Contribution of insulin to the translational control of protein synthesis in skeletal muscle by leucine

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Contribution of insulin to the translational control of protein synthesis in skeletal muscle by leucine. Am J Physiol Endocrinol Metab 282: E1092–E1101, 2002; 10.1152/ajpendo.00208.2001.—Enhanced protein synthesis in skeletal muscle after ingestion of a balanced meal in postabsorptive rats is mimicked by oral leucine administration. To assess the contribution of insulin to the protein synthetic response to leucine, food-deprived (18 h) male rats (~200 g) were intravenously administered a primed-constant infusion of somatostatin (60 μg + 3 μg kg−1·h−1) or vehicle beginning 1 h before administration of leucine (1.35 g·kg−1) or saline (control). Rats were killed 15, 30, 45, 60, or 120 min after leucine administration. Compared with controls, serum insulin concentrations were elevated between 15 and 45 min after leucine administration but returned to basal values by 60 min. Somatostatin maintained insulin concentrations at basal levels throughout the time course. Protein synthesis was increased between 30 and 60 min, and this effect was blocked by somatostatin.

Enhanced assembly of the mRNA cap-binding complex (composed of eukaryotic initiation factors eIF4E and eIF4G and hyperphosphorylation of the eIF4E-binding protein 1 [4E-BP1], the 70-kDa ribosomal protein S6 kinase [S6K1], and the ribosomal protein S6 [rp S6]) were observed as early as 15 min and persisted for at least 60 min. Somatostatin attenuated the leucine-induced changes in 4E-BP1 and S6K1 phosphorylation and completely blocked the change in rp S6 phosphorylation but had no effect on eIF4G·eIF4E assembly. Overall, the results suggest that the leucine-induced enhancement of protein synthesis and the phosphorylation states of 4E-BP1 and S6K1 are facilitated by the transient increase in serum insulin. In contrast, assembly of the mRNA cap-binding complex occurs independently of increases in insulin and, by itself, is insufficient to stimulate rates of protein synthesis in skeletal muscle after leucine administration.

amino acids; eukaryotic initiation factors; rats; somatostatin; time course

CONSUMPTION OF A PROTEIN-CONTAINING MEAL enhances the fractional rate of synthesis of total mixed proteins in skeletal muscle. The feeding-induced stimulation of protein synthesis requires the hormone insulin and an adequate supply of amino acids (14, 36, 37). The relative contribution of each of these regulatory factors to the increase in protein synthesis continues to be a topic of investigation and controversy. Some investigators favor the concept that amino acids independently regulate protein synthesis. However, amino acids can enhance pancreatic insulin release (11, 24), thereby complicating interpretation of results. Thus the stimulatory effect of amino acids on protein synthesis in skeletal muscle may be due in part to increases in circulating concentrations of insulin.

Numerous reports have established that, in skeletal muscle, the indispensable branched-chain amino acid leucine is unique in its ability to stimulate protein synthesis (3, 7, 8, 15, 22). Furthermore, the protein anabolic effect of leucine involves the initiation of mRNA translation (7, 22). We recently demonstrated that oral administration of leucine to food-deprived rats leads to hyperphosphorylation of the translational repressor eukaryotic initiation factor (eIF)4E-binding protein 1 (4E-BP1) and enhances the availability of the mRNA cap-binding protein eIF4E for binding eIF4G and assembly of the initiation complex known as eIF4F (2, 4). The eIF4F complex collectively serves to recognize, unfold, and guide the mRNA to the 43S preinitiation complex (30).

Additionally, leucine administration induces the hyperphosphorylation of the 70-kDa ribosomal protein S6 kinase (S6K1; see Refs 2 and 4). Phosphorylation of S6K1 is associated with its activation and results in hyperphosphorylation of ribosomal protein S6 (rp S6; see Refs 9 and 10). Activation of S6K1 facilitates the translation of a class of mRNAs containing terminal oligopyrimidine (TOP mRNAs) tracts at the 5′-end of the message (16). TOP mRNAs encode elements of the translational apparatus, including ribosomal proteins and elongation factors. Thus, by promoting the hyperphosphorylation of S6K1, leucine may enhance the synthesis of proteins involved in mRNA translation.

We previously reported that, when measured at 1 h, the stimulatory effects of oral leucine administration...
on both protein synthesis and translation initiation occurred without a concomitant increase in serum insulin concentration (2, 4). These reports suggested that the anabolic effect of leucine does not require an elevation in circulating insulin concentrations. However, since leucine has been shown to stimulate insulin secretion (24), the possibility exists that transient elevations in serum insulin occurred at an earlier time point and facilitated the protein synthetic response to leucine.

In the present study, time course changes in serum insulin concentrations were examined in food-deprived rats after a single oral bolus of leucine. Furthermore, the time course was defined for alterations in protein synthesis, the association of eIF4E with 4E-BP1 and eIF4G, and S6K1 phosphorylation. To examine the contribution of changes in serum concentrations of insulin to the increase in protein synthesis, a primed-constant infusion of somatostatin, an inhibitor of pancreatic hormone release, was administered intravenously to stabilize insulin at fasting values.

MATERIALS AND METHODS

Animals. The animal facilities and protocol were reviewed and approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine. Male Sprague-Dawley rats (~200 g) were maintained on a 12:12-h light-dark cycle with food (Harlan-Teklad Rodent Chow, Madison, WI) and water provided ad libitum. The food contained ~24% protein and 4% fat.

Placement of jugular catheters and infusion of somatostatin. In experiments in which somatostatin (Bachem, Torrance, CA) was used to inhibit insulin release after leucine administration, jugular catheters were inserted as described previously (31). Briefly, rats were anesthetized with an intraperitoneal injection of ketamine and xylazine (90 and 9 mg/kg, respectively), and sterile surgery was performed. A catheter was placed in the right jugular vein and advanced to the juncture of the inferior and superior vena cava. The catheter was secured in place with suture and exteriorized dorsally through a small skin incision at the base of the neck. The catheter was collared and secured with tape. Rats were returned to individual cages where food and water were provided ad libitum. Experiments were performed 3 days after surgery, at which time all rats had regained any lost body weight.

A primed-constant infusion of somatostatin (60 μg + 3 μg·kg⁻¹·min⁻¹) was administered intravenously via the jugular catheter beginning 30 min before oral administration of leucine or saline, as described below. Rats not administered somatostatin were infused with vehicle (0.155 M NaCl and 0.2% BSA) at the same rate (1.0 ml/h). The infusion was stopped briefly (<1 min) so that the rat could be gavaged (see below), and then the infusion was continued until sample collection. The dose of somatostatin was chosen because it had previously been shown to inhibit insulin release without significantly altering plasma glucose concentrations in the rat (21). In the studies reported herein, somatostatin maintained circulating insulin concentrations at fasting levels for at least 1 h after leucine administration, and it did not alter concentrations of the hormone when infused in food-deprived controls administered saline. Furthermore, previous studies have demonstrated that somatostatin does not adversely affect glucose uptake by skeletal muscle independent of the fall in plasma insulin concentrations (20).

Oral administration of leucine. In all experiments, rats were food deprived for 18 h and then administered saline (0.155 M) or 1.35 g l-leucine/kg body wt (prepared as 54.0 g/l-L-amino acid in distilled water) by oral gavage. The volume of saline or leucine administered was 2.5 ml/100 g body wt.

Administration of metabolic tracer and sample collection. A flooding dose (1.0 ml/100 g body wt) of [1-2,3,4,5,6-³H]phenylalanine (150 mM containing 100 μCi/ml) was injected intravenously 10 min before sample collection for the measurement of synthesis of total mixed proteins in skeletal muscle (13). Rats were killed by decapitation at 15, 30, 45, 60, or 120 min after leucine administration. Control rats were killed 30 min after receiving saline. Trunk blood was collected and centrifuged at 1,800 g for 10 min at 4°C to obtain serum. The left gastrocnemius and plantarius were excised as a unit and quickly frozen in liquid N₂ for the determination of muscle free amino acid concentrations, as described below. The contralateral hindlimb muscles were excised as a unit, weighed, and homogenized in 7 vol of buffer consisting of (in mM) 20 HEPES (pH 7.4), 100 KCl, 0.2 EDTA, 2 EGTA, 1 dithiothreitol, 50 sodium fluoride, 50 β-glycerophosphate, 0.1 phenylmethylsulfonyl fluoride, 1 benzamidine, and 0.5 sodium vanadate. An aliquot (0.5 ml) was used for the measurement of skeletal muscle protein synthesis, as described below. The remainder of the homogenate was immediately centrifuged at 10,000 g for 10 min at 4°C. The supernatant was used for measurement of translation initiation factor function and phosphorylation as described below.

Serum measurements. Serum insulin concentrations were analyzed using a commercial RIA kit for rat insulin (Linco Research, St. Charles, MO). Serum was analyzed for amino acids by derivatization with phenylisothiocyanate followed by HPLC analysis, as described previously (23).

Measurement of skeletal muscle amino acid concentrations. Frozen muscle samples were lyophilized and dissected free of visible blood and connective tissue. A portion (1–2 mg) of the sample was sonicated in 100 μl of deionized water using a high-intensity ultrasonic processor fitted with a stepped microtip (Thomas Scientific, Swedesboro, NJ), and the resulting homogenate was centrifuged for 3 min in an Eppendorf model 5415C centrifuge. The supernatant was analyzed for amino acids as described above for serum.

Measurement of skeletal muscle protein synthesis. Fractional rates of protein synthesis were estimated from the rate of radioactive phenylalanine incorporation into muscle protein by use of the specific radioactivity of serum phenylalanine as representative of the precursor pool (19). The elapsed time from injection of the metabolic tracer until homogenization of muscle was recorded as the actual time for incorporation of radiolabeled amino acid into protein.

Quantitation of 4E-BP1-eIF4E and eIF4G-eIF4E complexes. eIF4E was immunoprecipitated from 10,000-g supernatants of muscle homogenates with a monoclonal antibody to eIF4E (17). Next, samples were subjected to immunoblot analysis using polyclonal antibodies to either 4E-BP1 or eIF4G to determine the association of 4E-BP1 and eIF4G with eIF4E, respectively (17). Results were normalized to the amount of eIF4E in the immunoprecipitates.

Quantitation of phosphorylated and unphosphorylated eIF4E. The phosphorylated and unphosphorylated forms of eIF4E were separated by isoelectric focusing of 10,000-g supernatants on a slab gel and quantitated by protein immunoblot analysis, as described previously (17).

Examination of 4E-BP1 phosphorylation state. Aliquots of the 10,000-g supernatants of skeletal muscle were boiled for
10 min, cooled to room temperature, and then centrifuged at 10,000 g for 30 min at 4°C. The supernatants were then used for protein immunoblot analysis, as described previously (17). Briefly, 4E-BP1 resolves into multiple electrophoretic forms during SDS-PAGE depending on which, and how many, sites are phosphorylated (27, 28). Unlike the other two isoforms, the isoform exhibiting the slowest migration, referred to as the γ-form, does not bind to eIF4E, and thus the results are expressed as the ratio of 4E-BP1 in the γ-form to total.

**Phosphorylation of S6K1.** Phosphorylation of S6K1 was determined in 10,000-g supernatants by protein immunoblot analysis, as previously described (14). As discussed for 4E-BP1, resolution of S6K1 on SDS polyacrylamide gels results in the separation of the protein into multiple isoelectric forms with the slowest migrating forms representing hyperphosphorylated and the fastest migrating forms representing hypo- or dephosphorylated forms of the protein.

**Phosphorylation of S6K1 at Thr389.** Phosphorylation of S6K1 at Thr389 was determined in 10,000-g supernatants by protein immunoblot analysis, as previously described (17). Membranes were incubated with a rabbit polyclonal antibody, which specifically recognizes phosphorylation of S6K1 at Thr389 (New England Biolabs, Beverly, MA).

**Phosphorylation of rp S6.** Phosphorylation of rp S6 was determined in 10,000-g supernatants by protein immunoblot analysis, as previously described (18). Membranes were incubated with an anti-phosphopeptide antibody specific for phosphorylated rp S6 (a kind gift from Dr. Morris J. Birnbaum, Dept. of Medicine, University of Pennsylvania).

**Statistical analysis.** All data were analyzed by the STATISTICA statistical software package for Macintosh, volume II (StatSoft, Tulsa, OK). A one-way ANOVA was performed with the treatment group as the independent variable. When a significant interaction or overall effect was detected, differences among individual means were assessed with Duncan’s Multiple Range post hoc test. The level of significance was set at P < 0.05 for all statistical tests.

**RESULTS**

After oral administration of leucine, both serum and muscle concentrations of the amino acid were elevated within 15 min, reached maximum values between 30 and 45 min, and remained greater than control for at least 120 min (Fig. 1, A and B, respectively). Serum insulin concentrations were transiently elevated between 15 and 45 min after leucine administration and returned to control values by 60 min (Fig. 1C). Enhanced rates of protein synthesis were observed between 30 and 60 min after leucine administration and returned to baseline between 60 and 120 min (Fig. 1D).

Somatostatin infusion inhibited the increase in serum insulin concentrations observed in response to leucine administration, resulting in values that were not different from controls between 15 and 60 min (Fig. 2). Moreover, somatostatin blocked the stimulatory effect of leucine on muscle protein synthesis (Fig. 3). It is unlikely that the effect of somatostatin to block the stimulation of protein synthesis can be attributed to differences in leucine availability to skeletal muscle, because serum leucine concentrations were similarly elevated in control rats (−Leu = 158 ± 7 μmol/l vs. +Leu 2,695 ± 114 μmol/l, P < 0.05) and in rats infused with somatostatin (−Leu = 197 ± 19 μmol/l vs. +Leu 2,695 ± 114 μmol/l, P < 0.05).

![Fig. 1. Time course changes in serum insulin and leucine concentrations and free leucine content and protein synthesis in skeletal muscle of food-deprived rats after oral administration of leucine. A: serum leucine. B: free leucine in muscle. C: serum insulin. D: fractional rates of protein synthesis in skeletal muscle. Values are means ± SE; n = 4–10 rats. Means not sharing a superscript are different (P < 0.05). Phe: phenylalanine.](http://ajpendo.physiology.org/)

\[Leu\]
2,680 ± 205 μmol/l, P < 0.05) 30 min after oral administration of the amino acid.

Alterations in the phosphorylation state of 4E-BP1 preceded the stimulation of protein synthesis caused by leucine (Fig. 4A). Phosphorylation of 4E-BP1 was significantly elevated between 15 and 60 min after leucine administration, reaching a maximum at 30 min that was more than eightfold greater than control values. 4E-BP1 phosphorylation was not different from control values at 120 min. Enhanced phosphorylation of 4E-BP1 was associated with a decrease in the amount of the binding protein complexed to eIF4E, as suggested by coimmunoprecipitation experiments (Fig. 4B). After (15 min) leucine administration, the amount of inactive 4E-BP1-eIF4E complex was reduced to 60% of control values, and at 30 min the amount of 4E-BP1 associated with eIF4E was further reduced to ~25% of control. The binding of 4E-BP1 to eIF4E remained less than control for at least 1 h and returned to baseline by 120 min. The assembly of the eIF4G·eIF4E complex exhibited a reciprocal relationship compared with the association of 4E-BP1 with eIF4E. The amount of eIF4G bound to eIF4E was more than doubled by 15 min after leucine administration and reached a maximum between 30 and 45 min that was more than fourfold control values (Fig. 4C). At subsequent time points, eIF4G·eIF4E complex assembly returned toward control values; however, eIF4E remained greater than control values for the duration of the time course.

Infusion of somatostatin partially attenuated, but did not prevent, the leucine-induced hyperphosphorylation of 4E-BP1 (Fig. 5A). Regardless, the association of 4E-BP1 with eIF4E was reduced in rats infused with somatostatin after leucine administration to values that were equivalent to controls administered leucine (Fig. 5B). Assembly of the eIF4G·eIF4E complex was similarly enhanced in both control and somatostatin-treated rats after leucine administration (Fig. 5C). Thus assembly of the eIF4G·eIF4E complex occurred independently of increases in circulating concentrations of insulin and, by itself, was not sufficient to stimulate rates of synthesis of total mixed muscle proteins.

Some studies using cells in culture suggest that increases in eIF4E phosphorylation enhance mRNA cap-binding affinity and/or association with eIF4G. These changes are associated with enhanced rates of protein synthesis and cell growth in vitro (5, 25). Conversely, we previously showed that the phosphorylation of eIF4E decreases in skeletal muscle 1 h after leucine administration, even though rates of protein synthesis are elevated under these conditions (2, 4). In the present study, a transient decrease in eIF4E phosphorylation was observed between 30 and 45 min after leucine administration, suggesting that increases in eIF4E phosphorylation are not required to facilitate
enhanced rates of protein synthesis in skeletal muscle (Fig. 4D). However, although there was a trend for eIF4E to be reduced 30 min after leucine administration, the difference was not significant. Thus potential effects of somatostatin to alter the leucine-mediated decrease in eIF4E phosphorylation could not be established at this time point (Fig. 5D).

Phosphorylation of S6K1 was also rapidly induced after leucine administration (Fig. 6, A and B). By 15 min, S6K1 phosphorylation on Thr\(^{389}\), a residue whose phosphorylation is associated with increased activation of the kinase (6), was dramatically enhanced compared with control. Phosphorylation of S6K1 continued to increase until 30 min and thereafter returned to baseline between 60 and 120 min. The increase in S6K1 phosphorylation was associated with a rapid elevation in rp S6 phosphorylation (Fig. 6C). Phosphorylation of rp S6 was twofold greater than control by 15 min after leucine administration, more than threefold greater between 30 and 60 min, and remained elevated compared with control for the duration of the time course. Even at 120 min, rp S6 phosphorylation was twofold greater than control values. Thus rp S6 phosphorylation was sustained relative to S6K1 phosphorylation.

The leucine-induced phosphorylation of S6K1 was partially inhibited in rats infused with somatostatin compared with controls (Fig. 7, A and B). These alterations were most apparent when examining changes in the phosphorylation state of Thr\(^{389}\) (Fig. 7B). In fact, no phosphorylation of S6K1 on Thr\(^{389}\) was observed in two of the four samples obtained from rats infused with somatostatin and administered leucine. The attenuation of S6K1 phosphorylation caused by somatostatin was associated with an inhibition in the leucine-induced phosphorylation of rp S6 (Fig. 7C). Somatostatin infusion prevented the leucine-dependent increase in rp S6 phosphorylation, resulting in values that were not different from controls.

DISCUSSION

A number of studies indicate that dietary amino acids are essential for the enhanced rates of protein synthesis observed in skeletal muscle after food intake (14, 36, 37, 38). However, few studies have examined whether consumption of a large dose of a single amino acid can mimic the effects of feeding. It was recently reported that oral administration of leucine, alone or in combination with carbohydrate, restores rates of muscle protein synthesis in postabsorptive rats to values equivalent to those of freely fed controls 1 h after administration (2, 4). Moreover, leucine is as effective as a protein-containing meal in its ability to enhance recovery of muscle protein synthesis (unpublished observations). Hence, it appears that dietary leucine may mediate the anabolic effect of feeding on skeletal muscle protein synthesis. The results presented here suggest that, although leucine may independently signal to the translational apparatus to enhance rates of
protein synthesis in muscle, the stimulation is transient. Sustained increases may require the full complement of amino acids to ensure adequate substrate for protein synthesis. On the other hand, the duration of the stimulation of protein synthesis after leucine administration may be dependent on the changes in extracellular or intracellular leucine concentrations. As shown herein, rates of protein synthesis correlate with serum and muscle leucine concentrations. Thus the return of protein synthesis to baseline between 60 and 120 min may reflect a reduction in leucine delivery to skeletal muscle over time. Furthermore, elevations in serum insulin may be necessary to maintain enhanced rates of protein synthesis after oral administration of leucine, since somatostatin blocks the stimulatory effect of the amino acid on muscle protein synthesis.

Few studies have assessed the effect of increased amino acid concentrations on rates of protein synthesis in skeletal muscle while maintaining circulating insulin at fasting values. Watt and colleagues (34) used somatostatin to stabilize endogenous insulin in young pigs at fasting values while increasing amino acid concentrations through the intravenous administration of an amino acid mixture. The authors reported a stimulatory effect of amino acids alone on muscle protein synthesis, without the involvement of changes in circulating levels of pancreatic hormones. The reason for the apparent discrepancy between those results and the ones described herein are not readily apparent. One possible explanation is that the requirement for a transient rise in serum insulin to produce a stimulation of protein synthesis after amino acid administration varies with species. Another possible explanation is that the transient rise in insulin is not necessary to see the stimulatory effect of amino acids in younger animals. Indeed, prior studies have demonstrated that the response of protein synthesis in skeletal muscle to insulin decreases with development (35). Alternatively, differences in study design may explain the discrepancy. The experiments performed by Watt et al. (34) examined protein synthesis after infusion of a mixture of amino acids. In comparison, the study reported herein assessed changes in protein synthesis after administration of leucine alone. Therefore, the possibility cannot be eliminated that dietary amino acids other than leucine are required to see a protein synthesis response in the absence of a transient rise in serum insulin.

Several reports indicate that physiological increases in circulating insulin concentrations are not sufficient to stimulate protein synthesis in food-deprived rats (14, 36, 38, 32). When food-deprived rats are administered an oral bolus of carbohydrate alone, no change in protein synthesis is observed compared with starved
rats. In contrast, when food-deprived rats receive an isocaloric combination of carbohydrate plus leucine, rates of protein synthesis are stimulated nearly 40% and are equivalent to those of freely fed controls (2). Serum insulin concentrations in rats administered either the carbohydrate meal or the carbohydrate plus leucine meal were similar; hence, the enhanced rate of recovery cannot be attributed to a differential insulin response between the groups. Thus, although physiological increases in serum insulin do not independently stimulate protein synthesis in skeletal muscle of food-deprived rats, a transient increase in the circulating concentration of the hormone may be permissive for the leucine-induced stimulation of protein synthesis.

Accumulating evidence points to the protein kinase referred to as mammalian target of rapamycin (mTOR), which lies downstream of protein kinase B (also referred to as Akt) in the phosphoinositol 3-kinase signaling pathway, as a convergence point for both amino acid- and insulin-mediated effects on translation initiation. mTOR serves as a bifurcation point in the control of translation initiation, regulating the phosphorylation of both 4E-BP1 and S6K1. Experiments in HEK-293 cells demonstrate that insulin treatment induces phosphorylation of mTOR at Ser2448, a site that is considered crucial in the activation of the kinase (29). On the other hand, amino acid starvation reduces the phosphorylation of mTOR at the Ser2448 residue and makes the phosphorylation of this

![Fig. 6](image1.png)  
**Fig. 6.** Time course changes in the phosphorylation state of the 70-kDa ribosomal protein S6 kinase (S6K1) and ribosomal protein S6 (rp S6) in skeletal muscle of food-deprived rats after oral administration of leucine. A: phosphorylation of S6K1. Arrows indicate multiple electrophoretic forms of S6K1, with the most highly phosphorylated forms exhibiting the slowest electrophoretic mobility. B: phosphorylation of S6K1 on Thr389, a residue in which phosphorylation is associated with increased activation of the protein. Arrows indicate the location of the multiple electrophoretic forms of S6K1 as shown in A. C: phosphorylation of rp S6. Inset shows a representative immunoblot with phosphorylated rp S6 noted on right. Values are means ± SE; n = 6–10. Means not sharing a superscript are different (P < 0.05).

![Fig. 7](image2.png)  
**Fig. 7.** Phosphorylation states of the 70-kDa ribosomal protein S6K1 and rp S6 in skeletal muscle of food-deprived rats that were infused with somatostatin and administered leucine. Food-deprived rats received a primed-constant infusion of somatostatin (60 µg + 3 µg·kg⁻¹·min⁻¹ + Somatostatin) or were similarly infused with vehicle (0.155 mol/L NaCl and 0.2% BSA) via a jugular catheter. Later (1 h), one-half of the rats in each group were orally administered leucine. All measurements were made 30 min after leucine administration. A: phosphorylation of S6K1. Arrows indicate multiple electrophoretic forms of S6K1, with the most highly phosphorylated forms exhibiting the slowest electrophoretic mobility. B: phosphorylation of S6K1 on Thr389, a residue in which phosphorylation is associated with increased activation of the protein. Arrows indicate the location of the multiple electrophoretic forms of S6K1 as shown in A. C: phosphorylation of rp S6. Inset shows a representative immunoblot with phosphorylated rp S6 noted on right. Lane 1, −somatostatin rats that were food deprived; lane 2, + somatostatin rats that were food deprived; lane 3, + somatostatin rats that were orally administered leucine; lane 4, + somatostatin rats that were orally administered leucine. Values are means ± SE; n = 3–4. Means not sharing a superscript are different (P < 0.05).
site refractory to insulin. Recently the contribution of mTOR to the leucine-induced stimulation of protein synthesis and translation initiation was investigated (4). Food-deprived rats were injected intravenously with the immunosuppressant drug rapamycin, a specific inhibitor of mTOR, 2 h before oral leucine administration (4). It was reported that rapamycin completely prevents the leucine-induced hyperphosphorylation of both 4E-BP1 and S6K1. Thus available evidence suggests that leucine regulates translation initiation in the intact rat through a signaling pathway that involves mTOR. The results presented herein show that, although leucine enhances the phosphorylation of 4E-BP1 and S6K1 in the presence of fasting levels of insulin, a maximal response requires an indirect effect of leucine to cause a transient increase in the serum concentration of the hormone. Thus the possibility exists that mTOR may integrate both leucine- and insulin-mediated signals to enhance protein synthesis in skeletal muscle.

It is interesting to note that, although the results reported herein demonstrate that somatostatin partially inhibits the phosphorylation of 4E-BP1 after leucine administration, the association of eIF4E with eIF4G was not affected. There is some precedent for an enhanced association of eIF4G with eIF4E independent of alterations in 4E-BP1 phosphorylation or the association of eIF4E with 4E-BP1. In NIH/3T3 cells (26) or in Xenopus kidney cells (12) in culture, addition of serum to the medium enhances the association of eIF4E with eIF4G independent of alterations in the association of eIF4E with 4E-BP1. Furthermore, in perfused hindlimb preparations, elevating amino acid concentrations in the perfusate 10-fold increases the amount of eIF4G that immunoprecipitates with eIF4E from muscle extracts 8-fold but does not alter 4E-BP1 phosphorylation or the association of eIF4E with 4E-BP1 (33). Furthermore, leucine enhances the assembly of the eIF4G·eIF4E complex in the absence of increased 4E-BP1 phosphorylation in rats administered rapamycin (4). Thus it appears that the association of eIF4E with eIF4G can be regulated independently of changes in the phosphorylation state of 4E-BP1 through a pathway that does not involve mTOR.

Prior studies have suggested that the association of eIF4E and eIF4G may be rate limiting for enhanced rates of protein synthesis in skeletal muscle after leucine administration because changes in eIF4G·eIF4E complex formation closely mimic changes in muscle protein synthesis under these conditions (2–4). On the other hand, the results presented herein demonstrate that the leucine-induced increase eIF4G·eIF4E complex assembly is maintained in rats infused with somatostatin in the absence of a concomitant increase in protein synthesis. Therefore, some other step in translational control may be rate controlling for increasing protein synthesis under these conditions. Yoshizawa and colleagues (36) reported that feeding a diet containing 20% protein, but not a diet containing 0% protein, enhances initiation activity in muscles of mice fasted overnight. In contrast, elongation activity was increased in rats consuming either diet. In the present study, the ability of somatostatin to forestall the leucine-induced increase in protein synthesis could be due to a requirement for insulin in potentiating the elongation phase of mRNA translation once the initiation phase is stimulated.

Alternatively, the leucine-induced increase in eIF4G·eIF4E complex assembly in rats infused with somatostatin may be important in regulating the translation of a specific subpopulation of mRNAs. In cells in culture, enhanced formation of the eIF4G·eIF4E complex results in the preferential translation of those messages containing a high degree of secondary structure in the 5′-untranslated region. These include mRNAs that encode proteins that control cellular growth and development. For example, in L6 myoblasts, leucine readdition to the culture medium enhances the synthesis of ornithine decarboxylase (18). Such proteins may reflect only a small percentage of the total muscle proteins synthesized. Thus the assembly of eIF4G·eIF4E after leucine administration may function to promote the translation of a specific subset of mRNAs without significantly impacting the rate of synthesis of total mixed proteins in skeletal muscle.

In the present study, the changes in the phosphorylation state of rp S6 mimic alterations in protein synthesis. Phosphorylation of rp S6 regulates the synthesis of those messages containing TOP sequences at the 5′-end of the mRNA. Although TOP mRNAs may be derived from <200 genes, members of this family may account for up to 15% of total cellular mRNA (1). It follows, then, that alterations in the phosphorylation state of rp S6 may contribute to changes in the rates of protein synthesis by selectively modulating the translation of TOP mRNAs. However, it is unlikely that the selective inhibition of the translation of these messages could account for the 30% depression in global rates of protein synthesis observed in rats infused with somatostatin and administered leucine compared with controls. Therefore, the results suggest that some other step in the translational control of protein synthesis by leucine is affected in rats infused with somatostatin.

In conclusion, the results presented herein demonstrate that transient increases in serum insulin are permissive for the leucine-induced stimulation of protein synthesis in skeletal muscle. The transient rise in insulin also contributes to the hyperphosphorylation of 4E-BP1 and S6K1 caused by leucine. Because the phosphorylation of these proteins involves a signaling pathway that includes mTOR, the results suggest that mTOR may be a convergence point for both leucine- and insulin-mediated effects on certain steps in translation initiation. In addition, the results indicate that increases in the assembly of the eIF4G·eIF4E complex do not necessarily result in enhanced rates of protein synthesis. The latter finding suggests that another step in mRNA trans-
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