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## Enteral Leucine Supplementation Increases Protein Synthesis in Skeletal and Cardiac Muscles and Visceral Tissues of Neonatal Pigs through mTORC1-dependent Pathways

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### Abstract

Leucine (Leu) activates mammalian target of rapamycin (mTOR) to upregulate protein synthesis (PS). To examine enteral Leu effects on PS and signaling activation, 5-d-old piglets were fed for 24 h diets containing: 1) low protein (LP), 2) LP supplemented with Leu (LP+L), or 3) high protein (HP). PS in skeletal muscles, heart, liver, pancreas, and jejunum, but not kidney, were greater in LP+L than LP, but lower than HP. In longissimus dorsi muscle, protein kinase B phosphorylation was similar in LP and LP+L, but lower than HP. Although less than HP, p70 ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor (eIF) 4E binding protein 1 (4EBP1) association with regulatory associated protein of mammalian target of rapamycin was greater in LP+L than LP, resulting in higher S6K1 and 4EBP1 phosphorylation. Feeding LP+L versus LP decreased 4EBP1-eIF4E and increased eIF4E-eIF4G formation, but not to HP. Similar results were obtained for S6K1 and 4EBP1 phosphorylation in gastrocnemius, masseter, heart, liver, pancreas, and jejunum, but not kidney. eIF2 $\alpha$  and elongation factor 2 phosphorylation was unaffected by treatment. Our results suggest that enteral Leu supplementation of a low protein diet enhances PS in most tissues through mTOR complex 1 pathways.

### INTRODUCTION

Many babies are born preterm and despite improvements in their care, their extrauterine growth frequently falters (1–3). Although premature infants may be initially provided parenteral nutrition, the goal is to initiate and advance enteral feeding (4). However, enteral feedings is often limited by concern for the preterm infant's ability to metabolize nutrients and clinical complications related to feeding intolerance and necrotizing enterocolitis (5–7). Thus, enteral feeding may not achieve the level of protein required to sustain optimal growth.

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To identify strategies to optimize the nutrition of neonates, we have used the neonatal pig as a model of the human infant. We found that feeding stimulates PS in skeletal muscle of neonatal pigs (8), and this response is independently mediated by the rise in insulin and amino acids (AA) (9–10). Leucine is the most effective single AA to trigger the activation of translation initiation factors that regulate muscle PS (11–16), but the effects of leucine in visceral tissues are not well understood. We have shown that parenteral AA infusion in neonates stimulates the intracellular signaling proteins that regulate PS (17), however, less is known about the effects of enteral AA delivery on the activation of this pathway.

AA, especially leucine, serve as substrates for PS and also as nutrient signals to stimulate translation initiation (11–17). Unlike insulin, AA do not activate protein kinase B (PKB) but activate the mammalian target of rapamycin (mTOR) (16, 18), which functions in two distinct protein complexes (mTORC1 and mTORC2) (19). mTORC1, a major player for mRNA translation, consists of several components, including regulatory associated protein of mammalian target of rapamycin (raptor, an activator) and proline-rich Akt substrate of 40 kDa (PRAS40, an inhibitor) (20), whose association is crucial for mTORC1 activation. Activated mTORC1 promotes eukaryotic initiation factor (eIF) 4E-binding protein 1 (4EBP1) and ribosomal protein S6 kinase-1 (S6K1) phosphorylation. Phosphorylated 4EBP1 allows active eIF4E-eIF4G complex formation and activated S6K1 phosphorylates ribosomal protein S6. Both processes are crucial for translation initiation (21).

Other crucial steps for mRNA translation are the eukaryotic initiation factor (eIF2) pathway (22) and the peptide elongation process. Phosphorylation of eIF2 $\alpha$  (an inhibitor) is a limiting step in the eIF2 pathway while elongation factor 2 (eEF2) phosphorylation regulates elongation (23). Insulin and AA modulate eIF2 $\alpha$  and eEF2, resulting in PS progression.

Recently, we showed that enteral leucine supplementation of a low protein meal acutely stimulates PS (24). However, it is unclear whether the effects could be sustained by prolonged enteral leucine supplementation. Although prolonged intravenous leucine infusion alone can reduce circulating levels of essential AA, which can limit PS (17), whether this occurs with prolonged enteral leucine supplementation is unknown as a decline in other AA was not observed in our previous acute enteral leucine supplementation study (24). Therefore, the aims of this study were to determine whether feeding of a low protein diet supplemented with leucine for 24 h can sustain enhanced rates of muscle and visceral tissue PS in neonatal pigs to rates similar to those achieved with a high protein diet and to examine the mechanisms involved.

## MATERIALS AND METHODS

### Animals

Sows and piglets were managed as previously described (14, 15). After birth, piglets suckled ad libitum and were not given supplemental creep feed. At 2 d of age, indwelling catheters were surgically inserted into the jugular vein and carotid artery (25). The Animal Care and Use Committee of Baylor College of Medicine approved all experimental procedures. This study was conducted in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals*.

### Treatments and infusion

Overnight fasted 5-d-old piglets ( $2.3 \pm 0.1$  kg) were randomly assigned to 1 of the 3 dietary treatment groups: low protein (LP), low protein supplemented with leucine (LP+L), or high protein (HP) diets (Table 1 and 2;  $n = 7-9$  pigs). The LP+L diet provided an equivalent amount of leucine as the HP diet. All animals were gavage fed at a rate of  $40 \text{ ml} \cdot \text{kg}^{-1}$  at time 0 min and every 4 h for 24 h; the feeding was administered over a 15 min period. Protein provided in the meal was  $0.83$  and  $3.33 \text{ g} \cdot \text{kgbody weight (wt)}^{-1}$  for the LP and HP diets, respectively. Diets were isocaloric and contained the same lactose level. Blood samples were collected at intervals after feeding initiation for measurement of individual plasma AA, insulin, and glucose concentrations. At 25 h, piglets were injected with L[4-<sup>3</sup>H] phenylalanine to measure PS and killed 30 min later. Samples of longissimus dorsi, gastrocnemius, and masseter muscles, heart, liver, pancreas, kidney, and jejunum were obtained for measurements of PS rate and nutrient signaling activation.

### Plasma hormones and substrate assays

Blood glucose concentrations were analyzed using a YSI 2300 STAT Plus (Yellow Springs Instruments, Yellow Spring, OH). Plasma total branched-chain AA (BCAA) were analyzed by rapid enzymatic kinetic assay (26). Individual AA were measured by HPLC (PICO-TAG reverse-phase column; Waters, Milford, MA) (27). Plasma radioimmunoreactive insulin concentrations were measured using a porcine insulin radioimmunoassay kit (Linco, St. Louis, MO).

### Tissue PS in vivo

Fractional rates of PS were measured with a flooding dose of  $1.5 \text{ mmol} \cdot \text{kg body wt}^{-1}$  of L[4-<sup>3</sup>H]phenylalanine ( $18.5 \text{ MBq} \cdot \text{kg}^{-1}$  body weight; American Radiolabeled Chemicals Inc., St. Louis, MO) (28). Piglets were killed 90 min after the last meal because our previous meal feeding study (29) showed increased PS from 0.5 to 2 h after the meal. Tissue samples were immediately frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until analyzed.

### Protein immunoblot analysis

Proteins from tissue homogenates were separated on polyacrylamide gels (PAGE). Samples were run at the same time on triple-wide gels to eliminate inter-assay variation. Proteins were electrophoretically transferred to polyvinylidene difluoride transfer membranes (Pall Corporation, Pensacola, FA), incubated with primary antibodies, washed, and exposed to secondary antibody (16).

For normalization, immunoblotting performed with antiphospho-specific antibodies were stripped and reprobbed with corresponding nonphospho-specific antibodies. Blots were visualized and analyzed using a ChemiDoc-It Imaging System (UVP, Upland, CA). Primary antibodies were PKB (total and Ser<sup>473</sup>, Cell Signaling Technology Inc., Danvers, MA), mTOR (total and Ser<sup>2448</sup>, Cell Signaling), PRAS40 (Total and Thr<sup>246</sup>, Cell Signaling), 4EBP1 (total, Bethyl Laboratories Inc., Montgomery, TX and Thr<sup>70</sup>, Cell Signaling), eIF4G (total and Ser<sup>1180</sup>, Cell Signaling), S6K1 (total and Thr<sup>398</sup>, Cell Signaling), eIF2 $\alpha$  (Total and Ser<sup>51</sup>, Cell Signaling), and eEF2 (Total and Thr<sup>56</sup>, Cell Signaling).

### Quantification of protein-protein interaction

eIF4E-eIF4G and eIF4E-4EBP1 complexes were immunoprecipitated using an anti-eIF4E monoclonal antibody (Dr. Leonard Jefferson, Penn State University College of Medicine, Hershey, PA) followed by immunoblotting with 4EBP1 (Bethyl Laboratories) or eIF4G antibodies (16). For analysis of protein-protein interaction of members of the mTOR complex 1 (mTORC1), homogenates were immunoprecipitated using an anti-raptor antibody (Cell Signaling) (30). Western blot analysis using mTOR, S6K1, 4EBP1, and PRAS40 were conducted to determine interaction with raptor. Protein-protein interaction was normalized by eIF4E or raptor abundance.

### Calculations and statistics

Fractional rates of PS (Ks, percentage of protein mass synthesized in a day) were calculated as  $K_s (\%/day) = [(S_b/S_a) \times (1,440/t)] \times 100$ , where  $S_b$  (in  $dpm \cdot nmol^{-1}$ ) is the specific radioactivity of the protein-bound phenylalanine,  $S_a$  (in  $\cdot nmol^{-1}$ ) is the specific radioactivity of the tissue free phenylalanine,  $t$  is labeling time in min, and 1,440 is the min-to-d conversion (31).

Statistical analysis was carried out in SPSS (Version 17.0). A protected post hoc least significant differences t test was used to determine differences between groups if, after performing a 1-way ANOVA, a significant difference was found. Analysis of glucose and insulin across time was carried out with SPSS General Linear Model using repeated measures test for within-subject effects. Differences of  $< 0.05$  were considered significant and data are presented as means  $\pm$  SEM.

## RESULTS

### Plasma insulin, glucose, and AA

Plasma insulin concentrations increased over time ( $p < 0.05$ ) in a cyclic pattern with each bolus feed and returned to fasting levels by 4 h in all groups (Fig. 1A). Insulin did not differ between LP, LP+L, and HP except at 1530 min (90 min after the last meal) when insulin levels were higher in HP than LP and LP+L ( $p < 0.05$ ). In all groups, blood glucose concentrations over time were similar (Fig. 1B). BCAA concentrations changed with time in the three groups ( $p < 0.05$ ) in a cyclic pattern with each bolus feed and returned to baseline after 4 h, except for HP (Fig. 1C;  $p < 0.05$ ). BCAA concentrations at 1530 min were higher in LP+L than LP, but were lower than in HP ( $p < 0.05$ ).

Plasma levels of most essential and nonessential AA increased over time in the three groups ( $p < 0.05$ ; data not shown). Most nonessential AA at 1530 min were similar in LP and LP+L, but were greater in HP ( $p < 0.05$ ; Fig. 2A); plasma glutamine levels were higher in LP+L than LP ( $p < 0.05$ ). Most essential AA at 1530 min were higher in HP than LP and LP+L ( $p < 0.05$ ; Fig. 2B). Plasma isoleucine and valine concentrations differed between groups at 1530 min with lower levels in LP+L ( $p < 0.05$ ).

### PS and signaling components in fast-twitch, glycolytic muscle

In the longissimus dorsi, a muscle containing primarily fast-twitch, glycolytic fibers, PS was greater in HP than LP and LP+L (Fig. 3A), and the LP+L group was higher than in LP ( $p < 0.05$ ). PKB phosphorylation in longissimus dorsi muscle was higher in HP than LP and LP+L ( $p < 0.05$ ; Fig. 3B). Phosphorylation of S6K1 and 4EBP1 was greater in LP+L than LP ( $P < 0.05$ ) and further increased in HP ( $p < 0.05$ ; Fig. 3C and D). The association of mTOR with raptor and PRAS40 with raptor did not differ between groups (Fig. 4A and B). However, S6K1·raptor and the 4EBP1·raptor complexes were higher in LP+L than LP, but did not reach HP ( $p < 0.05$ ; Fig. 4C and D).

The abundance of 4EBP1·eIF4E in longissimus dorsi muscle was lower in LP+L than LP, but higher than HP ( $p < 0.05$ ; Fig. 5A). The abundance of the active eIF4E·eIF4G complex was greater ( $p < 0.05$ ) in LP+L than LP but was greater in HP ( $p < 0.05$ ; Fig. 5B). Phosphorylation of eIF2 $\alpha$  and eEF2 was unaffected by treatment (Fig. 5C and D).

### PS and signaling components in oxidative muscles

PS in gastrocnemius muscle, which contains mixed glycolytic and oxidative fibers, masseter muscle containing primarily oxidative fibers, and cardiac muscle which has oxidative fibers, were greater in LP+L than LP but were greatest in HP ( $p < 0.05$ ; Fig. 6). S6K1 and 4EBP1 phosphorylation in these tissues was greater in LP+L than LP ( $p < 0.05$ ) although highest in HP ( $p < 0.05$ ; Table 2). There were no treatment effects on eIF2 $\alpha$  and eEF2 phosphorylation (Table 3).

### PS and signaling components in visceral tissues

PS in liver, jejunum, and pancreas, but not kidney, was higher in LP+L than LP, but lower than HP ( $p < 0.05$ ; Fig. 7). Leucine supplementation increased S6K1 and 4EBP1 phosphorylation in visceral tissues, except kidney, although not to HP ( $p < 0.05$ ; Table 2). eIF2 $\alpha$  and eEF2 phosphorylation was unaffected by treatment (Table 3).

## DISCUSSION

Postnatal nutrition has a critical impact on the long-term outcome of preterm infants (32). Short-term decreases in dietary intake are inevitable consequences in the complex care of preterm infants (33). Preterm infants frequently have poor tolerance to enteral feeding and thus consume a lower volume of milk formula than that necessary to meet their protein requirements for adequate growth. Recently, we showed that feeding a meal of a low protein milk formula supplemented with leucine compared to a low protein formula meal alone acutely increases PS in most tissues to rates similar to that achieved with a high protein meal (24). In the current study, we wished to determine whether the anabolic response to leucine supplementation could be sustained for a more prolonged period. The results of the current study indicate that prolonged leucine supplementation of a milk formula with a low protein content can enhance PS compared to the low protein diet alone. Although the leucine-induced increase in PS was lower than that with a high protein milk formula, our findings suggest a potential beneficial effect of including leucine supplementation in the nutritional

management plan for sick premature infants. However, limitations of the study include the lack of a normal suckling pig control and the use of term rather than preterm piglets.

Previously, we showed that parenteral leucine infusion for 24 h reduced circulating levels of other AA and limited the leucine-induced increase in PS, likely because other AA were needed as substrates for PS (17). However, when the fall in other AA was prevented by performing an AA clamp, the leucine-induced increase in PS was sustained. Recently, we found that acute enteral leucine supplementation of a LP meal did not elicit a fall in other AA and increased PS similar to that of a HP meal (24). In the current study, consumption of the LP+L compared to LP diet for 24 h reduced circulating isoleucine and valine concentrations, likely due to activation of enzymes that metabolize BCAA, namely branched-chain aminotransferase and branched-chain  $\alpha$ -ketoacid dehydrogenase (34). Unlike in our previous acute enteral leucine supplementation study (24), the reduction in isoleucine and valine in the current study may be responsible for the failure to achieve maximum PS rates obtained with the HP diet. On the other hand, circulating insulin levels in the current study were higher in the HP than LP and LP+L groups, unlike our previous acute leucine supplementation study, and may have contributed to the higher PS rate, at least in muscle, but only in the HP group.

The positive effect of leucine administration on muscle PS in humans and animal models has been recognized (11–13, 35). In this study, we determined effects of leucine supplementation on muscles of different fiber types including longissimus dorsi (mainly glycolytic fibers), gastrocnemius (mixed glycolytic and oxidative fibers), masseter (oxidative fibers) and cardiac muscles (oxidative fibers). Consistent with our 24 h parenteral leucine infusion study (17), in the current study enteral leucine supplementation stimulated PS in muscles of different fiber types, although the leucine effect was less potent than the HP diet, which was used as a positive control to elicit maximum PS rates. In our previous study, acute leucine supplementation of a LP meal enhanced PS in these muscles comparable to the HP meal (24), likely due to maintenance of isoleucine and valine levels.

To better understand the overall anabolic effect of leucine supplementation in neonates, we determined PS rates in visceral tissues. We showed that leucine supplementation enhanced PS in liver, pancreas, and jejunum, but not kidney. In our previous 24 h parenteral leucine infusion study, the leucine-induced increase in PS was detected in liver and pancreas, although not jejunum or kidney, and only when AA availability was maintained (17). Differences between these two studies in the jejunum response could be due to the different route of leucine administration (i.e., parenteral versus enteral). The lack of effect in the kidney may be due to rerouting excess AA to gluconeogenesis (36).

To evaluate molecular mechanisms by which leucine stimulates PS *in vivo*, we determined the leucine-induced activation of signaling components leading to mRNA translation. More detailed study of signaling component activation was conducted in longissimus dorsi muscle. PKB activation is a hallmark for insulin-induced PS (37). Consistent with other studies (16, 18), we found that leucine supplementation had no effect on PKB activation in muscle. The recent consensus is that AA, particularly leucine, stimulate PS in an mTORC1-dependent pathway downstream of PKB (38) although the exact molecular mechanism is not



completely understood. Phosphorylation of two major targets of mTORC1, S6K1 and 4EBP1, has been used as a read-out for mTORC1 activation (38). Likewise, S6K1 and 4EBP1 association with mTORC1 through raptor is a bona fide indicator of mTORC1 activation (39, 40). Cell culture studies suggest that mTOR-raptor association positively and PRAS40-raptor association negatively regulates mTORC1 activation (41). In our study, leucine supplementation enhanced S6K1 and 4EBP1 association with raptor, which elicits increased phosphorylation of these two mTOR substrates. These results are consistent with cell culture studies that showed that, for mTORC1 to phosphorylate S6K1 and 4EBP1, raptor has to bind to them through their TOR signaling motif (39, 40). We did not find a leucine effect on raptor association with mTOR or PRAS40, which could be due to a transient nature of their interaction *in vivo*. A leucine-induced increase in S6K1 and 4EBP1 phosphorylation was also observed in muscles with different fiber types and visceral tissues, except kidney. Increased 4EBP1 phosphorylation leads to decreased association with eIF4E, allowing active eIF4G-eIF4E complex formation (38). In this study we found that leucine supplementation reduced 4EBP1-eIF4E complex formation and enhanced eIF4G-eIF4E complex formation.

In the current study, plasma glutamine levels were elevated in the LP+L compared to LP group suggesting *de novo* synthesis from exogenous leucine. Cell culture studies suggest that glutamine is needed for leucine-induced activation of mTORC1 (42), and thus, together with leucine, glutamine plays a crucial role in the regulation of PS.

Another potential signaling pathway mediating the leucine-induced stimulation of PS is the eIF2 pathway (22). In this study, we examined phosphorylation of eIF2 $\alpha$ , an inhibitor and key player in this pathway. We found that no effect of leucine on eIF2 $\alpha$  phosphorylation in all tissues studied. This result is consistent with the notion that leucine does not directly affect eIF2 $\alpha$  phosphorylation but eIF2 $\alpha$  phosphorylation status is affected by essential AA deficiency (43).

The elongation process in mRNA translation is another crucial step in the PS process (23). In cell culture studies, AA, especially leucine, activate eEF2, a marker for peptide chain elongation, by dephosphorylation (44). Thus, we determined whether leucine has similar effects *in vivo*. As in our previous study (16), leucine had no effect on eEF2 phosphorylation in all tissues studied. This suggests that regulation of elongation by AA *in vivo* is complex and may differ from responses found *in vitro*.

In summary, 24 h enteral leucine supplementation of a LP formula diet enhanced PS in most tissues of neonatal pigs in an mTORC1-dependent fashion. Although the leucine-induced increase in PS was less than that achieved with a HP diet, the results of our study suggest that leucine supplementation may have a beneficial effect on neonatal growth. Further studies are required to better understand the role of leucine and BCAA in the regulation of neonatal growth.

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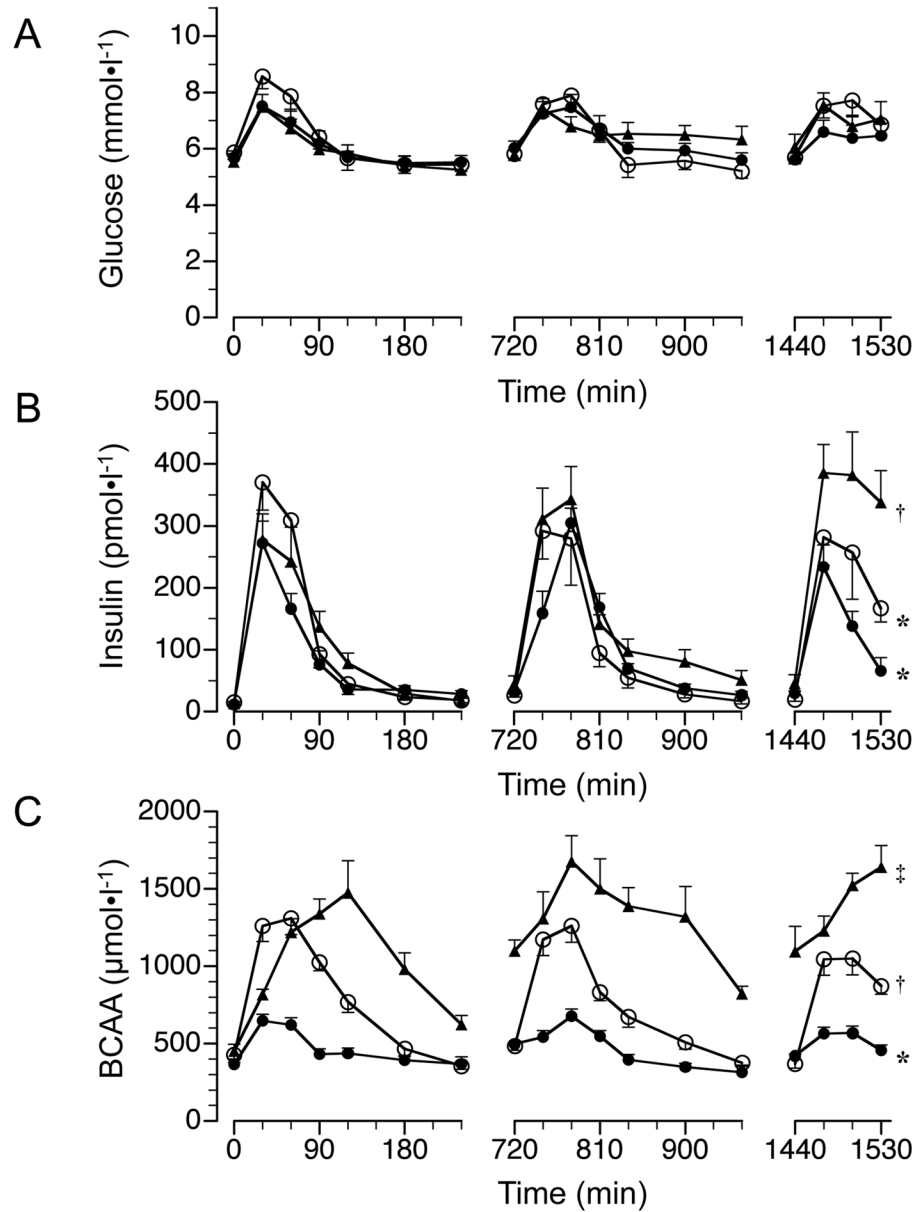
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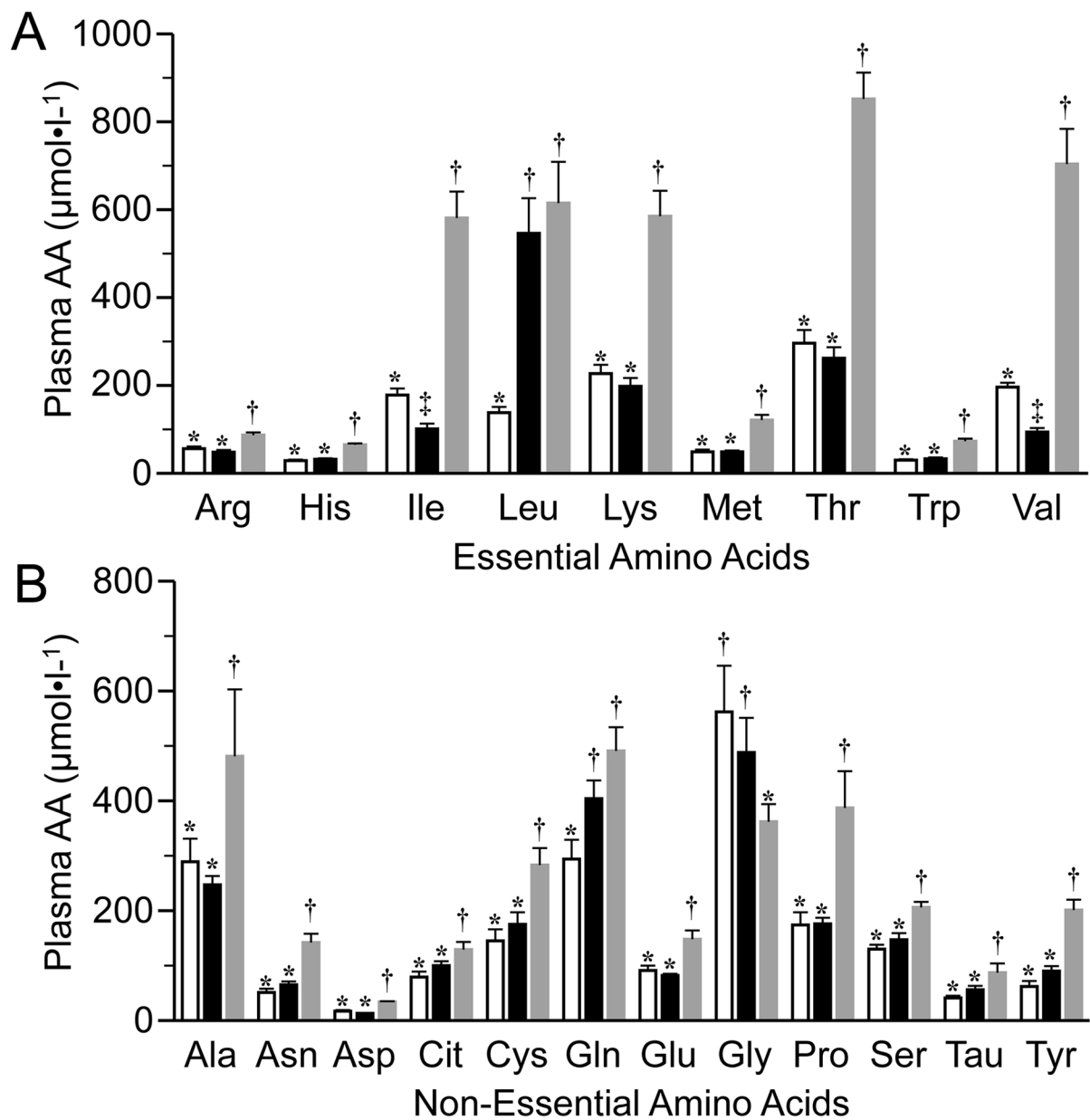


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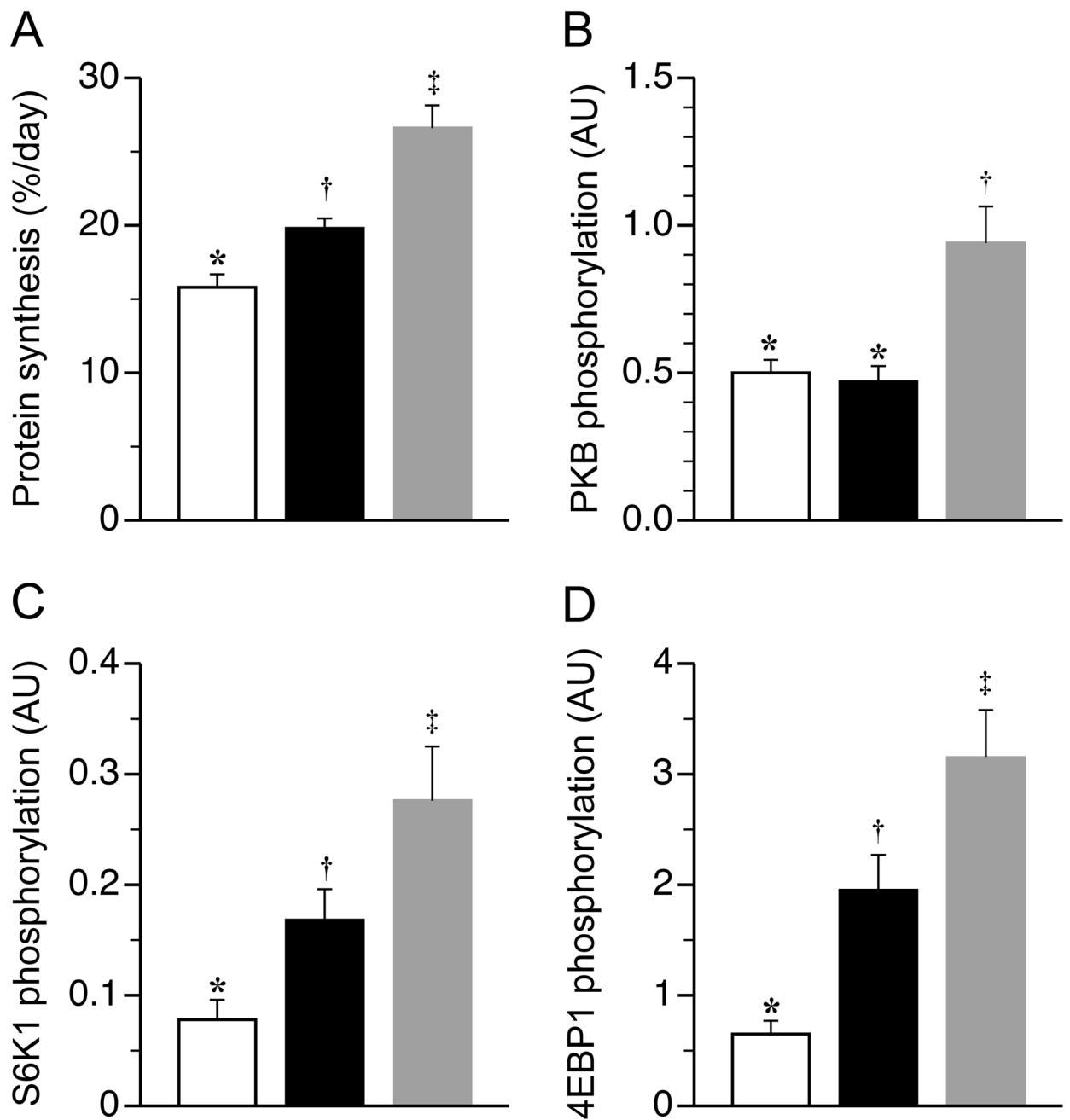


**Figure 1.** Circulating insulin (A), glucose (B), and BCAA (C) concentrations in piglets fed LP (●), LP +L (○), and HP (▲) diets every 4 h for 24 h. Values are means + SEM, n = 7–10 per treatment. Means without a common symbol differ,  $p < 0.05$ .

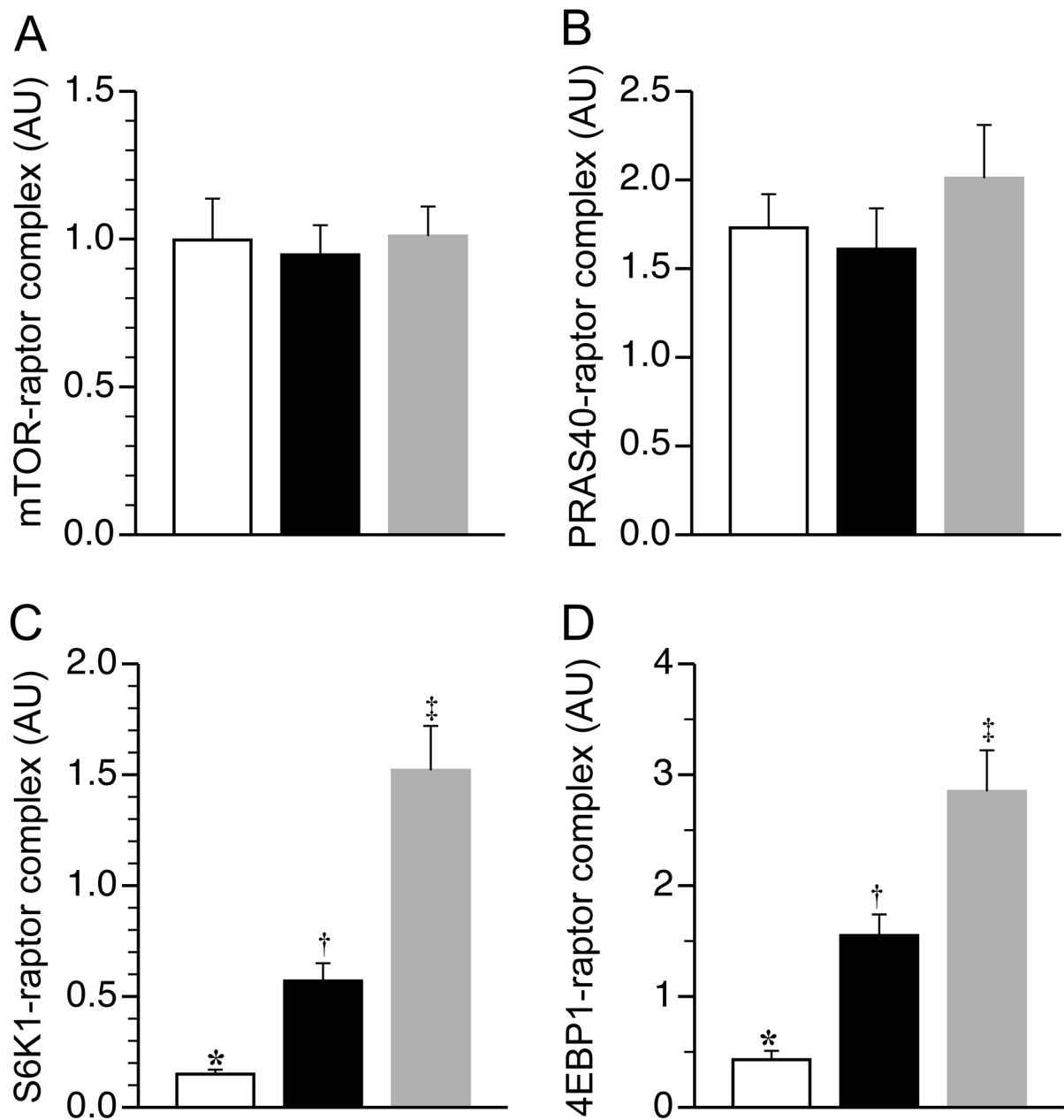


**Figure 2.**

Plasma concentrations of essential AA (A) and nonessential AA (B) in piglets fed LP ( $\square$ ), LP+L ( $\blacksquare$ ), or HP ( $\blacksquare$ ) diets for 1530 min. Values are means + SEM,  $n = 7-10$  per treatment. Means without a common symbol differ,  $p < 0.05$ .

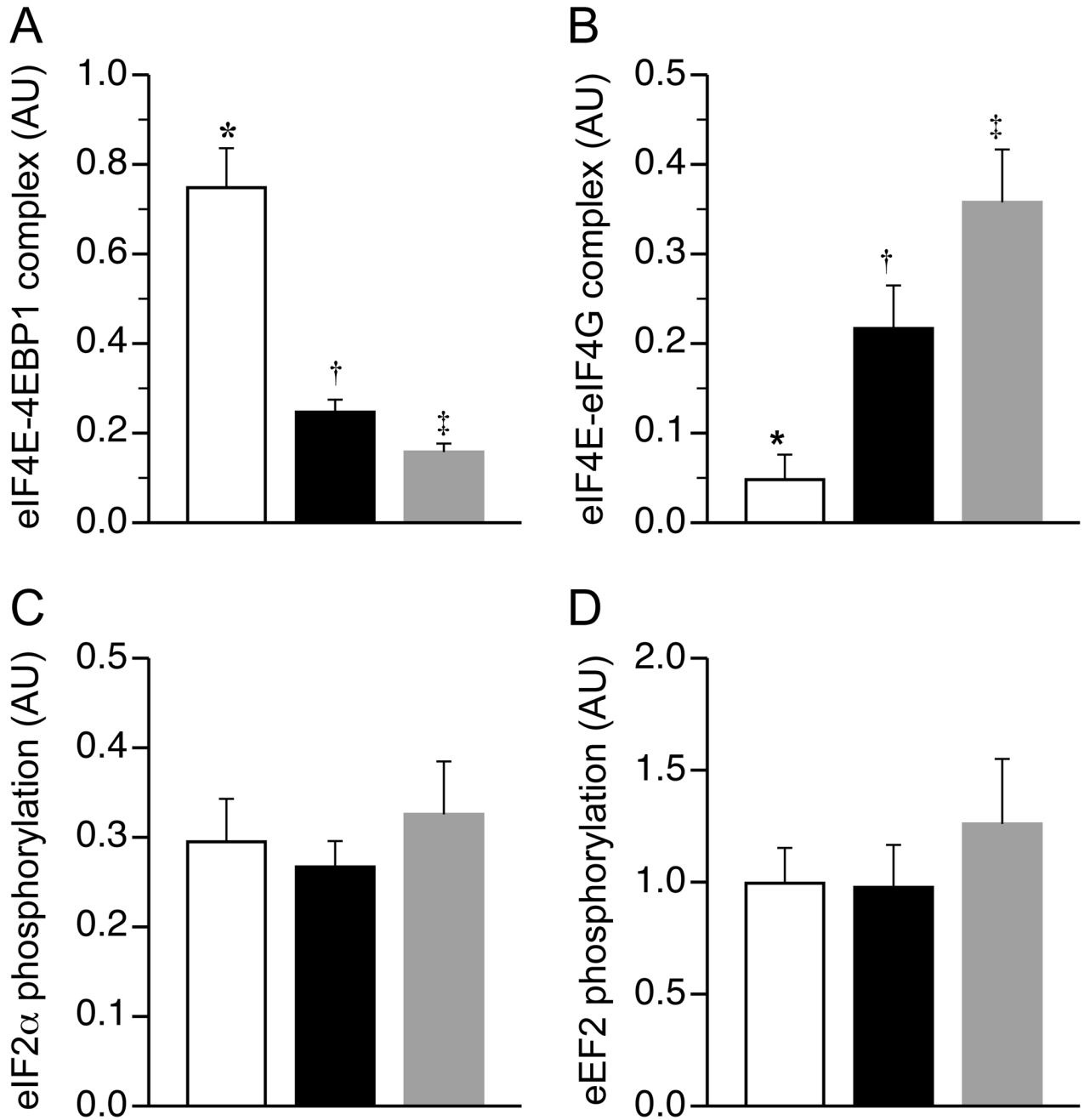


**Figure 3.** PS rates (A) and phosphorylation of PKB (B), S6K1 (C) and 4EBP1 (D) in longissimus dorsi muscle of piglets fed LP (□), LP+L (■), or HP (■) diets. Values are means + SEM, n = 7–10 per treatment. Means without a common symbol differed,  $p < 0.05$ .



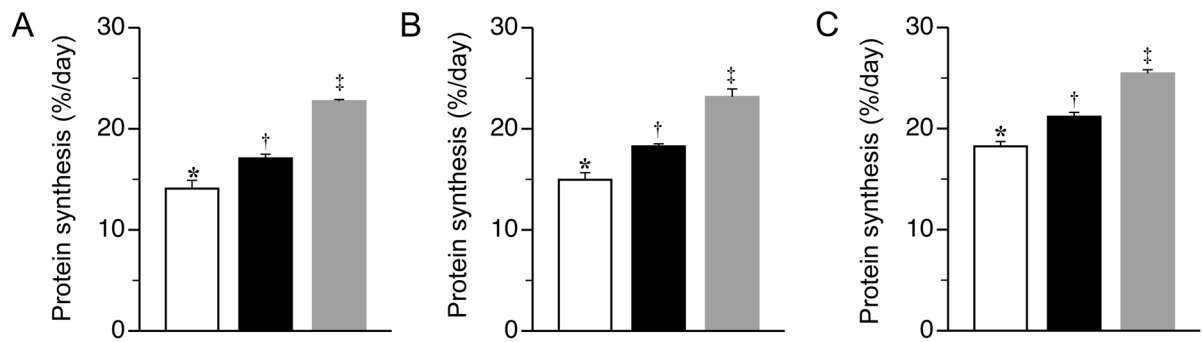
**Figure 4.** Abundance of raptor-mTOR (A), raptor-PRAS40 (B), raptor-S6K1 (C) and raptor-4EBP1 (D) complexes in longissimus dorsi muscle of piglets fed LP (□), LP+L (■), or HP (▒) diets. Values are means + SEM, n = 7–10 per treatment. Means without a common symbol differed,  $P < 0.05$ .





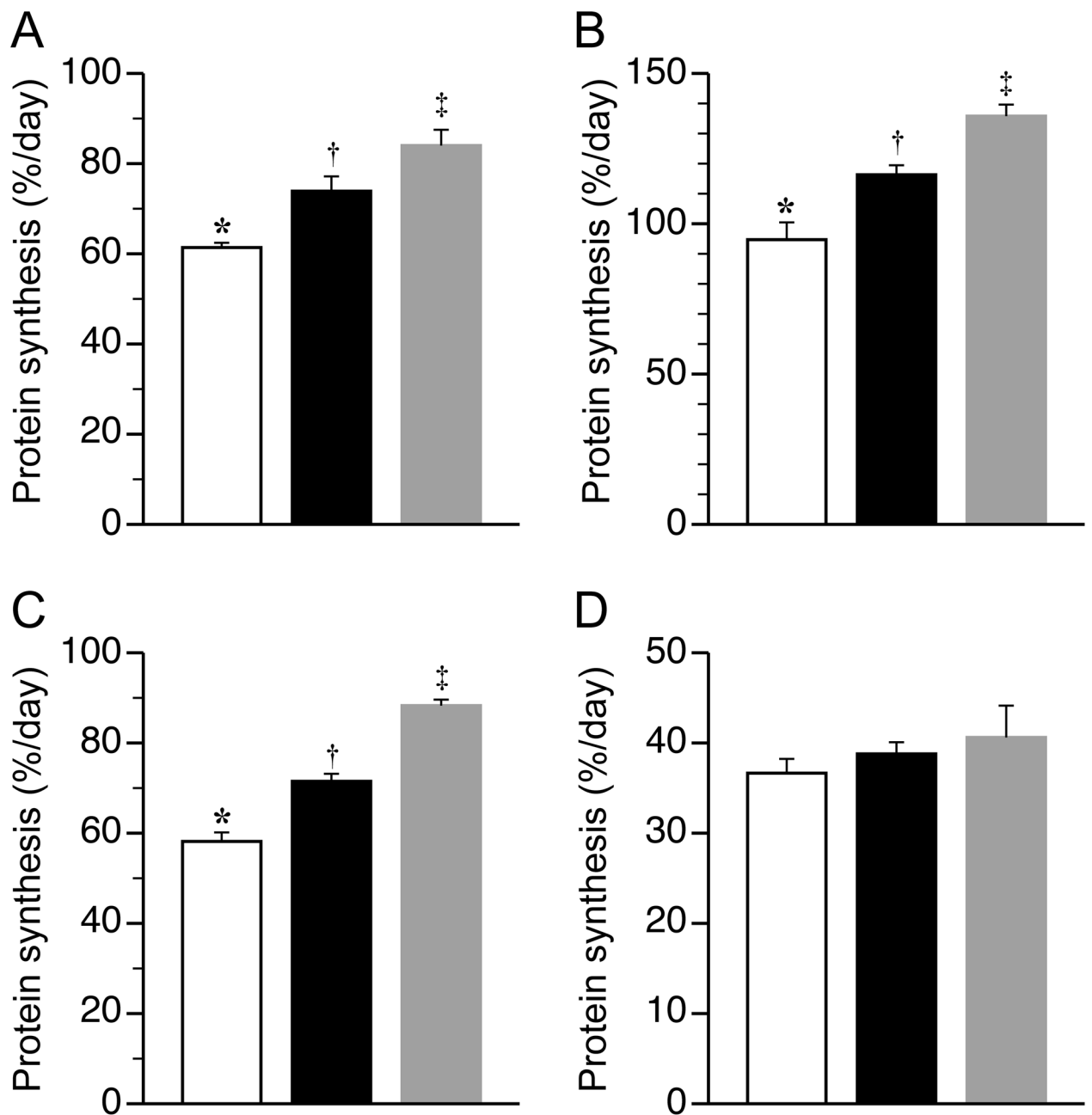
**Figure 5.**

Abundance of eIF4E-4EBP1 (A) and eIF4E-eIF4G (B), and phosphorylation of eIF2 $\alpha$  (C) and eEF2 (D) in longissimus dorsi muscle of piglets fed LP (□), LP+L (■), or HP (▒) diets. Values are means + SEM, n = 7–10 per treatment. Means without a common symbol differed,  $p < 0.05$ .



**Figure 6.**

PS rates in gastrocnemius (A), masseter (B), and heart (C) muscles of piglets fed LP (□), LP +L (■), or HP (■) diets. Values are means + SEM, n = 7–10 per treatment. Means without a common symbol differed,  $p < 0.05$ .



**Figure 7.** PS rates in liver (A), pancreas (B), jejunum (C), and kidney (D) of piglets fed LP (□), LP+L (■), or HP (■) diets. Values are means + SEM, n = 7–10 per treatment. Means without a common symbol differed,  $p < 0.05$ .

Table 1

## Composition of experimental diets

	LP	LP+L	HP
	<i>% of premix</i>		
<b>Ingredients</b>			
Whey Protein*	8.6	8.7	52
Lactose**	3.0	3.2	2.9
Fat Pak 80†	40.0	38.0	17
Water	44.0	42.0	24
Vitamin premix‡	0.35	0.35	0.35
Trace mineral premix‡	0.25	0.25	0.25
Xanthan Gum‡	1.0	1.0	1.0
Dicalcium	3.0	3.0	2.3
MgSO <sub>4</sub>	0.01	0.01	0.01
KCl	0.01	0.01	0.01
<b>Nutrition Composition§</b>			
Energy (kJ)	14,120	14,120	14,120
Protein %	8.3	8.3	42
Fat %	32	31	16
Lactose %	6.0	6.0	6.0
Leucine %	0.68	4.0	4.0

\* Hilmar™ 8000 (Hilmar Ingredients). Amino acid profile of whey, g/100 g whey: alanine, 2.7; arginine, 2.2; aspartic acid, 8.8; cystine/cysteine, 1.7; glutamic acid, 14.0; glycine, 1.5; histidine, 1.5; hydroxyproline, <0.1; isoleucine, 4.8; leucine, 9.1; lysine, 7.3; methionine, 1.7; phenylalanine, 2.7; proline, 6.2; serine, 4.8; threonine, 4.5; tryptophan, 1.2; tyrosine, 2.3; valine, 4.7.

\*\* International Ingredient Corporation.

† MSC Company.

‡ Dyets<sup>R</sup> Inc. Vitamin premix provided g/kg: Thiamine HCl, 0.1; riboflavin, 0.375; pyridoxine HCl, 0.1; Niacin, 1; calcium pantothenate, 1.2; folic acid, 0.13; biotin, 0.02; vitamin B12, 1.5; vitamin A palmitate, 0.8; vitamin D3, 0.05; vitamin E acetate, 8.8; menadione sodium bisulfite 0.08. Trace mineral premix provided g/kg: calcium phosphate, dibasic, 187; calcium carbonate, 279; sodium chloride, 85; potassium phosphate monobasic, 155; magnesium sulfate, anhydrous, 44; manganous carbonate, 0.93; ferric citrate, 10; Zinc carbonate, 1.84; cupric carbonate, 0.193; potassium iodate, 0.005; sodium selenite, 0.007.

§ Calculated nutrient composition.

**Table 2**

Phosphorylation of S6K1 and 4EBP1 in skeletal muscles and visceral tissues of piglets fed a LP, LP+P, or HP diet for 24 h.

Tissue	S6K1 (AU)			4EBP1 (AU)		
	LP	LP+L	HP	LP	LP+L	HP
Gastrocnemius	0.33 ± 0.03 *	0.63 ± 0.08 **	1.04 ± 0.11 †	0.45 ± 0.14 *	0.68 ± 0.09 **	0.95 ± 0.11 †
Masseter	0.22 ± 0.03 *	0.38 ± 0.04 **	0.66 ± 0.05 †	0.16 ± 0.06 *	0.34 ± 0.08 **	0.56 ± 0.12 †
Heart	0.31 ± 0.05 *	0.54 ± 0.06 **	0.79 ± 0.11 †	0.29 ± 0.09 *	0.57 ± 0.13 **	0.89 ± 0.14 †
Liver	0.24 ± 0.06 *	0.48 ± 0.09 **	0.79 ± 0.14 †	0.15 ± 0.06 *	0.32 ± 0.07 **	0.58 ± 0.11 †
Jejunum	0.22 ± 0.05 *	0.43 ± 0.10 **	0.66 ± 0.11 †	0.27 ± 0.07 *	0.53 ± 0.10 **	0.96 ± 0.09 †
Pancreas	0.31 ± 0.07 *	0.59 ± 0.12 **	0.90 ± 0.13 †	0.20 ± 0.06 *	0.52 ± 0.09 **	0.79 ± 0.13 †
Kidney	0.29 ± 0.09	0.37 ± 0.09	0.40 ± 0.09	0.59 ± 0.10	0.57 ± 0.12	0.69 ± 0.15

Values are means ± SEM, n = 7 per group. If ANOVA  $p < 0.05$ , post hoc test analysis was performed.

\*\*\*, † Means in a row with superscripts without a common symbol for either S6K1 or 4EBP1 differ,  $p < 0.05$ . AU, arbitrary units.

Phosphorylation of eEF2 and eIF2 $\alpha$  in other muscles and visceral tissues of piglets fed a LP, LP+P, or HP diet for 24 h.

**Table 3**

Tissue	eEF2 (AU)			eIF2 $\alpha$ (AU)		
	LP	LP+L	HP	LP	LP+L	HP
Gastrocnemius	0.87 $\pm$ 0.19	0.92 $\pm$ 0.09	1.01 $\pm$ 0.11	1.34 $\pm$ 0.15	1.19 $\pm$ 0.19	1.22 $\pm$ 0.11
Masseter	1.22 $\pm$ 0.22	1.31 $\pm$ 0.15	1.09 $\pm$ 0.16	1.26 $\pm$ 0.18	1.29 $\pm$ 0.13	1.17 $\pm$ 0.18
Heart	0.81 $\pm$ 0.16	0.93 $\pm$ 0.14	0.99 $\pm$ 0.16	0.99 $\pm$ 0.12	0.96 $\pm$ 0.15	0.94 $\pm$ 0.14
Liver	1.25 $\pm$ 0.12	1.38 $\pm$ 0.149	1.19 $\pm$ 0.15	0.77 $\pm$ 0.12	0.69 $\pm$ 0.09	0.82 $\pm$ 0.12
Jejunum	0.99 $\pm$ 0.13	1.16 $\pm$ 0.15	1.21 $\pm$ 0.16	0.91 $\pm$ 0.13	0.93 $\pm$ 0.10	0.99 $\pm$ 0.15
Pancreas	0.89 $\pm$ 0.15	0.93 $\pm$ 0.09	0.96 $\pm$ 0.11	0.86 $\pm$ 0.15	0.79 $\pm$ 0.09	0.81 $\pm$ 0.14
Kidney	1.17 $\pm$ 0.18	1.31 $\pm$ 0.17	1.37 $\pm$ 0.14	1.02 $\pm$ 0.11	0.93 $\pm$ 0.13	0.98 $\pm$ 0.12

Values are means  $\pm$  SEM, n = 7 per group. If ANOVA  $p < 0.05$ , post hoc test analysis was performed. AU, arbitrary units.