Regulation of cardiac and skeletal muscle protein synthesis by individual branched-chain amino acids in neonatal pigs


1United States Department of Agriculture/Agricultural Research Service, Children’s Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, Texas; and 2Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania

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Regulation of cardiac and skeletal muscle protein synthesis by individual branched-chain amino acids in neonatal pigs. Am J Physiol Endocrinol Metab 290: E612–E621, 2006. First published November 8, 2005; doi:10.1152/ajpendo.00402.2005.—Skeletal muscle grows at a very rapid rate in the neonatal pig, due in part to an enhanced sensitivity of protein synthesis to the postprandial rise in amino acids. An increase in leucine alone stimulates protein synthesis in skeletal muscle of the neonatal pig; however, the effect of isoleucine and valine has not been investigated in this experimental model. The left ventricular wall of the heart grows faster than the right ventricular wall during the first 10 days of postnatal life in the pig. Therefore, the effects of individual BCAA on protein synthesis in individual skeletal muscles and in the left and right ventricular walls were examined. Fasted pigs were infused with 0 or 400 μmol·kg⁻¹·h⁻¹ leucine, isoleucine, or valine to raise individual BCAA to fed levels. Fractional rates of protein synthesis and indexes of translation initiation were measured after 60 min. Infusion of leucine increased (P < 0.05) phosphorylation of eukaryotic initiation factor (eIF)4E-binding protein-1 and increased (P < 0.05) the amount and phosphorylation of eIF4G associated with eIF4E in longissimus dorsi and masseter muscles and in both ventricular walls. Leucine increased (P < 0.05) the phosphorylation of ribosomal protein (rp)S6 kinase and rpS6 in longissimus dorsi and masseter but not in either ventricular wall. Leucine stimulated (P < 0.05) protein synthesis in longissimus dorsi, masseter, and the left ventricular wall. Isoleucine and valine did not increase translation initiation factor activation or protein synthesis rates in skeletal or cardiac muscles. The results suggest that the postprandial rise in leucine, but not isoleucine or valine, acts as a nutrient signal to stimulate protein synthesis in cardiac and skeletal muscles of neonates by increasing eIF4E availability for eIF4F complex assembly.

NEONATES, A HIGHLY ANABOLIC POPULATION, use dietary amino acids efficiently for growth because they can increase protein synthesis (Kₚ) in response to feeding to a greater extent than mature animals (10, 15). In the neonate, the feeding-induced stimulation of protein synthesis occurs in virtually all tissues but is most pronounced in skeletal muscles containing primarily fast-twitch glycolytic fibers, where the response is mediated independently by the postprandial rise in both insulin and amino acids (13, 28, 29). This increase in protein synthesis in neonatal animals is associated with increased protein synthetic efficiency [KRNA (12, 15)], which is mainly 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 (16, 29). In skeletal muscle of neonates, feeding or the acute infusion of amino acids to raise levels to within the fed range increases the phosphorylation of the 70-kDa ribosomal protein (rp)S6 kinase (S6K1) as well as the phosphorylation of eukaryotic initiation factor (eIF)4E-binding protein-1 (4E-BP1). Phosphorylated 4E-BP1 releases eIF4E from the inactive 4E-BP1·eIF4E complex. In turn, the freed eIF4E is able to bind to eIF4G and eIF4A to form the active eIF4F complex, which mediates the binding of mRNA to the 43S ribosomal complex (29).

We (11, 13, 28, 29) have demonstrated that infusion of a balanced mixture of amino acids to mimic postprandial hyperaminoacidemia (7) results in activation of translation initiation factors and stimulation of protein synthesis in fast-twitch glycolytic skeletal muscles of neonatal pigs. It is not clear, however, whether this response is arbitrated by the increase in all amino acids or to the increase in an individual amino acid. Studies in cultured cells (19, 27, 31) and rats (1–3) suggest that leucine alone can increase protein synthesis in part by a mammalian target of rapamycin (mTOR)-dependent process that involves the phosphorylation of S6K1 and 4E-BP1 and eIF4F assembly. Because those studies were conducted in the presence of supraphysiologically concentrations of leucine, the physiological relevance of leucine to activate translation initiation factors and to stimulate protein synthesis was questionable. We addressed this issue in a recent study wherein a physiological increase in circulating leucine stimulated protein synthesis in the longissimus dorsi, a muscle that contains primarily fast-twitch glycolytic muscle fibers, in neonatal pigs (18). Furthermore, the leucine-induced stimulation of protein synthesis was mediated by increased activation of translation initiation factors. The effect of a physiological increase in circulating isoleucine or valine on protein synthesis in neonates has not been evaluated. Thus the main objective of this study was to compare the effects of a physiological rise in each of the individual branched-chain amino acids (BCAA) on protein synthesis in neonatal pigs. Furthermore, we wished to investigate the mechanism(s) involved in the BCAA stimulation of protein synthesis in skeletal and cardiac muscles.

Address for reprint requests and other correspondence: T. A. Davis, USDA/ARS Children’s Nutrition Research Center, Dept. of Pediatrics, Baylor College of Medicine, 1100 Bates St., Houston, TX 77030 (e-mail: tdavis@bcm.tmc.edu).

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The growth of the heart after birth is asymmetrical, which is primarily driven by the increased hemodynamic workload of the left compared with the right ventricle. Indeed, the left ventricular wall of the pig’s heart grows about three times faster than the right ventricular wall during the first 10 days of postnatal life (4, 30). The augmentation in mass of the left ventricular wall probably results from both increased hypertrophy and hyperplasia (4). The greater hypertrophy of the left ventricular wall compared with the right ventricular wall can be attributed mainly to an increase in the rate of protein synthesis rather than a change in the rate of protein degradation and is driven by both an enhanced efficiency of translation and an increased capacity for protein synthesis (30). The infusion of insulin in situ increases translational efficiency in the right ventricular wall but does not further enhance the high rate of translational efficiency in the left ventricular wall of 5-day-old pigs (8, 30). It is not known, however, whether the left ventricular wall is sensitive to direct nutrient stimulation. The potential to use BCAA therapy to increase protein synthesis in neonates at risk (e.g., extremely low birth weight infants) warrants the evaluation of cardiac and skeletal muscle responsiveness to BCAA administration. Thus an objective of the present study was to compare fast-twitch glycolytic and slow-twitch oxidative skeletal muscles, as well as the left and right ventricles, in their ability to stimulate protein synthesis and activate translation initiation factors in response to administration of individual BCAA.

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 before their due date. Sows and piglets were housed and managed as previously described (18). Piglets were studied at 5.8 ± 0.1 days of age weighing 2.0 ± 0.1 kg. The Animal Care and Use Committee of Baylor College of Medicine approved all experimental procedures. This study was conducted according to the National Research Council’s Guide for the Care and Use of Laboratory Animals.

Surgery. Immediately before anesthesia, each piglet received 54 μg/kg body wt atropine sulfate intramuscularly (Phoenix Pharmaceu-
ticals, St. Joseph, MO) and 2.27 mg/kg body wt enrofloxacin (Baytril, Bayer, Shawnee Mission, KS). An additional 4.54 mg of enrofloxacin were placed in the incision just before suturing. Anesthesia was induced with 5% isoflurane (AErrane; Baxter Healthcare, Deerfield, IL) and maintained with 2% isoflurane, both with 800 ml/min O2. The left external jugular vein and the left common carotid artery were surgically dissected, and an indwelling silicon catheter (HelixMark, 0.76 mm ID and 1.78 mm OD; Helix Medical, Carpenteria, CA) filled with heparinized saline (50 U/ml) was advanced posteriorly ~5 cm to the vena cava or the aortic arch, respectively. The jugular catheter was sutured to the scalene muscle and the carotid catheter to the sternothyroid and/or sternohyoid muscles. The distal end of each catheter was tunneled subcutaneously with a trocar and exteriorized between the dorsal neck region and the scalp. After recovering from anesthesia, piglets were returned to their respective sows until studied.

Treatments and infusion. Pigs were randomly assigned to one of four treatments and were infused with saline, leucine, isoleucine, or valine. All infusions were carried for 60 min as previously described (18). Infusion was initiated with a primed dose of 240 μmol/kg body wt for 10 min, followed by constant infusion at 400 μmol·kg⁻¹·h⁻¹ for each BCAA.

Tissue protein synthesis in vivo. Fractional rates of protein synthesis were measured with 10 ml/kg body wt of a flooding dose (1.5 nmol/kg body wt) of l-[4-¹⁴C]phenylalanine (0.5 mCi/kg body wt; Amersham Biosciences, Piscataway, NJ) injected 30 min before the infusion was ended, as previously described (20). At the end of the infusion, pigs were killed, and samples were obtained from the longissimus dorsi, which contains primarily fast-twitch muscle fibers, and the maeasteter muscle, which contains primarily slow-twitch muscle fibers, as well as the right and left ventricular walls. Samples were collected and immediately frozen in liquid nitrogen and stored at −70°C until analyzed, as previously described (14).

Protein synthesis (Kₚ; %protein mass synthesized in a day) was calculated as

\[ K_p = \frac{\%S_b - \%S_a}{t} \times \left[ \frac{(1.440 t)}{500} \right] \times 100 \]

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 minutes. The majority of RNA in tissues is ribosomal RNA; hence, RNA-to-protein ratio (mg RNA/g protein) was used as an estimate of protein synthetic capacity (Cₚ). RNA content was determined by spectrophotometric absorbency. Protein synthetic efficiency (KₚRNA) was estimated as the total protein synthesized in a day per total RNA (g protein·day⁻¹·g RNA⁻¹).

Blood glucose, plasma insulin, and amino acids. Blood samples were collected every 10 min throughout the study. Whole blood glucose concentration (YSI 2300 STAT Plus; Yellow Springs Instruments, Yellow Springs, OH) was determined immediately after sample collection. Plasma concentrations of insulin were determined by radioimmunoassay as previously described (7). Individual plasma amino acid concentrations were measured with an HPLC method (PICO-TAG reverse-phase column; Waters, Milford, MA) as previously described (15).

Protein immunoblot analysis. Proteins were electrophoretically separated in polyacrylamide gels (23). For each assay, all samples were run at the same time in triple-wide gels (C.B.S. Scientific, Del Mar, CA) to eliminate interassay variation. Proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA) and incubated with appropriate antibodies (all from Cell Signaling Technology, Beverly, MA, unless otherwise indicated). Blots were developed using an enhanced chemiluminescence kit (ECL, Amersham), visualized using ChemiDocIt (UVP, Upland, CA), and analyzed with LabWorks Image Acquisition and Analysis Software (UVP). Site-specific phosphorylation and total protein content were determined.

Quantiﬁcation of eIF4E-4E-BP1 and eIF4E-eIF4G complexes. These complexes were immunoprecipitated using an anti-eIF4E monoclonal antibody (22) from aliquots of fresh tissue homogenates (26). Briefly, samples were homogenized in seven volumes of buffer (in mM: 20 HEPES, 2 EGTA, 50 NaF, 100 KCl, and 0.2 EDTA, pH 7.4) containing Sigma P3840 Protease Inhibitor Cocktail (Sigma Chemical, St. Louis, MO) and centrifuged at 10,000 × g for 10 min at 4°C. Supernatants were incubated overnight at 4°C with constant rocking with anti-eIF4E antibody. Immunoprecipitates were recovered with goat anti-rabbit IgG magnetic beads (Polysciences, Warrington, PA), washed and resuspended in sample buffer as described elsewhere (22), and immediately subjected to protein immunoblot analysis using rabbit anti-4E-BP1 antibody or rabbit anti-eIF4G antibody, kindly provided by Dr. Richard E. Lloyd (Dept. of Molecular Virology and Microbiology, Baylor College of Medicine). Amounts of 4E-BP1 and eIF4G were corrected by the eIF4E recovered from the immunoprecipitate.

Quantiﬁcation of phosphorylated eIF4G in the eIF4E-eIF4G complex. Aliquots of immunoprecipitates were subjected to protein immunoblot analysis using a rabbit polyclonal antibody that recognizes site-specific phosphorylation of eIF4G at Ser¹¹⁰⁸.

Muscle and heart homogenates. Aliquots of supernatants obtained from tissue homogenates were diluted in sample buffer (23), boiled

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for 10 min, cooled to room temperature, frozen in liquid nitrogen, and stored at −70°C until protein immunoblot analyses.

Quantification of 4E-BP1 phosphorylation. Aliquots of homogenates were subjected to protein immunoblot analysis using a rabbit polyclonal antibody that recognizes site-specific phosphorylation of 4E-BP1 at Thr⁰⁰⁰ (Bethyl Laboratories, Montgomery, TX).

Quantification of S6K1 phosphorylation. Aliquots of homogenates were subjected to protein immunoblot analysis using a rabbit polyclonal antibody that recognizes site-specific phosphorylation of S6K1 at Thr³⁸⁹ or total S6K1 (Santa Cruz Biotechnology, Santa Cruz, CA). Phosphorylation of S6K1 was corrected by total S6K1.

Quantification of rpS6 phosphorylation. Aliquots of homogenates were subjected to protein immunoblot analysis using rabbit polyclonal antibodies that recognize site-specific phosphorylation of rpS6 at Ser²³⁵/²³⁶ and Ser²⁴⁰/²⁴⁴ or total rpS6. Phosphorylation of rpS6 was corrected by total rpS6.

Quantification of eukaryotic elongation factor-2 phosphorylation. Aliquots of homogenates were subjected to protein immunoblot analysis using rabbit polyclonal antibodies that recognize site-specific phosphorylation of eEF2 at Thr⁷⁰ (Fig. 2B) or total eEF2. Phosphorylation of eEF2 was corrected by total eEF2.

Statistical analyses. To determine the effect of treatment on fractional protein synthesis rates 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 randomized complete-block design (21). A comparison of the responsiveness of skeletal muscle (fast-twitch glycolytic vs. slow-twitch oxidative) and ventricular walls (right vs. left) to BCAA stimulation was performed nesting muscle (skeletal or cardiac) within treatment (Fig. 5). A combination of protein immunoblot analyses and Western analysis were made between skeletal and cardiac muscles. 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 (21). Least square means were compared using a t-test and Fisher adjustment by the PDIFF option of SAS (21).

RESULTS

Amino acids, glucose, and insulin in circulation. Plasma levels of glucose (P = 0.26) and insulin (P = 0.19), measured every 10 min, were not affected by the infusion of individual BCAA (data not shown). Infusion of each individual BCAA resulted in a marked increase (P < 0.05) in the plasma concentration of the corresponding BCAA over baseline (Table 1). Furthermore, leucine and valine were elevated within the postprandial physiological range [i.e., 2- to 4-fold above fasting levels (7)], although isoleucine infusion resulted in slightly higher values (i.e., 6-fold above baseline values). The infusion of one BCAA resulted in the decrease of another BCAA. More specifically, infusion of leucine reduced circulating isoleucine (P = 0.001) compared with baseline values. Similarly, circulating leucine was reduced compared with baseline values by the infusion of isoleucine (P = 0.04). Plasma concentrations of arginine (P = 0.22 to 0.79), proline (P = 0.10 to 0.57), serine (P = 0.12 to 0.88), aspartate (P = 0.10 to 0.68), asparagine (P = 0.11 to 0.93), glutamate (P = 0.36 to 0.93), glutamine (P = 0.12 to 0.90), glycine (P = 0.21 to 0.96), alanine (P = 0.18 to 0.52), histidine (P = 0.22 to 0.84), methionine (P = 0.43 to 0.59), tyrosine (P = 0.10 to 0.85), threonine (P = 0.28 to 0.86), and lysine (P = 0.15 to 0.73) were unaffected after 60 min of BCAA infusion (data not shown). However, infusion of isoleucine caused an increase in plasma methionine (P = 0.03) and a reduction in plasma threonine (P = 0.05).

Translation initiation factors. Phosphorylation at Thr⁰⁰⁰ (Fig. 1A) and the γ-isofrom (Fig. 1B) of 4E-BP1, the repressor protein of eIF4E, was increased (P < 0.03) by infusion of leucine in longissimus dorsi and masseter muscles, as well as in the right and left ventricular walls (P < 0.03; Fig. 1, C and D) compared with saline infusion. Infusion of isoleucine or valine did not change the 4E-BP1 phosphorylation at Thr⁰⁰⁰ or γ-isofrom in the skeletal and cardiac muscles (P = 0.22 to 0.87) compared with saline controls. A concomitant decrease in the amount of eIF4E associated with 4E-BP1, the repressor of 4E-BP1, the repressor of eIF4E complex was increased by leucine infusion in both skeletal muscles as well as in both ventricular walls (P = 0.003; Fig. 3A). Furthermore, the eIF4G·eIF4E complex tended to be higher (P = 0.08) in the masseter compared with the longissimus dorsi muscle. In the heart, infusion of leucine increased (P < 0.0001) eIF4G·eIF4E complex content in the left ventricular wall and tended to increase the amount of eIF4G·eIF4E complex in the right ventricular wall (P = 0.09; Fig. 3B) compared with saline infusion. Infusion of isoleucine also increased (P < 0.03) the phosphorylation of eIF4G at Ser¹¹⁰⁸ in the eIF4G·eIF4E complex in both skeletal muscles as well as in both ventricular walls (Figs. 4, A and B). The amount of eIF4G·eIF4E complex (Figs. 3A and 4A), as well as the phosphorylation of eIF4G at Ser¹¹⁰⁸ in the eIF4G·eIF4E complex (Figs. 3B and 4B), were unaffected by the infusion of isoleucine or valine in skeletal muscles (P = 0.22 to 0.99) and ventricular walls (P = 0.11 to 0.99) compared with saline infusion.

Phosphorylation of S6K1 at Thr³⁸⁹ was markedly increased (P < 0.0001) by leucine infusion in longissimus dorsi muscle but not in masseter muscle (P = 0.35) compared with saline-treated pigs (Fig. 5A). In the heart, phosphorylation of S6K1 at Thr³⁸⁹ was unaffected by leucine infusion (P = 0.35 to 0.79; Fig. 5B) compared with saline infusion. Similarly, phosphorylation of S6K1 at Thr³⁸⁹ was unaffected by the infusion of isoleucine or valine in skeletal muscles (P = 0.31 to 0.97; Fig. 5A) or ventricular walls (P = 0.42 to 0.75; Fig. 5B) compared with saline infusion. As observed for S6K1, phosphorylation of
rpS6 was also increased \((P = 0.003)\) by leucine treatment in longissimus dorsi muscle but not in masseter muscle \((P = 0.45; \text{Fig. 6A})\) compared with saline-treated pigs. In the heart, infusion of BCAA did not affect the phosphorylation of rpS6 \((P = 0.10\) to 0.95) with the exception of valine infusion, which reduced rpS6 phosphorylation in the right ventricular wall compared with saline infusion in the right ventricular wall \((P = 0.05)\) and leucine \((P = 0.07)\) and isoleucine infusion \((P = 0.03)\) in the left ventricular wall (Fig. 6B).

In skeletal muscle, phosphorylation of eEF2 at Thr\(^{56}\) was unaffected by BCAA treatment compared with saline-infused controls \((P = 0.11\) to 0.92; Fig. 7A). However, there was a numerical reduction in eEF2 phosphorylation at Thr\(^{56}\) in longissimus dorsi \((P = 0.11)\) and masseter \((P = 0.23)\) muscles of leucine-treated pigs compared with saline controls, as well as a numerical increase in eEF2 phosphorylation at Thr\(^{56}\) in the masseter \((P = 0.44)\) muscle of valine-treated pigs. In the ventricular walls, phosphorylation of eEF2 at Thr\(^{56}\) was not affected by the infusion of individual BCAA \((P = 0.44\) to 0.73; Fig. 7B).

**Protein synthesis.** \(K_s\), as well as \(K_{RNA}\) and \(C_s\) are summarized in Table 2. Infusion of leucine increased protein synthesis in the longissimus dorsi \((+28\%, P = 0.008)\), masseter \((+25\%, P = 0.01)\), and left ventricular wall \((+26\%, P = 0.003)\).
compared with saline infusion. In the right ventricular wall, a numerical increase in protein synthesis was obtained with leucine infusion (21%, \( P = 0.10 \)) compared with saline infusion. Infusion of isoleucine or valine did not increase (\( P = 0.11 \) to 0.93) protein synthesis over saline values in any tissue. To the contrary, infusion with valine tended to decrease (\( P = 0.06 \)) protein synthesis in longissimus dorsi. The stimulation of protein synthesis by leucine was not different between the longissimus dorsi and masseter muscles. In the heart, protein synthesis rates were higher (\( P = 0.04 \)) in the left ventricular wall compared with the right ventricular wall.

\( K_{RNA} \) in the longissimus dorsi was numerically increased in leucine-infused (22%, \( P = 0.14 \)) compared with saline-infused pigs. In the masseter and right and left ventricular walls, the \( K_{RNA} \) was increased (\( P = 0.09 \), 0.06, and 0.003, respectively) in response to leucine administration compared with saline-infused pigs. \( C_s \) was unaffected (\( P = 0.29 \) to 0.92) in skeletal muscle and cardiac tissues by individual BCAA infusion compared with saline controls. Nevertheless, \( C_s \) was higher (\( P < 0.05 \)) in the longissimus dorsi compared with the masseter of pigs infused with leucine.

**DISCUSSION**

We (10, 13, 28) have previously shown in neonatal pigs that fractional rates of protein synthesis increase in response to feeding. The protein synthetic response, which occurs in disparate tissues but is most profound in skeletal muscle (7, 10), is independently induced by the postprandial rise in insulin and amino acids (28). The amino acid-induced stimulation of skeletal muscle protein synthesis is modulated by the enhanced activation state of translation initiation factors that lead to increased eIF4G-eIF4E complex assembly (29). Recently, we (18) demonstrated that infusion of leucine alone, to increase its concentration in plasma to mimic postprandial levels, resulted in a stimulation of protein synthesis in skeletal muscle of neonatal pigs. Furthermore, the leucine-induced stimulation of protein synthesis was associated with enhanced phosphorylation of 4E-BP1, S6K1, and rpS6, and reduced the inactive 4E-BP1-eIF4E complex (18). In the present study, infusion of leucine also stimulated protein synthesis concomitantly with enhanced activation of translation initiation factors in cardiac and skeletal muscles. Infusion of isoleu-
cine and valine, however, failed to stimulate protein synthesis or translation initiation factor activation in any of the studied muscles.

**Effect of BCAA infusion on plasma amino acids.** Infusion of each individual BCAA resulted in a marked increase in the plasma concentration of the corresponding BCAA over baseline and saline-infused controls. Infusion of leucine and valine resulted in an approximately threefold increase in plasma levels, which falls within the postprandial range previously reported for neonatal pigs fed mature sow’s milk, colostrum, or formula (7). Despite the fact that isoleucine was infused on an equimolar basis to leucine and valine (i.e., 400 μmol·kg⁻¹·h⁻¹), plasma isoleucine levels increased approximately sixfold compared with baseline values. Plasma levels of leucine were reduced by isoleucine infusion for 60 min in neonatal pigs. This observation raises the possibility that any potential stimulatory effect of isoleucine on protein synthesis may have been impaired by the reduction in plasma leucine. It is not known whether the elevation of one BCAA affects intracellular transport or flux rates, as well as the incorporation into protein of the other two BCAA. Infusion of leucine for 60 min, however, did not reduce plasma concentrations of other essential amino acids. These observations are in agreement with our previous study (18). Similarly, infusion of isoleucine and valine for 60 min did not affect plasma concentrations of other essential amino acids.

**Effect of BCAA infusion on translation initiation factors.** A physiological increase in plasma leucine resulted in increased phosphorylation of 4E-BP1 on Thr³⁰ and the γ isoform, with a concomitant reduction in the 4E-BP1·eIF4E complex and an increase in the eIF4G·eIF4E complex in both skeletal muscles. Furthermore, phosphorylation of eIF4G in the eIF4G·eIF4E complex was also enhanced by the physiological increase in circulating leucine in both skeletal muscles. Interestingly, valine increased the amount of 4E-BP1·eIF4E complex in skel-

![Fig. 4. Phosphorylation of eIF4G at Ser¹¹⁰⁸ associated with eIF4E in skeletal muscles (A) and right and left VW (B) of the heart of neonatal pigs after 60 min of infusion with saline or 400 μmol·kg⁻¹·h⁻¹ leucine, isoleucine, or valine.](image)

![Fig. 5. Phosphorylation of 70-kDa ribosomal protein (rp)S6 kinase (S6K1) at Thr³⁸⁹ in skeletal muscles (A) and right and left VW (B) of the heart of neonatal pigs after 60 min of infusion with saline or 400 μmol·kg⁻¹·h⁻¹ leucine, isoleucine, or valine. Phosphorylation of S6K1 was corrected by total S6K1 content. The value from control pigs infused with saline was set at 1.0 (AU). Total S6K1 content was not different among treatments within tissue. Values are means ± pooled SE; n = 6–8 per treatment. a, b: means with different letters differ at P < 0.0007 in A, P < 0.03 in B.](image)
etal and cardiac muscles without a reciprocal reduction in eIF4G-eIF4E complex formation. Leucine enhanced the phosphorylation of 4E-BP1, as well as increased eIF4G-eIF4E complex formation and eIF4G phosphorylation in this active complex without a statistically significant reduction in the inactive 4E-BP1-eIF4E complex.

Leucine did not alter the phosphorylation state of S6K1 and rpS6 in either ventricle of the heart. Activation of S6K1 and rpS6 has been involved in the translational regulation of mRNAs containing a terminal oligopyrimidine tract. These

![Fig. 6. Phosphorylation of rpS6 at Ser235/236 and Ser240/244 in skeletal muscles (A) and right and left VW (B) of the heart of neonatal pigs after 60 min of infusion with saline or 400 μmol·kg⁻¹·h⁻¹ leucine, isoleucine, or valine. Phosphorylation of rpS6 was corrected by total rpS6. The value from control pigs infused with saline was set at 1.0 (AU). Total rpS6 content was not different among treatments within tissue. Values are means ± pooled SE; n = 6–8 per treatment. a, b: means with different letters differ at P < 0.02 in A, P < 0.05 in B. †Mean differs from left VW leucine at P = 0.07. ‡Mean differs from left VW isoleucine at P = 0.07.](Image)

![Fig. 7. Phosphorylation of eukaryotic elongation factor 2 (eEF2) at Thr56 in skeletal muscles (A) and right and left VW (B) of the heart of neonatal pigs after 60 min of infusion with saline or 400 μmol·kg⁻¹·h⁻¹ leucine, isoleucine, or valine. Phosphorylation of eEF2 was corrected by total eEF2. The value from control pigs infused with saline was set at 1.0 (AU). Total eEF2 content was not different among treatments within tissue. Values are means ± pooled SE; n = 6–8 per treatment. a, b: means with different letters differ at P < 0.05. †Mean differs from LD saline at P = 0.11.](Image)
mRNAs encode proteins involved in the protein synthetic machinery (17). Phosphorylation of S6K1 and rpS6 in the heart of mature rats has been reported to increase in response to oral administration of leucine that elevated plasma leucine to supraphysiological levels (24, 25). However, the experimental conditions of the present study are substantially different from those in previous reports. We elevated plasma leucine, within the postprandial range, in neonatal pigs via parenteral administration. Combined, these observations raise important questions about the involvement of S6K1 and rpS6 in the translation of mRNAs coding for components of the protein synthetic machinery as affected by nutrients, growth factors, and state of development of the animal. Furthermore, special consideration must be placed on the state of development of the neonatal heart, as well as the potential differential effects of long-term leucine administration on ribosome biosynthesis in cardiac and skeletal muscles.

Studies conducted in isolated adipocytes (19, 27) have suggested that isoleucine and valine can also activate translation initiation factors but to a lesser degree than leucine. These effects, however, have not been investigated in neonatal animals. In the present study, we found that an increase in circulating levels of isoleucine and valine failed to enhance translation initiation factor activation in the longissimus dorsi and masseter muscles, as well as in the left and right ventricular walls, of neonatal pigs. However, a potential stimulatory effect of isoleucine on the activation of translation initiation factors may have been blunted by the significant reduction in plasma leucine observed in isoleucine-treated animals compared with saline-treated animals. Infusion of valine significantly increased the 4E-BP1-eIF4E inactive complex in the longissimus dorsi, as well as in the right and left ventricular walls. Increased phosphorylation of eEF2 at Thr56 has been reported to reduce the rate of elongation in cultured cells (6). In the present study, phosphorylation of eEF2 at Thr56 was numerically lower in the longissimus dorsi and masseter muscle of leucine-infused pigs compared with controls. In the masseter muscle of valine-treated pigs, there was a numerical increase in the phosphorylation of eEF2 at Thr56 compared with controls. Therefore, a significant difference was obtained for the degree of phosphorylation of eEF2 at Thr56 between leucine-treated and valine-treated pigs. To the best of our knowledge, this is the first study to report the activation of translation initiation factors in skeletal and cardiac muscles of neonatal pigs in response to individual BCAA infusion. Collectively, these results indicate that leucine, but not isoleucine or valine, can enhance the activation of translation initiation factors in cardiac and skeletal muscles.

\[ \text{Effect of BCAA infusion on protein synthesis.} \]

We (28) have previously reported that the fractional rate of protein synthesis in longissimus dorsi, a muscle that contains primarily fast-twitch glycolytic muscle fibers, of neonatal pigs increases linearly when a balanced mixture of amino acids is infused. An amino acid-induced stimulation of protein synthesis has also been reported in the skeletal muscle of older pigs (33). Furthermore, the increase in protein synthesis in the skeletal muscle of neonatal pigs in response to amino acid infusion is mediated by translation initiation factor activation (29). More recently, we (18) reported that a physiological increase in circulating leucine alone can act as a nutrient signal to increase protein synthesis in the skeletal muscle of neonatal pigs, a response that was tissue specific, substrate dependent, and 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. Furthermore, skeletal muscle protein synthesis and the activation of translation initiation factors respond linearly to increased plasma levels of circulating leucine alone within the postprandial range (18). In the present study, we examined the efficacy of individual BCAA to stimulate protein synthesis in cardiac and skeletal muscles of neonatal pigs. The results indicate that a physiological increase in circulating leucine, but not isoleucine or valine, was sufficient to stimulate protein synthesis in skeletal muscles that contain either fast-twitch glycolytic or slow-twitch oxidative muscle fibers, as well as in the left ventricular wall of neonatal pigs.

The increase in protein synthesis in the longissimus dorsi muscle in response to leucine infusion was associated with increased phosphorylation of 4E-BP1, S6K1, and rpS6, reduced inactive 4E-BP1-eIF4E complex, increased active eIF4G-eIF4E complex assembly, and increased eIF4G phosphorylation in the active eIF4G-eIF4E complex. In the masseter muscle, as well as in the ventricular walls, we found no increase in the phosphorylation of S6K1 and rpS6.

### Table 2. \( K_s, K_{RNA}, \) and \( C_s \) in skeletal muscles and ventricular walls of neonatal pigs after 60 min of infusion with saline or 400 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) of leucine, isoleucine, or valine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Longissimus dorsi</th>
<th>Masseter</th>
<th>Right ventricular wall</th>
<th>Left ventricular wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Leucine</td>
<td>Isoleucine</td>
<td>Valine</td>
<td>Saline</td>
</tr>
<tr>
<td>( K_s ) %/day</td>
<td>13.8±c</td>
<td>17.6b</td>
<td>13.7±c</td>
<td>11.1†</td>
</tr>
<tr>
<td>( K_{RNA} ), g protein/day⁻¹/g RNA⁻¹</td>
<td>5.4b</td>
<td>6.0c±c</td>
<td>5.7b</td>
<td>4.8c</td>
</tr>
<tr>
<td>( C_s ), mg RNA/g protein</td>
<td>25.7±a§</td>
<td>27.3b</td>
<td>24.8a±c</td>
<td>24.5b</td>
</tr>
</tbody>
</table>

Values are means with pooled SE. \( K_s \), fractional rates of protein synthesis; \( K_{RNA} \), protein synthetic efficiencies; \( C_s \), protein synthetic capacities. a,b,c,d Mean effects differ at \( P < 0.05 \). †Differs from longissimus dorsi-saline and longissimus dorsi-isoleucine at \( P = 0.06 \). §Differs from longissimus dorsi-leucine at \( P = 0.07 \), and masseter-saline at \( P = 0.09 \). ‡Differs from masseter-isoleucine at \( P = 0.08 \), and masseter-valine at \( P = 0.06 \). *Differs from right ventricular wall-saline at \( P = 0.06 \) and right ventricular wall-isoleucine and left ventricular wall-saline at \( P = 0.07 \).
less, protein synthesis was significantly increased in the mas-
seter muscle, as well as in the left ventricular wall. These re-
results suggest that phosphorylation of S6K1 and rpS6 does not
appear to be involved in the leucine-induced stimulation of
global rates of protein synthesis in neonatal pigs. The effects of
leucine on the activation of translation initiation factors ob-
served in the current study resemble the effects of growth
factors, such as insulin and IGF-I, on the mTOR signaling
leading to protein synthesis that we have observed in previous
studies (3, 5, 27). Nevertheless, leucine can also act through an
mTOR-independent mechanism controlled by PKCε, which is
not activated by insulin (32).

The leucine-induced stimulation of protein synthesis was
accompanied by a significant increase in protein synthetic
efficiency in the left ventricular wall and a numeric increase in
protein synthetic efficiency in both skeletal muscles, as well as
the right ventricular wall. The greater hypertrophy of the left
ventricular wall compared with the right ventricular wall has
been associated with enhanced efficiency of translation (30). In
the present study, protein synthetic efficiency was significantly
higher in the left ventricular wall compared with the right
ventricular wall regardless of treatment. It has been previously
reported (8, 30) that, during the enhanced hypertrophy phase of
the left ventricular wall compared with the right ventricular
wall, the RNA content and ribosome formation is higher in the
left ventricular wall compared with the right ventricular wall of
pigs. In the present study, however, we found that protein
synthetic capacity did not differ between the left and right
ventricular walls. This discrepancy could be attributed to
differences in the experimental approaches. For example, re-
results from Camacho et al. (8) were obtained from free ventric-
ular walls perfused in situ, whereas we obtained our results
directly from nonmanipulated free ventricular walls. It has also
been reported that the difference in RNA content between the
right and left ventricular walls is not present at 10 days in
piglets (30). Therefore, the timing of sample collection and the
experimental approach used can potentially affect the mea-
sured RNA content of the tissue.

Collectively, results from this study indicate that leucine, but
not isoleucine or valine, at physiological levels, can act as a
nutrient signal to increase protein synthesis in skeletal muscles
as well as in the left ventricular wall of neonatal pigs. These
changes in protein synthesis were not dependent on changes in
circulating insulin levels, which is in agreement with our
previous report (18). The increases in protein synthesis in
cardiac and skeletal muscles were mediated by enhanced ac-
vation of translation initiation factors involved in the binding
of mRNA to the 43S ribosomal complex. Finally, the activation
of S6K1 and rpS6 does not appear to be involved in the
leucine-induced stimulation of global rates of protein synthesis
in neonatal pigs.

Perspectives. We (18) have recently reported that a physio-
logical increase in circulating leucine alone is sufficient to
stimulate protein synthesis in neonatal pig skeletal muscle that
contains primarily fast-twitch glycolytic muscle fibers. In the
present study, we found that leucine, but not isoleucine or
valine, can act as a nutrient signal to stimulate protein synthesis
in skeletal muscles that contain either fast-twitch glycolytic or
slow-twitch oxidative muscle fibers. However, leucine stimu-
lation of protein synthesis was higher in the left ventricular
wall compared with the right ventricular wall, suggesting the
potential for alterations in heart size during prolonged leucine
administration. In addition, the present study describes the
responsiveness of cardiac and skeletal muscles to short-term
administration (i.e., 60 min) of individual BCAA. We (18)
have previously reported that the leucine stimulation of protein
synthesis cannot be maintained during a longer period of
administration of leucine (i.e., 120 min), likely because of a
50% reduction in plasma essential amino acids. Moreover,
liver protein synthesis was unaffected by short-term leucine
infusion and was reduced by a longer period of infusion. There-
fore, studies specifically designed to determine the effect of
prolonged elevation of circulating leucine on tissue protein
synthesis, circulatin essential amino acids, and tissue mass
must be conducted before any potential use of BCAA therapy
can be implemented in neonates.

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