Leucine pulses enhance skeletal muscle protein synthesis during continuous feeding in neonatal pigs

Claire Boutry,1 Samer W. El-Kadi,1 Agus Suryawan,1 Scott M. Wheatley,1 Renán A. Orellana,1 Scot R. Kimball,2 Hanh V. Nguyen,1 and Teresa A. Davis1

1United States Department of Agriculture/Agricultural Research Service, Children’s Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston Texas; and 2Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania

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Boutry C, El-Kadi SW, Suryawan A, Wheatley SM, Orellana RA, Kimball SR, Nguyen HV, Davis TA. Leucine pulses enhance skeletal muscle protein synthesis during continuous feeding in neonatal pigs. Am J Physiol Endocrinol Metab 305: E620–E631, 2013. First published July 9, 2013; doi:10.1152/ajpendo.00135.2013.—In infants unable to maintain oral feeding can be nourished by orogastric tube feeding, using either continuous or intermittent bolus feeding. Continuous feeding is still indicated for some patients who are unable to feed normally (20). Although intermittent bolus feeding has been advocated because it mimics the hormonal profile of meal feeding, continuous feeding is still indicated for some patients due to feeding intolerance (20, 58). Recently, we demonstrated, using neonatal pigs as a model for the human neonate, that continuous feeding restricts muscle protein synthesis compared with intermittent bolus feeding in neonatal pigs. To determine whether leucine infusion can be used to enhance protein synthesis during continuous feeding, neonatal piglets received the same amount of formula enterally by orogastric tube for 25.25 h continuously (CON) with or without LEU or intermittently by bolus every 4 h (BOL). For the CON+LEU group, leucine pulses were administered parenterally (800 μmol·kg⁻¹·h⁻¹) every 4 h. Insulin and glucose concentrations increased after the BOL meal and were unchanged in groups fed continuously. LEU infusion during CON feeding increased plasma leucine after the leucine pulse and decreased essential amino acids compared with CON feeding. Protein synthesis in longissimus dorsi (LD), gastrocnemius, and soleus muscles, but not liver or heart, were greater in CON+LEU and BOL than in the CON group. Bolus feeding increased protein synthesis in the small intestine. Muscle S6K1 and 4E-BP1 phosphorylation and active eIF4E·eIF4G complex formation were higher in CON+LEU and BOL than in CON but AMPKα, AMPKγ, and eIF2 phosphorylation were unchanged. LC3-II-to-total LC3 ratio was lower in CON+LEU and BOL than in CON, but there were no differences in atrogen-1 and MuRF-1 abundance and FoxO3 phosphorylation. In conclusion, administration of leucine pulses during continuous orogastric feeding in neonates increases muscle protein synthesis by stimulating translation initiation and may reduce protein degradation via the autophagy-lysosome, but not the ubiquitin-proteasome pathway.

Thus, new strategies are needed to optimize the nutritional management of continuously fed neonates.

Feeding increases protein synthesis in all tissues of the neonate but particularly in skeletal muscle (13, 14). This effect is largely due to the rise in insulin and amino acid (AA) concentrations after feeding (15), and the response is dose dependent (56). The postprandial rise in insulin activates the phosphoinositide 3-kinase-protein kinase B (PKB)-mammalian target of rapamycin (mTOR) complex, whereas AA directly activate the protein kinase mTOR (17). Activation of mTOR increases the phosphorylation of S6 kinase-1 (S6K1), which enhances translation initiation (57). mTOR can also phosphorylate eukaryotic initiation factor 4E (eIF4E)-binding protein-1 (4E-BP1), which is a repressor protein that binds to eIF4E and blocks eIF4E·eIF4G complex formation. When 4E-BP1 is phosphorylated, eIF4E is released, which can then bind to eIF4G, forming a complex that upregulates mRNA binding to the 40S ribosomal subunit, thereby increasing translation initiation (65). Our recent studies in neonatal pigs suggest that intermittent bolus feeding enhances muscle protein synthesis by inducing a pulsatile pattern of AA- and insulin-stimulated translation initiation that is blunted by continuous feeding (32).

Several proteolytic systems regulate protein degradation; the ubiquitin-proteasome and autophagy-lysosome systems are thought to be the two most important (42, 63, 74). Insulin-stimulated PKB activity induces phosphorylation of the forkhead transcription factor (FoxO) family (42). FoxO1 and FoxO3 upregulate two muscle-specific ubiquitin ligases, muscle atrophy F-box (MAFbx; atrogin-1) and muscle-specific RING finger-1 (MuRF-1), which are proposed to be central to the control of muscle proteolysis (6). Autophagy-lysosomal protein degradation requires activation of the ubiquitin-like molecule microtubule-associated protein 1-light-chain 3 (LC3-II), followed by the formation of autophagosomes that fuse with lysosomes to form the mature autolysosomes in the final, rate-limiting step leading to autophagy. Although the ubiquitin-proteasome system appears largely insensitive to feeding frequency, we recently showed that LC3-II in muscle was downregulated in intermittent bolus-fed compared with continuous-fed or fasted neonatal pigs (26).

Leucine, a member of the branched-chain AA, is an essential AA that cannot be synthesized de novo by humans and other mammals (69). The principal function of AA is as substrates for protein synthesis; however, leucine is also considered a “signaling” AA that enhances protein synthesis in muscle (1, 11) and other tissues (47). Leucine has been shown to promote muscle anabolism in elderly humans (12, 40, 62) and during exercise (55, 59). Nevertheless, there are only a few studies...
assessing the effect of leucine during neonatal development, a period characterized by rapid growth, efficient use of dietary AA for protein deposition, and high rates of protein synthesis (16). Previously, our laboratory demonstrated that an acute parenteral infusion of leucine to raise circulating concentrations of leucine to fed levels increases protein synthesis in skeletal muscle of neonatal pigs (28). This effect is unique to leucine, as other branched-chain AA do not have this effect (29). However, the response to leucine is not sustained, likely due to a leucine-induced decrease in the circulating levels of other AA, a phenomenon which has been previously observed for essential AA (35, 73) and the branched-chain AA (36).

Recently, we demonstrated that the leucine-induced stimulation of protein synthesis can be sustained up to 24 h if the leucine-induced reduction in circulating AA is prevented (27, 75, 76). Furthermore, enteral leucine supplementation also increases skeletal muscle protein synthesis in neonatal piglets, and this effect is associated with an increase in the activation of mTOR and its downstream targets (52).

To determine whether leucine can be used as a nutritional supplement to promote protein anabolism in neonates who are continuously fed, the effect of leucine pulses delivered parenterally during 25.25 h of continuous orogastric feeding on protein synthesis was examined in neonatal pigs. We further sought to identify the intracellular signaling mechanisms involved. We hypothesized that administration of leucine pulses during continuous orogastric feeding would enhance skeletal muscle protein synthesis in neonatal piglets to the rate achieved by intermittent bolus feeding by stimulating translation initiation and suppressing protein degradation pathways.

**MATERIALS AND METHODS**

**Animals and surgery.** Experiments were carried out in accordance with the Animal Care and Use Committee of Baylor College of Medicine and were conducted in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals. Twenty 2-day-old piglets (2.0 ± 0.2 kg; Agricultural Headquarters of the Texas Dept. of Criminal Justice) were weaned from sows and housed in individual stainless steel cages in an environmentally controlled room. Piglets were fed a milk replacement formula ad libitum (Soweean Litter Life; Merrick’s, Middleton, WI) for 6 days and weighed every 2 days during the experiment. Three days before infusion, piglets (2.6 ± 0.3 kg) underwent surgery to have catheters placed in their left jugular vein and carotid artery. Surgical procedures were performed using sterile techniques under general anesthesia as described previously (13).

**Infusion and experimental protocol.** On the day of infusion, piglets (9 days old; 3.4 ± 0.4 kg) were placed in a sling restraint system and fitted with an orogastric tube. They were enterally fed by orogastric infusion a sow milk replacer formulated to meet NRC (1998) requirements of neonatal pigs at equal amounts (240 ml·kg body wt⁻¹·day⁻¹) for 25.25 h (Table 1). Piglets were randomly assigned to one of three groups (n = 6–7 per group): 1) continuous feeding (CON), 2) continuous feeding with leucine pulses (CON+LEU), and 3) bolus feeding (BOL). Continuous feeding (CON and CON+LEU groups) was provided at 10 ml·kg body wt⁻¹·h⁻¹ and the BOL feeding at 40 ml·kg body wt⁻¹ per bolus for 15 min every 4 h using a syringe pump. The enteral formula provided an equal and fixed amount of leucine (1.21 g·kg body wt⁻¹·day⁻¹). Pigs in the CON+LEU group also received a parenteral infusion of leucine in the jugular vein (0.63 g·kg body wt⁻¹·day⁻¹) for 1 h every 4 h.

Piglets were infused for 25.25 h in the CON and CON+LEU groups. BOL feeding and the infusion of LEU were performed every 4 h (at 0, 4, 8, 12, 16, 20 and 24 h). After 20 h of infusion, blood samples were collected from the carotid artery in heparinized tubes every 15 min for 2 h and every 1 h until 24 h of infusion to document hormone and substrate profiles over the course of the 4-h meal feeding period. Plasma was frozen at −20°C for subsequent analyses. Previously, we showed that the postprandial rise in protein synthesis is sustained from at least 0.5 to 2 h after a meal (32). We also showed that a twofold rise in plasma leucine levels is sufficient to stimulate protein synthesis (27), and pilot studies suggested that this could be achieved by pulsing with leucine during continuous feeding. Therefore, we opted to measure tissue protein synthesis rates (31) from 24.75 to 25.25 h by injecting piglets at 24.75 h with a flooding dose of [³H]-phenylalanine (1.5 mmol/kg body wt, 0.5 mCi/kg; American Radiolabeled Chemicals, St. Louis, MO) 30 min before organ collection (15). Plasma samples were collected 5, 15, and 30 min after [³H]-phenylalanine infusion and frozen for analysis. Piglets were euthanized at 25.25 h (i.e., 30 min after [³H]-phenylalanine infusion) with an injection of pentobarbital sodium (0.4 ml/kg body wt). Tissue samples were obtained from the longissimus dorsi (LD), gastrocnemius, and soleus muscles, heart, jejunum, and liver. They were promptly removed, rinsed, immediately frozen in liquid nitrogen, and stored at −70°C until analysis.

**Table 1. Composition of the experimental diet for a 4-h feeding period**

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>CON+LEU</th>
<th>BOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pork muscle, g·kg body wt⁻¹·4 h⁻¹</td>
<td>1.93</td>
<td>1.93</td>
<td>1.93</td>
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<tr>
<td>Lactose, g·kg body wt⁻¹·4 h⁻¹</td>
<td>0.36</td>
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<tr>
<td>Fat pack 80, g·kg body wt⁻¹·4 h⁻¹</td>
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<td>2.67</td>
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<tr>
<td>Minerals, g·kg body wt⁻¹·4 h⁻¹</td>
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<td>0.36</td>
<td>0.36</td>
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<tr>
<td>Vitamins, g·kg body wt⁻¹·4 h⁻¹</td>
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<td>0.08</td>
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</tr>
<tr>
<td>Xanthan gum, g·kg body wt⁻¹·4 h⁻¹</td>
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<tr>
<td>Water, g·kg body wt⁻¹·4 h⁻¹</td>
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<td>34.57</td>
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<tr>
<td>Total protein, g·kg body wt⁻¹·4 h⁻¹</td>
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<td>1.67</td>
<td>1.67</td>
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<tr>
<td>Total carbohydrate, g·kg body wt⁻¹·4 h⁻¹</td>
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<td>0.82</td>
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<td>Metabolizable energy, kcal·kg body wt⁻¹·4 h⁻¹</td>
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<td>Leucine, mg·kg body wt⁻¹·4 h⁻¹</td>
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<td>Valine, mg·kg body wt⁻¹·4 h⁻¹</td>
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<td>Lysine, mg·kg body wt⁻¹·4 h⁻¹</td>
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<td>Methionine, mg·kg body wt⁻¹·4 h⁻¹</td>
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<td>Cysteine, mg·kg body wt⁻¹·4 h⁻¹</td>
<td>45.6</td>
<td>45.6</td>
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<tr>
<td>Phenylalanine, mg·kg body wt⁻¹·4 h⁻¹</td>
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<tr>
<td>Threonine, mg·kg body wt⁻¹·4 h⁻¹</td>
<td>117.9</td>
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<tr>
<td>Tryptophan, mg·kg body wt⁻¹·4 h⁻¹</td>
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<tr>
<td>Leucine infused, mg·kg body wt⁻¹·4 h⁻¹</td>
<td>104.9</td>
<td></td>
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CON, continuously fed; CON+LEU, continuously fed and pulsed with leucine; BOL, bolus fed. *Nutribol, Middlesex, NJ. †Milk Specialties Global Animal Nutrition, Carpentersville, IL. §Dyets, Bethlehem, PA.
high-performance liquid chromatography using an anion exchange column (PA1 column; Dionex, Sunnyvale, CA) after sample preparation, as previously described (16). Fractions were collected, and the radioactivity associated with the phenylalanine peak was measured using Ultima gold (PerkinElmer, Waltham, MA) in a liquid scintillation counter (Tri-Carb 2500TR; Packard Instrument, Meriden, CT). Tissue fractional rate of protein synthesis (K_S, percentage of protein mass synthesized in a day) was calculated as $K_S = \frac{[\text{SA}_{\text{bound phe}}/\text{SA}_{\text{free phe}}] \times 1,440}{t} \times 100$, where $\text{SA}_{\text{bound phe}}$ and $\text{SA}_{\text{free phe}}$ (in dpm/nmol) are the specific radioactivity of the protein-bound and the tissue free phenylalanine, respectively, $t$ is the time of labeling in minutes and 1,440 is the minutes-to-day conversion. Protein content was determined by Pierce BCA assay (45) and total RNA by the method of Munro and Fleck (51) for the determination of both protein synthetic capacity ($C_S$) and efficiency ($K_{RNA}$). $C_S$ (in g RNA/mg protein) was estimated as RNA-to-protein ratio, because the majority of RNA in tissues is ribosomal. $K_{RNA}$ (in g protein·day$^{-1}$·g RNA$^{-1}$) is the total protein synthesized in a day per total RNA: $K_{RNA} = C_S/K_S$.

Protein immunoblot analysis. Proteins from LD muscle homogenates were subjected to Western blot analysis as previously described (70). Immunoblotting was performed using the following primary antibodies: S6K1 (Thr389, Millipore, Bedford, MA), 4E-BP1 (Thr46, Invitrogen, Carlsbad, CA), AMP-activated protein kinase-α (AMPKα; Thr78, Cell Signaling Technology, Danvers, MA), eIF2α (Ser51, Cell Signaling Technology), eukaryotic elongation factor 2 (eEF2; Thr56, Cell Signaling Technology), LC3 (Cell Signaling Technology), MuRF1 (R&D Systems, Minneapolis, MN), MAFbx (atrogin-1; ECM Biosciences, Versailles, KY), FoxO3 (Ser256, Cell Signaling Technology), and β-actin (Cell Signaling Technology). Blots were developed as previously described (26) and corrected with β-actin.

Quantification of eIF4E·eIF4G complex. The complex was immunoprecipitated as previously described (26). Amounts of eIF4E5G were corrected for the amount of eIF4E (Cell Signaling Technology) recovered in the immunoprecipitate.

Statistics. Data are expressed as means ± SE. The effects of the treatments (Ti) were analyzed using one-way ANOVA (SAS 9.1; SAS Institute, Cary, NC) for fractional protein synthesis rates and Western blots. Differences between groups for all other results were analyzed using mixed models for repeated-measure analysis, with the treatment and the time as independent, fixed factors and piglets as a random factor (version 9.1, SAS Institute). For each variable, the most appropriate matrix of covariance structures for random statements was selected based on Sawa’s Bayesian Information Criteria (BIC). The model with the smallest BIC among all competing models was deemed the best model. Post hoc tests were performed by using contrast analysis. A probability value of 0.05 was considered statistically significant.

RESULTS

Plasma glucose, insulin, and glucagon concentrations. Circulating hormone and substrate concentrations were determined over the last 4-h feeding cycle. BOL feeding resulted in sharp and transient increases in plasma glucose (Fig. 1A) and insulin (Fig. 1B) concentrations compared with CON and CON+LEU groups ($P < 0.05$). These concentrations remained elevated between 20.5 and 21 h of infusion for glucose and 20.25 and 21.5 h for insulin. In the CON+LEU group, there were no significant differences among time points. Plasma insulin concentration was greater ($P < 0.05$) for the CON+LEU compared with the CON group at 20 h only (Fig. 1B). There were no significant differences between the CON and CON+LEU groups for glucose concentrations (Fig. 1A). No significant differences between groups were observed for glucagon concentrations (Fig. 1C).

Fig. 1. Plasma glucose (A), insulin (B), and glucagon (C) concentrations in piglets continuously fed (CON), continuously fed and pulsed with leucine (CON+LEU), or bolus fed (BOL). Values are means ± SE ($n = 6$–7). Time is given as hours from initiation of infusion. Statistical effects (interaction of treatment by time, Ti × T) from a mixed model for repeated measures over time are reported for each variable when $P < 0.05$.
Plasma AA concentrations. BOL feeding increased essential (Fig. 2A) and nonessential (Fig. 2B) AA concentrations compared with CON feeding ($P < 0.05$). The responses were rapid and started 15 min and 30 min after the bolus meal for essential and nonessential AA, respectively. Plasma leucine concentrations (Fig. 3A) were significantly higher from 20.25 to 22 h of infusion in the CON+LEU group compared with the CON group. Leucine concentrations were also increased after BOL feeding ($P < 0.05$). A significant decrease in plasma isoleucine and valine concentrations occurred at 21.25 h after leucine infusion. The branched-chain AA concentrations increased for the BOL and CON+LEU groups but remained stable for the CON group ($P < 0.05$). There were significant increases in plasma phenylalanine (Fig. 4A), tryptophan (Fig. 4B), histidine (Fig. 4C), lysine (Fig. 4D), methionine (Fig. 4E), and threonine (Fig. 4F) concentrations after BOL feeding. The concentrations of these AA were constant in the CON-fed group. LEU infusion during CON feeding decreased plasma phenylalanine (Fig. 4A), tryptophan (Fig. 4B), and histidine (Fig. 4C) concentrations ($P < 0.05$). Plasma lysine (Fig. 4D), methionine

Fig. 2. Plasma essential (A) and nonessential (B) amino acid (AA) concentrations in piglets continuously fed (CON), continuously fed and pulsed with leucine (CON+LEU), or bolus fed (BOL). Values are means ± SE ($n = 6–7$). Time is given as hours from initiation of infusion. Statistical effects (interaction of $Tt \times T$) from a mixed model for repeated measures over time are reported for each variable when $P < 0.05$.

Fig. 3. Plasma leucine (A), isoleucine (B), valine (C), and branched-chain AA (D) concentrations in piglets continuously fed (CON), continuously fed and pulsed with leucine (CON+LEU), or bolus fed (BOL). Values are means ± SE ($n = 6–7$). Time is given as hours from initiation of infusion. Statistical effects (interaction of $Tt \times T$) from a mixed model for repeated measures over time are reported for each variable when $P < 0.05$. 

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Protein synthesis rates in tissues. Fractional protein synthesis rates, measured at 25.25 h (1.25 h after the last BOL meal at 24 h), were increased in the LD (Fig. 5A), gastrocnemius (Fig. 5B), and soleus (Fig. 5C) muscles (P < 0.05) and the small intestine (Fig. 5E) after BOL feeding compared with CON feeding (P < 0.05). The fractional protein synthesis rates in the heart (Fig. 5D) and liver (Fig. 5F) were unaffected by BOL feeding. The infusion of LEU during CON feeding increased protein synthesis rates in the LD, gastrocnemius, and soleus muscles (P < 0.05) but had no effect in the heart, small intestine, and liver. Protein synthesis rates in the LD, soleus, and intestine were greater in the BOL than in the CON+LEU group (P < 0.05).

Measurement of protein synthetic capacity (Cs) showed no effect of treatment (Table 2). However, both infusion of LEU during CON feeding and BOL feeding increased (P < 0.05) the efficiency with which the ribosomes translated mRNA into protein (KRNA) in the LD, gastrocnemius, and soleus muscles compared with CON feeding (Table 2). In the heart and small intestine, KRNA was significantly increased in the BOL feeding group only.

Fig. 4. Plasma phenylalanine (A), tryptophan (B), histidine (C), lysine (D), methionine (E), and threonine (F) concentrations in piglets continuously fed (CON), continuously fed and pulsed with leucine (CON+LEU), and bolus fed (BOL). Values are means ± SE (n = 6–7). Time is given as hours from initiation of infusion. Statistical effects (interaction of Tt × T) from a mixed model for repeated measures over time are reported for each variable when P < 0.05.
Translation initiation and degradation signaling. In the LD muscle, S6K1 (Fig. 6A) and 4E-BP1 (Fig. 6B) phosphorylation and active eukaryotic initiation factor (eIF) eIF4E-eIF4G complex formation (Fig. 6C), measured at 25.25 h (1.25 h after the last BOL meal), were significantly higher after BOL and CON+/LEU feedings compared with CON feeding. There were no significant differences between groups in the phosphorylation of AMPK (Fig. 7A), eIF2 (Fig. 7B), and eEF2 (Fig. 7C). FoxO3 phosphorylation (Fig. 8A) and atrogin-1 (Fig. 8B) and MuRF-1 (Fig. 8C) abundances were unaffected by treatment. However, the LC3-II-to-total LC3 ratio (Fig. 8D) was lower in the CON+/LEU and BOL groups than in the CON group (P < 0.05).

DISCUSSION
Recent studies from our laboratory using the piglet as a model for the human neonate showed that continuous feeding, compared with intermittent bolus feeding, by orogastric tube blunts protein synthesis in skeletal muscle (26, 32). Since continuous tube feeding is frequently used in infants with
intolerance to gastric bolus meals (20), we wished to identify a strategy to improve protein synthesis in neonates who are continuously fed. Since our previous studies suggested that the pulsatile rise in AA and insulin that occurs with bolus feeding elicits a rapid increase in protein synthesis (15) and that acute physiological elevations in leucine can also stimulate protein synthesis (28, 75), we hypothesized that leucine could be used as an agent to enhance protein synthesis during continuous feeding. Our results revealed that parenteral leucine pulses during continuous feeding increased protein synthesis rates in skeletal muscles of the neonate. These effects were associated with stimulation of mTOR complex 1 (mTORC1) signaling to proteins that regulate translation initiation and reduced autophagy-lysosome signaling.

In the present study, intermittent parenteral LEU pulses every 4 h for 25.25 h of CON feeding were associated with a 24% increase in skeletal muscle protein synthesis compared with CON feeding alone. However, this result was intermediate compared with the 56% increase in protein synthesis observed after BOL feeding and is consistent with our previously demonstrated stimulatory effect of BOL compared with CON feeding on muscle protein synthesis (26, 32). This greater stimulation of protein synthesis in the BOL compared with the CON+LEU group, despite the lower circulating concentration of leucine, could be explained by the higher level of plasma insulin after BOL feeding than in the CON+LEU group, as we have previously shown that neonatal muscle protein synthesis is more responsive to insulin than to leucine alone (56, 75). The stimulatory effect of leucine pulses on muscle protein synthesis in the CON+LEU group without any rise in circulating insulin suggests a noninsulin-dependent mechanism.

In the current study, the leucine-induced increase in muscle protein synthesis in the CON+LEU fed group compared with the CON group was accompanied by a reduction in circulating levels of the other branched-chain AA, isoleucine and valine. This phenomenon, called the "leucine paradox," occurs partly due to increased branched-chain AA oxidation through activation of the branched-chain-α-keto acid dehydrogenase complex
resulting in depletion of the keto acids from valine and isoleucine (64). Indeed, we have previously found that the enteral leucine-induced stimulation of muscle protein synthesis is blunted after 24 h and was associated with a fall in the circulating levels of the other branched-chain AA, which may become rate limiting for protein synthesis (71). Similarly, we have previously demonstrated that leucine administered parenterally acutely stimulates muscle protein synthesis but that this response is not maintained and is associated with a fall in circulating levels of essential AA (28). However, the leucine-induced stimulation of protein synthesis is sustained when circulating AA levels are maintained by infusion (75). In the current study, the circulating concentrations of most of the essential AA were also modestly reduced in response to leucine pulses, likely due to their utilization for protein synthesis (27, 28). Furthermore, the AA provided by the formula in the continuously fed piglets may have dampened the leucine-induced fall in the essential AA, which would have limited protein synthesis.

Leucine has been demonstrated to stimulate protein synthesis in skeletal muscle (69), but less is known about its effect in peripheral and visceral tissues. In rats, leucine supplementation increases protein synthesis in adipose tissue and liver (47). Studies in neonatal (52, 71) and weanling pigs (78) suggest that enteral leucine supplementation of a low-protein diet may increase protein synthesis in vital organs including the heart, kidney, liver, jejunum, pancreas, spleen, and stomach. However, parenteral infusion of leucine for 24 h had no effect in visceral tissue unless the leucine was infused with other essential AA (76). In the current study, leucine pulses during continuous feeding had no effect on protein synthesis in visceral tissues. It is possible that postprandial or pulsatile circulating AA concentrations are required when parenteral leucine is used to increase protein synthesis in visceral tissue.

Acute alterations in protein synthesis are driven primarily by changes in the activity of components of the translational machinery, whereas chronic alterations (i.e., hours to days) are commonly accompanied by changes in the level of ribosomes (14). In the current study, the leucine-induced increase in protein synthesis in all muscle types was driven by an increase in translational efficiency ($K_{\text{RNA}}$). Consistent with the lack of effect of leucine on protein synthesis in the heart, small intestine, and liver, leucine also had no effect on $K_{\text{RNA}}$ in these tissues. Although pigs were infused for a 25.25-h period, the leucine was delivered in pulses; thus, it is not surprising that the protein synthetic capacity ($C_S$) in all tissues, an estimate of the ribosome number (37), was not affected by leucine.

Although the exact mechanism by which leucine regulates muscle protein synthesis is not fully known, it is believed to occur primarily through the mTORC1-dependent activation of mRNA translation (1, 28, 67). The mechanism implicated is a cascade of intracellular signaling involving initiation and elongation factors that can be regulated by energy status (60), growth factors (insulin and IGF-I), and nutrient availability (18). Because in the current study the leucine-induced increase in protein synthesis was specific to skeletal muscle, did not differ between muscles of different fiber types, and was associated with an upregulation in translational efficiency, we examined the activation of signaling components downstream of mTORC1. We found that the pulsatile parenteral LEU infusion during 25.25 h of CON feeding increased translation

![Fig. 7. Phosphorylation of AMPKα (A), eIF2α (B), and eEF2 (C) in LD muscle of piglets continuously fed (CON), continuously fed and pulsed with leucine (CON+LEU), or bolus fed (BOL). Values are means ± SE (n = 6–7).](image-url)
initiation in skeletal muscle by stimulating S6K1 and 4E-BP1 phosphorylation and eIF4E·eIF4G active complex formation. These results are consistent with previous findings in piglets administered leucine parenterally and enterally (28, 30, 52, 68, 71, 75, 76). The activated or phosphorylated form of S6K1 is one of the key components of the protein synthetic machinery that is positively associated with an increased rate of translation initiation (25). Similarly important is the mTOR-induced phosphorylation 4E-BP1 at Thr37/46, Ser65, and Thr70, which liberates eIF4E and allows it to engage with eIF4G, promoting the eIF4F assembly, which is crucial for mRNA translation (33).

Cells have crucial mechanisms to cope with stressful insults, such as starvation, by modulating eIF2 status, leading to inhibition of translation initiation (21). As a result, the phosphorylation of eIF2α (Ser51) causes inhibition of protein synthesis. In the current study, LEU infusion during CON feeding had no effect on eIF2α phosphorylation, as we have previously shown during short-term enteral leucine supplementation (68, 71). However, eIF2α phosphorylation was reduced following 24 h of parenteral leucine infusion (75); thus, differences between this study and that of Wilson et al. (75) may involve the different modes of leucine administration, i.e., delivery of parenteral leucine by intermittent pulse vs. continuously. Phosphorylation of eIF2α has been reported to increase following AA or leucine starvation in cell and animal models (2, 19) but to be unaltered in response to leucine deprivation (3, 72) and following essential AA ingestion in human skeletal muscle (23). Thus, although the eIF2 pathway is crucial for translation initiation, this pathway may not be rate limiting for leucine-induced protein synthesis.

Protein synthesis consumes a high proportion of cellular energy, and the majority of the cellular energy is used for the polypeptide elongation process (41). This process is partly regulated by the eEF2 pathway (8). Phosphorylation of eEF2 has been reported to be increased during leucine deprivation in myoblast and myotubes (72) and reduced with leucine administration in cell culture (39, 61) in rats (77) and in humans (22, 34). In the current study, eEF2 phosphorylation was not affected by CON/LEU feeding compared with the CON and BOL groups. Indeed, we have previously shown no effect of leucine supplementation on eEF2 phosphorylation in neonatal pigs (68, 71, 75), suggesting that the elongation process is not a rate-limiting step in the leucine-induced stimulation of protein synthesis in neonatal muscle.

AMPK, an intracellular energy sensor, can inhibit mTOR via activation of the tuberous sclerosis complex (TSC)1-TSC2 (65). In vitro studies using C2C12 cells (24) and myocytes (39) and a recent study in rats (77) suggest that leucine decreases AMPK activation, at least in part, by decreasing the AMP-to-ATP ratio. In this study, pulsatile leucine infusion during continuous feeding had no effect on AMPKα phosphorylation, consistent with our previous studies which showed no effect of

Fig. 8. Phosphorylation of FoxO3 (A), abundance of atrogin-1 (B) and MuRF-1 (C), and the ratio of LC3-II to total LC3 (D) in LD muscle of piglets continuously fed (CON), continuously fed and pulsed with leucine (CON+LEU), or bolus fed (BOL). Values are means ± SE (n = 6–7). Statistical effects (Tt) are reported for each variable when P < 0.05 and values with different letters differ significantly (P < 0.05).
parenteral leucine or feeding on AMPK phosphorylation (32, 67, 75). Thus, the lack of change in AMPKα phosphorylation in response to administration of leucine pulses in continuously fed piglets suggests that the cellular AMP/ATP ratio was not altered.

There is limited evidence that leucine is a regulator of protein degradation in muscle (10). Although ingestion of essential AA has been reported to have no effect on protein breakdown (43), other studies have shown a reduction in protein degradation with branched-chain AA or leucine administration (44, 48, 49, 53, 54, 66). It has been reported that branched-chain AA and leucine modulate the ubiquitin-proteasome system by regulating MAFbx/atrogin-1 and MuRF-1 mRNA levels (4, 7, 38, 49) and the autophagy-lysosome system through alterations in the expression of LC3II (66), although evidence to the contrary has also been reported (49). In this study, LEU infusion during CON feeding did not have any effect on the ubiquitin-proteasome system, as indicated by the lack of change in the phosphorylation of FoxO3α and in the abundance of atrogin-1 and MuRF-1. However, the reduction in the ratio of LC3-II to total LC3 suggests downregulation of the autophagy-lysosome system in response to leucine supplementation in neonatal muscle. It has been previously shown that autophagy (LC3-II protein level) was increased by early weaning in piglets (79), suggesting that this system is activated by depletion of nutrients or reduced growth factors (50). In mammalian cells, rapamycin stimulates autophagy, indicating that mTOR activity also functions to suppress this pathway (5, 46).

In conclusion, the results of the current study suggest that parenteral administration of leucine pulses during continuous orogastric feeding increases muscle protein synthesis compared with continuous feeding alone. This leucine-induced increase in protein synthesis appears to be specific for skeletal muscle, and the mechanism implicated is a stimulation of the translation initiation pathway. Furthermore, the results suggest that leucine may reduce protein degradation via the autophagy-lysosome system. Although administration of leucine pulses during continuous feeding increased muscle protein synthesis rates beyond that achieved by continuous feeding alone, the rates reached in most muscles were not as great as those obtained by intermittent bolus feeding. Nonetheless, the results of this study are of direct relevance for infants and children who need continuous tube feeding, as they suggest that leucine pulses improve protein deposition in muscle. The long-term effect of leucine pulses during continuous feeding on growth needs further study.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

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REFERENCES
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