Leucine is a direct-acting nutrient signal that regulates protein synthesis in adipose tissue

CHRISTOPHER J. LYNCH, BRIAN J. PATSON, JOSHUA ANTHONY, ALAIN VAVAL, LEONARD S. JEFFERSON, AND THOMAS C. VARY
Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Received 26 February 2002; accepted in final form 6 May 2002

Lynch, Christopher J., Brian J. Patson, Joshua Anthony, Alain Vaval, Leonard S. Jefferson, and Thomas C. Vary. Leucine is a direct-acting nutrient signal that regulates protein synthesis in adipose tissue. Am J Physiol Endocrinol Metab 283: E503–E513, 2002. First published May 7, 2002; 10.1152/ajpendo.00084.2002.—In freshly isolated rat adipocytes, leucine or its analog norleucine activates the mammalian target of rapamycin (mTOR)-signaling pathway. This results in phosphorylation of the ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), two proteins involved in the initiation phase of protein synthesis. The purpose of the studies reported herein was to address the question of whether or not these in vitro effects of leucine and norleucine on adipocytes could be extended to the intact animal and to other tissues. To accomplish this, food-deprived (18 h) male Sprague-Dawley rats were orally administered solutions (2.5 ml/100 g body wt) containing normal saline (0.9% NaCl), a carbohydrate mixture (26.2% d-glucose and 26.2% sucrose), leucine (5.4%), or norleucine (5.4%). The protein synthetic responses of adipose tissue were measured and compared with those of other tissues. In addition, S6K1 and 4E-BP1 phosphorylation was measured, as was the plasma concentration of insulin and tissue ATP concentrations. Leucine administration stimulated protein synthesis in adipose tissue, gastrocnemius, and kidney but not in liver and heart. Norleucine stimulated protein synthesis in all of the tissues tested but, in contrast to leucine, without affecting plasma insulin concentrations. The carbohydrate meal had no effect on protein synthesis in any tissue tested but elicited a robust increase in plasma insulin. These findings provide support for a role of leucine as a direct-acting nutrient signal for stimulation of protein synthesis in adipose tissue as well as other select tissues. In adipose tissue, the effects of the different treatment conditions on the acute regulation of protein synthesis closely correlated with changes in phosphorylation of S6K1 and 4E-BP1; however, this correlation did not exist in all tissues examined. This result implies that leucine or norleucine may acutely stimulate protein synthesis, at least in some tissues, by a mechanism that is independent of both S6K1 and 4E-BP1 phosphorylation.

The branched-chain amino acids (leucine, isoleucine, valine) are the most abundant of the essential amino acids. In addition to being indispensable for life, the branched-chain amino acids are efficacious regulators of protein synthesis, protein degradation, and insulin secretion and synthesis (1, 11–15, 23, 31, 35, 50, 53–55, 59, 62, 68, 71, 76, 81, 82). Of the three branched-chain amino acids, leucine seems to be the most potent with regard to most of these effects and may therefore be the most physiologically relevant (45).

On the basis of acute in vitro studies, leucine appears to stimulate protein synthesis by activation of cytosolic cell-signaling pathways similar to those used by insulin (40, 57, 58). At least two signaling pathways appear to be important in this acute regulation of protein synthesis, a rapamycin-sensitive pathway involving the mammalian target of rapamycin (mTOR, a serine/threonine protein kinase) and an unknown rapamycin-insensitive pathway (16, 70). Activation of mTOR leads to phosphorylation of several substrates, including the ribosomal protein S6 kinase 1 (S6K1) and the translational repressor, eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), two proteins involved in the regulation of protein synthesis (28, 29, 52, 61, 63, 67, 73, 77). Phosphorylation of S6K1 and 4E-BP1 is associated with enhanced translation of mRNAs containing a polypyrimidine tract in their 5′-untranslated regions (UTR) or a highly structured 5′-UTR (for reviews see Refs. 17, 20, 63–65, 67). A subset of these types of mRNAs encodes for proteins that comprise elements of the protein synthetic machinery. Activation of mTOR has also been implicated in the elongation phase of protein synthesis and ribosome biogenesis (for review see Ref. 61). In adipose tissue, the mTOR-signaling pathway plays tissue-specific roles in differentiation of preadipocytes, adipose tissue morphology, hypertrophic growth, and leptin secretion (for review see Ref. 44). Furthermore, 4E-BP1, one of the aforementioned substrates of mTOR, appears to be a novel regulator of adipogenesis and metabolism (72).

The in vitro effects of leucine on protein synthesis, metabolism, and cell signaling has led to speculation that leucine may be a nutrient signal in adipose tissue (26, 44). Interestingly, plasma concentrations of leucine and other branched-chain amino acids are significantly ele-
vated in both rodent and human forms of obesity, which are characterized by excess adipose tissue growth (21, 22, 32, 56, 60). Although oral administration of leucine to food-deprived animals leads to an increase in protein synthesis associated with effects on S6K1 and 4E-BP1 in skeletal muscle (3), it is not known whether a similar relationship exists in adipose tissue or other peripheral tissues. To address this question, we have examined the effects of orally administered leucine on protein synthesis, S6K1 phosphorylation, and 4E-BP1 phosphorylation in adipose tissue and compared the results with several other tissues, including heart, gastrocnemius, kidney, and liver. In addition, we have examined the effects of oral administration of norleucine on the same parameters. Norleucine is of interest for several reasons. It is a relatively potent regulator of mTOR signaling in adipose tissue, analogous to leucine. Norleucine is also structurally related to leucine but does not charge leucyl tRNA. Last, previous studies have failed to observe an effect of norleucine in vivo are the result of the direct effect of norleucine, rather than a secondary effect mediated through a rise in plasma insulin.

The results of the present study provide support for the hypothesis that leucine is a direct-acting nutrient signal and that acute leucine administration can regulate mTOR signaling and protein synthesis in adipose tissue as well as in several other tissues. However, in some tissues, the acute effects of leucine and norleucine on protein synthesis cannot be explained by activation of S6K1 or phosphorylation of 4E-BP1.

**EXPERIMENTAL PROCEDURES**

**Animals and treatment protocol.** The Institutional Animal Care and Use Committee approved the animal protocol. Male Sprague-Dawley rats (100–125 g) were purchased from Charles River Laboratories (Cambridge, MA) and were maintained for ≥7 days before the start of the treatment protocol on Teklad rat chow and water ad libitum. Animals were food deprived for 18 h and then allocated to one of four treatment groups, as follows: saline (Sal, control), carbohydrate (CHO), norleucine (Nor), or leucine (Leu) administered. At time 0, the animals were administered one of the following solutions by oral gavage (2.5 ml/100 g body wt) according to their designated treatment group: saline (0.155 mol NaCl/l), carbohydrate (262.5 g/l D-glucose mixed with 262.5 g/l D-sucrose), norleucine (54.0 g/l l-norleucine), leucine (54.0 g/l l-leucine). The dose of leucine is equivalent to the amount of leucine consumed by rats of this age and strain during 24 h of free access to a commercial rodent diet (27). The amount of carbohydrate given represents ~15% of daily energy intake (27).

**Protein synthesis measurements by the flooding dose technique.** Twenty minutes after the gavage, a flooding dose of 1-[2,3,4,5,6-3H]phenylalanine (1 ml/100 g body wt of 150 mM phenylalanine containing 100 μCi/ml of the tritiated amino acid) was injected via the tail vein. Rats were killed by decapitation 10 min later (i.e., 30 min after oral administration). The 30-min time point was chosen on the basis of previous time course studies showing that plasma leucine concentrations peak at this point in time after gavage (data not shown). Blood was collected in heparinized Corex tubes and centrifuged at 1,800 g for 10 min at 4°C. The plasma was collected and frozen in aliquots. The following tissues were removed and frozen using aluminum clamps precooled to the temperature of liquid nitrogen: heart, gastrocnemius, epididymal adipose tissue, kidneys, and liver. The tissues were then stored frozen at −84°C. Subsequently, the tissue samples were powdered under liquid nitrogen with a mortar and pestle. Approximately 500 mg of the frozen powdered tissue were weighed and homogenized in 7 vol of homogenization buffer (in mM: 20 HEPES, pH 7.4, 2 EGTA, 50 NaF, 100 KCl, 0.2 EDTA, 50 β-glycerophosphate, 1 dithiothreitol, 0.1 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 1 benzamidine, 0.5 sodium vanadate, and 1 microcystin LR) with a Tissumizer homogenizer. These homogenates were used for Western blot analysis and protein synthesis measurements. For measurement of the rate of protein synthesis, ice-cold perchloric acid (HClO₄; 3.6% wt/vol final concentration) was mixed with a 0.5-ml aliquot of the ice-cold tissue homogenate. The mixture was centrifuged and then, after the supernatant was decanted, the pellet was washed 5 times with 3.6% HClO₄ to remove the perchloric acid. The pellet was further washed with acetone, followed by a mixture of chloroform-methanol (1:1 vol/vol) and then water. The pellet was then dissolved in 0.1 M NaOH, and aliquots were assayed for protein by the biuret method, with crystalline bovine serum albumin as a standard. Another aliquot was assayed for radioactivity by liquid scintillation spectrometry using the proper corrections for quenching (dpm). Rates of protein synthesis were calculated by dividing the amount of radioactivity incorporated into protein by the specific radioactivity of phenylalanine in the plasma per unit of time, as described in this laboratory previously (2, 41, 75). The assumption in using this technique to estimate the rate of protein synthesis in vivo is that the tissue phenylalanine concentration is elevated to a high concentration, thereby limiting any dilution effect of nonradioactive phenylalanine derived from proteolysis on the intracellular specific radioactivity. Under the condition of elevated plasma phenylalanine concentrations, the specific radioactivity of the plasma phenylalanine is assumed to be equal to the specific radioactivity of the tRNA-bound phenylalanine. Studies by McKee et al. (51) and Williams et al. (80) have shown that, at a perfusate phenylalanine concentration of 0.4 mM, the perfusate and intracellular and tRNA-bound phenylalanine have the same specific radioactivities within 10 min of the start of perfusion of the rat heart with radioisotopes. There were no significant differences in the plasma phenylalanine concentrations between different groups (Sal 1,011 ± 142; CHO 1,016 ± 143; Nor 1,380 ± 143; Leu 1,181 ± 132 nmol/ml; ANOVA: F = 1.604, P = 0.2185). The ratio of protein to gram of wet weight was not affected by the treatments. For the saline group, they were (mg protein/g wet wt): gastrocnemius 198.7 ± 4.614; heart 147.5 ± 8.711; liver 223.8 ± 7.153; kidney 60.4 ± 5.668; and adipose tissue 116.7 ± 7.625.

**Western blot analysis.** Another portion of the homogenate was centrifuged at 10,000 g for 10 min at 4°C. An aliquot of the resulting supernatant was reserved for protein assay, and the remainder was added to an equal volume of 2× Laemmli sodium dodecyl sulfate (SDS) sample buffer. The mixture was boiled for 3 min and centrifuged at 16,000 g for 4 min. For detection of total S6K1 and phosphorylation of S6K1 on T389, cytosolic proteins were separated on 7.5% acrylamide gels with reduced bisacrylamide concentration (30:0.19%) to improve separation of the various phosphorylated isoforms. After transfer to polyvinylidene difluoride (PVDF) membranes, the blots were probed using phosphospecific antibodies raised to a peptide corresponding to
T389 on S6K1 (Cell Signaling Technology, Beverly, MA). After this analysis, the antibodies were stripped from the PVDF membrane, and the membranes were reprobed with antibodies that recognized both phosphorylated and unphosphorylated forms of S6K1.

To examine 4E-BP1 concentration and phosphorylation, cytosolic proteins (100 μg) were separated on 15% acrylamide gels containing the same reduced bisacrylamide concentration. Under these conditions, 4E-BP1 resolved into three bands during electrophoresis: least phosphorylated and fastest migrating, α-; intermediate, β-; and slowest migrating most extensively phosphorylated, γ-form (38, 45).

Insulin, amino acids, and ATP assays. Insulin concentrations were measured by RIA using a kit from Linco Research (St. Charles, MO). The concentration of phenylalanine and other amino acids was determined by HPLC analysis of supernatants from trichloroacetic acid extracts of plasma (19). ATP concentrations in tissues were measured on neutralized HClO₄ tissue extracts with the standard spectrophotometric technique, as previously described (75).

Statistical analysis. ANOVA statistical analysis was performed using the INSTAT program with a Sidak posttest when ANOVA indicated a significant difference. Differences were considered significant when P < 0.05.

RESULTS

The average body weight of the animals in each group was not significantly different before receiving an oral bolus of either saline (152.8 ± 4.6), carbohydrate (147.9 ± 3.5), leucine (160.1 ± 3.8), or norleucine (156.6 ± 3.1). The effect of administration of saline (control), a carbohydrate meal, leucine, or norleucine on rates of protein synthesis is shown in Fig. 1. On the basis of the average standard deviations, we estimate that these measurements were sensitive enough to detect a significant change of ~30% or more. Rates of protein synthesis per gram wet weight in the saline control group were greatest in the liver, followed by the kidney, adipose tissue, heart, and gastrocnemius. The administration of carbohydrate did not lead to a significant stimulation of protein synthesis in any of the tissues examined, although there was a slight trend toward an increase in adipose tissue (Fig. 1). Leucine administration significantly stimulated protein synthesis in adipose tissue, gastrocnemius, and kidney only (Fig. 1). The incremental increase in protein synthesis was approximately twofold in adipose tissue and skeletal muscle. The lack of a statistically significant stimulatory effect of leucine on hepatic protein synthesis from food-deprived rats is in agreement with a recent report by Anthony et al. (4). Figure 1 also shows that a single oral dose of norleucine robustly stimulated protein synthesis not only in adipose tissue but also in heart and gastrocnemius muscle and in kidney and liver as well.

Fig. 1. Effects of oral administration of saline control, carbohydrate mixture (CHO), leucine, and norleucine on in vivo rates of protein synthesis. In vivo rates of protein synthesis were measured in different tissues from overnight-fasted rats 30 min after the oral administration of saline, CHO, or an isocaloric amount of norleucine or leucine. *Statistically significant difference, P < 0.05 vs. saline control.
To examine potential mechanisms responsible for stimulating protein synthesis, we measured plasma amino acid concentrations (Table 1). Carbohydrate administration was associated with an increase in plasma alanine concentration compared with saline and an increase in alanine and glycine concentrations compared with leucine administration. Relative to saline, leucine-treated animals had slightly reduced plasma alanine and asparagine concentrations and, as expected, highly elevated leucine concentrations. The elevations in plasma leucine were comparable to previous measurements made at 1 h after the same dose of leucine (4). Norleucine administration increased plasma norleucine to 740 μM. Surprisingly, norleucine treatment also raised plasma leucine concentrations to values observed with leucine administration. In contrast, valine and isoleucine concentrations were not elevated.

Phosphorylation of S6K1. Phosphorylation of ribosomal protein S6 is associated with preferential translation of messenger RNAs with a polypyrimidine tract in their 5′-UTR (for review see Ref. 20). Ribosomal protein S6 is phosphorylated by S6K1. The activity of S6K1 is itself enhanced by phosphorylation. Studies by Avruch and coworkers (6, 36, 78, 79) have shown that, although S6K1 is phosphorylated on multiple sites, the activity of S6K1 correlates only with phosphorylation of Thr389. To examine the potential role of S6K1 phosphorylation in the changes in protein synthesis observed in Fig. 1, samples from the four experimental groups were analyzed using techniques with antibodies raised to the phosphorylated form of Thr389 on S6K1. Based on the standard deviation observed and the number of animals analyzed, these measurements were generally sensitive enough to detect a 50% change in phosphorylation. Subsequently, blots were stripped and reprobed with an antibody that detects total S6K1. Analysis of these blots (data not shown) indicated that the concentration of S6K1 was ~25% higher in kidney, liver, and adipose tissue compared with heart and gastrocnemius. There was no significant difference in the amount of S6K1 among any of the conditions in any of the tissues examined (data not shown). For example, in adipose tissue, the values were (%mean saline values) 100 ± 5, 97 ± 6, 96 ± 4, and 104 ± 3 for Sal, CHO, Nor, and Leu groups, respectively.

In adipose tissue, there was a trend toward an increase of S6K1 Thr389 phosphorylation with carbohydrate administration compared with saline. However, the apparent increase was not statistically significant (Figs. 2 and 3). In contrast, both leucine and norleucine administration led to a significant stimulation of S6K1 phosphorylation in adipose tissue. A somewhat similar response was observed in gastrocnemius, except that the carbohydrate condition did cause a significant stimulation of S6K1 phosphorylation in the muscle. On the other hand, whereas carbohydrate and leucine administration resulted in significant increases in S6K1 phosphorylation were observed in other tissues examined. For example, there were few or no effects of norleucine on S6K1 phosphorylation in heart and liver, whereas carbohydrate and leucine administration resulted in significant increases in S6K1 phosphorylation (Figs. 2 and 3). Last, carbohydrate, norleucine, and leucine meals had no influence on S6K1 phosphorylation in kidney (Figs. 2 and 3).

The unexpected lack of effect of these treatments on kidney was further investigated using an antibody that recognizes both phosphorylated and unphosphorylated (total) S6K1 (Fig. 4). As seen in Fig. 4, multiple forms of S6K1 from adipose tissue and gastrocnemius (positive control) are resolved by SDS-PAGE. Furthermore, leucine administration results in the appearance of more phosphorylated forms with slower mobility during SDS-PAGE in the samples from adipose tissue shown. However, only the forms with the fastest mobility (hypophosphorylated forms) were observed in the kidney, in contrast to a positive control from gastrocnemius in which all four mobility forms are observed (Fig. 4). The failure to observe changes in phosphorylation in kidney were not the result of reduced amounts of S6K1, as similar concentrations of S6K1 are found in muscle and kidney.

Phosphorylation of 4E-BP1. The association of eukaryotic initiation factor (eIF)4E with the multiprotein translational complex, eIF4F, can be prevented by binding to 4E-BP1 and its family members (9, 25, 38, 39). Formation of the most highly phosphorylated form, which migrates as the γ-band, is associated with reduced binding to eIF4E and increased association of eIF4E with eIF4G. Previous studies on cells in culture and freshly isolated cells from tissue or perfused organ have shown that both leucine and insulin treatment is associated with increased phosphorylation of 4E-BP1 (9, 25, 28, 34, 37–40, 42, 45, 48, 57, 74, 82, 83). To evaluate the effects of our treatments on 4E-BP1 phos-

---

**Table 1. Amino acid concentrations of rat serum after gavage**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>424 ± 48</td>
<td>610 ± 45*</td>
<td>340 ± 32*c</td>
<td>517 ± 30</td>
</tr>
<tr>
<td>Arg</td>
<td>184 ± 31</td>
<td>231 ± 19</td>
<td>198 ± 31</td>
<td>188 ± 12</td>
</tr>
<tr>
<td>Asn</td>
<td>160 ± 21</td>
<td>193 ± 13</td>
<td>121 ± 16*</td>
<td>157 ± 8</td>
</tr>
<tr>
<td>Asp</td>
<td>52 ± 10</td>
<td>58 ± 4</td>
<td>48 ± 5</td>
<td>61 ± 6</td>
</tr>
<tr>
<td>Gly</td>
<td>517 ± 62</td>
<td>715 ± 90*</td>
<td>412 ± 54</td>
<td>588 ± 27</td>
</tr>
<tr>
<td>Gln</td>
<td>1,446 ± 144</td>
<td>1,465 ± 112</td>
<td>1,382 ± 121</td>
<td>1,510 ± 97</td>
</tr>
<tr>
<td>Glu</td>
<td>286 ± 45</td>
<td>335 ± 22</td>
<td>312 ± 29</td>
<td>387 ± 27</td>
</tr>
<tr>
<td>Ile</td>
<td>101 ± 10</td>
<td>116 ± 9</td>
<td>88 ± 7</td>
<td>77 ± 8</td>
</tr>
<tr>
<td>His</td>
<td>66 ± 20</td>
<td>86 ± 13</td>
<td>57 ± 18</td>
<td>68 ± 13</td>
</tr>
<tr>
<td>Leu</td>
<td>127 ± 15</td>
<td>148 ± 13</td>
<td>227 ± 99*</td>
<td>2390 ± 74*</td>
</tr>
<tr>
<td>Lys</td>
<td>320 ± 76</td>
<td>355 ± 28</td>
<td>266 ± 66</td>
<td>203 ± 46</td>
</tr>
<tr>
<td>Met</td>
<td>36 ± 11</td>
<td>30 ± 5</td>
<td>34 ± 13</td>
<td>31 ± 7</td>
</tr>
<tr>
<td>Nle</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>740 ± 46*</td>
</tr>
<tr>
<td>Phe</td>
<td>39 ± 2</td>
<td>38 ± 5</td>
<td>30 ± 1</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>Ser</td>
<td>388 ± 44</td>
<td>448 ± 23</td>
<td>335 ± 19b</td>
<td>436 ± 17</td>
</tr>
<tr>
<td>Thr</td>
<td>347 ± 45</td>
<td>511 ± 21</td>
<td>411 ± 30</td>
<td>478 ± 24</td>
</tr>
<tr>
<td>Val</td>
<td>167 ± 17</td>
<td>210 ± 12</td>
<td>146 ± 18</td>
<td>163 ± 13</td>
</tr>
<tr>
<td>Tyr</td>
<td>69 ± 11</td>
<td>71 ± 12</td>
<td>51 ± 11</td>
<td>78 ± 12</td>
</tr>
<tr>
<td>Trp</td>
<td>101 ± 13</td>
<td>118 ± 8</td>
<td>78 ± 15</td>
<td>97 ± 9</td>
</tr>
</tbody>
</table>

Values are means ± SE; nos. in parentheses are nos. of animals. CHO, carbohydrate; Nle, norleucine; ND, not detected. *P < 0.01 vs. saline; †P < 0.001 vs. CHO; ‡P < 0.05 vs. Nle; ‡‡P < 0.05 vs. CHO; §P < 0.05 vs. Leu; ¶P < 0.001 vs. CHO or saline; ‡§P < 0.05 vs. CHO or Nle; ‡¶P < 0.05 vs. CHO or Nle.
phorylation in vivo, we examined the degree to which leucine and norleucine modulate the percentage of 4E-BP1 in the γ-form in various organs. Figure 5 shows representative tissue blots from individual animals. The percentage of 4E-BP1 in the γ-form was quantitated and average responses were determined (Fig. 6). In adipose tissue and gastrocnemius, leucine and norleucine caused a significant stimulation of 4E-BP1 phosphorylation. Although a slight trend toward an increase in response to carbohydrate administration in adipose tissue and gastrocnemius was noted, these were not statistically significant and represented a <10% change in the percentage of the γ-form. Our measurements were generally sensitive enough to detect a 10% change in the percentage of 4E-BP1 in the γ-form. In heart and liver, carbohydrate administra-

Fig. 2. Phosphorylation of ribosomal protein S6 kinase-1 (S6K1) at Thr389. Tissue lysate proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF). Phosphorylation of S6K1 at Thr389 (phospho-S6K1) was examined using the phosphospecific antibody according to the manufacturer’s recommendations. Results show representative results from each tissue of rats gavaged with saline (S), CHO, norleucine (Nor), or leucine (Leu). A positive control (Pos; proteins from skeletal muscle removed from an ad libitum-fed rat 30 min after injection with 10 U insulin/200 g body wt) was run in each experiment, and this is shown with the kidney samples.

Fig. 3. Quantitation of the level of S6K1 phosphorylation at Thr389. The amount of S6K1 phosphorylated at Thr389 was quantitated by densitometry, as previously described in different tissues from rats gavaged with saline, CHO, norleucine, or leucine. Bars represent means ± SE from separate determinations of tissues from ≥6 animals. *Statistically significant difference, P < 0.05 vs. saline control.
tion as well as leucine and norleucine administration all caused a significant increase in 4E-BP1 phosphorylation. The concentration of 4E-BP1 in kidney was below the level of detection, and this makes it unlikely that 4E-BP1 plays a significant role in the protein synthesis response to oral administration of leucine and norleucine.

Fig. 4. Western blot analysis of total S6K1 and phosphorylation-associated shifts in electrophoretic mobility. Tissue lysate proteins were separated by SDS-PAGE under conditions optimized to separate unphosphorylated S6K1 from various phosphorylated forms of S6K1. Phosphorylation is associated with decreased electrophoretic mobility. After transfer of the proteins to PVDF, blots were probed with a commercial antibody ("Holo S6K1") that recognizes both phosphorylated and unphosphorylated S6K1, as previously described (24, 46). Representative samples are adipose tissue from saline- and leucine-treated rats and kidney from saline-, CHO-, norleucine-, and leucine-treated rats, as well as a positive control (Pos), which is gastrocnemius muscle from an ad libitum-fed rat injected with insulin as in Fig. 2.

Fig. 5. Effects of oral administration of saline, CHO, norleucine, and leucine on 4E-BP1 phosphorylation in peripheral tissues. Tissue lysate proteins were separated by SDS-PAGE under conditions optimized to separate unphosphorylated eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) from various phosphorylated forms, including the γ-form, as previously described. Blots were developed using enhanced chemiluminescence after incubation with a rabbit affinity-purified antibody to the COOH terminus of 4E-BP1.

Fig. 6. Quantitation of 4E-BP1 in γ-phosphorylation form. The amount of 4E-BP1 in the γ-form was quantitated, as previously described, in different tissues from rats gavaged with saline, CHO, norleucine, or leucine. Results are means ± SE from separate determinations of tissues from ≥6 animals. *Statistically different response from the saline control (P < 0.05).
E509

TISSUE-SPECIFIC EFFECTS OF LEUCINE/NORLEUCINE

Effects on plasma insulin and tissue ATP concentrations. Because insulin stimulates the mTOR-signaling pathway leading to phosphorylation of S6K1 and 4E-BP1 (for reviews see Refs. 29, 42, 67), the effects of carbohydrate administration on 4E-BP1 and S6K1 phosphorylation in heart, gastrocnemius, and liver could be secondary to the observed changes in plasma insulin. Carbohydrate administration caused a robust increase in plasma insulin concentration compared with saline-treated animals (Fig. 7). The effect of leucine on the plasma insulin concentration was variable. As a result, the averaged insulin concentration in the leucine group was not significantly different from that in either the Sal or CHO group. Unlike the results with leucine, none of the animals administered norleucine showed any changes in plasma insulin concentration (Fig. 2), despite the fact that norleucine administration also raised plasma leucine (Table 1). This represents a distinct difference between leucine and norleucine. Thus the effects of norleucine on protein synthesis and phosphorylation of S6K1 and 4E-BP1 cannot be attributed to elevations in plasma insulin concentrations.

Physiological changes in ATP concentrations might regulate mTOR, and those changes in ATP concentrations might be responsible for some of the nutritional regulation of mTOR (18). Therefore, we measured the ATP concentrations of tissues in response to various treatments (Table 2). ATP concentrations in various organs of the Sal group were similar to previously reported values in food-deprived rats (7). Feeding carbohydrate to food-deprived animals was associated with a significant increase in hepatic ATP concentrations. This is not surprising, because effects of carbohydrate feeding on hepatic ATP concentrations are well known (30). ATP was not affected in other tissues examined (Table 2). Thus liver may be a tissue where such regulation of protein synthesis may occur; however, it is unlikely this mechanism accounts for nutritionally related mTOR regulation in other tissues we examined, in which ATP was not affected by fasting and refeeding.

Oral administration of leucine or norleucine had no effect on hepatic ATP concentrations or ATP concentrations in other tissues examined. This is in agreement with in vitro findings (18). Thus the effects of amino acids on the mTOR-signaling pathway in vitro and in vivo seem to be independent of tissue ATP concentration.

DISCUSSION

The effects of adding leucine directly to adipocytes in vitro have suggested that it should be directly acting in vivo. However, to date, no reports have tested this hypothesis directly. In studies cited in this report (e.g., 1, 43, 47, 82), we compared the effects of leucine, norleucine, or carbohydrate administration to test the hypothesis that, in vivo, leucine is a direct-acting nutrient signal that stimulates protein synthesis in adipose tissue. In vivo, leucine is also an insulin secretagogue, and this has raised the question as to whether or not the effects of leucine in vivo are direct effects or rather reflect a leucine-mediated rise in plasma insulin

Table 2. Effects of carbohydrate, leucine, and norleucine on cellular ATP content in heart, adipose tissue, skeletal muscle, kidney, and liver

<table>
<thead>
<tr>
<th>Tissue</th>
<th>ATP Concentration, μmol/g wet wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline control</td>
</tr>
<tr>
<td>Heart</td>
<td>2.45 ± 0.29</td>
</tr>
<tr>
<td>Adipose</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>8.52 ± 0.66</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.84 ± 0.05</td>
</tr>
<tr>
<td>Liver</td>
<td>0.65 ± 0.04</td>
</tr>
</tbody>
</table>

Values shown are means ± SE. *Significantly different from the saline control (P < 0.05).
after feeding. Three lines of evidence seem to argue against a role of insulin in the leucine responses. First, insulin concentrations were elevated in some, but not all, of the leucine-treated rats, yet all of the animals had a protein synthesis response to leucine. Second, a significant rise in plasma insulin was provided by the carbohydrate meal, but these changes in insulin were not associated with significant changes in protein synthesis or phosphorylation of the factors in adipose tissue. Third, a leucine mimic in adipose tissue, norleucine, did not stimulate insulin secretion in any of the animals, in agreement with previous in vitro studies (43). However, norleucine did stimulate protein synthesis with equal efficacy compared with leucine. These findings argue against a role of insulin in these changes. This conclusion is strengthened by the observation that these responses can be observed in isolated cells (8, 25, 57, 82). Thus it is likely that leucine is a nutrient signal that acts directly on adipose tissue.

Comparison of the present results with previous in vitro studies. In the present study, the rise in plasma insulin elicited by carbohydrate administration did not correlate with enhanced rates of protein synthesis in vivo. This was a somewhat unexpected finding, because adding insulin to isolated adipocytes in vitro does significantly stimulate protein synthesis and the phosphorylation of factors such as 4E-BP1 and S6K1 (24, 25, 46, 49, 58, 66). One explanation for these different results has been the proposal that a rise in both plasma amino acids and insulin concentrations is necessary for insulin stimulation of protein synthesis (e.g., 5, 69). Thus our carbohydrate meal did not provide a rise in plasma amino acid concentrations and therefore would not be expected to stimulate protein synthesis. Alternatively, our ability to observe these changes may simply be greater in vitro, possibly in part because mTOR signaling is already stimulated in vivo, even in the control rats (e.g., in response to growth factors, cell-cell/cell-matrix interactions, or other regulators). This explanation is supported by comparison of the in vivo results presented here with many previous in vitro studies we have performed on freshly isolated adipocytes (24, 25, 45, 58). The level of 4E-BP1 phosphorylation and S6K1 phosphorylation is far less in adipocytes bathed in Krebs-Ringer buffer (or in the presence of 1× concentrations of amino acids) compared with adipose tissue from the saline-gavaged fasted rats. Addition of insulin in vitro to the isolated adipocytes brings the level of phosphorylation to approximately the same point as that observed in the in vivo controls (fasted rats administered saline). Thus it is possible that cell signaling pathways leading to protein synthesis in adipose tissue (which diverge from the insulin-signaling pathway leading to glucose transport) may already be stimulated in vivo, in contrast to the situation in vitro. If this is true, then a second question is how leucine and norleucine stimulate these end points in vivo if the signaling pathways upstream from mTOR are already quite stimulated. A logical explanation for this phenomenon is that amino acids activate mTOR signaling independently and additively from the signaling pathways used by insulin, and presumably other protein tyrosine kinase-linked receptors (58).

Role of S6K1 and 4E-BP1 in leucine and norleucine stimulation of protein synthesis. It has been known for many years that branched-chain amino acids, leucine in particular, stimulate protein synthesis (10, 11, 33). More recently, the potential role of mTOR, 4E-BP1, and S6K1 in this effect of the branched-chain amino acids has been proposed (for reviews see Refs. 17, 20, 63, 65). In this report, acute oral administration of leucine or norleucine stimulated protein synthesis and enhanced the phosphorylation state of S6K1 and 4E-BP1 in adipose tissue. In contrast, carbohydrate administration did not. Therefore, in adipose tissue, there appeared to be a close correlation between increased rates of protein synthesis and activation of mTOR signaling as reflected in the changes in 4E-BP1 and S6K1 phosphorylation. However, no such correlations could be drawn in the other tissues (muscle, heart, liver, and kidney) that we examined. For example, in heart and liver, administration of a carbohydrate meal stimulated S6K1 and 4E-BP1 phosphorylation but had no effect on protein synthesis. Likewise, rates of protein synthesis were accelerated by leucine or norleucine in kidney, but no increase in phosphorylation of S6K1 was observed. These observations suggest either that a tissue-specific effect of leucine exists or that leucine enhances protein synthesis through a mechanism that is independent of mTOR activation, particularly in those tissues. These conclusions add to an increasing set of observations suggesting that amino acids regulate protein synthesis through rapamycin-sensitive (e.g., mTOR, S6K1, and 4E-BP1) and rapamycin-insensitive pathways. Thus leucine may be able to acutely regulate protein synthesis through a rapamycin-insensitive pathway.

In conclusion, the results of the present study provide support for the idea that leucine is a direct-acting, orally active nutrient signal for adipose tissue protein synthesis regulation. The presence of leucine may signal the presence of a protein meal and/or the appropriate context in which to enhance protein synthesis in response to rises in the plasma insulin concentration. It is also demonstrated that norleucine may be more efficacious than leucine, at least with regard to the stimulation of protein synthesis. The effects of leucine and norleucine on phosphorylation of S6K1 and 4E-BP1 appear to be tissue specific. It is tempting to speculate, on the basis of this observation and other recent evidence (44), that different leucine recognition sites linked to mTOR signaling may exist and that these may have different pharmacological selectivity toward leucine analogs. An unexpected conclusion of our studies, however, is that the effects of leucine and norleucine on protein synthesis may be brought about either by tissue-specific mechanisms or by mechanisms that appear independent of mTOR signaling to S6K1 and 4E-BP1.
We thank Winston Irving, Trisha Garges, and Maggie McNitt for technical assistance.

This work was supported by grants from the Penn State Equal Opportunity Planning Committee (A. Vaval), the National Institutes of Health (DK-53843, C. J. Lynch; GM-39277 and AA-12814, T. C. Vary; DK-15658, L. S. Jefferson), and a grant from Solvay Pharmaceuticals, Germany.

REFERENCES


