Stimulation of Muscle Protein Synthesis by Prolonged Parenteral Infusion of Leucine Is Dependent on Amino Acid Availability in Neonatal Pigs\textsuperscript{1,2}

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Abstract

The postprandial rise in amino acids, particularly leucine, stimulates muscle protein synthesis in neonates. Previously, we showed that a 1-h infusion of leucine increased protein synthesis, but this response was not sustained for 2 h unless the leucine-induced decrease in amino acids was prevented. To determine whether a parenteral leucine infusion can stimulate protein synthesis for a more prolonged, clinically relevant period if baseline amino acid concentrations are maintained, overnight food-deprived neonatal pigs were infused for 24 h with saline, leucine (400 \(\mu\)mol·kg\(^{-1} \cdot \text{h}^{-1}\)), or leucine with replacement amino acids. Amino acid replacement prevented the leucine-induced decrease in amino acids. Muscle protein synthesis was increased by leucine but only when other amino acids were supplied to maintain euaminoacidemia. Leucine did not affect activators of mammalian target of rapamycin (mTOR), i.e., protein kinase B, AMP-activated protein kinase, tuberous sclerosis complex 2, or eukaryotic elongation factor 2. There was no effect of treatment on the association of mTOR with regulatory activators of mammalian target of rapamycin (mTOR), i.e., protein kinase B, AMP-activated protein kinase, tuberous sclerosis complex 1; PKB, protein kinase B; PRAS40, proline-rich Akt substrate of 40 kDa; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; PKB, protein kinase B; PRAS40, proline-rich Akt substrate of 40 kDa; raptor, regulatory associated protein of mammalian target of rapamycin; S6K1, ribosomal protein S6 kinase 1; TSC, tuberous sclerosis complex.

Introduction

The neonatal period is characterized by rates of growth and protein turnover that are higher than at any other period of postnatal development (1). However, most low birth weight (LBW)\textsuperscript{3} infants experience growth failure by discharge, despite the recent improvements in their nutritional management, and some remain small to adulthood (2,3). To improve nutritional management strategies for LBW infants, we have utilized neonatal piglets as a model of the human neonate to identify the mechanisms that regulate protein deposition. Our studies have shown that the enhanced capability of the neonate to promote protein accretion is a consequence of their highly efficient utilization of dietary amino acids for protein deposition (4). Feeding stimulates protein synthesis in most neonatal tissues; however, the postprandial increase is greatest in skeletal muscle (5), an observation that can be mimicked by independently raising circulating insulin or amino acid concentrations to levels associated with the fed state (6,7).

The amino acid leucine appears to be of particular importance in maintaining muscle protein synthesis rates (Ks). Oral administration of leucine increases rates of skeletal muscle protein synthesis in mature rats (8) and removal of this amino acid from a complete meal prevents stimulation of protein synthesis (9). Previously, our laboratory demonstrated that a 1-h parenteral infusion of leucine that raises circulating concentrations of leucine to fed levels increases protein synthesis in...
skeletal muscle of neonatal pigs (10). This anabolic effect appears to be unique for leucine, as increasing circulating levels of the other branched-chain amino acids, isoleucine and valine, within the physiological range does not alter muscle protein synthesis (11). However, the response to leucine is not sustained for 2 h, likely due to a leucine-induced decrease in the circulating levels of other amino acids. Indeed, hypoaminoacidemia can limit protein synthesis in adult animals (12) and humans (13,14) but appears to only blunt insulin’s stimulatory effect on muscle protein synthesis in the neonatal piglet (6). Recently, we demonstrated that when circulating amino acids are supplied to maintain euaminoacidemia, the leucine-induced stimulation of muscle protein synthesis can be maintained for 2 h (15).

The leucine-induced stimulation of protein synthesis is mediated by increased eukaryotic initiation factor (eIF) 4F complex formation, which regulates mRNA binding to the ribosome (16) and translation initiation (9). Formation of eIF4F is regulated by the rapamycin-sensitive mammalian target of rapamycin (mTOR) complex 1 (mTORC1), which is comprised of mTOR, regulatory associated protein of mammalian target of rapamycin (raptor), G-protein β subunit-like protein (GβL), and ras homolog enriched in brain (17,18). Insulin or amino acid activation of mTORC1 involves phosphorylation of proline-rich Akt substrate of 40 kDa (PRAS40), resulting in ribosomal protein S6 kinase 1 (S6K1) activation and eIF4F formation (19).

The rate-limiting step of eIF4F formation is availability of eIF4E, which can be sequestered by 4E binding protein (4E-BP1), preventing its association with the scaffolding protein eIF4G. Phosphorylation of 4E-BP1 by mTOR allows eIF4E, allowing eIF4F formation (20). Despite recognition of these mechanisms, little is known about how amino acids directly stimulate mTORC1 activation. Protein kinase B (PKB) and tuberous sclerosis complex 2 (TSC2), while involved in insulin-induced activation of mTORC1, appear unaffected by amino acids. A second, rapamycin-insensitive (17,18), mTOR complex, mTORC2 (comprised of mTOR, rictor, and GβL), activates PKB, allowing feedback stimulation of mTORC1 (21).

In addition to mRNA-ribosome binding, translation initiation requires initiator met-tRNA, binding to the start codon, a step mediated by eIF2 (20). Phosphorylation of the α-subunit of eIF2 reduces initiator met-tRNAi binding to the ribosome (20). Translation is also dependent upon rates of elongation. Although it has been suggested that the eukaryotic elongation factor 2 (eEF2) is a substrate of mTORC1, rapamycin treatment does not alter the phosphorylation of this factor (22).

To test the potential for the use of leucine supplementation to optimize the nutritional management of LBW infants, we aimed to determine whether parenteral infusion of leucine can promote elevated rates of skeletal muscle protein synthesis in the neonatal pig for a prolonged, clinically relevant duration of 24 h. Additionally, we wanted to examine in detail the activation of translation initiation and elongation factors involved in the leucine-mediated increase in protein synthesis at 24 h in the presence and absence of amino acid supplementation. Because in our previous studies we infused leucine acutely and our indices of translation initiation were limited, this will provide novel and important information regarding the sustainability of the effect of leucine on the activation of the signaling components of translation.

Materials and Methods

Pigs and study designs. Two multiparous crossbred (Yorkshire × Landrace × Hampshire × Duroc) pregnant sows (Agricultural Head-quarters, Texas Department of Criminal Justice, Huntsville, TX) were housed and managed as previously described (10). After farrowing, piglets resided with the sow and were studied at 5 d of age weighing 2.6 ± 0.1 kg. The Animal Care and Use Committee of Baylor College of Medicine approved all experimental procedures. This study was conducted in accordance with the NRC’s Guide for the Care and Use of Laboratory Animals.

At 1 d of age (2.1 ± 0.1 kg), catheters were placed in the left external jugular vein and left common carotid artery using sterile techniques under general anesthesia (Aerrane, Anaquest). After recovering from anesthesia, piglets were returned to their sows until studied.

Treatments and infusions. Overnight (12 h) food-deprived, 5-d-old piglets were randomly assigned to 1 of 3 treatment groups: saline, leucine, or leucine + amino acids (n = 6/treatment group) and infused parenterally for 24 h. Piglets assigned to the saline group were infused with sterile saline at 10 mL h⁻¹ throughout the infusion period. Piglets assigned to the leucine group were infused with leucine at 400 μmol kg⁻¹ h⁻¹, while piglets in the leucine + amino acid group were additionally infused with a balanced amino acid mixture⁴ (23), prepared devoid of leucine, to maintain circulating amino acid concentrations at baseline food-deprived levels. The infusion rate of the amino acid mixture was gradually increased at 10-min intervals from 0 to 0.4, 0.6, 0.85, 1.5, 1.85, 2.25, 2.7, and 2.85 mL h⁻¹ kg⁻¹ until the infusion rate of 2.85 mL h⁻¹ kg⁻¹ was achieved and maintained constant throughout as previously described (15). Blood samples were collected hourly for the first 2 h and then every 2 h thereafter to measure blood glucose concentrations (YSI 2300 STAT Plus, Yellow Springs Instruments). Plasma samples were collected and frozen at 0, 1, 2, 4, 8, 12, 16, 20, and 24 h for later analysis of insulin and individual amino acid concentrations.

Hormones and substrates. The concentrations of individual amino acids from frozen plasma samples were measured using a HPLC method (PICO-TOG reverse-phase column; Waters) as previously described (24). Plasma radioimmunoassay insulin concentrations were measured using a porcine insulin RIA kit (Linco).

Tissue protein synthesis in vivo. The fractional rate of protein synthesis was measured with a flooding dose of [¹⁴C]-phenylalanine injected 30 min prior to the end of the infusion (25). Piglets were killed at 24 h and samples were obtained from the longissimus dorsi muscle and immediately frozen in liquid nitrogen and stored at −70°C until analyzed as previously described (1). Previous studies have demonstrated that after a flooding dose of [¹³C]-phenylalanine is administered, 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 (26).

Protein immunoblot analysis. Proteins from longissimus dorsi muscle homogenates were separated on polyacrylamide gels (PAGE). For each assay, all samples were analyzed at the same time on triple-wide gels (C. B.S. Scientific) to eliminate inter-assay variation. Proteins were electro-photographically transferred to polyvinylidene difluoride transfer membranes (Pall), which were incubated with appropriate primary antibodies, washed, and exposed to an appropriate secondary antibody as previously described (27). Primary antibodies that were used in the immunoblotting were PKB (total and Ser473, Cell Signaling), AMP-activated kinase (AMPK) (total and Thr172, Cell Signaling), TSC2 (total, Santa Cruz Biotechnology, and Thr1462, Cell Signaling), raptor (total and Ser2448, Cell Signaling), mTOR (total and Ser2462, Cell Signaling), and Thr389, Cell Signaling).

⁴ Amino acid (Ajinomoto and Spectrum Chemicals) infusate composition (in mmol L⁻¹): L-alanine (27.3, 38% provided as L-alanyl-L-glutamine), L-arginine (20.1), L-aspartate (12.0), L-cysteine (6.2), L-glutamate (23.8), glutamine (17.1, 100% provided as L-alanyl-L-glutamine), glycine (54.3, 4% provided as glycyl-L-tyrosine), L-histidine (12.9), L-isoleucine (28.6), L-lysine (27.4), L-methionine (10.1), L-phenylalanine (12.1), L-proline (34.8), L-serine (23.8), L-threonine (2.0), L-tyrosine (7.2, 83% provided as glycyl-L-tyrosine), L-tryptophan (4.4), and L-valine (34.1).

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PRAS40 (total and Thr\(^{246}\), Cell Signaling), 4E-BP1 (total, Bethyl Laboratories, and Thr\(^{37}\), Cell Signaling), eIF4G (total and Ser\(^{1186}\), Cell Signaling), S6K1 (total and Thr\(^{389}\), Cell Signaling), eEF2α (total and Ser\(^{51}\), Cell Signaling), and eEF2 (total and Thr\(^{56}\), Cell Signaling).

Quantification of eIF4E - 4E-BP1 and eIF4E - eIF4G complexes. The eIF4E - 4E-BP1 and eIF4E - eIF4G complexes were immunoprecipitated from aliquots of fresh tissue homogenates using an anti-eIF4E monoclonal antibody (gift of Dr. Leonard Jefferson, Penn State University College of Medicine, Hershey, PA) as previously described (11).

Analysis of mTORC1 and mTORC2. To determine the association between mTOR and its partners (with raptor and GβL for mTORC1, or with rictor for mTORC2), muscle samples were homogenized in 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate buffer as described elsewhere (22,28).

Calculations and statistics. The fractional rate of protein synthesis (Ks) was calculated as previously (22). The majority of RNA in tissues is ribosomal; hence, the RNA:protein ratio (mg RNA × g protein\(^{-1}\)) was used as an estimate of protein synthetic capacity (Cs). Protein synthetic efficiency (Ks/Cs) was estimated as the total protein synthesized in 1 d/total RNA (g protein d\(^{-1}\)g RNA\(^{-1}\)).

ANOVA analysis was carried out in Minitab (version 13.31) using a general linear model to determine the main statistical differences. Where variances were unequal, ANOVA was performed assuming unequal variances. Between-group analysis was performed using Tukey multiple comparisons procedure. P < 0.05 was considered significant for all comparisons and data are presented as means ± SEM. Analysis of changes in circulating glucose, leucine, and insulin concentrations over the duration of the infusion period was carried out with SPSS general linear model using Greenhouse-Geisser test for within-subject effects.

Results

Plasma glucose, insulin, and amino acid concentrations. Circulating levels of glucose were unaffected by treatment and, although altered by time (P < 0.001), remained within the range of food-deprived piglets (Fig. 1A). Circulating insulin concentrations were increased in the leucine + amino acid group compared with the other treatment groups (P < 0.001); however, as with glucose levels, they remained within the range of food-deprived piglets (Fig. 1B). Plasma leucine concentrations were similar in all treatment groups at the beginning of the experimental period (Fig. 1C). The concentration in the 2 leucine-infused groups increased until ~12–16 h, when plasma concentrations in these 2 groups were ~4-fold those of control piglets (P < 0.001). The concentration of leucine in the 2 leucine infusion groups did not differ.

An infusion of leucine alone markedly decreased (P < 0.05; Fig. 2A) circulating concentrations of the essential amino acids with the exception of tryptophan. Infusion of replacement amino acids during the leucine infusion maintained circulating essential amino acids at control levels. For the nonessential amino acids (Fig. 2B), an infusion of leucine alone reduced the plasma concentrations of alanine (P < 0.01), glycine (P < 0.005), ornithine (P < 0.01), and serine (P < 0.01), with no effect of leucine infusion alone on the levels of the other nonessential amino acids. An infusion of leucine with replacement amino acids increased the plasma levels of asparagine (P < 0.001), glutamine (P < 0.001), proline (P < 0.01), and taurine (P < 0.005) above saline-infused controls, but the concentration of other nonessential amino acids did not differ from control levels.

Protein synthesis and K\(_{RNA}\). Measurement of muscle Ks showed an effect of treatment (P < 0.01; Table 1). An infusion of leucine alone for 24 h did not increase rates of protein synthesis above that of control pigs. However, infusion of leucine increased muscle Ks by 25% (P < 0.01) when other amino acids were maintained at control levels.

Measurement of the Cs showed no effect of treatment (Table 1). However, infusion of leucine over a 24-h period, when providing amino acids at replacement levels to maintain euaminoacidemia, increased the efficiency with which the ribosomes translated mRNA into skeletal muscle protein by 29% (P < 0.005) compared with saline-treated piglets (Table 1). In contrast, translational efficiency did not differ between pigs receiving saline and those treated with leucine alone for 24 h.

Signaling component activation. The phosphorylation of PKB on Ser\(^{437}\), AMPK on Thr\(^{172}\), and TSC2 on Thr\(^{1462}\) (Table 2), signaling components located upstream of the master kinase, mTOR, were unaffected by an infusion of leucine regardless of circulating amino acid concentration. In addition, leucine treatment did not alter the binding of mTOR with components of mTORC1 and mTORC2, i.e. raptor, GβL, or rictor (Table 3), irrespective of the amino acid concentration. However, long-term infusion of leucine alone tended to increase the phosphorylation and, hence, the activation of mTOR compared with saline-treated controls (P = 0.083; Table 2). Maintaining plasma
hypoaminoacidemia by amino acid replacement infusion did not alter the magnitude of the disassociation. Leucine increased the formation of the eIF4E·eIF4G complex compared with saline-treated controls \((P < 0.03); \text{Table 3}\), irrespective of amino acid supplementation. The phosphorylation of eIF4G on Ser\(^{1108}\) increased in response to leucine \((P < 0.05); \text{Table 2}\) and was not further increased by correction of the leucine-induced hypoaminoacidemia.

Long-term infusion of leucine tended to reduce the phosphorylation and hence increase the activity of eIF2\(\alpha\) \((P = 0.053); \text{Table 2}\), a phenomenon also independent of circulating amino acid concentrations. However, the dephosphorylation of eIF2\(\alpha\), which increases the elongation of translating peptides, showed no treatment effect (Table 2).

**Discussion**

To examine the potential for leucine supplementation as a useful adjunct in the nutritional management of LBW infants, we examined whether parenteral infusion of leucine can enhance protein synthesis in skeletal muscle of the neonatal pig for a prolonged, clinically relevant duration and whether maintenance of other amino acids at baseline levels is required. We showed that continuous parenteral infusion of leucine increased rates of protein synthesis in skeletal muscle of the neonatal pig for 24 h, if other amino acids were supplied to maintain euaminoacidemia. Importantly, leucine activated the mTORC1 pathway for prolonged periods; however, this was not sufficient to enable a sustained stimulation of muscle protein synthesis unless the leucine-induced hypoaminoacidemia was prevented.

Previously, we demonstrated that leucine infusion for 1 h increases protein synthesis in skeletal muscle of neonatal pigs (10). However, leucine did not stimulate muscle protein synthesis at 2 h and this was associated with a decrease in circulating levels of other essential amino acids, likely as they were utilized for protein synthesis (10,15). In the current study, infusion of leucine alone for 24 h was associated with a 50% reduction in circulating amino acids and an inability of leucine to stimulate muscle protein synthesis. However, when leucine was infused concurrently with a balanced amino acid mixture (devoid of leucine) sufficient to maintain circulating concentrations of the other essential and nonessential amino acids at baseline concentrations for 24 h, muscle protein synthesis was enhanced. By comparison, we have previously shown that raising insulin to the fed level for 2 h stimulated protein synthesis even in the presence of insulin-induced hypoaminoacidemia, although not to the level achieved when euaminoacidemia was maintained (6). This suggests that neonatal muscle protein synthesis is more responsive to insulin than to leucine alone. Leucine in the presence of euaminoacidemia modestly raised plasma insulin levels; nonetheless, insulin remained within the range of food-deprived piglets and thus the leucine-induced increase in neonatal muscle protein synthesis appears independent of insulin, in agreement with previous studies (6).

Although a reduction in the circulating concentrations of an individual amino acid such as glutamine (29), methionine, or threonine (30) can limit protein synthesis in some conditions, the circulating levels of most of the amino acids were reduced by leucine infusion in the current study and, thus, it is not possible to ascertain whether an individual amino acid potentially limited protein synthesis in muscle of leucine-infused neonatal pigs.

Chronic changes in protein synthesis are commonly accompanied by changes in ribosome abundance (31,32), whereas acute changes in protein synthesis are driven by changes in the

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**TABLE 1** Ks, \(K_{RNA}\), and Cs in skeletal muscle of neonatal piglets infused for 24 h with saline or leucine with and without replacement amino acids

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Leucine</th>
<th>Leu + AA (^2)</th>
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<tbody>
<tr>
<td>Ks, (%) d(^{-1})</td>
<td>12.43 ± 0.80(^a)</td>
<td>13.40 ± 0.75(^b)</td>
<td>16.51 ± 0.49(^b)</td>
</tr>
<tr>
<td>(K_{RNA}), g protein d(^{-1}) · g RNA (^{-1})</td>
<td>5.82 ± 0.34(^a)</td>
<td>6.05 ± 0.31(^b)</td>
<td>8.41 ± 0.50(^b)</td>
</tr>
<tr>
<td>Cs, (\mu)g RNA mg protein (^{-1})</td>
<td>21.38 ± 0.64</td>
<td>20.98 ± 0.70</td>
<td>20.98 ± 0.55</td>
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</table>

\(^1\) Values are mean ± SEM, \(n = 6\). Means in a row with superscripts without a common letter differ, \(P < 0.05\).

\(^2\) Leu + AA, Leucine with amino acids.
rate of translation of mRNA via alterations in peptide chain initiation (33). Because leucine was infused for a 24-h period, we wanted to determine whether the change in muscle protein synthesis was due to changes in ribosome abundance. However, the infusion of leucine, with or without replacement amino acids, did not affect ribosome abundance, but translational efficiency was enhanced in response to leucine when a concurrent infusion of amino acid was provided.

Previously, we demonstrated that the physiological increase in leucine by parenteral infusion for 2 h stimulated the activation of downstream effectors of mTOR in neonatal piglet muscle, despite the reduction in the circulating concentrations of essential amino acids and the failure of leucine to maintain the stimulation of muscle protein synthesis (10). When hypoaminoacidemia was prevented, there was no further increase in the activation of translation initiation factors (15). In the current study, we explored in more detail the role of the amino acid signaling pathway in the response to chronic leucine stimulation.

Although treatment with the mTORC1 inhibitor, rapamycin, prevents the leucine-induced stimulation of protein synthesis in mature rats (34) and neonatal pigs (22), the exact mechanism by which amino acids stimulate the mTOR pathway is unknown. Of the upstream regulators of mTOR, the best characterized is PKB, which mediates the insulin-associated response. However, PKB is not thought to mediate the amino acid-induced response (35,36), a finding confirmed in the current study. It has been proposed that amino acids may act by inhibiting the GAP activity of the negative mTOR regulator, TSC1/TSC2, toward ras homolog enriched in brain, a small guanine nucleotide-binding protein, and GTPase, which is thought to increase mTOR activity in its GTP form (37). Consistent with acute amino acid and leucine infusion studies (22,38), in the current study we showed no effect of chronic leucine infusion on TSC2 phosphorylation, suggesting that neither a short- nor long-term infusion of leucine mediates mTORC1 activation via this pathway. Not surprisingly, phosphorylation of AMPK, an energy sensor that stimulates TSC2 (37), was also unaltered by leucine infusion. Previously, we showed that AMPK is unaltered by food deprivation and feeding in neonatal pigs (38), indicating that AMPK may not be involved in the regulation of the mTOR pathway under physiological conditions.

Several binding proteins, including raptor, rictor, and GBL, are known to associate with mTOR (17,39); however, in the current study, long-term leucine infusion did not alter the binding of these proteins to mTOR. We speculate that the nature of the protein-protein interaction in mTORC1/2 is transient, so we could not detect changes in their interactions. Leucine treatment also did not alter phosphorylation of raptor, although raptor has been reported to be essential for mTORC1 signaling (39). However, raptor is phosphorylated by AMPK (40) and, because AMPK activation was not altered in the current study, no change in raptor phosphorylation would be expected.

Activation of mTORC1 regulates the downstream effectors PRAS40, S6K1, and 4E-BP1 (9,41). In the current study, leucine treatment increased S6K1 and 4E-BP1 phosphorylation, although PRAS40 phosphorylation was unaltered. Insulin- or amino acid-induced activation of mTORC1 is thought to lead to the phosphorylation and release of PRAS40 from raptor, allowing binding and phosphorylation of S6K1 and 4E-BP1 (41–43). Because Thr246 is phosphorylated by PKB and Ser183 is controlled by mTORC1 (41), it is not surprising that PRAS40 phosphorylation at Thr246 was unaltered in the current study, as leucine does not regulate PKB. The increased phosphorylation of S6K1 and 4E-BP1 and subsequent formation of the active eIF4E·eIF4G complex in response to prolonged leucine infusion is consistent with previous acute studies using parenteral leucine in neonatal pigs (10,15) and a gavage dose of leucine in adult rats (34). By contrast, supplementation of leucine via drinking water for 2 wk increased muscle protein synthesis in adult rats without altering S6K1 or 4E-BP1 activation (44).

Formation of the eIF4E·eIF4G complex is also dependent on eIF4F phosphorylation (9). In the current study, eIF4F phosphorylation was increased by leucine treatment, consistent with studies in rats where a leucine perfusion of the hind limb to 10× food-deprivation levels stimulated eIF4G phosphorylation and active complex formation (45). However, in the rat study, S6K1 and 4E-BP1 phosphorylation were unaltered, suggesting that leucine may be able to promote protein synthesis independently of mTOR. Indeed, studies have suggested that signaling through mTOR is insufficient to account for the leucine effect (34). In the current study, leucine stimulated the activation of mTOR and downstream signaling proteins during hypoaminoacidemia and there was no additional effect induced by euaminoacidemia. Thus, the leucine-induced activation of the mTOR signaling pathway was independent of circulating levels of other amino acids. However, the lack of effect of leucine on protein synthesis, unless hypoaminoacidemia was prevented, is indicative that

### TABLE 2
Phosphorylation status of various kinases involved in translation in skeletal muscle of neonatal pigs after 24 h of infusion with saline or leucine, with and without replacement amino acids

| Kinase | Control | Leucine | Leu + AA
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<tr>
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</thead>
<tbody>
<tr>
<td>PKB on Ser473</td>
<td>1.58 ± 0.42</td>
<td>1.57 ± 0.42</td>
<td>1.68 ± 0.42</td>
</tr>
<tr>
<td>AMPK on Thr172</td>
<td>3.38 ± 1.16</td>
<td>3.56 ± 0.70</td>
<td>3.83 ± 0.82</td>
</tr>
<tr>
<td>TSC2 on Thr1462</td>
<td>3.73 ± 0.86</td>
<td>4.09 ± 0.38</td>
<td>3.99 ± 0.68</td>
</tr>
<tr>
<td>mTOR on Ser2448</td>
<td>0.20 ± 0.05b</td>
<td>0.37 ± 0.07b</td>
<td>0.40 ± 0.04b</td>
</tr>
<tr>
<td>PRAS40 on Thr246</td>
<td>1.50 ± 0.14</td>
<td>1.52 ± 0.33</td>
<td>1.63 ± 0.25</td>
</tr>
<tr>
<td>Raptor on Ser792</td>
<td>1.30 ± 0.22</td>
<td>1.10 ± 0.18</td>
<td>1.03 ± 0.14</td>
</tr>
<tr>
<td>S6K1 on Thr1836</td>
<td>0.06 ± 0.02b</td>
<td>0.27 ± 0.08b</td>
<td>0.29 ± 0.09a</td>
</tr>
<tr>
<td>4E-BP1 on Thr70</td>
<td>0.03 ± 0.15b</td>
<td>1.27 ± 0.25b</td>
<td>1.47 ± 0.32a</td>
</tr>
<tr>
<td>eIF4G on Ser1180</td>
<td>0.43 ± 0.15b</td>
<td>0.95 ± 0.20a</td>
<td>1.26 ± 0.36a</td>
</tr>
<tr>
<td>eIF4E on Ser221</td>
<td>4.40 ± 1.28b</td>
<td>1.63 ± 0.52b</td>
<td>1.71 ± 0.48b</td>
</tr>
<tr>
<td>eEF2 on Thr56</td>
<td>4.47 ± 0.31</td>
<td>4.34 ± 0.32</td>
<td>3.98 ± 0.48</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM; n = 6. Means in a row with superscripts without a common letter differ, P < 0.05.
2 Leu + AA, Leucine and amino acids.

### TABLE 3
Association of mTOR and eIF4E with various proteins in skeletal muscle of neonatal pigs after 24 h of infusion with saline or leucine, with and without replacement amino acids

| Protein | Control | Leucine | Leu + AA
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>mTOR - rictor</td>
<td>2.31 ± 0.58</td>
<td>2.65 ± 0.50</td>
<td>2.46 ± 0.50</td>
</tr>
<tr>
<td>mTOR - raptor</td>
<td>3.83 ± 0.89</td>
<td>3.92 ± 1.20</td>
<td>3.34 ± 0.48</td>
</tr>
<tr>
<td>mTOR - GBL</td>
<td>2.89 ± 1.66</td>
<td>2.59 ± 1.39</td>
<td>2.64 ± 1.94</td>
</tr>
<tr>
<td>eIF4E - eIF4G</td>
<td>0.54 ± 0.16b</td>
<td>3.83 ± 1.22a</td>
<td>4.48 ± 1.01a</td>
</tr>
<tr>
<td>eIF4E - 4E-BP1</td>
<td>1.80 ± 0.20a</td>
<td>0.77 ± 0.17b</td>
<td>0.77 ± 0.16b</td>
</tr>
</tbody>
</table>

1 Values are mean ± SEM; n = 6. Means in a row with superscripts without a common letter differ, P < 0.05.
2 Leu + AA, Leucine and amino acids.
mTORC1 activation alone is not sufficient to stimulate protein synthesis.

This study also suggests that in muscle, long-term leucine treatment promotes initiator met-tRNA, binding to the start codon, as indicated by a reduction in eIF2α phosphorylation. This effect appears to be time dependent, because our early study showed that eIF2α phosphorylation and eIF2B activation in muscle are not altered acutely by provisions of a meal (46). Although hypoaminoacidemia alone reduced the activity of eIF2B and protein synthesis in muscle of mature swine (12), in the current study, leucine infusion even in the presence of hypoaminoacidemia likely increased the phosphorylation of eIF2B, as indicated by the reduced eIF2α phosphorylation.

Rates of protein synthesis can be altered by changes in translation elongation as well as translation initiation (16). In the current study, leucine did not alter the phosphorylation of eIF2α, suggesting the increase in protein synthesis in leucine-treated piglets is not a consequence of increased translation elongation.

In summary, the results of the current and previous studies (10,11,15) suggest that prolonged parenteral infusion of leucine induces sustained upregulation of the mTOR signaling pathway that regulates mRNA binding to the ribosome and eIF2 activity that regulates met-tRNA, binding to the ribosome in skeletal muscle of the neonate. These effects are independent of circulating levels of other amino acids, emphasizing the unique role of leucine in the stimulation of nutrient signaling. The leucine-induced stimulation of neonatal muscle protein synthesis, however, cannot be sustained beyond 1 h unless the leucine-induced hypoaminoacidemia is prevented. Thus, the leucine-mediated stimulation of protein synthesis is dependent on the availability of amino acids as substrates for protein synthesis. Importantly, the results suggest that changes in the molecular markers for translation, including those in the mTORC1 signaling pathway, do not always reflect the changes in protein synthesis. Nonetheless, the results are suggestive that leucine supplementation may be a potentially useful adjunct to the nutritional management of infants whose growth has been compromised, although further study is warranted.

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