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

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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 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 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 weighed at 2.0 ± 0.1 kg at 0.1 days of age. Animals were assigned to one of five treatments and infusion. Piglets were administered with saline, leucine, isoleucine, or valine. All infusions were carried for 60 min as previously described (14).

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 free 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 ChemiDoc It (UV, 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.

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

Tissue protein synthesis in vivo. Fractional rates of protein synthesis were measured with 10 ml/kg body wt of a floating dose (1.5 mmol/kg body wt) of l-[4-3H]phenylalanine (0.5 mCi/kg body wt; Amersham Biosciences, Piscataway, NJ) injected 30 min before 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 masseter 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 (18).

Protein synthesis (Ks; % protein mass synthesized in a day) was calculated as Ks (%/day) = ([S0/Sa] × (1.440/t)) × 100, where S0 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 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 (Cs). RNA content was determined by spectrophotometric absorbency. Protein synthetic efficiency (KnRNA) was estimated as the total protein synthesized in a day per total RNA (g protein ·day−1 ·g RNA−1).

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Quantification of phosphorylated eIF4G in the eIF4E-4E-BP1 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
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 Thr70 and total 4E-BP1 (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 rpS6.

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 eukaryotic elongation factor-2 (eEF2) at Thr56 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 8.2; 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 (srede, leucine, isoleucine, and valine). No statistical comparison was 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 Thr70 (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. 1C and D) compared with saline infusion. Infusion of isoleucine or valine did not change the 4E-BP1 phosphorylation at Thr70 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 (P < 0.03) in longissimus dorsi and masseter muscles (Fig. 2A), but not in the right and left ventricular walls (P = 0.26 and P = 0.19, respectively; Fig. 2B), was observed for leucine compared with saline infusion. Infusion of isoleucine did not affect the amount of 4E-BP1 associated with eIF4E in any tissue (P = 0.10 to 0.89; Fig. 2). Infusion of valine, however, increased the amount of 4E-BP1 associated with eIF4E in longissimus dorsi (P = 0.01; Fig. 2A) and right and left ventricular walls (P < 0.04; Fig. 2B) compared with saline infusion. The eIF4G-eIF4E complex was increased by leucine infusion in both skeletal muscles (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 leucine 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 (Fig. 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

<table>
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<td>163.3±4</td>
<td>202.3±3</td>
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Table 1. Plasma concentrations of BCAA in neonatal pigs at baseline and after 60 min of infusion with saline or 400 μmol·kg⁻¹·h⁻¹ leucine, isoleucine, or valine
rpS6 was also increased ($P = 0.003$) by leucine treatment in longissimus dorsi muscle but not in masseter muscle ($P = 0.45$; Fig. 6A) compared with saline-treated pigs. In the ventricular walls, phosphorylation of rpS6 at Thr$^{56}$ was not affected by the infusion of individual BCAA ($P = 0.44$ to 0.73; Fig. 6B).

**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-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).

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 ($P = 0.45$; Fig. 6A) compared with saline-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. 6B).

Fig. 1. Phosphorylation of eukaryotic initiation factor (eIF)4E-binding protein-1 (4E-BP1) at Thr$^{70}$ in skeletal muscles [longissimus dorsi (LD) and masseter (Mass); A] and right and left ventricular walls (VW; C) of the heart of neonatal pigs after 60 min of infusion with saline or 400 μmol·kg$^{-1}$·h$^{-1}$ leucine, isoleucine, or valine. Total 4E-BP1 in LD and Mass muscles (B), right and left VW (D), and positions of the α-, β-, and γ-isofoms. Total 4E-BP1 content was not different among treatments within tissue. Values are means ± pooled SE; $n = 6–8$ per treatment. a, b, c: means with different letters differ at $P < 0.03$ in B, $P < 0.001$ in D.

rpS6 was also increased ($P = 0.003$) by leucine treatment in longissimus dorsi muscle but not in masseter muscle ($P = 0.45$; 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 leucine 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).

Fig. 2. Association of 4E-BP1 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. Total 4E-BP1 was corrected by the eIF4E recovered from the immunoprecipitate. The value from control pigs infused with saline was set at 1.0 (AU). Total eIF4E content was not different among treatments within tissue. Values are means ± pooled SE; $n = 6–8$ per treatment. a, b, c: means with different letters differ at $P < 0.05$ in A, $P < 0.04$ in B. †Mean differs from right and left VW saline at $P = 0.09$. 

AJP-Endocrinol Metab • VOL 290 • APRIL 2006 • www.ajpendo.org
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 Thr⁷⁰ 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. eIF4G phosphorylation was corrected by the total eIF4G recovered from the immunoprecipitate. The value from control pigs infused with saline was set at 1.0 (AU). Total eIF4E 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 < 0.0001 in A; P < 0.03 in B.](image)

![Fig. 5. Phosphorylation of 70-kDa ribosomal protein (rp)S6 kinase (S6K1) at Thr⁴⁴⁹ 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. 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. †Mean differs from left VW isoleucine at P = 0.07.](image)
bral and cardiac muscles without a reciprocal reduction in eIF4G\textsuperscript{H18528}eIF4E complex formation. Leucine enhanced the phosphorylation of S6K1 and rpS6 in the fast-twitch glycolytic longissimus dorsi muscle but not in the slow-twitch oxidative masseter muscle. We (18) have previously reported increased phosphorylation of 4E-BP1, S6K1, and rpS6 and reduced 4E-BP1\textsuperscript{H18528}eIF4E complex content in the longissimus dorsi of neonatal pigs infused with leucine for 60 min. In addition, others (1–3) have also reported increased activation of translation initiation factors in response to oral administration of leucine in rats that resulted in supraphysiological concentrations of leucine in plasma. Moreover, a recent study (9) reported that a 3.5-fold increase in plasma leucine concentration in response to oral leucine administration increased phosphorylation of eIF4G and S6K1 and decreased the association of 4E-BP1 with eIF4E in rat skeletal muscle and maximally stimulated protein synthesis. Thus results reported herein for the activation of translation initiation factors by leucine in skeletal muscle are in agreement with several published reports. In the right and left ventricular walls, infusion of leucine caused a marked increase in the phosphorylation of 4E-BP1, as well as increased eIF4G\textsuperscript{H18528}eIF4E complex formation and eIF4G phosphorylation in this active complex without a statistically significant reduction in the inactive 4E-BP1\textsuperscript{H18528}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 Ser\textsuperscript{235/236} and Ser\textsuperscript{240/244} 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\textsuperscript{H11005}h\textsuperscript{H11002} 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.

Fig. 7. Phosphorylation of eukaryotic elongation factor 2 (eEF2) at Thr\textsuperscript{56} 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\textsuperscript{-1}\cdot h\textsuperscript{-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.
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 consistently 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.

**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 increases in protein synthesis in the skeletal muscle of neonatal pigs in response to amino acid infusion are 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. Nevertheless,
less, protein synthesis was significantly increased in the masseter muscle, as well as in the left ventricular wall. These 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 observed 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 PKCe, 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, results from Camacho et al. (8) were obtained from free ventricular 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 measured 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 activation 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 physiological 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 stimulation 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. Therefore, studies specifically designed to determine the effect of prolonged elevation of circulating leucine on tissue protein synthesis, circulating 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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