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Impaired translation initiation activation and reduced protein synthesis in weaned piglets fed a low-protein diet

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Abstract

Weanling mammals (including infants) often experience intestinal dysfunction when fed a high-protein diet. Recent work with the piglet (an animal model for studying human infant nutrition) shows that reducing protein intake can improve gut function during weaning but compromises the provision of essential amino acids (EAA) for muscle growth. The present study was conducted with weaned pigs to test the hypothesis that supplementing deficient EAA (Lys, Met, Thr, Trp, Leu, Ile and Val) to a low-protein diet may maintain the activation of translation initiation factors and adequate protein synthesis in tissues. Pigs were weaned at 21 days of age and fed diets containing 20.7, 16.7 or 12.7% crude protein (CP), with the low-CP diets supplemented with EAA to achieve the levels in the high-CP diet. On Day 14 of the trial, tissue protein synthesis was determined using the phenylalanine flooding dose method. Reducing dietary CP levels decreased protein synthesis in pancreas, liver, kidney and longissimus muscle. A low-CP diet reduced the phosphorylation of eukaryotic initiation factor (eIF) 4E-binding protein-1 (4E-BP1) in skeletal muscle and liver while increasing the formation of an inactive eIF4E·4E-BP1 complex in muscle. Dietary protein deficiency also decreased the phosphorylation of mammalian target of rapamycin (mTOR) and the formation of an active eIF4E·eIF4G complex in liver. These results demonstrate for the first time that chronic feeding of a low-CP diet suppresses protein synthesis in animals partly by inhibiting mTOR signaling. Additionally, our findings indicate that supplementing deficient EAA to low-protein diets is not highly effective in restoring protein synthesis or whole-body growth in piglets. We suggest that conditionally essential amino acids (e.g., glutamine and arginine) may be required to maintain the activation of translation initiation factors and optimal protein synthesis in neonates.

Keywords: Amino acids; Protein synthesis; mTOR signaling; Piglets; Weaning

1. Introduction

Weanling mammals (including infants) often experience intestinal dysfunction when fed a high-protein diet [1]. Recent work with the piglet, an excellent animal model for studying the nutrition of human infants, has shown that reducing dietary intake of protein can improve gut function during weaning [2]. However, this practice results in inadequate provision of essential amino acids (EAAs) for muscle growth [3]. Amino acids serve an important role in growth response by regulating protein turnover in various tissues. A deficiency and/or imbalance of an EAA can impair animal growth due to the repression of muscle protein synthesis [4]. Although it is known that a diet lacking EAA results in polyribosome disaggregation, the mechanism(s) responsible for the inhibition of liver and muscle protein synthesis is not clearly understood.

Feeding-induced stimulation of protein synthesis occurs in virtually all tissues of the neonate, but the postprandial rise in protein synthesis is most pronounced in skeletal muscle [5,6]. Increased protein synthesis in skeletal muscle is

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mediated by a postprandial increase in insulin and amino acids, whereas in liver, the response is mediated by amino acids alone [3]. The enhanced responsiveness of protein synthesis to stimuli in neonates is associated with increased translational efficiency, which is mediated primarily by the activation of translation initiation factors involved in the binding of mRNA to the 43S ribosomal complex and not by those involved in the binding of the initiator methionyl-tRNAi to the 40S ribosomal subunit [5,7]. In skeletal muscle and liver of neonates, feeding or acute infusion of amino acids to raise circulating levels to those within the physiological range in fed animals stimulates the phosphorylation of ribosomal protein S6 kinase (S6K1) and eukaryotic initiation factor (eIF)-4E-binding protein-1 (4E-BP1), which, in turn, releases eIF4E from the inactive 4E-BP1·eIF4E complex [5]. Free eIF4E binds to eIF4G and eIF4A to form the active eIF4F complex, which mediates the binding of mRNA to the 43S ribosomal complex [7]. Recent studies using cell cultures indicated that hyperphosphorylation of 4E-BP1 and S6K1, particularly by amino acids, involves a signaling pathway that includes the protein kinase mammalian target of rapamycin (mTOR) that is inhibited by the immunosuppressant drug rapamycin [8]. However, little is known about the effect of dietary amino acids on mTOR signaling or translation initiation in vivo.

In a previous study [9], the increase in translation initiation in both skeletal muscle and liver in response to feeding a diet composed of carbohydrate, lipid and protein was not associated with any detectable change in the activity or phosphorylation state of eIF2B, which regulates the binding of initiator methionyl-tRNAi to the 40S ribosomal subunit. Instead, changes in translation initiation were associated with alterations in the phosphorylation state of eIF4E and/or the association of eIF4E with eIF4G and 4E-BP1. However, it was not clear whether the stimulation of protein synthesis resulted from a simple increase in caloric intake or whether one component of the diet was the stimulus. The objective of the present study was to determine whether supplementing EAA to a low-protein diet for weanling pigs could be effective in maintaining adequate protein synthesis and to examine the underlying mechanism.

2. Materials and methods

2.1. Animals and diets

Crossbred piglets (n=18; Duroc×Landrace×Yorkshire) were weaned from sows at 21 days of age [8.24±0.67 kg body weight (BW)]. Three isonitrogenous and isocaloric diets were formulated to meet the National Research Council (NRC)-recommended requirements of true ileal digestible amino acids for pigs [10]. Piglets were fed one of three diets with different crude protein (CP) levels (20.7%, 16.7% or 12.7%; Table 1). The 20.7% CP diet was formulated without the addition of EAA (Lys, Met, Thr, Trp, Leu, Ile or Val). The other two diets were supplemented with the above EAA to provide digestible amino acids equal to the levels in the 20.7% CP diet (Table 1). True ileal digestible contents of Phe +Tyr and His were 0.92% and 0.32%, respectively, in the 12.7% CP diet; these amino acids met the current NRC requirements for 8- to 12-kg weaned pigs that gained <300 g/day [10] and, therefore, were not supplemented to low-CP diets. Pigs were housed individually in stainless steel metabolism pens (0.8×1.8 m) in an environmentally controlled room with an average temperature of 25°C. During a 4-day adaptation period, pigs were fed the diets prepared in wet-mash form with a water:mash ratio of 1:1. After a 10-day period of feeding, jugular catheters were inserted into pigs [12] after anesthesia was induced in piglets with a mixture of (−)-17-(cyclobutylmethyl)morphinan-3,14-diol[*S*-(*R*∗,*R*∗)]-2,3-dihydroxybutanedioate (butorphanol tartrate; 0.2 mg/kg), (±)-2-(2-chlorophenyl)-2-(methylamino)cyclohexanone (ketamine; 11 mg/kg), *N*-(2,6-dimethylphenyl)-5,6-dihydro-4*H*-1,3-thiazine-2-amine

Table 1
Composition and nutrient content of diets (as fed-basis)

Ingredients	Dietary CP (Crude protein) level, %		
	20.7	16.7	12.7
Corn	57.98	52.69	52.11
Soybean meal	21.50	13.60	4.20
Extruded soybean	6.00	6.00	6.00
Corn starch	0	11.80	20.52
Fish meal	5.00	5.00	5.00
Whey	6.00	6.00	6.00
L-Lysine-HCl (78.8%)	0	0.28	0.57
L-Methionine	0	0.11	0.20
L-Threonine	0	0.15	0.28
L-Tryptophan	0	0.05	0.08
L-Isoleucine	0	0.15	0.30
L-Leucine	0	0.29	0.53
L-Valine	0	0.16	0.31
Salt	0.35	0.35	0.35
Limestone	0.67	0.52	0.40
$CaHPO_4$	0.50	0.85	1.15
Premix[a]	2.00	2.00	2.00
Nutrient composition			
CP, %	20.7	16.7	12.7
Digestive energy, Mcal/kg	3.44	3.44	3.44
Ca, %	0.70	0.70	0.70
P, %	0.60	0.60	0.60
True ileal digestible Lys, %[b]	1.07	1.07	1.07
True ileal digestible Met+Cys, %[b]	0.58	0.58	0.58
True ileal digestible Thr, %[b]	0.71	0.71	0.71
True ileal digestible Trp, %[b]	0.21	0.21	0.21
True ileal digestible Ile, %[b]	0.74	0.74	0.74
True ileal digestible Leu, %[b]	1.60	1.60	1.60
True ileal digestible Val, %[b]	0.84	0.84	0.84

[a] Premix (Tianke Company, Guangzhou, China) supplied per kg diet: retinyl acetate, 11 445 IU; cholecalciferol, 1700 IU; all-*rac*-α-tocopheryl acetate, 43.75 IU; menadione, 3.12 mg; vitamin B_1, 1.87 mg; vitamin B_2, 6.25 mg; vitamin B_6, 5 mg; cyanocobalamin, 0.025 mg; riboflavin, 3.9 mg; choline, 500 mg; D-pantothenic acid, 9.37 mg; vitamin C, 288 mg; Fe, 165 mg; Cu, 60 mg; I, 0.9 mg; Se, 0.27 mg; Zn, 144 mg; Mg, 68 mg; Co, 0.3 mg.

[b] True ileal digestible amino acids were calculated on the basis of amino acid analyses in the single feedstuffs and coefficient [10,11].

hydrochloride (xylazine; 2.2 mg/kg] and 3[(cyclopentylhydroxyphenylacetyl)oxy]-1,1-dimethyl pyrrolidinium bromide (glycopyrrolate; 0.01 mg/kg) and maintained with 2% 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane) delivered with oxygen. Four days after the surgery, piglets were used for the measurement of tissue protein synthesis.

2.2. Hormone and substrate assays

Blood samples were collected from pigs at 1 h after the last meal and centrifuged at 3,000 rpm for 20 min at 4°C. The serum was stored at −20°C until analysis. Serum samples were analyzed using the porcine insulin RIA kit (Shanghai Biotechnologies, Shanghai, China). Double-antibody radioimmunoassays were used to determine serum insulin. Intra- and interassay variation coefficients were was 5.2% and 9%, respectively. The concentration of serum glucose (Shanghai Biotechnologies, China) was determined using a Beckman CX_4 (Beckman, Fullerton, CA, USA).

2.3. Measurement of protein synthesis

Tissue protein synthesis rates were measured in vivo using a modification of the flooding-dose technique [12], except that a stable isotope was used [13]. On day 14 post weaning, at 1 h after the last meal, all piglets were administered, through the jugular catheter, a flooding dose of L-phenylalanine (1.50 mmol/kg body weight) containing L-[ring-$^2H_5$]Phe (Cambridge Isotope Laboratories, Andover, MA, USA) at 40 mol% (0.60 mmol/kg BW) dissolved in sterile saline (154 mM). The iv injection was completed in 5–10 s. Venous blood samples were taken before and at 15, 30 and 45 min after injection to measure the isotopic enrichment of [^{2}H]phenylalanine in the extracellular free pool. Immediately after the last blood sample was collected, pigs were anaesthetised with an iv injection of sodium pentobarbital (50 mg/kg BW) and bled by exsanguination. 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 obtained. The collected duodenal tissue was the first 15-cm of small intestine. The ileal tissue was the 30-cm distal portion of the small intestine that ended at the ileocecocolic junction. Approximately a 30-cm segment at the middle of the small intestine was taken as jejunal tissue. Intestinal luminal contents were rinsed with sterile saline. The right longissimus dorsi muscle spanning the last five ribs, pancreas, kidneys and liver were also quickly removed and placed in liquid nitrogen. The exact time (min) of labeling was recorded between the end of the iv tracer injection and the placement of a tissue sample in liquid nitrogen.

The isotopic enrichment of L-[$^2H_5$] L-phenylalanine in the tissue free pool was determined according to the procedures of Wang et al. [13], except that the *n*-propyl heptafluorobutyrate derivative of L-phenylalanine was measured using a model 6890 GC linked to a 5973N quadruple MS set on Electron Ionization mode [14,15]. 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 FSR (%/d)=(S_a×1440×100%)/(S_b×t) where FSR is the percentage of protein synthesized in a day, S_a is the isotopic enrichment (mol%) of L-[ring-$^2H_5$] phenylalanine in the tissue protein-bound pool at time *t*, S_b is the observed isotopic enrichment (mol%) of L-[ring-$^2H_5$] phenylalanine in the free tissue pool at 15, 30 and 45 min and *t* is the exact time (min) of labeling measured between the end of the iv tracer injection and the placement of a tissue sample in liquid nitrogen. The isotopic enrichment of the tissue free L-[^{2}H]phenylalanine after a flooding dose of L-phenylalanine is in equilibrium with that of aminoacyl-tRNA and, therefore, is an appropriate measure of fractional synthesis rate [16].

2.4. Protein immunoblot analysis

Total 4E-BP1 and phosphospecific 4E-BP1 (Thr^{46}), total eIF4E and phosphospecific eIF4E (Ser^{209}), total S6K1 and phosphospecific S6K1 (Thr^{389}), phosphorylated (Ser^{2448}) and total mTOR, phosphorylated (Ser^{1108}) and total eIF4G polyclonal antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Frozen liver or muscle 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 DTT, 0.1 mM PMSF, 1 mM benzamidine and 0.5 mM sodium vanadate) with a Polytron homogenizer and centrifuged at 10,000×*g* for 10 min at 4°C. The supernatant was aliquoted into microcentrifuge tubes and 2× sodium dodecyl sulfate (SDS) sample buffer (2 ml of 0.5 M Tris, pH 6.8, 2 ml glycerol, 2 ml 10% SDS, 0.2 ml β-mercaptoethanol, 0.4 ml 4% solution of bromphenol blue and 1.4 ml water to a final volume of 8 ml) was added in a 1:1 ratio. The samples were boiled for 5 min and cooled on ice before being used for Western blot analysis. This procedure did not result in precipitation of the sample-SDS complex. The protein samples were separated by electrophoresis on a 7.5% (S6K1), 15% (4E-BP1) or 6% (mTOR) polyacrylamide gel. Proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with the respective primary polyclonal antibodies and a secondary antibody (Bio-Rad), conjugated with horseradish peroxidase and diluted to 1:10,000 in 1% milk. 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).

2.5. Quantification of 4E-BP1·eIF4E and eIF4G·eIF4E complexes

The 4E-BP1·eIF4E and eIF4G·eIF4E complexes were quantified as described previously [17,18]. Briefly, eIF4E

and 4E-BP1·eIF4E and eIF4G·eIF4E complexes were immunoprecipitated from aliquots of homogenized tissue supernatant with an anti-eIF4E polyclonal antibody (Santa Cruz Biotechnology). Antibody-antigen complexes were collected using magnetic beads and subjected to electrophoresis on a 7.5% or 15% polyacrylamide gel. Proteins were then electrophoretically transferred to a PVDF membrane. The blots were incubated with a rabbit polyclonal anti-eIF4E antibody, a rabbit polyclonal anti-4EBP1 antibody or a goat anti-eIF4G antibody for 12 h at 4°C. The phosphorylated forms of 4E-BP1 were measured after immunoprecipitation of 4E-BP1 from the tissue homogenates following centrifugation at 10,000×*g*. The various phosphorylated forms of 4E-BP1 were separated by SDS-polyacrylamide gel electrophoresis and analyzed by protein immunoblotting. The blots were developed using an enhanced chemiluminescence kit, and the autoradiographs were scanned as previously described.

2.6. Statistical analysis

Results are expressed as means±S.E.M. Statistical analysis of the data was performed using the GLM procedure of SAS [19]. Differences among treatment means were determined by the Student–Neuman–Keuls multiple comparison test. *P* values <.05 were taken to indicate statistical significance.

3. Results

3.1. Feed intake, growth performance and tissue weights

Feed intakes by pigs fed the 20.7, 16.7 and 12.7% CP diets did not differ during the experimental period (Table 2).

Table 2
Growth performance and relative tissue weights of weaned pigs fed low-protein diets supplemented with essential amino acids between 25 and 39 days of age

	Dietary CP content%			S.E.M.[a]	*P*
	20.7	16.7	12.7		
Growth performance					
Initial body weight, kg	7.82	7.65	7.80	0.24	.924
Final body weight, kg	12.01	11.52	11.50	0.08	.505
Average daily gain, g/d	299[b]	276[bc]	264[c]	0.01	.040
Daily feed intake, g/d	432	442	455	0.05	.120
Feed conversion, g/g weight gain/g feed	0.693[b]	0.624[c]	0.582[c]	0.13	.012
Tissue weights, g/kg body weight					
Liver	22.8	21.1	21.3	0.79	.297
Kidney	4.4	4.2	3.6	0.23	.078
Pancreas	1.7[b]	1.4[c]	1.3[c]	0.07	.003
Stomach	6.4	6.7	6.1	0.37	.572
Small intestine	23.4	22.8	23.1	1.35	.297
Large intestine	20.7	20.5	18.9	1.20	.515
Longissimus muscle	20.4	18.8	18.2	0.62	.068

[a] Values are means and pooled S.E.M., *n*=6/group. Means in a row without common superscripts differ (*P*<.05).

Table 3
Plasma concentrations (mg/100 ml) of amino acids in weaned piglets fed low-protein diets supplemented with essential amino acids[a]

	Dietary CP content%			S.E.M.[b]	*P*
	20.7	16.7	12.7		
Essential amino acids					
Arg	2.8[c]	2.0[d]	1.5[e]	0.24	<.01
His	1.0[c]	0.69[d]	0.56[d]	0.08	.04
Ile	1.6	1.7	1.9	0.01	.71
Leu	3.7	3.7	3.8	0.05	.91
Lys	2.69	3.09	3.10	0.13	.39
Met	0.81	0.73	0.99	0.09	.52
Thr	1.82	1.66	1.70	0.17	.67
Val	3.45	2.72	2.94	0.39	.26
Nonessential amino acids					
Ala	5.28	4.50	4.25	0.82	.11
Asp	6.36	4.35	4.47	0.66	.09
Cys	0.37	0.35	0.50	0.01	.68
Glu	3.10[c]	2.82[c]	1.85[d]	0.37	.03
Gly	4.85[c]	3.73[d]	3.48[d]	0.47	.02
Ser	1.29[c]	0.89[d]	0.67[d]	0.17	<.01
Tyr	1.59[c]	0.91[d]	1.00[d]	0.18	.03

[a] At 45 min post injection of phenylalanine.
[b] Values are means with pooled SEM, *n*=5–6/treatment. Means in a row without common superscripts differ (*P*<.05).

Weight gain (g/d) and feed conversion efficiency in pigs fed the 12.7% and 16.7% CP diets was lower (*P*<.05) than in pigs fed the 20.7% CP diet (control). The relative weights of pancreas (g/kg BW) decreased with lower protein intake (*P*<.001). The relative weights of kidney (*P*=.078) and longissimus muscle (*P*=.068) tended to decrease with the reduction of dietary CP levels. The relative weights of the liver, stomach, small intestine and large intestine did not differ among the three dietary groups.

3.2. Plasma insulin, glucose and amino acids

Concentrations of plasma insulin in pigs fed the 20.7%, 12.7% and 16.7% diet were 34.5, 31.6 and 34.6 μU/ml, respectively, at time 0 min (immediately before the injection of L-[ring-$^2H_5$]phenylalanine). The circulating insulin levels were not affected (*P*>.05) by dietary CP levels. The concentration of free phenylalanine in plasma increased

Table 4
Fractional rates of protein synthesis (%/day) measured in tissues of weaned piglets fed low-protein diets supplemented with essential amino acids

Tissue	Dietary CP content%			S.E.M.[a]	*P*
	20.7	16.7	12.7		
Small intestine (proximal)	59.7	54.3	51.5	3.2	.211
Small intestine (distal)	58.3	52.2	50.2	3.4	.242
Colon	42.3	44.2	38.6	1.9	.157
Pancreas	76.4[b]	65.8[c]	62.7[c]	2.3	.003
Liver	83.5[b]	65.5[c]	63.0[c]	4.2	.005
Kidney	36.1[b]	36.9[b]	24.1[c]	1.6	<.001
Longissimus muscle	11.8[b]	8.5[c]	7.1[c]	0.8	.003

[a] Values are means and pooled S.E.M., *n*=6/group. Means in a row without common superscripts differ, *P*< .05 or *P*< .01.

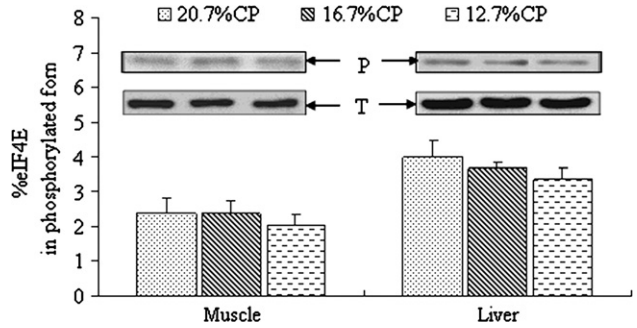

Fig. 1. Abundance and phosphorylation at Ser^{209} of eIF4E in longissimus muscle and liver in weaned piglets fed low-protein diets supplemented with essential amino acids. Values of the phosphorylated eIF4E were normalized for total eIF4E content in samples. Values are means and pooled SEM, n=5-6/group, P>.05. T, total protein; P, phosphorylated protein.

(P<.001) after iv injection of a flooding dose of phenylalanine, but plasma concentrations of free phenylalanine were not influenced by the time of post-injection (data not shown). Additionally, plasma concentrations of other free amino acids (Arg, His, Glu, Gly, Ser and Tyr) in pigs fed the 20.7% CP diet were higher than those in pigs fed the 16.7% or 12.7% CP diets (P< .05) (Table 3).

3.3. Tissue protein synthesis rates

Dietary protein level had no effect on protein synthesis in small intestine or colon (Table 4). The FSR in pancreas, liver and longissimus muscle was reduced as dietary CP levels were decreased (P< .01). In kidney, protein synthesis was lowest in piglets fed the 12.7% CP diet, compared with pigs fed the 16.7% or 20.7% CP diets (P<.001).

3.4. Activation of translation initiation factors

There were no differences among treatments in the percentage of phosphorylated eIF4E in muscle and liver

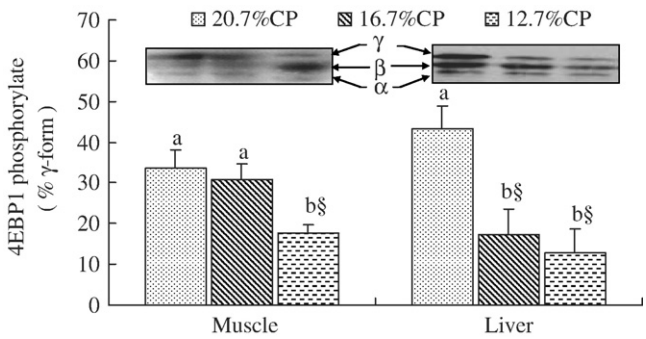

Fig. 2. Phosphorylation of 4E-BP1 in longissimus muscle and liver in weaned piglets fed low-protein diets supplemented with essential amino acids. Phosphorylation of 4E-BP1 was measured in homogenates of longissimus muscle and liver as described in Materials and methods. The graph represents the means of densitometric analysis of several individual immunoblots for γ-4E-BP/4E-BP1. Values are means and pooled S.E.M., n=5–6/group. §P<.01 vs the corresponding 20.7% CP group. a-b: Means sharing different letters within a tissue differ (P<.01).

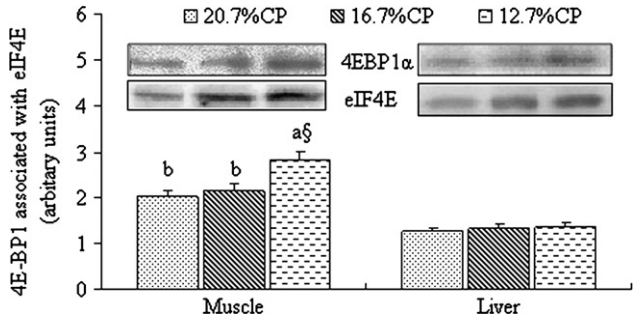

Fig. 3. Assembly of the 4E-BP1·eIF4E complex in longissimus muscle and liver in weaned piglets fed low-protein diets supplemented with essential amino acids. The amount of 4E-BP1α associated with eIF4E was assessed by immunoblotting equal amounts of protein from longissimus muscle and liver. The figure provided means of individual densitometric analysis of several immunoblot of 4E-BP1α associated with eIF4E as described in Materials and methods. Values are means and pooled S.E.M., n=5–6/group. §P<.01 vs the corresponding 20.7% CP group. a-b: Means sharing different letters within a tissue differ (P<.01).

(Fig. 1) or the amount of total and phosphorylated eIF4E (data not shown). 4E-BP1 can be resolved into multiple electrophoretic bands termed α, β and γ, which represent differentially phosphorylated forms of the protein. The most highly phosphorylated form, the γ-form, does not bind eIF4E. In longissimus muscle, pigs fed the 12.7% CP diet had the lowest abundance of 4E-BP1 in the γ-form, compared with the pigs fed the 16.7% or 20.7% CP diet (P<.01). In liver, the abundance of 4E-BP1 in the γ-form decreased with reduced dietary CP content (P<.01) and did not differ between pigs fed the 16.7% or 12.7% CP diets (Fig. 2). In longissimus muscle, the amount of inactive 4E-BP1·eIF4E complex in pigs fed the 12.7% CP diet was 38% and 32% higher (P< .01), respectively, than that in

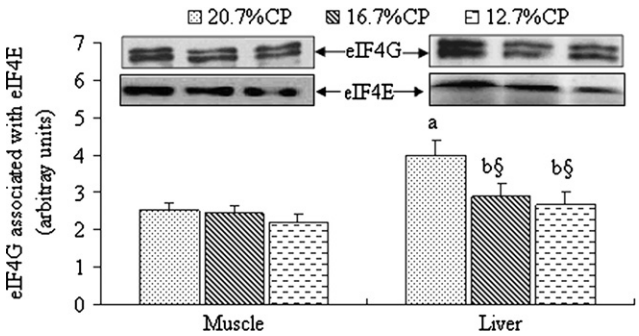

Fig. 4. The eIF4G·eIF4E complex in longissimus muscle and liver in weaned piglets fed low-protein diets supplemented with essential amino acids. Data was obtained for phosphorylated eIF4G by Western blot analysis using samples generated after immunoprecipitation of eIF4E obtained from longissimus muscle and liver. Equal amounts of protein in homogenates from longissimus muscle and liver and equal volumes of immunoprecipitated eIF4E were immunoblotted with antibodies specific for the phosphorylated form of eIF4G (Ser^{1108}). Values are means and pooled S.E.M., n=5–6/group. §P<.01 vs the corresponding 20.7% CP group. a-b: Means sharing different letters within a tissue differ (P<.01).

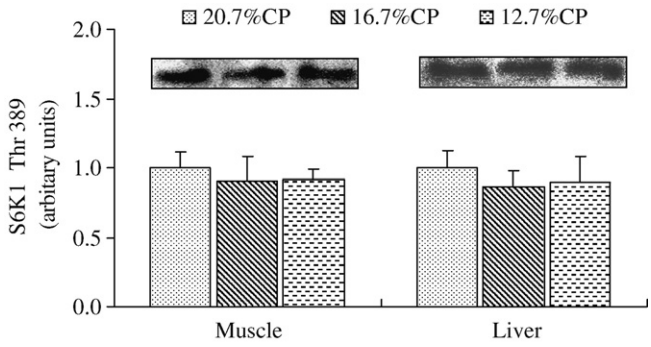

Fig. 5. Phosphorylation at Thr ^{389} of S6K1 in longissimus muscle and liver in weaned piglets fed low-protein diets supplemented with essential amino acids. The value from control pigs fed the 20.7% CP diet was set at 1.0 (arbitrary units). Values are means and pooled S.E.M., n=5–6/group, P>.05.

pigs fed the 16.7% or 20.7% CP diet (Fig. 3). Feeding low-CP diet had no effect on 4E-BP1·eIF4E in liver. However, the association of eIF4G with eIF4E in the liver of pigs fed the 12.7% CP diet was 33% and 30% lower (P<.01), respectively, compared with pigs fed the 16.7% CP or 20.7% CP diet. However, this result was not observed in longissimus muscle (Fig. 4).

The phosphorylation of S6K1 at Thr^{389} in both longissimus muscle and liver was unaffected by dietary CP content. However, S6K1 phosphorylation tended to decrease with reductions in dietary CP levels (Fig. 5). In longissimus muscle, the abundance state of mTOR and phosphorylation state of mTOR at Ser^{2448} were unaffected by protein intake. However, the phosphorylation state of mTOR (% total mTOR) in liver was lower in pigs fed low-protein diets (P<.01), compared with pigs fed the 20.7% CP diet (Fig. 6).

4. Discussion

The results of the current study indicate that feeding a low-protein diet (12.7% CP) to weanling piglets reduced growth performance even though the diet was supplemented with deficient EAA (Table 2). Thus, pigs fed a low-protein diet had a 12% lower weight gain and 16% lower feed efficiency, compared with pigs fed the control (20.7% CP) diet (Table 2). Similar reductions in the efficiency of feed utilization have been observed for pigs fed low-protein diets [11,20–27].

The low growth rate of young pigs fed a protein-deficient diet was likely due to a decrease in muscle protein deposition. The reduction in kidney weight in piglets fed low-protein diets may result, in part, from a reduced renal workload with respect to nitrogenous waste excretion, which was associated with a low rate of renal protein synthesis (Table 4). There was also a decrease in pancreas weight in pigs fed low-protein diets, which may be partly explained by a reduction in protein synthesis (Table 4). This response may reflect a reduced amount of pancreatic enzymes that would be required to digest the smaller quantity of consumed protein. The weight of the longissimus dorsi muscle tended to be reduced with a decline in CP levels during the 2-week experimental period, in agreement with the previous report that there was a positive relationship between dietary protein levels and muscle protein synthesis in young pigs [16]. In addition, a reduction in muscle protein synthesis rate in pigs fed the lower CP levels was observed in the current work (Table 2). Notably, a novel and interesting finding from the present study is that the reduction in protein synthesis with reduced CP levels occurred even when deficient EAA were supplemented to the protein-deficient diet (Table 4).

Insulin and amino acids play important roles in feeding-induced increase of protein synthesis in skeletal muscle of neonates [4,28,29]. The increase in protein synthesis results from the activation of translation initiation factors that regulate mRNA binding to the ribosomal complex [28,30]. However, the previous studies that delineated these effects utilized insulin–glucose–amino acid clamps to acutely infuse insulin and amino acids to piglets. The current study was the first to evaluate the chronic effects of low-protein diets supplemented with deficient EAA on protein synthesis and translation initiation factor activation in animals. The effect of feeding on protein synthesis in neonates is attributed to increased plasma concentration of circulating insulin and amino acids [29,30] that is associated with the activation of translation initiation factors along the insulin-signaling pathway [8]. In the present study, concentrations of insulin showed no change among the pigs fed the three diets. However, it is important to note that total concentrations of amino acids in plasma decreased with reduced dietary CP levels (Table 3). Thus, the reduction in tissue protein synthesis rates in weaned piglets fed low-protein diets is likely due to a decrease in amino acid availability. Recent findings indicate that concentrations of amino acids in plasma were positively correlated with protein synthesis in neonatal pigs [28,30].

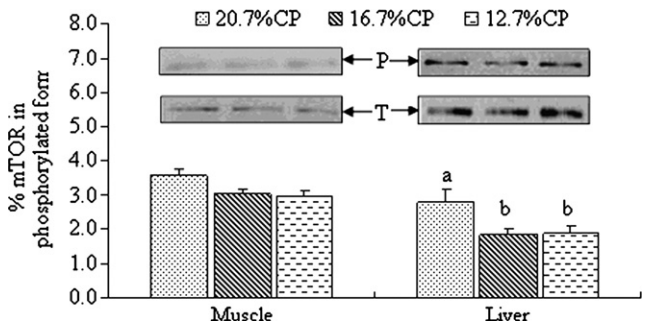

Fig. 6. Abundance and phosphorylation at Ser^{2448} of mTOR in longissimus muscle and liver in weaned piglets fed low-protein diets supplemented with essential amino acids. Values of the phosphorylated mTOR were normalized for total mTOR content in samples. Values are means and pooled S.E.M., n=5–6/group. a-b: Means sharing different letters within a tissue differ (P<.01). T, total protein; P, phosphorylated protein.

Therefore, the reduction in protein synthesis rate may be a necessary part of the metabolic adaptation to chronic protein insufficiency, therefore ensuring minimal nitrogen excretion and maximum utilization of dietary protein [31]. This would allow the organism to maintain a nitrogen balance and survive. In addition, crystalline amino acids are absorbed more rapidly than protein-bound amino acids in pigs [32]. This results in a temporary surplus of absorbed crystalline amino acids at sites of protein synthesis when pigs are fed diets supplemented with these nutrients once daily [32]. However, protein synthesis in the small intestine did not decrease with reduced protein intake (Table 4) probably because intestinal catabolism of dietary EAA was reduced during first-pass metabolism [33,34], and the 12.7% CP diet provided adequate amounts of amino acids to support protein synthesis in the gut. Interestingly, Frank et al. [35] did not observe consistent differences in protein synthesis in any tissue of pigs fed increasing levels of dietary protein. This result may be explained by the ability of the lowest protein diet to provide sufficient amounts of amino acids to maintain basal rates of protein synthesis during a short period of time.

The activation of mTOR, a major protein kinase that modifies translation initiation components, is regulated by nutrients and insulin [36–38]. The phosphorylation of mTOR on Ser^{2448}, which activates the kinase, is stimulated by both insulin and amino acids [38]. In this study, we found that phosphorylation of mTOR (% total mTOR) in liver was lower in pigs fed low-protein diets ($P<.01$), compared with pigs fed a 20.7% CP diet (Fig. 6). In contrast, the abundance and phosphorylation of mTOR were similar in longissimus muscle among the treatment groups, indicating tissue specificity of the response to dietary protein intake. S6K1 and 4E-BP1 (a translational repressor protein) act downstream of mTOR in the translation initiation pathway. mTOR controls the response of the translation initiation machinery to amino acids and growth factors via activation of S6K1 and 4E-BP1 [39]. Increase in S6K1 phosphorylation results in hyperphosphorylation of ribosomal protein S6 and, thus, facilitates the translation of mRNA to generate protein. In the present study, the levels of phosphorylated S6K1 were unaffected by dietary CP levels in longissimus muscle and liver, whereas previous reports of increased S6K1 phosphorylation were likely due to the rise in circulating levels of insulin [35].

A key step in translation is binding of the 43S preinitiation complex to mRNA [3]. This event is mediated by eIF4F, a complex of proteins that includes eIF4E. Assembly of the eIF4F complex is regulated by the phosphorylation or availability of eIF4E [4]. eIF4E phosphorylation status, indicated as a percentage of total eIF4E, was examined because it influences mRNA binding to the 43S preinitiation complex, resulting in increased protein synthesis in cell culture [40,41]. However, results of the present study indicate that dietary CP content had no effect on eIF4E phosphorylation in longissimus muscle and liver (Fig. 1). These results suggest that in vivo modulation of eIF4E phosphorylation status is unlikely to play a key regulatory step in muscle and hepatic protein synthesis in young pigs. Alternatively, an effect of dietary CP on eIF4E phosphorylation could have occurred at an earlier time point. It is noteworthy that most of the previous work on cell signaling was conducted with cultured cells in vitro under experimental conditions that may not be physiologically relevant and that little is known about mTOR signaling in animal tissues in vivo. We suggest that S6K1 plays a minor role in modulating the response of muscle protein synthesis to alterations in intakes of dietary protein.

One potential mechanism to account for decreased formation of the eIF4G·eIF4E complex involves a reduction in the availability of eIF4E bound to eIF4G. eIF4E availability is controlled through a family of 4E-binding proteins (4EBPs) [42], which function as translational repressors by competing with eIF4G for a common binding site on eIF4E [43]. 4EBP activity is regulated by phosphorylation; the nonphosphorylated isoforms of 4EBPs bind to eIF4E with high affinity and prevent it from binding to eIF4G and forming the translationally active eIF4F complex [43]. Conversely, the phosphorylation of 4EBPs reduces the binding affinity for eIF4E and relieves translational repression, as eIF4E is able to bind to eIF4G. In this study, reducing dietary CP levels decreased 4E-BP1 phosphorylation in longissimus muscle and liver (Fig. 2). Interestingly, the amount of inactive 4E-BP1·eIF4E complex increased with decreased dietary protein levels in longissimus muscle but not in liver (Fig. 3). The formation of an eIF4G-eIF4E complex was reduced with decreasing dietary CP levels in liver but not in longissimus muscle (Fig. 4), indicating a tissue-specific response.

To our knowledge, the results of the present study demonstrate, for the first time, that chronic feeding of a low-protein diet suppresses protein synthesis in extraintestinal tissues of animals, in part, by inhibiting mTOR signaling. Additionally, our findings indicate that supplementing deficient EAA to 12.7% CP diets was not effective in restoring protein synthesis or whole-body growth in weanling piglets. Thus, supplementing conditionally essential amino acids to low-protein diets may be necessary to maintain activation of translation initiation factors and optimal protein synthesis in tissues of young mammals. These amino acids may include glutamine, arginine and proline, which can be synthesized by weanling piglets [44,45], but synthesis rates are inadequate for supporting optimal function and growth of the neonates [46–48]. In support of this view, we found that dietary supplementation with arginine or glutamine stimulated whole-body growth in young pigs [49,50]. Future studies involving the piglet model as well as molecular and cellular biology techniques [51–54] are warranted to determine if supplementing conditionally essential amino acids to low-protein diets can enhance tissue protein synthesis and growth in young mammals.

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