Amino acid availability and age affect the leucine stimulation of protein synthesis and eIF4F formation in muscle

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Escobar J, Frank JW, Suryawan A, Nguyen HV, Davis TA. Amino acid availability and age affect the leucine stimulation of protein synthesis and eIF4F formation in muscle. Am J Physiol Endocrinol Metab 293: E1615–E1621, 2007. First published September 18, 2007; doi:10.1152/ajpendo.00302.2007.—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 because of the decrease in plasma amino acids (AA). In the present study, 7- and 26-day-old pigs were fasted overnight and infused with leucine (0 or 400 μmol·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 70-kDa ribosomal protein S6 kinase, ribosomal protein S6, 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 70-kDa ribosomal protein S6 kinase and ribosomal protein S6 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.

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

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 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 decrease with age (8, 10). The enhanced response of muscle protein synthesis to feeding appears to be largely responsible 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 a mammalian target of rapamycin-dependent process involving both 70-kDa ribosomal protein S6 kinase (S6K1) and 4E binding protein-1 (4E-BP1) phosphorylation and eukaryotic initiation factor 4F (eIF4F) formation (1, 2, 18). Our recent study (17) in neonatal pigs has demonstrated that muscle is responsive to stimulation by leucine but not by the other branched-chain amino acids (isoleucine and valine). 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). Our group (16) previously demonstrated that leucine administered parenterally at physiological levels acutely (60 min) stimulated muscle protein synthesis via translation of initiation factor activation. However, the stimulation of muscle protein synthesis by leucine was not maintained for longer periods (120 min) despite significant activation of translation initiation factors and was 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 S6K1, ribosomal protein S6 (rpS6), and the eukaryotic initiation factor (eIF) 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 whether the muscle protein synthetic responsiveness to leucine stimulation changes with age.

METHODS

Animals and housing. The Animal Care and Use Committee of Baylor College of Medicine approved all experimental procedures.

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This study was conducted according to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Research, National Research Council, Washington, DC: National Academy Press, 1996).

Four multiparous cross-bred (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 2 wk before their due date and housed as previously described (16). Sows had ad libitum access to food (laboratory diet 5084, PMI Nutrition International, Brentwood, MO) and water throughout the study. Pigs were injected intramuscularly with 100 mg of iron dextran (Phoenix Pharmaceuticals, 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.

Four days before the infusion studies, piglets were anesthetized, and in-dwelling 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 before infusion and placed in a sling restraint system. The carotid catheter was used to infuse saline, leucine, replacement amino acids, and l-[4-14C]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 amino acid for 120 min. Leucine infusion was initiated with a primed dose (148 μmol/kg) for 10 min, followed by a constant infusion of 400 μmol·kg⁻¹·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 amino acid required. Factors taken into consideration were body weight, baseline concentration of each essential amino acid, baseline whole body amino acid pool, the slope of the decreasing concentration of each essential amino acid in plasma during leucine infusion, final concentration of each essential amino acid after 120 min of leucine infusion, the increase in muscle protein synthesis by leucine, and the concentration of each essential amino acid in the balanced amino acid mixture (10). Replacement amino acid administration was initiated at 10 min, immediately after the ending of the leucine priming. The infusion rate of amino acids was adjusted at 10-min intervals until 90 min and held constant, at the 90-min rate, until the end of the study. Replacement amino acids were infused from 10 to 90 min as described by the following equation: AA rate = 3.21 × [1 + e⁻[BW - 44.44]/16.531], where AA rate is the amount of the balanced amino acid mixture without leucine (in ml) to be infused per minute 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-[4-14C]phenylalanine (0.5 mCi/kg body wt; Amersham Biosciences, Piscataway, NJ) injected 30 min before the infusion ended 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 (expressed as percent of protein synthesized in a day) was calculated as [S₀/Sₙ] × (1/44000) × 100, where S₀ is the specific radioactivity of the protein bound phenylalanine, Sₙ 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 g for 1 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 (CBS Scientific, Del Mar, CA) to eliminate interassay variation. Proteins were transferred to a polyvinylidine difluoride membrane (BioRad, Hercules, CA) and incubated with appropriate antibodies (all from Cell Signaling Technology, Beverly, MA, unless otherwise indicated). Blots were developed with an enhanced chemiluminescence kit (Amersham), visualized using ChemiDoc It (UVP, Upland, CA), and analyzed with LabWorks image acquisition and analysis software (UVP). Site-specific phosphorylation and total protein content were determined.

The 4E-BP1-elf4E and elf4E-elf4G complexes were immunoprecipitated overnight at 4°C using an anti-elf4E 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, Montgomery, TX), the aforementioned monoclonal anti-elf4E antibody, a rabbit polyclonal anti-elf4G antibody (Novus Biologicals, Littleton, CO), and a rabbit polyclonal antibody that recognizes site-specific phosphorylation of elf4G 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), total (Santa Cruz Biotechnology, Santa Cruz, CA) and phosphorylation levels at Thr389 of p70 S6K1, and total and phosphorylation levels at Ser235/236 and Ser240/244 of rpS6.

Statistical analyses. The pig was considered the experimental unit. ANOVA was performed with the general linear model procedure of SAS (release 8.02, SAS Institute, 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 with the use of a t-test and Fisher adjustment by the PDIIFF 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 ± 0.53 μU/ml) after the 120-min infusion of saline (2.47 ± 0.44 μU/ml), leucine (3.49 ± 0.61 μU/ml), or leucine with amino acids (3.41 ± 0.52 μU/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 (Fig. 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 methionine, threonine, and arginine (Figs. 1 and 2). Further-
more, 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 ($P = 0.06$), and glutamate ($P =$...
0.03), asparagine (P = 0.0004), and alanine (P = 0.002) were observed in 7-day-old pigs compared with 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 levels of 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), and 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 levels of 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 reductions of asparagine (P < 0.001) and phenylalanine (P = 0.04) were not prevented by the administration of replacement amino acids in 26-day-old pigs. In summary, the slope-ratio analyses by orthogonal contrast of the slopes obtained from multiple-linear regression curves indicated 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), compared with that shown in saline-infused controls (Figs. 3 and 4A). Infusion of leucine with replacement amino acids, compared with 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) (Figs. 3 and 4A). In addition, leucine infusion with replacement amino acids 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 with that shown in saline-infused controls (Fig. 4B and 5).

In 26-day-old pigs, infusion of leucine without replacement amino acids also increased the phosphorylation of 4E-BP1 (P < 0.04) and reduced the amount of the inactive 4E-BP1-eIF4E complex (P < 0.02), compared with that shown in 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), as well as increased the phosphorylation of S6K1 (P = 0.09) and rpS6 (P < 0.03) compared with saline-infused controls (Figs. 3 and 5). Unlike in 7-day-old pigs, infusion of leucine, with or without 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 with 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 with 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 initia-
tion 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., ~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 (16) in neonatal pigs in which 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, 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 present study was carefully calculated to supply both essential and nonessential amino acids to sustain the leucine-induced stimulation of muscle protein synthesis while maintaining euaminoacidemia at fasting levels. Although nonessential 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). Coinfusion of leucine with replacement amino acids resulted in an ~60% increase in muscle protein synthesis compared with that shown in saline-infused pigs, which is considerably higher than our previous results of ~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 with saline-infused controls was probably due to the nonlimiting abundance of both essential and nonessential 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 days old) but to a lesser degree than in younger pigs (i.e., 6 days 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 epitrochlearis muscles from 20-mo-old rats incubated with leucine at various physiological concentrations than in muscles from 6- to 8-mo-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 than in 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 the mammalian target of rapamycin 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 muscles 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.

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