A Physiological Rise in Plasma Leucine Stimulates Muscle Protein Synthesis in Neonatal Pigs by Enhancing Translation Initiation Factor Activation

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ABSTRACT

Protein synthesis in skeletal muscle of adult rats increases in response to oral gavage of supraphysiological doses of leucine. However, the effect on protein synthesis of a physiological rise in plasma leucine has not been investigated in neonates, an anabolic population highly sensitive to amino acids and insulin. Therefore, in the current study, fasted pigs were infused intra-arterially with leucine (0, 200 or 400 µmol·kg⁻¹·h⁻¹) and protein synthesis was measured after 60 or 120 min. Protein synthesis was increased in muscle, but not in liver, at 60 min. At 120 min, however, protein synthesis returned to baseline levels in muscle but was reduced below baseline values in liver. The increase in protein synthesis in muscle was associated with increased plasma leucine of 1.5- to 3-fold and no change in plasma insulin. Leucine infusion for 120 min reduced plasma essential amino acids levels. Phosphorylation of eukaryotic initiation factor (eIF) 4E binding protein-1 (4E-BP1), ribosomal protein (rp) S6 kinase, and rpS6 were increased, and the amount of eIF4E associated with its repressor, 4E-BP1, was reduced after 60 and 120 min of leucine infusion. No change in these biomarkers of mRNA translation was observed in liver. Thus, a physiological increase in plasma leucine stimulates protein synthesis in skeletal muscle of neonatal pigs in association with increased eIF4E availability for eIF4F assembly. This response appears to be insulin-independent, substrate-dependent, and tissue-specific. The results suggest that the branched-chain amino acid, leucine, can act as a nutrient signal to stimulate protein synthesis in skeletal muscle of neonates.

KEYWORDS

Nutrition; liver; amino acids; ribosomal protein S6 kinase; eukaryotic initiation factor-4E
Rapid growth, high rates of protein turnover and profound accretion of skeletal muscle protein are hallmarks of the neonatal period (10; 14). Previous studies in rats and pigs have shown that the fractional synthesis rate of skeletal muscle protein is very high immediately after birth and declines rapidly thereafter (6; 10). Dietary amino acids are used efficiently for protein deposition in neonates, a characteristic that also declines with development (12). Neonates use dietary amino acids efficiently for growth because they can increase protein synthesis in response to feeding to a greater extent than mature animals (6; 11). The feeding-induced stimulation of protein synthesis occurs in virtually all tissues of the neonate, however, the postprandial rise in protein synthesis is most pronounced in skeletal muscle. This response to feeding in skeletal muscle is mediated by the postprandial rise in insulin and amino acids whereas that in liver is mediated by amino acids (8; 25; 26).

The enhanced responsiveness of protein synthesis to stimuli in neonatal animals is associated with an increased efficiency of the translation process (7; 11). Furthermore, this increase in translational efficiency is primarily driven by enhanced 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-tRNA to the 40S ribosomal subunit (13; 26). In skeletal muscle and liver of neonates, feeding or the acute infusion of amino acids to raise levels to within the physiological fed range increases the phosphorylation of the 70 kDa ribosomal protein S6 kinase (S6K1) and the eukaryotic initiation factor (eIF) 4E binding protein-1 (4E-BP1), which in turn releases eIF4E from the inactive 4E-BP1-eIF4E complex. Thus, freed eIF4E binds to eIF4G and eIF4A to form the active eIF4F complex, which mediates the binding of mRNA to the 43S ribosomal complex (26; 27).
Although our studies have demonstrated that amino acids regulate the feeding-induced stimulation of protein synthesis in skeletal muscle and liver of neonatal pigs by changes in the activation of translation initiation factors that regulate mRNA binding to the ribosomal complex (25; 26), it is not clear whether this response to amino acids is mediated by the presence of higher levels of all amino acids or if it is attributable to specific effects of individual amino acids. 

*In vitro* studies suggest that leucine alone can stimulate protein synthesis by an mTOR-dependent process involving both S6K1 and 4E-BP1 phosphorylation, and eIF4F formation (15; 21; 31). The response to leucine is greater than the response to other amino acids, including the other branched-chain amino acids, isoleucine and valine. These studies, however, used leucine doses similar to a 4- to 10-fold increase in plasma leucine, compared to amino acid absence. Hence, the physiological relevance of these findings is questionable.

In a recent study, mature rats were gavage-fed the amount of leucine normally consumed in a 24-h period (1). Plasma leucine increased 12- to 18-fold and skeletal muscle protein synthesis was stimulated (1-3). The results showed that high doses of leucine stimulate protein synthesis by an mTOR-dependent process that involves S6K1 and 4E-BP1 phosphorylation, and eIF4F assembly (1; 2). In contrast, oral administration of leucine had no effect on global rates of protein synthesis in rat liver, but did promote S6K1 and 4E-BP1 phosphorylation (29). Therefore, our objectives were to determine whether a physiological increase in plasma leucine, similar to that observed after a meal (i.e., 2- to 4-fold increase over fasting levels), stimulates protein synthesis in neonatal pigs and if this response is modulated by changes in the activation of translation initiation factors that regulate the binding of mRNA to the ribosomal complex. We further wished to determine whether the responses differ in skeletal muscle and liver, and are time- and dose-dependent.
METHODS

Animals and housing. Five multiparous crossbred (Yorkshire × Landrace × Hampshire × Duroc) pregnant sows obtained from the Agriculture Headquarters of 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. Sows were housed in free-standing lactation crates in individual, environmentally controlled rooms. Sows had *ad libitum* access to food (Lab Diet 5084, PMI Nutrition International, LLC, Brentwood, MO) and water throughout the study. Within 24 h of birth, piglets were injected i.m. with 100 mg of iron dextran (Phoenix Pharmaceuticals, Inc. St. Joseph, MO). Piglets were allowed to nurse throughout the study, unless otherwise indicated, and were not supplemented with creep feed. Piglets were infused at 5.2 days (SD 1.5) of age weighing 2.0 kg (SD 0.4). The Animal Care and Use Committee of Baylor College of Medicine approved all experimental procedures. 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 (33). This study was conducted according to the National Research Council’s Guide for the Care and Use of Laboratory Animals.

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 and L[4-³H]phenylalanine whereas the jugular catheter was used for repeated blood sample collection. Pigs were randomly assigned to one of three constant leucine infusion rates of 0, 200 or 400 µmol·kg⁻¹·h⁻¹, and one of two infusion times of 60 or 120 min (n= 6, 8 and 7, respectively, for each time point). Piglets assigned to the 0 µmol leucine·kg⁻¹·h⁻¹ group were
infused with a 0.9% saline solution. During a 30-min basal period preceding leucine infusion, blood samples were collected and immediately analyzed for whole-blood glucose (YSI 2300 STAT Plus, Yellow Springs Instruments, Yellow Springs, OH) to establish the average basal concentration used to determine changes in blood glucose during the leucine infusion. Leucine infusion was initiated with a primed dose for 10 min, followed by the appropriate constant infusion rate. The prime dose of leucine received by piglets infused for 60 min was 80 µmol/kg for the 200 µmol·kg⁻¹·h⁻¹ group, and 240 µmol/kg for the 400 µmol·kg⁻¹·h⁻¹ group. The prime dose of leucine received by piglets infused for 120 min was 74 µmol/kg for the 200 µmol·kg⁻¹·h⁻¹ group, and 148 µmol/kg for the 400 µmol·kg⁻¹·h⁻¹ group. During the priming period, regardless of infusion length, saline infused piglets received an equal volume to the priming received by the 200 µmol·kg⁻¹·h⁻¹ group.

_Tissue protein synthesis in vivo._ Fractional rates of protein synthesis were measured with a flooding dose of L[4-³H]phenylalanine (Amersham Biosciences, Piscataway, NJ) injected 30 min before ending the infusion (16). We have demonstrated that after a flooding dose of phenylalanine is administered, the specific radioactivity of tissue-free phenylalanine is in equilibrium with the aminoacyl-tRNA specific radioactivity; thus, the tissue-free phenylalanine is a valid measure of the tissue precursor pool specific radioactivity (9). Protein synthesis (Ks expressed as percent of protein synthesized in a day) was calculated as: Ks (%/day) = [(Sb/Sa) × (1440/t)] × 100, where Sb is the specific radioactivity of the protein bound phenylalanine, Sa 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. Pigs were killed at 60 and 120 min, and longissimus dorsi muscle and liver tissue samples were
collected and immediately frozen in liquid nitrogen and stored at -70ºC until analyzed, as previously described (10).

**Blood glucose, and plasma insulin and amino acids.** Blood samples were collected every 10 min throughout the study. Whole-blood glucose concentration (YSI) was determined immediately after sample collection. To obtain plasma, whole-blood was collected in heparinized tubes, centrifuged at 10,000 × g for one min at room temperature, and stored at -20ºC until analyzed. Using a porcine insulin radioimmunoassay kit (Linco, St. Louis, MO) that used porcine insulin antibody and human insulin standards, plasma radio-immunoreactive insulin concentrations were measured as previously described (33). The concentrations of individual amino acids from frozen plasma samples obtained at 0, 30, 60, 90 and 120 min after the start of the leucine infusion, were measured with an HPLC method (PICO-TAG reverse-phase column, Waters, Milford, MA) as previously described (11).

**Protein immunoblot analysis.** Proteins were electrophoretically separated in polyacrylamide gels (20) and transferred to a PVDF membrane (BioRad, Hercules, CA), which was incubated with appropriate antibodies as previously described (13). Blots were developed using an enhanced chemiluminescense kit (ECL, Amersham), visualized using GeneGnome bioimaging system and analyzed using Gene Tools software (Syngene, Frederick, MD).

**Quantification of the 4E-BP1-eIF4E complex.** The association of eIF4E with 4E-BP1 was determined as previously described (19). The obtained supernatants were subjected to an overnight immunoprecipitation at 4ºC using a monoclonal antibody against eIF4E (19). Immunoprecipitates were subjected to protein immunoblot analysis, as described above, using a rabbit polyclonal anti-4E-BP1 antibody (Bethyl Laboratories Inc., Montgomery, TX) and the mouse monoclonal anti-eIF4E antibody.
Muscle and liver homogenates. Freshly collected longissimus dorsi muscle and liver tissue samples were homogenized, heated at 100°C for 10 min, cooled to room temperature and centrifuged at 10,000 × g for 10 min at 4°C. Supernatants were diluted in sample buffer (20), frozen in liquid nitrogen and stored at -70°C until protein immunoblot analyses.

Quantification of 4E-BP1 phosphorylation. An aliquot of muscle and liver homogenates were subjected to protein immunoblot analysis as previously described (13) using a rabbit polyclonal antibody that recognizes phosphorylation at Thr70 (Cell Signaling Technology, Inc., Beverly, MA).

Quantification of ribosomal protein S6 kinase (S6K1) phosphorylation. An aliquot of muscle and liver homogenates were subjected to protein immunoblot analysis as previously described (13) using a rabbit polyclonal antibody that recognizes p70 S6K1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Quantification of ribosomal protein S6 phosphorylation. An aliquot of muscle and liver homogenates were subjected to protein immunoblot analysis, as described above, using rabbit polyclonal antibodies that recognize phosphorylation at Ser235/236 and Ser240/244 of rpS6 (Cell Signaling Technology).

Statistical analyses. To determine the effect of treatment on fractional protein synthesis rate and the abundance of translation initiation factors, 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 (18), where the piglet was considered the experimental unit. An ANOVA for repeated measurements was used to analyze the concentration of whole-blood glucose, as well as plasma insulin and amino acids (18). Least squares means were compared using a t-test and Fisher adjustment by the PDIFF option of SAS (18). Finally, slope-ratio
analysis was used to compare multiple-linear regression curves of plasma amino acid concentration (plasma amino acid concentration vs. time) among leucine treatments (28). Data are presented according to the new guidelines of the American Physiological Society (5).

RESULTS

Amino acids, glucose and insulin in circulation. Plasma leucine levels in piglets infused with leucine at either 200 or 400 µmol·kg⁻¹·h⁻¹ were markedly increased \((P < 0.01)\) within 30 min of the start of the infusion and remained elevated throughout the experiment (Fig. 1). Furthermore, targeted plasma leucine levels within the fed range (i.e., 2- to 4-fold above fasting levels (4)) were achieved. Infusion of leucine, however, decreased \((P < 0.05)\) plasma levels of the other essential amino acids as the time of infusion progressed (Fig. 1B and 1C). 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. Results indicate linear reductions in plasma concentrations of isoleucine \((P = 0.0002)\), valine \((P = 0.0006)\), lysine \((P = 0.0005)\), methionine \((P = 0.07)\), and tyrosine \((P = 0.01)\) in piglets infused with 200 µmol·kg⁻¹·h⁻¹ of leucine compared to those piglets infused with saline, as time of infusion progressed. In piglets infused with 400 µmol·kg⁻¹·h⁻¹ of leucine, linear reductions in plasma concentrations of isoleucine \((P < 0.0001)\), valine \((P < 0.0001)\), lysine \((P < 0.0001)\), methionine \((P = 0.004)\), phenylalanine \((P = 0.02)\), threonine \((P = 0.03)\), tryptophan \((P = 0.02)\) and tyrosine \((P = 0.002)\) were observed compared to piglets infused with saline, as time of infusion progressed. On average, plasma concentrations of the aforementioned amino acids decreased ~50% by 120 min. Plasma concentrations of arginine \((P = 0.95)\), histidine \((P = 0.41)\), proline \((P = 0.16)\), serine \((P = 0.78)\), aspartate \((P = 0.83)\),
asparagine ($P = 0.20$), glutamate ($P = 0.99$), glutamine ($P = 0.84$), glycine ($P = 0.40$) and alanine ($P = 0.13$) were unaffected by leucine infusion. Plasma levels of phenylalanine and tyrosine after the isotope tracer was administered were not included in the statistical analysis because a large dose of phenylalanine was given along with L[4-H]phenylalanine to determine fractional rates of protein synthesis. Due to the high concentration in plasma of phenylalanine after the flooding procedure, we were unable to obtain separation of the tryptophan peak from the phenylalanine peak in our HPLC method. Blood glucose ($P = 0.78$; Fig. 2A) and plasma insulin ($P = 0.99$; Fig. 2B) were not different among treatments at any time during the 2-h leucine infusion.

Translation initiation factors. Phosphorylation of S6K1 increased in skeletal muscle at 60 min ($P < 0.0001$) and 120 min ($P = 0.02$) after the start of the 400-µmol·kg$^{-1}$·h$^{-1}$ leucine infusion, compared to saline infusion (Fig. 3A). Phosphorylation of S6, a target of S6K1, was also increased in skeletal muscle at 60 min ($P < 0.002$) and 120 min ($P = 0.01$) after the start of leucine infusion at 400 µmol·kg$^{-1}$·h$^{-1}$, compared to saline infusion (Fig. 3B). Phosphorylation of the repressor protein of eIF4E, 4E-BP1 at Thr70, in skeletal muscle was increased at 60 min ($P = 0.0002$) and 120 min ($P = 0.02$) after the start of leucine infusion at 400 µmol·kg$^{-1}$·h$^{-1}$, compared to saline infusion (Fig. 4A). A concomitant decrease in the amount of eIF4E associated with 4E-BP1 was observed at 60 min ($P = 0.006$) and 120 min ($P = 0.03$) after the start of leucine infusion at 400 µmol·kg$^{-1}$·h$^{-1}$, compared to saline infusion (Fig. 4B). Infusing piglets with 200 µmol·kg$^{-1}$·h$^{-1}$ of leucine, increased S6K1 phosphorylation in skeletal muscle after 60 min ($P = 0.04$) but not after 120 min ($P = 0.43$) of leucine infusion (Fig. 3A). However, phosphorylation of S6 (Fig. 3B) and 4E-BP1 (Fig. 4A) in skeletal muscle were not affected by leucine infusion of 200 µmol·kg$^{-1}$·h$^{-1}$ at 60 min ($P = 0.32$ and $P = 0.13$, respectively) or 120 min ($P = 0.27$ and $P =
0.39, respectively) compared to saline infused piglets. Similarly, eIF4E associated with 4E-BP1 in skeletal muscle was unaffected by leucine infusion at 200 µmol·kg⁻¹·h⁻¹ for 120 min ($P = 0.78$) but not at 60 min ($P = 0.09$). In skeletal muscle, phosphorylation of S6K1 ($P = 0.004$), S6 ($P = 0.008$), and 4E-BP1 ($P = 0.002$) increased linearly, whereas the association of eIF4E with 4E-BP1 decreased linearly ($P = 0.08$) in a leucine dose-dependent manner after 60 min of leucine infusion. Similarly, after 120 min of leucine infusion, phosphorylation of S6K1 ($P = 0.01$), S6 ($P = 0.07$), and 4E-BP1 ($P = 0.02$) increased linearly, whereas the association of eIF4E with 4E-BP1 decreased linearly ($P = 0.01$) in a leucine dose-dependent manner in skeletal muscle. In liver, S6K1 (Fig. 5A), S6 (Fig. 5B) and 4E-BP1 (Fig. 6A) phosphorylation or the association of eIF4E with 4E-BP1 (Fig. 6B) was unaffected by the infusion of either dose of leucine for 60 min ($P = 0.11$ to $0.97$) or 120 min ($P = 0.10$ to $0.99$).

Protein synthesis. After 60 min of leucine infusion, fractional rates of skeletal muscle protein synthesis were increased in piglets infused at 400 µmol·kg⁻¹·h⁻¹ (+33%, $P = 0.002$) but not in piglets infused at 200 µmol·kg⁻¹·h⁻¹ ($P = 0.50$) compared to saline infused piglets (Fig. 7A). After 120 min of leucine infusion, fractional rates of skeletal muscle protein synthesis were not different in piglets infused at 200 µmol·kg⁻¹·h⁻¹ ($P = 0.58$) or 400 µmol·kg⁻¹·h⁻¹ ($P = 0.34$) compared to saline infused piglets (Fig. 7A). In the liver, fractional rates of protein synthesis were not affected by 60 min of leucine infusion at 200 µmol·kg⁻¹·h⁻¹ ($P = 0.15$) or 400 µmol·kg⁻¹·h⁻¹ ($P = 0.85$) compared to saline infused piglets (Fig. 7B). However, after 120 min of leucine infusion, fractional rates of protein synthesis in the liver were markedly decreased, from saline infused piglets, at both 200 µmol·kg⁻¹·h⁻¹ (-14%, $P = 0.01$) and 400 µmol·kg⁻¹·h⁻¹ (-26%, $P < 0.0001$). Furthermore, fractional rates of protein synthesis in the liver were linearly reduced ($P < 0.0001$) in a leucine-dependent dose after 120 min of leucine infusion (Fig. 7B).
DISCUSSION

The human neonatal period is characterized by rapid growth, high rates of protein turnover and marked accretion of skeletal muscle (14). We have demonstrated that neonatal pigs are a population highly responsive to anabolic stimuli, such as feeding, insulin and amino acids (6; 8; 25; 34). When neonatal pigs are fed colostrum, mature sow’s milk or formula, protein synthesis increases in disparate tissues (4). This protein synthetic response to feeding is most profound in skeletal muscle (6). Similarly, infusion of a balanced amino acid mixture to mimic postprandial levels of plasma amino acids results in increased protein synthesis in skeletal muscle and liver, and this response to amino acids is independent of insulin (25; 27). The amino acid-induced stimulation of protein synthesis is modulated by enhanced translation initiation factors that lead to enhanced eIF4G•eIF4E complex assembly (26). In this study, we infused leucine to achieve levels within the fed physiological range, without eliciting an increase in circulating insulin, and found that leucine markedly stimulated protein synthesis in skeletal muscle by 60 min. This response to leucine involved modulation of translation initiation factors that regulate mRNA binding to the ribosomal complex. Infusion of leucine for 120 min, however, did not stimulate protein synthesis in skeletal muscle despite enhanced modulation of translation initiation factor activation. In the liver, protein synthesis was unaffected by leucine infusion for 60 min. Lengthening the time of leucine infusion resulted in a decrease in liver protein synthesis, without affecting translation initiation factor activity. This blunting of leucine-stimulated muscle protein synthesis and reduction in liver protein synthesis was associated with a 50% reduction in circulating essential amino acid levels and suggests that these amino acids became limiting as they were utilized for protein synthesis. Thus, leucine, at
physiological concentrations in plasma, can act as a nutrient signal to stimulate protein synthesis in skeletal muscle via modulation of the activation of translation initiation factors, but the response is substrate-dependent.

**Effect of leucine infusion on plasma amino acids.** As expected, infusion of leucine resulted in 1.5- to 3.0-fold increases in the plasma concentration of leucine, compared to baseline levels. This increase in circulating leucine was within the postprandial range observed when neonatal pigs are fed colostrum, mature sow’s milk or formula (4). However, the physiological increase in circulating leucine resulted in a reduction in the concentrations of the other branched-chain amino acids by 120 min of infusion. In our study, plasma concentrations of isoleucine and valine significantly decreased after 120 min of leucine infusion, and in a dose-dependent manner. The reduction in plasma amino acids after leucine infusion was not exclusive to the branched-chain amino acids. Slope-ratio analysis of plasma concentrations of individual amino acids indicated a significant linear decrease in plasma lysine, methionine, phenylalanine, threonine, tyrosine and tryptophan as the time of leucine infusion progressed and in a dose-dependent manner. The reduction in circulating essential amino acid concentrations with leucine infusion is consistent with studies in healthy humans subjects in which leucine levels were increased 4- to 6-fold for 2.5 to 7 hours and this resulted in a reduction in plasma concentrations of essential amino acids from 35 to 70% (17; 23).

**Effect of leucine infusion on translation initiation factors.** In the present study, a physiological increase in plasma leucine enhanced the phosphorylation of S6K1 and S6, and reduced the inhibitory effect of 4E-BP1 over eIF4E in muscle, but not in liver. Moreover, translation initiation factors in muscle were still activated after 120 min of leucine infusion. We have previously reported that infusion of a balanced mixture of amino acids, to increase all
circuiting amino acids to mimic the fed level, enhances the activation of S6K1, the assembly of
the active eIF4G•eIF4E complex, and dissociation of the inactive 4E-BP1•eIF4E complex as a
result of a lower inhibitory effect of 4E-BP1 over eIF4E in muscle of neonatal pigs (26).
Furthermore, the activation of translation initiation factors in muscle increase in an amino acid
dose-dependent manner (26). Previous studies have reported increased phosphorylation of
4E-BP1, reduced 4E-BP1•eIF4E complex assembly and enhanced eIF4G•eIF4E complex
assembly with supraphysiological increases (i.e., 16-fold increase over fasted controls) in
circulating levels of leucine in mature rats (1; 3; 29). To the best of our knowledge, however,
this is the first study to report enhanced activation of translation initiation factors in muscle in
response to a physiological increase in the plasma concentration of leucine in neonates.

In a recent study, mature rats were gavage-fed a bolus of leucine equivalent to the amount
of leucine consumed over a 24-hour period (2). This protocol resulted in a circulating leucine
concentration that was about 18-fold higher than controls after 30 min of bolus administration.
This increase in circulating leucine resulted in enhanced phosphorylation of 4E-BP1, S6K1 and
S6, a reduction in the 4E-BP1•eIF4E complex assembly, and an increase in the eIF4G•eIF4E
complex assembly in muscle. Two hours after the bolus of leucine, however, phosphorylation of
4E-BP1 and eIF4E, as well as the association of the 4E-BP1•eIF4E and eIF4G•eIF4E complexes
in muscle had returned to baseline levels, despite a circulating leucine concentration about 7-fold
above baseline levels. By contrast, phosphorylation of S6K1, S6 and 4E-BP1 was enhanced and
assembly of the inactive 4E-BP1•eIF4E complex was reduced in muscle of neonatal pigs after a
2-hour leucine infusion that resulted in circulating leucine concentration 2.8-fold higher than
baseline levels. These observations suggest that translation initiation factors that regulate mRNA
binding to the ribosomal complex are more sensitive to leucine stimulation in muscle of neonates than in muscle of mature animals.

We recently reported that infusion of a balanced mixture of amino acids, to increase all circulating amino acids to mimic the fed level, enhances the phosphorylation of S6K1 and 4E-BP1, as well as reduces 4E-BP1•eIF4E complex assembly in the liver of neonatal pigs in an amino acid dose-dependent manner (27). In the present study, however, translation initiation factors in liver of neonatal pigs were not affected by a physiological increase in circulating leucine. This suggests that additional amino acids, other than leucine, may be required for the stimulation of translation initiation factors in neonatal liver. However, increased phosphorylation of S6K1 and 4E-BP1 in liver has been reported in mature rats gavage-fed a bolus of leucine that resulted in a 18-fold increase in circulating leucine compared to controls with no change in global rates of protein synthesis (22; 29). Because neonatal animals are more sensitive to amino acid availability than mature rats (8), differences in the effect of leucine in studies using mature animals and neonates may be surprising. However, supraphysiological levels of leucine stimulated mTOR signaling in the liver of mature animals whereas physiological levels of leucine had no effect in neonatal liver in the present study. Therefore, the results suggest that the effect of leucine on translation initiation in liver may be dose-dependent. Collectively, results from this study indicate that a physiological increase in circulating leucine, acting as a nutrient signal, is sufficient to induce phosphorylation of S6K1, S6 and 4E-BP1, as well as a reduction in inactive 4E-BP1•eIF4E complex assembly in muscle, but not in liver, of neonatal pigs.

Effect of leucine infusion on protein synthesis. We have previously reported that both muscle and liver protein synthesis increase linearly when a balanced mixture of amino acids is
infused to neonatal pigs (26; 27). Others have also reported an amino acid-induced stimulation of protein synthesis in muscle of older pigs (32). Furthermore, the increases in protein synthesis in both muscle and liver, in response to amino acid infusion, are mediated by modulation of translation initiation factor activation (26; 27). In the present study, we examined the leucine-induced stimulation of protein synthesis in muscle and liver of neonatal pigs. Results indicate that a physiological increase in circulating leucine alone was sufficient to stimulate protein synthesis in muscle. This increase in muscle protein synthesis, which was mediated by increased phosphorylation of 4E-BP1, S6K1 and S6, as well as reduced 4E-BP1:eIF4E complex assembly, was significant only when neonatal pigs were infused for a short period of time (i.e., 60 min). When the infusion time was extended (i.e., 120 min), a numeric but not significant increase in muscle protein synthesis was measured, despite significant increases in 4E-BP1, S6K1 and S6 phosphorylation, as well as a significant reduction in assembly of the inactive 4E-BP1:eIF4E complex. This lack of response in muscle protein synthesis can likely be explained by the marked decline in circulating levels of several essential amino acids (~50%) after 120 min of leucine infusion. In previous studies using weaned, but growing rats, supraphysiological increases in circulating leucine (i.e., 16- to 18-fold from controls) have resulted in increases in muscle protein synthesis, which were mediated by enhanced activation of translation initiation factors (1-3; 22). Similarly, muscle protein synthesis was increased by leucine infusion in adult sheep (30).

Protein synthesis in liver is sensitive to circulating levels of plasma amino acids. In fact, protein synthesis in the liver increases linearly as concentrations of all amino acids are raised from below fasting levels to fed levels, a response that is mediated by enhanced phosphorylation of S6K1 and 4E-BP1, as well as reduced 4E-BP1:eIF4E complex assembly (27). Results
presented herein indicated that a physiological increase in plasma leucine was unable to stimulate protein synthesis or the phosphorylation of 4E-BP1, S6K1 and S6, as well as the dissociation of the 4E-BP1•eIF4E complex in liver. Similarly, protein synthesis in the liver was unaffected in rats experiencing a supraphysiological increase in circulating leucine (i.e., 18-fold from controls), despite enhanced activation of translation initiation factors (22; 29).

In the current study, infusion of leucine for 120 min reduced liver protein synthesis rates below baseline levels, in a leucine dose-dependent manner, while biomarkers of mRNA translation were unaffected. This reduction in liver protein synthesis was associated with a marked reduction in circulating levels of essential amino acids, suggesting that these amino acids had become limiting and unable to sustain the basal rate of liver protein synthesis. Similarly, liver protein synthesis was reduced by leucine infusion in adult sheep (30).

Collectively, results from this study indicate that leucine, at physiological levels, can act as a nutrient signal to increase protein synthesis in muscle, but not in liver, of neonatal pigs. These changes in protein synthesis were not dependent on changes in circulating insulin levels. Similarly, no changes in plasma insulin were reported in healthy men infused with leucine for 7 hours to raise plasma leucine levels 4-fold (24). By contrast, when a bolus of leucine is administered orally, leucine acts as an insulin secretagogue and can increase circulating insulin levels (e.g., about 3.0- to 4.5-fold) (2; 22). In the present study, a physiological increase in plasma leucine stimulated muscle protein synthesis of neonatal pigs and this response was not only tissue-specific and substrate-dependent, but was also insulin-independent in the sense that a physiological increase in circulating leucine did not increase plasma insulin and hence could not have contributed to the increase in muscle protein synthesis of neonatal pigs reported herein.
Perspectives. Previous studies from our group have demonstrated the ability of neonatal pigs to increase protein synthesis in both muscle and liver in response to a physiological increase in all circulating amino acids to mimic the fed state. In addition, several in vitro and in vivo studies have reported increased protein synthesis in response to supraphysiological doses of leucine. However, to the best of our knowledge, this is the first study to report a significant increase in protein synthesis in the muscle of neonatal pigs as a response to a physiological increase in circulating leucine. Thus, the results suggest that leucine, provided at physiological levels, can act as a nutrient-signal to stimulate protein synthesis in muscle of neonatal pigs and this response is time-specific and dose-dependent. When the infusion of leucine was prolonged, a numeric but not significant increase in muscle protein synthesis was measured despite significant increases in the activation of factors that regulate mRNA binding to the ribosomal complex. This lack of response in muscle protein synthesis can likely be explained by the marked decline in circulating levels of several essential amino acids. We speculate that these essential amino acids became limiting as they were used for protein synthesis and that protein synthesis in muscle of neonatal pigs would be increased during prolonged leucine infusion if circulating levels of essential amino acids were maintained at fasting levels. Nevertheless, studies specifically designed to maintain euaminoacidemia during the infusion of leucine must be conducted to address this issue.
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**FIGURE LEGENDS**

**Fig. 1.** Plasma concentrations of leucine, isoleucine and valine (A), lysine, methionine and threonine (B), and phenylalanine, tyrosine and tryptophan (C) at baseline and during 0, 200 and 400 µmol·kg⁻¹·h⁻¹ of leucine infusion. Plasma concentrations of phenylalanine, tyrosine and tryptophan are not reported after piglets were flooded with L[⁴⁻³H]phenylalanine to determine fractional rates of protein synthesis. Values are means (SD); n = 6-8 per treatment. * Means are different from baseline values (P < 0.05).

**Fig. 2.** Blood glucose (A) and plasma insulin (B) at baseline and during 0, 200 and 400 µmol·kg⁻¹·h⁻¹ of leucine infusion. Values are means (SD); n = 6-8 per treatment.

**Fig. 3.** Skeletal muscle phosphorylation of ribosomal protein S6 kinase (S6K1, A), and its target, ribosomal protein S6 (B) after 60 and 120 min of 0, 200 and 400 µmol·kg⁻¹·h⁻¹ of leucine infusion. Values are means (SD); n = 6-8 per treatment. Means with different superscripts differ at P < 0.04.

**Fig. 4.** Skeletal muscle phosphorylation of eukaryotic initiation factor (eIF) 4E binding protein-1 (4E-BP1, A), and the association of eIF4E with 4E-BP1 (B) after 60 and 120 min of 0, 200 and 400 µmol·kg⁻¹·h⁻¹ of leucine infusion. Values are means (SD); n = 6-8 per treatment. Means with different superscripts differ at P < 0.03.

**Fig. 5.** Liver phosphorylation of ribosomal protein S6 kinase (S6K1, A), and its target, ribosomal protein S6 (B) after 60 and 120 min of 0, 200 and 400 µmol·kg⁻¹·h⁻¹ of leucine infusion. Values are means (SD); n = 6-8 per treatment.
Fig. 6. Liver phosphorylation of eukaryotic initiation factor (eIF) 4E binding protein-1 (4E-BP1, A), and the association of eIF4E with 4E-BP1 (B) after 60 and 120 min of 0, 200 and 400 µmol·kg⁻¹·h⁻¹ of leucine infusion. Values are means (SD); n = 6-8 per treatment.

Fig. 7. Fractional rates of protein synthesis in skeletal muscle (A) and liver (B) after 60 and 120 min of 0, 200 and 400 µmol·kg⁻¹·h⁻¹ of leucine infusion. Values are means (SD); n = 6-8 per treatment. Means with different superscripts differ at $P < 0.03$. 
Figure 1

A

Baseline 0 200 400 \(\mu\text{mol\cdot kg}^{-1}\cdot \text{h}^{-1}\) Leucine

\[
\begin{array}{c}
\text{\mu M} \\
600 \\
500 \\
400 \\
300 \\
200 \\
100 \\
0 \\
0 \\
30 \\
60 \\
90 \\
120 \\
0 \\
30 \\
60 \\
90 \\
120 \\
0 \\
30 \\
60 \\
90 \\
120
\end{array}
\]

Leucine  Isoleucine  Valine

B

\[
\begin{array}{c}
\text{\mu M} \\
250 \\
150 \\
100 \\
50 \\
0 \\
0 \\
30 \\
60 \\
90 \\
120 \\
0 \\
30 \\
60 \\
90 \\
120 \\
0 \\
30 \\
60 \\
90 \\
120
\end{array}
\]

Lysine  Methionine  Threonine

C

\[
\begin{array}{c}
\text{\mu M} \\
250 \\
150 \\
100 \\
50 \\
0 \\
0 \\
30 \\
60 \\
90 \\
120 \\
0 \\
30 \\
60 \\
90 \\
120 \\
0 \\
30 \\
60 \\
90 \\
120
\end{array}
\]

Phenylalanine  Tyrosine  Tryptophan
Figure 2
Figure 3

A

S6K1 Phosphorylation
(% of control)

B

S6 Phosphorylation Ser235/236
Ser240/244 (% of control)

0 200 400 μmol·kg⁻¹·h⁻¹ Leucine

60 min 120 min
Figure 4

A

4E-BP1 Phosphorylation
Thr72(%) of control

B

dmE3A Associated with 4E-BP1(%) of control

0 200 400 μmol·kg⁻¹·h⁻¹ Leucine

0 200 400 μmol·kg⁻¹·h⁻¹ Leucine

60 min 120 min
Figure 5

A

B

[Graph showing phosphorylation levels with different conditions and time points]
Figure 6

A

4-E-BP1 Phosphorylation

Thr70 % of control

0 200 400 μmol·kg⁻¹·h⁻¹ Leucine

B

eIF4E Associated with 4E-BP1 (% of control)

60 min 120 min
Figure 7

(A) Skeletal muscle Ks (%/day)

(B) Liver Ks (%/day)

0 200 400 μmol·kg−1·h−1 Leucine

60 min 120 min

Significance letters indicate statistical differences.