Dietary protein and lactose increase translation initiation factor activation and tissue protein synthesis in neonatal pigs

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Dietary protein and lactose increase translation initiation factor activation and tissue protein synthesis in neonatal pigs. Am J Physiol Endocrinol Metab 290: E225–E233, 2006. First published September 6, 2005; doi:10.1152/ajpendo.00351.2005.—Protein synthesis and eukaryotic initiation factor (eIF) activation are increased in muscle and liver of pigs parenterally infused with amino acids and insulin. To examine the effects of enteral protein and carbohydrate on protein synthesis, pigs (n = 42, 1.7 kg body wt) were fed isocaloric milk diets containing three levels of protein (5, 15, and 25 g kg body wt⁻¹·day⁻¹) and two levels of lactose (low = 11 and high = 23 g kg body wt⁻¹·day⁻¹) from 1 to 6 days of age. On day 7, pigs were gavage fed after 4-h food deprivation, and tissue protein synthesis rates and biomarkers of mRNA translation were assessed. Piglet growth and protein synthesis rates in muscle and liver increased with dietary protein and plateaued at 15 g kg body wt⁻¹·day⁻¹ (P < 0.001). Growth tended to be greater in high-lactose-fed pigs (P = 0.07). Plasma insulin was lowest in pigs fed 5 g kg body wt⁻¹·day⁻¹ protein (P < 0.0001). Plasma branched-chain amino acids increased as protein intake increased (P < 0.0001). Muscle (P < 0.001) and liver (P ≤ 0.001) ribosomal protein S6 kinase-1 and eIF4E-binding protein phosphorylation increased with protein intake and plateaued at 15 g kg body wt⁻¹·day⁻¹. The results indicate that growth and protein synthesis rates in neonatal pigs are influenced by dietary protein and lactose intake and might be mediated by plasma amino acids and insulin levels. However, feeding protein well above the piglet’s requirement does not further stimulate the activation of translation initiation or protein synthesis in skeletal muscle and liver.

neonate; growth; insulin; ribosomal protein S6 kinase; eukaryotic initiation factor 4E-binding protein

DESPITE IMPROVEMENTS IN THE NUTRITIONAL MANAGEMENT of very low birth weight (VLBW) infants over the past decade (22, 23, 35), most are discharged weighing less than the 10th percentile of intrauterine growth standards (15). Many remain small into adulthood and some exhibit adverse, long-term developmental outcomes, including learning impairment and reduced work capacity (17, 36, 46, 48). However, consumption of a high-protein diet (4.3 vs. 2.25 g kg⁻¹·day⁻¹) can increase length and weight gain of VLBW infants (26–29), and a recent study demonstrated that whole body protein accretion and synthesis rates during the first days of life in VLBW infants are increased by parenteral feeding of 2.65 vs. 0.85 g amino acids·kg⁻¹·day⁻¹ (53). The invasiveness of the experimental methods needed to evaluate the effects of amino acids on protein synthesis rates in specific tissues necessitates the use of an animal model. The neonatal pig, due to similarities in physical size, physiology, and metabolism to human infants, has become a common research model for neonatal infants (6, 9, 25).

Feeding sow’s milk stimulates protein synthesis in all tissues of the neonatal pig (9), and this response can be reproduced with acute intravenous infusion of insulin and amino acids (10, 56, 57). Infusing insulin at doses that reproduce those observed under fed conditions, despite clamping essential amino acids and glucose at fasting levels, results in skeletal muscle protein synthesis rates equal to those observed in milk-fed pigs (11, 57). By use of this clamp technique, it was later demonstrated (41) that muscle protein synthesis dose-dependently increases as both insulin and amino acids levels increase. In fact, a plateau in protein synthesis in skeletal muscle was not achieved even at the highest infusion rate of amino acids (42). Although muscle protein synthesis is responsive to both insulin and amino acids, protein synthesis in liver and other visceral tissues is responsive only to amino acids (11).

The intracellular mechanisms by which insulin and amino acids regulate protein synthesis have been investigated by our laboratories (42, 43, 52) and others (50, 54, 55). Increases in protein synthesis are likely mediated through the insulin and nutrient-signaling pathways via a mammalian target of rapamycin (mTOR)-dependent processes (30). These signaling pathways include the phosphorylation of protein kinase B (PKB), a serine/threonine kinase that is activated by insulin (49). The 70-kDa ribosomal protein S6 kinase-1 (S6K1) and eukaryotic initiation factor (eIF)4E-binding protein-1 (4E-BP1) lie downstream of PKB and mTOR. Activation of S6K1 results in the phosphorylation of ribosomal protein S6, which may lead to an increase in translation of mRNAs containing a terminal oligopyrimidine (sequence adjacent to the m7GTP cap structure at the 5’ end of the message) (19, 39). Phosphorylation of the eIF4E repressor protein 4E-BP1 allows eIF4E to dissociate and bind to eIF4G to form the active eIF4F complex. This eIF4F complex mediates the binding of mRNA to the 40S ribosomal complex in the initiation of mRNA translation (21). Phosphorylation of S6K1 and 4E-BP1 have been shown to be positively correlated with both insulin and amino acid infusion levels within the fasting-to-fed range (42), and we postulate that the provision of higher levels of amino acids could further enhance eIF activation and protein synthesis.

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Recently, our laboratory (18) evaluated the influence of a high-protein diet on rates of tissue protein synthesis and translation initiation factor activation in neonatal pigs. The results indicated that chronic consumption of a high-protein diet beyond the known requirement (40) does not enhance protein synthesis or translation initiation factor activation. However, because of dietary formulation constraints, the high-protein diet yielded lower circulating insulin concentrations than in pigs receiving lower-protein diets. To address this limitation from the previous project, the present study was conducted to evaluate the effects of a wider range of consumption of dietary protein intakes fed at two different dietary lactose levels. The piglets were fed isonenergetic liquid diets that contained low, medium, or high levels of protein at two levels of lactose. The results indicate that growth and protein synthesis in neonatal pigs are influenced by dietary protein and lactose intake. Piglet growth rate and protein synthesis increased with protein intake and were associated with an increase in phosphorylation of biomarkers of mRNA translation. However, feeding protein well above the piglet's requirement does not further stimulate the activation of translation initiation or protein synthesis in skeletal muscle.

**METHODS**

**Animals.** Forty-two crossbred piglets (Yorkshire × Landrace × Hampshire × Duroc; Agriculture Headquarters, Texas Department of Criminal Justice, Huntsville, TX) were weaned from the sow at 1 day of age and had an average weight of 1,696 ± 61 g. Piglets were housed individually in stainless steel kennels and maintained at an ambient temperature of 33°C. The piglets were given one of six isocaloric diets with three different protein levels and two different lactose levels (low and high; Table 1) from 1 to 6 days of age. The low-, medium-, and high-protein diets provided 5, 15, and 25 g/kg body wt day⁻¹·day⁻¹, respectively. The medium-protein diet was formulated to meet or exceed all essential amino acid requirements to maximize growth of the piglet (40). The diets were provided in liquid form at a rate of 400 ml/kg body wt day⁻¹·day⁻¹ in a stainless steel bowl divided into five equal feedings per day. No additional water was provided. The diets were mixed in a Waring commercial blender (model 34BL22) before being offered to the piglets.

On day 2 of age, pigs were anesthetized, and a single jugular catheter was surgically inserted and then filled with heparinized saline by using sterile techniques (56). On day 7, after 4 h of food deprivation, pigs were placed awake in a sling restraint system. Piglets studied in the fed state were gavage-fed their respective diet at a rate of 80 ml·kg⁻¹·body wt⁻¹·feeding⁻¹ at 0 min. Blood samples were collected every 30 min from 0 to 90 min. At 90 min, the pigs were killed, and tissue samples were collected. The right longissimus dorsi muscle spanning the last five ribs and the liver, kidney, and brain were removed and weighed after euthanasia. Whole brain, liver, and both kidneys were also weighed. This protocol was approved by the Animal Care and Use Committee of Baylor College of Medicine and was conducted in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals.

**Tissue protein synthesis in vivo.** Tissue protein synthesis was measured in vivo using a modification of the flooding dose technique (20). At 60 min after feeding, pigs were injected via the jugular vein catheter with 10 ml/kg body wt of a flooding dose of phenylalanine (Amersham, Arlington Heights, IL), which provided 1.5 mmol phenylalanine/kg body wt and 1 mCi of [³H]phenylalanine/kg body wt. Samples of whole blood were taken 5, 15, and 30 min after the injection of [³H]phenylalanine for measurement of the specific radioactivity of the extracellular free pool of phenylalanine. Immediately after the 30-min blood sample was taken, pigs were given a lethal injection of pentobarbital sodium (50 mg/kg body wt). Fractional rates of protein synthesis (Kᵣ, percentage of protein mass synthesized in a day) for each tissue were calculated as

\[
Kᵣ \text{ (day⁻¹) = } \frac{[S_b/S_a] \cdot (T/t)}{100}
\]

where \(S_b\) (dpm/min) is the specific radioactivity of the protein-bound phenylalanine, \(S_a\) (dpm/min) is the specific radioactivity of the tissue free phenylalanine at the time of tissue collection and the linear regression of the blood-specific radioactivity of the animal at 5, 15, and 30 min against time, the constant \(T\) equals 1.440 min/day, and \(t\) is the time of labeling in minutes of the specific tissue. We (12) have previously demonstrated that the specific radioactivity of the tissue free phenylalanine after a flooding dose of phenylalanine is in equilibrium with the aminoacyl-tRNA specific radioactivity; hence, the

### Table 1. Composition of experimental diets

<table>
<thead>
<tr>
<th>Protein Level</th>
<th>Low Lactose</th>
<th>Medium Lactose</th>
<th>High Lactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient, g/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whey protein concentrate</td>
<td>0.3</td>
<td>28.7</td>
<td>57.2</td>
</tr>
<tr>
<td>Lactose*</td>
<td>7.7</td>
<td>7.7</td>
<td>7.5</td>
</tr>
<tr>
<td>Fat source†</td>
<td>43.6</td>
<td>28.6</td>
<td>13.5</td>
</tr>
<tr>
<td>Dried skim milk*</td>
<td>26.1</td>
<td>26.1</td>
<td>26.1</td>
</tr>
<tr>
<td>Other‡</td>
<td>7.3</td>
<td>6.7</td>
<td>6.1</td>
</tr>
<tr>
<td>Water</td>
<td>915.0</td>
<td>902.3</td>
<td>889.6</td>
</tr>
<tr>
<td>Calculated nutrient intake§</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude protein</td>
<td>5.0</td>
<td>15.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Lactose</td>
<td>10.8</td>
<td>10.8</td>
<td>10.8</td>
</tr>
<tr>
<td>Metabolizable energy</td>
<td>0.85</td>
<td>0.85</td>
<td>0.85</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.40</td>
<td>1.72</td>
<td>3.05</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.13</td>
<td>0.39</td>
<td>0.64</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.24</td>
<td>1.08</td>
<td>1.91</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.07</td>
<td>0.28</td>
<td>0.50</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.17</td>
<td>0.46</td>
<td>0.75</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.65</td>
<td>0.65</td>
<td>0.65</td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>0.48</td>
<td>0.48</td>
<td>0.48</td>
</tr>
</tbody>
</table>

*From International Ingredients (St. Louis, MO). †Advanced Fat Pak 80 (Milk Specialties, Dundee, IL). ‡Calcium phosphate, xanthan gum, sodium chloride, calcium carbonate, vitamin premix, mineral premix, potassium chloride, and magnesium sulfate. §Piglets were fed at a rate of 400 ml·kg⁻¹·body wt⁻¹·day⁻¹. Intakes are in MJ·kg⁻¹·body wt⁻¹·day⁻¹ for metabolizable energy and in g·kg⁻¹·body wt⁻¹·day⁻¹ for all other nutrients.
Hormone and substrate assays. The concentration of blood glucose was analyzed using a YSI 2300 STAT Plus (Yellow Springs Instruments, Yellow Springs, OH). Plasma total branched-chain amino acids (BCAA) were analyzed by rapid enzymatic kinetic assay (4). Plasma radioimmunoreactive insulin concentrations were measured using a porcine insulin radioimmunoassay kit (Linco, St. Charles, MO) that used porcine insulin antibody and human insulin standards. Commercially available kits were used to measure plasma urea nitrogen (PUN) (Biotron Diagnostics, Hemet, CA), creatinine (Cayman Chemicals, Ann Arbor, MI), and ammonia (Sigma, St. Louis, MO).

Protein immunoblot analysis. Proteins from muscle and liver homogenates were separated on polyacrylamide gels (PAGE) and then electrophoretically transferred to a polyvinylidene difluoride transfer membrane (Bio-Rad) as previously described (31, 33). The membrane was incubated with primary antibody overnight followed by a 1-h incubation with secondary antibody. The membrane was then washed with Tris-buffered saline-Tween 20 solution. Blots were developed using an enhanced chemiluminescence Western blotting kit (ECL-plus, Amersham), visualized using a GeneGenome bioimaging system, and analyzed using GeneTools software (Syngene, Frederick, MD).

Measurement of PKB phosphorylation. To determine PKB phosphorylation on Ser473, samples were subjected to SDS-PAGE, and the analyzed using GeneTools software (Syngene, Frederick, MD).

Muscle homogenates were centrifuged at 10,000 g for 10 min at 4°C and then frozen at −80°C until they were analyzed. The supernatants were diluted with SDS sample buffer and then subjected to protein immunoblot analysis as previously described (13, 31). The membranes were incubated with a polyclonal antibody that specifically recognizes PKB phosphorylated forms.

Examination of 4E-BP1 phosphorylation on Thr70. Aliquots of muscle homogenates (supernatants) were centrifuged at 4°C and then frozen at −80°C until they were analyzed. The supernatants were diluted with SDS sample buffer and then subjected to protein immunoblot analysis as previously described (13, 31). The membranes were incubated with a polyclonal antibody that specifically recognizes phosphorylation of 4E-BP1 at Thr70 (Cell Signaling).

Measurement of PKB phosphorylation. To determine PKB phosphorylation on Ser473, samples were subjected to SDS-PAGE, and the phosphorylation of PKB on Ser473 was determined by protein immunoblot analysis with an antibody that recognizes the protein only when it is phosphorylated on that residue or a separate antibody that recognizes total PKB. Antibodies were obtained from Cell Signaling (Beverly, MA). Data are expressed as arbitrary units.

RESULTS

Growth performance and organ and muscle weights. Pig body weights at 1 and 6 days of age are shown in Table 2. Pigs fed the medium- and high-protein diets were heavier than pigs fed the low-protein diet at 6 days of age (P = 0.03). The calculated average daily gain (ADG) of the pigs was also affected by protein level. The ADG of pigs on medium- and high-protein diets was greater than that of pigs on low-protein diets (P < 0.001). In addition, pigs fed high-lactose diets tended to have higher ADG than low-lactose-fed pigs (P = 0.07).

Organ and muscle weights are expressed as a function of pig body weight. Overall, both liver and brain weights were highest in low-protein-fed pigs compared with medium- and high-protein-fed pigs (P = 0.002). Liver weight was also greater in high-lactose-fed pigs compared with low-lactose-fed pigs (P < 0.001). Kidney weight was not affected by dietary protein or lactose level. The weight of the longissimus dorsi muscle was 21% greater in medium- and high-protein-fed pigs compared with low-protein-fed pigs (P < 0.001); however, there was no effect of lactose level (P = 0.79).

PUN, creatinine, ammonia, and lactate concentrations. PUN concentrations at 0 and 90 min increased with protein intake (Table 3; P < 0.001). Plasma creatinine concentrations at 0 min were not affected by dietary protein or lactose, but 90 min after the gavage, pigs given the low-protein diets had greater creatinine concentrations than high-lactose-fed pigs (P = 0.001). Ammonia concentrations were greatest in high-protein-fed pigs 90 min after feeding (P < 0.001). In addition, plasma lactate concentrations were 19% greater in high-lactose-fed pigs compared with low-lactose-fed pigs at 90 min, but this response was not significant (P = 0.12).

Plasma glucose, insulin, and BCAA. Plasma concentrations of glucose, insulin, and BCAA increased over time (P < 0.0001) and are presented in Fig. 1. Glucose concentrations in high-lactose-fed pigs were greater than in low-lactose-fed pigs (P = 0.001). In addition, plasma lactate concentrations were 19% greater in high-lactose-fed pigs compared with low-lactose-fed pigs at 90 min, but this response was not significant (P = 0.12).

Table 2. Growth and organ weights of neonatal piglets fed different levels of dietary protein and lactose

<table>
<thead>
<tr>
<th>Lactose Level</th>
<th>Protein level</th>
<th>Day 1 body wt</th>
<th>Day 6 body wt</th>
<th>ADG</th>
<th>Liver</th>
<th>Kidney</th>
<th>Brain</th>
<th>L. dorsi</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>Medium</td>
<td>High</td>
<td>1,656</td>
<td>1,698</td>
<td>1,727</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,675</td>
<td>2,001</td>
<td>2,053</td>
<td></td>
<td>1,656</td>
<td>1,684</td>
<td>1,670</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,656</td>
<td>1,684</td>
<td>1,670</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,686</td>
<td>2,097</td>
<td>2,100</td>
<td>193</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,656</td>
<td>1,684</td>
<td>1,670</td>
<td></td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,675</td>
<td>2,001</td>
<td>2,053</td>
<td></td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,686</td>
<td>2,097</td>
<td>2,100</td>
<td>193</td>
<td>0.98</td>
</tr>
</tbody>
</table>

- Values are means with a pooled SE; n = 6–8 per treatment. L, main effect of lactose level; P, main effect of dietary protein level; L X P, interaction between main effects of lactose and dietary protein levels; ADG, average daily gain; L. dorsi, longissimus dorsi. All pigs were fed 5 times/day at a rate of 400 ml/kg body wt • kg body wt −1 •day −1 from 1 to 6 days of age. After 4 h of food deprivation on day 7, pigs were gavage fed their respective diet at a rate of 80 ml/kg body wt at time 0. Days 1 and 6 body weights are in grams and ADG is in g/day. Organ (liver, kidney, and brain) and L. dorsi weights are expressed as g/kg body wt of day 7 food-deprived body weight.
There was no effect of dietary protein intake in the high-lactose group (time × lactose × protein, $P = 0.09$).

As shown in Fig. 1B, plasma insulin concentrations were greater in high-lactose-fed pigs compared with low-lactose-fed pigs ($P = 0.002$). Pigs fed low-protein diets had the lowest insulin concentrations regardless of lactose intake ($P < 0.0001$). In the low-lactose group, there was no difference in plasma insulin concentration between the medium- and high-protein-fed pigs. However, it is interesting that, in the high-lactose group, insulin concentrations increased with protein intake ($P = 0.02$) even though lactose intake and plasma glucose concentrations were not different.

Plasma BCAA concentrations increased with protein intake ($P < 0.0001$), but there was no main effect of lactose ($P = 0.14$; Fig. 1C). In the low-lactose group, there was no difference in BCAA concentrations at 30 or 60 min for medium- and high-protein-fed pigs; however, in the high-lactose group, plasma BCAA concentrations were 28% greater in high-

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**Table 3. PUN, creatinine, ammonia, and lactate of neonatal pigs fed different levels of dietary protein and lactose**

<table>
<thead>
<tr>
<th>Lactose Level</th>
<th>Low Lactose</th>
<th>High Lactose</th>
<th>$P$ Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein level</td>
<td>Low Medium High</td>
<td>Low Medium High</td>
<td>SE</td>
</tr>
<tr>
<td>PUN, mg/dl</td>
<td>8.6 18.9 30.8</td>
<td>14.0 19.0 34.4</td>
<td>2.0</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>8.6 19.0 35.2</td>
<td>15.1 19.9 32.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Ammonia, μmol/l</td>
<td>2.4 1.7 3.9</td>
<td>2.2 1.9 2.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Lactate, mg/dl</td>
<td>3.6 3.7 4.3</td>
<td>2.3 1.4 2.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Values are means with a pooled SE; $n = 6–8$ per treatment. PUN, plasma urea nitrogen. All pigs were fed 5 times per day at a rate of 400 ml/kg body wt$^{-1}$day$^{-1}$ from 1 to 6 days of age. After 4 h of food deprivation on day 7, pigs were gavage-fed their respective diet at a rate of 80 ml/kg body wt at time 0 min. Data represent the plasma concentrations from samples collected at 0 and 90 min.

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**Fig. 1.** Plasma glucose (A), insulin (B), and branched-chain amino acid (BCAA; C) concentrations in 7-day-old pigs gavage fed at time 0. Piglets had received their respective dietary treatment from 1 to 6 days of age. Diets consisted of 2 levels of lactose and 3 levels of protein. Values are means ± SE; $n = 6–8$ per treatment.
protein-fed pigs compared with medium-protein-fed pigs ($P = 0.02$).

**Insulin signaling protein and translation initiation factor activation.** The phosphorylations of PKB, S6K1, and 4E-BP1 in longissimus dorsi muscle and liver are shown in Fig. 2. In the longissimus dorsi, PKB phosphorylation was greater in high-lactose-fed pigs compared with low-lactose-fed pigs ($P = 0.002$; Fig. 2A). PKB phosphorylation in the longissimus dorsi of the high-lactose group was greater in medium- and high-protein-fed pigs compared with low-protein-fed pigs; however, there were no differences in PKB phosphorylation due to protein intake in the low-lactose group. This resulted in a lactose × protein interaction ($P = 0.03$). The phosphorylation of S6K1 ($P = 0.0005$; Fig. 2B) and 4E-BP1 ($P < 0.0001$; Fig. 2C) in the longissimus dorsi was higher in medium- and high-protein-fed pigs compared with low-protein-fed pigs. In addition, 4E-BP1 phosphorylation in the longissimus dorsi increased linearly with protein intake in the high-lactose group, whereas in the low-lactose group 4E-BP1 phosphorylation was greatest in medium-protein-fed pigs, resulting in the lactose × protein interaction ($P = 0.05$). Overall, liver PKB phosphorylation tended to be greater in the high-lactose group compared with the low-lactose group ($P = 0.10$; Fig. 2D). In addition, low-protein-fed pigs had the greatest phosphorylation of PKB in the liver compared with the medium- and high-protein-fed pigs ($P = 0.006$). As was observed in the longissimus dorsi, liver S6K1 ($P < 0.0001$; Fig. 2E) and 4E-BP1 ($P = 0.001$; Fig. 2F) phosphorylation increased and plateaued at the medium-protein intake.

**Fractional rates of tissue protein synthesis.** As shown in Fig. 3, protein synthesis in the longissimus dorsi, gastrocnemius, and masseter muscles and liver and kidney increased with protein intake and plateaued at the medium-protein level ($P = 0.0001$). These differences, due to dietary protein level, were most pronounced in skeletal muscle, regardless of fiber type. In the jejunum, pigs in the low-protein group and fed the high-protein diet had the greatest fractional rate of protein synthesis; however, pigs in the high-lactose group and fed the low-protein diet had the greatest fractional rate of protein synthesis (Fig. 3D). These differences resulted in the lactose × protein interaction ($P = 0.05$). In the pancreas, fractional rates of protein synthesis were highest in medium-protein-fed pigs followed by the high-protein-fed pigs ($P = 0.0002$; Fig. 3G).

**DISCUSSION**

Feeding stimulates protein synthesis in virtually all tissues in the neonatal pig (9), and this response can be reproduced with the intra-arterial infusion of insulin and amino acids (11). During these acute infusion studies, using insulin-glucose-amino acid clamp techniques, it was demonstrated that skeletal muscle protein synthesis is regulated by both insulin and amino acids, whereas visceral tissues are responsive only to amino acids (11). In our previous infusion studies (41), a full complement of amino acids was infused at a range to simulate from below fasting up to fed levels similar to concentrations observed in piglets that were fed mature sow’s milk. This range corresponds to BCAA concentrations from 250 to 1,000.

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**Fig. 2.** Phosphorylation of PKB, ribosomal protein S6 kinase (S6K1), and eukaryotic initiation factor 4E-binding protein (4E-BP1) in longissimus dorsi (A–C) and liver (D–F) of 7-day-old piglets 90 min after a gavage-fed meal. Piglets had received their respective dietary treatment from 1 to 6 days of age. Diets consisted of 2 levels of lactose and 3 levels of protein. Values are means ± SE; $n = 6–8$ per treatment.
Interestingly, over this range of amino acid infusion, fractional rates of protein synthesis in skeletal muscle were linearly increasing and did not plateau (42). In addition, activation of specific signaling proteins and translation initiation factors involved in the insulin- and nutrient-signaling pathways that regulate protein synthesis were correlated to insulin and BCAA concentrations (41, 42). The first objective of our present study was to determine the effects of a wide range of plasma amino acid concentrations by feeding increasing levels of dietary protein on the stimulation of the insulin- and nutrient-signaling cascades and tissue protein synthesis. Our second objective was to evaluate the effects of low insulin concentrations by feeding a low-lactose diet on the feeding-induced activation of translation initiation and protein synthesis. To meet these objectives, diets containing three different levels of dietary protein and two levels of lactose were fed from 1 to 6 days of age. Although we were able to demonstrate dietary protein-dependent increases in the stimulation of the insulin- and nutrient-signaling cascades and fractional rates of protein synthesis as a result of raising plasma BCAA concentrations from 500 to 1,000 \( \mu \text{mol/l} \) (Ref. 41), we also found that raising the BCAA concentration to 1,500 \( \mu \text{mol/l} \) did not yield further increases in signaling protein activation or protein synthesis.

### Growth and organ weights

In contrast to our previous work (41), this study evaluated piglets under more chronic conditions. The piglets were fed their respective diets for 6 days, thus allowing us to measure changes in growth and organ weights. The differences in day 6 body weight and ADG that were due to dietary protein were quite pronounced. Although piglets fed the low-protein diet had very low rates of body weight gain, there were no differences between the medium- and high-protein-fed piglets. This is likely because the medium-protein diet was formulated to meet or exceed all essential amino acid requirements for maximal growth of the piglet (40). The increases in growth rate and body weight were due to an increase in protein deposition. Longissimus dorsi weights were greatest in medium- and high-protein-fed piglets compared with the low-protein-fed piglets. Dietary lactose also tended to increase average daily gain, which was consistent with recently published work (38). This increase in daily gain
may be the result of higher rates of lipid deposition in high-
lactose-fed piglets. However, the increase in liver weight due
to higher levels of dietary lactose was not expected and can be
attributed to the piglets being fed high lactose and low protein.
Additionally, brain weight appeared to decrease with protein
intake, but it should be noted that these organ weights are
expressed as a function of body weight, and the differences in
the absolute weights of these tissues are much less pronounced.
In fact, there was no effect of dietary protein on the absolute
brain weight of the piglets.

**PUN, creatinine, ammonia, and lactate.** To address the
possibility of protein toxicity, measures of protein metabolism
were evaluated. Previous reports have shown that liver weight,
kidney weight, PUN, and plasma ammonia increase as protein
intake increases (7, 8, 14). As we observed previously (18),
PUN and ammonia concentrations increased with protein in-
take, whereas plasma creatinine and lactate were not affected
due to higher protein level. Plasma ammonia levels in the present
study were similar to those of sow-reared piglets (~60 µmol/l)
and less than that determined by Brunton et al. (5) to reflect
hyperammonemia (270 µmol/l). As in our recent study (18), no
detrimental effect of feeding very-high-protein diets to piglets
from 2 to 7 days of age was observed. This is likely due to the
piglets’ very high rate of protein synthesis compared with older
pigs (9, 13, 56, 57).

**Plasma glucose, insulin, and BCAA.** In our recently pub-
lished study (18) that evaluated the effects of dietary protein on
protein synthesis in piglets, plasma insulin concentrations were
lower in piglets fed high protein due to the lower inclusion rate
of lactose in the diet. Because muscle protein synthesis is very
sensitive to lower concentrations of insulin (41), it is possible
that the lower insulin concentration limited the effect of high-
protein intake on the fractional rates of protein synthesis (18).
Therefore, in the present study, the diets contained two different
lactose levels to provide two different levels of glucose to
stimulate insulin secretion. The low-lactose diet was designed
to yield very low plasma insulin concentrations; however, the
insulin concentrations in the low-lactose pigs fed medium and
high protein were stimulated adequately, such that fractional
rates of protein synthesis were not limited. Surprisingly,
plasma insulin concentrations did not parallel plasma glucose.
In fact, plasma insulin concentrations were influenced in two
ways by dietary protein. For both the low- and high-lactose-fed
groups, the pigs that were fed low-protein diets had very
similar insulin concentrations, and there was not a dramatic
rise in these concentrations with feeding, as was observed for
the medium- and high-protein-fed piglets. In this case, the low
levels of dietary protein might have blunted insulin secretion.
This type of response has been shown previously in rats fed
very low-protein diets and was due to a decrease in pancreatic
islet volume and insulin stores (44). Reis et al. (47) have shown
that pancreatic islets from rats fed a low-protein diet have
impaired secretory responses to glucose and that this is due to
an apparent decrease in the transcription of genes that encode
proteins involved in insulin secretion, specifically PKCo (16).
In contrast to the low levels of insulin that result from low
protein intake, piglets that were fed medium- and high-protein
diets had increased levels of plasma insulin. Subsequently,
these levels of protein intake appear to have increased insulin
secretion, despite the similarities in plasma glucose concentra-
tions. Although some amino acids are classified as glucogenic,
we observed no major differences in plasma glucose levels.
The concept that dietary protein can act as an insulin secreta-
gogue is controversial (34). However, leucine, when given
orally, has been shown to increase plasma insulin levels in
humans (3) and rats (2, 37).

As an indicator of total amino acids in the plasma, BCAA
concentrations were measured over time. In our previous
infusion studies, BCAA concentrations were raised to 1,000
µmol/l (11, 41, 42), a concentration similar to that observed in
piglets in the fed state consuming mature sow’s milk. O’Connor and colleagues (41, 42) demonstrated that fractional
rates of protein synthesis increased linearly with amino acid
infusion up to 1,000 µmol/l; however, higher levels were not
tested. Therefore, the dietary protein levels used in the present
study were designed to raise amino acids beyond those
achieved in the previous studies. In fact, we increased the
BCAA concentration to >1,500 µmol/l with the high-protein
diet. Similarly to our recent publication (18) where we in-
creased BCAA concentrations to 1,350 µmol/l, fractional rates of
protein synthesis in skeletal muscle were not increased
beyond that of the piglets that were fed medium-protein diets
whose BCAA concentrations were ~1,100 µmol/l.

**Insulin- and nutrient-signaling pathways.** Feeding stimu-
lates protein synthesis by activating the insulin- and nutrient-
signaling pathways through an mTOR-dependent process (30).
Components of these pathways include PKB, S6K1, and 4E-
BP1. Insulin activates PKB, a serine/threonine kinase that is
upstream of mTOR, linking phosphatidylinositol 3-kinase
(PI3K) activation to protein synthesis (49). Downstream of
mTOR in these signaling pathways are S6K1 and the repressor
protein 4E-BP1. Activated S6K1 will phosphorylate and acti-
vote ribosomal protein S6, which may lead to an increase in the
translation of mRNAs that encode the proteins of the transla-
tional apparatus, including ribosomal proteins and elongation
factors (19, 39). When the repressor protein 4E-BP1 becomes
phosphorylated, it will dissociate from eIF4E. This allows
eIF4E to bind to eIF4G and form the eIF4F complex, which
mediates the binding of mRNA to the 40S ribosomal subunit
in the initiation of translation (21).

Utilizing insulin-glucose-amino acid clamp studies, we previously
demonstrated that phosphorylation of PKB, S6K1, and 4E-BP1
is positively correlated with the intravenous infusion levels of
insulin and amino acids (41, 52). Specifically, the phosphory-
lation of PKB is correlated to insulin infusion level, and the
phosphorylation of S6K1 and 4E-BP1 is correlated with both
insulin and amino acid infusion levels. Amino acids stimulate
mTOR phosphorylation through an unknown pathway, leading
to the phosphorylation of S6K1 and 4E-BP1 (24, 45, 55). In
addition, phosphorylation of all of these proteins is positively
correlated with fractional rates of protein synthesis (41, 52).

Our laboratory (18) was the first to study the effects of feeding
graded levels of dietary protein on translation initiation factor
activation and protein synthesis. Yoshizawa et al. (58) compared
the response of feeding a protein-free diet vs. a 20% protein diet
and reported higher rates of protein synthesis and increased
4E-BP1 phosphorylation in skeletal muscle and liver of rats that
were fed the 20% protein diet. In our present feeding study, we
demonstrated that phosphorylation of S6K1 and 4E-BP1 in-
creased with protein intake from a low-protein to a medium-
protein diet, although phosphorylation did not continue to
increase in the piglets that were fed the high-protein diet.
Fractional rates of protein synthesis. The effect of feeding different levels of dietary protein was most pronounced in skeletal muscle. In the longissimus dorsi, gastrocnemius, and masseter muscles, fractional protein synthesis rates were lowest in the low-protein-fed pigs, with no differences between the medium- and high-protein-fed pigs. Protein synthesis rates in the skeletal muscle of neonatal pigs are very sensitive to low levels of plasma insulin (41), which may have contributed to the lower rates of protein synthesis in the low-protein-fed pigs. This same effect of dietary protein on protein synthesis was observed in the liver. Protein synthesis in the jejunum did not increase with protein intake, which was likely due to first-pass metabolism of the amino acids in the intestine (51), where the low-protein diet provided an adequate amount of protein to optimize protein synthesis in this tissue. In our previous feeding study, where different levels of dietary protein were fed, we did not observe consistent differences in protein synthesis in any tissues (18). This lack of a response was likely due to the lowest protein level fed in that study, providing a sufficient amount of amino acids to maximize protein synthesis.

The protein requirement of a 2-kg piglet is 16.9 g·kg body wt⁻¹·day⁻¹ to maximize growth rate (40). The low-, medium-, and high-protein diets provide 5, 15, and 25 g·kg body wt⁻¹·day⁻¹, respectively. Thus these diets were deficient, marginal, and in excess of the piglets’ requirements. The present recommended protein intake for a low-birth-weight infant (<2.5 kg body wt) consuming 120 kcal/day is ~4 g·kg body wt⁻¹·day⁻¹ (1, 32). Considering these differences in recommended protein intake and the fact that piglet growth rate is ~4 to 5 times greater than a human infant at the same weight, the low-, medium-, and high-protein diets used in this study for piglets would equate to protein intakes of 1.2, 3.6, and 5.9 g·kg body wt⁻¹·day⁻¹ for low-birth-weight infants.

Perspectives. This study was designed to relate the findings from our previously published acute, parenteral infusion studies that demonstrate that insulin and amino acids activate the insulin- and nutrient-signaling pathways, leading to an increase in protein synthesis, to responses that occur from chronic enteral feeding of different protein and carbohydrate levels. Here, we have established that the phosphorylation of PKB, S6K1, and 4E-BP1 is influenced by dietary protein and lactose level, which is consistent with our infusion studies (42). This feeding study also demonstrates that chronically feeding dietary protein to piglets beyond their known protein requirement (40) does not further stimulate the activation of the insulin- and nutrient-signaling pathways or protein synthesis in skeletal muscle. Furthermore, we demonstrated that these changes in the activation of nutrient-signaling proteins and translation initiation factors result in an increase in protein synthesis, leading to an increase in protein deposition. To date, our research indicates that the enteral diet should contain an adequate amount of carbohydrate to stimulate insulin secretion, as well as a sustained supply of essential amino acids to support the high anabolic drive for muscle protein synthesis of neonates throughout the postnatal period.

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REFERENCES


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