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Fed levels of amino acids are required for the somatotropin-induced increase in muscle protein synthesis

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Wilson FA, Suryawan A, Orellana RA, Nguyen HV, Jeyapalan AS, Gazzaneo MC, Davis TA. Fed levels of amino acids are required for the somatotropin-induced increase in muscle protein synthesis. *Am J Physiol Endocrinol Metab* 295: E876–E883, 2008. First published August 5, 2008; doi:10.1152/ajpendo.90423.2008.—Chronic somatotropin (pST) treatment in pigs increases muscle protein synthesis and circulating insulin, a known promoter of protein synthesis. Previously, we showed that the pST-mediated rise in insulin could not account for the pST-induced increase in muscle protein synthesis when amino acids were maintained at fasting levels. This study aimed to determine whether the pST-induced increase in insulin promotes skeletal muscle protein synthesis when amino acids are provided at fed levels and whether the response is associated with enhanced translation initiation factor activation. Growing pigs were treated with pST (0 or 180 $\mu g \cdot kg^{-1} \cdot day^{-1}$) for 7 days, and then pancreatic-glucose-amino acid clamps were performed. Amino acids were raised to fed levels in the presence of either fasted or fed insulin concentrations; glucose was maintained at fasting throughout. Muscle protein synthesis was increased by pST treatment and by amino acids (with or without insulin) ($P < 0.001$). In pST-treated pigs, fed, but not fasting, amino acid concentrations further increased muscle protein synthesis rates irrespective of insulin level ($P < 0.02$). Fed amino acids, with or without raised insulin concentrations, increased the phosphorylation of S6 kinase (S6K1) and eukaryotic initiation factor (eIF) 4E-binding protein 1 (4EBP1), decreased inactive 4EBP1·eIF4E complex association, and increased active eIF4E·eIF4G complex formation ($P < 0.02$). pST treatment did not alter translation initiation factor activation. We conclude that the pST-induced stimulation of muscle protein synthesis requires fed amino acid levels, but not fed insulin levels. However, under the current conditions, the response to amino acids is not mediated by the activation of translation initiation factors that regulate mRNA binding to the ribosomal complex.

translation initiation; growth hormone; mammalian target of rapamycin; eukaryotic initiation factor 4G

TREATMENT WITH SOMATOTROPIN (pST), also referred to as growth hormone, promotes weight gain and improves the efficiency with which dietary amino acids are used for growth (6, 7, 20, 47). Studies conducted in our laboratory have shown that treatment of pigs with pST for 7 days increases whole body protein accretion by minimizing postabsorptive protein loss and maximizing the gain of protein during the postprandial phase. However, the mechanism through which protein gain is stimulated by chronic pST treatment is unknown.

Previously, we have shown that 7 days of pST treatment increases muscle protein synthesis in the fed state only, with no effect observed during fasting (3). In the fed state, pigs treated with pST compared with diluent have higher circulating levels of insulin and glucose (17), which are known promoters of protein synthesis in young growing animals (12, 31, 38). Although the increase in circulating glucose and insulin levels with pST treatment has been attributed to insulin resistance (28, 53), kinetic studies indicate that reduced insulin-stimulated glucose uptake occurs in adipose tissue, not skeletal muscle (17, 53). Even though insulin is known to promote skeletal muscle protein synthesis in a dose-dependent manner (38), we have demonstrated, by using pancreatic-glucose-amino-acid clamps, that the pST-induced increase in muscle protein synthesis is not due to the pST-induced increase in insulin concentration (52). We postulated that the inability of increased circulating insulin levels to further enhance rates of protein synthesis in the skeletal muscle of pST-treated pigs might be a consequence of the limited amino acid supply induced by maintaining amino acids at fasting concentrations. Indeed, it has been shown that a high-protein diet is required to obtain the maximal effects of pST treatment (8).

Our previous studies have demonstrated that the effects of insulin and amino acids on muscle protein synthesis are additive until maximum rates of protein synthesis are achieved (10, 12, 38). Both insulin and amino acids also independently stimulate muscle protein synthesis (12, 38). Fed levels of amino acids promote protein synthesis rates in the muscle to levels similar to that observed in fed animals, even when insulin and glucose are maintained at fasting. Likewise, raising insulin to fed levels, even when amino acids and glucose are maintained at fasting, also increases rates of muscle protein synthesis.

Both insulin and amino acids promote protein synthesis through an increased activation of translation initiation in response to stimulation of the mammalian target of rapamycin (mTOR) pathway (19, 34, 39, 45). Insulin promotes mTOR kinase activity through activation of protein kinase B (PKB) (27, 50). PKB phosphorylates tuberous sclerosis complex (TSC) 2, inactivating the TSC1/TCS2 complex (34). TSC1/TSC2 inactivation suppresses its inhibition of Ras homolog enriched in brain (Rheb), a GTPase that activates mTOR. Activation of mTOR leads to the phosphorylation of the eukaryotic initiation factor (eIF) 4E-binding protein 1 (4EBP1), causing it to disassociate from eIF4E (37). This disassociation enables the formation of the active eIF4E·eIF4G complex (37), which brings the mRNA strand to the ribosome (24). Additionally, activation of mTOR increases the activity of the ribosomal

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protein S6 kinase (S6K1), an activator of S6 (24), a protein thought to promote mRNA translation of proteins involved in the regulation of translation (30), although recent studies have shown that S6 is not required for ribosomal protein translocation (42). The phosphorylation of 4EBP1 and S6K1 increases translation initiation efficiency and consequently, protein synthesis. The mechanism through which amino acids are able to promote mTOR activity is less well defined. It has been shown that amino acids are unable to alter the phosphorylation of TSC2 in neonatal pigs (45) and cell culture (2). However, cell culture studies have shown that amino acids are able to promote the binding of Rheb to mTOR (1), indicating that this may be one point of interaction between amino acids and the mTOR pathway.

This study aimed to determine whether the pST-induced stimulation of protein synthesis in skeletal muscle of fed pigs was a consequence of increased circulating insulin levels when amino acids were not limiting. Additionally, this study aimed to determine whether this increase in protein synthesis is modulated by activation of the mTOR pathway. Rapidly growing pigs were treated with either pST or diluent for 7 days, and then pancreatic-glucose-amino acid clamps were performed where insulin and amino acids were manipulated to achieve fasting and fed concentrations.

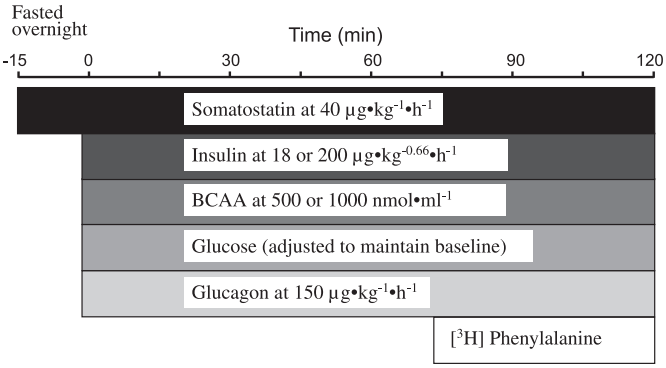

Fig. 1. Pancreatic-glucose-amino acid clamp protocol. Overnight-fasted diluent and somatotropin (pST)-treated pigs were injected with diluent or pST, respectively, 60 min before infusion. Fifteen minutes [time (t) = −15 min] before infusion, a primed, constant infusion of somatostatin was initiated. At t = 0 min, infusions of replacement glucagon and insulin commenced. Insulin was infused to reproduce fasting (5 μU/ml) or fed pST-treated (50 μU/ml) levels, amino acid concentrations were clamped at fasting [500 nmol branched-chain amino acid (BCAA)/ml] or fed (1,000 nmol BCAA/ml) levels, and glucose was clamped at baseline fasting levels. Fractional rates of protein synthesis were measured using a flooding dose of [^{3}H]phenylalanine. Blood samples were collected at 5-min intervals to clamp glucose and amino acids, with additional samples taken at baseline and 2 h for hormone and substrate analysis.

MATERIALS AND METHODS

Animals and design. Thirty-eight crossbred (Landrance × Yorkshire × Hampshire × Duroc) female pigs (Agricultural Headquarters, Texas Department of Criminal Justice, Huntsville, TX) weighing 12.0 ± 0.3 kg (8–10 wk) were housed in individual cages. Relatively high protein intakes are required to obtain the maximum promoting effects of pST (8); therefore, animals were fed a 24% protein diet (Producers Cooperative Association, Bryan, TX) at 6% of their body wt and provided water ad libitum. Pigs were adjusted to the diet for 7 days and then randomly assigned to one of two treatment groups, either diluent (saline) or recombinant pST (gift of Dr. Frank Dunshea) at a rate of 180 $\mu g \cdot kg$ body $wt^{-1} \cdot day^{-1}$ for 7 days. The dose of diluent or pST was divided into two daily injections (90 μg/kg body wt) and administered in the shoulder region. Body weights were measured daily, and the dietary intake and treatment doses were adjusted accordingly. Pigs treated with pST were offered the diet at 6% of their body weight per day, and diluent-treated pigs were pair-fed to the intake level of the pST-treated pigs to minimize any confounding effects of differences in food intake. Daily feed allowance was divided into two meals.

Before infusions (3–5 days), the pigs were fasted overnight, and the jugular vein and carotid artery of each pig were catheterized using sterile techniques under general anesthesia (Aerrane; Anaquest, Madison, WI) as described previously (9). After surgery, pigs were returned to their cages and resumed their regular treatment regimen. 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*.

Pancreatic glucose-amino acid clamps. Diluent and pST-treated pigs were fasted overnight (16 h) before the infusion studies. On the day of infusion, pigs were administered their daily dose of pST (180 μg/kg body wt) or diluent 60 min before infusion. Clamps were performed using techniques similar to those previously described (47, 54) (Fig. 1). Over a 30-min period before the initiation of the clamp, basal blood glucose (YSI 2300 STAT Plus; Yellow Springs Instruments, Yellow Springs, OH) and branched-chain amino acid (BCAA) concentrations were determined to establish the average fasting concentration to be used in the glucose-amino acid clamp procedure (54). The clamp was initiated with a primed (10 μg/kg), continuous (40 $\mu g \cdot kg^{-1} \cdot h^{-1}$; Bachem, Torrance, CA) somatostatin infusion to suppress endogenous insulin secretion and which concurrently suppresses glucagon secretion. After a 15-min infusion of somatostatin, an infusion of replacement glucagon (150 ng·kg body $wt^{-1} \cdot h^{-1}$; Eli Lilly, Indianapolis, IN) was initiated and continued to the end the clamp period. Insulin was infused at either 18 or 200 $ng \cdot kg^{-0.66} \cdot min^{-1}$ to reproduce the insulin concentrations normally present in the fasting state of untreated and pST pigs (5 μU/ml; fasting insulin) and the fed state of pST pigs (50 μU/ml; fed insulin) (47). Amino acids were clamped either at fasting levels (500 nmol BCAA/ml) or fed levels (1,000 nmol BCAA/ml) by using BCAA as an index for amino acid concentration. Glucose was maintained at fasting levels in all treatment groups. Thus, following 7 days of treatment with either diluent or pST, pigs were infused to achieve *1*) fasting insulin and fasting amino acids, *2*) fasting insulin and fed amino acids, and *3*) fed insulin and fed amino acids. Each pig was continuously infused for 2 h; arterial blood samples (0.5 ml) were obtained every 5 min and immediately analyzed for glucose and BCAA concentrations, as previously described (54). A 2.5-min enzymatic kinetic assay was used to determine total BCAA concentrations. A dextrose (Baxter Healthcare, Deerfield, IL) solution and a balanced amino acid mixture (containing all amino acids) (12) were infused to maintain glucose and amino acid values at the desired level. Additional blood samples (5 ml) were collected at baseline and 120 min for measurement of circulating insulin, plasma urea nitrogen (PUN), insulin-like growth factor I (IGF-I), pST, and individual amino acid concentrations.

Tissue protein synthesis in vivo. The fractional rate of protein synthesis was measured with a flooding dose of L-[4-^{3}H]phenylalanine injected 75 min after the initiation of the clamp procedure (23). Pigs were killed at 2 h, and samples of the longissimus dorsi muscle were collected and rapidly frozen. The specific radioactivity values of the protein hydrolysate, homogenate supernatant, and blood supernatant were determined as described previously (11). Previous studies have demonstrated that, after a flooding dose of [^{3}H]phenylalanine is administered, the specific radioactivity of tissue-free phenylalanine is in equilibrium with the aminoacyl-tRNA specific radioactivity; therefore, the tissue free phenylalanine is a valid measure of the tissue precursor pool specific radioactivity (14).

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Plasma hormones and substrates. The concentrations of individual amino acids from frozen plasma samples obtained at −15 and 120 min of the infusion were measured with a HPLC method (PICO-TAG reverse-phase column; Waters, Milford, MA) as previously described (14). Plasma radioimmunoreactive insulin and pST concentrations were measured using porcine insulin and growth hormone radioimmunoassay kits (Linco, St. Louis, MO). PUN concentrations were measured with the use of an end-point enzyme assay (Roche, Somerville, NJ) to determine the quantity of nitrogen in the urea. Plasma total IGF-I concentrations were measured with a two-site immunoradiometric assay with IGF-I-coated tubes (Diagnostic System Laboratories, Webster, TX).

Protein immunoblot analysis. Proteins from longissimus dorsi muscle homogenates were separated on polyacrylamide gels (PAGE). For each assay, all samples were run at the same time on triple-wide gels (C.B.S Scientific C., Del Mar, CA) to eliminate interassay variation. Proteins were electrophoretically transferred to polyvinylidene diflouride transfer membranes (Pall, Pensacola, FL), which were incubated with appropriate primary antibodies, washed, and exposed to an appropriate secondary antibody as previously described (15).

For normalization, immunoblotting preformed with antiphosphospecific antibodies were stripped in stripping buffer (Pierce Biotechnology, Rockford, IL) and reprobed with corresponding nonphospho-specific antibodies. Blots were developed using an enhanced chemiluminescence kit (GE health Sciences, Buckinghamshire, UK), visualized, and analyzed using a ChemiDoc-It Imaging System (UVP, Upland, CA). Primary antibodies that were used in the immunoblotting were 4EBP1 [total (Bethyl Laboratories, Montgomery, TX) and Thr^{70} (Cell Signaling, Boston, MA)], eIF4G (total and Ser^{1180}; Cell Signaling), and S6K1 (total and Thr^{398}; Cell Signaling).

Quantification of eIF4E·4EBP1 and eIF4E·eIF4G complexes. These complexes were immunoprecipitated using an anti-eIF4E monoclonal antibody (gift of Dr. Leonard Jefferson, Penn State University College of Medicine, Hershey, PA) from aliquots of fresh tissue homogenates (19). Briefly, samples were homogenized in 7 vol of buffer (in mM: 20 HEPES, 2 EGTA, 50 NaF, 100 KCl, and 0.2 EDTA, pH 7.4) containing Sigma P3840 Protease Inhibitor Cocktail (Sigma Chemical, St. Louis, MO) and centrifuged at 10,000 *g* for 10 min at 4°C. Supernatants were incubated overnight at 4°C, with constant rocking, with an anti-eIF4E antibody. Immunoprecipates were recovered with goat anti-mouse IgG magnetic beads (Polysciences, Warrington, PA), washed, resuspended in sample buffer as described previously (19), and immediately subjected to protein immunoblot analysis using rabbit anti-4EBP1 (Bethyl Laboratories) antibody or rabbit anti-eIF4G (Novus Biologicals, Littleton, CO). Amounts of 4EBP1 and eIF4G were corrected by the eIF4E recovered from the immunoprecipitate.

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

$$K_s(\%/\text{day}) = [(E_b/E_a) \times (1{,}440/t)] \times 100$$

where E_b [in disintegrations·min^{-1} (dpm)·$nmol^{-1}$] is the specific radioactivity of the protein-bound phenylalanine, E_a (in dpm/nmol) is the specific radioactivity of the tissue free phenylalanine at the time of tissue collection, t is the time of labeling in minutes, and 1,440 is the minutes-to-day conversion.

Two-way ANOVA was carried out using a general linear model to determine the effects of pST treatment, amino acid and/or insulin infusion, and the interaction between the two. When the interaction was statistically significant, analysis was performed using Tukey's multiple-comparison procedure to determine the effect of pST at a specific infusion period and the effect of amino acid/insulin infusion at a specific treatment. Where the interaction term was nonsignificant, the two-way ANOVA was repeated with the interaction term omitted, and, when the main effects were significant, analysis by Tukey's multiple-comparisons procedure was carried out. Probability values <0.05 were considered significant for all comparisons. Data are presented as means ± SE.

RESULTS

Animal weights. Average body weight did not differ significantly between pST and diluent-treated pigs at the initiation (15.8 ± 0.6 vs. 15.5 ± 0.6 kg, respectively) nor end (20.2 ± 0.7 vs. 18.9 ± 0.6 kg, respectively) of the treatment period. However, the daily weight gain was higher in pST-treated animals ($P < 0.001$) compared with diluent-treated pigs (0.57 ± 0.02 vs. 0.43 ± 0.02 kg, respectively). The efficiency with which feed was used for growth was higher in pigs treated with pST ($P < 0.001$) than in the diluent-treated animals, as reflected by their higher gain-to-feed ratios (0.66 ± 0.02 vs. 0.52 ± 0.03 kg gain/kg feed intake, respectively).

Plasma hormone and substrate concentrations. The plasma hormone and substrate concentrations shown in Tables 1 and 2 are mean values obtained at baseline and at the end of the infusion. The effectiveness of pST treatment was determined from the measurement of circulating PUN, IGF-I, and pST levels. Treatment with pST reduced PUN levels in the plasma by ~65% ($P < 0.001$) compared with diluent-treated pigs (Table 1). Pigs following the pST treatment regimen showed a 295% ($P < 0.001$) and 1,950% ($P < 0.001$) increase in plasma levels of IGF-I and pST, respectively (Table 1).

The effectiveness of the clamp in achieving the desired insulin, glucose, and amino acid concentrations is shown in Table 2. Targeted levels of both fasting and fed insulin were largely achieved in all groups. Glucose concentrations were maintained within 10% of fasting baseline levels in all groups; however, treatment with pST increased glucose levels in the plasma compared with diluent-treated animals ($P < 0.05$). BCAA and essential (EAA), nonessential (NEAA), and total (TAA) amino acid levels were successfully clamped at the predetermined level. BCAA, EAA, NEAA and TAA levels were doubled in the fed amino acid groups compared with preclamp and fasting levels. BCAA levels were lower in pST-treated animals than those receiving diluent ($P < 0.05$); however, this did not alter EAA, NEAA, nor TAA levels.

Glucose disposal rates. Net whole body glucose disposal rates were calculated from the average infusion rate of a 50% dextrose solution required to maintain circulating glucose levels within 10% of fasting baseline. Glucose disposal rates were raised by clamp treatment conditions ($P < 0.001$) due to the large increase in glucose uptake observed between the two fasting insulin groups and the fed insulin group ($P < 0.05$; Fig. 2). Glucose uptake was unaffected by increasing circulating amino acid concentrations to the fed level when insulin levels were

Table 1. *Plasma hormone and substrate concentrations in diluent and pST-treated pigs*

	Diluent	pST
PUN, mg/dl	11.7±1.0	7.6±0.5*
IGF-I, ng/ml	65.0±10.1	191.9±13.1*
pST, ng/ml	5.9±1.5	116.2±16.0*

Values are means ± SE; n = 12–14 pigs/treatment group. PUN, plasma urea nitrogen; IGF-I, insulin-like growth factor I; pST, somatotropin. *$P < 0.001$ compared with diluent-treated pigs. Significance determined by Tukey's multiple-comparison test.

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Table 2. *Plasma insulin, glucose, and amino acid concentrations in diluent and pST-treated pigs during pancreatic-glucose-amino-acid clamps*

			Target Insulin Levels, μU/ml		
	Treatment	Preclamp	Control	AA	Ins + AA
Insulin, μU/ml	Diluent	2.51±0.46	2.28±0.50	4.44±0.83	30.58±2.79†
	pST	3.83±0.63	4.75±0.76	7.56±2.66	31.84±2.93†
Glucose, mg/dl	Diluent	72.1±3.7	63.5±10.2	79.0±0.46	66.1±11.0
	pST*	81.0±3.0	90.1±13.9	93.7±7.4	87.1±8.4
BCAA, nmol/ml	Diluent	509.2±25.8	547.5±61.9	1,298.9±116.6†	1,335.7±114.3†
	pST	484.9±19.8	502.5±49.7	1,032.8±100.9†‡	1,171.3±23.4†‡
NEAA, nmol/ml	Diluent	2,178±122	2,181±135	4,130±342†	3,954±325†
	pST	2,265±107	2,176±233	3,661±296†	4,251±83†
EAA, nmol/ml	Diluent	859±50	755±48	2,060±118†	2,306±158†
	pST	841±36	744±88	1,851±101†	2,069±235†
TAA, nmol/ml	Diluent	3,037±118	2,936±162	6,189±394†	6,260±464†
	pST	3,106±128	2,921±313	5,512±394†	5,975±435†

Values are means ± SE; $n = 6–8$ pigs/treatment group. BCAA, branched-chain amino acids (Ile, Leu, Val); EAA, essential amino acids (Arg, His, Ile, Leu, Lys, Met, Thr, Trp, and Val); NEAA, nonessential amino acids (Ala, Asp, Cit, Glu, Gln, Gly, Orn, Pro, Ser, Tau, and Tyr); TAA, total amino acids (all EAA and NEAA). ANOVA indicated that infusion period altered insulin, BCAA, EAA, NEAA, and TAA ($P < 0.001$), and treatment with pST altered glucose ($P = 0.025$) and BCAA ($P = 0.026$) levels. Results from Tukey's multiple-comparison test: *Response in pST-treated pigs different from diluent-treated piglets at individual infusion periods ($P < 0.05$). †Change during infusion periods from preclamp value in either pST or diluent pigs ($P < 0.05$). ‡For glucose, there was no interaction term detected, however, treatment with pST increased glucose levels in the plasma compared to diluent-treated animals.

clamped at fasting. However, treatment with pST reduced glucose disposal rates compared with diluent-treated animals ($P < 0.05$), indicating that, on a whole body level, pST treatment induces insulin resistance for glucose metabolism.

Skeletal muscle protein synthesis. Fractional rates of skeletal muscle protein synthesis were increased by both raised circulating amino acid levels and pST treatment (Fig. 3). Increasing amino acids to fed levels, with or without increasing insulin to fed levels, increased the fractional rate of protein synthesis ($P < 0.05$). However, raising insulin levels in the presence of already high amino acid concentrations did not further enhance protein synthesis rates in the skeletal muscle, indicating that increasing amino acids to the fed state maximized protein synthesis rates. Treatment of pigs with pST increased rates of protein synthesis in skeletal muscle ($P < 0.05$), but this increase was mainly driven by the rise in protein synthesis rates in pST-treated pigs when amino acids were raised to fed levels ($P < 0.017$). This suggests that treatment with pST can promote rates of protein synthesis in skeletal muscle above that achieved by either amino acids and/or insulin when amino acid levels are maintained at fed concentrations.

Translation initiation factors. To determine if the increased rate of protein synthesis observed in pST-treated animals was mediated through an increase in translation initiation, Western blot analysis was performed. Both amino acids and insulin are able to promote the phosphorylation and activation of mTOR

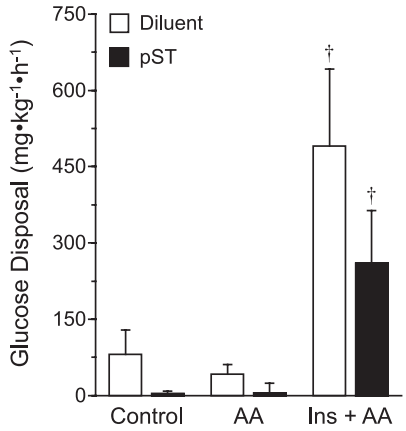

Fig. 2. Whole body net glucose disposal rates during pancreatic-glucose-amino acid clamps in diluent and pST-treated pigs. Pigs were infused with insulin to reproduce fasting (5 μU/ml) and fed (50 μU/ml) levels, and amino acids were clamped at either fasting (500 nmol BCAA/ml) or fed (1,000 nmol BCAA/ml) levels, whereas glucose was clamped at baseline fasting levels. Glucose disposal rates were calculated from the average rate of infusion of a 50% dextrose solution over the last hour of infusion. ANOVA indicated both a treatment [fasting, fed amino acid (AA), or fed insulin + AA] ($P < 0.001$) and pST ($P < 0.05$) effect. *Results of Tukey's multiple-comparison test: response to treatment with amino acids and/or insulin different from control group. Values are means ± SE; $n = 6–8$ pigs/group.

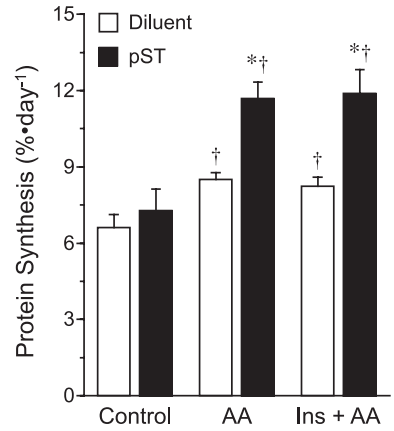

Fig. 3. Fractional rate of protein synthesis (K_s) in skeletal muscle of pigs during pancreatic-glucose-amino acid clamps in diluent and pST-treated pigs. Pigs were infused with insulin to reproduce fasting (5 μU/ml) and fed (50 μU/ml) levels, and amino acids were clamped at either fasting (500 nmol BCAA/ml) or fed (1,000 nmol BCAA/ml) levels, whereas glucose was clamped at baseline fasting levels. ANOVA indicated a treatment (fasting, fed AA, or fed insulin + AA) ($P < 0.001$) and a pST ($P < 0.001$) effect and an interaction ($P < 0.05$). Results of Tukey's multiple-comparison test: response to treatment with amino acids and/or insulin different from control group (*) and effect of pST-treatment different from diluent (†). Values are means ± SE; $n = 6–8$/group.

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(19, 34, 39, 45), a master kinase with the ability to promote the activation of several factors involved in translation initiation (24, 37). One such factor is S6K1, which, when activated by phosphorylation on the Thr^{389} residue, activates S6. Increasing circulating amino acids from fasting to fed levels, with insulin clamped at fasting, increased the phosphorylation of S6K1 ($P < 0.01$; Fig. 4*A*). However, raising both amino acids and insulin to fed levels did not increase S6K1 phosphorylation above that observed in animals with only raised amino acids. Treatment with pST did not alter levels of S6K1 phosphorylation. In addition, mTOR activation also promotes the phosphorylation of 4EBP1 on the Thr^{70} residue. As with S6K1, increasing amino acid concentrations to fed levels, while maintaining glucose and insulin at fasting, increased 4EBP1 phosphorylation ($P < 0.03$; Fig. 4*B*). Increasing circulating insulin to fed concentrations in the presence of fed amino acids did not further enhance 4EBP1 phosphorylation. Treatment with pST did not alter the phosphorylation of 4EBP1 in skeletal muscle, indicating that treatment with pST does not raise protein synthesis rates through activation of the mTOR pathway.

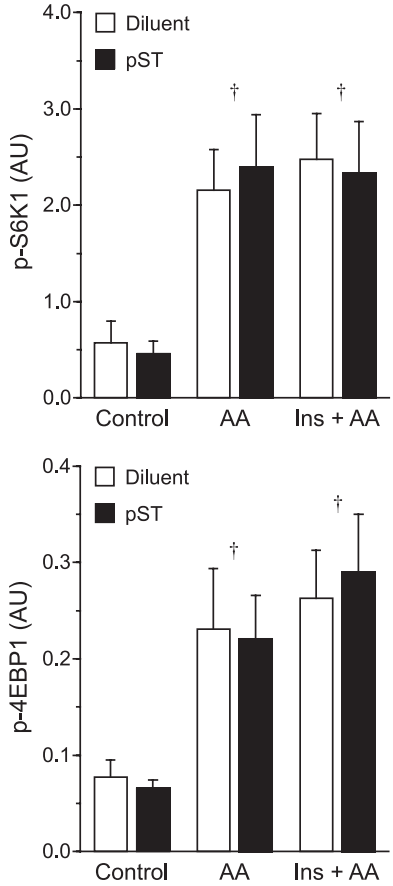

Fig. 4. Ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor (eIF) 4E-binding protein 1 (4EBP1) phosphorylation in skeletal muscle during pancreatic-glucose-amino acid clamps in diluent and pST-treated pigs. Pigs were infused with insulin to reproduce fasting (5 μU/ml) and fed (50 μU/ml) levels, and amino acids were clamped at either fasting (500 nmol BCAA/ml) or fed (1,000 nmol BCAA/ml) levels, whereas glucose was clamped at baseline fasting levels. ANOVA indicated a treatment (fasting, fed AA, or fed insulin + AA; $P < 0.02$) effect for both factors. *Results of Tukey's multiple-comparison test: response to treatment with amino acids and/or insulin different from control group. Values are means ± SE; $n = 6–8$/group.

Phosphorylation of 4EBP1 causes the disassociation of the inactive 4EBP1·eIF4E complex (37). Measurement of the abundance of the 4EBP1·eIF4E complex revealed that increasing the amino acid concentration in the blood either alone or in concurrence with insulin decreased the association of this complex ($P < 0.01$; Fig. 5*A*), with no difference in abundance detected between the fed amino acid groups ($P = 0.95$). Disassociation of eIF4E from 4EBP1 allows for the formation of the active eIF4G·eIF4E complex (37). Fed circulating amino acid concentrations, in the presence and absence of fed insulin, increased the association of the eIF4E·eIF4G complex ($P < 0.02$; Fig. 5*B*), with no significant effect measurable between the fasted and fed insulin levels when high amino acid were present. There was no effect of pST treatment on the association of either the inactive or the active complex compared with diluent-treated animals in the skeletal muscle. The association of the active complex is also dependent on the phosphorylation status of eIF4G. Increasing the amino acid concentrations in the circulation enhanced the phosphorylation, and hence activity, of eIF4G ($P < 0.04$; Fig. 6). However, raising the insulin concentration of the plasma in conjunction with amino acids did not increase phosphorylation above that observed with amino acids alone ($P > 0.74$). There was no effect of pST treatment on the phosphorylation status of eIF4G.

DISCUSSION

Treatment with pST increases skeletal muscle protein synthesis rates (3, 5–7), and the effect of pST is greater in the fed than in the fasted state. Compared with diluent-treated animals, pigs treated with pST have higher circulating postparandial insulin levels (17), an independent promoter of protein synthesis in the growing animal (12, 39). However, increased circulating insulin concentrations alone does not account for the pST-induced increase in protein synthesis (52). This study aimed to determine whether increased insulin concentrations in the presence of fed amino acid levels were required for the associated enhancement of skeletal muscle protein synthesis following pST treatment. Additionally, this study investigated whether the increase in protein synthesis was manifested by an increase in translation initiation. In the current study, we show that amino acids at fed concentrations are required to support the pST-enhanced rates of protein synthesis in skeletal muscle and that this effect appears to be independent of both insulin and the factors associated with translation initiation.

Growth and metabolic effects of pST. The results of the current study showed that treatment with pST for only 7 days was able to elicit the classic metabolic responses associated with pST treatment, i.e., increased weight gain and feed efficiency (20, 21, 43, 51), confirming the effectiveness of the pST treatment regimen. Treatment with pST also decreased circulating levels of PUN and increased plasma IGF-I. Treatment with pST has been demonstrated to reduce the activity of urea cycle enzymes (4) and hence urea synthesis (48), whereas increases in IGF-I plasma concentrations are well documented with treatment of pST (3, 26, 48).

Consistent with previous studies, pST treatment increased circulating glucose levels (17, 25, 28, 53) and reduced glucose disposal rates (16, 47). The increase in circulating glucose levels observed with pST treatment was previously thought to be a consequence of a reduction in glucose uptake by tissues in

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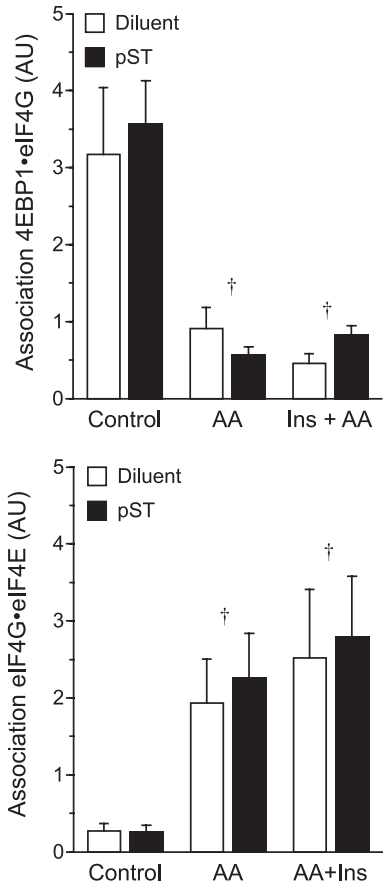

Fig. 5. Association of the 4EBP1·eIF4E and eIF4G·eIF4E complexes in skeletal muscle during pancreatic-glucose-amino acid clamps in diluent and pST-treated pigs. Pigs were infused with insulin to reproduce fasting (5 μU/ml) and fed (50 μU/ml) levels, and amino acids were clamped at either fasting (500 nmol BCAA/ml) or fed (1,000 nmol/BCAA ml) levels, whereas glucose was clamped at baseline fasting levels. ANOVA indicated a treatment (fasting, fed AA, or fed insulin + AA) ($P < 0.004$) effect for both complexes. *Results of Tukey's multiple-comparison test: response to treatment with amino acids and/or insulin different from control group. Values are means ± SE; $n = 6–8$/group.

response to insulin resistance (28). However, insulin resistance to glucose uptake has been shown to be tissue specific, with glucose uptake reduced in adipocytes of pST-treated animals and no effect observed in the skeletal muscle (17, 18, 53). The function of tissue-specific insulin resistance may be to aid the redistribution of nutrients for fat accretion to muscle deposition in pST-treated animals (18, 32). Therefore, in the current study, the increase in circulating glucose levels observed in response to a potential insulin resistance is not assumed to alter the glucose uptake response to insulin in muscle.

Protein synthesis rates. In this study, chronic pST treatment increased rates of muscle protein synthesis compared with diluent-treated pigs when amino acids were supplied to fed concentrations. Previous data indicate that treatment with pST promotes the redistribution of nutrients from fat accumulation to protein gain (6, 7, 20, 36), with increased rates of both whole body (25, 46) and skeletal muscle (6, 33, 43) protein synthesis. However, the increase in protein synthesis rates in response to treatment with pST in the current study was only observed when amino acid rates were raised to fed concentrations, in the presence or absence of high insulin levels, with no effect of pST treatment when animals were maintained in the fasting state. This is consistent with previous data from Bush et al. (3) where rates of protein synthesis in the muscle were increased in pST-treated animals only with refeeding after an overnight fast; no effect of pST was detected in fasted animals (3). Although feeding increases rates of muscle protein synthesis in animals that have been treated with pST for 1 wk, when rates of whole body protein synthesis were measured, an increase in both the fasted and fed state of pST-treated pigs was reported (47). This suggests that different mechanisms may alter pST-induced protein accretion rates in different tissues. In fact, protein synthesis in liver is increased in response to pST treatment in both the fed and fasted states, whereas pST-stimulated muscle protein synthesis only occurs in fed animals (3).

Increasing the amino acid concentration in the plasma to levels associated with feeding increased rates of muscle protein synthesis; however, increasing insulin concentrations in conjunction with increased levels of amino acids did not further enhance rates of protein synthesis in the muscle in either diluent or pST-treated pigs. This is consistent with previous data in neonatal pigs where increasing either insulin or amino acids independently increased rates of muscle protein synthesis to levels associated with feeding, whereas increasing both insulin and amino acids to fed levels showed no additional stimulation (12). In a recent study (52), we demonstrated using pancreatic-glucose-amino acid clamps that rates of muscle protein synthesis were not further increased by raising circulating concentrations of insulin from fed untreated levels (25 μU/ml) to plasma insulin levels seen in fed pST-treated animals (50 μU/ml) when pigs are treated with pST (52). Taken together, it appears that the pST-induced increase in circulating insulin concentrations does not mediate the pST-induced increase in muscle protein synthesis, but fed amino acids are required for the pST-induced increase in muscle protein synthesis. Interestingly, it has been shown that high rates of dietary protein are required for the maximal rate of pST-induced

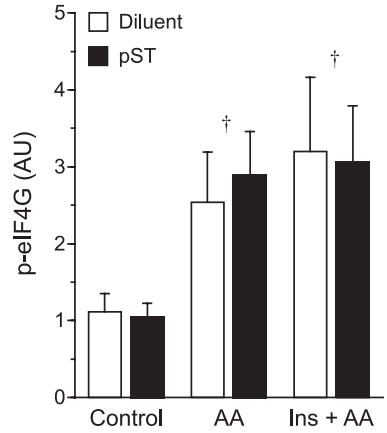

Fig. 6. eIF4G phosphorylation in skeletal muscle during pancreatic-glucose-amino acid clamps in diluent and pST-treated pigs. Pigs were infused with insulin to reproduce fasting (5 μU/ml) and fed (50 μU/ml) levels, and amino acids were clamped at either fasting (500 nmol BCAA/ml) or fed (1,000 nmol BCAA/ml) levels, whereas glucose was clamped at baseline fasting levels. ANOVA indicated a treatment (fasting, fed AA, or fed insulin + AA) effect ($P < 0.01$). *Results of Tukey's multiple-comparison test: response to treatment with amino acids and/or insulin different from control group. Values are means ± SE; $n = 6–8$/group.

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protein deposition, with diets containing less than 16% protein indicated to limit performance in pST-treated animals (8).

Translation initiation. Insulin and amino acids are among a group of anabolic agents known to promote rates of translation initiation through mTOR (13, 19, 22). In the current study, increasing the circulating amino acid concentration from fasting to fed levels increased the phosphorylation of both 4EBP1 and S6K1, downstream targets of mTOR activation (37, 44). Additionally, we observed that increasing the amino acid concentration from fasting to fed levels also decreased the association of the inactive 4EBP1·eIF4E complex and increased the formation of the active eIF4E·eIF4G complex. This is in agreement with previous studies conducted in cell culture, where increasing the amino acid concentration in the external medium led to high rates of 4EBP1 and S6K1 phosphorylation and hence increased formation of the active complex (41). On the other hand, amino acid deprivation leads to rapid dephosphorylation of 4EBP1 and S6K1, reducing formation of the active complex (49). Amino acids also have been shown to stimulate mTOR-mediated translation initiation in neonatal pigs (45).

In the current study, treatment with pST increased rates of muscle protein synthesis when amino acids were increased to fed concentrations. However, there was no additional increase in the factors involved in translation initiation or the association of the eIF4E·eIF4G complex in animals treated with pST when compared with diluent-treated pigs. We have shown, however, that, in fed growing pigs, treatment with pST increased 4EBP1 phosphorylation and eIF4E·eIF4G association (3). The mechanism that explains why feeding can induce phosphorylation of translation initiation factors in pST-treated animals, but not fed plasma levels of amino acids and insulin in pST-treated pigs, is unknown. The current data also contradict that of Hayashi and Proud (29), where cell culture experiments showed that pST treatment increased rates of protein synthesis in hepatoma cells via activation of the mTOR pathway. In that study, incubating pST-treated hepatocytes with either wortmannin (a phosphatidylinositol 3-kinase inhibitor) or rapamycin (a mTOR inhibitor) blocked phosphorylation of 4EBP1 and S6K1 (29). The difference between this cell culture experiment and the current study, however, could be tissue-specific differences, since activation of protein synthesis has been shown to differ between liver and muscle tissues in pigs treated with pST (3).

Therefore, the pST-induced increase in muscle protein synthesis appears to be dependent on attainment of plasma fed amino acid levels but independent of insulin concentrations and activation of the mTOR pathway. An alternative mechanism for pST-induced increase in muscle protein synthesis could be via increases in the activation of the eIF2B signaling pathway, which is involved in the binding of initiator methionyl-tRNA to the ribosome (40). However, amino acids have shown little or no effect on the activation of eIF2 in cell culture (49). Additionally, we previously showed that treatment of young pigs with pST for 7 days increased the activity of eIF2B in muscle but not in the liver, indicating that pST-mediated muscle protein synthesis may be a consequence of enhanced methionyl-tRNA binding to the ribosome (3). However, because eIF2B activity is not altered by feeding (15), the activity of this factor was not measured in the current study. Binding of pST to its own receptor, which activates the Janus-family tyrosine kinase 2 (35), may offer a potentially different pathway for pST activity in muscle. Alternatively, activation of translation elongation may account for the increase in protein synthesis observed with pST treatment. Leucine is known to reduce the phosphorylation and hence increase the activation of eukaryotic elongation factor 2 (19), a factor that can be activated by both mTOR-dependent and mTOR-independent pathways (2).

Perspectives

The results of the current study demonstrate that rates of muscle protein synthesis in young growing animals are increased by pST treatment. However, the pST-induced increase in muscle protein synthesis was only detected when circulating levels of amino acids were increased to fed concentrations, and this was not altered by the level of circulating insulin. The activity of the mTOR pathway, which is involved in translation initiation, was increased by amino acids but not by pST treatment, indicating that the pST-induced increase in muscle protein synthesis may be independent of mTOR activation of translation initiation.

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