

Supplementing L-leucine to a low-protein diet increases tissue protein synthesis in weanling pigs

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Abstract Recent work with young pigs shows that reducing dietary protein intake can improve gut function after weaning but results in inadequate provision of essential amino acids for muscle growth. Because acute administration of L-leucine stimulates protein synthesis in piglet muscle, the present study tested the hypothesis that supplementing L-leucine to a low-protein diet may maintain the activation of translation initiation factors and adequate protein synthesis in multiple organs of post-weaning pigs. Eighteen 21-day pigs (Duroc × Landrace × Yorkshire) were fed low-protein diets (16.9% crude protein) supplemented with 0, 0.27 or 0.55% L-leucine (total leucine contents in the diets being 1.34, 1.61 or 1.88%, respectively). At 35 days of age, protein synthesis was determined using the [^2H] phenylalanine flooding-dose technique. Additionally, total and phosphorylated levels of mammalian target of rapamycin (mTOR), ribosomal

protein S6 kinase 1 (S6K1), and eIF4E-binding protein-1 (4E-BP1) were measured in longissimus muscle and liver. Compared with the control group, dietary supplementation with 0.55% L-leucine for 2 weeks increased ($P < 0.05$): (1) the phosphorylated levels of S6K1 and 4E-BP1; (2) protein synthesis in skeletal muscle, liver, the heart, kidney, pancreas, spleen, and stomach; and (3) daily weight gain by 61%. Dietary supplementation with 0.27% L-leucine enhanced ($P < 0.05$) protein synthesis in proximal small intestine, kidney and pancreas. These novel findings provide a molecular basis for designing effective nutritional means to increase the efficiency of nutrient utilization for protein accretion in neonates.

Keywords Food · Weanling pigs · Protein synthesis · Tissues

Abbreviations

CP	Crude protein
eIF4E	Eukaryotic initiation factor 4E
4E-BP1	eIF4E-binding protein-1
FRS	Fractional synthesis rate
mTOR	Mammalian target of rapamycin
S6K1	Ribosomal protein S6 kinase 1

Introduction

Post-weaning diarrhea is a major problem in pork industrial production (Wang et al. 2009a, b; Tang et al. 2005, 2009; Deng et al. 2009). Reducing dietary crude protein (CP) level, as a strategy to solve the problem, has been found to limit the frequency and the severity of digestive problems in

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piglets (Ball and Aherne 1982; Kong et al. 2007). Recent work with the piglet, an excellent animal model for studying the nutrition of human infants (Deng et al. 2007a, b; Elango et al. 2009; Suryawan et al. 2009; Yin et al. 2009), has shown that reducing dietary intake of protein can improve gut function after weaning (Lalles et al. 2007). However, this method results in inadequate provision of amino acids for supporting tissue protein synthesis (Baker 2009; Deng et al. 2009). A strategy to overcome such a nutritional problem may be to supplement free amino acids to low-protein diets (Deng et al. 2007a, b; Hansen et al. 1993; Rotz 2004; Wu et al. 2009). However, little work has been conducted in this area of neonatal nutrition, despite a previous attempt to supplement histidine, isoleucine, and valine to low-protein diets for gilts (Figuerola et al. 2003). Identifying an amino acid that can stimulate protein synthesis in weanling piglets fed a reduced-CP diet is of enormous nutritional importance.

Leucine, as one of the essential amino acids, is of interest for addition to low-protein diets to regulate protein synthesis and animal growth because its biochemical actions include increasing the secretion of growth hormones (Wu 2009), regulating gene expression (Jobgen et al. 2009; Li et al. 2009a; Palii et al. 2009; Stipanuk et al. 2009), and repairing muscles (Dardevet et al. 2000; Tischler et al. 1982). Many studies have demonstrated that leucine can stimulate protein synthesis and inhibit protein degradation in skeletal muscle (Dardevet et al. 2000, 2002; Escobar et al. 2005; Tischler et al. 1982). Leucine has also been implicated to play a signaling role in enhancing the availability of specific eukaryotic initiation factors (Anthony et al. 2000), as well as augmenting the activity of proteins involved in mRNA translation (Davis and Fiorotto 2009; Wu et al. 2010a). This stimulatory effect of leucine is thought to be mediated partly via a mammalian target of rapamycin (mTOR)-dependent process that involves the phosphorylation of S6K1, 4E-BP1 and eIF4E assembly (Kimball and Jefferson 2006). However, little evidence is available regarding a beneficial effect on weaned pigs of supplementing leucine to a low-protein diet. Therefore, the primary objective of this work was to investigate the effect of leucine on growth performance and protein synthesis in multiple organs of weaned pigs fed a low-protein diet.

Materials and methods

Animals and diets

We conducted the experiment in accordance with the Chinese guidelines for animal welfare and it was approved by the animal welfare committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences. Eighteen

healthy pigs (Landrace $\times$ Yorkshire; 8.24 ± 0.67 kg body weight) were weaned at 21 days of age and housed individually in stainless steel metabolism pens (0.8×1.8 m) in environmentally controlled facilities (25°C) (Tan et al. 2009a, b). Immediately after removal from sows, piglets were assigned randomly into one of three corn and soybean meal-based diets supplemented with 0, 0.27, or 0.55% L-leucine (Table 1). There were six pigs per diet. The basal diet contained 16.9% crude protein (CP) which was 29% lower than that recommended by NRC (1998), and 1.34% leucine which just met NRC requirement. Thus, the total contents of leucine in the diets supplemented with 0.0, 0.27

Table 1 The composition and nutrient content of experimental diets

	1.34% Leu	1.61% Leu	1.88% Leu
Ingredients (%)			
Corn	50.56	50.29	49.41
Soybean meal	19.20	19.20	19.20
Corn starch	10.50	10.50	11.05
Whey	12.00	12.00	12.00
Fish meal	4.00	4.00	4.00
L-Leucine	0	0.27	0.55
L-Lysine-HCl (78.8%)	0.28	0.28	0.28
L-Methionine	0.11	0.11	0.11
L-Threonine	0.13	0.13	0.13
L-Tryptophan	0.05	0.05	0.05
L-Isoleucine	0.14	0.14	0.14
L-Valine	0.16	0.16	0.16
Salt	0.35	0.35	0.35
Limestone	0.22	0.22	0.22
CaHPO ₄	1.30	1.30	1.35
Premix	1.00	1.00	1.00
Total	100	100	100
Nutrient composition, %			
Crude protein	16.9	16.9	16.9
Digestible Lys	1.08	1.08	1.08
Digestible Met + Cys	0.58	0.58	0.58
Digestible Thr	0.70	0.70	0.70
Digestible Trp	0.22	0.22	0.22
Digestible Ile	0.76	0.76	0.76
Digestible Leu	1.34	1.61	1.88
Digestible Val	0.84	0.84	0.84
Total calcium	0.70	0.70	0.70
Total phosphorus	0.80	0.80	0.80
Digestible energy (Mcal/kg)	3.42	3.42	3.42

Premix supplied the following per kg complete diet: vitamin A, 11,445 IU; vitamin D₃, 1,700 IU; vitamin E, 43.75 mg; vitamin K₃, 3.12 mg; vitamin B₁, 1.87 mg; vitamin B₂, 6.25 mg; vitamin B₆, 5 mg; vitamin B₁₂, 0.025 mg; niacin, 25 mg; vitamin C, 288 mg; calcium pantothenate, 9.37 mg; folic acid, 0.62 mg; biotin, 0.45 mg; choline chloride, 500 mg; Fe, 165 mg; Cu, 60 mg; I, 0.90 mg; Se, 0.27 mg; Zn, 144 mg; Mg, 68 mg; Mn, 5.0 mg; and Co, 0.3 mg

and 0.55% leucine were 1.34, 1.61 and 1.88%, respectively. The piglets had free access to their respective diets. On each day, water was added to the diet in a 2:1 ratio. Additional water was available from a nipple drinker (Li et al. 2007; Ruan et al. 2007; Wu et al. 2008). At 32 days of age, all of the pigs were surgically inserted with a jugular catheter for blood sampling and isotope infusion, according to the technique of Li et al. (2008). Feed intake and body weight gain of the animal were recorded weekly (Yin et al. 2008).

Measurement of protein synthesis

Tissue protein synthesis rates were measured in vivo using a modification of the flooding-dose technique (Frank et al. 2007; Fan et al. 2006). Three days after the catheter insertion and at 1 h after the last meal, all piglets were injected, through the jugular catheter, with a flooding dose of L-phenylalanine (1.50 mmol/kg BW) containing L-[ring- $^2\text{H}_5$] phenylalanine at 40 mol% (0.60 mmol/kg BW) in sterile saline. The intravenous administration of phenylalanine was completed in 5–10 s. Venous blood samples (1 mL) were taken before injection and 15, 30, and 45 min after injection to measure the specific radioactivity of the extracellular free pool of phenylalanine. Blood samples were centrifuged at $3,000\times g$ for 10 min to obtain plasma (Kong et al. 2009; Li et al. 2009c; Wu et al. 2010b, c). Immediately after the last blood sample was collected, pigs were humanely killed (Deng et al. 2009, 2010). The entire intestine was then rapidly removed and dissected free of mesenteric attachments and placed on a smooth, cold surface tray. The duodenum, jejunum and ileum were separated. The duodenum was the first 30-cm small intestine beginning at the pyloric sphincter. An ileal portion of samples (the 30-cm distal portion of the small intestine ending at the ileocecolic junction) was also collected (Chen et al. 2007, 2009). Approximately 30 cm from the middle of the small intestine was taken as the jejunal tissue. Their luminal contents were removed by manually stripping out the contents followed by rinsing the lumen with saline (Haynes et al. 2009; Yin et al. 2001). At the same time, longissimus dorsi muscle (spanning the last five ribs on the right side), jejunum, pancreas, kidneys and liver were quickly removed and snap-frozen in liquid nitrogen. The exact time (min) of tracer labeling was recorded from the end of the intravenous injection of phenylalanine to the time of placing tissue samples in liquid nitrogen (Deng et al. 2009).

The isotopic enrichment of L-[$^2\text{H}_5$]phenylalanine in the tissue free pool was measured according to the procedures of Wang et al. (2007) except that the *n*-propyl heptafluorobutyrate derivative of phenylalanine analyzed using a model 6890 GC linked to a 5973N quadrupole MS set on Electron Ionization mode (MacKenzie 1987; Culea and Hachey 1995). Ions with mass-to-charge ratios of 91 and

96 were monitored and converted to percentage of molar enrichment (mol%) using calibration curves. Fractional rates of protein synthesis (FSR) for each tissue were calculated as: $\text{FSR (\%/day)} = (S_a \times 1,440 \times 100\%) / (S_b \times t)$ where FSR is the percentage of protein renewed in a day, S_a is the isotopic enrichment (mol%) of L-[ring- $^2\text{H}_5$] phenylalanine in the protein-bound pool of tissue at time t , S_b is the observed isotopic enrichment (mol%) of L-[ring- $^2\text{H}_5$]phenylalanine in the free pool of tissue at 15, 30, and 45 min, and t is the exact time (min) of tracer labeling. The isotopic enrichment of L-[$^2\text{H}_5$]phenylalanine in the free pool of tissue is in equilibrium with that in the aminoacyl-tRNA pool and is therefore an appropriate measure of fractional synthesis rate (Bregendahl et al. 2004).

Protein immunoblot analysis

Antibodies against total 4E-BP1, phosphorylated 4E-BP1 (Thr 70), total S6K1, phosphorylated S6K1 (Thr 389), total mTOR, and phosphorylated mTOR (Ser 2448) were purchased from Cell Signaling (Danvers, MA, USA). Frozen samples were powdered under liquid nitrogen using a mortar and pestle. The powdered tissue was homogenized in seven volumes of buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM NaF, 100 mM KCl, 0.2 mM EDTA, 50 mM β -glycerophosphate, 1 mM dithiothreitol, 0.1 mM phenylmethylsulphonylfluoride, 1 mM benzamidine, and 0.5 mM sodium vanadate) with a Polytron homogenizer and centrifuged at $10,000\times g$ for 10 min at 4°C. The supernatant fluid (0.5 mL) was aliquoted into microcentrifuge tubes containing 0.5 mL of $2\times$ sodiumdodecyl sulfate (SDS) sample buffer (2 mL of 0.5 M Tris, pH 6.8, 2 mL glycerol, 2 mL of 10% SDS, 0.2 mL of β -mercaptoethanol, 0.4 mL of a 4% solution of bromphenol blue, and 1.4 mL water to a final volume of 8 mL). The samples were boiled for 5 min and cooled on ice before being used for Western blot analysis. The protein samples were separated by electrophoresis on a 7.5% (S6K1), 15% (4E-BP1), or 6% (mTOR) polyacrylamide gel (Kang et al. 2010; Tan et al. 2010). Proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with the respective primary polyclonal antibodies and a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, Cell Signaling; 1:10,000 dilution in 1% milk) (Hou et al. 2010). Photographs of the membranes were taken using the Kodak Image Station 440, and densitometry was performed with Kodak 1D Network software (Eastman Kodak Company, New Haven, CT, USA).

Hormone and substrate assays in plasma

Plasma glucose was analyzed using a Beckman CX4 and reagents from Shanghai Biotechnologies Ltd. (Shanghai,

China) (He et al. 2009). Plasma insulin was measured using a porcine insulin RIA kit that used porcine insulin antibody and human insulin standards (Shanghai Biotechnologies Ltd., Shanghai, China) (Tan et al. 2009b). Plasma amino acids were determined using ion-exchange chromatography, as we previously described (Li et al. 2009b; Yin et al. 2009).

Statistical analysis

Values are expressed as mean $\pm$ SEM. Statistical analysis of the data was performed using the GLM procedure of SAS (SAS, 2002), followed by the Student–Neuman–Keuls test to determine treatment effects. Differences between the groups were considered significant when $P \leq 0.05$.

Results

Feed intake, performance, and tissue weights

Dietary supplementation with 0.27 and 0.55% leucine had no effect ($P > 0.05$) on feed intake (Table 2). Daily weight gains were 61 and 41% greater ($P < 0.05$) in weanling pigs supplemented with 0.55% leucine, respectively, compared with those supplemented with 0.0 and 0.27% leucine. Dietary supplementation with 0.27 and 0.55% leucine increased ($P < 0.05$) pancreas weight by 40 and 46%, respectively. Dietary supplementation with 0.27% leucine also enhanced ($P < 0.05$) the weights of stomach and liver.

Plasma insulin, glucose, and amino acids

Concentrations of insulin and glucose in plasma did not differ ($P > 0.05$) among either different time points after the intravenous administration of L-[ring- $^2\text{H}_5$]phenylalanine or

dietary treatments (Table 3). Concentrations of phenylalanine in plasma increased ($P < 0.001$) after the infusion of phenylalanine. At time 15 (baseline values), concentrations of plasma leucine were greater ($P < 0.05$) in leucine-supplemented pigs compared with the control group (Table 4). Opposite results were obtained for valine and isoleucine at times 30 and 45 min. Leucine supplementation has no effect ($P > 0.05$) on concentrations of other amino acids in plasma within 45 min after phenylalanine infusion except for threonine, lysine, alanine, glycine, and serine at time 30 min (Table 4).

Protein synthesis

Compared with the 0 and 0.27% leucine groups, dietary supplementation with 0.55% leucine increased ($P < 0.05$) protein synthesis in the heart, kidney, liver, skeletal muscle, pancreas, spleen, and stomach (Table 5). Dietary supplementation with 0.27% leucine enhanced ($P < 0.05$) protein synthesis in proximal small intestine, kidney and pancreas, compared with the control group (Table 4).

Phosphorylation of 4E-BP1

Phosphorylated levels of 4E-BP1 (Thr⁷⁰) and the γ -isoform of 4E-BP1, the repressor protein of eIF4E, are shown in Fig. 1. The 4E-BP1 protein, upon phosphorylation, can be resolved into α -, β -, and γ -forms. The γ -form is the predominant phosphorylated form of the protein and does not bind eIF4E (Romanelli et al. 2002). Compared with the control group, dietary supplementation with 0.55% leucine enhanced ($P < 0.05$) the phosphorylation state of 4E-BP1 in both skeletal muscle and liver (Fig. 1) while dietary supplementation with 0.27% leucine had no effect ($P > 0.05$).

Table 2 Growth and organ weights of weaning piglets fed different levels of dietary leucine

Variable	Leucine level			Pooled SEM	P value
	1.34%	1.61%	1.88%		
Initial body weight, kg	8.39	8.42	8.28	0.75	0.990
Final body weight, kg	11.3	11.7	12.9	0.71	0.040
Average daily gain, g	206 ^b	236 ^b	332 ^a	10	0.048
Daily feed intake, g/day	455	442	432	0.05	0.120
Stomach weight/BW, g/kg	8.89 ^b	10.8 ^a	10.2 ^{ab}	0.58	0.062
Heart weight/BW, g/kg	6.37	6.87	6.19	0.56	0.552
Pancreas weight/BW, g/kg	2.38 ^b	3.34 ^a	3.48 ^a	0.29	0.036
Liver weight/BW, g/kg	31.9 ^b	39.8 ^a	35.8 ^{ab}	1.75	0.027
Kidney weight/BW, g/kg	3.94	4.90	4.58	0.66	0.548
Lung weight/BW, g/kg	15.2	15.5	18.1	1.11	0.195
SI weight/BW, g/kg	16.9	16.8	17.7	1.87	0.958
LI weight/BW, g/kg	69.0	73.4	77.1	12.6	0.907

^{a,b} Mean within a row without a common superscript differ ($P < 0.05$)

BW Body weight, LI Large intestine, SI Small intestine

Table 3 Concentrations of insulin and glucose in the plasma of weanling piglets fed different levels of dietary leucine at 15, 30 and 45 min after intravenous administration of [ring-²H₅]phenylalanine

	Dietary leucine content			Pooled SEM	<i>P</i> value
	1.34%	1.61%	1.88%		
Insulin (mU/L)					
15 min	34.6	31.5	34.1	3.6	0.89
30 min	38.4	33.4	34.7	5.4	0.34
45 min	39.3	37.5	35.1	2.9	0.73
Glucose (mmol/L)					
15 min	4.5	4.2	4.3	0.22	0.84
30 min	4.7	4.4	4.1	0.24	0.66
45 min	5.0	5.8	5.7	0.18	0.79

Phosphorylation of S6K1

Dietary supplementation with 0.55% leucine increased ($P < 0.05$) the phosphorylation level of S6K1(Thr³⁸⁹) in both skeletal muscle and liver, compared with the control group (Fig. 2). Dietary supplementation with 0.27% leucine modestly increased ($P < 0.05$) the phosphorylation level of S6K1 in the liver but had no effect ($P > 0.05$) on skeletal muscle, compared with the control group (Fig. 2).

Phosphorylation of mTOR

Total mTOR levels in skeletal muscle and liver (Fig. 3), as well as phosphorylated mTOR levels in skeletal muscle (Fig. 4) did not differ ($P > 0.05$) among pigs fed the different levels of dietary leucine (Fig. 3). However, the phosphorylated level of mTOR (Ser²⁴⁴⁸) was higher ($P < 0.05$) in the liver of pigs supplemented with 0.55% leucine compared with the control group (Fig. 4).

Discussion

Edmonds and Baker (1987) reported that supplementing 1, 2 and 4% L-leucine to a corn and soybean meal-based diet (20% CP) for 16 days did not affect growth performance in weanling pigs, whereas dietary supplementation with 6% leucine reduced weight gain and food intake. This failure of detecting a beneficial effect of leucine on young pigs was likely due to a high CP content and an imbalance among branched-chain amino acids in the basal diet. Here, we reported for the first time that supplementing 0.55% L-leucine to a low-protein diet (16.9% CP) for 14 days increased weight gain of weanling pigs (Table 2). Additionally, the leucine treatment enhanced protein synthesis (Table 5) and mTOR signaling (Fig. 1) in both skeletal muscle and liver of weanling pigs. These results confirmed

Table 4 Concentrations of amino acids in the plasma of weanling piglets fed different levels of dietary leucine at 15, 30 and 45 min after intravenous administration of [ring-²H₅]phenylalanine

Amino acids	Leucine content			Pooled SEM	<i>P</i> value
	1.34%	1.61%	1.88%		
Essential amino acids (μmol/L)					
Valine					
0 min	70.9	76.5	88.8	13.9	0.659
15 min	66.6	74.0	66.6	7.5	0.478
30 min	87.0 ^a	69.2 ^{ab}	57.8 ^b	8.5	0.101
45 min	91.4 ^a	51.7 ^b	63.7 ^b	8.6	0.026
Threonine					
0 min	70.9	77.3	80.2	12.8	0.598
15 min	60.5	74.0	66.6	7.0	0.478
30 min	69.2 ^{ab}	87.0 ^a	57.8 ^b	8.2	0.101
45 min	91.4 ^a	51.7 ^b	63.7 ^b	8.4	0.026
Leucine					
0 min	222	231	262	18.2	0.310
15 min	177 ^b	226 ^a	229 ^a	14.2	0.047
30 min	247	224	254	12.5	0.754
45 min	232	213	263	10.9	0.391
Isoleucine					
0 min	58.6	43.8	53.0	5.7	0.235
15 min	48.6	36.2	40.9	7.5	0.525
30 min	42.4 ^a	41.6 ^{ab}	35.4 ^b	2.3	0.014
45 min	41.7 ^a	31.2 ^b	30.8 ^b	3.0	0.049
Lysine					
0 min	143 ^b	119 ^b	218 ^a	9.0	0.020
15 min	141	205	131	14.1	0.265
30 min	196 ^a	93.7 ^b	165 ^a	7.5	0.005
45 min	176	152	150	7.9	0.503
Nonessential amino acids (μmol/L)					
Alanine					
0 min	164 ^b	269 ^a	285 ^a	15.6	0.008
15 min	77.8	77.0	113	16.8	0.269
30 min	56.6	59.4	79.4	8.0	0.144
45 min	95.4 ^a	59.4 ^b	58.1 ^b	4.6	<0.001
Glycine					
0 min	348 ^b	445 ^{ab}	522 ^a	22.9	0.019
15 min	494	424	516	24.8	0.521
30 min	318 ^a	308 ^a	247 ^b	8.5	0.050
45 min	265 ^b	297 ^{ab}	309 ^a	10.8	0.047
Serine					
0 min	89.6	94.1	100	9.8	0.748
15 min	210	232	205	13.1	0.796
30 min	112 ^a	92.4 ^{ab}	89.7 ^b	6.1	0.046
45 min	212	203	215	10.2	0.673
Proline					
0 min	35.0	37.2	38.3	6.7	0.938
15 min	43.9	36.8	42.4	6.9	0.748

Table 4 continued

Amino acids	Leucine content			Pooled SEM	P value
	1.34%	1.61%	1.88%		
30 min	35.7	36.0	29.1	3.4	0.308
45 min	36.0	34.2	35.2	5.7	0.974
Glutamate + Glutamine					
0 min	528	576	539	26.7	0.682
15 min	481	485	515	21.1	0.951
30 min	568	560	477	26.3	0.719
45 min	458	454	493	17.4	0.881
Aspartate					
0 min	29.9 ^b	36.5 ^a	37.4 ^a	1.8	0.034
15 min	30.4	33.7	33.2	5.4	0.897
30 min	31.1	44.9	31.0	4.7	0.106
45 min	39.9	36.1	31.7	5.1	0.544

^{a,b} Mean within a row without a common superscript differ ($P < 0.05$)

and extended the previous in vivo findings that an increase in circulating levels of leucine within physiological ranges stimulated the phosphorylation of both 4E-BP1 and S6K1 in skeletal muscle, liver, and intestine (Escobar et al. 2005; Rhoads and Wu 2009).

Leucine has long been known to stimulate protein synthesis and inhibit protein degradation in incubated skeletal muscle (Tischler et al. 1982). Intensive in vivo studies have extended these in vitro seminal findings and identified that elevated concentrations of leucine in plasma resulting from oral administration or dietary supplementation also increased muscle protein synthesis in young rats (Anthony et al. 2000) and neonatal pigs (Escobar et al. 2005) under physiological conditions. It is now clear that leucine increases muscle protein synthesis by activating the mTOR signaling pathway (Meijer and Dubbelhuis 2004). The

results of this study indicated that chronic dietary supplementation with leucine promoted the growth of not only skeletal muscle but also the liver and gastrointestinal tract (Table 2) in young pigs.

Evidence from studies with cultured cells suggests that mTOR is an upstream kinase responsible for phosphorylating both 4E-BP1 and S6K1 (a threonine/serine kinase) in cultured cells (Asnaghi et al. 2004; Um et al. 2004). Phosphorylation of mTOR on Ser²⁴⁴⁸ is positively related to the activity of mTOR (Lynch et al. 2002; Yonezawa et al. 2004). S6K1 phosphorylates ribosomal protein S6 and promotes the translation of mRNA containing a TOP motif (Um et al. 2004). Additionally, phosphorylation of 4E-BP1 can partly regulate the dissociation of 4E-BP1 with eIF4E, thereby releasing eIF4E for initiation of protein synthesis (Gingras et al. 1999; Raught and Gingras 1999, 2001; Vary and Lynch 2005). Consistent with this notion, dietary supplementation with leucine stimulated the phosphorylation of mTOR, 4E-BP1 and S6K1 proteins in the muscle and liver of young pigs (Figs. 1, 2, and 4). Of particular interest, a novel and important finding from the present study is that the leucine treatment enhanced the phosphorylation of both 4E-BP1 and S6K1 in skeletal muscle despite a lack of change in the phosphorylation of mTOR (Fig. 4). Thus, under physiological conditions in young pigs, 4E-BP1 and S6K1 may be activated independent of changes in mTOR phosphorylation. L-Leucine may directly stimulate 4E-BP1 and S6K1 phosphorylation in cells. It is unlikely that the action of leucine in vivo is mediated by insulin, because plasma levels of this hormone did not differ between control and leucine-supplemented piglets (Table 3). Notably, leucine can be transaminated extensively in skeletal muscle to produce α -keto-isocaproate, glutamate, glutamine, and alanine (Wu and Thompson 1988; Wu et al. 1989). Some of these products may directly phosphorylate both 4E-BP1 and S6K1 in muscle.

Table 5 Fractional rates of protein synthesis (%/day) in tissues of weanling piglets fed different levels of dietary leucine

Organ	Leucine content in the diet			Pooled SEM	P value
	1.34%	1.61%	1.88%		
Cecum	43.1	44.5	47.6	1.8	0.251
Colon	51.4	49.7	45.0	3.4	0.443
Heart	30.8 ^b	27.7 ^b	36.6 ^a	1.8	0.018
Distal small intestine	59.1 ^b	70.5 ^{ab}	73.2 ^a	4.1	0.082
Proximal small intestine	60.9 ^b	74.2 ^a	66.0 ^b	2.6	0.006
Kidney	45.1 ^c	57.0 ^b	66.2 ^a	2.5	0.001
Liver	75.5 ^b	74.4 ^b	86.5 ^a	2.9	0.031
Lung	58.0	64.6	68.9	4.1	0.220
Skeletal muscle	9.0 ^b	9.5 ^b	11.9 ^a	0.5	0.020
Pancreas	53.6 ^c	64.0 ^b	74.6 ^a	2.1	<0.001
Spleen	57.0 ^b	56.8 ^b	73.3 ^a	4.3	0.039
Stomach	57.7 ^b	53.8 ^b	72.7 ^a	2.7	0.002

^{a,b} Mean within a row without a common superscript differ ($P < 0.05$)

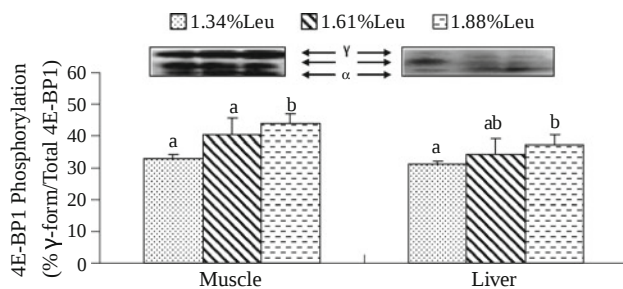


Fig. 1 Abundance of phosphorylated 4E-BP1 (Thr⁷⁰) in longissimus muscle and liver of weanling piglets fed different levels of dietary leucine. Data are mean $\pm$ SEM for $n = 6$. Values not sharing the same letter differ ($P < 0.05$)

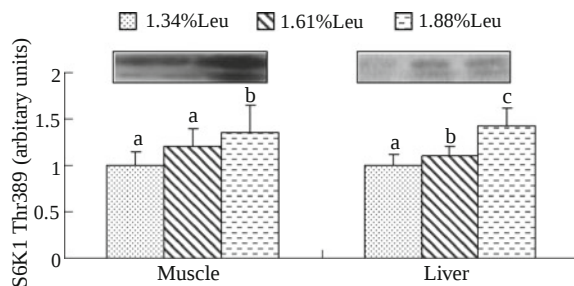


Fig. 2 Abundance of phosphorylated S6K1 (Thr³⁸⁹) in longissimus muscle and liver of weanling piglets fed different levels of dietary leucine. Data are mean $\pm$ SEM for $n = 6$. Values not sharing the same letter differ ($P < 0.05$)

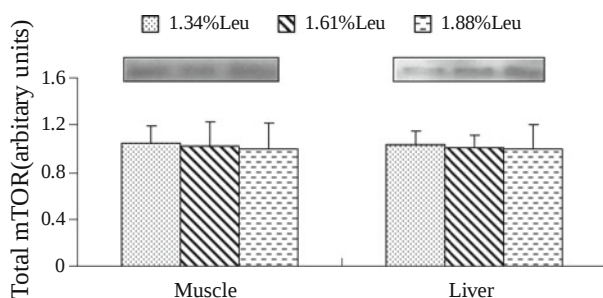


Fig. 3 The abundance of total mTOR in longissimus muscle and liver of weanling piglets fed different levels of dietary leucine. Data are mean $\pm$ SEM for $n = 6$

Sow milk provides large amounts of branched-chain amino acids to suckling piglets (Kim and Wu 2009). However, when the neonates are removed from their mothers, they respond to the physiological stress with a reduction in food intake (Flynn et al. 2009) and impaired growth of skeletal muscle (Han et al. 2009). Although feeding a low-protein diet appears to be effective in ameliorating the intestinal dysfunction, a reduced provision of dietary amino acids limits tissue protein synthesis (Wu 2009). The observations of the present study and those of

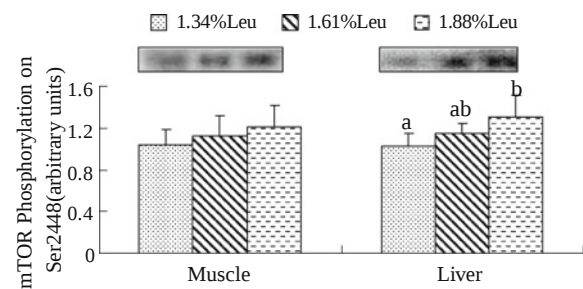


Fig. 4 Abundance of phosphorylated mTOR (Ser²⁴⁴⁸) in longissimus muscle and liver of weanling piglets fed different levels of dietary leucine. Data are mean $\pm$ SEM for $n = 6$. Values not sharing the same letter differ ($P < 0.05$)

others (Escobar et al. 2005) clearly indicate that leucine has potential for activating the machinery for protein synthesis in the skeletal muscle of young pigs. However, due to antagonism among branched-chain amino acids and an imbalance between leucine and threonine in the diet (Dien et al. 1954; Gatnau et al. 1995; Harper et al. 1954), dietary supplementation with leucine alone reduced plasma concentrations of isoleucine, valine, and threonine in piglets (Table 4). Interestingly, the extent of reduction in these three essential amino acids did not appear to limit the effect of leucine on stimulating protein synthesis in skeletal muscle, liver and distal small intestine. Because protein synthesis in tissues of young pigs is very sensitive to circulating levels of threonine (Wang et al. 2007), chronic dietary supplementation with leucine plus isoleucine, valine and threonine to a low-protein diet may hold great promise for enhancing their growth performance. Future studies involving a large number of young pigs are necessary to test this novel hypothesis.

In conclusion, dietary supplementation with 0.55% L-leucine increased the phosphorylation of 4E-BP1 and S6K1 in skeletal muscle and liver, as well as the growth performance of young pigs fed a low-protein diet. Our results also indicate that supplemental L-leucine exerted these effects without affecting mTOR phosphorylation in skeletal muscle. These novel findings provide a molecular basis for designing effective nutritional means to increase the efficiency of nutrient utilization for muscle and whole-body growth in neonates.

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References

- Anthony JC, Anthony TG, Kimball SR et al (2000) Orally administered leucine stimulates protein synthesis in skeletal muscle of postabsorptive rats in association with increased eIF4F formation. *J Nutr* 130:139–145
- Asnaghi L, Bruno P, Priulla M et al (2004) mTOR: a protein kinase switching between life and death. *Pharmacol Res* 50:545–549
- Baker DH (2009) Advances in protein-amino acid nutrition of poultry. *Amino Acids* 37:29–41
- Ball RO, Aherne FX (1982) Effect of diet complexity and feed restriction on the incidence and severity of diarrhea in early-weaned pigs. *Can J Anim Sci* 62:907–913
- Bregendahl K, Liu L, Cant JP et al (2004) Fractional protein synthesis rates measured by an intraperitoneal injection of a flooding dose of L-[ring-²H₅]phenylalanine in pigs. *J Nutr* 134:2722–2728
- Chen L, Yin YL, Jobgen WS, Jobgen SC, Knabe DA, Hu WX, Wu G (2007) In vitro oxidation of essential amino acids by jejunal mucosal cells of growing pigs. *Livest Sci* 109:19–23
- Chen LX, Li P, Wang JJ et al (2009) Catabolism of nutritionally essential amino acids in developing porcine enterocytes. *Amino Acids* 37:143–152
- Culea M, Hachey D (1995) Determination of multi-labeled serine and glycine isotopomers in human plasma by isotope dilution negative-ion chemical ionization mass spectrometry. *Mass Spectrom* 9:655–659
- Dardevet D, Sornet C, Balage M et al (2000) Stimulation of in vitro rat muscle protein synthesis by leucine decreases with age. *J Nutr* 130:2630–2635
- Dardevet D, Sornet C, Bayle G et al (2002) Postprandial stimulation of muscle protein synthesis in old rats can be restored by a leucine-supplemented meal. *J Nutr* 132:95–100
- Davis TA, Fiorotto ML (2009) Regulation of muscle growth in neonates. *Curr Opin Nutr Metab Care* 12:78–85
- Deng D, Huang RL, Li TJ et al (2007a) Nitrogen balance in barrows fed low-protein diets supplemented with essential amino acids. *Livest Sci* 109:220–223
- Deng D, Li AK, Chu WY et al (2007b) Growth performance and metabolic responses in barrows fed low-protein diets supplemented with essential amino acids. *Livest Sci* 109:224–227
- Deng D, Yin YL, Chu WY et al (2009) Impaired translation initiation activation and reduced protein synthesis in weaned piglets fed a low-protein diet. *J Nutr Biochem* 20:544–552
- Deng J, Wu X, Bin S, Li TJ, Huang R, Liu Z, Liu Y, Ruan Z, Deng Z, Hou Y, Yin YL (2010) Dietary amylose and amylopectin ratio and resistant starch content affects plasma glucose, lactic acid, hormone levels and protein synthesis in splanchnic tissues. *Zeitschrift für Tierphysiologie Tierernährung und Futtermittelkunde* 94:220–226
- Dien LTH, Ravel JM, Shive W (1954) Some inhibitory interrelationships among leucine, isoleucine and valine. *Arch Biochem Biophys* 49:283–291
- Edmonds MS, Baker DH (1987) Amino acid excesses for young pigs: effects of excess methionine, tryptophan, threonine or leucine. *J Anim Sci* 64:1664–1669
- Elango R, Ball RO, Pencharz PB (2009) Amino acid requirements in humans: with a special emphasis on the metabolic availability of amino acids. *Amino Acids* 37:19–27
- Escobar J, Frank JW, Suryawan A et al (2005) Physiological rise in plasma leucine stimulates muscle protein synthesis in neonatal pigs by enhancing translation initiation factor activation. *Am J Physiol* 288:E914–E921
- Fan MZ, Chiba LI, Matzat PD, Yang X, Yin YL, Mine Y, Stein HH (2006) Measuring synthesis rates of nitrogen-containing polymers by using stable isotope tracers. *J Anim Sci* 84(E. Suppl.): E79–E93
- Figuerola JL, Lewis AJ, Miller PS et al (2003) Growth, carcass traits, and plasma amino acid concentrations of gilts fed low-protein diets supplemented with amino acids including histidine, isoleucine, and valine. *J Anim Sci* 81:1529–1537
- Flynn NE, Bird JG, Guthrie AS (2009) Glucocorticoid regulation of amino acid and polyamine metabolism in the small intestine. *Amino Acids* 37:123–129
- Frank JW, Escobar J, Nguyen HV et al (2007) Oral N-carbamoylglutamate supplementation increases protein synthesis in skeletal muscle of piglets. *J Nutr* 137:315–319
- Gatnau R, Zimmerman DR, Nissen SL et al (1995) Effects of excess dietary leucine and leucine catabolites on growth and immune responses in weanling pigs. *J Anim Sci* 73:159–165
- Gingras AC, Gygi SP, Raught B (1999) Regulation of 4E-BP1 phosphorylation: a novel 2-step mechanism. *Gene Dev* 13:1422–1437
- Han J, Liu YL, Fan W et al (2009) Dietary L-arginine supplementation alleviates immunosuppression induced by cyclophosphamide in weaned pigs. *Amino Acids* 37:643–651
- Hansen JA, Knaba DA, Burgoon KG (1993) Amino acid supplementation of low-protein sorghum-soybean meal diets for 5- to 20-kilogram swine. *J Anim Sci* 71:452–458
- Harper AE, Benton DA, Winje ME et al (1954) Leucine-isoleucine antagonism in the rat. *Arch Biochem Biophys* 51:523–526
- Haynes TE, Li P, Li XL et al (2009) L-Glutamine or L-alanyl-L-glutamine prevents oxidant- or endotoxin-induced death of neonatal enterocytes. *Amino Acids* 37:131–142
- He QH, Kong XF, Wu GY et al (2009) Metabolomic analysis of the response of growing pigs to dietary L-arginine supplementation. *Amino Acids* 37:199–208
- Hou YQ, Wang L, Ding BY et al (2010) Dietary α -ketoglutarate supplementation ameliorates intestinal injury in lipopolysaccharide-challenged piglets. *Amino Acids*. doi:10.1007/s00726-010-0473-y
- Jobgen W, Fu WJ, Gao H et al (2009) High fat feeding and dietary L-arginine supplementation differentially regulate gene expression in rat white adipose tissue. *Amino Acids* 37:187–198
- Kang P, Toms D, Yin Y, Cheung Q, Gong J, De Lange K, Li J (2010) Epidermal growth factor-expressing *Lactococcus lactis* enhances intestinal development of early-weaned pigs. *J Nutr* 140:806–811
- Kim SW, Wu G (2009) Regulatory role for amino acids in mammary gland growth and milk synthesis. *Amino Acids* 37:89–95
- Kimball SR, Jefferson LS (2006) New functions for amino acids: effects on gene transcription and translation. *Am J Clin Nutr* 83:500S–507S
- Kong XF, Wu GY, Liao YP et al (2007) Effects of Chinese herbal ultra-fine powder as a dietary additive on growth performance, serum metabolites and intestinal health in early-weaned piglets. *Livest Sci* 108:272–275
- Kong XF, Yin YL, He QH et al (2009) Dietary supplementation with Chinese herbal powder enhances ileal digestibilities and serum concentrations of amino acids in young pigs. *Amino Acids* 37:573–582
- Lalles JP, Bosi P, Smidt H et al (2007) Weaning—a challenge to gut physiologists. *Livest Sci* 108:82–93

- Li P, Yin YL, Li DF et al (2007) Amino acids and immune function. *Br J Nutr* 98:237–252
- Li TJ, Dai QZ, Yin YL et al (2008) Dietary starch sources affect net portal appearance of amino acids and glucose in growing pigs. *Animal* 2:723–729
- Li XL, Bazer FW, Gao HJ et al (2009a) Amino acids and gaseous signaling. *Amino Acids* 37:65–78
- Li P, Kim SW, Li XL et al (2009b) Dietary supplementation with cholesterol and docosahexaenoic acid affects concentrations of amino acids in tissues of young pigs. *Amino Acids* 37:709–716
- Li L, Peng H, Zhang B, Wu G, Yin Y, Yang K, Li T, Li A, Hou Z, Zhang P, Liu C (2009c) The effect of dietary addition of a polysaccharide from *Atractylodes macrophala* koidz on growth performance, immunoglobulin concentration and IL-1 β expression in weaned pigs. *J Agric Sci* 147:625–631
- Lynch CJ, Hutson SM, Patson BJ et al (2002) Tissue-specific effects of chronic dietary leucine and norleucine supplementation on protein synthesis in rats. *Am J Physiol* 283:E824–E835
- MacKenzie SL (1987) Gas chromatographic analysis of amino acids as the n-heptafluorobutyl isobutyl esters. *Assoc Official Anal Chem* 70:151–160
- Meijer AJ, Dubbelhuis PF (2004) Amino acid signaling and the integration of metabolism. *Biochem Biophys Res Commun* 313:397–403
- Palii SS, Kays CE, Deval C et al (2009) Specificity of amino acid regulated gene expression: analysis of gene subjected to either complete or single amino acid deprivation. *Amino Acids* 37:79–88
- Raught B, Gingras AC (1999) eIF4E is regulated at multiple levels. *Int J Biochem Cell Biol* 31:43–57
- Raught B, Gingras AC, Sonenberg N (2001) The target of rapamycin (TOR) proteins. *Proc Natl Acad Sci USA* 98:7037–7044
- Rhoads JM, Wu G (2009) Glutamine, arginine, and leucine signaling in the intestine. *Amino Acids* 37:111–122
- Romanelli A, Dreisbach VC, Blenis J (2002) Characterization of phosphatidylinositol 3-kinase-dependent phosphorylation of the hydrophobic motif site Thr(389) in p70 S6 kinase 1. *J Biol Chem* 277:40281–40289
- Rotz CA (2004) Management to reduce nitrogen losses in animal production. *J Anim Sci* 82:119S–137S
- Ruan Z, Zang G, Yin YL, Li TJ, Huang RL, Kim SW, Wu GY, Deng ZY (2007) Dietary requirement of true digestible phosphorus and total calcium for growing pigs. *Asian Aust J Anim Sci* 20:1236–1242
- SAS (Statistical Analysis System Inc) (2002) SAS/STAT user's guide, version 9. SAS Institute, Cary
- Stipanuk MH, Ueki I, Dominy JE et al (2009) Cysteine dioxygenase: a robust system for regulation of cellular cysteine levels. *Amino Acids* 37:55–63
- Suryawan A, O'Connor PMJ, Bush JA et al (2009) Differential regulation of protein synthesis by amino acids and insulin in peripheral and visceral tissues of neonatal pigs. *Amino Acids* 37:97–104
- Tan BE, Li XG, Kong XF et al (2009a) Dietary L-arginine supplementation enhances the immune status in early-weaned piglets. *Amino Acids* 37:323–331
- Tan BE, Yin YL, Liu ZQ et al (2009b) Dietary L-arginine supplementation increases muscle gain and reduces body fat mass in growing-finishing pigs. *Amino Acids* 37:169–175
- Tan B, Yin Y, Kong X et al (2010) L-Arginine stimulates proliferation and prevents endotoxin-induced death of intestinal cells. *Amino Acids* 38:1227–1235
- Tang ZR, Yin YL, Nyachoti CM, Huang RL, Li TJ, Yang C, Yang X, Gong J, Peng J, Qi DS, Xing JJ, Sun ZH, Fan MZ (2005) Effect of dietary supplementation of chitosan and galacto-mannan-oligosaccharide on serum parameters and the insulin like growth factor-I mRNA expression in early-weaned piglets. *Domest Anim Endocrinol* 28:430–441
- Tang ZR, Yin YL, Zhang Y, Huang R, Sun Z, Li T, Chu W, Kong X, Li L, Geng M, Tu Q (2009) Effects of dietary supplementation with an expressed fusion peptide bovine lactoferricin–lactoferrampin on performance, immune function and intestinal mucosal morphology in piglets weaned at age 21 d. *Bri J Nutr* 101:998–1005
- Tischler ME, Desautels M, Goldberg AL (1982) Does leucine, leucyl-transfer RNA, or some metabolite of leucine regulate protein synthesis and degradation in skeletal and cardiac muscle? *J Biol Chem* 257:1613–1621
- Um SH, Frigerio F, Watanabe M (2004) Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* 431:200–205
- Vary TC, Lynch CJ (2005) Meal feeding enhances formation of eIF4F in skeletal muscle: role of increased eIF4F availability and eIF4G phosphorylation. *Am J Physiol* 290:E631–E642
- Wang X, Qiao SY, Yin YL et al (2007) A deficiency or excess of dietary threonine reduces protein synthesis in jejunum and skeletal muscle of young pigs. *J Nutr* 137:1442–1446
- Wang XQ, Ou DY, Yin JD et al (2009a) Proteomic analysis reveals altered expression of proteins related to glutathione metabolism and apoptosis in the small intestine of zinc oxide-supplemented piglets. *Amino Acids* 37:209–218
- Wang WW, Qiao SY, Li DF (2009b) Amino acids and gut function. *Amino Acids* 37:105–110
- Wu G (2009) Amino acids: metabolism, functions, and nutrition. *Amino Acids* 37:1–17
- Wu G, Thompson JR (1988) The effect of ketone bodies on alanine and glutamine metabolism in isolated skeletal muscle from the fasted chick. *Biochem J* 255:139–144
- Wu G, Thompson JR, Sedgwick G et al (1989) Formation of alanine and glutamine in chick (*Gallus domesticus*) skeletal muscle. *Comp Biochem Physiol* 93B:609–613
- Wu X, Ruan Z, Zhang G, Hou YQ, Yin YL, Li TJ, Huang RL, Kong XF, Chu WY, Chen LX, Hou YQ, Ruan Z, Gao B, Chen LX (2008) True digestibility of phosphorus in different resources of phosphorus ingredients in growing pigs. *Asian Aust J Anim Sci* 21:107
- Wu G, Bazer FW, Davis TA et al (2009) Arginine metabolism and nutrition in growth, health and disease. *Amino Acids* 37:153–168
- Wu G, Bazer FW, Burghardt RC et al (2010a) Impacts of amino acid nutrition on pregnancy outcome in pigs: mechanisms and implications for swine production. *J Anim Sci* 88:E195–E204
- Wu X, Yin YL, Li TJ, Wang L, Ruan Z, Liu ZQ, Hou YQ (2010b) Dietary protein, energy and arginine affect LAT1 expression in forebrain white matter differently. *Animal*. doi:10.1017/S1751731110000534
- Wu X, Ruan Z, Gao Y, Yin Y, Zhou X, Wang L, Geng M, Hou Y, Wu G (2010c) Dietary supplementation with L-arginine or N-carbamylglutamate enhances intestinal growth and heat shock protein-70 expression in weanling pigs fed a corn- and soybean meal-based diet. *Amino Acids*. doi:10.1007/s00726-010-0538-y
- Yin YL, Huang RL, Zhong HY (1993) Comparison of the ileorectal anastomosis and conventional method for the measurement of ileal digestibility of protein sources and mixed diets in growing pigs. *Anim Feed Sci Technol* 42:297–308
- Yin YL, Baidoo SK, Schulze H, Simmins PH (2001) Effect of supplementing diets containing hullless barley varieties having different levels of non-starch polysaccharides with β -glucanase and xylanase on the physiological status of gastrointestinal tract and nutrient digestibility of weaned pigs. *Livest Prod Sci* 71:97–107

- Yin YL, Huang C, Wu X, Li T, Huang R, Kang P, Hu Q, Chu W, Kong X (2008) Nutrient digestibility response to graded dietary levels of sodium chloride in weanling pigs. *J Sci Food Agric* 88:940–944
- Yin FG, Liu YL, Yin YL et al (2009) Dietary supplementation with Astragalus polysaccharide enhances ileal digestibilities and serum concentrations of amino acids in early weaned piglets. *Amino Acids* 37:263–270
- Yonezawa K, Yoshino KI, Tokunaga C et al (2004) Kinase activities associated with mTOR. *Curr Top Microbiol Immunol* 279:271–282