Physiological rise in plasma leucine stimulates muscle protein synthesis in neonatal pigs by enhancing translation initiation factor activation

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Protein synthesis in skeletal muscle of adult rats increases in response to feeding to a greater extent than amino acids efficiently for growth because they can increase also declines with development (12). Neonates use dietary rapidly thereafter (6, 10). Dietary amino acids are used effi-

specific. The results suggest that the branched-chain amino acid

effect on protein synthesis of a physiological rise in plasma leucine

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

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-tRNAi 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 whether it is attributable to specific effects of individual amino acids. In vitro studies suggest that leucine alone can stimulate protein synthesis by a mammalian target of rapamycin (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 with 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-
derpendent process that involves S6K1 and 4E-BP1 phosphor-
ylation, and eIF4F assembly (1, 2). In contrast, oral adminis-
tration 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 deter-
mine 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 whether 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 2 wk 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, Brentwood, MO) and water throughout
the study. Within 24 h of birth, piglets were injected intramuscularly
with 100 mg of iron dextran (Phoenix Pharmaceuticals, 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 by use
of sterile techniques (33). This study was conducted according to
the National Research Council’s Guide for the Care and Use of Labora-
tory Animals.

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, and t-[4-3H]phenylalanine,
whereas the jugular catheter was used for repeated blood sample
collections. To obtain plasma, whole blood was collected in
(collection. Pigs were randomly assigned to one of three constant
infusion rates. 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 syn-
thesis were measured with a flooding dose of t-[4-3H]phenylalanine
(Amersham Biosciences, Piscataway, NJ) injected 30 min before the
end of the infusion (16). We (9) have demonstrated that, after a
flooding dose of phenylalanine is administered, the specific radioac-
tivity of tissue-free phenylalanine is in equilibrium with the amino-
acyl-tRNA specific radioactivity; thus the tissue-free phenylalanine is
a valid measure of the tissue precursor pool specific radioactivity.

Protein synthesis (Kₚ, expressed as %protein synthesized in a day) was
calculated as: $Kₚ = (Sₑ/Sₚ) \times (1,440/t) \times 100$, where Sₑ is
the specific radioactivity of the protein-bound phenylalanine; Sₚ 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 minutes. 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 −70°C 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 sam-
ple collection. To obtain plasma, whole blood was collected in
heparinized tubes, centrifuged at 10,000 g for 1 min at room temper-
ature, and stored at −20°C until analyzed. By use of a porcine insulin
radioimmunoassay kit (Linco, St. Louis, MO) that used porcine insulin
antibody and human insulin standards, plasma radioimmuno-
reactive 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 (Bio-Rad, Hercules, CA), which was incubated with ap-
propriate antibodies, as previously described (13). Blots were devel-
oped using an enhanced chemiluminescence kit (ECL, Amersharm),
visualized using a 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 immunoprecipi-
tation at 4°C using a monoclonal antibody against eIF4E (19). Im-
munoprecipitates were subjected to protein immunoblot analysis,
as described above, using a rabbit polyclonal anti-4E-BP1 antibody
(Bethyl Laboratories, Montgomery, TX) and the mouse monoclonal
anti-eIF4E antibody.

Muscle and liver homogenates. Freshly collected longissimus dorsi
muscle and liver tissue samples were homogenized, centrifuged at
10,000 g for 10 min at 4°C, heated at 100°C for 10 min, and
cooled to room temperature. Supernatants were diluted in sample buffer (20),
frozen in liquid nitrogen, and stored at −70°C until protein immuno-
blot analysis.

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

Quantification of S6K1 phosphorylation. An aliquot of muscle and
liver homogenates was subjected to protein immunoblot analysis as
previously described (13), using a rabbit polyclonal antibody that
recognizes phosphorylation at pThr³⁸⁹⁸ (Santa Cruz Biotechnology, Santa Cruz,
CA).

Quantification of rpS6 phosphorylation. An aliquot of muscle and
liver homogenates was 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 frac-
tional 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, Cary, NC) for ran-

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domized 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 square means were compared using a t-test and Fisher adjustment by the PDIF 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 (Fig. 1A). Infusion of leucine, however, decreased (∆P < 0.05) plasma levels of the other essential amino acids as the time of infusion progressed (Fig. 1, B and C). 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 leucine, compared with those piglets infused with saline, as time of infusion progressed. In piglets infused with 400 μmol·kg⁻¹·h⁻¹ 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 with 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 reported after piglets were flooded with L-[4-³H]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 with saline infusion (Fig. 3A). Phosphorylation of S6, a target of S6K1, was also increased in skeletal muscle at 60 min (∆P < 0.0002) and 120 min (∆P = 0.01) after the start of leucine infusion at 400 μmol·kg⁻¹·h⁻¹ compared with 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 with 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 with saline infusion (Fig. 4B). Infusing piglets with 200 μmol·kg⁻¹·h⁻¹ 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 was not
affected by leucine infusion of 200 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) at 60 min \((P = 0.32\) and \(P = 0.13\), respectively\) or 120 min \((P = 0.27\) and \(P = 0.39\), respectively\) compared with saline-infused piglets. Similarly, eIF4E associated with 4E-BP1 in skeletal muscle was unaffected by leucine infusion at 200 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) 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 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) \((+33\%, P = 0.002)\) but not in piglets infused at 200 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) \((P = 0.50)\) compared with 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 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) \((P = 0.58)\) or 400 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) \((P = 0.34)\) compared with saline-infused piglets (Fig. 7A). In the liver, fractional rates of protein synthesis were not affected by 60 min of leucine infusion at 200 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) \((P = 0.15)\) or

![Blood glucose (A) and plasma insulin (B) at baseline and during 0, 200, and 400 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) leucine infusion. Values are means (SD); \( n = 6–8 \) per treatment.](image1)

![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 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) leucine infusion. Values are means (SD); \( n = 6–8 \) per treatment. Means with different superscripts differ at \( P < 0.04 \).](image2)

![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 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) leucine infusion. Values are means (SD); \( n = 6–8 \) per treatment. Means with different superscripts differ at \( P < 0.03 \).](image3)
400 μmol·kg\(^{-1}\)·h\(^{-1}\) (P = 0.85) compared with 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 with 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 human subjects in whom leucine levels were increased four- to sixfold for 2.5 to 7 h, 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 (26) have previously 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 and reduces 4E-BP1·eIF4E complex assembly in the liver of neonatal pigs in an amino acid dose-dependent manner. 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 an 18-fold increase in circulating leucine compared with 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 (26, 27) have previously reported that protein synthesis in both muscle and liver increases linearly when a balanced mixture of amino acids is infused to neonatal pigs. 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 present study, infusion of leucine for 120 min reduced liver protein synthesis rates below baseline levels in a leucine dose-dependent manner, whereas 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 h to raise plasma leucine levels fourfold (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., ~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 (8, 25–27) 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 newborn pigs and that 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 because 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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