Feeding stimulates protein synthesis in muscle and liver of neonatal pigs through an mTOR-dependent process

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1Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033; and 2United States Department of Agriculture/Agricultural Research Service, Children’s Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030

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Kimball, Scot R., Leonard S. Jefferson, Hahn V. Nguyen, Agus Suryawan, Jill A. Bush, and Teresa A. Davis. Feeding stimulates protein synthesis in muscle and liver of neonatal pigs through an mTOR-dependent process. Am J Physiol Endocrinol Metab 279: E1080–E1087, 2000.—Protein synthesis is repressed in both skeletal muscle and liver after a short-term fast and is rapidly stimulated in response to feeding. Previous studies in rats and pigs have shown that the feeding-induced stimulation of protein synthesis is associated with activation of the 70-kDa ribosomal protein S6 kinase (S6K1) as well as enhanced binding of eukaryotic initiation factor eIF4E to eIF4G to form the active eIF4F complex. In cells in culture, hormones and nutrients regulate both of these events through a protein kinase termed the mammalian target of rapamycin (mTOR). In the present study, the involvement of mTOR in the feeding-induced stimulation of protein synthesis in skeletal muscle and liver was examined. Pigs at 7 days of age were fasted for 18 h, and then one-half of the animals were fed. In addition, one-half of the animals in each group were administered rapamycin (0.75 mg/kg) 2 h before feeding. The results reveal that treating 18-h fasted pigs with rapamycin, a specific inhibitor of mTOR, before feeding prevented the activation of S6K1 and the changes in eIF4F complex formation observed in skeletal muscle and liver after feeding. Rapamycin also ablated the feeding-induced stimulation of protein synthesis in liver. In contrast, in skeletal muscle, rapamycin attenuated, but did not prevent, the stimulation of protein synthesis in response to feeding. The results suggest that feeding stimulates hepatic protein synthesis through an mTOR-dependent process involving enhanced eIF4F complex formation and activation of S6K1. However, in skeletal muscle, these two processes may account for only part of the stimulation of protein synthesis, and thus additional steps may be involved in the response.

neonate; growth; eukaryotic initiation factor 4E; 4E-binding protein 1; S6K1

IN BOTH CELLS IN CULTURE and animals in vivo, protein synthesis is stimulated by a variety of hormones and nutrients. In particular, insulin and amino acids independently activate the initiation phase of mRNA translation, an effect that involves enhanced binding of both mRNA and initiator methionyl-tRNA (met-tRNA) to the 40S ribosomal subunit (reviewed in Ref. 42). The met-tRNA, binding step in translation initiation is mediated by eukaryotic initiation factor eIF2, which binds to the 40S ribosomal subunit as a GTP-eIF2-met-tRNA ternary complex (reviewed in Refs. 35, 39). After binding to the 40S ribosomal subunit, the guanosine triphosphate (GTP) bound to eIF2 is hydrolyzed to guanosine diphosphate (GDP), and eIF2 is released from the 40S ribosomal subunit as a binary complex with GDP. Before binding met-tRNA, and participating in another round of initiation, the GDP bound to eIF2 is exchanged for GTP through the action of a second initiation factor, termed eIF2B. However, recent in vivo studies in both rats and pigs suggest that eIF2B activity may not be regulated by changes in nutritional status (15, 50).

The mRNA binding step in translation initiation is mediated by a complex of three initiation factors, termed eIF4F, that is composed of eIF4E, the protein that binds to the m7GTP cap at the 5′-end of the mRNA, eIF4A, an RNA helicase, and eIF4G, a scaffolding protein that, in addition to binding eIF4A and eIF4E, also binds to the ribosome through interactions with eIF3. The best characterized mechanism for regulating mRNA binding to the 40S ribosomal subunit involves the reversible association of eIF4E with the translational repressor, 4E-binding protein 1 (4E-BP1). Binding of 4E-BP1 to eIF4E blocks the association of eIF4E with eIF4G, thereby preventing the binding of mRNA to the 40S ribosomal subunit. 4E-BP1 binding to eIF4E is modulated by phosphorylation of 4E-BP1 on multiple Ser and Thr residues, where hyperphosphorylation precludes and hypophosphorylation is permissive for binding.

In cells in culture, insulin stimulates binding of eIF4E to eIF4G (3, 30, 31, 44), an effect mediated by phosphorylation of 4E-BP1, which results in dissoci-
tion of the 4E-BP1–eIF4E complex, freeing eIF4E to bind to eIF4G (reviewed in Ref. 22). Phosphorylation of 4E-BP1 requires activation of phosphatidylinositol (PI) 3-kinase and protein kinase B (PKB) (21, 43) but additionally involves another protein kinase, referred to as the mammalian target of rapamycin (mTOR) (5, 18). mTOR serves as a bifurcation point in insulin signaling, with both 4E-BP1 and the 70-kDa ribosomal protein S6 kinase (S6K1) being downstream of the kinase. Inhibition of mTOR by the bacterially derived macro-lide immunosuppressant, rapamycin, obviates phosphorylation of both 4E-BP1 and S6K1 (5, 18). Like insulin, amino acids, and, in particular Leu, promote phosphorylation of 4E-BP1 and binding of eIF4E to eIF4G (24, 28, 40, 45, 48). However, in contrast to insulin, amino acids do not activate PI 3-kinase but do require mTOR to be active for phosphorylation of 4E-BP1 and S6K1 to occur (24, 40, 45).

In animals subjected to a short (18-h) fast, protein synthesis is repressed in both skeletal muscle and liver (1, 6, 10, 12, 13). The stimulation of protein synthesis that occurs after feeding of fasted animals is associated with phosphorylation of 4E-BP1, release of eIF4E from the inactive 4E-BP1–eIF4E complex, and increased binding of eIF4E to eIF4G (15, 50). However, no change in eIF2B activity is observed in response to feeding. In the present study, we asked whether activation of S6K1 and binding of eIF4E to eIF4G are required for the stimulation of protein synthesis caused by feeding in the neonate. We chose the neonatal pig as the animal model, because the activation of S6K1, formation of the eIF4F complex, and rates of protein synthesis are highly responsive to feeding, and because these effects are more pronounced the younger the animal (10, 15). We show that, administered 2 h before feeding, rapamycin has differential effectiveness in preventing the feeding-induced stimulation of protein synthesis in skeletal muscle and liver. Thus activation of S6K1 and/or formation of the eIF4F complex is a required component of the feeding-induced stimulation of protein synthesis in liver in neonatal pigs. In contrast, activation of S6K1 and eIF4F complex formation accounts for only part of the stimulation in muscle protein synthesis.

MATERIALS AND METHODS

Animals. Two crossbred (Landrace × Yorkshire × Hampshire × Duroc) pregnant sows (Agriculture Headquarters, Texas Dept. 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 (5084, PMI Feeds, Richmond, IN) and provided with water ad libitum. After farrowing, piglets remained with the sow and were not given supplemental creep feed. Three days before the study was performed, piglets were anesthetized, and catheters were surgically inserted into a jugular vein as described previously (10). Piglets were returned to the sow until fasting was initiated.

At 7 days of age, piglets within each litter were randomly assigned to one of two treatment groups and were either (1) fasted for 18 h or (2) fed for 1.5 h after an 18-h fast. The two treatment groups were further subdivided, and one-half of the animals in each subgroup were administered rapamycin (0.75 mg/kg in 5% dimethyl sulfoxide) into the jugular vein catheter. In fasted pigs, rapamycin was injected at the end of the 18-h fasting period, and pigs were killed 2 h later. In fed pigs, rapamycin was injected 2 h before feeding, and pigs were killed 3.5 h later. Pigs were provided with water throughout the fasting period. Pigs that were fed after the 18-h fast were given two gavage feeds of 30 ml/kg body wt of porcine mature milk (University of Nebraska, Lincoln, NE) at 60-min intervals. Pigs were killed, and samples of longissimus dorsi 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.

Materials. Enhanced chemiluminescence (ECL) detection reagents and horseradish peroxidase-conjugated sheep antimmune immunoglobulin G (IgG) and donkey anti-rabbit IgG were purchased from Amersham Life Sciences. Polyvinylidenefluoride (PVDF) membrane was obtained from Bio-Rad. And C-H phenylalanine was obtained from Amersham Pharmacia Biotech, Piscataway, NJ. Antibodies against S6K1 were purchased from Santa Cruz Biotechnology.

Measurement of protein synthesis in skeletal muscle and liver. The fractional rate of protein synthesis was measured with a flooding dose of 1-[14C]phenylalanine (19) injected 30 min before the pigs were killed. The specific radioactivities of the protein hydrolysate, homogenate supernatant, and blood supernatant were determined as described previously (14). The fractional rate of protein synthesis (K, percentage of protein mass synthesized in a day) was calculated as K = (Sb/Sa) × (1,400/t) × 100, where Sb is the specific radioactivity of the protein-bound phenylalanine, Sa is the specific radioactivity of the tissue-free phenylalanine at the time of the tissue collection and the linear regression of the blood specific radioactivity of the animal at 5, 15, and 30 min against time, and t is the time of labeling in minutes. We have demonstrated (11) that the specific radioactivity of the muscle free phenylalanine, after a flooding dose of the amino acid is administered, is in equilibrium with the aminocyl tRNA specific radioactivity and therefore provides an equally valid measure of fractional synthesis rate.

Protein immunoblot analysis. Blots were developed using an Amersham ECL Western Blotting Kit as described previously (32). Films were scanned using a Microtek ScanMaker V scanner connected to a Macintosh PowerMac 9600 computer. Images were obtained using the ScanWizard Plugin (Microtek) for Adobe Photoshop and quantitated using NIH Image software.

Quantitation of 4E-BP1–eIF4E and eIF4G–eIF4E complexes. The association of eIF4E with 4E-BP1 or eIF4G was quantitated by the previously described method (28). Briefly, eIF4E and the 4E-BP1–eIF4E and eIF4G–eIF4E complexes were immunoprecipitated from aliquots of tissue extracts using an anti-eIF4E monoclonal antibody. The antibody–antigen complex was collected by incubation for 1 h with goat antimouse Biomag IgG beads (PerSeptive Diagnostics). Protein bound to the beads was eluted by resuspending the beads in SDS sample buffer and boiling the sample for 5 min. The beads were collected by centrifugation, and the supernatants were subjected to electrophoresis either on a 7.5% polyacrylamide gel for quantitation of eIF4G or on a 15% polyacrylamide gel for quantitation of 4E-BP1 and eIF4E. Proteins were then electrophoretically transferred to a PVDF membrane as described previously (32). The membranes were incubated with a mouse anti-human eIF4E antibody, a rabbit anti-rat 4E-BP1 antibody, or a rabbit anti-human eIF4G.
into protein as described under MATERIALS AND METHODS. Hatched bars in the legend to Fig. 1 represent fasted and solid bars represent fed animals. Data are means ± SE for 4 animals/condition. Values not sharing the same letter are significantly different, P < 0.05.

Analysis of 4E-BP1 phosphorylation state. Aliquots of tissue extracts were heated at 100°C for 10 min, cooled to room temperature, and then centrifuged at 10,000 g for 10 min at 4°C. The supernatants were diluted with SDS sample buffer and then subjected to protein immunoblot analysis using a rabbit anti-human 4E-BP1 antibody as described previously (30). Previous studies have shown that phosphorylation of 4E-BP1 causes a decrease in the electrophoretic mobility of the protein on SDS-polyacrylamide gels (25, 37). Thus 4E-BP1 present in tissue extracts is separated into multiple electrophoretic forms during SDS-PAGE, with the more slowly migrating forms representing more highly phosphorylated 4E-BP1. Because it is the hyperphosphorylated γ-form that does not bind to eIF4E, results are presented as the percentage of 4E-BP1 present in the γ-form.

S6K1 phosphorylation. An aliquot of tissue homogenate was combined with an equal volume of SDS sample buffer, and the diluted samples were subjected to electrophoresis on a 7.5% polyacrylamide gel (36). The samples were then analyzed by protein immunoblot analysis using a rabbit anti-rat S6K1 polyclonal antibody as described above.

Statistical analyses. All data were analyzed using a one-way analysis of variance (ANOVA) with fasted non-rapamycin-treated animals as the independent variable. When a significant overall effect was observed, differences among individual means were assessed by the Tukey-Kramer Comparisons Test. The level of significance was set at P < 0.05 for all statistical tests.

RESULTS

As shown in Fig. 1, protein synthesis in skeletal muscle of pigs fasted for 18 h was stimulated 62% 1.5 h after feeding. Rapamycin, administered to fasted ani-

mals, had no significant effect on muscle protein synthesis. However, when administered 2 h before feeding, rapamycin markedly attenuated the stimulation of protein synthesis caused by feeding, although it did not completely prevent the increase. Thus the majority of the stimulation of muscle protein synthesis caused by feeding is mediated by a rapamycin-sensitive process.

In a recent study (15), we observed that the stimulation of protein synthesis in skeletal muscle caused by feeding neonatal pigs that had been fasted overnight was associated with increased formation of the eIF4G-eIF4E complex. In the present study, we confirm the observation that feeding promotes association of eIF4G with eIF4E, and we extend it to show that, in the presence of rapamycin, feeding no longer stimulates this association (Fig. 2). Although not statistically significant, it is noteworthy that, even in the presence of rapamycin, binding of eIF4G to eIF4E tended to increase after feeding. Rapamycin had no effect on eIF4G-eIF4E complex formation in fasted animals, although it should be noted that the amount of eIF4G bound to eIF4E in muscle from fasted animals is already low and near the threshold of sensitivity for the assay. Thus a decrease in binding likely could not have been detected.

The observed changes in eIF4E binding to eIF4G were inversely proportional to alterations in 4E-BP1 association with eIF4E (Fig. 3). Thus the amount of 4E-BP1 bound to eIF4E in muscle extracts from fed animals was ~15% of that in extracts from fasted
animals. Rapamycin prevented the decline in 4E-BP1 binding to eIF4E associated with feeding but did not promote association beyond that observed in muscle from fasted animals. Furthermore, rapamycin had no effect on 4E-BP1-eIF4E binding in muscle from fasted animals.

As shown in Fig. 4, the decline in 4E-BP1 binding to eIF4E caused by feeding was associated with phosphorylation of 4E-BP1. On SDS-polyacrylamide gels, 4E-BP1 can be resolved into multiple electrophoretic forms (denoted α, β, and γ), representing different phosphorylated forms of the protein (37). The α- and β-forms of 4E-BP1 both bind to eIF4E and should therefore both reduce formation of the eIF4F complex. The γ-form does not bind to eIF4E and so allows formation of the eIF4F complex. In particular, in muscle from fasted animals, 4E-BP1 was predominantly present in the hypophosphorylated α-form, whereas, in muscle from fed animals, ~80% of the protein was in the hyperphosphorylated, γ-form. As expected, rapamycin prevented completely the feeding-induced stimulation of 4E-BP1 phosphorylation.

In addition to promoting eIF4F formation, feeding also stimulates phosphorylation of S6K1 (20). In the present study, the effect of rapamycin on the feeding-induced phosphorylation of S6K1 phosphorylation was examined. During electrophoresis on SDS-polyacrylamide gels, S6K1 resolves into multiple electrophoretic forms based on the amount of phosphate present on the protein. Similar to 4E-BP1, hyperphosphorylated forms exhibit decreased and hypophosphorylated forms increased mobility (47). As shown in Fig. 5, feeding promoted formation of the more slowly migrating, highly phosphorylated forms of S6K1. Rapamycin was without effect on basal phosphorylation under fasting conditions but prevented completely the stimulation of S6K1 phosphorylation caused by feeding.

In addition to stimulating protein synthesis in skeletal muscle, feeding a fasted animal promotes protein synthesis in liver (10, 49, 50). To determine whether the stimulation of protein synthesis in liver was blocked by rapamycin, as was shown above for muscle, 18-h fasted animals were treated with rapamycin before feeding. As shown in Fig. 6A, in the absence of rapamycin, feeding caused a significant stimulation in liver protein synthesis. Rapamycin had no significant effect on liver protein synthesis in fasted animals, although there was a trend toward lower rates after treatment. Furthermore, in contrast to skeletal muscle, where rapamycin did not completely prevent the stimulation of protein synthesis, in liver, rapamycin obviated the increase in protein synthesis caused by feeding.

To establish that the changes in liver protein synthesis were associated with changes in translation initiation, phosphorylation of 4E-BP1 and S6K1 was examined. As described above for skeletal muscle, feeding 18-h fasted animals caused a dramatic increase in the amount of 4E-BP1 in the hyperphosphorylated γ-form (Fig. 6B). Thus, in fasted animals, 4E-BP1 was not significantly different, P < 0.05.
almost exclusively in the hypophosphorylated α-form; no γ-form was detected. Feeding caused a shift in 4E-BP1 distribution such that 50% of the protein was in the hyperphosphorylated γ-form. Rapamycin prevented completely the shift caused by feeding. In liver of fasted animals, S6K1 was already partially in hyperphosphorylated forms, and feeding promoted further phosphorylation (Fig. 5B). Treating fasted animals with rapamycin caused a shift in S6K1 distribution such that the majority of the protein was present in the fastest migrating, hypophosphorylated form. In addition, rapamycin blocked the hyperphosphorylation of S6K1 caused by feeding. Overall, the results suggest that feeding stimulates protein synthesis in liver entirely through a rapamycin-dependent process.

**DISCUSSION**

The aim of the present study was to examine the contribution of increased eIF4F complex formation and/or S6K1 activity in controlling protein synthesis after feeding. We chose an animal model that is highly responsive to the stimulatory action of feeding on fractional protein synthesis rates, eIF4F complex formation, and S6K1 activation. One way to examine the contribution of eIF4F and S6K1 is to prevent the changes in these factors and determine whether protein synthesis is still enhanced by feeding. Herein, we used rapamycin in an attempt to specifically block the effects of feeding on protein synthesis in skeletal muscle and liver. Rapamycin is a specific inhibitor of the protein kinase mTOR (reviewed in Ref. 17). However, rapamycin does not directly inhibit mTOR but instead forms an inhibitory complex with the immunophilin referred to as FK506 binding protein (FKBP12) (7, 8, 41). Like rapamycin, FK506 is an immunosuppressant that binds to FKBP12, but unlike rapamycin, the FK506-FKBP12 complex does not inhibit mTOR.

The importance of mTOR in regulating translation initiation was first demonstrated in yeast, where loss of TOR function was shown to cause an inhibition of translation initiation with characteristics reminiscent of cells subjected to nutrient deprivation (4). Subsequent studies in mammalian cells confirmed the importance of mTOR in mediating the signal generated both by provision of amino acids to amino acid-deprived cells (24, 40, 45, 48) and by treatment of serum-deprived cells with insulin or insulin-like growth factor
mRNAs that have long, highly structured 5′-UTRs may reflect the proportion of actively translating mRNAs, as well as the translation of the majority of cellular proteins. In both L6 myoblasts (33) and Swiss 3T3 cells (27), where global protein synthesis is insensitive to rapamycin, the macrolide obviates the stimulation of translation of mRNAs harboring the TOP sequence. Rapamycin also potently inhibits the translation of ornithine decarboxylase mRNA, which has a long, highly structured 5′-UTR (46). The translation of mRNAs with highly structured 5′-UTRs is regulated by eIF4F, whereas translation of TOP mRNAs is regulated by activation of S6K1 (reviewed in Ref. 16). Because mTOR activity is essential for enhanced formation of the eIF4F complex and activation of S6K1 by amino acids and insulin, inhibition of mTOR by rapamycin would be expected to have a selective effect in modulating translation of such messages. Thus we hypothesize that the differential sensitivity of L6 myoblasts and NIH 3T3 cells may be a result of a greater proportion of mRNAs in NIH 3T3 cells having highly structured 5′-UTRs and/or TOP sequences. Alternatively, eIF4F and/or S6K1 activity may be limited in NIH 3T3 compared with L6 myoblasts, which would increase the apparent sensitivity to rapamycin.

In the present study, rapamycin completely blocked the feeding-induced stimulation of global protein synthesis in liver. In contrast, in skeletal muscle, rapamycin attenuated but did not completely prevent the stimulation. However, rapamycin was equally effective at preventing changes in eIF4F complex formation and S6K1 phosphorylation in both liver and skeletal muscle. Similarly, Dardevet et al. (9) report that, in incubated muscle preparations, rapamycin attenuates but does not prevent insulin- or IGF-I-stimulated protein synthesis but does block S6K1 activation. These results suggest that feeding stimulates hepatic protein synthesis through activation of eIF4F and/or S6K1. Moreover, although activation of eIF4F and S6K1 accounts for part of the stimulation of muscle protein synthesis, it probably does not account entirely for the effect.

The mechanism responsible for the rapamycin-insensitive stimulation of muscle protein synthesis is unknown. In PC12 pheochromocytoma cells, nerve growth factor- and epidermal growth factor-enhanced binding of eIF4G to eIF4E is prevented by an inhibitor of the mitogen-activated protein (MAP) kinase signaling pathway (34). Likewise, in HEK293 cells, two structurally unrelated inhibitors of the MAP kinase pathway block eIF4G association with eIF4E caused by stimulation of cells with phorbol esters (26). These results suggest that formation of the eIF4F complex cannot be regulated only through the mTOR signaling pathway but also through the MAP kinase pathway. Interestingly, in the present study, there is a trend toward greater binding of eIF4G to eIF4E after feeding in rapamycin-treated pigs, although the difference is not significant. Furthermore, we have recently found that rapamycin attenuates but does not completely prevent the feeding-induced binding of eIF4G to eIF4E in skeletal muscle of fasted rats (2). Thus, in part, feeding may promote eIF4F complex formation through the MAP kinase signaling pathway. It must also be considered that the proportion of mRNAs represented by the TOP family or containing highly structured 5′-UTRs may be greater in liver than in skeletal muscle, which would increase the apparent sensitivity of liver protein synthesis to rapamycin. Overall, it is clear that the mechanism by which feeding stimulates protein synthesis in skeletal muscle through a rapamycin-insensitive pathway is an important topic that should be addressed in future studies.

In summary, in the present study, we show that the feeding-induced stimulation of protein synthesis in liver of neonatal pigs is exquisitely sensitive to rapamycin. The stimulation is associated with increased phosphorylation of 4E-BP1, which promotes dissociation of the 4E-BP1-eIF4E complex and subsequent binding of eIF4E to eIF4G. The stimulation is also associated with enhanced phosphorylation of S6K1. Furthermore, we show that the stimulation of protein synthesis in skeletal muscle caused by feeding in neonatal pigs is only partially prevented by rapamycin. In that tissue, modulation of eIF4F and S6K1 does not account entirely for the changes in protein synthesis. Thus feeding in the neonate enhances protein synthesis via both shared and tissue-specific mechanisms.

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