Activation by insulin and amino acids of signaling components leading to translation initiation in skeletal muscle of neonatal pigs is developmentally regulated

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Suryawan A, Orellana RA, Nguyen HV, Jeyapalan AS, Fleming JR, Davis TA. Activation by insulin and amino acids of signaling components leading to translation initiation in skeletal muscle of neonatal pigs is developmentally regulated. Am J Physiol Endocrinol Metab 293: E1597–E1605, 2007. First published September 18, 2007; doi:10.1152/ajpendo.00307.2007.— Insulin and amino acids act independently to stimulate protein synthesis in skeletal muscle of neonatal pigs, and the responses decrease with development. The purpose of this study was to compare the separate effects of fed levels of INS and AA on the activation of signaling components leading to translation initiation and how these responses change with development. Overnight-fasted 6- (n = 4/group) and 26-day-old (n = 6/group) pigs were studied during 1) eugluclidean-eugluclemic-euaminoacidemic conditions (controls), 2) eugluclidean-eugluclemic-hyperaminoacidic clamp conditions, and 3) euaminoacidemic conditions. INS, but not AA, increased the phosphorylation of protein kinase B (PKB) and tuberous sclerosis 2 (TSC2). Both INS and AA increased protein synthesis and the phosphorylation of a mammalian target of rapamycin (mTOR), ribosomal protein S6 kinase-1, and eukaryotic initiation factor (eIF)4E-binding protein 1 (4E-BP1), and these responses were higher in 6-day-old compared with 26-day-old pigs. Both INS and AA decreased the binding of 4E-BP1 to eIF4E and increased eIF4E binding to eIF4G; these effects were greater in 6-day-old than in 26-day-old pigs. Neither INS nor AA altered the composition of mTORC1 (raptor, mTOR, and GβL) or mTORC2 (rictor, mTOR, and GβL) complexes. Furthermore, neither INS, AA, nor age had any effect on the abundance of Rheb and the phosphorylation of AMP-activated protein kinase and eukaryotic elongation factor 2. Our results suggest that the activation by insulin and amino acids of signaling components leading to translation initiation is developmentally regulated and parallels the developmental decline in protein synthesis in skeletal muscle of neonatal pigs.

neonates; mammalian target of rapamycin; tuberous sclerosis; raptor; protein synthesis

THE NEONATAL PERIOD IS CHARACTERIZED by rapid growth that is supported by a high rate of skeletal muscle protein synthesis (6, 13). The profound accretion of skeletal muscle protein is in part due to the ability of neonatal muscle to markedly increase protein synthesis in response to feeding, a response that declines rapidly with development (7, 10, 11). Although a postprandial rise in protein synthesis occurs in virtually all tissues of the body in the neonate, it is most pronounced in skeletal muscle (7). In skeletal muscle, stimulation of protein synthesis is independently modulated by the postprandial rise in insulin and amino acids, whereas the response in other tissues, such as liver, is mediated by amino acids (9, 27, 28).

The molecular mechanism by which insulin stimulates protein synthesis has been well studied; however, the amino acid-signaling pathway leading to the mRNA translation is less well known. Much of the information on the molecular mechanism by which amino acids stimulate protein synthesis was generated from mammalian cell culture studies (31), and little is known about the role of these signaling components in the intact animal. Recently, we found that, in neonatal pigs, amino acids stimulate skeletal muscle protein synthesis by activating signaling components downstream, but not upstream, of protein kinase B (PKB) (41). These results support data from cell culture studies and further suggest that, to induce protein synthesis, both insulin and amino acids utilize a common signaling pathway downstream of PKB.

To stimulate protein synthesis, insulin initiates its signal by activating the insulin receptor (IR) and IR substrate-1 (IRS-1), followed by the activation of phosphoinositide 3-kinase (PI 3-kinase) (4, 14). Activated PI 3-kinase then stimulates the activation of downstream effector molecules such as phosphoinositide-dependent kinase-1 and PKB (4). PKB phosphorylates and inactivates an inhibitor of cell growth, tuberin [also known as tuberous sclerosis complex 2 (TSC2)], thereby inactivating the function of the TSC1/2 (19, 22, 23), resulting in the activation of Rho and the phosphorylation of AMP-activated protein kinase and eukaryotic elongation factor 2. Our results suggest that the activation by insulin and amino acids of signaling components leading to translation initiation is developmentally regulated and parallels the developmental decline in protein synthesis in skeletal muscle of neonatal pigs.

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mTOR. Under energy starvation conditions, AMP-activated protein kinase (AMPK) phosphorylates TSC2 and enhances its activity, resulting in the inhibition of mTOR activation and a reduction in protein synthesis (5, 18, 23). A recent study (32) suggests that amino acids regulate mTOR by activating a signaling component(s) downstream of the TSC1/TSC2 complex. Furthermore, raptor, an mTOR regulatory protein, has been shown to be an important component of mTOR that transmits amino acid signals (17, 20).

mTOR, a key player that relays growth factor and amino acid signaling, is a serine/threonine kinase that controls many aspects of cellular physiology, including transcription, translation, cell size, and autophagy (1). Recent studies suggest that mTOR exists in two heteromeric complexes, mTOR complex (mTORC)1 and mTORC2 (20, 35). mTORC1, a rapamycin-sensitive complex, has been shown (17, 26) to consist of the three proteins mTOR, GβL, and raptor and largely control translation and cell growth in response to amino acids. mTORC2, a rapamycin insensitive complex, consists of mTOR, GβL, and rictor and has been shown (3) to control actin cytoskeleton dynamics and to activate PKB. Amino acids regulate raptor-mTOR binding, resulting in the activation of the downstream effectors of the mTORC1 complex, i.e., S6K1 and 4E-BP1 (20).

Using the neonatal pig as our animal model, we (12, 39, 40) previously demonstrated that the enhanced feeding-induced stimulation of muscle protein synthesis in the neonate is associated with increased activation of insulin-signaling components leading to mRNA translation. These responses decrease with development, in parallel with the decline in muscle protein synthesis (12, 39, 40). However, there is little information on the individual contribution of insulin and amino acids to the regulation of skeletal muscle protein synthesis in the neonate and how these responses change with development. Therefore, the purpose of the present study was to determine the molecular mechanism by which insulin and amino acids regulate skeletal muscle protein synthesis in neonatal pigs and the effect of development on these responses.

MATERIALS AND METHODS

Animals and housing. Two crossbred (Landrace × Yorkshire × Duroc × Hampshire) pregnant sows (Agriculture Headquarters, Texas Department of Criminal Justice, Huntsville, TX) were housed in lactation crates in individual, environmentally controlled rooms 2 wk before being farrowed. Sows were fed a commercial diet (no. 5084; PMI Feeds, Richmond, IN) and provided water ad libitum. After being farrowed, piglets remained with the sow but were not allowed access to the sow’s diet. A total of 28 piglets from four litters, weighing 2.8 kg, were studied at 7 and 26 days of age, respectively. Three days prior to the experiment, piglets were anesthetized for sterile catheter insertion into a jugular vein and carotid artery. Piglets were then returned to the sow and allowed to suckle freely until they were studied. The protocol was approved by the Animal Care and Use Committee of Baylor College of Medicine and was conducted in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals.

Treatments and infusion. Pigs were placed unanesthetized in a sling restraint system after a 12-h fast. Pigs were assigned to one of three treatment groups: 1) euinsulinemic-euglycemic-euaminoacidemic conditions (control), 2) euinsulinemic-euglycemic-hyperaminoacidemic clamp, and 3) hyperinsulinemic-euglycemic-euaminoacidemic clamp. Body weights were similar in each treatment group at each age studied. During a 30-min basal period, blood samples were obtained and immediately analyzed for glucose (YSI 2300 STAT Plus; Yellow Springs Instruments, Yellow Springs, OH) to establish the average basal concentration of blood glucose to be used in the subsequent euglycemic clamp procedure (27, 28). Plasma samples were analyzed for total branched-chain amino acids (BCAA) by use of a rapid enzymatic kinetic assay to establish the average basal concentration of BCAA to be used in the subsequent euaminoacidemic or hyperaminoacidemic clamp procedure. The clamps were initiated with a primed, constant (12 ml/h) infusion of insulin (Eli Lilly, Indianapolis, IN) at 0 or 100 ng·kg⁻¹·min⁻¹. To either maintain insulin at the fasted level or raise insulin to the fed level, venous blood samples (0.2 ml) were obtained every 5 min and immediately analyzed for glucose and BCAA concentrations. The infusion rate of dextrose (Baxter Healthcare, Deerfield, IL) was adjusted as necessary to maintain the blood glucose concentration within ±10% of the average basal concentration. Euaminoacidemia was obtained by adjusting the infusion rate of an amino acid mixture to maintain the plasma BCAA concentration within 10% of the fasting level. Hyperaminoacidemia was obtained by infusing a balanced amino acid mixture (9) to raise plasma BCAA concentrations to twofold the fasting level to reproduce the level of amino acids present in the fed state.

Tissue protein synthesis in vivo. Fractional rates of protein synthesis were measured with a flooding dose of L-[4-¹⁴C]phenylalanine (Amersham Biosciences, Piscataway, NJ) injected 30 min before the end of the infusion (15). Protein synthesis (Ks expressed as %protein synthesized in a day) was calculated as: Ks (%/day) = ([S0/S1] × (1,440/r)) × 100, where S0 is the specific radioactivity of the protein-bound phenylalanine, S1 is the specific radioactivity of the free phenylalanine for the labeling period determined from the value of the animal at the time of tissue collection and corrected by the linear regression of the blood-specific radioactivity of the animal against time, and r is the time of labeling in minutes. Pigs were killed at 120 min, and longissimus dorsi muscle samples were collected and immediately frozen in liquid nitrogen and stored at −70°C until they were analyzed as previously described (39).

Tissue extraction and immunoblot analysis. Freshly collected longissimus dorsi muscle tissue samples were homogenized and centrifuged at 10,000 g for 10 min at 4°C (39). Supernatants were diluted in sample buffer, frozen in liquid nitrogen, and stored at −70°C until analysis. Equal amounts of protein samples were electrophoretically separated in polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) that was incubated with appropriate primary antibodies, washed, and exposed to an appropriate secondary antibody as previously described (39).

For normalization, immunoblots performed with anti-phosphospecific antibodies were stripped in stripping buffer (Pierce Biotechnology, Rockford, IL) and reprobed with the corresponding nonphosphospecific antibodies. Blots were developed using an enhanced chemiluminescence kit (Amersham), visualized, and analyzed using a ChemiDoc-It Imaging System (UVP, Upland, CA). Primary antibodies that were used in the immunoblotting were PKB (total and Ser733; Cell Signaling Technology, Beverly, MA), AMPKα (total and Thr172; Cell Signaling Technology), TSC2 (total and Thr1462; Cell Signaling Technology), Rheb (total; Cell Signaling Technology), mTOR (total and Ser2448; Cell Signaling Technology), S6K1 (total and Thr389; Cell Signaling Technology), 4E-BP1 (total; Cell Signaling Technology, Montgomerie, TX, and Thr46; Cell Signaling Technology), and eIF2 (total and Thr56; Cell Signaling Technology).

Quantification of eIF4E-4EBP1 and eIF4E·eIF4G complexes. These complexes were immunoprecipitated using an anti-eIF4E monoclonal antibody (gift of Dr. Leonard Jefferson, Pennsylvania State University College of Medicine, Hershey, PA) from aliquots of fresh tissue homogenates (12). Briefly, samples were homogenized in seven volumes of buffer (in mM: 20 HEPS, 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-4E-BP1 antibody. Immunoprecipitates were recovered with goat anti-rabbit IgG magnetic beads (Polysciences, Warrington, PA), washed and resuspended in sample buffer as described elsewhere (12), and immediately subjected to protein immunoblot analysis using rabbit anti-4E-BP1 (Cell Signaling Technology) antibody or rabbit anti-4E-F4 (Bethyl Laboratories). Amounts of 4E-BP1 and eIF4G were corrected by the eIF4E recovery from the immunoprecipitate.

Analysis of mTORC1 and mTORC2. To determine the association between mTOR and its partners (with raptor and GβL for the mTORC1 or with rictor and GβL for the mTORC2), muscle samples were homogenized in CHAPS buffer as described by Williamson et al. (43). The CHAPS buffer consisted of 40 mM HEPES, pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM β-glycerophosphate, 40 mM NaF, 1.5 mM sodium vanadate, 0.3% CHAPS, 0.1 mM PMSF, 1 mM benzamidine, and 1 mM DTT. The homogenate was mixed on a platform rocker for 30 min at 4°C and then centrifuged at 1,000 g for 3 min (4°C). The supernatant containing 500 μg of protein was combined with 2 μl of anti-mTOR antibody (Cell Signaling Technology) and mixed on a platform rocker overnight at 4°C. Following the incubation the immune complexes were isolated with a goat anti-mouse BioMag IgG (PerSeptive Diagnostics) bead slurry. The magnetic bead complexes were collected using a magnetic stand, washed twice with CHAPS buffer, and then washed once in CHAPS buffer containing 200 instead of 120 mM NaCl and 60 instead of 40 mM HEPES. The precipitates were rinsed with 100 μl of 1× SDS sample buffer and then boiled for 5 min and centrifuged to collect the supernatant. The samples were subjected to SDS-PAGE followed by immunoblotting with anti-raptor antibody, anti-rictor antibody, and anti-GβL antibody (all from Cell Signaling Technology). The mTOR-protein complexes were normalized by the amount of total mTOR in the precipitates.

Statistics. Two-way ANOVA was used to assess the effect of insulin, amino acids, age, and their interaction on fractional protein synthesis rate and the activation of signaling components leading to translation initiation. When significant interactions were detected, the value in each treatment group for each age was compared with the control value by use of t-tests. Probability values of P < 0.05 were considered statistically significant. Data are presented as means ± SE.

RESULTS

The effectiveness of the clamp technique is crucial for the overall outcome of these experiments. As shown in Table 1, we achieved our objectives. Plasma glucose was maintained at baseline fasting levels in all treatments. In the amino acid group the plasma BCAA concentrations, which we used as indicators of plasma amino acid levels, were increased twofold to the fed level, whereas in other treatment groups BCAA concentrations were maintained at fasting levels. Likewise, in the insulin group the plasma insulin concentration was increased to the fed level, whereas in other treatment groups plasma insulin was maintained at the fasting level. Furthermore, we found that glucose and insulin levels were significantly higher in 26- compared with 6-day-old pigs (P = 0.005 and 0.008, respectively). These results suggest that insulin sensitivity is reduced in 26- compared with 6-day-old pigs, in agreement with our previous studies (44, 45), which showed that insulin sensitivity of glucose and amino acid disposal decrease with age.

Table 1. Plasma glucose, BCAA, and insulin concentrations in 6- and 26-day-old neonatal pigs

<table>
<thead>
<tr>
<th></th>
<th>Baseline†</th>
<th>Control</th>
<th>AA</th>
<th>INS</th>
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<tbody>
<tr>
<td>Glucose, mg/dl</td>
<td>26 days</td>
<td>67.3 ± 5.9</td>
<td>62.5 ± 3.3</td>
<td>76.2 ± 3.8</td>
</tr>
<tr>
<td>BCAA, nmol/ml</td>
<td>6 days</td>
<td>433 ± 100</td>
<td>470 ± 51</td>
<td>1,139 ± 47†</td>
</tr>
<tr>
<td>Insulin, μU/ml</td>
<td>6 days</td>
<td>2.65 ± 0.5</td>
<td>2.07 ± 0.7</td>
<td>2.26 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>26 days</td>
<td>3.97 ± 0.9†</td>
<td>5.35 ± 0.8†</td>
<td>4.45 ± 0.72</td>
</tr>
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</table>

Values are means ± SE; n = 4–6. BCAA, branched-chain amino acids; AA, response to euinsulinemic-euglycemic-hyperinsulinemic clamp; INS, response to hyperinsulinemic-euglycemic-euaminoacidic clamp; †P < 0.05 compared with baseline; ‡P < 0.05 compared with 6 days.

In our previous studies (12, 39) we showed that feeding increases the fractional rate of protein synthesis in skeletal muscle through the activation of the signaling pathway leading to mRNA translation, and this effect is reduced with development. However, the individual effect of amino acids and insulin on this process has not been fully elucidated. Thus, the present studies were designed to identify signaling components that regulate mRNA translation in skeletal muscle of neonatal pigs that are induced by a physiological rise in insulin and amino acids. Furthermore, we wished to determine whether the effects of insulin and amino acids on the activation of signaling components decrease with development.

First, we investigated the individual effects of amino acids and insulin on the fractional rate of protein synthesis in longissimus dorsi muscle, a muscle that contains primarily fast-twitch muscle fibers, of 6- and 26-day-old pigs that were infused with either insulin or amino acids after overnight fasting (Fig. 1A). Amino acids alone, as well as insulin alone, stimulated skeletal muscle protein synthesis (P < 0.05), and these effects were higher in 6- than in 26-day-old pigs (P < 0.05).

We (37–39) have previously found that the activation of early steps in the insulin-signaling pathway upstream of PKB is induced by feeding. In this study, we investigated the separate effects of amino acids and insulin on the phosphorylation of PKB at Ser473, an important component of the insulin-signaling pathway (Fig. 1B). Insulin, but not amino acids, significantly increased the phosphorylation of PKB in skeletal muscle (P < 0.05), and this response decreased with development (P < 0.05). Moreover, PKB abundance was significantly higher in skeletal muscle of 6- compared with 26-day-old pigs (P < 0.05; data not shown).

AMPK, a kinase that is activated by falling energy status as a result of starvation for carbon sources or other stresses, inhibits the mTOR pathway by phosphorylating TSC2, thereby inhibiting protein synthesis and cell growth (5, 18). To determine whether AMPK activation is affected by amino acids or insulin, the phosphorylation of AMPKα at Thr172, a crucial site for its activity, was analyzed with immunoblotting. In skeletal muscle neither amino acids nor insulin altered the phosphorylation state of AMPKα at Thr172 (Fig. 2A).

The TSC1/2 complex functions as a GTPase-activating protein toward Rheb, a positive regulator of mTOR (2). Recent
studies show that Rheb binds and activates mTOR following stimulation by amino acids and growth factors. Cell culture studies suggest that stimulation of the AMPK pathway enhances TSC1/2 activation, resulting in inhibition of protein synthesis, whereas the PI 3-kinase-PKB pathway suppresses TSC1/2 activation. In this study, we determined the effects of amino acids and insulin on the phosphorylation state of TSC2 at Thr1462, a site that is phosphorylated by PKB and results in the deactivation of the TSC1/2 complex activity toward Rheb. As shown in Fig. 2B, insulin, but not amino acids, induced TSC2 phosphorylation, and this effect was higher in muscle of 6- than in 26-day-old pigs. TSC2 abundance was significantly lower in younger pigs (*P* < 0.05; data not shown). Furthermore, we tried to determine the association between Rheb and mTOR but did not detect Rheb in the mTOR immunoprecipitate. We did, however, determine the Rheb abundance, which was similar in the two age groups (Fig. 2C).

Raptor and GβL are members of the mTORC1 (20, 35). Although GβL-mTOR interaction does not affect mTOR ac-
tivity, the state of raptor binding to mTOR induces mTOR activity toward its substrates S6K1 and 4E-BP1. Rictor, on the other hand, is a member of the mTOR2 that is activated by growth factors (3, 35). One of the major functions of mTORC2 is to induce PKB phosphorylation at Ser473. In this study, we determined the separate effect of amino acids and insulin on the protein-protein interaction of the components of mTORC1 and mTORC2. As shown in Fig. 3, neither amino acids nor insulin influenced the interaction of mTOR with raptor, mTOR with GβL, or mTOR with rictor in skeletal muscle.

mTOR is considered a master protein kinase that is regulated independently by insulin, amino acids, and energy sufficiency and participates in the control of components of protein synthesis that are responsible for cell growth (1). Because one of the mechanisms for regulation of mTOR activity involves its phosphorylation at Ser^{2448}, a residue that is present in a putative repressor domain (31), in this study we determined mTOR activation by measuring phosphorylation at this site. As shown in Fig. 4A, both amino acids and insulin enhanced mTOR phosphorylation (P < 0.05), and the effects were significantly higher in skeletal muscle of 6- than in 26-day-old pigs (P < 0.05). Furthermore, mTOR abundance was also significantly higher in skeletal muscle of 6- than in 26-day-old pigs (P < 0.05; data not shown).

mTOR regulates mRNA translation in response to amino acids and growth factors via activation of its downstream substrates S6K1 and 4E-BP1 (3, 33, 37). To determine the separate effects of amino acids and insulin on the activation of these important effectors of mTOR, the phosphorylation state of S6K1 at Thr^{398} and 4E-BP1 at Thr^{70} was analyzed with immunoblotting. Both amino acids and insulin significantly enhanced the phosphorylation of S6K1 and 4E-BP1 in skeletal muscle (Fig. 4, B and C, respectively), and their effects on the phosphorylation of these mTOR effectors were significantly higher in 6- than in 26-day-old pigs.

One of the major factors that regulates the formation of an active eIF4E-eIF4G complex is 4E-BP1 (25, 42). In the hypophosphorylated state, this protein binds eIF4E to form an inactive complex. Conversely, in the hyperphosphorylated state, 4E-BP1 detaches from eIF4E, allowing eIF4G to assemble an active eIF4E-eIF4G complex and initiate mRNA translation (42). To determine the effects of amino acids and insulin on the assembly of an active eIF4E-eIF4G complex and an inactive eIF4E-4EBP1 complex, immunoprecipitation and immunoblot analysis were performed. As shown in Fig. 5, A and B, both amino acids and insulin increased the formation of the active eIF4E-eIF4G complex (P < 0.05) and inhibited the formation of an inactive eIF4E-4EBP1 complex. These effects were greater in skeletal muscle of 6- than in 26-day-old pigs (P < 0.05).

Translation elongation in mammalian cells requires two eukaryotic elongation factors (eEFs), eEF1 and eEF2 (34). eEF2 mediates the translocation of the ribosome by three nucleotides along mRNA after the addition of each new amino acid. Phosphorylation of eEF2 on Thr^{56} impairs its ability to bind ribosomes, thus inactivating this protein (32, 34). In this study, we determined the effects of amino acids and insulin on the phosphorylation of eEF2 at Thr^{56}. As shown in Fig. 6, neither amino acids nor insulin altered the phosphorylation of eEF2 in skeletal muscle.

**DISCUSSION**

The rapid growth of skeletal muscle during the neonatal period is supported by an elevated rate of skeletal muscle protein synthesis (6, 13). We (12, 39) have previously shown that feeding increases skeletal muscle protein synthesis through the activation of signaling components leading to mRNA translation, and this feeding effect decreases with development.
In the present study, we utilized clamp techniques (hyperaminoacidemic or hyperinsulinemic euglycemic clamps) to dissect the individual effect of amino acids and insulin on skeletal muscle protein synthesis and the signaling components leading to mRNA translation in 6- and 26-day-old pigs. We confirm our previous findings that amino acids and insulin independently stimulate skeletal muscle protein synthesis, and these responses decrease with development. Furthermore, our results suggest that the stimulation of muscle protein synthesis by insulin and amino acid is due to the activation of the amino acid as well as growth factor-signaling pathways.

Due to the simplistic nature of the cell culture system, most studies determining the effects of amino acids or growth factors on the signaling pathway leading to mRNA translation (31, 32) have been conducted using mammalian cell cultures. However, the complete understanding of the mechanism regulating these processes in intact animals (in vivo) is lacking. To our knowledge, there are no published studies determining the independent role of amino acids or insulin on the activation of many of the signaling components leading to mRNA translation in neonates. Therefore, the purpose of the current study was to examine the independent role of amino acids and insulin in the developmental regulation of protein synthesis in skeletal muscle and to investigate some of the underlying mechanisms in vivo.

In our previous study, we found that the postprandial rise in skeletal muscle protein synthesis was in part due to the acti-
vation of the early steps of the insulin-signaling pathway, i.e., IR, IRS-1, PI 3-kinase, and PKB, and the activation of translation initiation factors, including mTOR, S6K1, 4E-BP1, and the eIF4F complex of proteins (12, 21, 39). The first step to dissect the molecular mechanism by which amino acids and insulin stimulate skeletal muscle protein synthesis in vivo was to determine PKB phosphorylation as an indicator of the activation of the early steps of the insulin-signaling pathway. Our results show that insulin, but not amino acids, increased PKB phosphorylation at Ser473 in skeletal muscle. Although it is generally expected that insulin activates PKB, these results are consistent and complement previous in vitro (3) and in vivo studies (31). During insulin stimulation, PKB phosphorylates TSC2 at Thr1462, resulting in the decreased ability of TSC1/TSC2 to inhibit the mTORC1 complex (22). Our results showed that insulin, but not amino acids, stimulated TSC2 phosphorylation, and this effect was significantly higher in skeletal muscle of 6- than in 26-day-old pigs. Similarly, TSC2 abundance was higher in older pigs, consistent with the higher TSC1/TSC2 activation we observed in our previous study (40). Our results are consistent with data from cell culture studies (32) indicating that amino acids fail to phosphorylate TSC2 and alter TSC1/TSC2 activation. Unlike PKB, AMPK acts as a kinase that activates TSC1/TSC2, resulting in mTOR inhibition (16, 18). We found that neither insulin, amino acids, nor age affected AMPK phosphorylation in skeletal muscle. However, we cannot discount the possibility that, in the current model, AMPK may not be activated.

Rheb, a small GTPase protein, functions as an important mediator between TSC1/TSC2 and the mTORC1 (2). To activate the mTORC1, Rheb binds directly to the mTOR catalytic domain, allowing mTORC1 to attain an active configuration. Furthermore, cell culture studies showed that both insulin and amino acids induce the binding of Rheb to mTORC1, resulting in the activation of this complex. In the current study, we attempted to verify that the Rheb interaction with mTORC1 is stimulated by a physiological rise in either amino acids or insulin in neonatal muscle. However, we did not detect the presence of Rheb in the mTOR immunoprecipitant. Nevertheless, we found that Rheb protein abundance was similar in both age groups, and, as expected, Rheb abundance did not change with acute insulin or amino acid treatment.

mTOR is part of two distinct multiprotein complexes, mTORC1 (mTOR, GβL, and raptor), which is sensitive to rapamycin, and mTORC2 (mTOR, GβL, and rictor), which is rapamycin insensitive (3). Using isolation conditions that preserved the integrity of the mTORC1 and mTORC2 protein complexes, we sought to determine the effect of amino acids and insulin on the interaction of these complexes. Our data showed that neither amino acids nor insulin influenced the protein-protein interactions in the mTORC1 and mTORC2 complexes in neonatal muscle. Although the significance of these interactions is poorly understood, a recent study (30) showed that rapamycin disturbs mTORC1 activation by removing raptor from the complex. Furthermore, in cell culture conditions, amino acid deprivation causes more raptor to bind to the mTORC1, resulting in the specific mTORC1 configuration that allows inhibition of mTOR activation toward downstream effectors (20). Due to the complexity of mTORC1 and mTORC2 regulation, more studies need to be performed to improve our understanding of the role of amino acids and growth factors in mTOR activation.

In the translation initiation pathway, mTOR relays its signal to S6K1 and 4E-BP1 (1). Furthermore, mTOR controls the response of the translation initiation machinery to amino acids and growth factors via activation of S6K1 and 4E-BP1 (1, 31). In these studies, we found that both the insulin- and amino acid-induced phosphorylation of mTOR as well as mTOR abundance decrease with development. We previously showed a developmental reduction of the feeding-induced phosphorylation of S6K1 and 4E-BP1 in skeletal muscle (12). In these studies we found that both insulin and amino acids induced S6K1 phosphorylation at Thr429 and 4E-BP1 phosphorylation at Thr70, and their effects were significantly higher in skeletal muscle of 6- than in 26-day-old pigs. The effect of amino acids in the regulation of these factors is consistent with data from cell culture studies (31).

Assembly of the eIF4E-eIF4G complex has a central role in the regulation of translation initiation (25, 42). The formation of this complex is partly regulated by 4E-BP1, which competes with eIF4G for binding with eIF4E. Furthermore, the phosphorylation of 4E-BP1 at Ser65 and Thr70 is sufficient to prevent binding to eIF4E (25). Our previous study showed that feeding enhanced the formation of an active eIF4E-eIF4G complex and reduced the formation of inactive eIF4E-4E-BP1 complex. These responses decline with development in skeletal muscle (12). In the present study, we observed similar effects of both amino acids and insulin, suggesting that neonatal skeletal muscles are sensitive to amino acid and growth factor stimulation of translation initiation activation.

The elongation phase of mRNA translation is the stage at which the polypeptide is assembled and requires a substantial amount of metabolic energy (32, 34). Thus, it is not surprising that, in skeletal muscle of adult rats, 2 days of starvation induced a marked reduction in the abundance of eEF2, a major
player in elongation. eEF2 is inactivated by phosphorylation in response to stimuli that increases energy demand or reduces its supply. Conversely, amino acids and growth factors stimulate the activation of eEF2 through the mTOR-signaling pathway (32). In this study, we found that neither amino acids, insulin, nor age affected eEF2 phosphorylation in skeletal muscle.

In summary, the present study demonstrates that many of the amino acid- and insulin-signaling components that are involved in the regulation of protein synthesis in skeletal muscle are activated by either amino acid or insulin, and the effects are developmentally regulated. Most of the previous data on the activation of signaling components leading to mRNA translation were obtained from cell culture studies. The majority of the data generated in the current in vivo study are consistent with in vitro findings. To demonstrate that the physical interaction of the members of the mTORC1 and mTORC2 that affects amino acid- or growth factor-induced activation in vivo conditions seems difficult, likely due to the complexity of in vivo environments. In conclusion, the results suggest that the postprandial rise in amino acids in stimulated skeletal muscle protein synthesis through the activation of insulin-signaling components leading to mRNA translation, whereas the postprandial rise in amino acids regulates this process through the activation of mTOR-signaling pathways downstream of TSC2. Nevertheless, there is a possibility of mTOR-independent effects of amino acids. Importantly, the developmental changes in these signaling components and in ribosome number (8) likely contribute to high rates of protein synthesis and rapid gain in skeletal muscle mass in neonates.

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