Dietary amino acids stimulate muscle protein synthesis after food intake (Gautsch et al. 1998, Yoshizawa et al. 1995 and 1998). This anabolic effect may be attributed in part to an increase in amino acid supply to the muscle, thereby augmenting substrate availability for peptide synthesis. Additionally, individual amino acids may function as nutritional signaling molecules that regulate mRNA translation. Indeed, we recently demonstrated that oral administration of leucine independently stimulates protein synthesis in muscle in association with enhanced rates of translation initiation (Anthony et al. 1999 and 2000). The remaining branched-chain amino acids (BCAA), isoleucine and valine, are similar in structure to leucine, and like leucine, are degraded extensively in skeletal muscle. Therefore, dietary isoleucine or valine may also signal independently for enhanced rates of translation initiation in muscle. Whether these BCAA exhibit anabolic potential similar to that of leucine remains to be determined.

A principal site in the regulation of translation initiation involves the binding of mRNA to the 40 S ribosome [reviewed by Pain (1996) and Voorma et al. (1994)]. Oral administration of leucine facilitates this process by increasing the availability of eukaryotic initiation factor 4E (eIF 4E), a protein that binds the m’GTP cap present at the 5’-end of the mRNA, for binding eIF4G, a large, 220-kDa polypeptide that functions as a scaffold for eIF4E, the mRNA (via association with eIF4E) and the ribosome (via association with eIF3) (Anthony et al. 2000). The increase in eIF4E availability is due in part to the leucine-dependent hyperphosphorylation of the translational repressor, eIF4E-binding protein 1 (4E-BP1). Increased phosphorylation of 4E-BP1 decreases its affinity for eIF4E, thereby facilitating the association of eIF4E with eIF4G.

Increased activity of the 70-kDa ribosomal protein S6 kinase (S6K1) has been implicated in stimulating protein synthesis under conditions that promote 4E-BP1 phosphorylation (Sonenberg 1996). We demonstrated previously that oral administration of leucine enhances the phosphorylation state of S6K1 (Anthony et al. 2000). Because phosphorylation of the...
kinase is associated with its activation (Cheatham et al. 1994, Chung et al. 1994), our previous observations suggest the involvement of S6K1 in stimulating protein synthesis after oral administration of leucine. The ability of leucine to promote the hyperphosphorylation of both 4E-BP1 and S6K1 suggests a common signaling pathway through which the amino acid upregulates translational efficiency.

Recent studies using cells in culture indicate that the hyperphosphorylation of 4E-BP1 and S6K1 by amino acids, and leucine, in particular, involves a signaling pathway that includes the protein kinase mTOR that is inhibited by the immunosuppressant drug rapamycin (Kimball et al. 1999, Patti et al. 1998, Xu et al. 1998). Therefore, leucine may stimulate translation initiation by modulating the activity of mTOR in vivo. The involvement of mTOR in stimulating protein synthesis in skeletal muscle after oral administration of leucine remains to be determined.

The objectives of the present study were twofold: 1) to determine whether leucine is unique among the BCAA in its ability to stimulate protein synthesis in skeletal muscle of food-deprived rats; and 2) to investigate whether changes in muscle protein synthesis after leucine administration involve a signaling pathway that includes mTOR. To investigate the role of mTOR signaling in the stimulation of protein synthesis and translation initiation in vivo, food-deprived rats were injected intravenously with the immunosuppressant drug rapamycin, a specific inhibitor of mTOR, before leucine administration.

MATERIALS AND METHODS

Animals and experimental design. 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-h light/dark cycle with food (Harlan-Teklad Rodent Chow, Madison, WI) and water provided freely. The food contained ~24% protein and 4% fat.

**Study 1.** Food-deprived (18 h) rats were assigned randomly to one of the following four dietary treatments: control (Con), or administered 1.35 g/kg body weight L-valine (Val), L-isoleucine (Ile) or L-leucine (Leu) by oral gavage. The dose for each amino acid was 2.5 mL/100 g body weight (prepared as 54.0 g/L in distilled water). Control rats were fed 2.5 mL saline/100 g body weight (0.155 mol/L). After amino acid administration, rats were returned to their cages where they were permitted free access to water only. The amount of each amino acid administered was equivalent to the amount of leucine consumed by rats of this age and strain during 24 h (Gautsch et al. 1998) of free access to an AIN-93 powdered diet (Harlan-Teklad, Madison, WI).

**Study 2.** Rats were food-deprived for 16 h and then randomly administered 0.75 mg rapamycin (Rap)/kg body weight (Calbiochem-Novabiochem, La Jolla, CA) or an equal volume of excipient (Con; 0.155 mol/L NaCl, 2% v/v ethanol) via the tail vein. Two hours later, one half of the rats in the Rap and Con groups were orally adminis-
tered 1.35 g/kg body weight as described in Study 1 (RapLeu and ConLeu, respectively). Rats not receiving leucine were gavaged with 2.5 mL saline/100 g body weight (0.155 mol/L).

**Administration of metabolic tracer and sample collection.** A flooding dose (1.0 mL/100 g body weight) of L-[2,3,4,5,6-3H] phenylalanine (150 mmol/L, containing 3.70 GBq/L) was injected via the tail vein 50 min after oral administration of amino acids for the measurement of synthesis of total mixed proteins in skeletal muscle (Garlick et al. 1980). Exactly 1 h after oral administration of amino acids, rats were killed by decapitation. Trunk blood was collected and centrifuged at 1800 × g for 10 min at 4°C to obtain serum. The gastrocnemius and plantaris muscles were excised as a unit, weighed and homogenized in 7 volumes of buffer consisting of (in mmol/L) 20 N, N', N'-tetraacetic acid, 1 dithiothreitol, 50 sodium fluoride, 50 β-glycerophosphate, 0.1 phenylmethylsulfonyl fluoride, 1 benzami-
dine 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 mea-
urement of eIF4 distribution and phosphorylation as described below.

**Serum measurements.** Serum insulin concentrations were ana-
lyzed using a commercial RIA kit for rat insulin (Linco Research, St. Charles, MO). Serum was analyzed for amino acids by derivatizing with phenylisothiocyanate and HPLC analysis as described previously (Mather and Nolan 1997). Serum measurements were performed on a commercial RIA kit for rat insulin (Linco Research, St. Charles, MO).

**Measurement of skeletal muscle protein synthesis.** Fractional rates of skeletal muscle protein synthesis were estimated from the rate of incorporation of radioactive phenylalanine into muscle protein using the specific radioactivity of serum phenylalanine as representa-
tive of the precursor pool (Kimball et al. 1992). The elapsed time from injection of the metabolic tracer until homogenization of muscles was recorded as the actual time for incorporation of labeled amino acid into protein (~13 min).

The phosphorylated and unphosphorylated forms of eIF4E were sep-
charged by isoelectric focusing of 10,000 × g supernatants on a slab gel and quantitated by protein immunoblot analysis as described previ-
ously (Kimball et al. 1997).

**Examination of 4E-BP1 phosphorylation state.** The phosphorylated and unphosphorylated forms of eIF4E were sep-
parated by isoelectric focusing of 10,000 × g supernatants on a slab gel and quantitated by protein immunoblot analysis as described previ-
ously (Kimball et al. 1997).

**Phosphorylation of S6K1.** Phosphorylation of S6K1 was deter-
mined in 10,000 × g supernatants by protein immunoblot analysis as previously described (Gautsch et al. 1998).

**Phosphorylation of S6K1 at Thr389.** Phosphorylation of S6K1 at Thr389 was determined in 10,000 × g supernatants by protein immunoblot analysis as described previously (Kimball et al. 1997). Mem-
branes were incubated with a rabbit polyclonal antibody, which specifically recognizes phosphorylation of S6K1 at Thr389 (New En-
gland Biolabs, Beverly, MA).

**Statistical analysis.** All data were analyzed by the STATISTI-
CA statistical software package for the Macintosh, version III (StatSoft, Tulsa, OK). All data are expressed as means ± SEM. If there were no differences among individual means were assessed with Duncan’s Multiple Range post-hoc test. In Study 2, a two-way ANOVA was performed to assess main vs. interaction effects with leucine administration and drug treatment as independent variables. If no significant interaction was detected, a one-way ANOVA was performed with treatment group (leucine + drug) 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**

In Study 1, oral administration of valine (Val), isoleucine (Ile) or leucine (Leu) to food-deprived rats did not alter circulating insulin concentrations compared with food-de-
prived controls (Table 1). In contrast, provision of valine, isoleucine or leucine elevated serum concentrations of the administered amino acid (Table 1). Additionally, leucine ad-

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ministration reduced circulating concentrations of isoleucine and valine compared with control rats. This reduction of serum isoleucine and valine after leucine administration may reflect an increase in uptake of those amino acids to support enhanced rates of protein synthesis (Table 1). Leucine was unique among the BCAA in its ability to stimulate skeletal muscle protein synthesis in vivo. Provision of leucine stimulated protein synthesis 65% compared with control rats. In contrast, neither valine nor isoleucine administration affected rates of protein synthesis.

Leucine was also most effective among the BCAA in its ability to increase the amount of eIF4E available for active eIF4G-eIF4E complex formation (Fig. 1). Leucine administration reduced the amount of the inactive 4E-BP1-eIF4E complex to 17% of values observed in controls (Fig. 1A). Concomitantly, the association of eIF4G with eIF4E was fourfold greater in rats fed leucine compared with food-deprived rats (Fig. 1C). This increase in the availability of eIF4E for binding eIF4G resulted from hyperphosphorylation of 4E-BP1. 4E-BP1 phosphorylation was fivefold greater in rats fed leucine compared with controls (Fig. 1B).

Although isoleucine administration was ineffective at stimulating protein synthesis, it did result in alterations in eIF4E availability. 4E-BP1 phosphorylation was 2.5-fold greater in rats fed isoleucine compared with saline-treated rats (Fig. 1B), and the amount of 4E-BP1 in the eIF4E immunoprecipitate was reduced to 65% of control values (Fig. 1A). Furthermore, the association of eIF4E with eIF4G doubled and was statistically intermediate between control rats and rats administered leucine (Fig. 1C). In contrast, valine administration did not alter either 4E-BP1 phosphorylation or the association of eIF4E with either 4E-BP1 or eIF4G. These results indicate that although oral administration of isoleucine promotes increased

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Insulin (pmol/L)</th>
<th>Valine (μmol/L)</th>
<th>Isoleucine (nmol phe/(mg protein h))</th>
<th>Leucine (nmol phe/(mg protein h))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>57 ± 3.0 (5)</td>
<td>170 ± 12b (3)</td>
<td>86 ± 4b (3)</td>
<td>129 ± 8b (3)</td>
</tr>
<tr>
<td>Valine</td>
<td>66 ± 7.5 (5)</td>
<td>9685 ± 493a (3)</td>
<td>116 ± 15b (3)</td>
<td>137 ± 18b (3)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>129 ± 30 (5)</td>
<td>188 ± 19b (3)</td>
<td>6146 ± 1909a (3)</td>
<td>125 ± 17b (3)</td>
</tr>
<tr>
<td>Leucine</td>
<td>104 ± 24 (5)</td>
<td>61 ± 9c (3)</td>
<td>21 ± 4c (3)</td>
<td>2086 ± 109a (3)</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM (n).
2 Means in a column not sharing a superscript are different (P < 0.05).
3 All measurements were made 1 h after amino acid administration.

**FIGURE 1** Amount of eukaryotic initiation factor (eIF) 4E-binding protein (4E-BP1) and eIF4G associated with eIF4E and phosphorylation state of 4E-BP1 and eIF4E in skeletal muscle of food-deprived rats (Con) or 1 h after oral administration of valine (Val), isoleucine (Ile) or leucine (Leu). (A) Amount of 4E-BP1 associated with eIF4E. Insert shows a representative immunoblot with positions of eIF4E and α- and β-forms of 4E-BP1 noted to the right. (B) Amount of 4E-BP1 in the γ-phosphorylated form as a percentage of the total 4E-BP1. Insert shows a representative immunoblot with positions of α-, β- and γ-forms of 4E-BP1 noted to the right. The most highly phosphorylated form, i.e., the γ-form, exhibits the slowest electrophoretic mobility and does not bind eIF4E. Therefore, phosphorylation of 4E-BP1 was expressed as the percentage of the protein in the γ-form. (C) Amount of eIF4G associated with eIF4E. Insert shows a representative immunoblot with eIF4G and eIF4E noted to the right. (D) Amount of phosphorylated eIF4E as a percentage of total eIF4E. Insert shows a representative immunoblot with phosphorylated [eIF4E(P)] and unphosphorylated forms of eIF4E noted to the right. Values are means ± SEM; n = 5–6. Means not sharing a superscript are different, P < 0.05.
Availability of eIF4E, these alterations in translation initiation do not lead to a stimulation of protein synthesis.

The effect of BCAA administration on eIF4E phosphorylation was also examined. Phosphorylation of eIF4E in cells in culture has been shown to be increased under a variety of conditions in which rates of translation initiation are accelerated (Sonenberg 1996). In Study 1, we did not observe any significant differences among treatment groups in the percentage of eIF4E in the phosphorylated form (Fig. ID). However, the absolute values obtained for eIF4E phosphorylation in rats fed leucine are less than those observed in the other treatment groups. Additionally, leucine administration resulted in an inhibition of eIF4E phosphorylation in Study 2. Further, we demonstrated previously that leucine reduces the amount of eIF4E in the phosphorylated form (Anthony et al. 2000). Collectively, these observations indicate that leucine administration reduces the amount of eIF4E in the phosphorylated form.

To further evaluate the effects of BCAA administration on translation initiation, we examined the relative abilities of leucine, isoleucine and valine to enhance phosphorylation of S6K1. During SDS-PAGE, S6K1 resolves into multiple electrophoretic forms, with increased phosphorylation corresponding to decreased electrophoretic mobility. The slowest migrating electrophoretic forms represent 56K1 phosphorylated on multiple residues including Thr389, a residue whose phosphorylation is associated with increased activation of the protein (Burnett et al. 1998). After food deprivation, the kinase became hypophosphorylated and, only the fastest migrating electrophoretic forms were observed (Fig. 2). Leucine was most effective among the BCAA in its ability to stimulate phosphorylation of S6K1 (Fig. 2A), particularly on Thr389 (Fig. 2B). Moreover, isoleucine administration also promoted phosphorylation of the kinase on Thr389 although to a lesser extent than leucine. Finally, administration of valine did not alter S6K1 phosphorylation compared with control rats.

In Study 2, food-deprived rats were injected with rapamycin before leucine administration to investigate whether the leucine-dependent stimulation of muscle protein synthesis involves mTOR signaling. Neither leucine administration nor rapamycin treatment had any effect on circulating concentrations of insulin and there were no differences among treatment groups (Table 2). In contrast, both leucine administration and drug treatment raised serum leucine concentrations (Table 2). Moreover, rapamycin independently elevated serum 3-methylhistidine concentrations (Table 2). Circulating 3-methylhistidine concentrations have been shown to correlate with rates of myofibrillar protein breakdown (Nagasawa et al. 1996). Therefore, the increase in serum leucine values in rats fed rapamycin may be due in part to enhanced degradation of myofibrillar proteins. Further studies are required to determine the involvement of mTOR in the regulation of muscle protein breakdown.

Leucine administration stimulated protein synthesis in skeletal muscle irrespective of drug treatment (Table 2). Protein synthesis rates in rats administered leucine alone were 42% greater than in food-deprived controls. Similarly, leucine also stimulated protein synthesis 35% in rats injected with rapamycin (Rap vs. RapLeu).

On the other hand, rapamycin treatment reduced rates of protein synthesis independently of leucine administration. Protein synthesis rates in rats treated with rapamycin and then administered leucine were only 72% of those in rats fed

### TABLE 2

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Insulin (μmol/L)</th>
<th>Leucine (μmol/L)</th>
<th>3-Methylhistidine (nmol phe/(mg protein · h))</th>
<th>Protein synthesis (nmol phe/(mg protein · h))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>95 ± 14 (7)</td>
<td>152 ± 11g (4)</td>
<td>8.76 ± 0.53 (4)</td>
<td>1.09 ± 0.11bc (7)</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>93 ± 11 (8)</td>
<td>203 ± 5c (4)</td>
<td>10.81 ± 1.13 (4)</td>
<td>0.83 ± 0.08bc (8)</td>
</tr>
<tr>
<td>Control + Leucine</td>
<td>104 ± 24 (8)</td>
<td>1794 ± 156 (4)</td>
<td>7.92 ± 0.57 (4)</td>
<td>1.55 ± 0.12 (8)</td>
</tr>
<tr>
<td>Rapamycin + Leucine</td>
<td>84 ± 23 (7)</td>
<td>3109 ± 2159 (4)</td>
<td>11.07 ± 1.08 (4)</td>
<td>1.12 ± 0.05c (8)</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM (n).
2 Means in a column not sharing a superscript are different (P < 0.05, one-way ANOVA).
3 All measurements were made 1 h after leucine administration.
4 Two-way ANOVA indicated significant main effects for leucine administration and drug treatment (P < 0.05) without significant interaction.
5 The asterisk indicates main effect of drug treatment (P < 0.05, two-way ANOVA).
6 Food-deprived rats were administered rapamycin (Rapamycin) or an equal volume of excipient (Control) via the tail vein. Two hours later, one half of the rats in the Rapamycin and Control groups were orally administered leucine (Rapamycin + Leucine and Control + Leucine, respectively).

![Phosphorylation of the 70-kDa ribosomal protein S6 kinase (S6K1) in skeletal muscle of food-deprived rats treated with rapamycin and then orally administered leucine](image-url)
leucine alone and were equal to those of food-deprived controls administered saline. Additionally, rapamycin also tended to inhibit protein synthesis in food-deprived rats (Con vs. Rap; P = 0.058). These results suggest that the leucine-dependent stimulation of muscle protein synthesis is rapamycin sensitive in part and involves mTOR.

Administration of leucine increased the availability of eIF4E for binding eIF4G (Fig. 3). Leucine reduced the association of 4E-BP1 with eIF4E to 50% of that in food-deprived rats (Fig. 3A). The leucine-dependent inhibition of eIF4E binding 4E-BP1 was associated with 4E-BP1 phosphorylation values that were fourfold greater than those of food-deprived controls (Fig. 3B). Consistent with the above data, eIF4G·eIF4E complex formation was more than doubled in rats fed leucine (Fig. 3C). Finally, leucine reduced the amount of eIF4E in the phosphorylated form compared with food-deprived rats.

Conversely, rapamycin treatment reduced the availability of eIF4E for binding eIF4G. Rapamycin administration completely blocked the leucine-dependent inhibition of 4E-BP1·eIF4E complex formation (Fig. 3A). Rapamycin administration was also associated with reduced 4E-BP1 phosphorylation (Fig. 3B). 4E-BP1 phosphorylation in drug-treated rats (Rap and RapLeu) was < 10% of that in rats fed leucine alone and significantly lower than that in food-deprived controls. Furthermore, rapamycin treatment reversed the effects of leucine on formation of the active eIF4G·eIF4E complex, resulting in values that were 50% less than those of ConLeu and not different than those of food-deprived controls (Fig. 3C). Additionally, rapamycin also inhibited the association of eIF4E with eIF4G in rats not receiving leucine (Con vs. Rap).

Finally, rapamycin reversed the leucine-dependent phosphorylation of S6K1 (Fig. 4). Leucine promoted hyperphosphorylation of the kinase and resulted in bands with the slowest electrophoretic mobility (Fig. 4A). The ability of leucine to stimulate phosphorylation of S6K1 was ablated in the presence of rapamycin. This was particularly evident when examining the phosphorylation of Thr389 (Fig. 4B). Phosphorylation of Thr389 was observed only in rats administered leucine alone. Little or no phosphorylation of this residue was observed in any other treatment group. These observations indicate that rapamycin reverses the leucine-dependent phosphorylation of S6K1 in skeletal muscle.

**DISCUSSION**

Remarkable progress has been made in the past few years in identifying insulin- and growth factor-induced modulators of translation initiation. However, investigations into the upstream signaling events that culminate in changes in mRNA translation by amino acids have been limited. Studies using cells in culture indicate that although amino acids can directly initiate signaling pathways that modulate translational effi-
study, we demonstrated for the first time that rapamycin inhibits leucine-dependent hyperphosphorylation of 4E-BP1 and S6K1 in skeletal muscle in vivo. These results indicate that oral administration of leucine increases the availability of eIF4E for active eIF4G·eIF4E complex formation as well as the activity of S6K1 through mTOR signaling in skeletal muscle.

Although mTOR signaling appears requisite for increasing the availability of eIF4E and the activity of S6K1, the data indicate that the leucine-dependent increase in skeletal muscle protein synthesis involves additional intracellular signaling pathways. For example, iso-leucine administration was able to increase 4E-BP1 and S6K1 phosphorylation without altering rates of protein synthesis. Additionally, leucine was able to stimulate protein synthesis in rats treated with rapamycin even though the absolute increase in protein synthesis was not as great as that seen in rats administered excipient. Finally, there was no statistical interaction between leucine administration and drug treatment on rates of protein synthesis (Table 2).

Collectively, the results presented here suggest that signaling through mTOR alone is not sufficient to explain the leucine-dependent stimulation of muscle protein synthesis in postabsorptive rats. Therefore, leucine administration may regulate additional steps in translation initiation.

The physiologic consequences of modifying the phosphorylation state of eIF4E remain to be determined. Studies using cells in culture suggest that an increase in eIF4E phosphorylation enhances mRNA cap-binding affinity and/or association with eIF4G. These changes augment rates of protein synthesis and cell growth (Bu et al. 1993, Minich et al. 1994). In contrast, experiments in vivo demonstrate that eIF4E phosphorylation either does not change, or increases and then decreases after food deprivation and refeeding, respectively (Yoshizawa et al. 1997 and 1998). Furthermore, we demonstrated previously that oral administration of leucine results in a net dephosphorylation of eIF4E compared with food-deprived rats (Anthony et al. 2000). The results of the present study support these findings because the proportion of eIF4E in the phosphorylated form was reduced in rats administered leucine. The basis for the leucine-dependent decrease in eIF4E phosphorylation is unknown; however, the data presented here suggest that this inhibition is rapamycin sensitive, implicating mTOR in the regulation of eIF4E phosphorylation.

In conclusion, these data suggest that leucine is unique among the BCAA in its ability to stimulate protein synthesis in muscle of food-deprived rats. Further, leucine was also most effective in enhancing translation initiation by increasing the availability of eIF4E for formation of the active eIF4G·eIF4E complex and through hyperphosphorylation of S6K1. Administration of rapamycin inhibited the stimulatory effects of leucine on both protein synthesis and translation initiation. These results demonstrate that leucine-dependent stimulation of translation initiation in food-deprived rats occurs via a rapamycin-sensitive pathway and likely involves mTOR. However, the ability of iso-leucine to hyperphosphorylate 4E-BP1 and S6K1 in the absence of increased rates of protein synthesis as well as the ability of leucine to stimulate protein synthesis in drug-treated rats would indicate that mTOR signaling alone does not account for the stimulatory effect of leucine on muscle protein synthesis.

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**LITERATURE CITED**


