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Translational Control of Protein Synthesis in Muscle and Liver of Growth Hormone-Treated Pigs

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GH treatment increases protein deposition and the efficiency of dietary protein used for growth. To identify the mechanisms that regulate tissue protein synthesis in response to exogenous GH treatment, fully fed, growing swine were treated with GH for 7 d. Fasted and fed pigs were infused with [1-^{13}C]leucine to determine protein synthesis rates, and translation initiation factor activity levels were measured in skeletal muscle and liver. Feeding increased protein synthesis and translational efficiency in both muscle and liver of control and GH-treated pigs, and this was associated with increased 4E-BP1 and S6 kinase 1 phosphorylation, decreased association of eukaryotic initiation factor (eIF) 4E with 4E-BP1, and increased association of eIF4E with eIF4G. GH increased muscle protein synthesis and translational efficiency in fed pigs. GH increased liver protein synthesis of fasted and fed pigs in association with increased ribosome number. In muscle, but not liver, GH increased eIF2B activity and 4E-BP1 phosphorylation in both the fasted and fed state and increased the association of eIF4E with eIF4G in the fed state. We conclude that GH increases muscle protein synthesis in the fed state, in part, via mechanisms that enhance the binding of mRNA and methionyl-tRNA to the 40S ribosomal subunit, whereas GH increases liver protein synthesis in the fasted and fed states by increasing ribosome number. The results further indicate that the GH-induced protein synthetic response is dependent upon nutritional state and is tissue specific. (*Endocrinology* 144: 1273–1283, 2003)

EXOGENOUS GH administration increases protein deposition, decreases fat accretion, and improves nitrogen retention in domestic animals. GH treatment also enhances the efficiency with which dietary protein is used for growth (1, 2). Studies in our laboratory have demonstrated that GH treatment in growing swine enhances metabolic efficiency by minimizing protein loss during fasting and maximizing protein gain during meal absorption, thereby improving protein balance (2, 3). Our recent results suggest that GH administration increases protein synthesis in the hindquarters area in the fed condition (4), but the specific effect in muscle was not determined, and the mechanism governing the increase in protein synthesis was not identified.

Most research in GH-deficient (5, 6), as well as normal, mature animals and adult humans (7–9), suggests that GH treatment increases protein deposition by stimulating whole body and muscle protein synthesis. For example, acute GH infusion (*i.e.* 6–7 h) in adult humans increases muscle protein synthesis (8), although differing results have been reported (10). Chronic GH treatment (*i.e.* 14–20 d) in cattle and swine increases amino acid uptake by the hindquarter (11) and protein synthesis in muscle (4, 7). However, the underlying mechanisms by which chronic GH treatment enhances muscle protein synthesis and subsequent protein deposition are not clearly defined.

Abbreviations: eIF, Eukaryotic initiation factor; 4E-BP1, eIF4E binding protein 1; KICA, α-[1-^{13}C]ketoisocaproic acid; met-tRNA$_i$, initiator methionyl-tRNA; mTOR, mammalian target of rapamycin; PKB, protein kinase B; PI 3-kinase, phosphatidylinositol 3-kinase; PUN, plasma urea nitrogen; S6K1, S6 kinase 1; SDS, sodium dodecyl sulfate.

The rate at which protein synthesis occurs depends upon the capacity and efficiency of the translation process. Acute alterations in protein synthesis are driven primarily by changes in translation initiation (12, 13), whereas long-term alterations result from increases in ribosome number (14, 15). Two key processes can regulate acute changes in translation initiation. The first regulatory process is the binding of initiator methionyl-tRNA (met-tRNA$_i$) to the 40S ribosomal subunit to form the 43S preinitiation complex via mediation of eukaryotic initiation factor (eIF) 2 (16). The eIF2-mediated met-tRNA$_i$ binding to the 40S subunit is further regulated by the activity of eIF2B, which exchanges GDP for GTP on eIF2 (16). The second regulatory process in translation initiation is the binding of mRNA to the 43S preinitiation complex via mediation of the assembly of the eIF4F complex of proteins (17–19). Phosphorylation (20) and availability (21) of eIF4E regulate the formation of the eIF4F complex. Early studies suggested that phosphorylation of eIF4E increases its association with eIF4G and eIF4A and its affinity to bind to the m^{7}GTP cap at the 5′ end of mRNA (20, 22), whereas more recent studies indicate that the phosphorylation of eIF4E reduces the affinity to bind to the m^{7}GTP cap at the 5′ end of mRNA (23, 24) and potentially is not required for translation (23–25). Availability of eIF4E is regulated by its association with 4E-BP1, a repressor protein that competes with eIF4G for binding to eIF4E (21). The phosphorylation of 4E-BP1 in the inactive eIF4E·4E-BP1 complex results in a decreased affinity of eIF4E for 4E-BP1, and the release of eIF4E enhances the binding of eIF4E to eIF4G to form the active eIF4E·eIF4G complex (26). These translational events

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may be regulated by changes in the phosphatidylinositol 3-kinase (PI3-kinase)/S6 kinase 1 (S6K1) signaling pathway (27).

Binding of GH to the GH receptor causes dimerization of the receptor (28) with subsequent phosphorylation of two Janus kinase molecules (29). Through this signaling pathway, GH indirectly activates insulin receptor substrate-1 and -2 (30), PI3-kinase independent of insulin receptor substrate (31), and factors downstream of PI-3 kinase such as protein kinase B (PKB) (32) and S6K1 (33). Anabolism-inducing agents, such as food (34–36), insulin (35, 37), IGF-I (38, 39), and amino acids (40, 41), have been shown to induce changes in various components of the signaling pathways leading to translation initiation as well as changes in the overall rates of protein synthesis. However, information is lacking with regard to the anabolic effect of GH on the regulation of translation initiation *in vivo*, specifically the activity of key initiation factors in skeletal muscle.

Therefore, the objective of this study was to determine whether GH increases protein deposition by increasing protein synthesis in skeletal muscle and liver in the fasted and fed states. We further wished to identify the regulatory mechanisms underlying the effect of GH by measuring ribosome number and the activity of key translation initiation factors. Studies were performed in rapidly growing pigs (~25 kg) in which protein intake and GH treatment were rigorously controlled over a 7-d treatment period and during a 6-h isotope infusion study to ensure steady-state conditions in the fasted and fed states.

Materials and Methods

Animals and dietary intake

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*. Twenty-four crossbred (Landrace x Yorkshire x Hampshire x Duroc) female pigs were purchased from the Agriculture Headquarters at the Texas Department of Criminal Justice (Huntsville, TX). The pigs were received at the Baylor College of Medicine animal facility at 8–10 wk of age, weighing approximately 10 kg. During a 2-wk acclimation period, pigs were fed a 24% high protein, dry diet (Producers Cooperative Association, Bryan, TX) at a rate of 6% of body weight per day, thus ensuring approximately 90% of *ad libitum* intake for pigs of this age was consumed (42). Pigs were offered food in two equal amounts (one-half the total amount of feed, twice daily) each day at 0800 and 1500 h. Pigs generally consumed all the food presented to them; unconsumed food was accounted for when estimating daily food intake and feed efficiency. Water was continuously available.

Surgery

Following the 2-wk orientation period, pigs (n = 24) were fasted overnight and a carotid artery, jugular vein, and duodenum were catheterized using sterile techniques and general anesthesia as previously described (4, 43). Intravenous antibiotics (enrofloxacin, 2.5–5.0 $mg \cdot kg^{-1}$) were administered daily to prevent infection, and im injections of a mild pain reliever (butorphanol tartate, 0.01 $mg \cdot kg^{-1}$) were given 1 d following surgery.

Experimental design

Pigs were weight-matched and randomly assigned to either the control (saline, n = 12) or recombinant porcine GH (Southern Cross Biotech, Melbourne, Australia) group (n = 12) at a concentration of 150 $\mu g \cdot kg^{-1} \cdot d^{-1}$ for a 7-d period. This dose has been shown to be effective in increasing protein deposition and reducing blood urea nitrogen concentrations in domestic animals (1–3, 11, 44). The dose of GH was divided into two equal daily injections (75 $\mu g \cdot kg^{-1}$) and administered into the hindquarter musculature concurrent with the feeding sessions. Control pigs received equal volume injections of sterile saline. To minimize the confounding effect of differences in feed intake, pigs were pair-fed to the level of the GH-treated group during the 7-d treatment period.

Infusions

Overnight fasted pigs were given their final injection of GH (150 $\mu g \cdot kg^{-1} \cdot d^{-1}$), and secured in a swine hammock (Walter Terry Distributor, Inc., Houston, TX). Pigs were randomized to either a fasted or fed group. To ensure a fully fed state throughout the infusion period, pigs were infused intraduodenally for 7 h with a nutrient solution (11 $ml \cdot kg^{-1} \cdot h^{-1}$) containing 24% protein beginning 1 h before the onset of the tracer infusion. The pigs in the fasted group received an intraduodenal saline solution at the same rate.

To quantify tissue fractional protein synthesis rates, a primed (15 $\mu mol \cdot kg^{-1}$), continuous infusion (15 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) of [1-^{13}C]leucine (Cambridge Isotope Laboratories, Andover, MA) was administered into the carotid artery. Venous blood samples (1.0 ml) for analysis of isotopic enrichments of plasma [1-^{13}C]leucine and α-[1-^{13}C]ketoisocaproic acid (KICA), and whole blood glucose were obtained at baseline, every 60 min during the first 4 h of the infusion period, and every 30 min during the last 2 h of tracer infusion. Venous blood samples (1.0 ml) for analysis of IGF-I, plasma urea nitrogen (PUN), insulin, and glucagon concentrations were obtained at baseline, 3 h, and 6 h.

Tissue extraction

At the end of the 6-h infusion study, pigs were killed by exsanguination under anesthesia (pentobarbital sodium), at which time tissue samples from longissimus dorsi and liver were rapidly removed and frozen in liquid nitrogen before storage at −80 C for analysis of tissue fractional synthesis rates. Fresh longissimus dorsi and liver tissue samples were also extracted for determination of translation initiation factor activity.

Hormone and substrate concentrations

Heparinized blood (1.5 ml) samples were obtained and immediately analyzed for blood glucose concentration by a glucose oxidase reaction (Yellow Springs Instruments, Yellow Springs, OH; 2300 STAT Plus). The remainder of the blood was centrifuged at 3000 × *g* for 15 min at 4 C, and the plasma was stored at −80 C until analyzed for IGF-I, PUN, insulin, and glucagon concentrations. Plasma IGF-I concentrations were analyzed in duplicate via two-site immunoradiometric assay with prior extraction (Diagnostic Systems Laboratories, Inc., Webster, TX). PUN concentrations were analyzed in duplicate via an end-point colorimetric assay (Ortho-Clinical Diagnostics, Inc., Rochester, NY). Plasma insulin concentrations were analyzed in duplicate by RIA (Linco Research, Inc., St. Charles, MO). Plasma glucagon concentrations were analyzed in duplicate via RIA (Linco Research, Inc.).

Fractional rate of protein synthesis

Fractional rates of tissue protein synthesis were measured following the 6-h infusion of [1-^{13}C]leucine. Briefly, frozen muscle and liver samples (~100 mg) were homogenized in perchloric acid (0.2 M), centrifuged (3000 × g, 4 C, 15 min), and the supernatant, constituting the precursor pool, was removed. The hydrolysate, constituting the bound pool, was washed in perchloric acid (10 ml), resuspended in HCl (6 N), and incubated (36 h at 100 C). Plasma and tissue precursor and tissue bound pools of [1-^{13}C]leucine were isolated via cation exchange chromatography (AG-50W resin, Bio-Rad Laboratories, Inc., Hercules, CA). Analysis of plasma and tissue [1-^{13}C]leucine precursor and tissue bound pools were conducted via heptaflurobutyric anhydride derivatization, and the isotopic enrichment of derivatized [1-^{13}C]leucine was determined by negative chemical ionization gas chromatography mass spectrometry (Hewlett-Packard Co., Palo Alto, CA; 5890 Series II GC equipped with a Europa Orchid 20/20 stable isotope analyzer) by monitoring the mass-

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to-charge ratio of ions at 349/350. Plasma α-KICA was isolated via cation exchange chromatography (AG-50W resin, Bio-Rad Laboratories, Inc.). To each eluant of plasma α-KICA, 10 N sodium hydroxide and 0.36 M hydroxylamine hydrochloric acid were added. Samples were heated for 30 min at 60 C and cooled in an ice bath. The pH was adjusted to less than 2 by adding 6 N HCl. Ketoacids were extracted with ethylacetate and dried under nitrogen gas at room temperature. The α-KICA was derivatized by adding *N*-methyl-*N*-*t*-butyl dimethylsilyl trifluoracetamide + 1% *t*-butyl dimethyl-chlorosilane (MTBSTFA + 1% TBDMCS; Regis Chemical, Morton Grove, IL) and stored in a desiccator overnight until analysis. The isotopic enrichment of α-[1-^{13}C]KICA was determined via electron ionization gas chromatography mass spectrometry (Hewlett-Packard Co. 5989 B GC-mass spectrometer equipped with a Hewlett-Packard Co. 5890 Series II GC) by monitoring the mass-to-charge ratio of ions at 316/317.

The fractional rates of protein synthesis (K_s) were determined by using the isotopic enrichment of leucine in the precursor pool (*i.e.* intracellular leucine) and protein-bound pool. Estimates of protein synthesis were also determined using plasma leucine and α-KICA as the precursor pool. Fractional rates of protein synthesis (K_s, percent protein mass synthesized per day) were calculated as K_s (%/d) = $[(E_a/E_b) \times (1440/t) \times 100$, where E_a is the enrichment of the tissue [1-^{13}C]leucine precursor pool, E_b is the enrichment of the protein-bound leucine, and t is the time of labeling in minutes. Most of the RNA in tissues is ribosomal RNA (45), and therefore the RNA-to-protein ratio (μg RNA·mg protein^{-1}) was used as an estimate of ribosome number, *i.e.* protein synthetic capacity (C_s). Protein synthetic efficiency (K_{RNA}) was estimated as the total protein synthesized per total RNA (G protein·d^{-1}·g RNA^{-1}).

Measurement of eIF2B activity

The eIF2B activity in muscle and liver supernatants was measured as the exchange of [^{3}H]GDP bound to eIF2 for unlabeled GDP or GTP, as previously described (27). Briefly, an eIF2·[^{3}H]GDP binary complex was formed in the absence of magnesium chloride. The eIF2·[^{3}H]GDP complex was then stabilized by the addition of magnesium to a final concentration of 2 mM. The eIF2·[^{3}H]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.

Protein immunoblot analysis

Proteins in polyacrylamide gels (PAGE) were electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.) as previously described (16). The membranes were then incubated with primary antibody (Amersham Life Sciences, Arlington, Heights, IL) for 1 h at room temperature. Blots were developed using an ECL Western blotting kit (Amersham Life Sciences) as previously described (16), visualized using a GeneGnome bioimaging system, and analyzed using GeneTools software (Syngene). Results are expressed as arbitrary units, which represent the integrated pixel intensity of the band being analyzed.

Quantitation of 4E-BP1·eIF4E and eIF4G·eIF4E complexes

The association of eIF4E with 4E-BP1 or eIF4G was quantitated as described previously (27). Briefly, eIF4E and the 4E-BP1·eIF4E and eIF4G·eIF4E complexes were immunoprecipitated using an anti-eIF4E monoclonal antibody. The immunoprecipitates were resuspended in sodium dodecyl sulfate (SDS) sample buffer, and the samples were boiled for 5 min. The samples were then centrifuged, and 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 polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.) as described above. The membranes were incubated with a mouse antihuman eIF4E antibody, a rabbit antirat 4E-BP1 antibody, or a rabbit antihuman eIF4G antibody. The antibodies against 4E-BP1 and eIF4G were raised against the recombinant rat and human proteins, respectively, by the method described previously (46). The blots were then developed using an ECL Western blotting kit (Amersham Life Sciences) as described above.

Examination of 4E-BP1 phosphorylation

Aliquots of muscle and liver homogenates 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 (16). Previous studies have shown that phosphorylation of 4E-BP1 causes a decrease in the electrophoretic mobility of the protein on SDS-PAGE (17). Thus, 4E-BP1 present in tissue extracts was separated into multiple electrophoretic forms during SDS-PAGE, with the more slowly migrating forms representing more highly phosphorylated 4E-BP1.

Measurement of eIF4E phosphorylation

The phosphorylated and unphosphorylated forms of eIF4E in tissue extracts were separated by isoelectric focusing on a slab gel and were quantitated by protein immunoblot analysis with a monoclonal antibody against eIF4E, as previously described (27).

Determination of S6K1 phosphorylation

Muscle and liver 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. The samples were then analyzed by protein immunoblot analysis by use of rabbit antirat S6K1 polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), as described above.

Measurement of PKB phosphorylation

Muscle and liver samples were homogenized in seven volumes of buffer and then centrifuged at 10,000 × *g* for 10 min at 4 C. One membrane was incubated with an anti-PKB antibody (New England Biolabs, Inc., Beverly, MA). The second membrane was incubated with a rabbit polyclonal antibody that recognizes the phosphorylation of PKB on Ser^{473} (New England Biolabs, Inc.).

Statistics

A general linear model of ANOVA was used to assess the effect of somatotropin treatment, feeding, and the interaction between treatment and feeding over the 6-h infusion period. When significant interactions were detected, *t* tests were performed to detect significant differences between treatment groups. ANOVA with repeated measures showed no difference across time for hormonal and substrate concentrations, thus the averages across time are presented. Results are presented as means ± SD. $P \leq 0.05$ was considered statistically significant.

Results

Animal growth

Body weight measured at the end of the 7-d treatment period (23.68 ± 2.91 *vs.* 22.64 ± 2.03 kg, respectively) did not differ significantly between GH-treated and control pigs. The rate of daily weight gain during the treatment period (47.68 ± 12.3 *vs.* 38.23 ± 11.64 g·kg^{-1}·d^{-1}, respectively) tended to be ($P < 0.06$) higher in GH-treated *vs.* controls. Feed efficiency, as reflected by gain-to-feed ratio (0.84 ± 0.18 *vs.* 0.72 ± 0.17, respectively), also tended ($P < 0.06$) to be higher in the GH-treated *vs.* control pigs. These data indicate that the efficiency with which dietary proteins were used for growth was enhanced by GH treatment in growing pigs, consistent with previous studies (1–4, 42). On the day of the infusion, control and GH-treated pigs were randomized into four separate infusion groups: control fasted, control fed, GH

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fasted, and GH fed. There was no significant difference in body weights among the four treatment groups (control fasted, 22.77 ± 2.06 kg; control fed, 22.73 ± 1.97 kg; GH fasted, 24.00 ± 3.77 kg; and GH fed, 23.85 ± 2.28 kg) on the experimental testing day.

Hormone and substrate concentrations

To verify the effectiveness of GH treatment in growing pigs, we measured circulating concentrations of IGF-1, PUN, glucose, glucagon, and insulin concentrations (Table 1). As expected, IGF-I concentration was significantly ($P < 0.001$) higher (fasted, +132%; fed, +244%) in the GH-treated groups compared with controls, and in the GH group was higher ($P > 0.01$) in the fed (+62%) than in the fasted state. The increase in IGF-I concentration in the GH-treated pigs indicated not only the effectiveness of the treatment, but an activation of the somatotropic axis as well. Consistent with our previously published data (2, 3), there was a significant ($P < 0.001$) decrease (fasted, −43%; fed, −43%) in PUN concentrations in the GH-treated pigs compared with controls, indicating an improvement in nitrogen retention in GH-treated pigs in the fed state. There was no significant effect of nutritional state on PUN in either control or GH groups.

Circulating insulin levels were significantly ($P < 0.001$) higher in the fed condition in both treatment groups (control, +642%; GH, +1017%). There was no significant effect of treatment on insulin levels in the fasted condition. However, the GH-fed group exhibited a significantly ($P < 0.002$) higher (+43%) insulin concentration *vs.* controls in the fed state, consistent with our previous studies (2, 47). Glucose concentrations increased (control, +52%; GH, +60%) significantly ($P < 0.04$) with feeding in both groups. There was no significant ($P > 0.05$) effect of GH treatment on glucose concentration. Circulating glucagon concentrations were unaffected by either treatment or nutritional state.

Fractional rates of protein synthesis

It has been previously observed that plasma α-KICA may not be a reliable indicator of muscle intracellular leucine when insulin levels are elevated because the arterial α-KICA-to-vein leucine ratio decreases at high insulin levels (48). Because GH induces a diabetogenic response (*i.e.* elevated insulin concentrations; Refs. 2 and 47), isotopic enrichment of intracellular [1-^{13}C]leucine was used to estimate both muscle and liver protein synthesis rates under metabolic steady-state conditions. In muscle, feeding significantly increased protein synthesis rates in both control (+39%) and GH-treated pigs (+110%; Fig. 1). GH significantly increased muscle protein synthesis rates, but only in the fed state (+47%). In liver, feeding significantly elevated protein synthesis rates in both control (+15%) and GH-treated pigs (+21%). GH significantly increased liver synthesis rates in both the fasted (+21%) and fed conditions (+28%; Fig. 1).

Protein synthetic capacity and translational efficiency

The protein synthetic capacity, C_s, as indicated by the RNA-to-protein ratio, was determined in muscle and liver samples. In muscle, there was no significant effect of feeding or GH on C_s (Fig. 2A). However, in liver, GH significantly increased the C_s in the fasted (+20%) and fed (+18%) state (Fig. 2A), consistent with our GH-induced increase in liver protein synthesis in the fasted and fed conditions (Fig. 1). The translational efficiency, K_{RNA}, was estimated as the total protein synthesized per total RNA. In muscle, feeding increased the K_{RNA} in both control (+30%) and GH-treated (+100%) pigs (Fig. 2B). GH significantly increased the K_{RNA} in muscle, but only in the fed (+30%) condition, consistent with our GH-induced increase in muscle protein synthesis in the fed state (Fig. 1). In liver, feeding increased the K_{RNA} in control (+28%) and GH-treated (+24%) pigs, whereas no significant effect of GH treatment on K_{RNA} was observed (Fig. 2B).

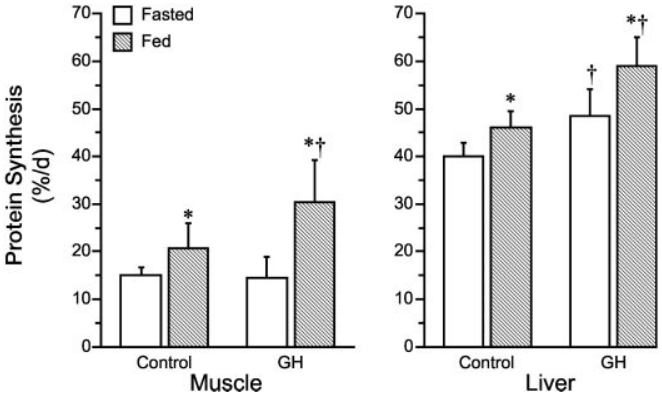

FIG. 1. Muscle and liver fractional protein synthesis rates in control and GH-treated pigs in the fasted and fed state. Values are mean ± SD; n = 6/treatment/nutritional state. *, $P < 0.05$ effect of feeding; †, $P < 0.05$ effect of GH.

TABLE 1. Hormone and substrate concentrations in venous circulation of control and GH-treated pigs in the fasted and fed states

	Control		GH	
	Fasted	Fed	Fasted	Fed
IGF-I	149.2 ± 60.9	162.9 ± 51.1	347.3 ± 127.6[a]	560.7 ± 53.2[a,b]
Insulin	4.5 ± 1.1	33.4 ± 3.4[b]	5.2 ± 2.0[a]	58.1 ± 10.5[a,b]
Glucagon	117.8 ± 26.9	115.7 ± 20.9	109.9 ± 58.7	126.9 ± 17.8
PUN	12.6 ± 3.7	15.5 ± 2.3	7.2 ± 2.3[a]	8.8 ± 2.7[a]
Glucose	76.4 ± 6.2	116.5 ± 27.0[b]	79.2 ± 13.0	127.3 ± 29.0[b]

Values are mean ± SD; n = 6/treatment/nutritional state. IGF-I concentration measured in ng·ml^{-1}; insulin in μU·ml^{-1}; glucagon in pg·ml^{-1}; and PUN and glucose in mg·dl^{-1}. [a], Significant difference ($P < 0.05$) from corresponding control value. [b], Significant difference ($P < 0.05$) from corresponding fasted condition.

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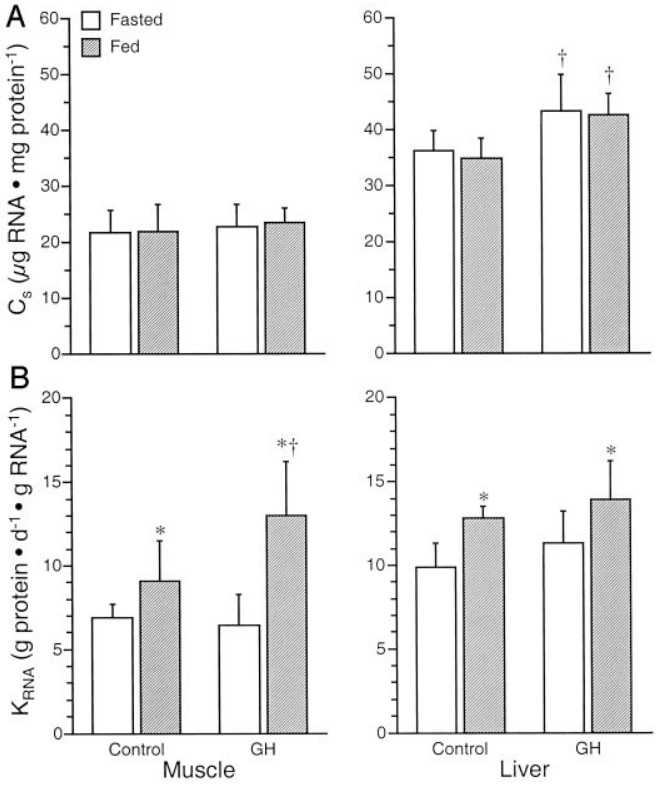

FIG. 2. Muscle and liver protein synthetic capacity (C_s; A) translational efficiency (K_{RNA}; B) in control and GH-treated pigs in the fasted and fed state. Values are mean ± SD; n = 6/treatment/nutritional state. *, $P < 0.05$ effect of feeding; †, $P < 0.05$ effect of GH.

eIF2/eIF2B activity

The eIF2-mediated binding of met-tRNA$_i$ to the 40S ribosomal subunit is regulated by modulation of the activity of eIF2B, a factor required for the exchange of GDP for GTP on eIF2 (41). Feeding had no significant effect on eIF2B activity in skeletal muscle (Fig. 3A). However, GH treatment significantly ($P < 0.01$) increased eIF2B activity in muscle of fasted (+56%) and fed (+54%) swine. In liver, neither feeding nor GH treatment had a significant effect on eIF2B activity (Fig. 3A). This suggests a tissue-specific action of GH treatment on eIF2B activity.

eIF4E function

eIF4E plays a critical role in the binding of mRNA to the 43S preinitiation complex (18, 19). The assembly of the eIF4F complex is regulated by the phosphorylation state of the translational repressor protein, eIF4E-binding protein 1 (4E-BP1). The availability of eIF4E can be regulated through changes in the amount of eIF4E bound to 4E-BP1 (17). Examination of the electrophoretic mobility of different phosphorylated forms (α, β, and γ) of the resolved protein during SDS-PAGE enabled the analysis of changes in the phosphorylation of 4E-BP1. The most highly phosphorylated and slowest migrating form of the protein (the γ-form) does not bind to eIF4E and thus allows for the formation of the eIF4F complex. The α- and β-forms both bind to eIF4E, thus reducing the formation of the eFI4F complex. In skeletal muscle, feeding increased ($P < 0.001$) the phosphorylation of 4E-BP1 in both control (+550%) and GH-treated (+420%) pigs (Fig. 3B). GH treatment also increased ($P < 0.02$) the phosphorylation of 4E-BP1 in muscle in both the fasted (+138%) and fed (+90%) conditions. In liver, feeding increased ($P < 0.05$) the phosphorylation of 4E-BP1 in both control (+130%) and GH-treated (+547%) pigs (Fig. 3B). However, there was no effect of GH treatment on the phosphorylation of 4E-BP1 in liver. To further define the mechanism by which GH regulates eIF4E function, we examined the phosphorylation state of eIF4E in skeletal muscle and liver. Neither feeding nor GH treatment affected the phosphorylation state of eIF4E either in muscle or liver of growing pigs in this study (data not shown).

Association of eIF4E with 4E-BP1 and eIF4G

Phosphorylation of 4E-BP1 in cell culture, *in situ*, and *in vivo* decreases the association of 4E-BP1 with eIF4E, thereby allowing eIF4E to bind with eIF4G (17, 35, 36). To determine the amount of 4E-BP1 associated with eIF4E, eIF4E was immunoprecipitated with an anti-eIF4E antibody, followed by immunoblot analysis with an anti-4E-BP1 antibody. In skeletal muscle, in conjunction with a feeding-induced increase in the phosphorylation of 4E-BP1, feeding induced a significant ($P < 0.001$) reduction in the association of eIF4E with 4E-BP1 in control (−45%) and GH-treated (−50%) pigs (Fig. 4A). There was no significant effect of GH treatment on the association of eIF4E with 4E-BP1 even though GH treatment increased the phosphorylation of 4E-BP1 (Fig. 4A). Similarly in liver, feeding decreased the association of eIF4E with 4E-BP1 in control (−38%) and GH-treated (−24%) pigs. There was no significant effect of GH treatment on the association of eIF4E with 4E-BP1.

The amount of eIF4G bound to eIF4E was also determined in the same eIF4E immunoprecipitates. It is known that anabolic agents (*i.e.* feeding, amino acids, insulin, IGF-I) increase the association of eIF4E with eIF4G in an active complex that binds to mRNA and the 43S preinitiation complex (35, 36, 39, 40). In skeletal muscle, feeding significantly ($P < 0.004$) increased the association of eIF4E with eIF4G in control (+287%) and GH-treated (+618%) pigs (Fig. 4B). GH treatment further increased the association of eIF4E with eIF4G, but in only the fed condition (+287%). In liver, feeding increased the association of eIF4E with eIF4G in control (+167%) and GH-treated (+590%) pigs (Fig. 4B), whereas GH treatment had no effect. Together, these results suggest that acute increases in muscle and liver protein synthesis in response to feeding are, in part, due to changes in the activity of translation initiation factors that regulate the binding of mRNA to the 43S preinitiation complex. Furthermore, a tissue-specific effect of GH treatment on muscle protein synthesis exists through regulation of the binding of mRNA to the 43S preinitiation complex.

S6K1 phosphorylation

In this study, the effect of 7 d of GH treatment in fasted and fed swine on the phosphorylation of S6K1 was also examined. Phosphorylation of S6K1 is associated with its activation. Through protein immunoblot analysis, we determined

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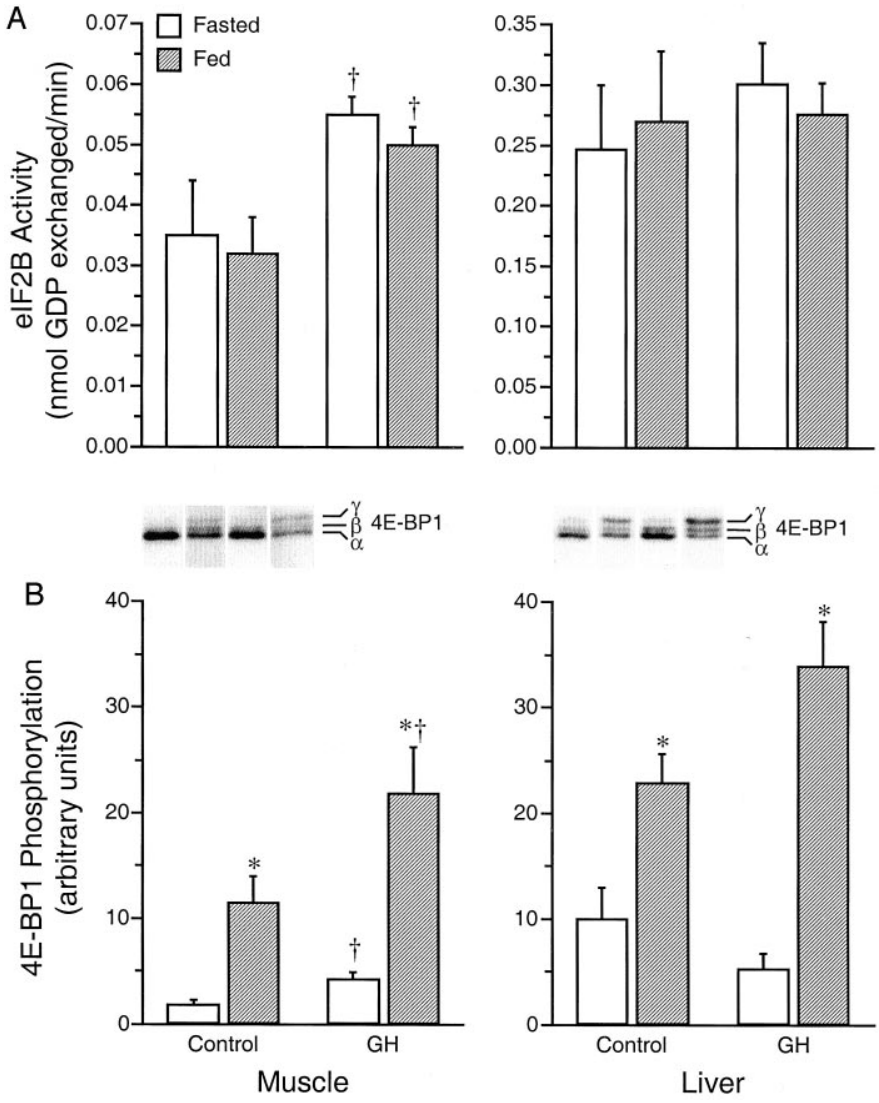

FIG. 3. Eukaryotic initiation factor 2B (eIF2B) activity (A) and 4E binding protein 1 (eIF4E-BP1) phosphorylation (B) in skeletal muscle and liver of fasted and fed, control and GH-treated pigs. Values are mean ± SD; n = 6/treatment/nutritional state. *, $P < 0.05$ effect of feeding; †, $P < 0.05$ effect of GH.

the phosphorylation state of S6K1 in skeletal muscle and liver extracts. Similar to that observed for 4E-BP1 during electrophoresis, S6K1 also resolves into multiple electrophoretic forms depending on the amount of phosphate present on the protein; hyperphosphorylated forms exhibit decreased and hypophosphorylated forms exhibit increased mobility (48). It has been shown that feeding decreases the electrophoretic mobility of S6K1 in skeletal muscle and liver of young piglets (35, 36). In skeletal muscle of rapidly growing pigs, the hyperphosphorylated form of S6K1 was significantly increased ($P < 0.02$) after feeding in both control (+90%) and GH-treated pigs (+110%; Fig. 5A). There was a trend ($P = 0.09$) for GH to increase the phosphorylation of S6K1, but only in the fed condition. In liver of rapidly growing pigs, feeding increased ($P < 0.05$) S6K1 phosphorylation in both control (+20%) and GH-treated pigs (+16%) with no significant effect of GH treatment (Fig. 5A). These data suggest that the feeding-induced stimulation of protein synthesis involves the activation of the S6K1 pathway in both skeletal muscle and liver. Furthermore, a tissue-specific effect of GH-induced stimulation of protein synthesis likely exists that involves the activation of the S6K1 pathway.

PKB phosphorylation

To examine signaling upstream of S6K1, we measured the phosphorylation of PKB in muscle and liver following 7 d of GH treatment in fasted and fed swine. The phosphorylation state of Ser^{473} was examined by Western blot analysis by use of an antiphosphopeptide antibody that recognized PKB only when phosphorylated at Ser^{473}. Feeding significantly increased the phosphorylation of PKB in muscle of control (+176%) and GH-treated (+260%) pigs and in liver of GH-treated (+200%) pigs (Fig. 5B). GH treatment had no significant effect on PKB phosphorylation.

Discussion

This is the first study to examine the mechanisms that regulate the effects of GH treatment on skeletal muscle and

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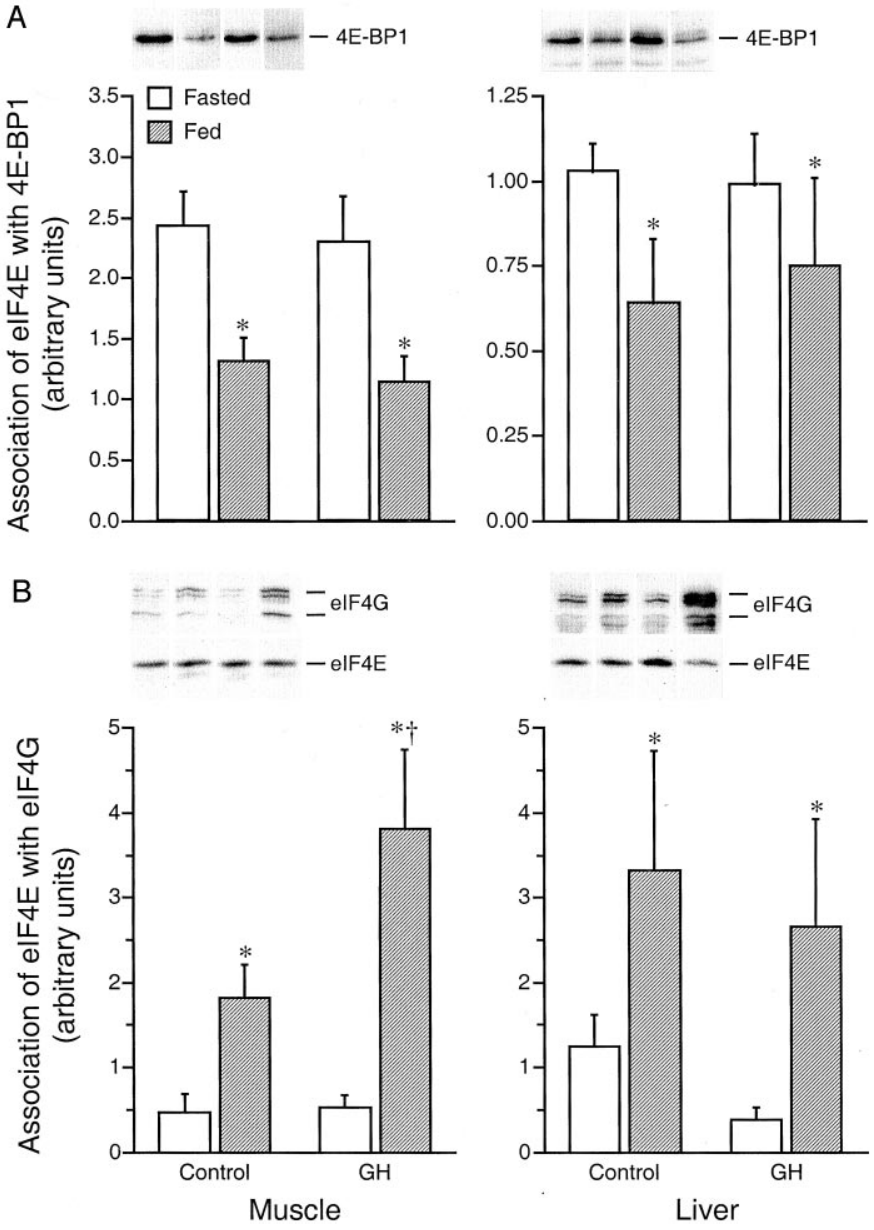

FIG. 4. Association of eIF4E with 4E-BP1 (A) and eIF4G (B) in skeletal muscle and liver of fasted and fed, control and GH-treated pigs. Values are mean ± SD; n = 6/treatment/nutritional state. *, $P < 0.05$ effect of feeding. †, $P < 0.05$ effect of GH.

liver protein synthesis at the level of translation initiation. The results of this study indicated that 7 d of GH treatment in growing pigs significantly increased skeletal muscle protein synthesis, but this increase was only apparent in the postprandial condition and was due to an increase in the efficiency of translation. The changes in muscle protein synthesis in the postprandial state were dependent upon those translation initiation factors that regulate the binding of both mRNA and initiator met-tRNA$_i$ to the ribosomal complex. We further observed a tissue-specific response to 7 d of GH treatment as GH significantly elevated liver protein synthesis in both the postabsorptive and postprandial conditions, unlike that of skeletal muscle. This increase in protein synthesis was due to an increase in ribosome number and not to a change in the processes that regulate translation initiation.

GH-stimulated protein synthesis

The increase in skeletal muscle protein synthesis in the fed state in our current study is supported by recent studies in our laboratory (4), demonstrating that 7 d of GH treatment increased (+43%) protein synthesis in the hindquarter of growing pigs in the postprandial state. The increase in amino acid extraction and utilization for protein synthesis in the hindquarter were mediated largely by an increase in blood flow (+63%) to the hindquarter. Although the results of the current study in young pigs show an increase in muscle protein synthesis only in the postprandial state by GH, the findings of earlier studies, which were conducted in mature humans, suggested that GH increases muscle protein synthesis in the fasting state (8). In a growing animal, changes

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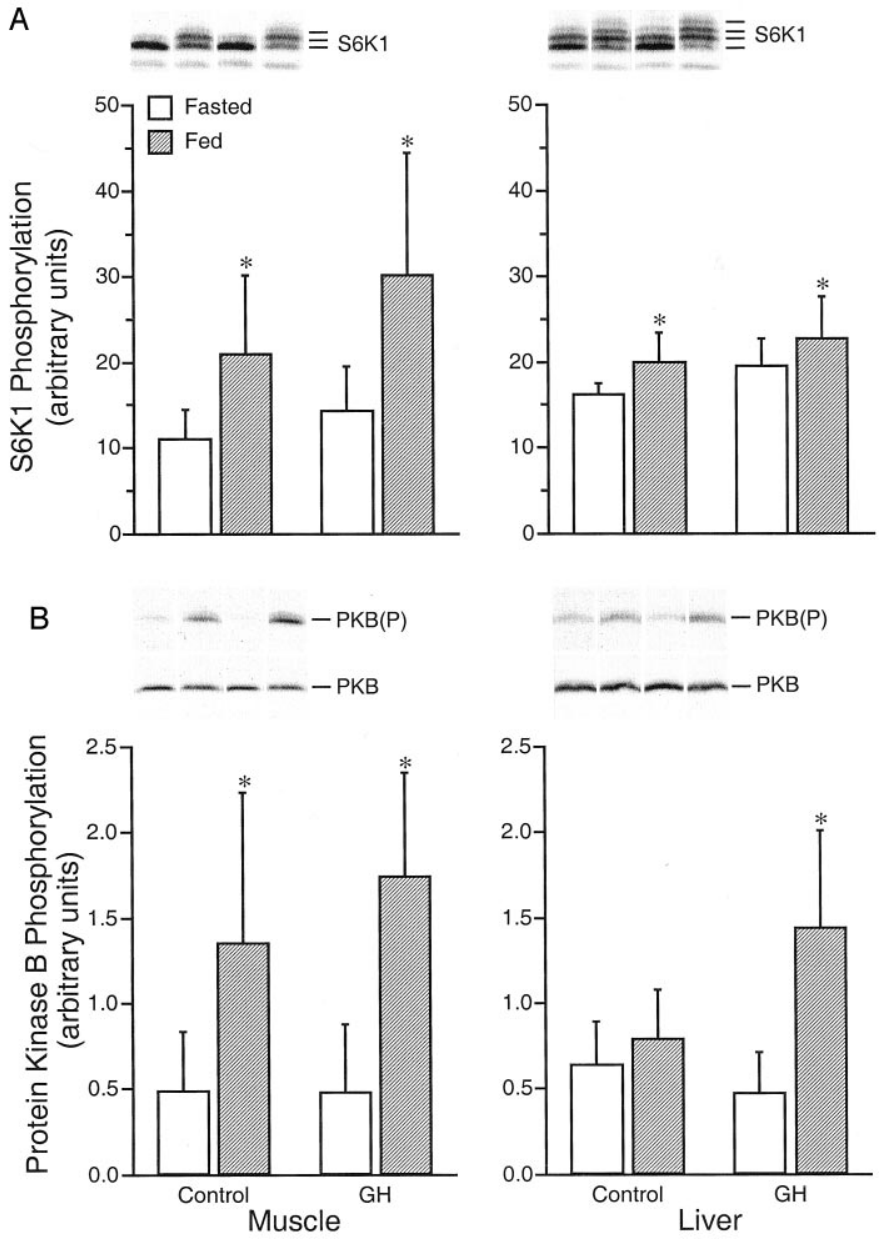

FIG. 5. Phosphorylation of S6K1 (A) and protein kinase B (B) in skeletal muscle and liver of fasted and fed, control and GH-treated pigs. Values are mean ± SD; n = 6/treatment/nutritional state. *, $P < 0.05$ effect of feeding.

in muscle protein synthesis are very sensitive to feeding (43) and amino acid stimulation (49), as indicated in the current study, in which feeding increased muscle protein synthesis by 39%. In fact, previous research has shown that a high protein diet (*i.e.* 20–24%) provides greater stimulus for a GH-induced increase in muscle protein synthesis and subsequent protein accretion (1, 9). One possible explanation for the lack of increase in muscle protein synthesis with GH treatment in the fasted condition was the lack of substrate availability. In the current study, we carefully controlled the nutritional state of the animal by supplying all the macro- and micro-nutrients directly into an intraduodenal catheter, thereby ensuring a fully fed state. Although feeding also increased protein synthesis in the liver (+15%), feeding did not seem to be an influential factor in affecting GH-induced protein synthesis in the liver, as GH increased protein synthesis in both the fasted and fed states. Thus, the interactive effect of GH and feeding on protein synthesis appears to be tissue specific.

Numerous studies suggest that administration of GH is associated with a diabetogenic effect by increasing insulin and/or glucose concentration. In the present study, feeding increased circulating insulin and glucose concentrations in both control and GH-treated swine. GH further increased insulin concentrations in the postprandial state, with no concurrent change in glucose concentration, indicative of a state of insulin resistance for glucose metabolism (47) in the GH-treated swine. Because insulin mediates the feeding-induced increase in skeletal muscle protein synthesis in young, growing swine (50), it is plausible that the GH-induced stimula-

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tion of protein synthesis in skeletal muscle of fed pigs may be due, in part, to the GH-induced increase in insulin concentration. Previous studies incorporating pancreatic glucose-amino acid clamps (3) have indicated that although GH treatment reduces the responsiveness of whole body glucose metabolism to insulin, GH does not alter the responsiveness of whole body protein metabolism to insulin. However, whole body protein synthesis is a compilation of protein synthesis rates in numerous tissues, most of which are not as responsive as skeletal muscle to circulating levels of insulin. Further studies involving GH-stimulated tissue protein synthesis incorporating hyperinsulinemic-euglycemic-euaminoacidemic clamps are warranted.

GH significantly increased circulating IGF-I concentrations in both the fasted and fed conditions compared with controls, indicative of a stimulation of the somatotropic axis by GH treatment in growing swine, as previously reported (2, 51, 52). IGF-I concentrations were significantly higher in the fed than in the fasted condition with GH treatment. Previous studies have suggested an increase in muscle protein synthesis, but not liver protein synthesis, in very young, growing pigs infused with physiological levels of IGF-I (53). Current dogma holds that the anabolic effects of GH are mediated by IGF-I produced by the liver or locally synthesized (53). However, whether the GH-induced increase in protein synthesis is due to the GH-induced increase in IGF-I remains to be determined.

Mechanisms regulating GH-stimulated protein synthesis

The rate at which protein synthesis occurs depends upon the capacity and efficiency of the translational process. Chronic alterations in protein synthesis are thought to be a result of an increase in the number of ribosomes (14, 15), whereas acute changes in protein synthesis are thought to be due to changes in translation initiation (12, 13). In the current study, the feeding-induced stimulation of protein synthesis in both skeletal muscle and liver was driven by an increase in translational efficiency, *i.e.* the amount of protein synthesized per ribosome. Interestingly, the GH-induced increase in muscle protein synthesis in the fed state was also due to an increase in translational efficiency. In contrast to muscle, the GH-induced increase in liver protein synthesis in both the postabsorptive and postprandial states was driven by an increase in ribosome number rather than translational efficiency. Thus, the effect of GH treatment on the mechanisms driving the increase in muscle and liver protein synthesis appears to be tissue specific.

Changes in translation initiation factor activity have been induced by several different anabolic stimuli (*i.e.* feeding, insulin, amino acids, and IGF-I) in *in vitro* (17, 41), *in situ* (39, 54), and *in vivo* systems (34–36, 40). However, the mechanisms regulating the GH-induced increase in protein synthesis have not been determined previously in an *in vivo* model. A major regulatory process in translation initiation is the binding of mRNA to the 43S preinitiation complex via mediation of the assembly of the eIF4F complex of proteins (17, 18). Phosphorylation (20) or availability (21) of eIF4E regulates the formation of the eIF4F complex. Phosphorylation of eIF4E increases its association with eIF4G and eIF4A and influenced its binding to the m^7GTP cap at the 5′ end of mRNA (20, 22, 24). Availability of eIF4E is regulated by its association with 4E-BP1, a repressor protein that competes with eIF4G to bind to eIF4E (21). The phosphorylation of 4E-BP1 in the inactive eIF4E·4E-BP1 complex results in a decreased affinity of eIF4E for 4E-BP1, and this release of eIF4E enhances the binding of eIF4E to eIF4G to form the active eIF4E·eIF4G complex (26).

It was not unexpected to observe that feeding increased skeletal muscle and liver protein synthesis in our current study, as previous studies from our laboratory in neonatal swine have demonstrated similar results (34, 43, 49). These changes in protein synthesis in muscle and liver were regulated by increases in the activation of key translation initiation factors in both control and GH-treated swine, including increased phosphorylation of S6K1 kinase and 4E-BP1, decreased formation of the inactive eIF4E·4E-BP1 complex, and increased formation of the active eIF4E·eIF4G complex, as we have shown previously in control neonatal pigs (35, 36). Changes in muscle and liver protein synthesis were not, however, regulated by changes in eIF4E phosphorylation.

In the current study, GH increased skeletal muscle protein synthesis only in the postprandial condition. This increase in GH-stimulated muscle protein synthesis was regulated by increases in the phosphorylation of 4E-BP1 and an increase in the formation of the active complex eIF4E·eIF4G. Similar increases in 4E-BP1 phosphorylation and active eIF4E·eIF4G formation have been induced by insulin, IGF-I, or amino acid administration in growing animals (36, 38, 39). IGF-I has been shown recently to increase eIF4E to eIF4G binding in skeletal muscle of the mature rat (38) and both skeletal muscle and liver of the fetal lamb (39), although in the current study, the GH-induced increase in the association of eIF4E with eIF4G was present in skeletal muscle but not in liver. Whether the GH-induced increases in the activation of factors involved in the binding of mRNA to the ribosomal complex in muscle involve regulation by amino acids, insulin, or IGF-I remains to be determined.

A second major regulatory step in translation initiation is the binding of met-$tRNA_i$ to the 40S ribosomal subunit to form the 43S preinitiation complex via mediation of eIF2 (16). The eIF2-mediated met-$tRNA_i$ binding to the 40S subunit is further regulated by the activity of eIF2B, which exchanges GDP for GTP on eIF2 (16). In the current study, GH increased the activity of eIF2B in muscle but not liver. This suggests that the GH-induced increase in protein synthesis in muscle, but not liver, involves enhanced binding of met-$tRNA_i$ to the 40S ribosomal complex.

We further examined the activation by GH of factors involved in a signal transduction pathway leading to translation initiation, *i.e.* PKB and S6K1 phosphorylation. Insulin has been shown to increase muscle protein synthesis via a PI3-kinase/PKB pathway, which involves the phosphorylation of S6K1 and 4E-BP1 (36, 54). Amino acids are known to stimulate protein synthesis *in vivo* by interacting with the signaling pathway involving mammalian target of rapamycin (mTOR) (40), which is downstream of PI-3 kinase and PKB and upstream of 4E-BP1 and S6K1 phosphorylation. Inhibition of mTOR attenuates the feeding-induced assembly of both eIF4F and S6K1 activation (35). In the current study, feeding increased PKB phosphorylation in muscle of

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control and GH-treated pigs and in liver of GH-treated pigs. However, there was no effect of GH treatment on PKB phosphorylation in either muscle or liver of fasted or fed pigs. We did, however, observe a trend for GH in the fed state to increase S6K1 phosphorylation in muscle, consistent with the GH-induced increase in the phosphorylation of 4E-BP1. This would suggest that GH activates factors in the insulin/IGF-I signal transduction pathway that are downstream of PKB and/or mTOR. Whether this response involves alterations in the expression of GH, IGF-I, or insulin receptors remains to be determined.

Perspectives

There seem to be fundamental differences between skeletal muscle and liver in the GH-induced regulation of protein synthesis. This leads to a tissue-specific response of GH treatment in which increases in the efficiency of the translational process, via enhanced translation initiation factor activity, drive the GH-induced increase in muscle protein synthesis in the postprandial state, whereas elevations in ribosome number, not translational efficiency, drive the GH-induced increases in liver protein synthesis in both the postabsorptive and postprandial states. Furthermore, GH-induced stimulation of skeletal muscle protein synthesis in the postprandial state was regulated by mechanisms involving translation initiation by increasing the factors associated with the binding of both mRNA and met-tRNA$_i$ to the ribosomal complex such that enhanced phosphorylation of 4E-BP1, association of eIF4E with eIF4G, and eIF2B activity occurred, whereas the GH-induced increase in liver protein synthesis was not mediated by changes in translation initiation. Nevertheless, further study is needed to delineate the role of insulin, amino acids, and IGF-I in the tissue-specific effects of GH on protein synthesis.

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