Published in final edited form as:

Am J Physiol Endocrinol Metab. 2007 December ; 293(6): E1615–E1621. doi:10.1152/ajpendo.00302.2007.

Amino Acid Availability and Age Affect the Leucine Stimulation of Protein Synthesis and eIF4F Formation in Muscle

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Abstract

We have previously shown that a physiological increase in plasma leucine for 60- and 120-min increases translation initiation factor activation in muscle of neonatal pigs. Although muscle protein synthesis is increased by leucine at 60 min, it is not maintained at 120 min, perhaps due to the decrease in plasma amino acids (AA). In the current study, 7- and 26-day-old pigs were fasted overnight and infused with leucine (0 or 400 $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) for 120 min to raise leucine within the postprandial range. The leucine was infused in the presence or absence of a replacement AA mixture (without leucine) to maintain baseline plasma AA levels. AA administration prevented the leucine-induced reduction in plasma AA in both age groups. At 7 days, leucine infusion alone increased eukaryotic initiation factor (eIF) 4E binding protein-1 (4E-BP1) phosphorylation, decreased inactive 4E-BP1-eIF4E complex abundance, and increased active eIF4G-eIF4E complex formation in skeletal muscle; leucine infusion with replacement AA also stimulated these, as well as S6K1, rpS6, and eIF4G phosphorylation. At 26 days, leucine infusion alone increased 4E-BP1 phosphorylation and decreased the inactive 4E-BP1-eIF4E complex only; leucine with AA also stimulated these, as well as S6K1 and rpS6 phosphorylation. Muscle protein synthesis was increased in 7-day-old (+60%) and 26-day-old (+40%) pigs infused with leucine and replacement AA, but not with leucine alone. Thus, the ability of leucine to stimulate eIF4F formation and protein synthesis in skeletal muscle is dependent on AA availability and age.

Keywords

Neonate; translation initiation; infusion; parenteral; eukaryotic initiation factor 4G; amino acids

INTRODUCTION

The neonatal period is characterized by rapid growth and elevated rates of protein synthesis and protein accretion of skeletal muscle proteins (11;15). The rate of protein synthesis rate is very high immediately after birth and declines rapidly with development (8;11). Feeding stimulates protein synthesis in virtually all tissues of neonatal animals but it is most prominent in skeletal muscle, where the response is independently mediated by the postprandial increase in circulating insulin and amino acids (10;24;25). These responses decreases with age (8;10). The enhanced response of muscle protein synthesis to feeding appears to be largely responsible

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for the more efficient use of dietary amino acids for protein deposition during the neonatal period (13).

Studies conducted *in vitro* and *in vivo* indicate that leucine stimulates protein synthesis by an mTOR-dependent process involving both S6K1 and 4E-BP1 phosphorylation and eIF4F formation (1;2;18). Our recent studies in neonatal pigs have demonstrated that muscle is responsive to stimulation by leucine, but not the other branched-chain amino acids, isoleucine and valine (17). A physiological rise in plasma leucine alone can stimulate muscle protein synthesis in neonatal pigs through the activation of translation initiation factors (16;17). Leucine stimulates protein synthesis in neonatal skeletal muscles containing primarily fast-twitch and slow-twitch fibers, as well as in cardiac muscle (17). We previously demonstrated that leucine administered parenterally at physiological levels acutely (60 min) stimulates muscle protein synthesis via translation initiation factor activation. However, the stimulation of muscle protein synthesis by leucine is not maintained for longer periods (120 min) despite significant activation of translation initiation factors and is associated with significant reductions (~50%) in plasma essential amino acids (16). Similar reductions in plasma essential amino acids have been reported during acute infusion of insulin, a potent anabolic hormone that increases muscle protein synthesis in neonatal animals (30).

The increased responsiveness of muscle protein synthesis to anabolic stimuli in young animals is associated with an increased efficiency of the translation process (9;12). Moreover, this increase in translational efficiency is mainly driven by the enhanced activation of translation initiation factors involved in the binding of mRNA to the 43S ribosomal complex (14;25). In muscle, the acute infusion of amino acids increases the phosphorylation of the 70 kDa ribosomal protein S6 kinase (S6K1), ribosomal protein S6 (rpS6), and the eukaryotic initiation factor (eIF) 4E binding protein 4E-BP1, which in turn releases eIF4E from the inactive 4E-BP1-eIF4E complex. Consequently, freed eIF4E binds to eIF4G and eIF4A to form the active eIF4F complex, which mediates the binding of mRNA to the 43S ribosomal complex (25).

Because of the significant decline in plasma essential amino acids during leucine infusion in our previous study (16), our objective was to determine the effect of replacement amino acid administration during a 120-min leucine infusion on muscle protein synthesis in neonatal pigs. Furthermore, we wished to determine if the muscle protein synthetic responsiveness to leucine stimulation changes with age.

METHODS

Animals and housing

Four multiparous crossbred (Yorkshire × Landrace × Hampshire × Duroc) pregnant sows from the Texas Department of Criminal Justice (Huntsville, TX) were brought to the animal facility of the Children's Nutrition Research Center two weeks before their due date, and housed as previously described (16). Sows had ad libitum access to food (Lab Diet 5084, PMI Nutrition International, LLC, Brentwood, MO) and water throughout the study. Pigs were injected i.m. with 100 mg of iron dextran (Phoenix Pharmaceuticals, Inc. St. Joseph, MO) within 24 h of birth. Pigs were allowed to nurse throughout the study, unless otherwise indicated, and were not supplemented with creep feed. Pigs were studied at 7.0 ± 0.1 days of age weighing 2.2 ± 0.1 kg, and 26.5 ± 0.4 days of age weighing 5.2 ± 0.3 kg. The Animal Care and Use Committee of Baylor College of Medicine approved all experimental procedures. This study was conducted according to the National Research Council's Guide for the Care and Use of Laboratory Animals. Four days before the infusion studies, piglets were anesthetized and indwelling catheters were surgically inserted into the jugular vein and carotid artery using sterile techniques as previously described (17).

Treatments and infusion

Piglets were food-deprived for 12–14 h prior to infusion and placed in a sling restraint system. The carotid catheter was used to infuse saline, leucine, replacement AA, and L[4-³H] phenylalanine and the jugular catheter was used for repeated blood sample collection. Pigs were randomly assigned to one of three treatments consisting of infusion of saline, leucine or leucine with replacement AA for 120 min. Leucine infusion was initiated with a primed dose (148 $\mu\text{mol/kg}$) for 10 min, followed by a constant infusion of 400 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$. During the priming period, saline infused pigs received an equal volume of saline as those receiving leucine. Mathematical modeling of data from our previous study (16) was used to determine the amount of replacement AA required. Factors taken into consideration were: body weight, baseline concentration of each essential AA, baseline whole body AA pool, the slope of the decreasing concentration of each essential AA in plasma during leucine infusion, final concentration of each essential AA after 120 min of leucine infusion, the increase in muscle protein synthesis by leucine, and the concentration of each essential AA in the balanced AA mixture (10). Replacement AA administration was initiated at 10 min, immediately after the ending of the leucine priming. The infusion rate of AA was adjusted at 10-min intervals until 90 min and held constant, at the 90 min rate, until the end of the study. Replacement AA were infused from 10 to 90 min as described by the following equation: $\text{AA rate} = 3.21 \div \{1 + e^{[-(BW-44.44) \div 16.53]}\}$. Where: “AA rate” is the amount of the balanced AA mixture without leucine, in ml, to be infused per min; and “BW” is the body weight of each pig in kg.

Tissue protein synthesis in vivo

Fractional rates of protein synthesis were measured using a 10 ml/kg body wt flooding dose (1.5 mmol/kg body wt) of L[4-³H]phenylalanine (0.5 mCi/kg body wt; Amersham Biosciences, Piscataway, NJ) injected 30 min before ending the infusion as previously described (19). Pigs were killed at the end of the infusion and samples were obtained from the longissimus dorsi muscle. Samples were collected and immediately frozen in liquid nitrogen and stored at -70°C until analyzed, as previously described (11). Protein synthesis (Ks expressed as percent of protein synthesized in a day) was calculated as: $\text{Ks (\%/day)} = [(S_b/S_a) \times (1440/t)] \times 100$, where S_b is the specific radioactivity of the protein bound phenylalanine, S_a is the specific radioactivity of the tissue-free phenylalanine for the labeling period, determined from the value of the animal at the time of tissue collection, corrected by the linear regression of the blood specific radioactivity of the animal against time, and t is the time of labeling in min.

Plasma insulin and amino acids

Blood samples were collected at 0, 30, 60, 90 and 120 min after the start of the infusion in heparinized tubes, centrifuged at $10,000 \times g$ for one min at room temperature, and stored at -20°C until analyzed. Plasma insulin concentrations were measured with a porcine-specific radioimmunoassay kit (Linco, St. Louis, MO) as previously described (30). The concentrations of individual amino acids from frozen plasma samples were measured with an HPLC method (PICO-TAG reverse-phase column, Waters, Milford, MA) as previously described (3).

Protein immunoblot analysis

Proteins were electrophoretically separated in polyacrylamide gels (22). For each assay, all tissue samples were run together in triple-wide gels (C.B.S. Scientific C, Del Mar, CA) to eliminate inter-assay variation. Proteins were transferred to a PVDF membrane (BioRad, Hercules, CA) and incubated with appropriate antibodies (all from Cell Signaling Technology, Inc., Beverly, MA, unless otherwise indicated). Blots were developed using an enhanced chemiluminescence kit (ECL, Amersham), visualized using ChemiDocIt (UVP, Inc., Upland, CA) and analyzed with LabWorks Image Acquisition and Analysis Software (UVP). Site-specific phosphorylation and total protein content were determined.

The 4E-BP1-eIF4E and eIF4E-eIF4G complexes were immunoprecipitated overnight at 4°C using an anti-eIF4E monoclonal antibody (21) from aliquots of fresh tissue homogenates as previously described (17;23). Immunoprecipitates were subjected to protein immunoblot analysis, as described above, using a rabbit polyclonal anti-4E-BP1 antibody (Bethyl Laboratories Inc., Montgomery, TX), the aforementioned monoclonal anti-eIF4E antibody, a rabbit polyclonal anti-eIF4G antibody (Novus Biologicals, Inc., Littleton, CO) and a rabbit polyclonal antibody that recognizes site-specific phosphorylation of eIF4G at Ser1108.

Fresh muscle tissue samples were homogenized, diluted in sample buffer (22), boiled for 10 min, cooled to room temperature, frozen in liquid nitrogen, and stored at -70°C until protein immunoblot analyses as previously described (17). Aliquots of homogenates were subjected to protein immunoblot analysis using rabbit polyclonal antibodies that recognize total 4E-BP1 (Bethyl Laboratories, Inc. Montgomery, TX), total (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and phosphorylation at Thr389 of p70 S6K1, and total and phosphorylation at Ser235/236 and Ser240/244 of rpS6.

Statistical analyses

The pig was considered the experimental unit. Analysis of variance (ANOVA) was performed using the GLM procedure of SAS (release 8.02, SAS Institute Inc., Cary, NC) for randomized complete-block design (20) to test the effect of treatment on fractional rates of protein synthesis and the activation of translation initiation factors. An ANOVA for repeated measurements was used to analyze the concentration of plasma amino acids (20). Least squares means were compared using a *t*-test and Fisher adjustment by the PDIF option of SAS (20). Finally, slope-ratio analysis was used to compare multiple-linear regression curves of plasma amino acid concentration (plasma amino acid concentration vs. time) among treatments (20;27) Data and *p*-values are presented according to the guidelines of the American Physiological Society (4).

RESULTS

Plasma insulin and amino acids

Plasma insulin concentrations were not different ($P = 0.52$) from baseline ($3.02 \pm 0.53 \mu\text{U/ml}$) after the 120-min infusion of saline ($2.47 \pm 0.44 \mu\text{U/ml}$), leucine ($3.49 \pm 0.61 \mu\text{U/ml}$), or leucine with amino acids ($3.41 \pm 0.52 \mu\text{U/ml}$) and were unaffected by age. Infusion of leucine resulted in a marked increase ($P < 0.01$) in its plasma concentration over baseline in 7-day-old (Fig. 1A) and 26-day-old pigs (Figure 2A). Furthermore, the achieved plasma levels in both ages were within the postprandial physiological range [i.e., 2- to 4-fold above fasting levels (3)]. Infusion of leucine decreased plasma levels of essential amino acids as the time of infusion progressed with the exception of Met, Thr, and Arg (Fig. 1 and Fig. 2). Furthermore, slope-ratio analysis by orthogonal contrast of the slopes obtained from multiple-linear regression curves of plasma amino acid concentration vs. time of infusion was performed for all treatments within age. Linear reductions in plasma concentrations of isoleucine ($P < 0.0001$), valine ($P = 0.0003$), lysine ($P = 0.0002$), methionine ($P = 0.02$), histidine ($P = 0.06$), phenylalanine ($P < 0.0001$), arginine ($P = 0.03$), proline ($P = 0.06$), threonine ($P = 0.06$), glutamate ($P = 0.03$), asparagine ($P = 0.0004$), and alanine ($P = 0.002$) were observed in 7-day-old pigs compared to pigs infused with saline, as time of infusion progressed; whereas tryptophan ($P = 0.15$), tyrosine ($P = 0.35$), serine ($P = 0.88$), aspartate ($P = 0.19$), glutamine ($P = 0.26$), and glycine ($P = 0.49$) remained unchanged. During leucine infusion with replacement amino acids, plasma levels of isoleucine ($P = 0.12$), lysine ($P = 0.78$), glutamate ($P = 0.43$), arginine ($P = 0.83$), threonine ($P = 0.26$), tryptophan ($P = 0.79$), tyrosine ($P = 0.86$), serine ($P = 0.50$), alanine ($P = 0.84$), and aspartate ($P = 0.41$) were not different from control pigs infused with saline. However, infusion of leucine with replacement amino acids caused an increase in plasma levels of methionine ($P = 0.09$), histidine ($P = 0.06$), proline ($P < 0.0001$), glutamine ($P = 0.006$),

and glycine ($P = 0.01$). Furthermore, the leucine-induced reduction of valine ($P = 0.01$), asparagine ($P = 0.05$), and phenylalanine ($P = 0.0002$) was not prevented by the administration of replacement amino acids in 7-day-old pigs.

In 26-day-old pigs, infusion of leucine caused a reduction of plasma concentration of isoleucine ($P = 0.0006$), valine ($P = 0.002$), lysine ($P = 0.003$), methionine ($P = 0.04$), histidine ($P = 0.006$), asparagine ($P = 0.02$), phenylalanine ($P = 0.02$), arginine ($P = 0.01$), tryptophan ($P = 0.007$), serine ($P = 0.02$), and glycine ($P = 0.03$); whereas glutamate ($P = 0.12$), proline ($P = 0.31$), alanine ($P = 0.67$), threonine ($P = 0.39$), tyrosine ($P = 0.21$), aspartate ($P = 0.25$), glutamine ($P = 0.50$) remain unchanged. During leucine infusion with replacement amino acids, plasma levels of isoleucine ($P = 0.47$), valine ($P = 0.43$), methionine ($P = 0.86$), glutamate ($P = 0.20$), arginine ($P = 0.15$), alanine ($P = 0.28$), threonine ($P = 0.40$), tryptophan ($P = 0.98$), tyrosine ($P = 0.11$), serine ($P = 0.77$), and glycine ($P = 0.31$) were not different from control pigs infused with saline. However, infusion of leucine with replacement amino acids caused an increase in plasma levels of lysine ($P = 0.002$), histidine ($P = 0.003$), proline ($P = 0.01$), aspartate ($P < 0.0001$), and glutamine ($P = 0.07$). Furthermore, the leucine-induced reduction of asparagine ($P < 0.001$), and phenylalanine ($P = 0.04$) was not prevented by the administration of replacement amino acids in 26-day-old pigs. In summary, the slope-ratio analysis by orthogonal contrast of the slopes obtained from multiple-linear regression curves indicate that an increase in plasma leucine causes a reduction in plasma essential amino acids, which can be prevented or ameliorated by administration of replacement amino acids in both ages.

Translation initiation factors

In longissimus dorsi muscle of 7-day-old pigs, infusion of leucine without replacement amino acids increased the phosphorylation of 4E-BP1 ($P < 0.02$), reduced the amount of inactive 4E-BP1-eIF4E complex ($P < 0.005$), and increased the formation of active eIF4G-eIF4E complex ($P < 0.001$), as compared to saline-infused controls (Fig. 3 and Fig 4A). Infusion of leucine with replacement amino acids, compared to controls, also increased 4E-BP1 phosphorylation ($P < 0.02$), decreased the inactive 4E-BP1-eIF4E complex content ($P < 0.005$), and increased the active eIF4G-eIF4E complex formation ($P < 0.001$) (Fig. 3 and Fig. 4A). In addition, leucine infusion with replacement also increased the phosphorylation of eIF4G in the active eIF4G-eIF4E complex ($P < 0.002$) as well as S6K1 ($P < 0.05$) and rpS6 ($P < 0.003$), compared to saline-infused controls (Fig. 4B and Fig. 5).

In 26-day-old pigs, infusion of leucine without replacement amino acids increased the phosphorylation of 4E-BP1 ($P < 0.04$) and reduced the amount of the inactive 4E-BP1-eIF4E complex ($P < 0.02$), compared to saline-infused controls (Fig. 3). Infusion of leucine with replacement amino acids also increased 4E-BP1 phosphorylation ($P < 0.04$) and reduced the amount of the inactive 4E-BP1-eIF4E complex ($P < 0.02$) in addition to increasing the phosphorylation of S6K1 ($P = 0.09$), and rpS6 ($P < 0.03$), compared to saline-infused controls (Fig. 3 and Fig. 5). Unlike in 7-day-old pigs, infusion of leucine, with or without of replacement leucine, did not increase formation of the active eIF4G-eIF4E complex in 26-day-old pigs (Fig. 4).

Protein synthesis

Infusion of leucine alone for 120 min failed to increase protein synthesis in the longissimus dorsi muscle of 7- and 26-day-old pigs ($P = 0.21$ and 0.88 , respectively) compared to saline-infused controls (Fig. 6). However, protein synthesis was increased in 7-day-old ($+60\%$, $P < 0.02$) and 26-day-old ($+40\%$, $P < 0.006$) pigs during leucine infusion with replacement amino acids compared to pigs infused with saline (i.e., controls) and leucine alone.

DISCUSSION

Rapid growth, high rates of protein turnover, and marked accretion of skeletal muscle are hallmarks of the human neonatal period (15). Similarly, neonatal pigs are also a highly anabolic population very responsive to stimuli, such as feeding, insulin, and amino acids (8;10;24;31). Feeding of neonatal pigs with colostrum, mature sow's milk, or formula increases protein synthesis in disparate tissues (3). However, this response in protein synthesis to feeding is most profound in skeletal muscle (8). Infusion of a balanced amino acid mixture to mimic postprandial levels of plasma amino acids results in increased rates of protein synthesis in skeletal muscle and liver of neonatal pigs (24;26). In addition, a comparable muscle protein synthetic response can be obtained by infusing leucine intra-arterially to mimic postprandial levels of this amino acid (16). However, muscle protein synthesis is not responsive to plasma increases of isoleucine and valine within the physiological postprandial range (17). Furthermore, the muscle protein synthetic response to amino acids is independent of insulin and is modulated by enhanced activation of translation initiation factors (16;17;25). The leucine-induced stimulation of protein synthesis appears to be substrate-dependent because a 120-min infusion of leucine reduces plasma essential amino acids (i.e., about 50% reduction) and is unable to stimulate protein synthesis despite profound activation of translation initiation factors (16). Thus, the main objective of this study was to quantify the leucine-induced stimulation of muscle protein synthesis with and without a replacement dose of a balanced amino acid mixture. In addition, we tested the efficacy of leucine to stimulate muscle protein synthesis in older pigs.

In this study, we confirmed our previous results in neonatal pigs where infusion of leucine for 120 min stimulated the activation of translation initiation factors, reduced plasma concentrations of essential amino acids, and failed to stimulate muscle protein synthesis (16), a process that uses essential amino acids, which the neonatal body of the pig is not capable of synthesizing *de novo*, to maintain the leucine-induced increase in muscle protein synthesis. A significant increase in muscle protein synthesis can be measured when leucine is infused for a shorter period (i.e., 60 min) without significant changes in plasma concentrations of essential amino acids (16;17). Thus, the leucine-induced reduction in plasma essential amino acids is likely due to the increase in muscle protein synthesis that occurs earlier during the infusion period. The replacement dose of amino acids used in the current study was carefully calculated to supply both essential and non-essential amino acids to sustain the leucine-induced stimulation of muscle protein synthesis while maintaining euaminoacidemia at fasting levels. Although non-essential amino acids can be synthesized *de novo* by the body of neonatal pigs from readily available carbon skeletons, they were included in the replacement dose to avoid the energetic cost of their synthesis.

Results from this study confirm that a physiological increase in plasma leucine activates translation initiation factors in muscle regardless of infusion time (i.e., 60 or 120 min) (16; 17). Co-infusion of leucine with replacement amino acids resulted in about 60% increase in muscle protein synthesis compared to saline-infused pigs, which is considerably higher than our previous results of about 28% (17) and 33% (16) during a shorter (i.e., 60-min) leucine infusion. However, this increase in muscle protein synthesis is comparable to results obtained in our laboratory during hyperinsulinemia while maintaining euaminoacidemia at fasting levels (10;25). Therefore, we speculate that the higher increase obtained in this study compared to saline-infused controls was probably due to the non-limiting abundance of both essential and non-essential amino acids for muscle protein synthesis.

We have previously reported that feeding (8), insulin (31), and a balanced amino acid mixture (10) can stimulate muscle protein synthesis in older pigs (i.e., 26-day-old) but to a lesser degree than in younger pigs (i.e., 6-day-old). Likewise, results presented herein indicate that a

physiological increase in plasma leucine stimulated muscle protein synthesis in older pigs to a lesser degree than in younger pigs. In rats, protein synthesis was lower in 20-month-old epitrochlearis muscles incubated with leucine at various physiological concentrations compared to muscles from 6- to 8-month-old rats (6). In old rats and humans, supraphysiological concentrations of leucine appear capable of stimulating muscle protein synthesis to the same degree as physiological concentrations in younger subjects (7;28). In the present study, plasma leucine at the end of the infusion was higher in 26-day-old pigs compared to 7-day-old pigs and protein synthesis was lower for the older pigs. We have previously reported that muscle protein synthesis in neonatal pigs responds linearly to plasma concentration of amino acids (25) and leucine (16). Thus, we speculate that the stimulation of muscle protein synthesis by leucine in 26-day old pigs could have been less if plasma levels of leucine would have been comparable to those achieved in 7-day-old pigs. The age-dependent reduction in the stimulation of muscle protein synthesis to feeding, amino acids, and leucine has been associated with reduced intracellular anabolic signaling. We have shown an age-dependent reduction in the activation of components of the signaling pathway governed by mTOR leading to translation initiation in muscle of pigs in response to feeding (29). Similar age-dependent reductions in anabolic signaling components have been reported in response to amino acids (5) and leucine (6). In the present study, we found again age-dependent reductions in the signaling proteins that lead to translation initiation. Collectively, results from this study and others (1;2;5;6;16;17) indicate that leucine can act as a nutrient signal to stimulate protein synthesis in skeletal muscle of animals and humans of diverse ages. However, the protein synthetic response of muscle to leucine stimulation is dependent on the availability of plasma essential amino acids and age.

ACKNOWLEDGMENTS

We thank W. Liu and J. Fleming for technical assistance; D. E. Miller and J. C. Stubblefield for care of animals; and L. F. Weiser for secretarial assistance.

GRANTS

This work is a publication of the United States Department of Agriculture/Agricultural Research Service (USDA/ARS) Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine. This project has been funded in part by National Institutes of Health (NIH) grants AR-44474 (T. A. Davis) and by the USDA/ARS under Cooperative Agreement no. 6250510000-33 (T. A. Davis). This research was also supported in part by NIH Training Grant T32 HD-07445. The contents of this publication do not necessarily reflect the views or policies of the U.S. Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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Figure 1.

Plasma amino acid concentrations in 7-day-old pigs at baseline and after 120 min of infusion with saline (Saline) or $400 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of leucine with (Leucine+AA) and without (Leucine) replacement amino acids. Values are means $\pm$ SEM; $n = 5-6$ per treatment. Plasma concentrations of phenylalanine are not reported after piglets were flooded with $L[^3\text{H}]$ phenylalanine to determine fractional rates of protein synthesis. * Means are different from baseline values, $P < 0.05$; + means are different from baseline values, $P < 0.10$.

Figure 2.

Plasma amino acid concentrations in 26-day-old pigs at baseline and after 120 min of infusion with saline (Saline) or $400 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of leucine with (Leucine+AA) and without (Leucine) replacement amino acids. Values are means $\pm$ SEM; $n = 5-6$ per treatment. Plasma concentrations of phenylalanine are not reported after piglets were flooded with $L[^3\text{H}]$ phenylalanine to determine fractional rates of protein synthesis. * Means are different from baseline values, $P < 0.05$; + means are different from baseline values, $P < 0.10$.

Figure 3.

Phosphorylation of eukaryotic initiation factor (eIF) 4E binding protein-1 (4E-BP1) (**A**) and association of 4E-BP1 with eIF4E (**B**) in longissimus dorsi of 7- and 26-day-old pigs after 120 min of infusion with saline (Saline) or 400 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of leucine with (Leucine+AA) and without (Leucine) replacement amino acids. Total 4E-BP1 content was not different among treatments within age. Total 4E-BP1 was corrected by the eIF4E recovered from the immunoprecipitate. Values are means $\pm$ pooled SEM; $n = 5-6$ per treatment. **A.** Means with different letters differ at $P < 0.02$ in 7-day-old pigs, and $P < 0.04$ in 26-day-old pigs; * mean differs from 26-day-old Leucine-infused pigs at $P = 0.10$. **B.** The value from control pigs

infused with saline was set at 1.0 (AU); means with different letters differ at $P < 0.005$ in 7-day-old pigs, and $P < 0.02$ in 26-day-old pigs.

Figure 4.

Association of eukaryotic initiation factor (eIF) 4G with eIF4E (**A**) and phosphorylation of eIF4G at Ser1108 associated with eIF4E (**B**) in longissimus dorsi of 7- and 26-day-old pigs after 120 min of infusion with saline (Saline) or 400 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of leucine with (Leucine +AA) and without (Leucine) replacement amino acids. Total eIF4G was corrected by the eIF4E recovered from the immunoprecipitate. The value from control pigs infused with saline was set at 1.0 (AU) within age. Total eIF4E content was not different among treatments within age. Values are means $\pm$ pooled SEM; $n = 5-6$ per treatment. **A.** Means with different letters differ at $P < 0.001$ in 7-day-old pigs. **B.** Means with different letters differ at $P < 0.002$ in 7-day-old

pigs; * mean differ from 7-day-old pigs infused with Saline at $P = 0.07$ and Leucine+AA at $P = 0.09$.

Figure 5.

Phosphorylation of the 70-kDa ribosomal protein S6 kinase (S6K1) at Thr389 (**A**) and phosphorylation of ribosomal protein (rp) S6 at Ser235/236 and Ser240/244 (**B**) in longissimus dorsi of 7- and 26-day-old pigs after 120 min of infusion with saline (Saline) or 400 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of leucine with (Leucine+AA) and without (Leucine) replacement amino acids. Phosphorylation of S6K1 and rpS6 were corrected by total S6K1 and rpS6, respectively. The value from control pigs infused with saline was set at 1.0 (AU) within age. Total S6K1 and rpS6 contents were not different among treatments within age. Values are means $\pm$ pooled SEM; $n = 5-6$ per treatment. **A.** Means with different letters differ at $P < 0.05$ in 7-day-old pigs; * mean differs from 26-day-old Saline- and Leucine-infused pigs at $P = 0.10$. **B.** Means

with different letters differ at $P < 0.003$ in 7-day-old pigs, and $P < 0.03$ in 26-day-old pigs; * mean differs from 7-day-old Leucine-infused pigs at $P = 0.08$; + mean differs from 26-day-old Leucine-infused pigs at $P = 0.07$.

Figure 6.

Fractional rates of protein synthesis in longissimus dorsi of 7- and 26-day-old pigs after 120 min of infusion with saline (Saline) or $400 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of leucine with (Leucine+AA) and without (Leucine) replacement amino acids. Values are means $\pm$ pooled SEM; $n = 5-6$ per treatment. Means with different letters differ at $P < 0.02$ in 7-day-old pigs, and $P < 0.006$ in 26-day-old pigs. * Mean differs from 26-day-old Leucine-infused pigs at $P = 0.06$.