Impact of prolonged leucine supplementation on protein synthesis and lean growth in neonatal pigs

Daniel A. Columbus,* Julia Steinhoff-Wagner,* Agus Suryawan, Hanh V. Nguyen, Adriana Hernandez-Garcia, Marta L. Fiorotto, and Teresa A. Davis

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

Submitted 24 February 2015; accepted in final form 28 July 2015

Columbus DA, Steinhoff-Wagner J, Suryawan A, Nguyen HV, Hernandez-Garcia A, Fiorotto ML, Davis TA. Impact of prolonged leucine supplementation on protein synthesis and lean growth in neonatal pigs. Am J Physiol Endocrinol Metab 309: E601–E610, 2015. First published August 4, 2015; doi:10.1152/ajpendo.00089.2015.—Most low-birth weight infants born at <1500 g are discharged from hospital weighing less than their age-appropriate weight (17). Due to general feeding intolerance and concerns regarding a reduced ability to metabolize excess protein leading to increased risk of hyperammonemia, metabolic acidosis, and necrotizing enterocolitis (1, 26), the advancement in protein intake is often delayed, although enhanced growth can be achieved with an increase in parenteral amino acid infusion (27, 52) or dietary protein intake (10, 29, 30).

POOR GROWTH IN THE NEONATAL PERIOD is associated with an increased risk for developing metabolic syndrome, obesity, short stature, and neuronal deficits later in life (6, 26, 47). Despite improvements in the nutritional management of low-birth weight infants, many are discharged from hospital weighing less than their age-appropriate weight (17). Due to general feeding intolerance and concerns regarding a reduced ability to metabolize excess protein leading to increased risk of hyperammonemia, metabolic acidosis, and necrotizing enterocolitis (1, 26), the advancement in protein intake is often delayed, although enhanced growth can be achieved with an increase in parenteral amino acid infusion (27, 52) or dietary protein intake (10, 29, 30).

Our previous studies in the neonatal piglet, a well-accepted model for the study of nutrient metabolism in human infants (46), demonstrated that the increase in muscle protein synthesis in response to feeding is due to the postprandial rise in amino acids and insulin (16, 45, 57). In particular, leucine, a branched-chain amino acid, has been shown to have unique anabolic properties as a nutrient signal that stimulates protein synthesis (2, 3, 36). Leucine infusion increases muscle protein synthesis (19), and this effect can be extended for at least 24 h when the supply of amino acids is maintained (20).

Short-term leucine supplementation increases muscle protein synthesis in neonatal (50, 53) and weaned pigs (58) as well as human adults (11, 12) fed low-protein meals. Moreover, the effect of leucine on protein synthesis in neonates is similar to feeding a high-protein meal (53). Whether this effect of leucine supplementation can be sustained in the long term to enhance muscle growth and lean gain is not known.

The stimulation of protein synthesis by leucine is mediated through the mammalian target of rapamycin (mTOR) complex 1 signaling pathway. The mTOR signaling cascade through which amino acids stimulate protein synthesis has been well characterized (31). Briefly, activation of mTOR results in phosphorylation and activation of the 70-kDa ribosomal protein S6 kinase 1 (S6K1), which then phosphorylates ribosomal protein S6. mTOR also phosphorylates the eukaryotic initiation factor 4E (eIF4E), which can then form the active eIF4G-eIF4E complex. Phosphorylation of ribosomal protein S6 and the formation of the active eIF4G-eIF4E complex stimulate translation initiation and, therefore, protein synthesis.

The objective of this study was to determine the effect of long-term leucine supplementation on protein synthesis and body composition of neonatal pigs fed a restricted-protein diet. We also sought to identify the intracellular signaling pathways involved. We hypothesized that supplementation of a restricted-protein diet with leucine would enhance lean growth to that of a high-protein diet by upregulating skeletal muscle protein synthesis due to the action of leucine on the mTOR signaling pathway.

MATERIALS AND METHODS

Animals and housing. The experimental protocol was approved by the Institutional Animal Care and Use Committee, Baylor College of Medicine, and was conducted in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals. Neonatal pigs (Yorkshire × Landrace × Duroc × Hampshire) were obtained from a commercial farm (Burton, TX) at 3 days of age and an initial body weight of 1.8 ± 0.3 kg. The pigs were housed...
individually in stainless-steel cages in an environmentally controlled room at 84°F at the Comparative Nutrition Research Facility (Houston, TX) and received a milk replacement formula ad libitum (Soweeena Litter Life; Merrick’s, Middleton, WI) for 3 days. Two days prior to the experimental period, piglets underwent surgery to insert a catheter into the left jugular vein and a feeding tube into the stomach. All surgical procedures were performed using sterile technique under general anesthesia, as described previously (15).

Experimental design. At 5 days of age, piglets were randomly assigned to one of three whey-based milk replacement diets (n = 14–16/diet) formulated to differ in dietary protein and leucine content, 1) high protein (HP), 2) restricted protein (RP), and 3) restricted protein with leucine (RPL), as shown in Table 1. The HP diet was formulated to meet or exceed nutrient requirements of neonatal pigs (44). The RP diet was formulated to contain half the amount of protein present in the HP diet and to be below protein requirements (44). Leucine was added to the RPL diet to match the amount of leucine present in the HP diet. Diets were kept isoenergetic by adjusting the amount of fat. To limit any confounding effect of carbohydrate intake on plasma insulin concentrations, the amount of daily carbohydrate intake was kept constant by adjusting the dietary lactose content. All piglets were fed their respective diet for 21 days via gastric feeding delun over 20 min. Piglets were weighed every 3 days during the study, and the amount of feed was adjusted to account for the change in body weight. Dual-energy X-ray absorptiometry (DEXA) for body composition analysis (Hologic Delphi-Am software version 11.2) was performed on days 0 and 20 of the study. Body composition data were assigned to one of three whey-based milk replacement diets.

Table 1. Ingredient composition of experimental diets and daily nutrient supply for a 21-day feeding study

<table>
<thead>
<tr>
<th>Ingredient, g/kg</th>
<th>RP</th>
<th>RPL</th>
<th>HP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey protein concentrate</td>
<td>45.0</td>
<td>45.0</td>
<td>91.7</td>
</tr>
<tr>
<td>Casein, hydrolyzed</td>
<td>11.2</td>
<td>11.2</td>
<td>22.5</td>
</tr>
<tr>
<td>Corn oil</td>
<td>48.7</td>
<td>48.7</td>
<td>26.3</td>
</tr>
<tr>
<td>Fat Pak 80</td>
<td>10.8</td>
<td>10.8</td>
<td>5.8</td>
</tr>
<tr>
<td>Lactose</td>
<td>19.1</td>
<td>19.1</td>
<td>14.4</td>
</tr>
<tr>
<td>Xanthan gum</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Vitamin premix</td>
<td>2.1</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Mineral premix</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>5.4</td>
<td>5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Water</td>
<td>853</td>
<td>848</td>
<td>827</td>
</tr>
</tbody>
</table>

Daily nutrient supply

<table>
<thead>
<tr>
<th>Item</th>
<th>RP</th>
<th>RPL</th>
<th>HP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein, g·kg body wt−1·day−1</td>
<td>11.2</td>
<td>12.0</td>
<td>22.5</td>
</tr>
<tr>
<td>Total carbohydrate, g·kg body wt−1·day−1</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Total fat, g·kg body wt−1·day−1</td>
<td>14.2</td>
<td>14.2</td>
<td>8.6</td>
</tr>
<tr>
<td>Metabolizable energy, kcal·kg body wt−1·day−1</td>
<td>193</td>
<td>201</td>
<td>193</td>
</tr>
</tbody>
</table>

Amino acids, g·kg body wt−1·day−1

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>RP</th>
<th>RPL</th>
<th>HP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>1.17</td>
<td>2.36</td>
<td>2.36</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.63</td>
<td>0.63</td>
<td>1.27</td>
</tr>
<tr>
<td>Valine</td>
<td>0.61</td>
<td>0.61</td>
<td>1.23</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.91</td>
<td>0.91</td>
<td>1.84</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.24</td>
<td>0.24</td>
<td>0.48</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.39</td>
<td>0.39</td>
<td>0.78</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.60</td>
<td>0.60</td>
<td>1.20</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.17</td>
<td>0.17</td>
<td>0.33</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.28</td>
<td>0.28</td>
<td>0.57</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.24</td>
<td>0.24</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Fig. 1. Plasma insulin (A), glucagon (B), and glucose (C) in piglets fed a high-protein (HP), restricted-protein (RP), or restricted-protein diet supplemented with leucine (RPL) for 21 days. Values are least square means ± SE (n = 14–16). Time (T) is given as minutes from feeding. Statistical effects of T from a mixed model for repeated measurements over T are reported for each variable when P < 0.05.

On day 21, piglets were either fasted or fed prior to tissue collection to determine the effect of feeding on protein synthesis and mTOR activation (n = 7–8/condition). Blood samples were collected into heparinized tubes immediately prior to feeding and at 15, 30, 60, 75, 90, 120, 180, and 240 min postmeal to determine postprandial hor-
mone and substrate profiles. Plasma was collected by centrifugation at 2,000 g for 2 min and stored at −20 °C until further analysis. Tissue samples were obtained from the longissimus dorsi (LD), gastrocnemius, and soleus muscles, liver, heart, and small intestine (jejunum) in the fasting (fasted, 5.25 h postfeeding) condition or 75 min after feeding (fed) to quantify protein synthesis and mTOR signaling in response to a meal. All tissue samples were removed as quickly as possible following euthanasia, rinsed, weighed, and immediately frozen in liquid nitrogen. Tissue samples were stored at −80°C until analysis.

Plasma hormone and substrate assays. Whole blood glucose (Infinity Glucose Oxidase TR-15221; Thermo Scientific, Rockford, IL), plasma insulin (Porcine Insulin Radioimmunoassay No. PI-12K; Linco Research, St. Louis, MO) and plasma glucagon (Glucagon Radioimmunoassay No. GL-32K; Millipore, St. Charles, MO) were determined using commercially available kits. Plasma levels of individual amino acids were determined using the PICO-TAG reverse-phase column HPLC (Waters, Milford, MA) method, as described previously (16).

Tissue protein synthesis. In vivo tissue protein synthesis rates were determined with a flooding dose of L-[4-3H]phenylalanine (1.5 mmol/kg body wt, 0.5 mCi/kg; American Radiolabeled Chemicals, St. Louis, MO) given 30 min prior to tissue collection. Samples of heparinized whole blood were collected 5, 15, and 30 min after tracer infusion and stored at −20°C until further analysis. Fractional rates of protein synthesis (Ks) were calculated as Ks (%/day) = [(Sb/Sa) × (1,440/t)] × 100, where Sb and Sa are the specific radioactivity (disintegrations/min) of the protein-bound and tissue-free phenylalanine pools, respectively, and t is the time of labeling (min) of the specific tissue. Sb is corrected for the linear regression of the blood-specific radioactivity of each pig 5, 15, and 30 min after injection of the tracer (14).

Protein immunoblot analysis. Proteins from LD muscle homogenates were subjected to Western blot analysis, as previously described (18). Immunoblotting was performed using the following primary antibodies: 4E-BP1 (1:4,000, no. A300-501A; Bethyl Laboratories, Montgomery, TX), phospho-4E-BP1 Thr308 (1:1,000, no. 9275; Biosource, Waltham, MA), Akt (1:1,000, no. 9272; Cell Signaling Technology), S6K1 (1:1,000, sc-230; Santa Cruz Biotechnology, Dallas, TX), microtubule-associated protein-1 light chain 3 (LC3; 1:1,000, no. sc-223; Santa Cruz Biotechnology, Dallas, TX), GAPDH (1:4,000, no. ACR001PT; Acris Antibodies, San Diego, CA), atrogin-1; 1:1,000, no. AP2014; ECM Biosciences, Versailles, KY), muscle RING finger 1 (MuRF1; 1:700, no. AF5366; R & D Systems, Minneapolis, MN), S6K1 (1:1,000, no. sc-230; Santa Cruz Biotechnology, Dallas, TX), and phospho-S6K1 Thr389 (1:2,000, no. 9275; Cell Signaling Technology). Membranes were then washed with Tris-buffered saline with 0.1% Tween 20 and incubated in appropriate secondary antibody for 1 h at room temperature before detection with an enhanced chemiluminescence kit (GE Healthcare UK, Buckinghamshire, UK) and analyzed using a ChemiDoc-It Imaging System (UVI, Upland, CA). Phosphorylated forms of the signaling proteins were normalized to the total abundance of the respective proteins. Total abundance of proteins was normalized to GAPDH.

The abundance of the active eIF4G-eIF4E complex was determined as previously described (18). Briefly, the complex was immunoprecipitated using an anti-eIF4E monoclonal antibody (gift of Dr. Leonard Jefferson, College of Medicine, Pennsylvania State University) from aliquots of fresh tissue homogenates. The isolated complex was then analyzed for total eIF4E (1:4,000, no. 9742; Cell Signaling Technology) and total eIF4G (1:1,000, no. 07-1800; Millipore) by protein immunoblotting. The abundance of eIF4G was corrected for the abundance of eIF4E. mTOR signaling phosphorylation and formation of the active eIF4G-eIF4E complex were calculated as the fold change from fasted levels.

Statistical analyses. Data are presented as least square means ± SE. All data were analyzed using the mixed-model procedure (PROC MIXED) of the SAS statistical program (SAS 9.3; SAS Institute, Cary, NC), with diet and block included as fixed effects and pig included as a random effect, where appropriate. A correction for repeated measurements was included for data collected over time. Differences between means were determined using the Tukey test and were considered significantly different at P ≤ 0.05. Main effect and time point comparison P values are reported here in the text, and P values for overall significance of diet, time, or the interaction of diet and time are reported in the figures when significant.

RESULTS

Hormones and substrates. For all diets, there was a rapid and transient increase in plasma insulin concentrations (Fig. 1A) within 15 min of feeding that returned to baseline within 2 h of the meal. There was no effect of diet or feeding on plasma glucagon (P > 0.05; Fig. 1B) or glucose concentration (P > 0.05; Fig. 1C). Plasma concentrations of total indispensable amino acids (Fig. 2A) increased with feeding at 60–75 min with all diets (P < 0.05), regardless of diet. There was no effect of time or diet on total dispensable amino acid concentrations (P > 0.05; Fig. 2B). Supplementation of the RP diet with leucine increased plasma leucine concentrations (Fig. 3A) to a level similar to the HP diet (P < 0.05) from 60 to 180 min postfeeding. Feeding the HP diet increased the concentrations of isoleucine (Fig. 3B) and valine (Fig. 3C) above the levels in RP- and RPL-fed pigs (P < 0.05), and there was no effect of leucine supplementation on either valine or isoleucine concen-
Body weight and body composition. Body weight increased over time among all dietary groups, with HP-fed pigs attaining a higher body weight than RP-fed pigs by day 15 of the study (P < 0.05; Fig. 4). Overall weight gain (Fig. 5A) was highest for the HP-fed pigs compared with pigs fed the RP diet (P < 0.05), with RPL-fed pigs having intermediate body weight gain. Overall lean mass gain was greater in the HP-fed pigs (P < 0.05) compared with the RP diet, with the RPL diet resulting in intermediate values (Fig. 5B). There was no diet effect on fat mass gain (P > 0.05; Fig. 5C).

Tissue weight and tissue protein synthesis. Feeding the HP diet increased the weight of the LD muscle, heart, liver, and kidney (P < 0.05; Table 2) but not gastrocnemius and soleus muscles or small intestine (P > 0.05) compared with RP-fed pigs. The weights of the LD, heart, liver, and kidney in the RPL group were intermediate to values for the RP and HP groups.

Tissue protein synthesis rates (K_S) in the LD, gastrocnemius, and soleus muscles (Fig. 6) increased with feeding for all diets (P < 0.05). In the fed state, K_S for the LD was greater in both HP and RPL diets compared with RP (P < 0.05); however, there was no effect of leucine supplementation on gastrocnemius or soleus K_S (P > 0.05). The K_S of heart was increased only with feeding of the HP diet (P < 0.05; Fig. 7), with no effect of diet or feeding for the kidney or liver (P > 0.05; Fig. 7).

Translation initiation factor and protein degradation signaling. In the LD, feeding increased the phosphorylation of Akt, 4E-BP1, and S6K1 and formation of the active eIF4G·eIF4E complex (P < 0.05; Fig. 8). The addition of leucine to the RP diet further increased phosphorylation of 4E-BP1 and formation of the eIF4G·eIF4E complex in the fed state. There was no significant effect of diet on S6K1 or Akt phosphorylation in the LD (P > 0.05). Neither feeding nor diet affected abundance of the degradation factors MuRF1 or atrogin-1 (P > 0.05; Fig. 9). For all pigs, feeding decreased the ratio of LC3-II to total LC3 (P < 0.05), and the response was similar across all diets (P > 0.05; Fig. 9).

DISCUSSION

Advancement of protein feeding is delayed in many low-birth weight infants due to concerns regarding intolerance to higher protein intake that can increase the risk of developing aminoacidemia, ammonia toxicity, and kidney failure (1, 26). Leucine has been shown to increase muscle protein synthesis in pigs (7, 19), rats (3, 36), and humans (11, 12, 24), although the effect of long-term supplementation with leucine on lean gain is not known. In the current study, we sought to determine
leucine resulted in a slight increase in tissue weight of only the
synthesis, the effect of leucine on lean gain in the current study
and thereby enhance lean tissue gain in the neonatal piglet
increase muscle protein synthesis in an mTOR-dependent man-
whether chronic leucine supplementation of a RP diet could
21 days in piglets fed a HP, RP, or RPL diet for 21 days. Values are least
Fig. 5. Change in body weight, lean body mass, and fat body mass from 0 to
whether chronic leucine supplementation of a RP diet could
increase muscle protein synthesis in an mTOR-dependent man-
and thereby enhance lean tissue gain in the neonatal piglet
LD muscle and visceral tissues (heart, liver, and kidney), and
this increase resulted in minimal changes in overall body
weight and lean tissue mass as determined by DEXA. Our data
demonstrated that leucine supplementation increased protein
synthesis in an mTOR-dependent manner in the LD muscle.
Thus, leucine has the potential to improve muscle protein
synthesis when protein intake is limited; however, this effect
may be restricted to certain tissues, as there was no increase in
response to leucine supplementation beyond the general re-
sponse to feeding in mTOR activation or rates of protein
synthesis in other muscles or visceral tissues sampled. It has
been suggested that the response of skeletal muscle to anabolic
signals such as leucine and insulin is dependent on the major
fiber type present (22) and that the response is more profound
in muscles containing primarily fast-twitch glycolytic fibers
(23). The muscles sampled in the current study differ in the
major muscle fiber type present, with LD being composed
primarily of fast-twitch glycolytic fibers, gastrocnemius being
composed of mixed glycolytic and oxidative fibers, and soleus
composed mainly of slow-twitch oxidative fibers (50). The
differential effect of leucine on muscle protein synthesis and
mTOR signaling observed in the current study may be indic-
itive of this fiber type-specific response.

The RP and RPL diets were formulated to be below the total
protein requirement of 16 g·kg body wt⁻¹·day⁻¹ for pigs (44),
which was confirmed by the reduced growth observed in pigs
fed the RP diet compared with HP-fed pigs. Both Torrazza et
Fig. 5. Change in body weight, lean body mass, and fat body mass from 0 to
21 days in piglets fed a HP, RP, or RPL diet for 21 days. Values are least
square means ± SE (n = 14–16). Values with different letters differ signifi-
antly (P < 0.05).

LD muscle and visceral tissues (heart, liver, and kidney), and
this increase resulted in minimal changes in overall body
weight and lean tissue mass as determined by DEXA. Our data
demonstrated that leucine supplementation increased protein
synthesis in an mTOR-dependent manner in the LD muscle.
Thus, leucine has the potential to improve muscle protein
synthesis when protein intake is limited; however, this effect
may be restricted to certain tissues, as there was no increase in
response to leucine supplementation beyond the general re-
sponse to feeding in mTOR activation or rates of protein
synthesis in other muscles or visceral tissues sampled. It has
been suggested that the response of skeletal muscle to anabolic
signals such as leucine and insulin is dependent on the major
fiber type present (22) and that the response is more profound
in muscles containing primarily fast-twitch glycolytic fibers
(23). The muscles sampled in the current study differ in the
major muscle fiber type present, with LD being composed
primarily of fast-twitch glycolytic fibers, gastrocnemius being
composed of mixed glycolytic and oxidative fibers, and soleus
composed mainly of slow-twitch oxidative fibers (50). The
differential effect of leucine on muscle protein synthesis and
mTOR signaling observed in the current study may be indic-
itive of this fiber type-specific response.

The RP and RPL diets were formulated to be below the total
protein requirement of 16 g·kg body wt⁻¹·day⁻¹ for pigs (44),
which was confirmed by the reduced growth observed in pigs
fed the RP diet compared with HP-fed pigs. Both Torrazza et
et al. (53) and Suryawan et al. (50) reported that short-term
leucine supplementation of a low-protein diet increased muscle
protein synthesis in neonatal pigs. The diets used in those
studies were of an ingredient composition similar to the ones
used here; however, the protein restriction achieved in the
current study, although not as severe as in the previous studies,
was more chronic and may explain why leucine did not have the
same anabolic effect.

It has been suggested that dietary indispensable amino acid
content is a primary factor that determines the variability in
rates of muscle protein synthesis in response to intake of
different protein sources (33, 55). Of the indispensable amino
acids, the leucine content of a protein source in particular is
thought to drive the peak response of muscle protein synthesis
to ingestion of a protein (43, 51, 54). Both whey and casein,
the protein sources used in the diets of the current study, are rich
in indispensable amino acids, and the branched-chain amino
acid content, including leucine, in milk-based protein sources
is particularly high. In a study by Frank et al. (21), a dose
response in protein synthesis to dietary protein in neonatal pigs

<table>
<thead>
<tr>
<th>Muscle</th>
<th>RP</th>
<th>RPL</th>
<th>HP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Longissimus dorsi</td>
<td>53.1 ± 3.3ab</td>
<td>61.4 ± 4.2a</td>
<td>68.1 ± 3.8a</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>15.4 ± 1.2b</td>
<td>13.7 ± 1.4b</td>
<td>16.0 ± 1.3b</td>
</tr>
<tr>
<td>Soleus</td>
<td>3.74 ± 0.27b</td>
<td>3.70 ± 0.23b</td>
<td>4.76 ± 0.29b</td>
</tr>
<tr>
<td>Heart</td>
<td>24.2 ± 1.1b</td>
<td>26.5 ± 1.2b</td>
<td>28.7 ± 1.2b</td>
</tr>
<tr>
<td>Liver</td>
<td>173 ± 7.8b</td>
<td>186 ± 9.2b</td>
<td>213 ± 8.5b</td>
</tr>
<tr>
<td>Kidney</td>
<td>19.8 ± 1.8b</td>
<td>22.9 ± 2.1b</td>
<td>27.2 ± 1.9b</td>
</tr>
<tr>
<td>Small intestine</td>
<td>254 ± 15.3b</td>
<td>275 ± 20.3</td>
<td>392 ± 16.8</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 14–16. Tissue weight is expressed in g.
Labeled means in a row without a common letter differ; P < 0.05.
was demonstrated, but this response was not enhanced further by dietary protein intake above requirements (i.e., when post-prandial plasma branched-chain amino acid concentration increased to between 1,000 and 1,500 μM). Therefore, the excess leucine in the RP diet may have been sufficient to attain the maximum rate of protein synthesis achievable with the dietary protein restriction and thus limit the benefit of additional leucine supplementation.

Previous studies have reported a reduction in other indispensable amino acids with leucine supplementation (50, 56, 62).

Fig. 6. Protein synthesis (KS) in longissimus dorsi (A), gastrocnemius (B), and soleus (C) in piglets fed a HP, RP, or RPL for 21 days. Protein synthesis was determined in either the fasted or fed state. Values are least square means ± SE (n = 5–6). Values with different letters differ significantly (P < 0.05).

Fig. 7. KS in heart (A), kidney (B), and liver (C) in piglets fed a HP, RP, or RPL diet for 21 days. KS was determined in either the fasted or fed state. Values are least square means ± SE (n = 5–6). Values with different letters differ significantly (P < 0.05).
which is most likely due to the utilization of these amino acids to support the increase in muscle protein synthesis. In particular, the plasma concentration of the other branched-chain amino acids isoleucine and valine can show substantial reductions and may become limiting for protein synthesis. It has been hypothesized that excess leucine may increase branched-chain amino acid catabolism through activation of the major branched-chain amino acid-metabolizing enzymes branched-chain aminotransferase and branched-chain α-ketoacid dehydrogenase (56). The absence of a decrease in the plasma concentration of either isoleucine or valine following the addition of leucine in this study was also reported by Torrazza et al. (53) using diets of similar composition. Moreover, in the study by Yin et al. (58), the fall in isoleucine and valine did not appear to limit protein synthesis, as supplementation with leucine still resulted in increased weight gain. This suggests that the fall in branched-chain amino acids may be dependent on diet composition or degree of leucine supplementation and may not always be a limiting factor for protein synthesis. Additionally, Churchward-Venne et al. (12) observed that intracellular levels of the branched-chain amino acids remained constant regardless of a fall in plasma levels. This suggests that intracellular levels of amino acids may be more important than plasma levels for mTOR signaling and protein synthesis, which is supported by recent advances in our understanding of amino acid effects on mTOR (59). The fall in plasma levels of amino acids is likely due to the uptake of amino acids to maintain intracellular amino acid concentrations.

In the neonatal animal, insulin is also a potent anabolic stimulus that acts in an mTOR-dependent manner to regulate protein synthesis. Insulin initiates a signaling cascade independent of the amino acid signaling pathway. The binding of insulin to its receptor activates phosphoinositide 3-kinase and phosphoinositide-dependent kinase 1, which in turn leads to activation of Akt and inactivation of the mTOR repressor tuberous sclerosis complex 1 and 2 (31). In the current study, the rapid postprandial rise in insulin following a meal resulted in an increase in the phosphorylation of Akt, which did not differ across diets. In previous studies, we demonstrated a maximum response in protein synthesis at a plasma insulin concentration of ~10 μU/ml (16, 45). In the current study, the increase in insulin following a meal peaked at 50 μU/ml and remained above the previously determined threshold at the time protein synthesis and mTOR signaling were measured. Therefore, the increase in insulin due to a meal may have masked the effect of leucine on protein synthesis in the majority of muscles. At plasma insulin levels of less than 10 μU/ml, the infusion of amino acids has been shown to further increase the activation of mTOR signaling (45). In the current study, plasma leucine levels were elevated within 30 min of the RPL diet being fed and remained above those in the RP diet for at least 180 min postfeeding. Therefore, it is possible that leucine supplementation may have resulted in higher protein synthesis.

**Fig. 8.** Phosphorylation of Akt (A), 4E-binding protein (4E-BP1; B), active eukaryotic initiation factor (eIF)-4E-eIF4G complex (C), and phosphorylation of ribosomal protein S6 kinase 1 (S6K1; D) in longissimus dorsi muscle of piglets fed a HP, RP, or RPL diet for 21 days. Phosphorylation calculated as fold change from fasted piglets. Values are least square means ± SE (n = 5–6). Values with different letters differ significantly (P < 0.05).
with different letters differ significantly ($P < 0.05$). Values are least square means $\pm$ SE ($n = 5–6$). Values with different letters differ significantly ($P < 0.05$).

Fig. 9. Abundance of atrogin-1 (A), muscle RING finger 1 (MuRF1; B), and light chain 3 (LC3)-II/total LC3 ratio (C) in longissimus dorsi muscle of piglets fed a HP, RP, or RPL diet for 21 days. Abundance calculated as fold change from fasted piglets. Values are least square means $\pm$ SE ($n = 5–6$). Values with different letters differ significantly ($P < 0.05$).

synthesis rates in RPL-fed pigs only during those times that plasma leucine was elevated, but after insulin had returned to near basal levels. The effect of insulin on protein synthesis has also been shown to be tissue specific, with muscle responsive to both insulin and amino acids but visceral tissue responding only to amino acid supply (16). This differential response to anabolic signals across tissues may account for the greater response in visceral tissue weight gain with leucine supplementation than in muscle tissue, in which insulin will have maximized protein synthesis regardless of dietary leucine content.

Previous studies have shown that the anabolic response to feeding rapidly declines with age in the pig (15), rat (5), and human (14, 40). Indeed, in the pig, the muscle protein synthesis response to a meal is reduced by 26 days of age (15). The response to individual anabolic signals such as insulin and amino acids is also reduced in muscle of older animals (13, 16, 49, 57). It is possible that the stimulatory effect of leucine on muscle protein synthesis was greater earlier in the study when the pig exhibited a greater sensitivity to anabolic stimuli and was blunted at the time protein synthesis and mTOR activation were determined at 26 days of age. In addition, the developmental decline in the response of muscle protein synthesis to anabolic signals was more pronounced for skeletal muscle than for visceral tissue (15), which may explain the lack of an effect of leucine on the weight of the majority of muscles sampled, but not the visceral tissues. A diminishing response to leucine with age could explain the small difference in lean gain observed in the current study and suggests that there may be a limited age range during which nutritional therapies may be effective for improving lean gain in neonatal animals and humans.

Lean tissue gain is the net result of the balance between anabolic (protein synthesis) and catabolic (protein degradation) processes within the muscle, with gain in protein mass resulting from a higher rate of protein synthesis than degradation (34). Two of the main degradation pathways in skeletal muscle are the ubiquitin-proteasome and autophagy-lysosome pathways (28). In muscle, the ubiquitin-proteasome system involves the upregulation of two ubiquitin ligases, MuRF1 and atrogin-1, that mark proteins for degradation by the proteasome. The autophagy-lysosome system involves the activation of LC3, with the relative abundance of LC3-II to total LC3 considered an indicator of increased autophagy. It has been shown previously that protein degradation is reduced by branched-chain amino acids (35, 37–39) and leucine (9, 41, 42).

Although protein breakdown rates were not measured in the current study, indicators of the ubiquitin-proteasome pathway or lysosome-autophagy pathway showed no effect of dietary protein content or leucine supplementation. Moreover, feeding had no effect on the abundance of atrogin-1 and MuRF1 but produced a significant decrease in the abundance of LC3-II. These results are similar to those reported by Sugawara et al. (48), where leucine supplementation in rats fed a protein-free diet had no effect on the abundance of atrogin-1 or MuRF1 but resulted in a decrease in the abundance of LC3-II. This effect of leucine on LC3-II was not observed in the current study but may be the result of differences in feeding modality (i.e., meal vs. ad libitum feeding) and dietary protein content (i.e., low protein vs. protein free) between the studies. Indeed, in a study by Boutry et al. (7), a significant decrease in LC3-II was observed when a leucine pulse was administered to continuously fed pigs, a situation that closely resembles ad libitum feeding with respect to plasma nutrient and hormone levels. This provides further evidence for the importance of autophagy during times of amino acid starvation and indicates that autophagy is likely more responsive to changes in overall nutrient availability than...
individual amino acids (28, 39). Thus, leucine may have very little effect on protein degradation through the autophagy-lysosome pathway in the meal-fed pigs, as autophagic degradation may be maximally downregulated due to the effect of feeding alone. There have been a number of studies examining the effect of deletion of MuRF1 and atrogin-1 in mice. These studies observed no effect of the deletion of these genes on early postnatal growth (8) or muscle degradation in response to nutrient deprivation (4); however, muscle atrophy that occurs during many disease states and periods of muscle disuse appears to be related to the upregulation of MuRF1 and atrogin-1. This suggests that MuRF1 and atrogin-1 may not be critical in the regulation of lean tissue gain in healthy neonates.

In conclusion, the results of the current study suggest that long-term supplementation with leucine of a diet with a restricted protein content has limited benefit on the accretion of lean tissue and body weight gain in neonatal pigs that are meal-fed milk protein-based diets. Leucine resulted in minor increases in body weight, although the weight of the longissimus dorsi muscle, heart, liver, and kidney increased. This limited effect of leucine appears to be due to the anabolic effectiveness of postprandial hyperinsulinemia that independently maximized protein synthesis and a lack of restriction in dietary-indispensable amino acid content with the formulation of diets used in this study. Furthermore, the results suggest that leucine supplementation has no effect on protein degradation through either the ubiquitin-proteasome or autophagy-lysosome pathway. Although leucine has the potential to improve lean gain in neonatal animals, this may be dependent on the indispensable amino acid content of the diet, and thus further studies in which both indispensable and dispensable amino acids are restricted are warranted.

ACKNOWLEDGMENTS

We gratefully acknowledge the contributions of D. G. Burrin and C. Boutry to the development of this project, R. D. Almonacri and B. P. Scull for technical assistance, M. Kao and D. P. Ferguson for animal assistance, R. Shypailo and M. Laurent for performing DEXA measurements, and the staff of the Comparative Nutrition Research Facility for animal care.

GRANTS

This work is a publication of the US Department of Agriculture (USDA)/Agricultural Research Service (ARS) Children’s Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine. Financial support for this project was provided by National Institute of Child Health and Human Development Grant HD-072891, USDA National Institute of Food and Agriculture Grant 2013-67015-20438, National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-044474, and USDA/ARS Grant 6250-51000-055. The contents of this publication do not necessarily reflect the views and policies of the USDA, nor does the mention of trade names, commercial products, or organizations imply endorsement by the US Government.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

20. Escobar J, Frank JW, Suryawan A, Nguyen HV, Davis TA. Amino acid availability and age affect the leucine stimulation of protein synthesis


