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. McDaniels, 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 (p70(S6K)), we infused healthy adults with leucine alone (n = 6), insulin alone (n = 6), or both leucine and insulin (n = 6) for 2 h. p70(S6K) and protein kinase B (PKB) serine473 phosphorylation were measured in vastus lateralis muscles. Plasma leucine increased from ∼116 to 343 μmol/l during the leucine-alone and leucine + insulin infusions. Plasma insulin increased to ∼400 pmol/l during the insulin-alone and leucine + insulin infusions and was unchanged during the leucine-alone infusion. Phosphorylation of p70(S6K) increased 4-fold in response to leucine alone, 8-fold in response to insulin alone, and 18-fold after the leucine + insulin infusion. Insulin-alone and leucine + insulin infusions increased PKB phosphorylation, but leucine alone had no effect. These results show that physiological concentrations of leucine and insulin activate a key mediator of protein synthesis in human skeletal muscle. They suggest that leucine stimulates protein synthesis through a nutrient signaling mechanism independent of insulin, raising the possibility that administration of branched-chain amino acids may improve protein synthesis in insulin-resistant states.

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 (p70(S6K)) 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).

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 p70(S6K), but not 4EBP-1, in human skeletal muscle (16). In various cell types, p70(S6K) 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), phosphatidylinositol (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 model systems. Whether leucine can activate mTOR in human skeletal muscle to cause p70(S6K) phosphorylation and increase translation is unknown.

In this study, we sought to determine whether leucine and insulin affect p70(S6K) phosphorylation in human skeletal muscle. To determine whether these agents act through the same or different pathways, we also assayed the phosphorylated form of PKB (to assess activation of insulin signaling) and performed infusions of leucine and insulin in combination. Our results show that leucine and insulin activate p70(S6K) through distinct pathways in human muscle, raising the possibility that modulating nutrient-signaling pathways may represent an innovative strategy for improving skeletal muscle metabolism.
the vastus lateralis muscle, infusion and immediately after the infusion from the con-eral hand, another catheter was inserted for sampling. The remaining supernatant was stored at

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 ante-cubital 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 hyperinsu-linemic 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 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 ± 2</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>(\text{VO}_{2\text{max}}, \text{mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1})</td>
<td>36.6 ± 4.7</td>
<td>44.4 ± 6.9</td>
</tr>
</tbody>
</table>

Values are means ± SD. BMI, body mass index. \(\text{VO}_{2\text{max}}\), maximal oxygen uptake. Body fat was determined by dual-energy X-ray absorptiometry.

Table 2. Plasma leucine concentrations

<table>
<thead>
<tr>
<th>Condition</th>
<th>Plasma Leucine ((\mu\text{mol/l}))</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<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 aprotonin. Homogenates were analyzed for p70S6K and serine\(^{73}\) (Ser\(^{73}\)-phosphorylated PKB by SDS-PAGE and Western blotting, as described previously (15, 36, 37).

Plasma analyses. Arterialized venous samples were subjected to centrifugation, and the supernatant was analyzed for plasma glucose with a YSI 2300 Stat Plus analyzer (Yellow Springs Instrument, Yellow Springs, OH) at the time of sampling. The remaining supernatant was stored at ~80°C. Plasma samples were analyzed for insulin by radioimmunoassay (24). Plasma leucine was measured by high-performance liquid chromatography in samples obtained before the infusion and in samples obtained during the last 1 min of the 2-h infusion.

Statistical analyses. Analysis of variance was performed to analyze data between the leucine alone, insulin alone, and leucine + insulin infusion protocols. A Tukey’s post hoc test was performed when analysis of variance revealed a significant difference \(P < 0.05\).

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. Plasma leucine increased about threefold during the leucine-alone and leucine + insulin infusions (Table 2). Leucine concentrations achieved by infusion were within the range associated with eating a high-protein meal (7). Plasma glucose concentration was maintained at ~89 mg/dl during each of the three infusion protocols (Table 3). As expected, plasma insulin was elevated during the insulin-alone and leucine + insulin infusions (Fig. 1). Leucine infusion alone had no effect on plasma insulin levels (C, Fig. 1). At high concentrations, leucine can

Table 3. Plasma glucose concentrations

<table>
<thead>
<tr>
<th>Condition</th>
<th>Plasma Glucose (mg/dl)</th>
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<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>
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Values are means ± SE and represent the average glucose concentration during the 2-h infusion.
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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, 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, 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 are ∼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 Ser 473 phosphorylation, whereas leucine had no effect on PKB Ser 473 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

Fig. 1. Plasma insulin concentrations during the infusion protocols. *Significant difference between levels during the infusion of leucine alone (○) and insulin alone (●) (P < 0.01); †significant difference between insulin levels during the infusion of insulin alone (●) and leucine + insulin (▼) (P < 0.05). Values represent means ± SE; n = 6 for each data point.

Fig. 2. Phosphorylated ribosomal protein p70 S6 kinase (p70S6K) before (filled bars) and immediately after (open bars) a 2-h infusion of leucine alone (left), insulin alone (middle), or leucine + insulin (right). *Significant difference between baseline and postinfusion samples (P ≤ 0.01); †significant difference compared with either of the remaining 2 open bars (P < 0.01). Data are expressed as the percentage of total p70S6K in the phosphorylated state and represent means ± SE; n = 6 for each condition. Insets: photographs of representative Western blots of muscle after leucine alone (left), insulin alone (middle), and insulin + leucine (right) infusions. For each photo, the left lane represents the baseline sample and the right lane the postinfusion sample.
Leucine and insulin appear to have a synergistic effect on p70S6k 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 p70S6k 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 p70S6k to improve protein synthesis and muscle function in insulin-resistant states.

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