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Regulation of Translation Initiation by Insulin and Amino Acids in Skeletal Muscle of Neonatal Pigs

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ABSTRACT

Previous studies have shown that intravenous infusion of insulin and/or amino acids reproduces the feeding-induced stimulation of muscle protein synthesis in neonates, and that insulin and amino acids act independently to produce this effect. The goal of the current study was to delineate the regulatory roles of insulin and amino acids on muscle protein synthesis in neonates by examining translational control mechanisms, specifically the eukaryotic translation initiation factors (eIFs) which enable coupling of initiator methionyl-tRNA_i and mRNA to the 40S ribosomal subunit. Insulin secretion was blocked by somatostatin in fasted 7-day-old pigs (n=8-12/group), insulin was infused to achieve plasma levels of ~0, 2, 6, and 30 $\mu\text{U}/\text{ml}$, and amino acids were clamped at fasting or fed levels, or, at the high insulin dose, below fasting. Both insulin and amino acids increased the phosphorylation of ribosomal protein S6 kinase (S6K1) and the eIF4E binding protein, 4E-BP1, decreased the binding of 4E-BP1 to eIF4E, increased eIF4E binding to eIF4G, and increased fractional protein synthesis rates, but did not affect eIF2B activity. In the absence of insulin, amino acids had no effect on these translation initiation factors, but increased protein synthesis rates. Raising insulin from below fasting to fasting levels generally did not alter translation initiation factor activity, but raised protein synthesis rates. The phosphorylation of S6K1 and 4E-BP1 and the amount of 4E-BP1 bound to eIF4E and eIF4E bound to eIF4G were correlated with insulin level, amino acid level, and protein synthesis rate. Thus, insulin and amino acids regulate muscle protein synthesis in skeletal muscle of neonates by modulating the availability of eIF4E for 48S ribosomal complex assembly, although other processes also must be involved.

Key Words: Protein synthesis, nutrition, growth, eukaryotic initiation factor 4E, S6K1

INTRODUCTION

The neonatal period is characterized by high rates of protein deposition and growth (9, 13, 19), with the most rapid gains in protein mass occurring in skeletal muscle (58). Previous studies in neonatal rats and pigs have revealed high efficiency rates of dietary protein utilization (11), that is likely due to the higher protein synthesis rates of neonates in response to feeding than those of older animals (4, 10). The feeding-induced stimulation of protein synthesis occurs in virtually all tissues of the neonate, but is most dramatic in skeletal muscle. The developmental decline in the protein synthesis response to feeding in muscle parallels the fall in the feeding-induced activation of translation initiation factors that regulate the binding of mRNA to the 40S ribosomal subunit (4, 12, 27, 31).

Both insulin and amino acids play key roles in the regulation of skeletal muscle protein synthesis in the neonate (6, 7, 55, 56). Our studies in neonatal pigs have shown that insulin stimulates whole body amino acid disposal, and that the insulin sensitivity and responsiveness of amino acid disposal decreases with development (55). Raising insulin to fed levels by infusion increases muscle protein synthesis to within the range normally present in the fed state, even when amino acids and glucose are maintained at fasting levels (6, 56). The infusion of amino acids at a dose that reproduces fed state amino acid levels, alone or concurrent with insulin infusion, increases protein synthesis in skeletal muscle of the neonate, and the magnitude of the amino acid-stimulated increase is similar to that which occurs with insulin infusion alone and to that which occurs in response to feeding (7). These responses to insulin and amino acids decrease with development. Taken together, the results imply that insulin and amino acids may be interacting with the same signalling pathway within skeletal muscle of the neonate. Using pancreatic-glucose-amino acid

clamps, we recently demonstrated that both insulin and amino acids independently stimulate muscle protein synthesis in the neonate (44). However, the intracellular mechanisms by which insulin and amino acids regulate muscle protein synthesis were not explored.

Rapid changes in the rate of protein synthesis, including those due to insulin and amino acid stimulation, are regulated by changes in the rate of translation of mRNA via alterations in the rate of peptide chain initiation (21, 30, 36). The first important regulatory step in translation initiation is the binding of initiator methionyl-tRNA_i (met-tRNA_i) to the 40S ribosomal subunit, a reaction that is mediated by eukaryotic initiation factor (eIF)₂, and results in the formation of the 43S preinitiation complex (33, 46). The eIF₂ mediated binding of met-tRNA_i to the 40S subunit is regulated by modulation of the activity of eIF₂B, which exchanges GDP for GTP on eIF₂ (34). The second regulatory process in translation initiation is the binding of mRNA to the 43S preinitiation complex, which is mediated by the eIF₄F complex of proteins (36, 47, 49). Proteins comprising the eIF₄F complex are eIF₄A, an RNA helicase, eIF₄E, the protein that binds to the m⁷GTP cap structure at the 5'-end of mRNA, and eIF₄G, a scaffolding protein that binds to the 40S ribosomal subunit. Phosphorylation (43) and availability (45) of eIF₄E regulate the assembly of the eIF₄F complex. Phosphorylation of eIF₄E increases its association with eIF₄G and eIF₄A and influences its affinity for the m⁷GTP cap at the 5' end of mRNA (40, 42, 43). Availability of eIF₄E for assembly of the eIF₄F complex is limited by its binding to 4E-BP1, a repressor binding protein that competes with eIF₄G for binding to eIF₄E (45). Phosphorylation of 4E-BP1 results in decreased affinity for eIF₄E and thus, enhanced formation of the active eIF₄G•eIF₄E complex (18). These translation initiation events may be modulated through the phosphatidylinositol 3- kinase/mammalian target of rapamycin (mTOR)/70-kDa ribosomal protein S6 Kinase (S6K) 1 signal transduction pathway (29).

Previous studies in our laboratory have shown that the postprandial increase in protein synthesis in the neonate (4) is associated with an increase in translational efficiency via marked increases in the activation of translation initiation factors involved in the binding of mRNA, but not met-tRNA_i, to the 40S ribosomal subunit (12, 27, 31). Thus, in the neonate, feeding increases the phosphorylation of S6K1 and the repressor protein, 4E-BP1, thereby releasing eIF4E from the inactive 4E-BP1•eIF4E complex, and increasing the association of eIF4E with eIF4G. All of these responses to feeding decline with development in skeletal muscle (12) in parallel with the developmental decline in the postprandial activation of the insulin signalling pathway (50). These postprandial changes in translation initiation in the neonate are rapamycin-dependent (31). However, feeding does not alter eIF2B activity, which regulates the binding of met-tRNA_i to the 40S ribosomal subunit, although eIF2B activity declines with development (12).

In the current study, we wished to determine whether insulin and amino acids independently regulate skeletal muscle protein synthesis in the neonate by modulation of the activation of specific translation initiation factors. Specifically, we wished to determine whether insulin and amino acids independently regulate the binding of mRNA to the 43S preinitiation complex, mediated through the formation of the eIF4F complex. To address these issues, pancreatic-glucose-amino acid clamps were used in fasted neonatal pigs to block insulin secretion, insulin was infused to achieve levels simulating less than fasting, fasting, intermediate, and fed conditions, and amino acids were clamped at fasting, fed, or (at the high insulin dose only) below fasting levels. The results showed that the stimulation of protein synthesis by insulin and amino acids involves mechanisms that regulate the binding of mRNA to the 40S ribosomal subunit as well as pathways that are independent of the activation of these translation initiation factors.

METHODS

Animals. Eleven multiparous crossbred (Yorkshire x Landrace x Hampshire x Duroc) sows (Agriculture Headquarters, Texas Department of Criminal Justice Huntsville, TX) were housed in lactation crates in individual, environmentally controlled rooms, maintained on a commercial diet (5084, PMI Feeds, Richmond, IN), and provided water ad libitum throughout the lactation period. After farrowing, piglets remained with the sow and were not given supplemental creep feed. Piglets were studied at 5-8 days of age (2.1 ± 0.4 kg). Three to 5 days before the infusion study, pigs were anaesthetized, and catheters were surgically inserted into a jugular vein and a carotid artery, using sterile techniques as described previously (55). Piglets were returned to the sow until studied. The previously described protocol (44) was approved by the Animal Care and Use Committee of Baylor College of Medicine. The study was conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Pancreatic-glucose-amino acid clamps. The clamp procedure has been previously described by O'Connor et al. (44). Briefly, pigs were placed awake in a sling restraint system following an overnight fast. The average basal concentration of blood glucose (YSI 2300 STAT Plus, Yellow Springs Instruments, Yellow Springs, OH) and plasma branched-chain amino acid (BCAA) concentrations (3) to be used in the subsequent pancreatic-glucose-amino acids clamp procedure were established during a 30-minute basal period. The clamp was initiated with a primed ($20 \mu\text{g}/\text{kg}$), continuous ($100 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{hr}^{-1}$) somatostatin (BACHEM, Torrance, CA) infusion. After a 10-min infusion of somatostatin, a continuous infusion of replacement glucagon ($150 \text{ng}\cdot\text{kg}^{-1}\cdot\text{hr}^{-1}$; Eli Lilly, Indianapolis, IN) was initiated and continued to the end of the clamp period. Insulin was infused at

0, 10, 22, or 110 ng•kg^{0.66}•min⁻¹ to achieve plasma insulin concentrations of ~ 0, 2, 6, and 30 μU/ml to simulate less than fasting, fasting, intermediate, and fed insulin levels (4). At each dose of insulin, amino acids were clamped at either the fasting (500 nmol BCAA/ml) or fed (1000 nmol BCAA/ml) level by monitoring the BCAA every 5 minutes and adjusting the infusion rate of a balanced amino acid mixture (7) to maintain the plasma BCAA concentration within 10% of the desired level (6, 7). At the highest insulin dose only, amino acids were also allowed to fall below fasting levels by omitting the amino acid clamp. Blood glucose concentrations were measured at 5-min intervals and the dextrose infusion rate was adjusted to maintain the glucose concentration at a constant value (14). Blood samples also were taken at intervals for later determination of circulating insulin, glucagon, and individual essential and nonessential amino acid concentrations (11).

Tissue protein synthesis in vivo. The fractional rate of protein synthesis was measured with a flooding dose of L-[4-³H]phenylalanine (17) injected 90 min after the initiation of the clamp procedure for a 30-min labelling period. Pigs were killed at 2 h, samples of longissimus dorsi were collected and rapidly frozen, and fractional rates of protein synthesis were determined as previously described (9).

Plasma hormones and substrates. The concentrations of individual amino acids from frozen plasma samples obtained at 0 and 90 min of the insulin infusions were measured with an HPLC method (PICO-TAG reverse-phase column, Waters, Milford, MA) as previously described (11). Plasma radioimmunoreactive insulin concentrations were measured using a porcine insulin radioimmunoassay kit (Linco, St. Louis, MO) that used porcine insulin antibody and human insulin standards. Plasma radioimmunoreactive glucagon concentrations were measured using a porcine

glucagon radioimmunoassay kit (Linco, St. Louis, MO) that used porcine glucagon antibody and human glucagon standards.

Protein immunoblot analysis. Blots were developed using an Amersham enhanced chemiluminescence (ECL) Western Blotting Kit, as described previously (33). Chemiluminescence was measured using a Gene Genome imaging system (Syngene) and quantitated using GeneTools software (Syngene). Results are expressed as arbitrary units, which represent the integrated pixel intensity of the band being analyzed.

Examination of 4E-BP1 phosphorylation on Thr⁷⁰. Aliquots of muscle homogenates (supernatants) 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, as described previously (12, 33). The membranes were incubated with a polyclonal antibody that specifically recognizes phosphorylation of 4E-BP1 at Thr⁷⁰.

Quantitation of 4E-BP1•eIF4E and eIF4G•eIF4E complexes. The association of eIF4E with 4E-BP1 or eIF4G was quantitated as described previously (12, 34). Briefly, eIF4E was immunoprecipitated from 10,000-g supernatants of muscle homogenates using a monoclonal antibody to eIF4E (28). Next, proteins in the immunoprecipitate were resolved by SDS-polyacrylamide gel electrophoresis and then transferred to PVDF membranes. The membranes were then probed with either anti-4E-BP1 or anti-eIF4G antibodies and subsequently developed using an ECL Western Blotting Kit (Amersham Pharmacia Biotech). The horseradish peroxidase coupled to the anti-rabbit secondary antibody was then inactivated by incubating the blot in 15% hydrogen peroxide for 30 min at room temperature and the membranes were reprobed with the monoclonal

anti-eIF4E antibody (32). Values obtained using the anti-4E-BP1 and anti-eIF4G antibodies were normalized for the amount of eIF4E in the immunoprecipitates.

Measurement of eIF4E phosphorylation and content. The phosphorylated and unphosphorylated forms of eIF4E in tissue extracts were separated by isoelectric focusing of 10,000-g supernatants on a slab gel and were quantitated by protein immunoblot analysis with a monoclonal antibody against eIF4E, as previously described (12, 28).

Measurement of S6K1 phosphorylation. Muscle homogenates were combined with an equal volume of SDS sample buffer, and the diluted samples were subjected to electrophoresis on a 7.5% polyacrylamide gel (37). The samples were then subjected to protein immunoblot analysis by use of rabbit anti-rat S6 kinase polyclonal antibodies, as previously described (12).

Measurement of eIF2B activity. eIF2B activity in fresh muscle postmitochondrial supernatants was measured as the exchange of [³H]GDP bound to eIF2 for unlabeled GDP or GTP, as previously described (12, 26). Briefly, an eIF2•[³H] GDP binary complex was formed in the absence of magnesium chloride. The eIF2•[³H] GDP complex was then stabilized by the addition of magnesium chloride to a final concentration of 2 mM. The eIF2•[³H] GDP complex was incubated with samples containing eIF2B in the presence of a 100-fold molar excess of unlabeled, HPLC-purified GTP at 30°C for various times. The reaction mixture was filtered through a nitrocellulose filter, the filters were washed, and radioactivity bound to the filter was quantitated using a liquid scintillation counter.

Calculations and statistics. The fractional rate of protein synthesis (K_s ; percentage of protein mass synthesized in a day) was calculated as:

$$K_s (\%/day) = [(S_b/S_a) \times (1,440/t)] \times 100$$

where S_b (dpm/min) is the specific radioactivity of the protein-bound phenylalanine, S_a (dpm/min) is the specific radioactivity of the tissue-free phenylalanine at the time of 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 labelling in minutes. We have previously demonstrated that the specific radioactivity of the tissue-free phenylalanine after a flooding dose of phenylalanine is in equilibrium with the aminoacyl-tRNA specific radioactivity; hence, the tissue-free phenylalanine reflects the specific radioactivity of the tissue precursor pool. (8).

Analysis of variance (general linear modelling) was used to assess the effect of insulin, amino acids, and their interaction, and to determine whether or not there was a linear and/or quadratic relationship between insulin or BCAA and translation initiation factor activity, and between translation initiation factor activity and protein synthesis. Student's t-test was used to test for specific differences between groups. To determine the effectiveness of the clamp procedure, amino acid and insulin concentrations in each treatment group were compared with their basal concentrations by use of the t test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Infusions, hormones and substrates. Fasted 7-day-old pigs were infused with somatostatin (to block insulin secretion), glucagon (at replacement levels), and glucose (as needed to maintain fasting levels). Insulin was infused at four doses to achieve levels that simulated: 1) less than fasting, 2) fasting, 3) intermediate between fasting and fed, and 4) fed conditions. As shown in Table 1, targeted plasma insulin levels, i.e., 0, 2, 6, and 30 $\mu\text{U/ml}$, were largely achieved in all treatment groups. Amino acids were clamped at fasting or fed levels, resulting in BCAA of

approximately 500 and 1000 nmol/ml, respectively; at the highest insulin dose, amino acids were also allowed to fall to less than the fasting levels of about 250 nmol/ml. Concentrations of total, essential, nonessential, and branched chain amino acids as compared with baseline (0 time) values are shown in Table 1. Circulating glucose and glucagon concentrations were largely maintained at baseline fasting levels during the infusion of somatostatin, glucagon, insulin, and/or amino acids (data not shown).

Insulin dose response of translation initiation factors during euaminoacidemia and hyperaminoacidemia. We have recently demonstrated in neonatal pigs that insulin and amino acids independently stimulate protein synthesis in skeletal muscle (44). The purpose of the current study was to identify the translational control mechanisms involved in this response. For the purpose of comparison, we have included the previously reported (44) muscle protein synthesis data herein. Figure 1A compares the dose response effect of insulin on muscle protein synthesis in the presence of fasting and fed amino acid levels. There was a progressive increase in protein synthesis rates as the concentration of insulin was increased ($P < 0.005$). Amino acids increased muscle protein synthesis ($P < 0.05$) at each dose of insulin, including the 0 $\mu\text{U}/\text{ml}$ insulin/ml dose; at the highest insulin dose, there was a tendency for amino acids to stimulate muscle protein synthesis ($P = 0.06$). Thus, there was a dose-response effect of insulin on muscle protein synthesis in the presence of fasting and fed amino acid levels. The effects of insulin and amino acids were additive until maximal rates of protein synthesis were achieved at fed insulin and amino acid levels.

To examine the underlying mechanisms that independently regulate the stimulation of protein synthesis by insulin and amino acids in neonatal muscle, the dose-response effect of insulin, in the presence of fasting and fed amino acid levels, on translation initiation factor content and/or

phosphorylation was examined. The binding of met-tRNA_i to the 40S ribosomal subunit is primarily regulated by changes in eIF2B activity (44). The response of guanine nucleotide exchange activity of eIF2B to insulin doses of ~ 0, 2, 6, and 30 μ U/ml, when amino acids were either at fasted or fed levels, was measured. There were no differences in eIF2B activity regardless of insulin or amino acid dose (Fig. 1B).

Studies in cell culture have shown that eIF4E plays a critical role in the binding of mRNA to the 43S preinitiation complex (47, 49) and that the function of eIF4E may be influenced by either its phosphorylation state or its availability (43, 45). To determine whether insulin and/or amino acids affected eIF4E phosphorylation status, the amount of eIF4E present in the phosphorylated form, as a percentage of the total eIF4E, was examined. There was no significant effect of insulin or amino acids on eIF4E phosphorylation (data not shown). The availability of eIF4E can be regulated through changes in the amount of eIF4E bound to 4E-BP1 such that phosphorylation of 4E-BP1 causes disassembly of the 4E-BP1•eIF4E complex (32). Phosphorylation of 4E-BP1 at the Thr⁷⁰ site has been shown to be important in regulating its association with eIF4E (31, 32). Both insulin ($P < 0.005$) and amino acids ($P < 0.01$) stimulated 4E-BP1 phosphorylation at the Thr⁷⁰ site (Fig. 1C). There was a tendency for an interaction between the effects of insulin and amino acids on 4E-BP1 phosphorylation ($P=0.08$). Examination of specific differences among individual groups revealed that the effect of insulin on 4E-BP1 phosphorylation occurred at the intermediate ($P < 0.05$) and high ($P < 0.01$) doses of insulin, in the presence of hyperaminoacidemia. The effect of amino acids on 4E-BP1 phosphorylation occurred at the highest dose of insulin ($P < 0.05$).

Phosphorylation of 4E-BP1 in cells in culture and in vitro decreases the association of 4E-BP1 with eIF4E, thereby allowing eIF4E to bind to eIF4G (12, 32, 38). To examine the effect of

amino acids on the insulin dose response of 4E-BP1 modulation, eIF4E was immunoprecipitated with an anti-eIF4E antibody, followed by immunoblot analysis with an anti-4E-BP1 antibody. Both insulin ($P < 0.001$) and amino acids ($P < 0.05$) decreased the amount of 4E-BP1 present in the eIF4E immunoprecipitate (Fig. 1D). Though there was no interaction between the effects of insulin and amino acids, by analysing individual treatment groups, insulin's effect on the association of eIF4E with 4E-BP1 occurred at the highest dose of insulin ($P < 0.05$) and tended to occur at the intermediate dose ($P < 0.10$) in the presence of euaminoacidemia. In the presence of hyperaminoacidemia, insulin's effect on the association of eIF4E with 4E-BP1 occurred at the low basal ($P < 0.05$), intermediate ($P < 0.01$), and high ($P < 0.001$) insulin doses. The effect of amino acids on the association of eIF4E with 4E-BP1 occurred at the low and intermediate doses of insulin ($P < 0.05$), and tended to occur at the high dose ($P < 0.10$).

It is the reversible binding of eIF4G to eIF4E that primarily regulates the binding of mRNA to the 40S ribosomal subunit (31, 22,35). In the present study, the amount of eIF4G bound to eIF4E was measured in the same eIF4E immunoprecipitates used to quantitate 4E-BP1 bound to eIF4E. Both insulin ($P < 0.001$) and amino acids ($P < 0.05$) increased the amount of eIF4G associated with eIF4E (Fig. 1E) and there was significant interaction of insulin and amino acids on the amount of the active complex ($P < 0.01$). Analysing differences between individual treatment groups revealed the effect of insulin on the amount of eIF4G associated with eIF4E occurred at the highest dose of insulin in the presence of euaminoacidemia ($P < 0.001$). In the presence of hyperaminoacidemia, insulin tended to increase the association at the low basal insulin dose ($P < 0.10$), and significantly increased the association of eIF4G with eIF4E at the intermediate ($P < 0.05$), and highest insulin

doses ($P < 0.001$). Amino acids increased the association of eIF4G with eIF4E at the low and intermediate doses of insulin ($P < 0.05$).

Both 4E-BP1 and S6K1 are downstream of mTOR, and thus dependent upon the activity of this kinase for activation. Through protein immunoblot analysis, we determined the phosphorylation state of S6K1, the hyperphosphorylated form results in decreased mobility of the compound during SDS-polyacrylamide gel electrophoresis (54). Insulin ($P < 0.001$) and amino acids ($P < 0.01$) increased S6K1 phosphorylation (Fig. 1F), and there was no interaction between the effects of insulin and amino acids on S6K1 phosphorylation. Examination of individual treatment groups revealed that S6K1 phosphorylation was increased at the highest dose of insulin in the presence of euaminoacidemia ($P < 0.005$) and hyperaminoacidemia ($P < 0.001$). Amino acids further increased S6K1 phosphorylation at the highest insulin dose ($P < 0.05$).

Amino acid dose response of translation initiation factors. Figure 2 shows the dose response effect of below fasting, fasting, and fed amino acid levels, when insulin is infused to simulate the fed level, on translation initiation factor modulation. We have previously shown (4) that muscle protein synthesis rates increase as circulating amino acid levels are increased from below fasting to the fasting level ($P < 0.001$) and tend to increase further when amino acids are increased to fed levels ($P = 0.06$); and the data are presented here (Fig. 2A) for comparison. Amino acids had no significant effect on eIF2B activity. When amino acids were increased from below fasting to fasting levels, in the presence of fed insulin concentrations, S6K1 phosphorylation and eIF4G•eIF4E association were increased ($P < 0.005$). When amino acids were raised from fasting to fed levels, S6K1 phosphorylation and 4E-BP1 phosphorylation were increased and 4E-BP1•eIF4E association were reduced ($P < 0.05$).

Effect of insulin in the absence of an amino acid clamp on translation initiation factors.

We recently demonstrated that the stimulation of muscle protein synthesis by insulin does not require concurrent amino acid stimulation in the neonate (44). In the current study, translation initiation factor activity was examined when insulin was infused to the fed level, in the presence of fasting (500 nmol BCAA/ml) and less than fasting (250 nmol BCAA/ml) amino acid levels, and the results were compared to the basal fasting insulin and amino acid condition. Similar to the effects on fractional protein synthesis rates (Fig. 3A), insulin increased the amount of eIF4G associated with eIF4E in the absence of an amino acid clamp ($P < 0.001$; Fig. 3E), although not to the level seen when amino acids were clamped at fasting levels ($P < 0.001$). Insulin increased S6K1 phosphorylation in the presence ($P < 0.001$), but not in the absence of an amino acid clamp (Fig. 3F). Although there were tendencies for similar changes in 4E-BP1 phosphorylation (Fig. 3C) and the association of 4E-BP1 with eIF4E (Fig. 3D), these were not statistically significant. eIF2B activity was unaffected by amino acids (Fig. 3B).

Correlation of translation initiation factor activity with plasma insulin concentration during euaminoacidemia and hyperaminoacidemia. We have previously shown a positive curvilinear relationship between protein synthesis and insulin that is influenced by amino acids (44); and the data are shown here for comparison (Fig. 4A). In the current study, translation initiation factor activity and plasma insulin concentrations were related as indicated by the significance of first and second degree polynomial terms (Fig. 4C-4F). There was a positive linear relationship between insulin and S6K1 phosphorylation ($P < 0.001$), 4E-BP1 phosphorylation ($P < 0.05$), and eIF4G•eIF4E content ($P < 0.01$) and a negative linear relationship between insulin and 4E-BP1•eIF4E content ($P < 0.001$). The relationship between translation initiation factor activity and

insulin was influenced by amino acids for eIF4G•eIF4E association ($P < 0.005$) and 4E-BP1•eIF4E association ($P < 0.01$). Amino acids tended to influence the relationship between insulin and both 4E-BP1 phosphorylation and S6K1 phosphorylation (Fig. 4C and 4F; $P < 0.08$). There was no relationship (neither linear nor quadratic) between eIF2B activity and plasma insulin levels (Fig. 4B).

Correlation of translation initiation factor activity with BCAA during hyperinsulinemia. In the presence of fed insulin levels, there was a significant positive linear correlation of plasma BCAA with protein synthetic rates, S6K1 phosphorylation, and 4E-BP1 phosphorylation ($P < 0.01$, $P < 0.001$, and $P < 0.04$, respectively; Fig. 5). A negative linear correlation occurred between BCAA and 4E-BP1•eIF4E complex content ($P < 0.006$). BCAA correlated with the amount of active eIF4G•eIF4E complex in a quadratic manner ($P < 0.02$), and there was a tendency for BCAA and protein synthesis rates to be related in a quadratic manner ($P < 0.10$). No significant correlation existed between BCAA and eIF2B activity.

Correlation of muscle protein synthesis rates and translation initiation factor modulation. The relationship between skeletal muscle protein synthesis rates and translation initiation factor activity in all pigs was examined (Fig. 6). The relationships between protein synthesis rates and phosphorylation of 4E-BP1 ($P < 0.001$), 4E-BP1•eIF4E ($P < 0.02$), and eIF4G•eIF4E ($P < 0.01$) were quadratic. There was a positive linear relationship between protein synthesis rates and S6K1 phosphorylation ($P < 0.001$). There was no significant linear or quadratic relationship between protein synthesis rates and eIF2B activity.

DISCUSSION

Insulin has long been recognized for its key role in the regulation of protein synthesis in growing animals (16, 22, 24). Furthermore, previous studies from our laboratory have demonstrated that both insulin and amino acids play critical roles in the feeding-induced stimulation of protein synthesis in the neonate (5, 7, 56). The results of a recent study (44) using pancreatic–glucose-amino acid clamps suggest that insulin and amino acids independently stimulate skeletal muscle protein synthesis in neonates, and that the effects of insulin and amino acids are additive until maximal rates are achieved. Because the enhanced feeding-induced stimulation of muscle protein synthesis in the neonate involves the activation of key translation initiation factors (12, 31), we wished to define the roles of insulin and amino acids in this process. The results of the current study show that both insulin and amino acids stimulate neonatal muscle protein synthesis via mechanisms that are associated with increased 4E-BP1 and S6K1 phosphorylation and enhanced assembly of the eIF4G•eIF4E complex that regulates the binding of mRNA to the 43S preinitiation complex, as well as by mechanisms that are independent of this pathway.

Effects of amino acids on insulin dose response of translation initiation. A major site in the regulation of translation initiation is the binding of met-tRNA_i to the 40S ribosomal subunit, an event mediated by eIF2 (33, 46). This step is regulated by eIF2B, which exchanges GTP for GDP on eIF2 (34). Studies in cell culture suggest that insulin and amino acids increase eIF2B activity (15, 25, 28, 53). Furthermore, studies in diabetic animals suggest that insulin plays an important role in increasing eIF2B activity (23). In the current study, neither insulin nor amino acids had any effect on eIF2B activity. However, this was not unexpected, as previous *in vivo* studies in both mature rats

and neonatal pigs suggest that eIF2B activity may not be regulated by changes in nutritional status (12, 31, 57).

A second important regulatory step in translation initiation is the binding of mRNA to the 43S preinitiation complex, which is mediated by eIF4F, a complex of proteins that includes eIF4E. Assembly of the eIF4F complex can be regulated by the phosphorylation (43) or availability (46) of eIF4E. The eIF4E phosphorylation status, as a percentage of the total eIF4E, was examined in the current study, as it has been shown in cell culture studies to influence mRNA binding to the 43S preinitiation complex, with ultimate increases in protein synthesis (42, 43, 47, 49). However, no effect of insulin and/or amino acids on eIF4E phosphorylation was observed in this study. These results extend our previous study findings showing that feeding has no effect on eIF4E phosphorylation in the neonate. Thus, *in vivo*, modulation of eIF4E phosphorylation status is not a key regulatory step in the feeding-induced stimulation of muscle protein synthesis by insulin and amino acids in neonatal pigs.

The most well characterized regulatory step involved in the mRNA binding process is the reversible association of eIF4E with the translational repressor, 4E-BP1, which competes with eIF4G to bind to eIF4E. Phosphorylation of 4E-BP1 results in a decreased affinity of eIF4E for 4E-BP1, and an enhanced binding of eIF4E to eIF4G (18). 4E-BP1 phosphorylation at the Thr⁷⁰ site was stimulated by both insulin and amino acids. However, the hormonal effect occurred only at elevated levels of insulin in the presence of hyperaminoacidemia, and an effect of amino acids was present only at the highest dose of insulin. In contrast, protein synthesis rates were increased at each insulin dose, with a further increase in protein synthesis achieved at each dose of insulin by raising amino acids to simulate fed levels (44). It is noteworthy that the large variation in results may have

precluded us from observing an effect of insulin and amino acids on 4E-BP1 phosphorylation at the Thr⁷⁰ at the lower doses. However, the findings suggest that low doses of insulin increase muscle protein synthesis independent of 4E-BP1 phosphorylation. Furthermore, the results suggest that the increase in 4E-BP1 phosphorylation by amino acids requires insulin. This finding is consistent with a study in mature diabetic rats that showed that leucine enhanced skeletal muscle protein synthesis with no increase in 4E-BP1 phosphorylation, and that leucine increased 4E-BP1 phosphorylation only in the presence of a high dose of insulin (2). However, a study in mature nondiabetic rats revealed only partial attenuation of leucine-induced 4E-BP1 phosphorylation by somatostatin infusion, and a failure of leucine to stimulate muscle protein synthesis in the presence of somatostatin (1). The mechanism for the differences in the results using diabetes vs. somatostatin to reduce circulating insulin levels in mature rats may be model- or dose-dependent. In adult rats (39), amino acids, but not insulin, increased 4E-BP1 phosphorylation, whereas in a recent study in fetal lambs, an elevation in insulin increased 4E-BP1 (48). Together, the results suggest a developmental decline in insulin-stimulated 4E-BP1 phosphorylation may exist.

An important regulatory step involved in the mRNA binding process is the association of eIF4E with eIF4G following the dissociation of 4E-BP1 from eIF4E. This results in active eIF4F complex assembly, thus enabling mRNA binding to the 43S preinitiation complex (18). Previous studies in mature rats and neonatal pigs have shown an increase in the active eIF4G•eIF4E complex in response to refeeding (12, 31, 57). In the current study, both insulin and amino acids decreased the 4E-BP1•eIF4E complex assembly. This effect of insulin was most profound in the presence of the higher doses of insulin in the presence of euaminoacidemia, but occurred at all doses of insulin in the presence of hyperaminoacidemia. Both insulin and amino acids increased the amount of eIF4G

bound to eIF4E. However, the effect of insulin only occurred at the highest dose during euaminoacidemia, and occurred at the intermediate and high doses of insulin during hyperaminoacidemia. This finding contrasts with a stimulatory response of muscle protein synthesis to all doses of insulin, and suggests that mechanisms other than an enhanced assembly of the active eIF4G•eIF4E complex must be involved in the stimulation of muscle protein synthesis by insulin. Because amino acids increased the amount of eIF4G bound to eIF4E at the low and intermediate doses of insulin, this suggests that amino acid-stimulated eIF4G•eIF4E assembly requires at least basal fasting insulin levels. However, the presence of variation may have precluded the observation of an effect of insulin at lower doses. In mature diabetic rats, leucine increased protein synthesis and tended to increase eIF4G•eIF4E formation (2). However, somatostatin treatment in adult rats suppressed leucine-induced protein synthesis, but had no effect on 4E-BP1•eIF4E or eIF4G•eIF4E content (1).

S6K1 phosphorylation status is primarily mTOR-dependent, as is the repressor protein, 4E-BP1 (29, 52). Previous studies in neonatal pigs have shown that feeding, through its activation of mTOR, causes hyperphosphorylation of S6K1 (12, 31). Increases in the phosphorylation of S6K1 result in hyperphosphorylation of ribosomal protein S6, and thus facilitate the translation of mRNAs containing terminal oligopyrimidine tracts at the 5' end, which encode elements of the translation apparatus, including ribosomal proteins and elongation factors (35, 41). Therefore, activation of S6K1 appears to increase the synthesis of proteins involved in mRNA translation. In the present study, hyperinsulinemia increased S6K1 phosphorylation, and hyperaminoacidemia also had a stimulatory effect on S6K1. However, the effect of amino acids on S6K1 phosphorylation was present only at the higher doses of insulin. Our findings are consistent with the findings in mature

rats and adult humans showing that increases in S6K1 activation by amino acids were facilitated by increases in insulin levels (1, 20), but contrast with another study in adult rats which showed that insulin did not enhance amino acid-stimulated S6K1 phosphorylation (39). Whether the differing conclusions involve differences in age, model, or dose is not discernable, but they suggest that a potentially interdependent mechanism for S6K1 activation by insulin and amino acids may exist. Nonetheless, the results of the current study implicate S6K1 dependent and independent pathways for both insulin-and amino acid-stimulated protein synthesis in skeletal muscle of the neonate.

Amino acid dose response of translation initiation factors. We have recently shown that amino acids increase muscle protein synthesis rates in a dose response manner in neonatal muscle. This current work constitutes the first study to determine whether there is a dose response effect of amino acids on translation initiation factor activity. In the presence of fed insulin levels, amino acids increased S6K1 phosphorylation, 4E-BP1 phosphorylation, and eIF4G•eIF4E content, and reduced 4E-BP1 • eIF4E content as amino acids were increased from below fasting, to fasting, and/or fed levels. These data highlight the importance of amino acids in regulating the availability of eIF4E for 48S ribosomal complex assembly in skeletal muscle of neonates. The lack of effect of reducing plasma amino acid concentrations to half of fasting levels on eIF2B activity in the current study contrasts with the reduction in eIF2B activity in the hindlimb of adult rats perfused with a leucine-deprived medium (51). This finding suggests that amino acids do not play a critical role in the modulation of eIF2B activity *in vivo*.

Effect of insulin on translation initiation factors in the absence of an amino acid clamp. We have previously shown that insulin stimulates muscle protein synthesis in neonates in the presence of fasting and below fasting amino acids levels (44). In the current study, eIF4G•eIF4E association was

also increased by insulin in the absence of an amino acid clamp, and in the presence of fasting level amino acid clamp. However, the lack of a statistically significant effect of insulin on the activity of other translation initiation factors when amino acids were below fasting levels and, in some cases, in the presence of fasting amino acid levels is surprising and emphasizes the importance of amino acids to the postprandial stimulation of translation initiation. The results further suggest that insulin-dependent and -independent pathways are involved in the regulation of skeletal muscle protein synthesis in the neonate.

Correlations. Examination of the relationships between translation initiation factor activity and plasma insulin level, in the presence of euaminoacidemia and hyperaminoacidemia, showed a linear relationship between plasma insulin levels and S6K1 phosphorylation, 4E-BP1 phosphorylation, 4E-BP1•eIF4E content, and eIF4G•eIF4E complex assembly, but a curvilinear relationship between plasma insulin and protein synthesis rates (44). This suggests that while higher levels of insulin may increase translation initiation factor activity, no further increases in protein synthesis in skeletal muscle of the neonate may occur. This conclusion is supported by the curvilinear relationship between muscle protein synthesis and the activity of most translation initiation factors. Furthermore, the relationships between plasma insulin and muscle protein synthesis, 4E-BP1 phosphorylation, 4E-BP1•eIF4E association, eIF4G•eIF4E content, and S6K1 phosphorylation were influenced by the amino acid level in the blood. This suggests that amino acids augment the insulin-induced activation of translation initiation factor activity in skeletal muscle of the neonate. Furthermore, the relationship between skeletal muscle translation initiation activity and circulating amino acid concentration, in the presence of hyperinsulinemia, revealed a linear relationship of amino acids with S6K1 phosphorylation, 4E-BP1 phosphorylation, and 4E-

BP1•eIF4E formation, but a curvilinear relationship with the amount of eIF4G•eIF4E complex.

Protein synthesis rates were related linearly and tended to relate in a quadratic manner to amino acid levels, suggesting that higher amino acid concentrations may have no further effect on eIF4G•eIF4E content or protein synthesis. In contrast, eIF2B activity showed no relationship with plasma insulin or amino acid levels or with protein synthesis rates, consistent with the findings of previous feeding studies in neonatal pigs in which no effect on eIF2B activity was found after refeeding (12, 31). Together the results suggest that modulation of binding of mRNA, but not met-tRNA_i, to the 40S ribosomal subunit plays a critical role in the post-prandial stimulation of skeletal muscle protein synthesis in the neonate.

Perspectives. In a recent study using pancreatic-glucose-amino acid clamps, we demonstrated the independent stimulatory roles of insulin and amino acids on skeletal muscle protein synthesis in the neonate (44). Herein, we show that, although neither insulin nor amino acids altered eIF2B activity or eIF4E phosphorylation, both insulin and amino acids increased phosphorylation of S6K1 and 4E-BP1, resulting in the release of eIF4E from the inactive association with 4E-BP1, and allowing eIF4G to bind to eIF4E forming an active complex. However, the response to insulin was present only at doses greater than fasting levels and the effect of amino acids on translation initiation factor activity was seen only in the presence of insulin. The results suggest that insulin and amino acids stimulate muscle protein synthesis in the neonate via mechanisms that are associated with enhanced 4E-BP1 and S6K1 phosphorylation and eIF4F complex formation, and by mechanisms independent of this pathway.

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FIGURE LEGENDS

Fig. 1: Fractional protein synthesis rates (A) and translation initiation factor activity (B-F) in longissimus dorsi muscle of 7-day-old pigs at ~0, 2, 6, and 30 $\mu\text{U/ml}$ plasma insulin levels during euaminoacidemia (500 nmol BCAA/ml) and hyperaminoacidemia (1000 nmol BCAA/ml). Values are means \pm SE; n=8-12 per treatment group. Insulin increased fractional protein synthesis rates ($P<0.005$), 4E-BP1 phosphorylation at Thr⁷⁰ ($P<0.005$), eIF4G • eIF4E content ($P<0.001$), and S6K1 phosphorylation ($P<0.05$) and decreased eIF4E • 4E-BP1 content ($P<0.001$). Amino acids increased fractional protein synthesis rates ($P<0.05$), 4E-BP1 phosphorylation at Thr⁷⁰ ($P<0.01$), eIF4G • eIF4E content ($P<0.05$), and S6K1 phosphorylation ($P<0.05$) and decreased eIF4E • 4E-BP1 content ($P<0.05$). *Statistically significantly different from ~0 $\mu\text{U/ml}$ insulin dose within the same amino acid (AA) level ($P<0.05$). †Significantly different from euaminoacidemic group within same insulin group ($P<0.05$).

Fig. 2: Translation initiation activity in longissimus dorsi muscle of 7-day-old pigs in the presence of hyperinsulinemia (~30 $\mu\text{U/ml}$) is compared during hypoaminoacidemia (250 nmol BCAA/ml), euaminoacidemia (500 nmol BCAA/ml), or hyperaminoacidemia (1000 nmol BCAA/ml) respectively). Values are means \pm SE; n=8-12 per treatment group. Amino acids increased protein synthesis rates ($P<0.05$). *Significantly different from hyperinsulinemic-hypoaminoacidemic group ($P<0.05$). †Significantly different from hyperinsulinemic-euaminoacidemic group ($P<0.05$).

Fig. 3: Translation initiation factor activity in longissimus dorsi muscle of 7-day-old pigs in the presence of hyperinsulinemia (~30 $\mu\text{U/ml}$) and either euaminoacidemia or hypoaminoacidemia (500 or 250 nmol BCAA/ml) is compared to basal fasting condition (~2 μU insulin/ml and 500 nmol BCAA/ml). Values are means \pm SE; n=8-12 per treatment group. *Significantly different from basal

fasting condition ($P < 0.05$). †Significantly different from hyperinsulinemic- euaminoacidemic group ($P < 0.05$).

Fig. 4: Correlations of muscle protein synthesis rates (A) and translation initiation factor activity (B-F) with plasma insulin levels in the presence of hyperaminoacidemia, and euaminoacidemia in 7-day-old pigs. Continuous line signifies correlation during hyperaminoacidemia and dotted line signifies correlation during euaminoacidemia.

Fig. 5. Correlations of muscle protein synthesis rates (A) and translation initiation factor activity (B-F) with plasma branched chain amino acid (BCAA) concentration in 7-day-old pigs.

Fig 6. Correlations of protein synthesis rates with translation initiation factor activity in skeletal muscle of 7-day-old pigs.

Table 1. Plasma insulin and amino acid levels in response to insulin and amino acid infusion during pancreatic- glucose- amino acid clamps in 7-day-old pigs.

Insulin/ Amino Acids	AA level	Baseline	Insulin group			
			0 μ U/ml	2 μ U/ml	6 μ U/ml	30 μ U/ml
Insulin	Fasting	1.5 \pm 0.2	0.7 \pm 0.2*	2.4 \pm 0.2	5.6 \pm 0.6*	30.8 \pm 4.2*
	Fed	1.7 \pm 0.2	0.9 \pm 0.2*	2.7 \pm 0.3	6.2 \pm 0.2*	29.0 \pm 3.4*
	<Fasting	1.8 \pm 0.2				24.0 \pm 2.0*
TAA	Fasting	3245 \pm 53	3014 \pm 156	3079 \pm 241	3060 \pm 149	2947 \pm 123
	Fed	3172 \pm 74	5546 \pm 497*	5227 \pm 232*	4649 \pm 392*	4697 \pm 117*
	<Fasting	3354 \pm 134				1851 \pm 167*
BCAA	Fasting	544 \pm 19	507 \pm 47	506 \pm 64	534 \pm 58	510 \pm 23
	Fed	560 \pm 25	888 \pm 55*	1072 \pm 74*	873 \pm 58*	887 \pm 37*
	<Fasting	601 \pm 52				259 \pm 25*
EAA	Fasting	1126 \pm 35	1105 \pm 88	1121 \pm 122	1158 \pm 92	1074 \pm 62
	Fed	1165 \pm 41	1968 \pm 132*	2210 \pm 160*	1936 \pm 93*	1907 \pm 60*
	<Fasting	1243 \pm 79				602 \pm 73*
NEAA	Fasting	2116 \pm 76	1905 \pm 97	1954 \pm 157	1904 \pm 107	1874 \pm 82
	Fed	2007 \pm 50	3575 \pm 420*	3017 \pm 124*	2712 \pm 146*	2790 \pm 89*
	<Fasting	2107 \pm 87				1248 \pm 110*

Plasma concentrations of insulin are in μ U/ml, and amino acids (total, branched-chain, essential, and nonessential) are in nmol/ml. Values are means \pm SE; n=8-12 per group. * Significantly different

from baseline (0 time) values ($P < 0.05$).

Figure 1

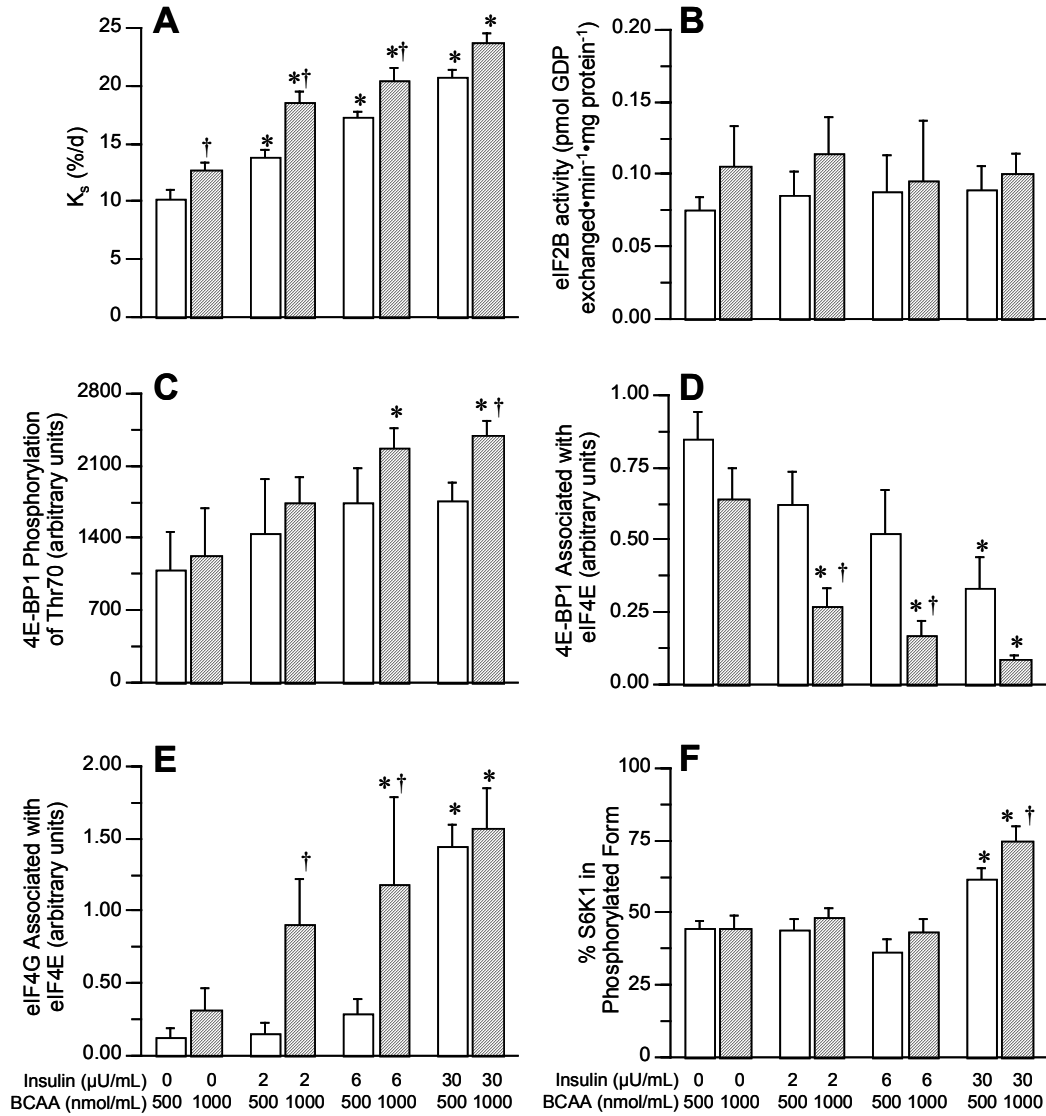


Figure 2

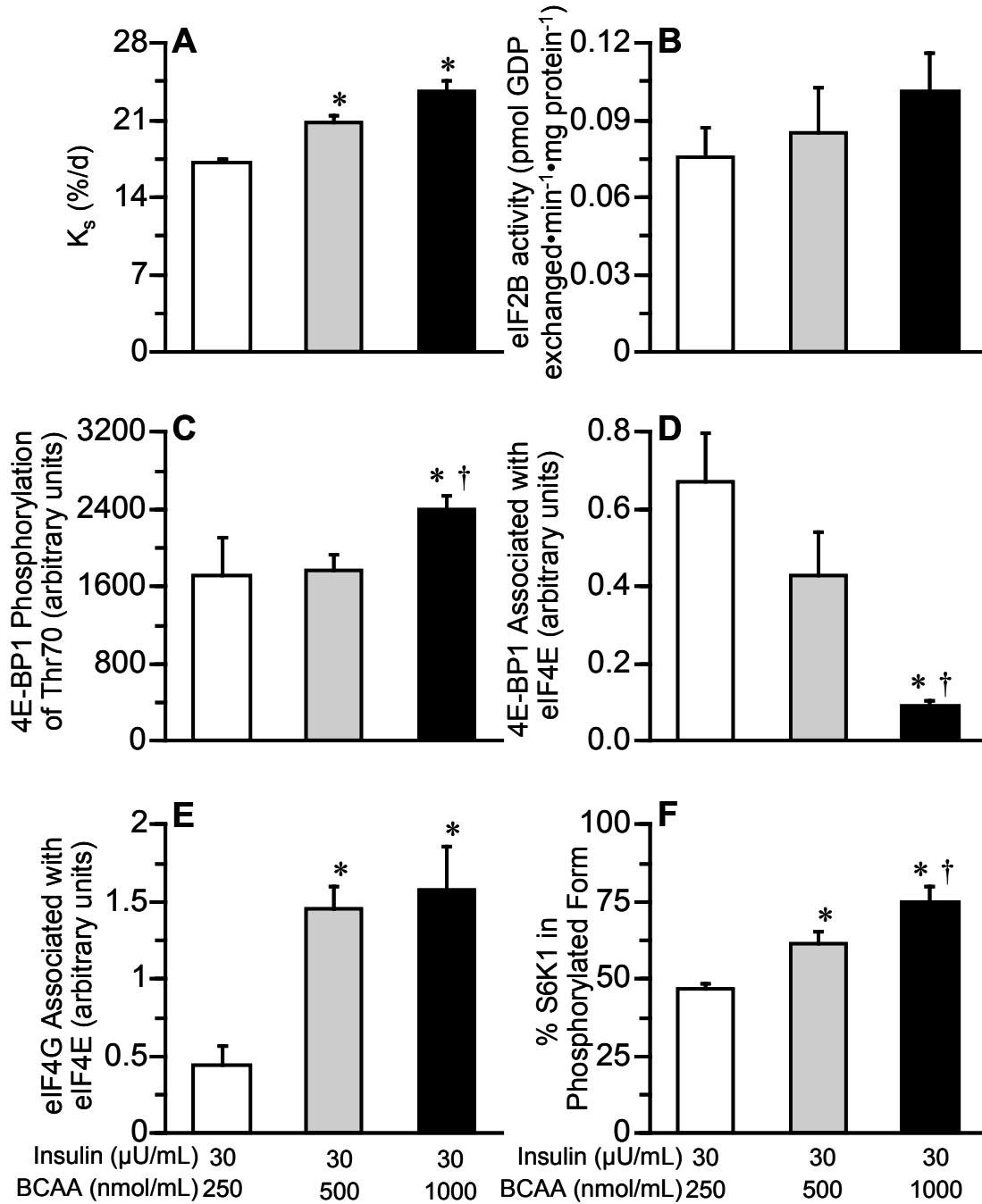


Figure 3

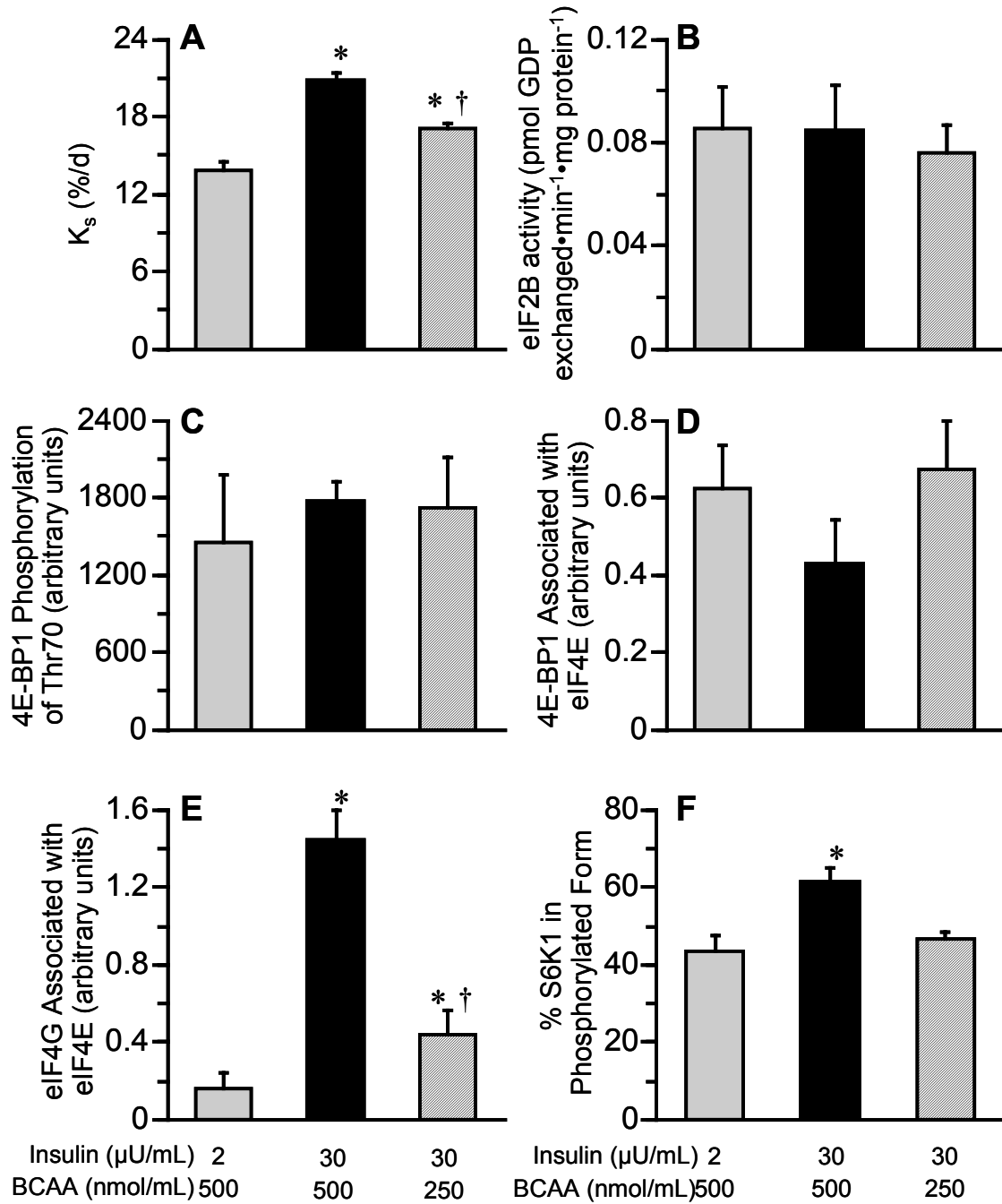


Figure 4

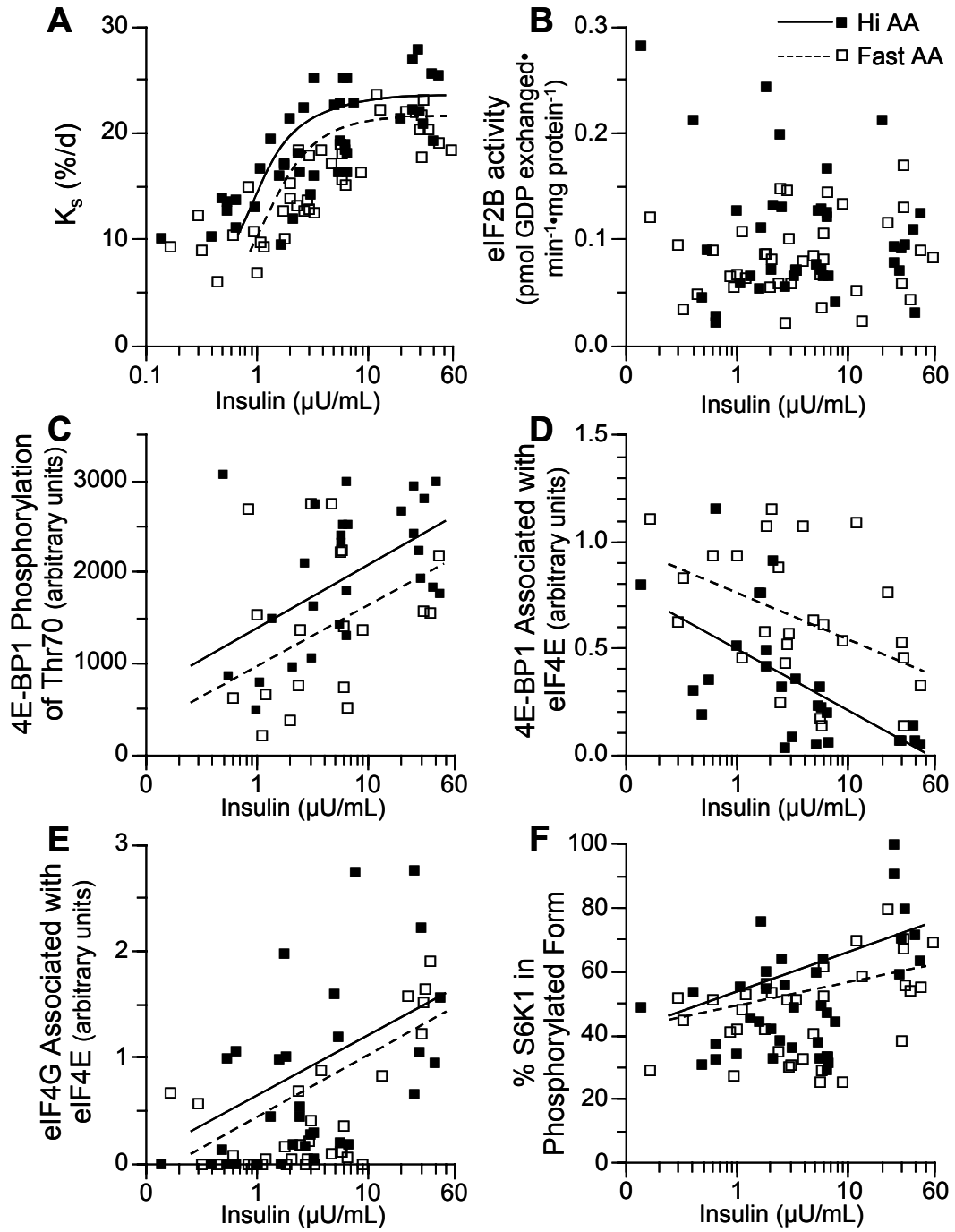


Figure 5

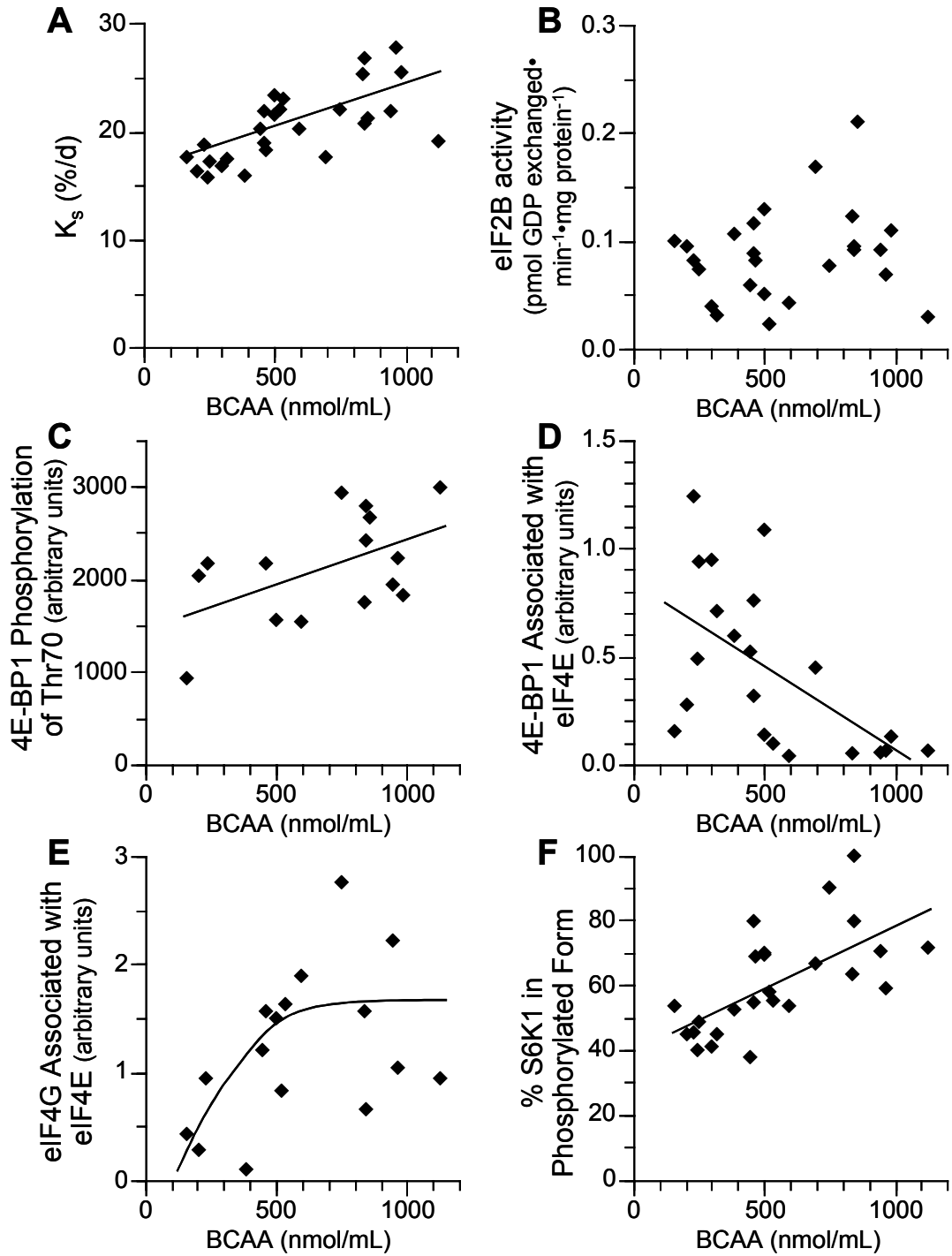


Figure 6

