Leucine and insulin activate p70 S6 kinase through different pathways in human skeletal muscle

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Greiwe, Jeffrey S., Guim Kwon, Michael L. McDaniel, and Clay F. Semenkovich. Leucine and insulin activate p70 S6 kinase through different pathways in human skeletal muscle. Am J Physiol Endocrinol Metab 281: E466–E471, 2001.—Amino acids and insulin have anabolic effects in skeletal muscle, but the mechanisms are poorly understood. To test the hypothesis that leucine and insulin stimulate translation initiation in human skeletal muscle by phosphorylating 70-kDa ribosomal protein S6 kinase (p70S6k), we infused healthy adults with leucine alone (n = 6), insulin alone (n = 6), or both leucine and insulin (n = 6) for 2 h. p70S6k and protein kinase B (PKB, also known as Akt) (30). However, mTOR can also be activated independently of insulin. Branched-chain amino acids, specifically leucine, are the most potent stimulators of mRNA translation in rat skeletal muscle (1, 2) and in various cell lines (27, 36).

The anabolic effects of insulin have been studied for decades (26), but new participants in this process continue to be identified. ATM, the protein that is mutated in the disease ataxia telangiectasia, is stimulated by insulin in cultured cells to phosphorylate 4EBP-1 (38), an observation that may help explain the insulin resistance and abnormalities of growth seen in this disease. However, 4EBP-1 may not be critical for insulin-stimulated protein synthesis in skeletal muscle. Recent data suggest that physiological levels of insulin cause phosphorylation of p70S6k, but not 4EBP-1, in human skeletal muscle (16). In various cell types, p70S6k has been shown to be phosphorylated by a kinase known as the mammalian target of rapamycin (mTOR, also called FRAP). mTOR, a member of the ATM-related kinase family, can be activated by the insulin receptor signaling cascade through sequential involvement of insulin receptor substrate-1 and -2 (IRS-1 and -2), phosphatiidylinositol (PI) 3-kinase, and protein kinase B (PKB, also known as Akt) (30). However, mTOR can also be activated independently of insulin. Branched-chain amino acids such as leucine appear to activate this kinase in cultured cells through a nutrient-signaling pathway (27, 28), an observation that helps explain why amino acids have striking effects on protein synthesis in insulin-resistant states.

protein synthesis; translation initiation; hyperinsulinemia; mammalian target of rapamycin; insulin resistance

BOTH AMINO ACIDS AND INSULIN have anabolic effects on human skeletal muscle, but the underlying mechanisms remain elusive. Increasing amino acid availability by intravenous or oral administration increases amino acid transport and protein synthesis in human skeletal muscle (5, 23, 29, 34). Amino acids can also increase mRNA translation (independently of merely serving as substrates for synthesis) through a rapamycin-sensitive pathway involving two key regulatory proteins, 70-kDa ribosomal protein S6 kinase (p70S6k) and eukaryotic initiation factor 4E-binding protein-1 (4EBP-1, also known as PHAS-I) (1, 2, 18, 21, 32, 39). Branched-chain amino acids, specifically leucine, are the most potent stimulators of mRNA translation in rat skeletal muscle (1, 2) and in various cell lines (27, 36).

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Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Women (n = 8)</th>
<th>Men (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>21 ± 1</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>Height, cm</td>
<td>165 ± 9</td>
<td>182 ± 9</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>60.7 ± 9</td>
<td>80.5 ± 14.6</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22.3 ± 1.8</td>
<td>24.0 ± 2.8</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>27 ± 5</td>
<td>15 ± 7</td>
</tr>
<tr>
<td>VO₂max, ml·kg⁻¹·min⁻¹</td>
<td>36.6 ± 4.7</td>
<td>44.4 ± 6.9</td>
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</tbody>
</table>

Values are means ± SD. BMI, body mass index. VO₂max, maximal oxygen uptake. Body fat was determined by dual-energy X-ray absorptiometry.

EXPERIMENTAL PROCEDURES

Subjects. Subjects were healthy young women and men with no known disease or metabolic disorders. Body composition was assessed for each subject by dual-energy X-ray absorptiometry as described previously (19). Each subject provided written consent before participation. The study was approved by the Washington University Human Studies Committee.

Maximal oxygen consumption. To characterize individual fitness levels, maximal oxygen consumption was measured during a continuous, incremental treadmill test to exhaustion, as described previously (14). At least two of the following criteria were met for each subject: plateau in oxygen consumption with increasing work rate, heart rate ≥ 10 beats/min of age-predicted maximal heart rate, and a respiratory exchange ratio > 1.10.

Infusion protocols. Subjects reported to the General Clinical Research Center at the Washington University School of Medicine in the morning after an overnight fast. The subjects were randomly assigned to receive a 2-h infusion of leucine alone, insulin alone, or leucine + insulin in combination. Before each infusion, a catheter was inserted into an antecubital vein and served as the infusion line. In the contralateral hand, another catheter was inserted for sampling. The hand was kept in a 65°C box to provide arterialized venous samples. Subjects remained in a semisupine position during the entire procedure.

Muscle tissue samples were obtained before the start of the infusion and immediately after the infusion from the contralateral leg. Biopsies were obtained from the lateral head of the vastus lateralis muscle, ~10–18 cm proximal to the patella. After administration of lidocaine, an incision (~1 cm) was made in the skin and fascia covering the muscle, and a Bergstrom needle was used to obtain ~75–100 mg of tissue. The tissue was immediately rinsed with sterile saline, blotted dry, trimmed of any visible fat, and quickly frozen in separate aliquots in liquid nitrogen. Samples were then stored at −80°C until subsequent analysis.

The leucine infusion protocol consisted of infusing leucine at a constant rate of 1 g/h for 2 h. Plasma glucose was monitored every 5 min during the 1st h and every 15 min during the 2nd h.

The insulin infusion protocol consisted of a 2-h hyperinsulinemic euglycemic clamp. Insulin was infused in an exponentially decreasing manner until a rate of 40 mU·m⁻²·min⁻¹ was achieved. Plasma glucose concentration was measured every 5 min, and a 20% dextrose solution was infused to maintain plasma glucose at ~90 mg/dl.

The 2-h leucine + insulin infusion protocol consisted of a combination of the euglycemic hyperinsulinemic clamp and the leucine infusion, as described.

Table 2. Plasma leucine concentrations

<table>
<thead>
<tr>
<th>Condition</th>
<th>Plasma Leucine (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine infusion</td>
<td>119 ± 11</td>
</tr>
<tr>
<td>Leucine + insulin infusion</td>
<td>113 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Significant difference from the baseline concentration (P < 0.001). The infusion sample was obtained during the last minute of the 2-h infusion.

Muscle tissue analyses. Muscle tissue was homogenized in a freshly prepared buffer containing (in mM): 20 Tris-HCl (pH 7.5), 100 potassium chloride, 100 sodium fluoride, 1 EDTA, 50 β-glycerophosphate, and 1 sodium orthovanadate, and 10 mg/ml phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin hemisulfate, and 10 mg/ml aprotinin. Homogenates were analyzed for p70S6K and serine⁷³⁷ (Ser⁷³⁷)-phosphorylated PKB by SDS-PAGE and Western blotting, as described previously (15, 36, 37).

RESULTS

Subject characteristics are shown in Table 1. Eight women and 10 men participated in this study. Responses to leucine alone (2 women and 4 men), insulin alone (3 women and 3 men), and leucine + insulin infusion (3 women and 3 men) were similar for women and men; hence, data from both genders were pooled.

Table 3. Plasma glucose concentrations

<table>
<thead>
<tr>
<th>Condition</th>
<th>Plasma Glucose (mg/dl)</th>
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</thead>
<tbody>
<tr>
<td>Insulin infusion</td>
<td>89 ± 0.9</td>
</tr>
<tr>
<td>Leucine infusion</td>
<td>89 ± 1.0</td>
</tr>
<tr>
<td>Leucine + insulin infusion</td>
<td>88 ± 0.6</td>
</tr>
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Values are means ± SE and represent the average glucose concentration during the 2-h infusion.
stimulate insulin secretion (12). However, previous investigators using leucine infusion conditions identical to those in the present study also reported no effect on plasma insulin levels (11). Plasma insulin levels were modestly but significantly higher during the leucine + insulin infusion (¶, Fig. 1) compared with the infusion of insulin alone (●, Fig. 1), an unexpected observation because leucine alone had no effect on insulin concentrations (○, Fig. 1).

The infusion of leucine alone, insulin alone, and leucine + insulin each caused a significant increase in p70S6k phosphorylation. Insets in Fig. 2 show the typical appearance of p70S6k and its phosphorylated isoforms on Western blots after 2-h infusions of leucine alone, insulin alone, and leucine + insulin. Phosphorylation of p70S6k retards its electrophoretic mobility when subjected to SDS-PAGE. We quantified p70S6k phosphorylation by measuring the ratio of the slower-migrating phosphorylated forms to the total mass of p70S6k detected on Western blots. In the fasted condition (before infusion), 3.5 ± 1.0% of total p70S6k was in the phosphorylated state. p70S6k phosphorylation increased 4-, 8-, and 18-fold in response to leucine alone, insulin alone, and leucine + insulin infusion, respectively (Fig. 2). If the effects of leucine and insulin were merely additive, infusing them in combination would be expected to cause a 12- to 14-fold increase in p70S6k phosphorylation. A twelvefold increase would be predicted by combining the results from leucine alone (4-fold) and insulin alone (8-fold), but insulin levels were ~20% higher when insulin is infused with leucine compared with insulin infusion alone (Fig. 1). With the assumption that the higher levels of insulin under these conditions have physiological effects on p70S6k that are linear (which may not be correct on the basis of the PKB data below), the increase associated with leucine + insulin could be estimated at 14-fold. However, p70S6k phosphorylation was increased 18-fold (Fig. 2, open bar, far right), suggesting that leucine and insulin act in synergy to activate p70S6k.

Insulin alone and leucine + insulin each caused the same twofold increase in PKB Ser473 phosphorylation, whereas leucine had no effect on PKB Ser473 phosphorylation (Fig. 3). These results indicate that insulin-stimulated p70S6k phosphorylation involves the activation of PKB. They also suggest that the higher levels of plasma insulin detected in the leucine + insulin infusions (Fig. 1) did not result in greater signaling through the insulin receptor cascade, because PKB phosphorylation was not increased to a greater extent in the leucine + insulin compared with the insulin-alone protocols.

The infusion of leucine alone stimulated p70S6k phosphorylation (Fig. 2) but did not affect plasma insulin levels (Fig. 1) and had no effect on PKB activation (Fig. 3). These results indicate that leucine acts independently of insulin.

DISCUSSION

p70S6k is a key regulatory protein involved in the initiation of mRNA translation. In this study, we provide evidence that physiological concentrations of leucine and insulin activate this protein by different mechanisms in human skeletal muscle. Insulin phosphorylates PKB, a known activator of p70S6k and an important regulator of insulin-stimulated cell growth (33). Leucine activates p70S6k without affecting PKB and has a synergistic effect when combined with insulin. Taken together, the data are consistent with the existence of distinct pathways for stimulating muscle...
protein synthesis in human skeletal muscle (Fig. 4), one driven by insulin (or other growth factors) and another mediated by signaling through amino acids such as leucine.

Increasing amino acid availability increases amino acid transport and protein synthesis in human skeletal muscle (5, 23, 29, 34). In addition to increasing substrate availability, amino acids activate p70\textsuperscript{S6k} and 4EBP-1 (PHAS-I) in several cell types (8, 27, 36) and in rat (1, 2, 32, 39) and pig (18) skeletal muscle. This effect is mediated by mTOR (2, 18, 27, 30, 36). In the present work, we present evidence that leucine stimulates p70\textsuperscript{S6k} phosphorylation despite the lack of change in plasma insulin concentration or PKB activation. In contrast to insulin, which requires PKB for mTOR activation, amino acids activate mTOR independently of PKB activation (2, 18, 27, 30, 36). The nature of this activation may be complex. Recent data indicate that both mTOR and p70\textsuperscript{S6k} shuttle between the nucleus and cytoplasm (17). Our findings in human skeletal muscle could thus be explained either by an increase in the intrinsic kinase activity of mTOR after leucine infusion or by increased availability of either mTOR or p70\textsuperscript{S6k} at cytoplasmic sites.

Our results do not preclude the possibility that adequate basal levels of insulin are required for leucine activation of p70\textsuperscript{S6k}. Studies in diabetic mice support the notion that basal insulin may be necessary for priming protein synthesis (31). However, the effects of leucine clearly occur independently of the elevated levels of insulin required to stimulate PKB phosphorylation in muscle. Elevated levels of insulin are known to stimulate protein synthesis in human skeletal muscle (4, 6). Physiological hyperinsulinemia stimulates p70\textsuperscript{S6k} phosphorylation without affecting 4EBP-1 phosphorylation in human skeletal muscle (16). In cultured cells, p70\textsuperscript{S6k}, but not 4EBP-1, can shuttle between the nucleus and cytoplasm (17), providing additional evidence that these proteins are regulated differently. Our results suggest that insulin-stimulated phosphorylation of skeletal muscle p70\textsuperscript{S6k} is mediated by signals (see Fig. 4) generated by the insulin receptor or other growth factor receptors such as the insulin-like growth factor-I receptor. Consistent with this concept, wortmannin, a known inhibitor of PI 3-kinase, and rapamycin, a known inhibitor of mTOR, block insulin-stimulated phosphorylation of p70\textsuperscript{S6k} in rat skeletal muscle (9, 10, 13, 27). Insulin activation of mTOR is mediated by PKB in various cell types (30, 35).

Leucine and insulin appear to have a synergistic effect on p70\textsuperscript{S6k} phosphorylation (Fig. 2). Plasma insulin levels were ~20% higher (Fig. 1) during the leucine + insulin infusion compared with the insulin infusion alone, but the induction of PKB phosphorylation was not higher in leucine + insulin-infused muscle (Fig. 3), making it unlikely that the difference in insulin levels was responsible for the synergistic effect of leucine + insulin. One potential explanation is that insulin promotes increased blood flow to skeletal muscle (3, 6). Muscle protein synthesis is positively correlated with blood flow (5). Insulin also increases amino acid uptake into skeletal muscle (4, 34), which would increase the availability of leucine to activate p70\textsuperscript{S6k} and promote translation.

The finding of higher insulin levels when leucine and insulin were infused together was surprising, especially because leucine alone did not increase plasma insulin (Fig. 1). These findings suggest that leucine impairs insulin clearance when insulin concentrations are elevated. Under normal conditions, insulin is cleared from the plasma mostly by receptor-dependent protein degradation in liver and muscle. Glomerular
filtration and tubular transport in the kidney play a smaller role. Although no data directly address the relationship between insulin clearance and leucine levels, there is evidence that leucine acutely suppresses protein degradation in both liver and muscle (22, 25), making it possible that leucine alters the degradation of insulin under conditions of insulin excess.

Insulin resistance is associated with common conditions including type 2 diabetes, the postoperative state, trauma, pregnancy, and infections. Although the relationship between muscle function and insulin resistance has not been defined, many of these conditions are characterized by weakness, which is poorly understood and inadequately treated. For example, handgrip strength appears to be negatively associated with markers of insulin sensitivity (20). Our results indicate that leucine activates a key protein in muscle protein synthesis independently of insulin signaling. Future studies will determine whether the administration of branched-chain amino acids such as leucine can activate skeletal muscle 70S6k to improve protein synthesis and muscle function in insulin-resistant states.

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