Tissue-specific effects of chronic dietary leucine and norleucine supplementation on protein synthesis in rats

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POSTPRANDIAL INCREASES in the plasma concentration of amino acids after a mainly protein-containing meal may provide a signal for accelerating protein synthesis (9, 10, 25, 37, 38, 51). The mammalian target of rapamycin (mTOR)-signaling pathway has been proposed as one potential target for mediating these effects. In adipocytes, the efficacy of amino acids in activating mTOR signaling appears to be related to their structural similarity to leucine. Thus leucine and norleucine are posited to be agonists at a common leucine recognition site in adipocytes, LeuRa (48, 49). Short-term administration of leucine stimulates protein synthesis by enhancing mRNA translation initiation through an increase in the number of polysomes and an increased rate of formation of the 40S initiation complex (24). These actions improve the efficiency of the mRNA translation initiation cycle.

Insulin and branched-chain amino acids (BCAA) influence protein synthesis by activating the serine/threonine kinase mTOR, which then stimulates downstream targets such as the translation repressor, eukaryotic initiation factor 4E (eIF4E)-binding protein-1 (4E-BP1, or PHAS-I) and the 70-kDa ribosomal protein kinase, S6K1 [for review see Gingras et al. (30)]. Leucine stimulates the hyperphosphorylation of 4E-BP1 (27, 57, 71), resulting in its release from eIF4E, thus allowing the initiation cycle to proceed more efficiently. Multisite phosphorylation of S6K1 is associated with acute changes in synthesis of a subset of proteins that may lead to subsequent changes in global protein synthesis. Notably, phosphorylation of S6 is associated with increased translation of messenger RNA species with terminal oligopyrimidine (TOP) tracts at the 5′-cap. Because many TOP-containing mRNAs encoded for proteins are components of the protein synthetic machinery, it is expected that persistent activation of mTOR would lead to increases in protein synthetic capacity (55); however, this hypothesis has not been rigorously tested in vivo. In fact, although leucine regulates protein synthesis acutely, it...
is not known whether or not chronic oral supplementation of leucine stimulates rates of protein synthesis. The need to better understand leucine metabolism arises from studies that suggest a leucine metabolite or leucine metabolism, rather than leucine itself, may be the signal for activation of mTOR (27, 49, 57 and compare 63, 70). The first step in leucine metabolism is reversible, transamination of leucine to α-ketoisocaproate catalyzed by the branched-chain aminotransferase isoenzymes [mitochondrial (BCATm) and cytosolic (BCATc)]. BCATm is expressed ubiquitously (3, 15, 32, 42–44), whereas BCATc is found primarily in neural tissue (40). The next step is irreversible oxidative decarboxylation of the branched-chain α-keto acids to produce the corresponding branched-chain acyl-CoA derivatives catalyzed by the mitochondrial branched-chain α-keto acid dehydrogenase (BCKD) enzyme complex. The mammalian BCKD complex contains multiple copies of three enzymes: a branched-chain α-keto acid dehydrogenase (E1) composed of 2α and 2β subunits, a dihydrolipoyl transacylase (E2), and a dihydrolipoyl dehydrogenase (E3) (35). The activity of the complex within a tissue is regulated by phosphorylation–dephosphorylation catalyzed by a specific kinase and phosphatase. The phosphorylation state of the complex is controlled primarily by the activity of the BCKD kinase; phosphorylation of S293 on the E1-α subunit results in inactivation (22, 35, 58). Depending on the tissue, activity state is influenced by hormones, diabetes, exercise, starvation, acidosis, or low dietary protein feeding (for review see Ref. 54). The kinase can be inhibited directly in vitro by the keto acid of leucine, which in turn results in activation of the BCKD complex. This may explain the activation of BCKD in skeletal muscle after leucine injection (28, 35, 36). Although it is apparent that the enzymes involved in the initial steps in leucine metabolism are present in adipose tissue, their relative level of expression compared with other tissues is not known.

It is important to understand the effects of chronic elevations in leucine, because concentrations of BCAAs are chronically elevated in human and animal forms of obesity and adipose tissue appears to be highly responsive to leucine (18, 23). For example, it is not known whether chronic exposure to excess leucine or leucine mimetics (norleucine) results in changes in protein synthetic capacity. Alternatively, the levels and activity of enzymes involved in either the cell-signaling response to leucine or the metabolism of leucine might adapt to a chronic increase in plasma leucine concentrations. Therefore, in this study, we have examined the effects of chronic, continuous elevations in plasma leucine by use of a new model of chronic leucine or norleucine supplementation. Norleucine was used because it is a structural analog of leucine that we have shown can stimulate mTOR signaling and protein synthesis in vitro and in vivo (48–50). In contrast to leucine, acutely administered norleucine does not stimulate insulin secretion and is not incorporated into protein. A continuous supply of these amino acids was provided in the drinking water. Using this model, we have determined the effect of chronic leucine or norleucine supplementation on postprandial protein synthesis in adipose tissue as well as in muscle, heart, liver, and kidney. Plasma hormone concentrations and tissue RNA levels were examined as potential mediators of the effects on protein synthesis. The tissue-dependent expression of proteins involved in the mTOR-signaling pathway and leucine catabolism in adipose tissue were compared with expression of these proteins in the other tissues. The results show that chronic supplementation of leucine or norleucine stimulates postprandial protein synthesis in responsive tissues without affecting levels of signaling proteins or BCAA catabolic enzymes. The protein synthesis responses displayed a higher degree of tissue specificity compared with the acute effects of leucine on protein synthesis [e.g., in the preceding study (50)]. The likelihood that this may reflect differences in the mechanisms mediating the acute effects of leucine administered to a fasting animal and the chronic effects of leucine in ad libitum-fed animals reported in the present communication is discussed.

EXPERIMENTAL PROCEDURES

Animals and treatment protocol. The Institutional Animal Care and Use Committee approved the animal protocol. Male Sprague-Dawley rats were purchased from Charles River and maintained at our facility for ≥7 days before the start of the treatment protocol. The light cycle began at 7 AM and the dark cycle began at 7 PM, with rats fed ad libitum with measurements made 2–4 h after the beginning of the light cycle. Two identical studies with rats were conducted in which animals were allocated to one of the following three groups: control (6 rats), leucine supplemented (8 rats), and norleucine supplemented (6 rats). Animals were caged in pairs to reduce anxiety-induced changes in food intake. Two experiments were conducted that initially had a planned experimental design of six animals per group. Two extra rats were placed in the leucine group. The mean starting body weights of animals in each group were not statistically different (control: 96.2 ± 2.6 g, n = 12; leucine supplemented: 97.8 ± 1.8, n = 16; norleucine supplemented: 96.2 ± 2.4, n = 12).

Each cage had two ceramic food containers in separate corners of the cage to facilitate ad libitum feeding. Food remaining in dishes and crumbs that fell through the cage mesh were weighed daily starting 1–2 days before day 0. Food consumption was calculated as food consumed per cage and divided by two. Water or leucine analog-containing water (114 mM leucine or norleucine) was provided starting on day 0. The amount of fluid consumed was measured daily. A drinking bottle was also always hung on an empty cage per cage rack. This allowed an estimate of the amount of water lost each day from dripping due to placing of the drinking bottle or handling of the wheeled cage rack (generally this was 1–2 ml). This amount lost was subtracted from the water consumption measurements. Net water consumption was measured per rat cage and divided by two.

Protein synthesis. Protein synthesis measurements were made on the morning of the 12th day of dietary supplementation. Food and water/supplement were provided until the time of anesthetization. Thus, in contrast to the previous study on fasted rats (50), these measurements were made in ad libitum-fed rats. The animals were judged to be in the postprandial phase on the basis of the presence of food in
their stomachs and elevated insulin concentrations. Rates of protein synthesis in vivo were estimated using the flooding-dose method to measure the incorporation of radioactive phenylalanine into protein. This method has been described previously and characterized in our laboratory (65–67). Briefly, an incision was made in the neck of anesthetized animals (Nembutal, 50 mg/kg body wt) for the placement of PE-50 catheters in the carotid artery. A bolus of L-[3H]phenylalanine (0.2 mCi/ml·1·μmol−1, 30 μCi/100 g body wt, 1 ml/100 g body wt) was infused as a bolus intravenously. Ten minutes after injection of the radioisotope, an arterial blood sample (3 ml) was taken for measurement of phenylalanine concentrations and radioactivity. The concentration of phenylalanine and other amino acids was determined by HPLC analysis of supernatants from trichloroacetic acid extracts of plasma (19). In addition, the radioactivity in the phenylalanine peak was measured to determine the specific activity of L-[3H]phenylalanine in the blood.

Gastrocnemius muscle, heart, kidney, liver, and epididymal adipose tissue were excised and frozen between clamps precooled in liquid nitrogen, weighed, and stored at −84°C. The frozen tissue was powdered under liquid nitrogen and then stored at −84°C for later measurements as described in the following section.

Measurements of incorporation of radioactivity in proteins. Approximately 0.5–0.5 g of frozen powdered tissue was homogenized in 2 ml of ice-cold 3.6% (wt/vol) perchloric acid (HClO4) and centrifuged. The supernatant was decanted, and the pellet was washed a minimum of five times with 3.6% (wt/vol) HClO4 to remove any acid-soluble radioactivity. The pellet was 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. The assumption in the use of this technique to estimate the rate of protein synthesis in vivo is that the tissue phenylalanine concentration is elevated to 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 (−1.3 ± 0.9 mM), 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. (52) and Williams et al. (69) have shown that, at a perfusate phenylalanine concentration of 0.4 mM, the perfusate and intracellular tRNA-bound phenylalanine have the same specific radioactivity within 10 min of the start of perfusion with radioisotopes.

BCKD complex activity. Extraction of the BCKD complex from tissues (50–100 mg tissue) was performed essentially as described by Block et al. (5) by use of the modification in Ref. 15. BCKD activity was measured by release of 14CO2 from α-keto-[1-14C]isocaproate. Total BCKD complex activity, which is an estimate of enzyme amount, was measured after activation of a separate aliquot of the same sample in the presence of MnCl2 and lambda protein phosphatase (4). The activation state of BCKD is the ratio of actual activity before activation to total activity obtained after activation by phosphatase treatment. A unit of activity was defined as 1 nmol 14CO2 formed/min at 37°C.

Hormone assays. Insulin and leptin concentrations were measured by RIA with a kit from Linco Research (St. Charles, MO). Liver and serum concentrations of insulin-like growth factor (IGF) were assayed according to Fan et al. (21).

Western blot analysis. For Western blotting of cytosolic proteins, the frozen, powdered tissue was 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 DTT, 0.1 PMSF, 1 benzamidine, 0.5 sodium vanadate, and 1 μM microcin LR) with a Polytron homogenizer. For mitochondrial proteins and mTOR, 0.4% 3-(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) was included in the homogenization buffer to release mitochondrial proteins. The homogenate was centrifuged at 10,000 g for 10 min at 4°C, and the pellet was discarded. An aliquot of the supernatant was reserved for protein assay, and the rest was added to an equal volume of 2× Laemmli sodium dodecyl sulfate (SDS) sample buffer. The mixtures were boiled for 3 min and centrifuged at 16,000 g for 4 min.

To detect mTOR, proteins were separated on a 5% Bio-Rad (Hercules, CA) Criterion Tris-glycine gel and transferred to PVDF for 3 h at 50 V in transfer buffer (10 mM CAPS, pH 11.0, 10% methanol, and 0.1% SDS) by use of a platinum electrode Bio-Rad Criterion blotter. Positive controls for mTOR in Western blots were rat brain lysates (100 μg/lane) and a recombinant FLAG-tagged version of human TOR [aka FK506 rapamycin-associated protein (FRAP)] expressed in SF-9 insect cells. The FRAP/FLAG 1392 baculovirus transfer vector described previously (7, 8) was obtained as a generous gift from Dr. Stuart L. Schreiber (Boston, MA). Immunoblotting was performed using an antibody (MTAB5), produced in our laboratory, directed against a keyhole limpet hemocyanin-linked peptide, (C)-QREPMEKQPQWYRHT FEE, representing an NH2-terminal sequence (residues 221–40) of RAPT, a rat form of mTOR. PVDF membranes were incubated in a 1:1,000 dilution for 1 h at room temperature and then overnight at 4°C. Bands were detected with an enhanced chemiluminescence ECL Western Blotting Kit from Amersham Pharmacia (Piscataway, NJ).

To examine 4E-BP1 concentration and phosphorylation, cytosolic proteins (100 μg) were separated on 15% acrylamide gels containing a reduced bisacrylamide concentration that allows the electrophoretic resolution of 4E-BP1 into three bands: least phosphorylated and fastest migrating, α; intermediate, β; and slowest migrating and most extensively phosphorylated, γ (45, 49). Formation of the most highly phosphorylated form, which migrates as the γ-band, correlates with decreased binding to eIF4E. For detection of total S6K1 and phosphorylation of S6K1 on T389, cytosolic proteins were separated on a 7.5% Bio-Rad Criterion Tris-glycine gel. After transfer to PVDF, the blots were probed using an antibody to S6K1 (Cell Signaling Technology, Beverly, MA).

BCAT isoenzyme-specific antisera was raised in rabbits as described in Wallin et al. (68). Purified recombinant human BCATm (13) was used as antigen. For preparation of the affinity-purified BCATm antibodies, human BCATm-Sepharose was prepared by coupling the purified human recombinant BCAT isoenzyme to Affigel 10 support (Bio-Rad, Richmond, CA) according to the manufacturer’s directions. The BCAT antisera generated against E1 of the purified rat liver BCAT complex was a gift from Dr. Yoshi Shimomura (Nagoya, Japan). This antisera recognizes the E1α, E1β, and E2 BCAT subunits.

BCAT kinase-specific antisera was raised in rabbits with the use of purified recombinant human BCAT kinase as antigen. Affinity-purified antibodies were obtained by chro-
matography on a recombinant BCKD kinase-AH-Sepharose 4B column resin and prepared as recommended by the supplier (Amersham Pharmacia Biotech). Serum was saturated with 50% ammonium sulfate and the precipitate harvested by centrifugation. The precipitate was dissolved in PBS and applied to the column. After extensive washing of the column with PBS, the anti-BCKD kinase antibodies were eluted with 4 M urea and 0.5 M NaCl in 0.1 M sodium acetate buffer, pH 4.0. The affinity-purified antibodies were dialyzed against 50% glycerol-water and stored in aliquots at −85°C. These procedures were carried out at 4°C.

The BCKD E1α, the BCKD kinase, and the BCATm proteins in the tissue supernatant were separated on 10% Bio-Rad Criterion Tris-glycine gels and then electrophoretically transferred to PVDF membrane at 100 V for 45 min in transfer buffer (10 mM CAPS, pH 11.0, and 10% methanol). The resulting PVDF membranes were blocked with 5% (wt/vol) skim milk in Tris-buffered saline-Tween-20 and incubated with their respective antibodies as follows: rabbit anti-BCKD E1α (1:1,000 dilution), rabbit anti-BCKD kinase serum (1:1,000 dilution), or rabbit anti-BCATm (1:1,000 dilution) for 1 h at room temperature. Specific bands were detected using an ECL Western Blotting Kit from Amersham Pharmacia. NIH Image 1.61 was used to perform densitometry of ECL-exposed X-ray films.

**Statistical analysis.** The feeding study was performed twice with similar results. At least six animals were examined per condition for each of the Western blotting studies and other studies. ANOVA statistical analysis was performed using the INSTAT program and a Student-Newman-Keuls post test where appropriate.

**RESULTS**

The early work of Harper and colleagues (33, 34) demonstrated adverse effects on growing animals of a low-protein diet containing an inordinately large amount of a single amino acid. These adverse effects included decreased food intake when norleucine or leucine was added directly to the chow. However, it has also been shown that, when fed a normal diet, rats prefer norleucine (62) or leucine (59) solutions over water. Therefore, in this study, young growing rats were fed a commercial diet containing 24.5% protein, and leucine or norleucine supplements (114 mM) were added directly to the chow. However, it has also been shown that, when fed a normal diet, rats prefer norleucine (62) or leucine (59) solutions over water. Therefore, in this study, young growing rats were fed a commercial diet containing 24.5% protein, and leucine or norleucine supplements (114 mM) were supplied in the drinking water.

Figure 1 shows the total daily sum of the leucine and norleucine consumed from both food and liquid over the course of the experiment. Only the norleucine-supplemented animals received significant amounts of norleucine. The leucine and norleucine in the rat chow were 2.04 and 0%, respectively. Before supplementation, the amount of the leucine consumed was similar in each group (−0.28–0.30 g/100 g body wt −1·day −1). Leucine intake per 100 g body wt stayed about the same for control rats over the course of the experiment. In contrast, the total amount of leucine plus norleucine consumed from water and food in the two supplemented groups doubled within a day of adding leucine or norleucine to the drinking water. This difference in the leucine plus norleucine intake between the two experimental groups and the control group was maintained over the entire protocol period (Fig. 1).

**Effects of dietary supplementation on body weight, food intake, and water consumption.** All groups gained weight at similar rates over the course of the experiment (Fig. 2, top). A trend of increased body weight in the leucine- and norleucine-supplemented groups was noted over the last few days of the study; however, this was not statistically significant. Figure 2 (middle) shows that, overall, neither leucine nor norleucine supplementation had adverse effects on food intake; indeed, food intake was similar in the three groups over the protocol period. The fact that food intake was equivalent among the groups allows us to evaluate the independent effects of leucine and norleucine supplementation, even though the rats were in the postprandial phase when the measurements were made. In agreement with previous palatability studies (59, 62), adding leucine or norleucine to the water did not significantly diminish fluid intake (Fig. 2, bottom). Although there were statistically significant differences in the amount of fluid consumed by rats provided one of the amino acid solutions on some individual days, these differences were not consistent (Fig. 2, bottom).

**Plasma hormone and amino acid concentrations.** To determine whether leucine or norleucine supplementation affected hormones associated with energy metabolism or growth, circulating serum concentrations of insulin, IGF-I, and leptin were measured at the time of death (postprandial state), and the results are summarized in Table 1. Chronic amino acid supplementation had no significant effect on serum hormone concentrations, nor were hepatic tissue levels of IGF-I affected.
Table 2. Amino acids concentrations of rat serum

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Control</th>
<th>Leucine Supplementation</th>
<th>Norleucine Supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>525 ± 7</td>
<td>638 ± 72</td>
<td>673 ± 90</td>
</tr>
<tr>
<td>Arg</td>
<td>245 ± 8</td>
<td>268 ± 23</td>
<td>283 ± 29</td>
</tr>
<tr>
<td>Asn</td>
<td>44 ± 7</td>
<td>44 ± 3</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>Asp</td>
<td>37 ± 5</td>
<td>46 ± 4</td>
<td>46 ± 8</td>
</tr>
<tr>
<td>Gly</td>
<td>337 ± 29</td>
<td>387 ± 37</td>
<td>347 ± 61</td>
</tr>
<tr>
<td>Gln</td>
<td>915 ± 107</td>
<td>1,182 ± 86</td>
<td>1,289 ± 172</td>
</tr>
<tr>
<td>Glu</td>
<td>99 ± 15</td>
<td>106 ± 6</td>
<td>90 ± 7</td>
</tr>
<tr>
<td>Ile</td>
<td>88 ± 12</td>
<td>117 ± 16</td>
<td>101 ± 19</td>
</tr>
<tr>
<td>Leu</td>
<td>160 ± 15</td>
<td>235 ± 19*</td>
<td>173 ± 24</td>
</tr>
<tr>
<td>Lys</td>
<td>413 ± 45</td>
<td>482 ± 40</td>
<td>407 ± 41</td>
</tr>
<tr>
<td>Met</td>
<td>48 ± 6</td>
<td>61 ± 5</td>
<td>58 ± 7</td>
</tr>
<tr>
<td>Phe</td>
<td>51 ± 6</td>
<td>70 ± 6</td>
<td>60 ± 9</td>
</tr>
<tr>
<td>Pro</td>
<td>162 ± 23</td>
<td>243 ± 44</td>
<td>252 ± 64</td>
</tr>
<tr>
<td>Ser</td>
<td>326 ± 23</td>
<td>414 ± 30</td>
<td>358 ± 21</td>
</tr>
<tr>
<td>Thr</td>
<td>224 ± 27</td>
<td>322 ± 48</td>
<td>271 ± 42</td>
</tr>
<tr>
<td>Val</td>
<td>189 ± 26</td>
<td>280 ± 45</td>
<td>239 ± 51</td>
</tr>
<tr>
<td>Tyr</td>
<td>75 ± 11</td>
<td>129 ± 21*</td>
<td>75 ± 14</td>
</tr>
<tr>
<td>BCAA</td>
<td>437 ± 43</td>
<td>695 ± 67*</td>
<td>561 ± 85</td>
</tr>
<tr>
<td>Total1</td>
<td>3,319 ± 447</td>
<td>4,972 ± 372*</td>
<td>4,847 ± 579</td>
</tr>
</tbody>
</table>

Values are means ± SE, μM. BCAA, branched-chain amino acids.
*P < 0.05 vs. control, ANOVA with Student-Newman-Keuls post test. Results pooled from 2 experiments. Sufficient plasma was not available from all of the animals in the two experiments for all of the plasma analyses. However, the results shown are for ≥11 rats per condition. 1Total was calculated only for samples in which Glu and Gln were available.

Rats were determined, and the results are shown in Table 2. Differences in the effects of leucine and norleucine supplementation were observed. Serum leucine (47%) and Tyr (72%) concentrations were significantly higher in the leucine-supplemented group compared with the control group. The rises in leucine are equivalent to increases observed in obesity (18, 23). Although the results did not reach statistical significance, plasma Ala, Asp, Gln, Ile, Pro, and Val were elevated compared with control animals in both the leucine- and norleucine-supplemented groups. The sum of the BCAA concentrations and the total amino acid concentrations were both significantly higher in the leucine-supplemented group compared with control animals, whereas differences did not reach statistical significance in the norleucine group.

Protein synthesis. Next, we measured rates of protein synthesis in selected rat tissues after chronic dietary supplementation. In contrast to the previous study (50), which utilized fasted rats, these animals were in the postprandial phase, as judged by the food present in their small bowel and food in their stomachs. Consequently, the measured rates of protein synthesis in the control group were higher than those observed in fasted rats (Fig. 3; compare with Ref. 50). In the norleucine- and leucine-supplemented groups, rates of protein synthesis were significantly higher than these already elevated (i.e., due to feeding) control rates in adipose tissue, gastrocnemius muscle, and liver (Fig. 3). In terms of percent increase, the effect of leucine or norleucine supplementation on protein synthesis was most dramatic on adipose tissue (277 and 377%, respectively). Although measured rates of protein syn-
thesis also appeared higher in heart and kidney than those in the control group, the data are not statistically different (Fig. 3). Supplementation did not affect tissue total RNA concentrations (data not shown); therefore, the mechanism of the increase in protein synthesis seems to involve an increase in translational efficiency.

**Tissue distribution and effects of leucine and norleucine supplementation on the mTOR-signaling pathway.** Little information is available on the relative tissue distribution of components of the mTOR cell-signaling pathway or the effect of chronic leucine supplementation on these. Therefore, tissue concentrations of mTOR, 4E-BP1, and S6K1 as well as the level of 4E-BP1 and S6K1 phosphorylation were determined. For mTOR, we used both a commercial anti-FRAP antibody from Stress Gen Biotechnologies (not shown) and a newly developed MTAB5 antibody from our laboratory (Fig. 4). Both detected the same ~240-kDa band in tissue lysates from baculovirus-infected Sf-9 cells expressing a recombinant human mTOR with an amino-terminal epitope tag MDYKDDDDK (Fig. 4 top, lane R). Furthermore, both antibodies detected a band with similar electrophoretic mobility in lysates from rat brain (known to contain high concentrations of mTOR) as well as the tissues pertinent to this study (Fig. 4, top, lane B). Because these antibodies were directed against two entirely different regions in mTOR, it is likely that the immunoreactive band represents mTOR. In Fig. 4 (bottom), the relative amount of mTOR per milligram of soluble tissue lysate protein is presented. Each of the lanes in Fig. 4 (top) was loaded with either 50 or 100 μg of tissue, and the loading differences were equalized to prepare the bar graphs shown in Fig. 4 (bottom) and in subsequent figures. Most tissues, with the exception of adipose tissue, expressed similar amounts of mTOR per milligram of soluble tissue protein (Fig. 4, bottom). Adipose tissue contained the most mTOR per milligram of soluble tissue protein compared with other peripheral tissues examined. Neither leucine nor norleucine supplementation had any significant effect on the content of mTOR (Table 3).

As shown in Fig. 5, the content of 4E-BP1 in heart, liver, gastrocnemius, and adipose tissue was approximately the same. However, renal content was approximately five times lower than the other peripheral tissues.
tissues examined (Fig. 5). This is in agreement with the preceding study (50) in younger rats, in which 4E-BP1 could not be detected in kidney. There were no significant differences among the total (i.e., α-, β-, and γ-forms combined) amounts of 4E-BP1 in any tissue (Table 3). The percentages of 4E-BP1 in the γ-form are shown in Table 4. The percentage of 4E-BP1 in the γ-form in the control tissues was higher than in the preceding study, again consistent with the animals being in the postprandial rather than the fasted state. No further increase in 4E-BP1 phosphorylation was caused by the supplements at the time these measurements were taken (Table 4). Compared with the other tissues, adipose tissue had the highest percentage of 4E-BP1 in the γ-form. This is consistent with the observation that adipose tissue also had the highest concentration of mTOR per milligram of solubilized tissue protein.

Figure 6 shows a representative Western blot and a graph of the tissue distribution of S6K1, corrected for the amount of protein loaded. Multiple electrophoretic forms corresponding to multisite phosphorylation were observed as previously reported (e.g., Ref. 26). The concentration of S6K1 was ~25% higher in kidney, liver, and adipose tissue compared with heart and gastrocnemius. The amounts of total S6K1 (Table 3) or S6K1 phosphorylated on T389 (elevated consistent with postprandial state, data not shown) in the tissues were the same in the control and leucine- and norleucine-supplemented groups.
Effects of chronic leucine and norleucine supplementation on enzymes involved in leucine metabolism. Leucine injection and dietary protein content have been shown to affect BCATm and/or BCKD activity and expression in several tissues (1, 5, 16, 35, 56, 60, 64). Therefore, the effect of chronic administration of excess leucine or norleucine on the key enzymes involved in the initial steps of leucine metabolism was examined using immunoblotting to determine levels of BCATm, BCKD subunit, and BCKD kinase proteins. BCKD activity was also measured.

Figure 7 shows a representative Western blot and the tissue distribution of BCATm in control tissues. As reported previously (43), BCATm is not found in adult rat liver. The pattern of BCATm enzyme protein levels agrees with the reported distribution of BCATm activity in heart, kidney, and gastrocnemius (2, 39, 43). Measurement of BCATm in adipose tissue (corrected for protein loading) reveals levels of BCATm equivalent to those observed in kidney. This result is significant, because kidney is one of the tissues with high BCATm activity (64). Dietary supplementation with leucine or norleucine had no statistically significant effect on the tissue BCATm concentrations (Table 5). Representative blots showing the tissue distribution of BCKD subunits and BCKD kinase are shown in Figs. 8 and 9. The levels of BCKD subunits were examined using an antibody to the E1-α-subunit that also recognizes E1β and E2 subunits. Relative levels of E1α protein were similar in liver and kidney (Table 5). Adipose tissue had nearly the same level of E1α protein as that found in heart muscle. BCKD subunit proteins were lowest in gastrocnemius (Fig. 8). Interestingly, the blots also revealed that levels of E2 and E1β relative to E1α exhibit tissue-specific differences.

The comparison of tissue levels of BCATm and BCKD subunits and BCKD kinase in the controls and leucine- and norleucine-supplemented groups is summarized in Table 5. As observed with proteins of the mTOR-signaling pathway, dietary supplementation

<table>
<thead>
<tr>
<th>Protein and Tissue</th>
<th>Control</th>
<th>Leucine</th>
<th>Norleucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCATm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>100 ± 4</td>
<td>92 ± 4</td>
<td>93 ± 2</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>100 ± 8</td>
<td>119 ± 7</td>
<td>120 ± 6</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>100 ± 5</td>
<td>84 ± 9</td>
<td>80 ± 11</td>
</tr>
<tr>
<td>Kidney</td>
<td>100 ± 4</td>
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<td>101 ± 4</td>
</tr>
<tr>
<td>Liver</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BCKD E1-α</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>100 ± 9</td>
<td>113 ± 10</td>
<td>124 ± 8</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>100 ± 9</td>
<td>104 ± 8</td>
<td>119 ± 5</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>100 ± 17</td>
<td>101 ± 20</td>
<td>96 ± 9</td>
</tr>
<tr>
<td>Kidney</td>
<td>100 ± 2</td>
<td>100 ± 4</td>
<td>170 ± 5</td>
</tr>
<tr>
<td>Liver</td>
<td>100 ± 5</td>
<td>106 ± 5</td>
<td>97 ± 5</td>
</tr>
<tr>
<td>BCAD kinase</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Heart</td>
<td>100 ± 23</td>
<td>80 ± 6</td>
<td>119 ± 9</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>100 ± 24</td>
<td>129 ± 21</td>
<td>118 ± 32</td>
</tr>
<tr>
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<td>76 ± 7</td>
<td>82 ± 6</td>
</tr>
<tr>
<td>Kidney</td>
<td>100 ± 22</td>
<td>102 ± 17</td>
<td>116 ± 14</td>
</tr>
<tr>
<td>Liver</td>
<td>100 ± 15</td>
<td>99 ± 10</td>
<td>106 ± 7</td>
</tr>
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</table>

Results are means and SE, expressed as percentage of control. BCATm, mitochondrial branched-chain aminotransferase; BCKD E1α, E1α subunit of branched-chain α-keto acid dehydrogenase; ND, not determined. Western blotting results were quantitated from ≥6 animals per group.
with leucine or norleucine had no statistically significant effect on the tissue BCATm concentrations (Fig. 7) or levels of E1-α subunit (Fig. 8) or BCKD kinase (Fig. 9). Consistent with results from Western blotting, no statistically significant differences in BCAT or BCKD activity and activity state were found between control and leucine-supplemented or control and norleucine-supplemented groups (data not shown). Adipose tissue activity was not measured, because we did not have sufficient quantities of tissue.

The activity of BCKD kinase is thought to control the activity state of the BCKD complex (35). As suggested by measurements of BCKD activity state and kinase mRNA levels in rat and other species (61), skeletal muscle contained the highest levels of BCKD kinase protein, whereas liver had the lowest levels of BCKD kinase protein, whereas liver had the lowest levels of BCKD kinase protein. Lower adipose tissue concentrations of BCKD kinase were also found in adipose tissue. In some tissues, Western blotting of whole tissue lysates for the kinase revealed “doublets” around 43–46 kDa (Fig. 9). Relatively few Western blots of the kinase are available in the literature, but in mitochondria extracts, only a single band has been observed (e.g., Ref. 46), although a doublet was detected in the recombinant kinase preparation reported by Popov et al. (58) and transgene studies reported by Doering and Danner (17). Because protease inhibitors were present in the extraction buffer, the higher molecular weight band may represent BCKD kinase that still contains the mitochondrial targeting signal.

**DISCUSSION**

In this study, we have developed a model for chronic supplementation in rats with leucine or norleucine that does not affect food intake or growth. Using this model, we have shown that leucine or norleucine stimulates protein synthesis in a tissue-selective manner and that the tissue responses differ from those reported in food-deprived rats orally administered leucine or norleucine (50). In our chronic model, animals were fed the experimental diets for 12 days, and protein synthesis was measured in ad libitum-fed animals in the postprandial phase. With chronic supplementation, protein synthesis was elevated in adipose tissue, liver, and skeletal muscle, but not in kidney or heart. In the preceding, acute study (50), leucine and norleucine had different effects on protein synthesis: leucine administration stimulated protein synthesis in adipose tissue, muscle, and kidney, whereas norleucine was effective in all tissues (50). The tissue-specific differences in effects of leucine and norleucine supplementation in these two models suggest that there may be varied pathways by which amino acids such as leucine affect protein synthesis in body tissues and/or that the mechanisms involved in leucine’s acute and chronic effects on protein synthesis occur by different mechanisms. This idea is in agreement with recent studies showing that the acute affects of leucine are mediated by both rapamycin-sensitive and rapamycin-insensitive pathways (12, 53). The rapamycin-sensitive pathway involves E1-BP1, S6K1, and mTOR; however, little is known about the rapamycin-insensitive pathway.

In the present study, insulin concentrations were high in the control animals and in the supplemented animals, as was the degree of 4E-BP1 and S6K1 phosphorylation. These findings are consistent with the postprandial state of the animals. These parameters were much lower in controls from the preceding acute study, in which the animals were food deprived. Thus, in the chronic study, leucine and norleucine were able to stimulate protein synthesis above the already high levels of protein synthesis caused by ad libitum feeding alone. These effects were not associated with further increases in plasma insulin or IGF concentrations and therefore probably represent direct effects of the supplements on the affected target tissues. The apparent lack of effect of chronic supplementation on mTOR-signaling proteins may be related to the time at which the measurements were made (i.e., maximally stimulated by the postprandial state). Presumably, differences would be seen at other times of day, when the animals were drinking but not yet eating; however, further studies are required to determine the exact mechanism responsible for the increase in protein synthesis we observed.

**Tissue specificity and comparison of adipose tissue to other tissues.** Persistent activation of mTOR and downstream targets of mTOR have been linked to an increase protein synthetic capacity (for reviews see Refs. 20, 55). It is anticipated, therefore, that the cumulative effects of consuming leucine and norleucine in the water may be an increase in protein synthetic capacity in certain tissues. The stimulation of protein synthesis by chronic leucine administration was sur-
prisingly tissue specific. Thus protein synthesis in heart and kidney was unaffected by leucine or norleucine supplementation, in contrast to the effects in other tissues. There are at least four possible explanations for this tissue specificity, that is, for the lack of response in heart and kidney. The first possible explanation for the tissue specificity is that the supplementation for 12 days may lead to downregulation of an important component(s) of the leucine-signaling pathway in heart and or kidney. If such adaptation does occur, it seems unlikely that it is due either to changes in the components of mTOR signaling or to the leucine metabolic pathways that we examined, because these did not change appreciably. Further studies will be required to evaluate this possibility once more information develops about how leucine activates mTOR signaling and as more information develops on the rapamycin-insensitive pathway. The second possibility is that either heart or kidney may already be maximally stimulated by ad libitum feeding. This seems particularly likely in heart, because we observed that S6K1 was stimulated strongly by the carbohydrate feeding in the preceding study (50), in contrast to other tissues where the control carbohydrate meal had no effect on S6K1. Third, heart and kidney may be poor responders, because they do not express the proteins coupling the presence of leucine to activation of the mTOR-signaling pathway or overexpress proteins antagonizing the signaling, such as phosphatases. In particular, kidney was noted to have comparably low levels of 4E-BP1. We also noted a rather different pattern of S6K1 responses in kidney and heart compared with gastrocnemius and adipose tissue in our previous, acute study (50). Although both heart and kidney showed an acute protein synthesis response to leucine and norleucine in fasted rats, no S6K1 response to either carbohydrate, leucine, or norleucine gavage was observed in kidney. Thus kidney may become an ideal tissue in which to examine the rapamycin-insensitive effects of leucine on protein synthesis. Similarly, S6K1 from heart did not show increased phosphorylation in response to oral leucine administration, as did S6K1 from muscle and adipose tissue. Last, leucine metabolism should also be considered. Kidney and heart express a high concentration of both BCATm and BCKD relative to other tissues. The resulting high flux through leucine metabolic pathways might diminish the ability of leucine to regulate mTOR signaling.

Adipose tissue gave the most robust response in protein synthesis to chronic leucine or norleucine supplementation. Thus the role of dietary amino acids as metabolic substrates in adipose tissue may be underappreciated. Adipose tissue also had the greatest level of 4E-BP1 phosphorylation. This may possibly be related to the finding that, compared with other peripheral tissues, adipose tissue expressed the greatest amount of mTOR per milligram of solubilized tissue protein.

Per gram tissue wet weight, adipose tissue, and skeletal muscle had equivalent capacities for protein synthesis. However, skeletal muscle represents 35–40% of body weight, so it has a larger impact on whole body protein synthesis compared with adipose tissue. Although there was not sufficient adipose tissue to make BCKD activity measurements, previous studies in our laboratory (49) and earlier studies (31) have demonstrated the capacity of adipocytes to transaminate leucine, and the results in Fig. 7 show that BCATm levels per milligram of detergent-solubilized lysate protein are high in adipose tissue. Furthermore, studies by Goodman’s group (Frick and colleagues (28, 29)) showed that the fat cell dehydrogenase is readily regulated by insulin and the ketoacid of leucine, presumably through BCKD kinase. Thus, although lower concentrations of the kinase and dehydrogenase are generally observed in adipose tissue, they seem to be in an appropriate ratio to allow nutritional regulation. Thus adipocytes may be an excellent model system in which to elucidate the mechanism of mTOR regulation by leucine.

Tissue-specific expression of enzymes involved in leucine catabolism and leucine as a potential nutrient signal. It is not entirely clear whether leucine or the transamination metabolite α-ketoisocaproate mediates the effects on mTOR signaling. We (27, 49) and others (63) found leucine to be more efficacious than α-ketoisocaproate in skeletal muscle and adipocytes. On the other hand, Patti et al. (57) and Xu et al. (70) reported that α-ketoisocaproate was more efficacious in different cell lines. Attempts have been made to address this question by inhibiting BCATm (27, 49, 57, 70). A limiting factor is that specific inhibitors of the first reversible step in leucine metabolism are not available, and the available inhibitors can affect ATP concentrations within the cells because they are least potent against BCATm (75, 86). This is important, because ATP concentration may affect mTOR activity due to its relatively low K_m for ATP (14).

Although it is recognized that this is still an open question, the tissue-dependent expression of BCATm, BCKD, and BCKD kinase would seem ideally suited to allow leucine to operate as a nutritional signal in liver and peripheral tissues. BCATm is not found in adult rat liver (39). Because mTOR signaling is regulated by leucine in freshly isolated rat hepatocytes (6), the absence of BCATm would facilitate its role as a nutritional signal there. The first step in leucine metabolism takes place primarily in extrahepatic tissues such as skeletal muscle, which releases considerable amounts of α-ketoisocaproate (41, 44). In kidney, muscle, or adipose tissue, either dietary leucine or α-ketoisocaproate may serve as a nutrient signal, as all possess considerable BCAT activity. Skeletal muscle expresses a disproportionately large amount of BCKD kinase relative to the amount of BCKD, thus limiting oxidation and promoting α-ketoisocaproate release (15). In liver, there is a disproportionately high concentration of the dehydrogenase relative to the BCKD kinase. Thus liver may be important for oxidizing circulating ketoacid that escapes extrahepatic metabolism and removing the leucine/α-ketoisocaproate signal. A second benefit of having little or no hepatic BCATm activ-
ity but very active BCKD activity in the liver is to ensure that dietary leucine reaches peripheral tissues in sufficiently high concentrations to perform its function as a nutrient signal for the presence of amino acids in a meal. Thus, without BCATm in liver, leucine will be spared from so-called “first-pass” metabolism. As mentioned in the previous studies (47, 50), the lack of effect of norleucine on insulin secretion suggests that different mechanisms may be responsible for the effects of BCAAs/α-keto acids in islet cells [e.g., described by Xu et al. (70)] and other peripheral tissues. Future studies will be required to determine whether leucine and/or α-ketoisocaproate mediate these effects and for a complete understanding of the complex signaling pathways that mediate the effects of the nutritional signaling molecules.

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