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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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Abstract

Insulin (INS) and amino acids (AA) 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-day-old (n=4/group) and 26-day-old (n=6/group) pigs were studied during: 1) euinsulinemic-euglycemic-euaminoacidemic conditions (controls), 2) euinsulinemic-euglycemic-hyperaminoacidemic clamps (AA), and 3) hyperinsulinemic-euglycemic-euaminoacidemic clamps (INS). 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 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 to 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 kinase (AMPK) and eukaryotic elongation factor 2 (eEF2). 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.

Keywords

Insulin; amino acids; neonates; mammalian target of rapamycin; tuberous sclerosis

INTRODUCTION

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 in order 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 insulin receptor 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 (PDK-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 Rheb, followed by the induction of mTOR. The mammalian target of rapamycin (mTOR) regulates mRNA translation by phosphorylating two of its effectors, ribosomal protein S6 kinase 1 (S6K1) and eIF4E-binding protein-1 (4E-BP1) (1,3,24,36). Phosphorylated 4E-BP1 releases eIF4E from the inactive eIF4E-4EBP1 complex, allowing the formation of the active eIF4G-eIF4E complex (25,39).

The amino acid signaling pathway leading to stimulation of mRNA translation is still elusive. However, data from cell culture studies indicate that mTOR integrates amino acid and growth factor signals via multiple mechanisms, including phosphorylation of the protein kinase S6K1 and the translation initiation factor 4E-BP1, resulting in modulation of protein synthesis and cell growth (31,32). Less is known about the mechanism by which amino acids, such as amino acids or glucose, modulate the activation of signaling components upstream of 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 suggests that amino acids regulate mTOR by activating a signaling component(s) downstream of the TSC1/TSC2 complex (32). 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, mTORC1 and mTORC2 (20,35). mTORC1, a rapamycin sensitive complex, has been shown to consist of the three proteins, mTOR, GβL and raptor, and largely control translation and cell growth in response to amino acids (17,26). mTORC2, a rapamycin insensitive complex, consists of mTOR, GβL, and rictor, and has been shown to control actin cytoskeleton dynamics and to activate PKB (3). 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 neonates 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 farrowing. Sows were fed a commercial diet (no. 5084; PMI Feeds, Richmond, IN) and provided water ad libitum. After farrowing, 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 and 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 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 (AA), and 3) hyperinsulinemic-euglycemic-euaminoacidemic clamp (INS). 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^{-0.66} · min⁻¹. To either maintain insulin at the fasted level or raise insulin to the fed level, venous blood samples (0.2 ml) were acquired 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-[4-³H] phenylalanine (Amersham Biosciences, Piscataway, NJ) injected 30 min before the end of the infusion (15). Protein synthesis (K_s expressed as % protein synthesized in a day) was calculated as: K_s (%/day) = $[(S_b/S_a) \times (1,440/t)] \times 100$, where S_b is the specific radioactivity of the protein-bound phenylalanine; S_a is the specific radioactivity of the tissue free phenylalanine for the labeling period, determined from the value of the animal at the time of tissue collection,

corrected by the linear regression of the blood specific radioactivity of the animal against time; and t is the time of labeling in minutes. Pigs were killed at 120 min, and longissimus dorsi muscle samples were collected and immediately frozen in liquid nitrogen and stored at -70°C until analyzed, as previously described (39).

Tissue extraction and immunoblot analysis

Freshly collected longissimus dorsi muscle tissue samples were homogenized, 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 (PVDF) membrane (Bio-Rad, Hercules, CA), which was incubated with appropriate primary antibodies, washed, and exposed to an appropriate secondary antibody as previously described (39).

For normalization, immunoblotting performed with antiphospho-specific antibodies were stripped in stripping buffer (Pierce Biotechnology, Rockford, IL) and reprobed with the corresponding nonphospho-specific 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: PKB (total and Ser 473, Cell Signaling Technology, Inc., Beverly, MA), AMPK α (total and Thr 172, Cell Signaling), TSC2 (total and Thr 1462, Cell Signaling), Rheb (total, Cell Signaling), mTOR (total and Ser 2448, Cell Signaling), S6K1 (total and Thr 398, Cell Signaling), 4E-BP1 (total, Bethyl Laboratories, Montgomery, TX, and Thr 70, Cell Signaling), and eEF2 (total and Thr 56, Cell Signaling).

Quantification of eIF4E-4EBP1 and eIF4E-eIF4G complexes

These complexes were immunoprecipitated using an anti-eIF4E monoclonal antibody (gift of Dr. Leonard Jefferson, Penn 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 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 (12), and immediately subjected to protein immunoblot analysis using rabbit anti-4E-BP1 (Cell Signaling) antibody or rabbit anti-eIF4G (Bethyl Laboratories). Amounts of 4E-BP1 and eIF4G were corrected by the eIF4E recovered from the immunoprecipitate.

Analysis of mTORC1 and mTORC2 complexes

To determine the association between mTOR and its partners (with raptor and G β L for the mTORC1 complex, or with rictor and G β L for the mTORC2 complex), muscle samples were homogenized in CHAPS buffer according to Williamson et al (43). The CHAPS buffer consisted of 40 mM HEPES, pH 7.5, 120mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM β -glycerolphosphate, 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) 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 once in CHAPS buffer containing 200 mM instead of 120 mM NaCl and 60 mM

instead of 40 mM HEPES. The precipitates were rinsed with 100 μ l of 1X 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 (Cell Signaling), anti-riCTOR antibody (Cell Signaling) and anti-G β L antibody (Cell Signaling). 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 $\pm$ SE. To determine the effectiveness of the clamp procedure, amino acid, glucose, and insulin concentrations in each treatment group were compared to their basal concentrations by use of *t*-tests. Data are presented as means $\pm$ 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, was increased two-fold to the fed level while 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, while 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-d compared to 6-d-old-pigs ($P = 0.005$ and 0.008 , respectively). These results suggest insulin sensitivity is reduced in 26-d compared to 6-d-old pigs, in agreement with our previous studies which showed that insulin sensitivity of glucose and amino acid disposal decreases with age (44,45).

In our previous studies, 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 (12,39). 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 have previously found that the activation of early steps in the insulin signaling pathway upstream of PKB is induced by feeding (37–39). In this study, we investigated the separate effects of amino acids and insulin on the phosphorylation of PKB at Ser 473, 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-d compared to 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 AMPK α at Thr 172, a crucial site for its activity, was analyzed using immunoblotting. In skeletal muscle, neither amino acids nor insulin altered the phosphorylation state of AMPK α at Thr 172 (Fig. 2A).

The TSC1/2 complex functions as a GTPase-activating protein towards 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 complex activation. In this study, we determined the effects of amino acids and insulin on the phosphorylation state of TSC2 at Thr 1462, 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 G β L are members of the mTORC1 complex (20,35). While G β L-mTOR interaction does not affect mTOR activity, 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 mTORC2 complex which is activated by growth factors (3,35). One of the major functions of mTORC2 is to induce PKB phosphorylation at Ser 473. 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 G β L, 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 were analyzed using immunoblot analysis. Both amino acids and insulin significantly enhanced the phosphorylation of S6K1 and 4E-BP1 in skeletal muscle (Figs. 4B and 4C, 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 hypo-phosphorylated state, this protein binds eIF4E to form an inactive

complex. Conversely, in the hyper-phosphorylated 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. 5A and 5B, 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 eEFs (eukaryotic elongation factors), 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 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 (12,39). 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 have been conducted using mammalian cell cultures (31,32). 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 activation 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 Ser 473 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 (29,41) studies demonstrating that amino acids stimulate protein synthesis through the activation of signaling components downstream of PKB. Furthermore, our results

demonstrate that PKB abundance and the activation of PKB by insulin in skeletal muscle decrease with development.

During insulin stimulation, PKB phosphorylates TSC2 at Thr 1462 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 which indicated that amino acids fail to phosphorylate TSC2 and alter TSC1/TSC2 activation (32). 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 complex (2). In order to activate the mTORC1 complex, 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 complex, 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 showed that rapamycin disturbs mTORC1 activation by removing raptor from the complex (30). Furthermore, in cell culture conditions, amino acid deprivation causes more raptor to bind to the mTORC1 complex, 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 Thr 389 and 4E-BP1 phosphorylation at Thr 70 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 Ser 65 and Thr 70 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 neither amino acids, insulin, or 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 complexes 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 insulin stimulates skeletal muscle protein synthesis through the activation of insulin signaling components leading to mRNA translation, while 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 rate of protein synthesis and rapid gain in skeletal muscle mass in neonates.

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REFERENCES

1. Asnaghi L, Bruno P, Priulla M, Nicolin A. mTOR: a protein kinase switching between life and death. *Pharmacol Res* 2004;50:545–549. [PubMed: 15501691]
2. Avruch J, Hara K, Lin Y, Liu M, Long X, Ortiz-Vega S, Yonezawa K. Insulin and amino-acid regulation of mTOR signaling and kinase activity through the Rheb GTPase. *Oncogene* 2006;25:6361–6372. [PubMed: 17041622]
3. Avruch J, Lin Y, Long X, Murthy S, Ortiz-Vega S. Recent advances in the regulation of the TOR pathway by insulin and amino acids. *Curr Opin Clin Nutr Metab Care* 2005;8:67–72. [PubMed: 15586002]
4. Bevan P. Insulin signalling. *J Cell Sci* 2001;114:1429–1430. [PubMed: 11282018]

5. Carling D. AMP-activated protein kinase: balancing the scales. *Biochimie* 2005;87:87–91. [PubMed: 15733742]
6. Chien PFW, Smith K, Watt PW, Scrimgeour CM, Taylor DJ, Rennie MJ. Protein turnover in the human fetus studied at term using stable isotope tracer amino acids. *Am J Physiol Endocrinol Metab* 1993;265:E31–E35.
7. Davis TA, Burrin DG, Fiorotto ML, Nguyen HV. Protein synthesis in skeletal muscle and jejunum is more responsive to feeding in 7- than in 26-day-old pigs. *Am J Physiol Endocrinol Metab* 1996;270:E802–E809.
8. Davis TA, Fiorotto ML, Beckett PR, Burrin DG, Reeds PJ, Wray-Cahen D, Nguyen HV. Differential effects of insulin on peripheral and visceral tissue protein synthesis in neonatal pigs. *Am J Physiol Endocrinol Metab* 2001;280:E770–E779. [PubMed: 11287360]
9. Davis TA, Fiorotto ML, Burrin DG, Reeds PJ, Nguyen HV, Beckett PR, Vann RC, O'Connor PM. Stimulation of protein synthesis by both insulin and amino acids is unique to skeletal muscle in neonatal pigs. *Am J Physiol Endocrinol Metab* 2002;282:E880–E890. [PubMed: 11882508]
10. Davis TA, Fiorotto ML, Nguyen HV, Reeds PJ. Enhanced response of muscle protein synthesis and plasma insulin to food intake in suckled rats. *Am J Physiol Regul Integr Comp Physiol* 1993;265:R334–R340.
11. Davis TA, Fiorotto ML, Nguyen HV, Reeds PJ. Protein turnover in skeletal muscle of suckling rats. *Am J Physiol Regul Integr Comp Physiol* 1989;257:R1141–R1146.
12. Davis TA, Nguyen HV, Suryawan A, Bush JA, Jefferson LS, Kimball SR. Developmental changes in the feeding-induced stimulation of translation initiation in muscle of neonatal pigs. *Am J Physiol Endocrinol Metab* 2000;279:E1226–E1234. [PubMed: 11093908]
13. Denne SC, Kalhan SC. Leucine metabolism in human newborns. *Am J Physiol Endocrinol Metab* 1987;253:E608–E615.
14. Di Guglielmo GM, Drake PG, Baass PC, Authier F, Posner BI, Bergeron JJ. Insulin receptor internalization and signalling. *Mol Cell Biochem* 1998;182:59–63. [PubMed: 9609114]
15. Garlick PJ, McNurlan MA, Preedy VR. A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of [³H]phenylalanine. *Biochem J* 1980;192(2):719–723. [PubMed: 6786283]
16. Gonzalez AA, Kumar R, Mulligan JD, Davis AJ, Weindruch R, Saupe KW. Metabolic adaptations to fasting and chronic caloric restriction in heart, muscle, and liver do not include changes in AMPK activity. *Am J Physiol Endocrinol Metab* 2004;287:E1032–E1037. [PubMed: 15251868]
17. Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, Tokunaga C, Avruch J, Yonezawa K. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* 2002;110:177–189. [PubMed: 12150926]
18. Hardie DG. New roles for the LKB1—>AMPK pathway. *Curr Opin Cell Biol* 2005;17:167–173. [PubMed: 15780593]
19. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 2003;115:577–590. [PubMed: 14651849]
20. Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. mTOR interacts with raptor to form a amino acid-sensitive complex that signals to the cell growth machinery. *Cell* 2002;110:163–175. [PubMed: 12150925]
21. Kimball SR, Farrell PA, Nguyen HV, Jefferson LS, Davis TA. Developmental decline in components of signal transduction pathways regulating protein synthesis in pig muscle. *Am J Physiol Endocrinol Metab* 2002;282:E585–E592. [PubMed: 11832361]
22. Krymskaya VP. Tumour suppressors hamartin and tuberlin: intracellular signalling. *Cell Signal* 2003;15:729–739. [PubMed: 12781866]
23. Kwiatkowski DJ, Manning BD. Tuberous sclerosis: a GAP at the crossroads of multiple signaling pathways. *Hum Mol Genet* 2005;14:R251–R258. [PubMed: 16244323]
24. Martin DE, Hall MN. The expanding TOR signaling network. *Curr Opin Cell Biol* 2005;17:158–166. [PubMed: 15780592]
25. Niedzwiecka A, Stepinski J, Darzynkiewicz E, Sonenberg N, Stolarski R. Positive heat capacity change upon specific binding of translation initiation factor eIF4E to mRNA 5' cap. *Biochemistry* 2002;41:12140–12148. [PubMed: 12356315]

26. Nobukuni T, Joaquin M, Roccio M, Dann SG, Kim SY, Gulati P, Byfield MP, Backer JM, Natt F, Bos JL, Zwartkruis FJ, Thomas G. Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc Natl Acad Sci USA* 2005;102:14238–14243. [PubMed: 16176982]
27. O'Connor PM, Bush JA, Suryawan A, Nguyen HV, Davis TA. Insulin and amino acids independently stimulate skeletal muscle protein synthesis in neonatal pigs. *Am J Physiol Endocrinol Metab* 2003;284:E110–E119. [PubMed: 12388131]
28. O'Connor PM, Kimball SR, Suryawan A, Bush JA, Nguyen HV, Jefferson LS, Davis TA. Regulation of neonatal liver protein synthesis by insulin and amino acids in pigs. *Am J Physiol Endocrinol Metab* 2004;286:E994–E1003. [PubMed: 14761876]
29. O'Connor PM, Kimball SR, Suryawan A, Bush JA, Nguyen HV, Jefferson LS, Davis TA. Regulation of translation initiation by insulin and amino acids in skeletal muscle of neonatal pigs. *Am J Physiol Endocrinol Metab* 2003;285:E40–E53. [PubMed: 12637260]
30. Oshiro N, Yoshino K, Hidayat S, Tokunaga C, Hara K, Eguchi S, Avruch J, Yonezawa K. Dissociation of raptor from mTOR is a mechanism of rapamycin-induced inhibition of mTOR function. *Genes Cells* 2004;9:359–366. [PubMed: 15066126]
31. Proud GC. Regulation of mammalian translation factors by amino acids. *Eur J Biochem* 2002;269:5338–5349. [PubMed: 12423332]
32. Proud GC. Role of mTOR signalling in the control of translation initiation and elongation by amino acids. *Curr Top Microbiol Immunol* 2004;279:215–244. [PubMed: 14560960]
33. Romanelli A, Dreisbach VC, Blenis J. Characterization of phosphatidylinositol 3-kinase-dependent phosphorylation of the hydrophobic motif site Thr(389) in p70 S6 kinase 1. *J Biol Chem* 2002;277:40281–40289. [PubMed: 12183455]
34. Ryazanov AG. Elongation factor-2 kinase and its newly discovered relatives. *FEBS Lett* 2002;514:26–29. [PubMed: 11904175]
35. Sabatini DM. mTOR and cancer: insights into a complex relationship. *Nat Rev Cancer* 2006;6:729–734. [PubMed: 16915295]
36. Shima H, Pende M, Chen Y, Fumagalli S, Thomas G, Kozma SC. Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. *EMBO J* 1998;17:6649–6659. [PubMed: 9822608]
37. Suryawan A, Davis TA. Developmental regulation of protein kinase B activation is isoform specific in skeletal muscle of neonatal pigs. *Pediatr Res* 2005;58:719–724. [PubMed: 16189199]
38. Suryawan A, Davis TA. Protein-tyrosine-phosphatase 1B activation is regulated developmentally in muscle of neonatal pigs. *Am J Physiol Endocrinol Metab* 2003;284:E47–E54. [PubMed: 12388170]
39. Suryawan A, Nguyen HV, Bush JA, Davis TA. Developmental changes in the feeding-induced activation of the insulin-signaling pathway in neonatal pigs. *Am J Physiol Endocrinol Metab* 2001;281:E908–E915. [PubMed: 11595645]
40. Suryawan A, Escobar J, Frank JW, Nguyen HV, Davis TA. Developmental regulation of the activation of signaling components leading to translation initiation in skeletal muscle of neonatal pigs. *Am J Physiol Endocrinol Metab* 2006;291:E849–E859. [PubMed: 16757550]
41. Suryawan A, O'Connor PM, Kimball SR, Bush JA, Nguyen HV, Jefferson LS, Davis TA. Amino acids do not alter the insulin-induced activation of the insulin signaling pathway in neonatal pigs. *J Nutr* 2004;134:24–30. [PubMed: 14704288]
42. Vary TC, Lynch CJ. Meal feeding enhances formation of eIF4F in skeletal muscle: role of increased eIF4E availability and eIF4G phosphorylation. *Am J Physiol Endocrinol Metab* 2006;290:E631–E642. [PubMed: 16263769]
43. Williamson DL, Bolster DR, Kimball SR, Jefferson LS. Time course changes in signaling pathways and protein synthesis in C2C12 myotubes following AMPK activation by AICAR. *Am J Physiol Endocrinol Metab* 2006;291(1):E80–E89. [PubMed: 16760336]
44. Wray-Cahen D, Beckett PR, Nguyen HV, Davis TA. Insulin-stimulated amino acid utilization during glucose and amino acid clamps decreases with development. *Am J Physiol Endocrinol Metab* 1997;273(1):E305–E314.

45. Wray-Cahen D, Nguyen HV, Burrin DG, Beckett PR, Fiorotto ML, Reeds PJ, Wester TJ, Davis TA. Response of skeletal muscle protein synthesis to insulin in suckling pigs decreases with development. *Am J Physiol Endocrinol Metab* 1998;275(1):E602–E609.

Figure 1.

Fractional rates of protein synthesis (A) and the phosphorylation of PKB at Ser 473 (B) in longissimus dorsi muscle of 6- and 26-day-old pigs in response to euinsulinemic-euglycemic-euaminoacidemic conditions (C), euinsulinemic-euglycemic-hyperaminoacidemic clamps (AA), and hyperinsulinemic-euglycemic-euaminoacidemic clamps (INS). Values of the phosphorylation of PKB were normalized for PKB content in samples. Values are means $\pm$ SEM; $n = 4-6$ per treatments. Means with different letters differ at $P < 0.05$.

Figure 2.

The phosphorylation of AMPK at Thr 172 (A), the phosphorylation of TSC2 at Thr 1462 (B), and the abundance of Rheb (C) in longissimus dorsi muscle of 6- and 26-day-old pigs in response to euinsulinemic-euglycemic-euaminoacidemic conditions (C), euinsulinemic-euglycemic-hyperaminoacidemic clamps (AA), and hyperinsulinemic-euglycemic-euaminoacidemic clamps (INS). Values of the phosphorylation of AMPK or TSC2 were normalized for AMPK or TSC2 content in samples. Values are means $\pm$ SEM; $n = 4-6$ per treatments. Means with different letters differ at $P < 0.05$.

Figure 3.

The raptor-mTOR association (A), the GβL-mTOR association (B), and the rictor-mTOR association (C) in longissimus dorsi muscle of 6- and 26-day-old pigs in response to euinsulinemic-euglycemic-euaminoacidemic conditions (C), euinsulinemic-euglycemic-hyperaminoacidemic clamps (AA), and hyperinsulinemic-euglycemic-euaminoacidemic clamps (INS). Values of all complexes were normalized for mTOR content in the immunoprecipitant (the amounts of mTOR in the immunoprecipitant were similar in both ages). Values are means $\pm$ SEM; $n = 4-6$ per treatments. Means with different letters differ at $P < 0.05$.

Figure 4.

The phosphorylation of mTOR at Ser 2448 (A), the phosphorylation of S6K1 at Thr 389 (B) and the phosphorylation of 4E-BP1 at Thr 70 in longissimus dorsi muscle of 6- and 26-day-old pigs in response to euinsulinemic-euglycemic-euaminoacidemic conditions (C), euinsulinemic-euglycemic-hyperaminoacidemic clamps (AA), and hyperinsulinemic-euglycemic-euaminoacidemic clamps (INS). Values of the phosphorylation of mTOR or S6K1 or 4E-BP1 were normalized for mTOR, S6K1, or 4E-BP1 content in samples. Values are means $\pm$ SEM; $n = 4-6$ per treatments. Means with different letters differ at $P < 0.05$.

Figure 5.

The formation of an active complex of eIF4E-eIF4G (A) and the formation of an inactive complex of eIF4E-4EBP1 in longissimus dorsi muscle of 6- and 26-day-old pigs in response to euinsulinemic-euglycemic-euaminoacidemic conditions (C), euinsulinemic-euglycemic-hyperaminoacidemic clamps (AA), and hyperinsulinemic-euglycemic-euaminoacidemic clamps (INS). Values of both complexes were normalized for eIF4E content in the immunoprecipitant (the amounts of eIF4E in the immunoprecipitant were similar in both ages). Values are means $\pm$ SEM; $n = 4-6$ per treatments. Means with different letters differ at $P < 0.05$.

Figure 6.

The phosphorylation of eEF2 at Thr 56 in longissimus dorsi muscle of 6- and 26-day-old pigs in response to euinsulinemic-euglycemic-euaminoacidemic conditions (C), euinsulinemic-euglycemic-hyperaminoacidemic clamps (AA), and hyperinsulinemic-euglycemic-euaminoacidemic clamps (INS). Values of the phosphorylation of eEF2 were normalized for eEF2 content in samples. Values are means $\pm$ SEM; $n = 4-6$ per treatments. Means with different letters differ at $P < 0.05$.

Table 1
Plasma glucose, amino acid and insulin concentrations

	Age	Baseline ^I	Control	AA	INS
Glucose (mg/dl)	6d	67.3 ± 5.9	62.5 ± 3.3	76.2 ± 3.8	72.7 ± 6.3
	26d	97.1 ± 5.8 [†]	96.8 ± 7.6 [†]	98.2 ± 3.9 [†]	83.1 ± 5.3 [†]
BCAA (mmol/ml)	6d	433 ± 100	470 ± 51	1139 ± 47 [*]	549 ± 110
	26d	442 ± 46	487 ± 47	1075 ± 50 [*]	477 ± 14
Insulin (μU/ml)	6d	2.65 ± 0.5	2.07 ± 0.7	2.26 ± 0.6	28.3 ± 7.6 [*]
	26d	3.97 ± 0.9 [†]	5.35 ± 0.8 [†]	4.45 ± 0.7 [†]	23.2 ± 2.5 [*]

Values are means ± SE, N = 4–6.
^IThe baseline values are the data collected at 0 min.
^{*}P < 0.05 compared to baseline.
[†]P < 0.05 compared to 6 d.