Developmental regulation of the activation of signaling components leading to translation initiation in skeletal muscle of neonatal pigs

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Suryawan, Agus, Jeffery Escobar, Jason W. Frank, Hanh V. Nguyen, and Teresa A. Davis. Developmental regulation of the activation of signaling components leading to translation initiation in skeletal muscle of neonatal pigs. Am J Physiol Endocrinol Metab 291: E849–E859, 2006. First published June 6, 2006; doi:10.1152/ajpendo.00069.2006.—The rapid growth of neonates is driven by high rates of skeletal muscle protein synthesis. This high rate of protein synthesis, which is induced by feeding, declines with development. Overnight-fasted 7- and 26-day-old pigs either remained fasted or were refed, and the abundance and phosphorylation of growth factor- and nutrient-induced signaling components that regulate mRNA translation initiation were measured in skeletal muscle and liver. In muscle, but not liver, the activation of inhibitors of protein synthesis, translation initiation was initiated by the binding of insulin to its receptor. This activates the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1), followed by the activation of phosphoinositide 3-kinase (PI 3-kinase; Fig. 1) (17). Activated PI 3-kinase then stimulates the activation of downstream effector molecules such as phosphoinositide-dependent kinase-1 (PDK-1) and protein kinase B (PKB) (17, 23). PKB phosphorylates and inactivates an inhibitor of cell growth, tuberin [also known as tuberous sclerosis complex 2 (TSC2)], thereby inactivating the function of the tuberous sclerosis complex 1/2 (TSC1/2) (28) and inducing the activation of mammalian target of rapamycin (mTOR).

mTOR regulates mRNA translation by phosphorylating two of its effectors, ribosomal protein S6 kinase 1 (S6K1) and eIF4E-binding protein-1 (4E-BP1) (1). Insulin signaling can be attenuated by the action of a number of phosphatases, including protein tyrosine phosphatase-1B (PTP1B), which dephosphorylates the IR and IRS-1; phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which inactivates PI3-kinase; and protein phosphatase 2A (PP2A), which acts on PKB and S6K1. Although the IR and IRS-1 are activated by phosphorylation on tyrosine residues, insulin signaling activation is attenuated by serine/threonine (Ser/Thr) phosphorylation of the IR and IRS-1 (18, 62).

The nutrient-induced signaling pathway leading to the activation of translation initiation factors is less well understood. mTOR has been shown to integrate nutrient and growth factor signals via multiple mechanisms, including phosphorylation of the protein kinase S6K1 and the translation factor 4E-BP1, resulting in modulation of protein synthesis and cell growth (43). Less is known about the mechanisms by which nutrients such as amino acids or glucose modulate the activation of signaling components upstream of mTOR (Fig. 1). 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 (24). Cell culture studies have shown that TSC2 is an important nutrient sensor required for the downregulation of mTOR activation following glucose deprivation (24). Although the involvement of amino acids in the activation of mTOR is well recognized, the mechanism by which amino acids regulate mTOR signaling is unclear (38). Recent findings indicate that raptor, an mTOR regulatory protein, is an important component upstream of mTOR that transmits amino acid signals. Amino acids regulate raptor-mTOR binding, resulting in the activation of the downstream effectors of mTOR, i.e., S6K1 and 4E-BP1 (21).

The neonatal period is characterized by high growth rates, with the most rapid gain occurring in skeletal muscle (8, 16, 61). The profound accretion of skeletal muscle mass 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 (9, 13, 14). 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 (9). The stimulation of protein synthesis in skeletal muscle 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 (12, 39, 40, 48).

The insulin-signaling pathway leading to the activation of translation initiation is initiated by the binding of insulin to its receptor. This activates the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1), followed by the activation of phosphoinositide 3-kinase (PI 3-kinase; Fig. 1) (17). Activated PI 3-kinase then stimulates the activation of downstream effector molecules such as phosphoinositide-dependent kinase-1 (PDK-1) and protein kinase B (PKB) (17, 23). PKB phosphorylates and inactivates an inhibitor of cell growth, tuberin [also known as tuberous sclerosis complex 2 (TSC2)], thereby inactivating the function of the tuberous sclerosis complex 1/2 (TSC1/2) (28) and inducing the activation of mammalian target of rapamycin (mTOR).

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Fig. 1. Growth factor and nutrient-signaling pathway leading to the stimulation of protein synthesis. IR, insulin receptor; PTP1B, protein tyrosine phosphatase-1B; IRS-1/2, insulin receptor substrate-1/2; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PI 3-K, phosphoinositide-3 kinase; PDK-1, phosphoinositide-dependent kinase-1; AMPK, AMP-activated protein kinase; PKB, protein kinase B; TSC1/2, tuberous sclerosis complex 1/2; mTOR, mammalian target of rapamycin; 4E-BP1, eIF4E-binding protein-1; S6K1, ribosomal protein S6 kinase-1.

The majority of studies that examined the regulation of insulin and nutrient-signaling pathways leading to translation initiation were conducted either in vitro or in cell culture systems, and little is known about the role of these signaling components in the whole animal. Using the neonatal pig as our animal model, we (15, 41, 50) 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. Our studies have shown that the activation of positive regulators of insulin signaling (IR, IRS-1, PI 3-kinase, and PKB) are induced by feeding in skeletal muscle (48, 50). These responses decrease with development in parallel with the decline in muscle protein synthesis (48, 50, 58). Furthermore, the activity of PTP1B, an inhibitor of the insulin-signaling pathway, increases with development, consistent with the developmental decline in insulin sensitivity during the early postnatal period (49). Here, we report a more detailed study of the role of development in the feeding-induced activation and protein abundance of signaling components that are upstream of mTOR, as well as downstream targets of mTOR, in skeletal muscle and liver of neonatal pigs. Signaling components that were examined include those that are considered negative regulators of growth factor/nutrient-signaling pathways that lead to translation initiation (PTEN, PP2A, AMPK, TSC1/2, and Ser/Thr phosphorylation of IR and IRS-1) and those that are positive regulators of translation initiation (raptor, mTOR, S6K1, and 4E-BP1).

METHODS

Animals. 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 24 piglets from four litters, weighing ~2 and 8 kg, were studied at 7 and 26 days of age, respectively. Pigs within each litter were randomly assigned to one of two treatment groups (n = 6 per age group per treatment group), and were either (1) fasted for 18 h or (2) fed for 1.5 h after an 18-h fast. Water was provided throughout the fasting period. Pigs that were fed after the 18-h fast were given two gavage administrations of 30 ml/kg body wt of mature porcine milk (University of Nebraska, Lincoln, NE) at 60-min intervals. Pigs in the fasting group were killed after 18 h of fasting, and pigs in the fed group were killed 30 min after the second gavage feeding. Samples of longissimus dorsi muscle and liver were rinsed in ice-cold saline and rapidly frozen. 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.

Muscle and liver homogenates. Freshly collected longissimus dorsi muscle and liver samples were homogenized and centrifuged at 10,000 g for 10 min at 4°C. Supernatants were diluted in sample buffer (41), frozen in liquid nitrogen, and stored at −70°C until protein immunoblot analysis.

Protein immunoblot analysis. Proteins were electrophoretically separated in polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA), which was incubated with appropriate antibodies as previously described (50). Blots were developed using an enhanced chemiluminescence kit (Amersham), visualized, and analyzed using a ChemiDoc-It imaging system (UVP, Upland, CA).

Determination of IR/IRS-1 Ser/Thr phosphorylation. Ser/Thr phosphorylation of the IR and IRS-1 is considered to be a negative regulator of the insulin-signaling pathway (18, 62). IR or IRS-1 in muscle and liver samples were immunoprecipitated using anti-IR or anti-IRS-1 antibody, respectively (50), followed by immunoblotting using anti-phospho-Ser/Thr antibody. Values obtained using the anti-phospho-Ser/Thr antibody were normalized for the total amount of IR or IRS-1 present in the immunoprecipitant.

Analysis of PTEN. Phosphorylation of PTEN on Ser380, a residue critical for inhibition of PTEN activity, was assessed by Western blot analysis using an antibody specific for PTEN phosphorylated on Ser380. The membranes were stripped and reprobed with an anti-PTEN antibody that recognizes both the phosphorylated and unphosphorylated forms of the protein. Values obtained using the anti-phospho-PTEN antibody were normalized for the total amount of PTEN present in the sample.

Analysis of PP2A. Phosphorylation of PP2A on Tyr207, a residue critical for inhibition of PP2A activity, was assessed by Western blot analysis using an antibody specific for PP2A phosphorylated on Ser207. The membranes were stripped and reprobed with an anti-PP2A antibody that recognizes both the phosphorylated and unphosphorylated forms of the protein. Values obtained using the anti-phospho-PP2A antibody were normalized for the total amount of PP2A present in the sample.

Analysis of AMPK. Phosphorylation of AMPKα on Thr172, a residue critical for AMPK activation, was assessed by Western blot analysis using an antibody specific for AMPKα phosphorylated on Thr172. The membranes were stripped and reprobed with an anti-AMPKα antibody that recognizes both the phosphorylated and unphosphorylated forms of the protein. Values obtained using the anti-phospho-AMPKα antibody were normalized for the total amount of AMPKα present in the sample.

Analysis of TSC2. Phosphorylation of TSC2 on Thr1462, a residue that is phosphorylated by PKB and blunts TSC1/2 complex activation, was assessed by Western blot analysis using an antibody specific for TSC2 phosphorylated on Thr1462. The membranes were stripped and reprobed with an anti-TSC2 antibody that recognizes both the phosphorylated and unphosphorylated forms of the protein. Values ob-
tained using the anti-phospho-TSC2 antibody were normalized for the total amount of TSC2 present in the sample.

**Analysis of mTOR.** Phosphorylation of mTOR on Ser2448, a residue located in a repressor domain, was assessed by Western blot analysis using an antibody specific for mTOR phosphorylated on Ser2448. The membranes were stripped and reprobed with an anti-mTOR antibody that recognizes both the phosphorylated and unphosphorylated forms of the protein. Values obtained using the anti-phospho-mTOR antibody were normalized for the total amount of mTOR present in the sample.

**Analysis of S6K1.** Phosphorylation of S6K1 was assessed by Western blot analysis using an antibody specific for S6K1 when it is phosphorylated at the activating residue, Thr389. The membranes were stripped and reprobed with an anti-S6K1 antibody that recognizes both the phosphorylated and unphosphorylated forms of the protein. Values obtained using the anti-phospho-S6K1 antibody were normalized for the total amount of S6K1 present in the sample.

**Analysis of 4E-BP1.** Phosphorylation of 4E-BP1 was assessed by Western blot analysis using an antibody specific for 4E-BP1 when it is phosphorylated at Thr70. The membranes were stripped and reprobed with an anti-4E-BP1 antibody that recognizes both the phosphorylated and unphosphorylated forms of the protein. Values obtained using the anti-phospho-4E-BP1 antibody were normalized for the total amount of 4E-BP1 present in the sample.

**Quantification of the abundance of raptor and mTOR-raptor complex.** To determine the abundance of raptor and the mTOR-raptor complex, tissues were processed to assure that the mTOR and raptor association was intact (21). Briefly, tissues were homogenized in ice-cold *buffer A* (20 mM Tris, 20 mM NaCl, 1 mM EDTA, 20 mM β-glycerophosphate, 5 mM EGTA, 1 mM dithiothreitol, and 10 μM inhibitor cocktail; Sigma) and centrifuged at 1,000 g for 20 min at 4°C. To determine raptor abundance, an equal amount of protein samples (supernatants) were subjected to SDS-PAGE followed by immunoblotting using an anti-raptor antibody. To quantify mTOR-raptor complex, this protein complex was immunoprecipitated from the supernatant. Briefly, samples (500 μg of supernatant) were immunoprecipitated with an anti-mTOR antibody overnight, and immunoprecipitates were washed twice with *buffer A* containing 0.5 M NaCl and twice with *buffer B* (10 mM HEPES, 50 mM β-glycerophosphate, 50 mM NaCl, pH 7.4). Proteins in the immunoprecipitate were resolved by SDS-PAGE and then transferred to PVDF membranes. The membranes were then probed with an anti-raptor antibody and then developed using an enhanced chemiluminescence Western Blotting kit (Amersham). The membranes were stripped and reprobed with an anti-mTOR antibody. Values obtained using the anti-raptor antibody were normalized for the amount of mTOR present in the sample.

**Statistics.** Values shown are means ± SE. Statistical evaluation of the data was performed using analysis of variance to determine the effect of feeding, age, and their interaction. Unpaired two-tailed *t*-tests were also performed to examine the specific effect of each treatment group. Differences between means were considered significant at *P* < 0.05.

**RESULTS**

Mounting evidence indicates that Ser/Thr phosphorylation of the IR and IRS-1 protein plays a critical, but negative, role in insulin signaling (25). Here, we determined the Ser/Thr phosphorylation of the IR and IRS-1 in longissimus dorsi muscle, a muscle that contains primarily fast-twitch muscle fibers, and in liver of 7- and 26-day-old pigs that were either fasted overnight or re-fed for 1.5 h after an overnight fast. As shown in Fig. 2, A and B, neither feeding nor age altered the phosphorylation of the IR or IRS-1 on Ser/Thr residues in skeletal muscle or liver, suggesting that the enhanced activation of early steps of the insulin-signaling pathway in skeletal muscle of neonatal pigs (50) does not involve developmental changes in Ser/Thr phosphorylation of the IR or IRS-1.

PTEN is a major phosphatase that downregulates PI 3-kinase activity and is therefore a negative regulator of protein synthesis (19). Protein abundance of PTEN was lower in muscle of 7- compared with 26-day-old pigs (*P* < 0.05; Fig. 3A). Because
phosphorylation at the PTEN tail (Ser\(^{380}\), Thr\(^{382}\), and Thr\(^{383}\)) inhibits its activity (57); we determined indirectly the activation of PTEN by measuring the PTEN phosphorylation state at the Ser\(^{380}\) residue. As shown in Fig. 3B, the phosphorylation of PTEN at Ser\(^{380}\) was significantly higher in skeletal muscle of 7- than in 26-day-old pigs \((P < 0.05)\), suggesting that PTEN is less active in younger pigs. By contrast, both PTEN abundance and PTEN phosphorylation in the liver were similar in 7- and 26-day-old pigs (Fig. 3, C and D). There was no effect of feeding on the abundance or phosphorylation of PTEN in skeletal muscle or liver.

PP2A is an important phosphatase that negatively regulates the phosphorylation state of PKB (5, 54), S6K1, and 4E-BP1 (42). In the present study, surprisingly, the protein abundance of PP2Ac (catalytic subunit) was significantly higher in skeletal muscle of 7- than in 26-day-old pigs \((P < 0.05);\) Fig. 4A). Studies show that the catalytic subunit of PP2A is inactivated in part by phosphorylation of Tyr\(^{307}\) by receptor and non-receptor protein tyrosine kinases (6, 7). Therefore, in the present study tissue protein extracts were analyzed by immunoblotting using an antibody that recognizes total PP2A or an antibody that binds to PP2Ac only when it is phosphorylated at Tyr\(^{307}\). As shown in Fig. 4B, the phosphorylation state of PP2Ac at Tyr\(^{307}\) was relatively high in skeletal muscle of 7-day-old pigs and undetectable in skeletal muscle of 26-day-old pigs. By contrast, both the abundance and the phosphorylation state of PP2Ac in liver were similar in both age groups (Fig. 4, C and D). There was no effect of feeding on the abundance or phosphorylation of PP2Ac in skeletal muscle or liver.

AMPK, a kinase that is activated by falling energy status as a result of starvation for a carbon source or other stresses, inhibits the mTOR pathway by phosphorylating TSC2, thereby inhibiting protein synthesis and cell growth (32). To determine whether this regulator is affected by fasting or age, the protein abundance of AMPK\(\alpha\) and its phosphorylation on Thr\(^{172}\), a crucial site for its activity, were analyzed with immunoblotting. In skeletal muscle (Fig. 5, A and B) and liver (Fig. 5, C and D), neither the AMPK\(\alpha\) abundance nor the phosphorylation state of AMPK\(\alpha\) at Thr\(^{172}\) was affected by fasting or age.

The TSC1/2 complex functions as a GTPase-activating protein towards Rheb, a major positive regulator of mTOR (29). Whereas cell culture studies suggest that stimulation of the AMPK pathway enhances TSC1/2 complex activation, resulting in inhibition of protein synthesis, the PI 3-kinase-PKB pathway suppresses TSC1/2 complex activation (29). In this study, we determined the protein abundance and 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. As shown in Fig. 6A, TSC2 abundance was significantly lower in skeletal muscle of 7- than in 26-day-old pigs \((P < 0.05)\). The phosphorylation of TSC2 at Thr\(^{1462}\) was induced by feeding \((P < 0.05)\) and was significantly higher \((P < 0.05)\) in skeletal muscle of 7- than in 26-day-old pigs (Fig. 6B), suggesting that the TSC1/2 complex is less active in younger pigs. The abundance and the phosphorylation state of TSC2 in the liver were not determined.

Raptor is an mTOR-interacting protein that plays an important role in regulating mTOR function (21, 26). Deleting 293T cells of raptor by using siRNA decreases cell size comparable with that produced by using siRNA to deplete cells of mTOR (26). This suggests that raptor, like mTOR, is crucial for cell growth. As shown in Fig. 7A, the protein abundance of raptor was significantly higher \((P < 0.05)\) in skeletal muscle of 7- than in 26-day-old pigs. By contrast, the protein abundance of raptor in the liver was similar in both ages (Fig. 7C). Because increasing the mTOR-raptor complex enhances the kinase

![Fig. 3. Protein abundance of PTEN and phosphorylation of Ser\(^{380}\) on PTEN in skeletal muscle (A and B) and liver (C and D) of fasted and fed pigs at 7 and 26 days of age. Animals were either fasted for 18 h (open bars) or refed for 1.5 h after an 18-h fast (gray bars). Values of the phosphorylation of PTEN were normalized for PTEN content in samples. Values are means \pm SE in arbitrary densitometric units; \(n = 5/\)group. *Effect of age \((P < 0.05)\).](E852_CHANGES_IN_SIGNALING_COMPONENTS_IN_NEONATAL_TISSUES)
activity of mTOR (21), in the present study, we determined raptor and mTOR interaction by immunoprecipitation and immunoblotting. Using a specific buffer (devoid of nonionic detergent) that was designed to capture an intact mTOR-raptor complex (21), we were able to demonstrate the interaction between raptor and mTOR in vivo. As shown in Fig. 7B, the abundance of the mTOR-raptor complex was significantly higher in skeletal muscle of 7- than in 26-day-old pigs (P < 0.05), which is consistent with a higher mTOR activation in younger pigs (see below). There was no effect of feeding on raptor abundance or the interaction of mTOR with raptor.

Fig. 4. Protein abundance of PP2A and phosphorylation of Tyr307 on PP2A in skeletal muscle (A and B) and liver (C and D) of fasting and fed pigs at 7 and 26 days of age. Animals were either fasted for 18 h (open bars) or refed for 1.5 h after an 18-h fast (gray bars). Values of the phosphorylation of PP2Ac (catalytic subunit) were normalized for PP2Ac content in samples. Values are means ± SE in arbitrary densitometric units; n = 5/group. *Effect of age (P < 0.05).

Fig. 5. Protein abundance of AMPKα and phosphorylation of Thr172 on AMPKα in skeletal muscle (A and B) and liver (C and D) of fasting and fed pigs at 7 and 26 days of age. Animals were either fasted for 18 h (open bars) or refed for 1.5 h after an 18-h fast (gray bars). Values of the phosphorylation of AMPKα were normalized for AMPKα content in samples. Values are means ± SE in arbitrary densitometric units; n = 5/group.
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 (2). In the present study, we found that the abundance of mTOR was significantly higher \( (P < 0.05) \) in skeletal muscle of 7- than in 26-day-old pigs (Fig. 8A). Furthermore, because one of the mechanisms for regulation of mTOR activity involves phosphorylation of the protein at Ser\(^{2448} \), a residue that is present in a putative repressor domain (45), we examined indirectly mTOR activity by measuring phosphorylation at this site. The phosphorylation state of mTOR at Ser\(^{2448} \) was enhanced by feeding \( (P < 0.05) \) and was significantly higher \( (P < 0.05) \) in skeletal muscle of 7- than in 26-day-old pigs (Fig. 8B). There was a tendency \( (P = 0.055) \) for the response to feeding to be greater in the younger pigs. By contrast, both the abundance and the phosphorylation state of mTOR at Ser\(^{2448} \) in liver were similar in both age groups and were unaffected by feeding (Fig. 8, C and D).

The mTOR kinase regulates the translational machinery in response to nutrients and growth factors via phosphorylation of S6K1 and phosphorylation of 4E-BP1 (1). To examine the effect of feeding and age on the activation of these crucial effectors of mTOR, the protein abundance of S6K1 and 4E-BP1 and the phosphorylation state of S6K1 at Thr\(^{398} \) and 4E-BP1 at Thr\(^{70} \) were analyzed by immunoblotting. The protein abundance of both S6K1 and 4E-BP1 were not affected by age in either tissue (skeletal muscle; Figs. 9A and 10A, respectively, and liver; Figs. 9C and 10C, respectively). By contrast, feeding significantly enhanced the phosphorylation states of both S6K1 and 4E-BP1 in skeletal muscle and liver \( (P < 0.05) \), and the response was significantly higher in skeletal muscle \( (P < 0.05; \text{Figs. } 9B \text{ and } 10B, \text{ respectively}) \), but not in liver (Figs. 9D and 10D, respectively), of 7- than in 26-day-old pigs. In skeletal muscle, there was an interaction between age and feeding \( (P < 0.05) \).
DISCUSSION

Ethical considerations precluded the measurement of tissue protein synthesis in the human infant; therefore, we utilized the neonatal pig as an animal model because the neonatal pig is similar to the human infant in anatomy, developmental physiology, and metabolism (35). We have shown that the high rate of protein synthesis, and thus growth, in skeletal muscle of the neonate is due to an elevated ribosome content (9, 11, 14) as well as an increased efficiency with which ribosomes translate...
mRNA in response to the postprandial rise in insulin and amino acids (12, 39, 58). Therefore, in previous studies (15, 27, 41, 48 - 50), we explored the effect of feeding and development on the activation of early steps of the insulin-signaling pathway and the activation of translation initiation factors leading to the stimulation of protein synthesis. We found that the postprandial rise in skeletal muscle protein synthesis in the neonatal pig is regulated by the activation of insulin-signaling components, i.e., IR, IRS-1, PI 3-kinase, PKB, and PTP1B, and the activation of translation initiation factors, including mTOR, S6K1, 4E-BP1, and the eIF4F complex of proteins. However, the detailed study of some of the signaling components that are involved in the activation of nutrient- and growth factor-signaling pathways had not been performed. The results of the present study fill in some of the gaps that were missing from our previous studies and clarify the important role of a number of signaling components in the developmental regulation of the feeding-induced stimulation of muscle protein synthesis in neonates.

In this study, the Ser/Thr phosphorylation of the IR and IRS inhibits the activation of insulin signaling (62). In fact, Ser/Thr phosphorylation of the IR and IRS represents one of the potential mechanisms responsible for the development of insulin resistance in vivo, and its reversal is a potential target for the treatment of diabetes (47). In a previous study, we (50) demonstrated that the activation of early steps in the insulin signaling pathway, i.e., IR, IRS-1, and PI 3-kinase, is very high in skeletal muscle of neonatal pigs and decreases with development. Therefore, we hypothesized that Ser/Thr phosphorylation of the IR and IRS would be lower in the youngest pigs. Our results show that Ser/Thr phosphorylation of the IR and IRS-1 in muscle and liver did not change with development and was unaffected by feeding. We speculate that the regulation of insulin signaling by this event occurs primarily in abnormal physiological conditions such as oxidative stress (18) or diabetes (25, 47).

PTEN has phosphatase activity against lipid substrates and, in particular, against the D3-phosphorylated position of phosphoinositide-3,4,5-trisphosphate, a direct product of PI 3-kinase (31). Thus PTEN is a negative regulator that antagonizes the effects of activated PI 3-kinase in the nutritionally controlled insulin pathway, thereby reducing protein synthesis and restraining cell and animal growth (19). Activation of PTEN is regulated by the balance of phosphorylation and dephosphorylation.
ylation of the protein at the COOH-terminal domain, which appears to be important for the stability and the function of the protein (53, 57). PTEN is phosphorylated at three residues (Ser^380, Thr^382, and Thr^383). When PTEN is phosphorylated at the COOH-terminal domain the enzyme is inactive, whereas dephosphorylated PTEN is in an active form. We showed that PTEN abundance was lower in skeletal muscle, but not in the liver, of 7- than in 26-day-old pigs. Furthermore, the phosphorylation of PTEN at Ser^380 was significantly higher in skeletal liver, of 7- than in 26-day-old pigs. Furthermore, the phosphor-
itation of PTEN abundance was lower in skeletal muscle, but not in the dephosphorylated PTEN is in an active form. We showed that the COOH-terminal domain the enzyme is inactive, whereas phosphorylation on Tyr^307 at a catalytic subunit (PP2Ac) by level of regulation of PP2A activity involves the tyrosine (32). The regulation of PP2A activity is highly complex. One known to be modulated by PP2A, including PKB and S6K1 regulation of phosphorylation of signaling proteins in eukary-
oughtic cells (33). The activity of more than 30 protein kinases are known to be modulated by PP2A, including PKB and S6K1 (32). The regulation of PP2A activity is highly complex. One level of regulation of PP2A activity involves the tyrosine phosphorylation on Tyr^307 at a catalytic subunit (PP2Ac) by cellular tyrosine kinases (30). The tyrosine phosphorylation at this site inhibits PP2A activity (7). Furthermore, PP2A activation is also regulated by methylation. Several studies (52, 59) demonstrated that methylation acts as molecular switch that controls the assembly of PP2A holoenzymes. Hence, like phosphorylation, methylation regulates protein-protein interactions and the recruitment of regulatory proteins into PP2A complexes (59). In this study, surprisingly, we found that the abundance of PP2Ac was significantly higher in skeletal muscle, but was similar in the liver, of 7- compared with 26-day-
old pigs. However, the phosphorylation of PP2A on Tyr^307 in skeletal muscle was high in 7-day-old pigs and undetectable in muscle of 26-day-old pigs. There was no effect of age in liver. Although it is tempting to speculate that PP2A activation is higher in skeletal muscle of younger pigs, analyses of PP2A methylation and protein-protein interactions need to be performed. Future studies will address these possible mechanisms.

AMPK, a sensor of cellular energy, is activated by rising AMP levels as a result of starvation for a carbon source or other stress (22). Activation of AMPK requires phosphorylation of Thr^172 within the catalytic subunit of AMPK by LKB1 (4). Furthermore, AMPK inhibits the mTOR pathway by phosphorylating TSC2, thus inhibiting cell growth during times of stress (24). In this study, we determined whether AMPK abundance or phosphorylation was altered by 18-h fasting or by development. We found that fasting did not alter AMPK protein abundance or phosphorylation on Thr^172 in skeletal muscle or liver and that there were no differences in the abundance or phosphorylation of AMPK in 7- and 26-day-old pigs. These results are in agreement with a recent study by Gonzalez et al. (20), who reported that neither 24-h fasting nor chronic caloric restriction of adult mice altered the activity of AMPK in either skeletal muscle or liver. These results suggest that AMPK may not be involved in the regulation of the mTOR pathway during normal physiological conditions, although extreme stress may deplete cellular AMP levels and increase AMPK activity.

PP2A is a phosphatase that plays a major role in the regulation of phosphorylation of signaling proteins in eukaryotic cells (33). The activity of more than 30 protein kinases are known to be modulated by PP2A, including PKB and S6K1 (32). The regulation of PP2A activity is highly complex. One level of regulation of PP2A activity involves the tyrosine phosphorylation on Tyr^307 at a catalytic subunit (PP2Ac) by cellular tyrosine kinases (30). The tyrosine phosphorylation at this site inhibits PP2A activity (7). Furthermore, PP2A activation is also regulated by methylation. Several studies (52, 59) demonstrated that methylation acts as molecular switch that controls the assembly of PP2A holoenzymes. Hence, like phosphorylation, methylation regulates protein-protein interactions and the recruitment of regulatory proteins into PP2A complexes (59). In this study, surprisingly, we found that the abundance of PP2Ac was significantly higher in skeletal muscle, but was similar in the liver, of 7- compared with 26-day-old pigs. However, the phosphorylation of PP2A on Tyr^307 in skeletal muscle was high in 7-day-old pigs and undetectable in muscle of 26-day-old pigs. There was no effect of age in liver. Although it is tempting to speculate that PP2A activation is higher in skeletal muscle of younger pigs, analyses of PP2A methylation and protein-protein interactions need to be performed. Future studies will address these possible mechanisms.

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The TSC1 and TSC2 gene products, TSC1 and TSC2, form a tumor suppressor complex, TSC1/2, that integrates inputs from multiple signaling cascades to inactivate the small GTPase, Rheb, and thereby inhibit mTOR-dependent cell growth (36). Cell culture studies indicate that TSC1/2 complex activation is regulated by both growth factors and amino acids (24). Although the mechanism by which amino acids regulate TSC1/2 complex activation is unknown, recent data suggest that growth factors activate this complex through PKB-dependent phosphorylation of TSC2 on Thr^1462 (51). Our results show that TSC2 abundance in skeletal muscle increased with development and that the feeding-induced increase in the phosphorylation of TSC2 on Thr^1462 in skeletal muscle was greater the younger the animal. Thus the results suggest that the TSC1/2 complex is an important signaling component of the nutrient/ growth factor pathway leading to transition initiation and that the feeding-induced changes in the activation of this complex are developmentally regulated.

The activation of mTOR, a major protein kinase that modulates translation initiation components, is regulated by nutrients and insulin (2, 32). The phosphorylation of mTOR on Ser^2448, which activates the kinase, is stimulated by both insulin and/or amino acids (34). Furthermore, the association of raptor with mTOR appears to be essential for TOR signaling and the binding of mTOR with 4E-BP1 and S6K1 (60). In this study, we found that both the abundance and the phosphorylation of mTOR on Ser^2448 were significantly higher in skeletal muscle of 7- than in 26-day-old pigs and that the feeding-induced increase in mTOR phosphorylation in skeletal muscle decreased with development. Furthermore, the abundance of raptor and the association of raptor with mTOR were also higher in skeletal muscle of the younger pigs. By contrast, the abundance of mTOR and raptor as well as the phosphorylation of mTOR were similar in liver of both ages, indicating tissue specificity of the responses. The higher mTOR abundance, mTOR phosphorylation, and raptor-mTOR complex in skeletal muscle, but not in liver, of the younger pigs is consistent with the higher activation of the mTOR pathway in neonatal muscle and the lack of change in mTOR activation in liver as shown in previous studies (27).

The well-known targets of mTOR in the translation initiation pathway are S6K1 and a translational repressor protein, 4E-BP1 (1). mTOR controls the response of the translation initiation machinery to amino acids and growth factors via activation of S6K1 and inhibition of 4E-BP1 (1). We previously showed a developmental reduction in the feeding-induced phosphorylation of S6K1 and 4E-BP1, as indicated by changes in the hyperphosphorylation of the proteins (15). In the present study, we wished to examine more closely the effect of development on the site-specific phosphorylation of the proteins as well as on the abundance of these proteins. The phosphorylation of S6K1 at Thr^389 is correlated with its activity (44). In this study, we found that feeding increased S6K1 phosphorylation at Thr^389 in both skeletal muscle and liver, but the response decreased with age only in the muscle. The S6K1 abundance in both tissues was similar in both age groups. In skeletal muscle, S6K2 can compensate for some of S6K1 functions (55). By contrast, liver S6K2 can fully compensate for the S6K1-induced phosphorylation of rpS6, thus indicating tissue specificity of the kinase (46). This could explain the absence of a developmental effect on S6K1 in liver.
The binding of eIF4E to 4E-BP1 is regulated by phosphorylation of 4E-BP1, with phosphorylation being associated with a decrease in the amount of the inactive 4E-BP1-eIF4E complex (53). Furthermore, the phosphorylation of 4E-BP1 at Ser65 and Thr70 is sufficient to prevent binding to eIF4E (37). We found that the phosphorylation of 4E-BP1 at Thr70 was significantly higher in skeletal muscle of 7- than in 26-day-old pigs and was similar in liver of both age groups. Feeding significantly increased 4E-BP1 phosphorylation in both tissues of all age groups, and the response was greater in muscle of 7- than in 26-day-old pigs. The abundance of 4E-BP1 was not affected by either feeding or development. The higher activation of both S6K1 and 4E-BP1 in skeletal muscle of younger pigs is consistent with the higher activation of mTOR and signaling components upstream of mTOR (15, 26, 48).

In this study, we used liver as a comparison to skeletal muscle. Although feeding stimulates protein synthesis in liver, as it does in other tissues of the neonate (9, 10), this effect does not change with development (9). Therefore, it is not surprising that, in the present study, we found no effect of development on the activation of all of the signaling components that we measured in the liver.

In summary, the present study demonstrates that many of the nutrient- and insulin-signaling components that are involved in the regulation of protein synthesis in skeletal muscle are developmentally regulated. Most of the previous information on these signaling components was generated from cell culture studies; however, our present study indicates that a number of the signaling components play important roles in the regulation of translation initiation in the in vivo condition. Importantly, our study shows that, in skeletal muscle, the activation of negative regulators of protein synthesis, i.e., PTEN, TSC2, and perhaps PP2A, is low, and the activation of positive regulators, i.e., mTOR, S6K1, and 4E-BP1, is high in the younger pigs, which is consistent with the elevated rates of protein synthesis in neonatal muscle. The lack of effect of feeding on the activation of some of these signaling components is likely due to the time at which we harvested the tissues (56), whereby activation may have occurred at an earlier time point. The developmental changes in the abundance and activation of these signaling components and the change in ribosome number (11) likely contribute to the high rate of protein synthesis and more rapid gain in skeletal muscle mass in neonates.

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