Leucine stimulates protein synthesis in skeletal muscle of neonatal pigs by enhancing mTORC1 activation

Agus Suryawan, Asumthia S. Jeyapalan, Renan A. Orellana, Fiona A. Wilson, Hanh V. Nguyen, and Teresa A. Davis

United States Department of Agriculture/Agriculture Research Service Children’s Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, Texas

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Suryawan A, Jeyapalan AS, Orellana RA, Wilson FA, Nguyen HV, Davis TA. Leucine stimulates protein synthesis in skeletal muscle of neonatal pigs by enhancing mTORC1 activation. Am J Physiol Endocrinol Metab 295: E868–E875, 2008.—Skeletal muscle of the neonate grows at a rapid rate due in part to an enhanced sensitivity to the postprandial rise in amino acids, particularly leucine. To elucidate the molecular mechanism by which leucine stimulates protein synthesis in neonatal muscle, overnight-fasted 7-day-old piglets were treated with rapamycin [an inhibitor of mammalian target of rapamycin (mTOR) complex (mTORC1)] for 1 h and then infused with leucine for 1 h. Fractional rates of protein synthesis and activation of signaling components that lead to mRNA translation were determined in skeletal muscle. Rapamycin completely blocked leucine-induced muscle protein synthesis. Rapamycin markedly reduced raptor-mTOR association, an indicator of mTORC1 activation. Rapamycin blocked the leucine-induced phosphorylation of mTOR, S6 kinase 1 (S6K1), and eukaryotic initiation factor (eIF)4E-binding protein-1 (4E-BP1) and formation of the eIF4E:eIF4G complex and increased eIF4E::eIF4B complex abundance. Rapamycin had no effect on the association of mTOR with rictor, a crucial component for mTORC2 activation, or G protein β-subunit-like protein (GβL), a component of mTORC1 and mTORC2. Neither leucine nor rapamycin affected the phosphorylation of AMP-activated protein kinase (AMPK), PKB, or tuberous sclerosis complex (TSC)2, signaling components that reside upstream of mTOR. Eukaryotic elongation factor (eEF)2 phosphorylation was not affected by leucine or rapamycin, although current dogma indicates that eEF2 phosphorylation is mTOR dependent. Together, these in vivo data suggest that leucine stimulates muscle protein synthesis in neonates by enhancing mTORC1 activation and its downstream effectors.

mRNA translation; eukaryotic initiation factor 4G; AMP-activated protein kinase; raptor; rictor

The neonatal period is characterized by rapid growth that is supported by a high rate of protein synthesis (3). However, most infants that are born at low birth weight are discharged weighing less than the 10th percentile for age despite improvements in their nutritional management (9). Some remain small to adulthood and exhibit adverse long-term developmental outcomes including reduced work capacity (27). To improve strategies for the nutritional management of low-birth-weight infants, we have used the neonatal pig as a model of the human neonate so as to identify the mechanisms that regulate protein deposition in neonates. Our studies have shown that the profound accretion of skeletal muscle protein is in part due to the ability of neonatal muscle to markedly increase protein synthesis in response to feeding, a response that declines rapidly with development (5). In skeletal muscle, the feeding-induced stimulation of protein synthesis is independently modulated by the rise in insulin and amino acids (6, 24). Recently, we demonstrated (10–12) that the postprandial rise in leucine alone, but not isoleucine or valine alone, stimulates skeletal muscle protein synthesis in neonatal pigs and this response decreases with age.

Signaling through the mammalian target of rapamycin (mTOR) plays a significant role in cell growth regulation including protein synthesis (4). Recent studies have revealed that mTOR exists in two protein complexes (Fig. 1): mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (4). mTORC1 is rapamycin sensitive and consists of mTOR, raptor, and G protein β-subunit-like protein (GβL). This complex is activated by amino acids (especially leucine), hormones/growth factors, and energy signals (17). mTORC1 regulates mRNA translation by phosphorylating two of its effectors, ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor (eIF)4E-binding protein-1 (4E-BP1) (4). Phosphorylated 4E-BP1 releases eIF4E from the inactive eIF4E::eIF4B complex, allowing the formation of the active eIF4G::eIF4E complex (29). mTORC1 has also been implicated in the regulation of eukaryotic elongation factor (eEF)2 activation (38). The second protein complex, mTORC2, is rapamycin insensitive and consists of mTOR, rictor, and GβL (4). Recent biochemical studies suggest that one major function of mTORC2 is to activate the growth factor-regulated protein kinase B (PKB) (28).

The molecular mechanisms by which amino acids or leucine modulate the activation of mTORC1 in vivo are unknown. Recent studies suggest that amino acids or leucine stimulate mTORC1 independent of PKB, AMP-activated protein kinase (AMPK), and tuberous sclerosis complex (TSC1)/TSC2 activation (4). Furthermore, studies conducted in cell culture suggest that the activation of mTORC1 is partly regulated by the protein-protein interaction among members of mTORC1, in particular the interaction of mTOR with raptor (16). However, we (31) and others (37) have found that feeding stimulates the activation of mTORC1, especially in vivo. The authors assert that feeding induces an augmented interaction of mTORC1 with raptor that is independent of PKB (28).
protein synthesis in neonatal pigs by enhancing translation initiation factor activation. However, detailed study of the effect of physiological levels of leucine on the activation of mTORC1 leading to the stimulation of protein synthesis in skeletal muscle of the neonate has not been conducted previously. Therefore, our objective was to determine the molecular mechanisms by which leucine modulates mTORC1 activation in vivo by using rapamycin, a potent inhibitor of mTORC1. Previously, we demonstrated (21) that the feeding-induced stimulation of muscle protein synthesis in neonatal pigs was only partially inhibited by rapamycin.

MATERIALS AND METHODS

Animals and housing. Two crossbred (Landrace × Yorkshire × Duroc × Hampshire) pregnant sows (Agriculture Headquarters, Texas Dept. of Criminal Justice, Huntsville, TX) were housed in lactation crates in individual environmentally controlled rooms 2 wk before farrowing. Sows were fed a commercial diet (no. 5084, PMI Feeds, Richmond, IN) and provided water ad libitum. After farrowing, piglets remained with the sow but were not allowed access to the sow’s diet. A total of 23 piglets from 3 litters, weighing ~2 kg, were studied at 7 days of age. Three days before the experiment, piglets were anesthetized for sterile catheter insertion into a jugular vein and carotid artery. Piglets were then returned to the sow and allowed to suckle freely until being studied. The protocol was approved by the Animal Care and Use Committee of Baylor College of Medicine and was conducted in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals.

Treatments and infusion. Piglets were fasted for 12–14 h before infusion and placed in a sling restraint system. The carotid catheter was used to infuse saline, leucine, rapamycin, and L-[4-3H]phenylalanine, whereas the jugular catheter was used for repeated blood sample collection. Pigs were randomly assigned to one of four treatment groups: (1) saline (control), (2) saline + rapamycin, (3) leucine, and (4) leucine + rapamycin. Piglets assigned to the rapamycin groups were injected with a rapamycin solution (0.75 mg/kg in 5% dimethyl sulfoxide) 1 h before the initiation of the leucine infusion; other pigs were injected with diluent. Leucine infusion was initiated with a primed dose (148 μmol/kg) for 10 min, followed by a constant infusion of leucine at 400 μmol·kg⁻¹·h⁻¹ for 1 h. Previous studies (11) have shown that a two- to threefold elevation in plasma leucine concentration, similar to that observed with feeding, is achieved by this rate of leucine infusion. During the priming and constant infusion period, saline-infused pigs received a volume of saline equal to that of those receiving leucine.

Tissue protein synthesis in vivo. Fractional rates of protein synthesis were measured with a modification of the flooding dose method (14). At 30 min before the end of the infusion, pigs were injected with 10 ml/kg body wt of a flooding dose of phenylalanine (Amersham Biosciences, Piscataway, NJ), which provided 1.5 mM phenylalanine/kg body wt and 0.5 mCi L-[4-3H]phenylalanine/kg body wt. Samples of whole blood were taken 5, 15, and 30 min after the injection for measurement of the specific radioactivity of the extra-cellular free pool of phenylalanine. Pigs were killed at 60 min, and longissimus dorsi muscle samples were collected, immediately frozen in liquid nitrogen, and stored at −70°C until being analyzed, as previously described (8). Protein synthesis (Kₑ expressed as % protein synthesized in a day) was calculated as

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K_e = \left( \frac{S_b}{S_a} \right) \times \left( \frac{1}{(1.440/t)} \right) \times 100
\]

where \(S_a\) is the specific radioactivity of the tissue free phenylalanine for the labeling period, determined from the value for the animal at the time of tissue collection, corrected by the linear regression of the blood specific radioactivity of the animal against time; and \(t\) is the time of labeling in minutes. Previous studies have demonstrated that after a flooding dose of L-[4-3H]phenylalanine is administrated, the specific radioactivity of tissue free phenylalanine is in equilibrium with the aminoacyl tRNA specific radioactivity, and therefore the tissue free phenylalanine is a valid measure of the tissue precursor pool specific radioactivity (7).

Tissue extraction and immunoblot analysis. Freshly collected longissimus dorsi muscle tissue samples were homogenized and centrifuged at 10,000 g for 10 min at 4°C. Supernatants were diluted in sample buffer, frozen in liquid nitrogen, and stored at −70°C until analysis. Equal amounts of protein samples were electrophoretically separated in polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA), which was incubated with appropriate primary antibodies, washed, and exposed to an appropriate secondary antibody as previously described (32).

For normalization, immunoblots performed with anti-phospho-specific antibodies were stripped in stripping buffer (Pierce Biotechnology, Rockford, IL) and reprobed with the corresponding non-phospho-specific antibodies. Blots were developed with an enhanced chemiluminescence kit (Amersham Biosciences), visualized, and analyzed with a ChemiDoc-It Imaging System (UVP, Upland, CA). Primary antibodies that were used in the immunoblotting were PKB (total and Ser473, Cell Signaling Technology, Beverly, MA), AMPK-α (total and Thr172, Cell Signaling Technology), TSC2 (total and Thr1462, Cell Signaling Technology), mTOR (total, Ser2448, and Ser2481, Cell Signaling Technology), S6K1 (total and Thr389, Cell Signaling Technology), 4E-BP1 (total, Bethyl Laboratories, Montgomery, TX, and Thr70, Cell Signaling Technology), and eEF2 (total and Thr56, Cell Signaling Technology).
Quantification of elf4E-eIF4E-BP1 and elf4E-eIF4G complexes. These complexes were immunoprecipitated with an anti-eIF4E monoclonal antibody (gift of Dr. Leonard Jefferson, Penn State University College of Medicine, Hershey, PA) from aliquots of fresh tissue homogenates. Briefly, samples were homogenized in seven volumes of buffer (in mM: 20 HEPES, 2 EGTA, 50 NaF, 100 KCl, and 0.2 EDTA, pH 7.4) containing Sigma P3840 Protease Inhibitor Cocktail (Sigma, St. Louis, MO) and centrifuged at 10,000 g for 10 min at 4°C. Supernatants were incubated overnight at 4°C with constant rocking with anti-eIF4G antibody. Immunoprecipitates were recovered with goat anti-rabbit IgG magnetic beads (Polysciences, Warrington, PA), washed and resuspended in sample buffer as described elsewhere (8), and immediately subjected to protein immunoblot analysis using rabbit anti-4E-BP1 (Cell Signaling Technology) or rabbit anti-eIF4G (Bethyl Laboratories) antibody. Amounts of 4E-BP1 and eIF4G were corrected by the eIF4E recovered from the immunoprecipitate.

Analysis of mTORC1 and mTORC2. To determine the association between mTOR and its partners (raptor and GβL for the mTORC1 complex or rictor and GβL for the mTORC2 complex), muscle samples were homogenized in 5-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) buffer according to Williamson et al. (39). The CHAPS buffer consisted of (in mM) 40 HEPES, pH 7.5, 120 NaCl, 1 EDTA, 10 pyrophosphate, 10 β-glycerophosphate, 40 NaF, 1.5 sodium vanadate, 1 PMSF, 1 benzamidine, and 1 DTT with 0.3% CHAPS. The homogenate was mixed on a platform rocker for 30 min at 4°C and then centrifuged at 1,000 g for 3 min (4°C). The supernatant containing 500 μg of protein was combined with 2 μl of anti-mTOR antibody (Cell Signaling Technology) and mixed on a platform rocker overnight at 4°C. After the incubation, the immune complexes were isolated with a goat anti-mouse BioMag IgG (PerSeptive Diagnostics, Cambridge, MA) bead slurry. The magnetic bead complexes were collected with a magnetic stand and washed twice with CHAPS buffer and once in CHAPS buffer containing 200 mM NaCl instead of 120 mM NaCl and 60 mM instead of 40 mM HEPES. The precipitates were rinsed with 100 μl of 1 × SDS sample buffer and then boiled for 5 min and centrifuged to collect the supernatant. The samples were subjected to SDS-PAGE followed by immunoblotting with anti-raptor, anti-rictor antibody, and anti-GβL antibody (all from Cell Signaling Technology). The mTOR-protein complexes were normalized by the amount of total mTOR in the precipitates.

Statistics. All data were analyzed by one-way analysis of variance (ANOVA) with saline-treated animals as the independent variable. When a significant overall effect was observed, differences among individual means were assessed by the Tukey-Kramer comparisons test. Probability values of P < 0.05 were considered statistically significant. Data are presented as means ± SE.

RESULTS
Fractional protein synthesis rates. Several laboratories have demonstrated that leucine administration increases in vivo skeletal muscle protein synthesis (1, 2). Recently, we also provided evidence (11) that leucine administration at physiological levels stimulates protein synthesis in skeletal muscle of neonatal pigs. In this study, we examined whether treatment with rapamycin could suppress the leucine-induced stimulation of protein synthesis in skeletal muscle of neonatal pigs. As shown in Fig. 2, leucine infusion, compared with saline infusion, increased the fractional rates of protein synthesis in skeletal muscle (P < 0.05). Administration of rapamycin completely blocked the stimulatory effect of leucine on skeletal muscle protein synthesis. Because it is important to take into account any possible effect of rapamycin on the basal fasting rate of protein synthesis, rapamycin was also administered to saline-infused pigs. We found that rapamycin administration to saline-infused pigs did not reduce the fractional rate of skeletal muscle protein synthesis below the basal fasting level.

mTORC1 and mTORC2 activation. We previously showed (11) that administration of leucine to neonatal pigs increases skeletal muscle protein synthesis through the activation of translation initiation factors. However, the mechanism underlying the effect of leucine on mTORC1 was not investigated. Therefore, in this study we wished to determine whether physiological changes in circulating leucine levels modulate the activation of signaling components upstream of mTOR (AMPK, PKB, and TSC2) and the protein-protein interactions between members of mTORC1 and mTORC2 and whether the response is affected by rapamycin treatment. We found that neither leucine infusion nor rapamycin treatment affected the phosphorylation of AMPK, PKB, or TSC2 (Fig. 3).

In cell culture (17), rapamycin’s potency for dissociating the interaction of raptor with mTOR is increased under leucine-rich conditions, and this is correlated with a decrease in mTORC1 kinase activity. Therefore, we hypothesized that rapamycin’s ability to disturb the raptor-mTOR interaction would be greater in skeletal muscle of leucine-infused piglets than in saline-infused piglets. As shown in Fig. 4A, rapamycin severely destabilized the raptor-mTOR complex. Although there was no significant difference between the presence or absence of leucine in the potency of rapamycin in perturbing the interaction of raptor with mTOR, there was a tendency (P = 0.20) for the rapamycin-induced dissociation of the raptor-mTOR complex to be greater in the leucine-treated group compared with the control group. Furthermore, rapamycin did not affect the GβL-mTOR complex (Fig. 4B) or the rictor-mTOR complex (mTORC2: Fig. 4C).

Activation of mTOR and its downstream signaling components. Next we wished to investigate the effect of rapamycin on the leucine-induced activation of mTOR and its downstream components leading to the stimulation of skeletal muscle protein synthesis in neonatal pigs. In this study we determined mTOR activation by measuring mTOR phosphorylation at Ser2448 and Ser2481. Leucine enhanced mTOR phosphorylation at both sites (P < 0.05), and this effect was abolished by rapamycin (Fig. 5).

mTOR regulates mRNA translation in response to nutrients/leucine and growth factors via activation of its downstream substrates, S6K1 and 4E-BP1 (4). To determine the effects of leucine and rapamycin on the activation of these important effec-
tors of mTOR, the phosphorylation states of S6K1 at Thr389 and 4E-BP1 at Thr479 were analyzed with immunoblot analysis. Leucine significantly enhanced the phosphorylation of S6K1 and 4E-BP1 in skeletal muscle (P < 0.05) (Fig. 6, A and B, respectively), and its effects on the phosphorylation of these mTOR effectors were completely abolished by rapamycin.

One of the major factors that regulate the formation of an active eIF4E·eIF4G complex is 4E-BP1 (29). In the hypophosphorylated state, this protein binds eIF4E to form an inactive complex. Conversely, in the hyperphosphorylated state, 4E-BP1 detaches from eIF4E, allowing eIF4G to assemble an active eIF4E·eIF4G complex and initiate mRNA translation. To determine the effects of leucine and rapamycin on the assembly of an active eIF4E·eIF4G complex and an inactive eIF4E·4E-BP1 complex, immunoprecipitation and immunoblot analysis were performed. As shown in Fig. 7, leucine increased the formation of the active eIF4E·eIF4G complex (P < 0.05) and inhibited the formation of an inactive eIF4E·4E-BP1 complex (P < 0.05). The leucine-induced formation of the active eIF4E·eIF4G complex was completely prevented by rapamycin (Fig. 7A). Likewise, the
inhibitory effect of leucine on the formation of the inactive eIF4E/H1852/4E-BP1 complex was eliminated by rapamycin (Fig. 7B).

Translation elongation in mammalian cells requires two eEFs, eEF1 and eEF2. eEF2 mediates the translocation of the ribosome by three nucleotides along the mRNA after the addition of each new amino acid. Phosphorylation of eEF2 on Thr56 impairs its ability to bind ribosomes, thus inactivating this signaling protein and inhibiting mRNA translation (18). In this study, we determined the effects of leucine on the phosphorylation of eEF2 at Thr56. As shown in Fig. 8, neither leucine nor rapamycin altered the phosphorylation of eEF2 in skeletal muscle.

**Effect of rapamycin on protein abundance of signaling components.** Treatment with rapamycin, which downregulates mTORC1 activation, has been associated with increased autophagy, one of the intracellular protein degradation pathways (4). To determine whether short-term exposure to rapamycin alters the degradation of several signaling proteins, we measured their protein abundance. We found that the protein abundance of all signaling components measured in the present study was not affected by short-term exposure to rapamycin (data not shown).

**DISCUSSION**

Amino acids, especially the branched-chain amino acid leucine, serve not only as precursors for protein synthesis but also as signals that activate protein synthesis via activation of intracellular signal transduction pathways that regulate mRNA translation (19). However, despite tremendous efforts, the molecular mechanisms by which amino acids regulate mRNA translation remain largely unknown. Studies using different cell culture systems have reported conflicting results. Early data suggest that TSC1 and TSC2 are required for amino acid-induced signaling through mTOR (34). Other studies indicate that rhabdomyosarcoma activation, but not TSC1/2 activation, is required for amino acid control of mTOR activation (30). A recent study suggested that amino acid input into mTOR...
2 h, the leucine-induced stimulation of muscle protein synthesis is maintained. In addition, we demonstrated that the stimulation of the mTOR signaling pathway and protein synthesis in muscle by leucine, like that by feeding, insulin, and balanced amino acid mixtures, decreases with age (8, 10, 33). In this study, we wished to further characterize the acute action of leucine by utilizing rapamycin, a potent inhibitor of mTOR.

Amino acids, especially leucine, regulate signaling through mTOR and thereby control a number of components of the protein synthetic machinery, including initiation and elongation factors (26). Rapamycin, a well-characterized mTOR inhibitor, has been widely used to study mTOR functions including protein synthesis (21). Previously, we showed (21) that rapamycin completely blocked the feeding-induced stimulation of protein synthesis in liver but only attenuated 60% of the feeding-induced stimulation of skeletal muscle protein synthesis in neonatal pigs. However, in the present study, infusion of leucine to raise circulating levels of leucine to fed levels, similar to those achieved in previous feeding studies (5, 13), stimulated muscle protein synthesis, and this was completely blocked by rapamycin.

While there is evidence of the involvement of the TSC1/2 complex in the amino acid-induced activation of mTORC1 (34), in the present in vivo study we did not find any effect of physiological levels of leucine on the phosphorylation of TSC2 and its kinases, PKB and AMPK. This is consistent with our previous finding (33) that fed amino acid levels have no effect on AMPK, PKB, or TSC2 phosphorylation, although raising insulin to fed levels increased PKB and TSC2 phosphorylation but not AMPK. To the best of our knowledge, this is the first in vivo study to examine the effect of leucine on the activation of TSC1/TSC2 complex.

Mechanistic studies using cell cultures showed that amino acids regulate the interaction of raptor with mTOR, resulting in the activation of mTORC1 and its downstream effectors, S6K1 and 4E-BP1 (4). A recent cell culture study indicates that under nutrient- or leucine-rich conditions, the mTOR-raptor association is destabilized, resulting in the activation of mTOR (16). However, our in vivo studies in skeletal muscle (31, 33) and other studies in heart (37) showed that although both feeding signaling was not mediated by the TSC1/2 complex or rheb but by a class 3 phosphatidylinositol 3OH-kinase (PI3K) or human vacuolar protein sorting 34 (hVps34) (23). Our recent work (33) demonstrated that a physiological rise in amino acids in vivo had no effect on the phosphorylation of AMPK, PKB, and TSC2 in skeletal muscle of neonatal pigs, suggesting that amino acids transmit their signal downstream of the TSC1/2 complex.

Our in vivo studies have revealed that leucine is effective in stimulating protein synthesis in skeletal muscle of the neonate, although the response to leucine is less than that to a complete amino acid mixture (11). The leucine-induced stimulation of skeletal muscle protein synthesis involves the activation of signaling components downstream of mTOR leading to mRNA translation. We further demonstrated that the acute (1 h) leucine-induced stimulation of protein synthesis is not maintained (2 h), despite continued activation of the mTOR signaling pathway, because circulating concentrations of essential amino acids fall to less than fasting levels as they are utilized as substrates for protein synthesis (10). However, when circulating amino acid levels are maintained at baseline levels for
and amino acid infusion increase S6K1 and 4E-BP1 activation, no change in the interaction of mTOR with raptor is detected. In this study, leucine infusion also did not have any effect on the interaction of raptor with mTOR. Thus further study of the association of mTOR and raptor is required.

Rapamycin, like nutrient deprivation, inhibits the activation of downstream effectors of mTOR, but exactly how the drug perturbs mTOR function is unknown (4). A recent study showed that one of the actions of rapamycin is to interfere with the ability of raptor to effectively present substrates (such as S6K1 and 4E-BP1) to mTOR (4). In support of this model, we found that rapamycin significantly reduced the interaction of raptor with mTOR in both control and leucine-treated groups. In cell culture conditions, addition of leucine to nutrient-poor medium enhanced the destabilizing effect of rapamycin on the raptor-mTOR complex (17). Interestingly, we found that there was a tendency ($P = 0.20$) for the rapamycin-induced dissociation of the raptor-mTOR complex to be greater in the leucine-treated group compared with the control group.

GβL is a crucial member of mTORC1 because its binding to the kinase domain of mTOR stabilizes the interaction of raptor with mTOR (4). Furthermore, mTOR, GβL, and rictor are members of mTORC2, a rapamycin-insensitive complex (4). While we were able to detect both GβL and rictor in mTOR immunoprecipitants, their interactions were not affected by rapamycin treatment in vivo. To the best of our knowledge, this is the first in vivo study to examine the effect of rapamycin on leucine regulation of the integrity of mTORC1 and mTORC2.

Several lines of evidence suggest that leucine induces the activation of mTOR and its downstream substrates (S6K1 and 4E-BP1) that control in part the step in translation initiation involving the binding of mRNA to 40S ribosomal subunit (20). In this study, we found that in fasting pigs rapamycin did not reduce the phosphorylation of mTOR, S6K1, and 4E-BP1 below the basal level. In addition, rapamycin completely blocked the leucine-induced phosphorylation of mTOR, S6K1, and 4E-BP1 in skeletal muscle. Furthermore, rapamycin completely blunted the eIF4G association with eIF4E and restored the association between 4E-BP1 and eIF4E. Most of these observations are in agreement with a study from Vary et al. (36).

Recent studies suggest that one of the key roles of mTOR is to regulate the activation of elongation factors (25). Previously, we found (12, 33) that neither amino acids nor leucine alters eEF2 phosphorylation. Interestingly, rapamycin did not have any effect on the phosphorylation of eEF2. Since eEF2 is thought to be one of the major substrates of mTOR (25), the lack of effect of rapamycin on eEF2 phosphorylation is surprising, but it could be due to the physiological dose of leucine used in this study.

In summary, in the present study we showed that the leucine-induced stimulation of protein synthesis and the activation of signaling components leading to mRNA translation in skeletal muscle of the neonate are sensitive to rapamycin. This contrasts with a study published while we were preparing this manuscript, which showed that the feeding-induced activation of 5′ mRNA translation is resistant to rapamycin in fetal liver (hepatocytes) but not adult liver (15). In the present study, rapamycin blocked leucine-induced phosphorylation of mTOR, S6K1, and 4E-BP1 and the formation of active eIF4E-eIF4G complex in neonatal muscle, in agreement with data from cell culture studies (17, 22). However, study of the effect of leucine on the protein interaction of members of the mTORC1 complex is more challenging because we were unable to detect an effect of leucine on raptor-mTOR association in vivo although we did show a tendency toward leucine-induced loosening of the raptor-mTOR association, a condition that has been documented in cell culture studies (17). Nevertheless, it is clear that our understanding of the molecular mechanisms by which leucine regulates the signaling function of mTORC1 in vivo is incomplete. Considering the potential significance of leucine’s action as a nutrient signal to stimulate muscle protein synthesis in vivo, further studies to elucidate its mechanistic functions are warranted.

REFERENCES