ingestion.

Following an increase in amino acid levels, both extracellular (34,35) and intracellular (4,36) amino acid availability have been proposed to regulate the increase in human skeletal muscle protein synthesis. Furthermore, although the precise mechanisms are still under investigation, the amino acid availability within these pools has been linked to mTORC1 activity (37,38). Therefore, we measured extracellular (blood) and intracellular amino acid availability via phenylalanine and leucine concentration to determine whether potential differences in amino acid availability as a result of rapamycin administration could explain the blunted mTORC1 signaling and inhibition of
skeletal muscle protein synthesis. We observed that a similar increase in both extracellular and intracellular phenylalanine and leucine availability occurred during both trials and that similar concentrations were maintained throughout each trial, suggesting rapamycin administration did not impair amino acid absorption into circulation or transport into the muscle. Therefore, the blunting of mTORC1 signaling and skeletal muscle protein synthesis with prior rapamycin treatment cannot be explained by differences in either extracellular or intracellular amino acid availability.

Following amino acid ingestion, skeletal muscle protein synthesis rates did not drop below basal values during the rapamycin trial. Additionally, work in animal skeletal muscle has demonstrated that rapamycin does not alter basal protein synthesis rates (19,20). These data suggest that either an unidentified pathway is able to maintain translational processes at basal rates (potentially involving 4E-BP1) or that rapamycin does not interfere with basal activation of mTORC1 and its influence on protein synthesis. With respect to the latter, recent data have demonstrated that activation of mTORC1 likely involves its translocation within the cell, a process facilitated by Rag proteins that bind mTORC1 via raptor (39,40). Rapamycin administration, therefore, may have little influence on basal mTORC1 activation, because mTORC1 translocation has already occurred. On the other hand, the inhibitory effect that is observed following a stimulus (i.e. amino acid ingestion) may be due to a rapamycin-dependent inhibition of increased mTORC1 translocation, possibly through decreased mTORC1-raptor association (31). However, such an aim is beyond the scope of the current investigation and therefore further research is necessary to more clearly define the mechanism by which rapamycin inhibits amino acid-induced mTORC1 activation.

The characteristics of rapamycin provide a unique research strategy that is commonly utilized to investigate the relevance of the mTORC1 signaling pathway in response to a given stimulus. However, in addition to its use for mechanistic research, rapamycin has been documented to have clinical benefits as well. For instance, rapamycin has been used as treatment for various clinical conditions (41–43) and is commonly prescribed to patients following organ transplants (44,45). Although our purpose was not to examine the clinical applicability of rapamycin treatment (or mTORC1 inhibition), the results of our study, as well as previous data from our laboratory showing rapamycin blocks resistance exercise-induced increases in muscle protein synthesis (25), could have important implications for various clinical populations that are treated with rapamycin (or various other mTORC1 inhibitors), specifically as it relates to the maintenance and/or restoration of muscle mass in these individuals. However, whether an inhibition of muscle protein synthesis is observed at varying prescription doses (44) clearly warrants further investigation.

In summary, administration of rapamycin prior to the ingestion of EAA blocks the increase in skeletal muscle protein synthesis and inhibits or attenuates the activation of key downstream components of the mTORC1 signaling pathway in healthy young individuals. These data suggest that the stimulation of human skeletal muscle protein synthesis in response to elevated EAA levels requires a functional mTORC1 signal. Furthermore, the findings from this investigation provide insight that can be used in a manner to develop better evidence-based nutritional therapies to counter muscle wasting and decreased muscle function that is associated with numerous debilitating clinical conditions.
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Literature Cited


