Branched Chain Amino Acids Activate Messenger Ribonucleic Acid Translation Regulatory Proteins in Human Skeletal Muscle, and Glucocorticoids Blunt This Action

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Branched Chain Amino Acids Activate Messenger Ribonucleic Acid Translation Regulatory Proteins in Human Skeletal Muscle, and Glucocorticoids Blunt This Action*

ZHENQI LIU, LINDA A. JAHN, WEN LONG, DAVID A. FRYBURG, LIPING WEI, AND EUGENE J. BARRETT

Department of Internal Medicine, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

ABSTRACT
Branched chain amino acids (BCAA) are particularly effective anabolic agents. Recent in vitro studies suggest that amino acids, particularly leucine, activate a signaling pathway that enhances messenger ribonucleic acid translation and protein synthesis. The physiological relevance of these findings to normal human physiology is uncertain. We examined the effects of BCAA on the phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (eIF4E-BP1) and ribosomal protein S6 kinase (p70S6K) in skeletal muscle of seven healthy volunteers. We simultaneously examined whether BCAA affected urinary nitrogen excretion and forearm skeletal muscle protein turnover and whether the catabolic action of glucocorticoids could be mediated in part by inhibition of the action of BCAA on the protein synthetic apparatus.

In humans, amino acid infusions stimulate muscle protein synthesis (1, 2) and enhance the anabolic effect of insulin (2). In vitro, the branched chain amino acids (BCAA), including valine, leucine, and isoleucine, display particularly important anabolic actions on both muscle protein synthesis and degradation (3–6). In humans, infusion of BCAA alone slows skeletal muscle protein degradation, but does not increase protein synthesis (7, 8). This is perhaps not surprising, as the BCAA also inhibit whole body protein degradation and thereby decrease the arterial concentrations of other essential and nonessential amino acids (7). This decline may blunt any effect of infused BCAA on protein synthesis by limiting substrate availability and/or interfering with nutrient signaling by other amino acids. As the blood flow to resting muscle is low, any decreased availability of amino acids from muscle proteolysis might not be readily replaced from plasma.

Recently, several laboratories have shown that amino acids, especially leucine, stimulate the phosphorylation of several key proteins involved in the regulation of protein synthesis at the level of messenger ribonucleic acid (mRNA) translation in Chinese hamster ovary (CHO) (9, 10) and hepatoma cells (11). These proteins include eukaryotic initiation factor 4E-binding protein 1 (eIF4E-BP1, or PHAS-I) and the 40S ribosomal protein S6 kinase (p70S6K) among others (10). Both eIF4E-BP1 and p70S6K have been identified as important elements in the signal transduction pathways responsible for the regulation of protein synthesis (12, 13). Recently, we and others have observed that infusion of mixture of amino acids (14) or protein feeding (15, 16) can stimulate the phosphorylation of eIF4E-BP1, and p70S6K in vivo in rat skeletal muscle. Leucine deprivation is associated with decreased protein synthesis, decreased phosphorylation of eIF4E-BP1 and p70S6K, and decreased eIF4E availability in cultured L6 myoblasts (17). Oral administration of leucine to rats enhances protein synthesis independently of increased plasma insulin (18) and increases eIF4E availability for eIF4E:elf4F complex formation and p70S6K phosphorylation (19). Data for humans are lacking, and this translational regulation may apply to the physiological regulation of skeletal muscle in humans.

Translation initiation is a complex process comprising a set of reactions that leads to the formation of an 80S initiation complex (20). The step in initiation involving elf4E-mediated binding of mRNA to the 43S preinitiation complex to form the 48S preinitiation complex may be rate limiting for protein synthesis (21). elf4E is among the least abundant translation initiation factors, and changes in elf4E availability are as-


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sociated with increases or decreases in rates of protein synthesis (22, 23). When eIF4E is bound to eIF4E-BP1 it is not available to mediate the binding of mRNA to the 43S preinitiation complex. The phosphorylation of eIF4E-BP1 in response to insulin or other growth factors promotes its dissociation from eIF4E (24).

p70S6K is a 70-kDa serine/threonine kinase that is critical in both cell cycle progression through G1 and the translation of a subpopulation of mRNAs containing a terminal oligopyrimidine sequence near the 5' cap (25–27). Phosphorylation of S6 by p70S6K increases 5'-terminal oligopyrimidine mRNA translation. These mRNAs encode ribosomal proteins, initiation and elongation factors that play an important role in protein synthesis (13, 26). Although not fully elucidated, the signaling pathway that mediates eIF4E-BP1 and p70S6K phosphorylation involves activation of the mammalian target of rapamycin, a kinase downstream of phosphatidylinositol 3-kinase in the insulin signaling cascade (28, 29).

Glucocorticoid excess is associated with negative nitrogen balance in both experimental animals and humans (30–32). Glucocorticoid excess not only enhances proteolysis, but also inhibits protein synthesis and the transport of amino acids into muscle (33). In addition to its well known antagonism of insulin-mediated glucose disposal, it causes resistance to insulin’s antiproteolytic action in muscle (34). Whether it also affects the anabolic action of infused BCAA is not known, and the mechanism(s) by which glucocorticoids exert their catabolic action in humans is uncertain.

The major purpose of the present study was to examine whether BCAA can regulate activation of the protein synthesis pathway by phosphorylating eIF4E-BP1 and p70S6K in human skeletal muscle in vivo and, if so, whether glucocorticoid excess can blunt or abolish this effect. We used the phenylalanine kinetic method to assess protein synthesis and degradation in the forearm (35). The results showed that BCAA significantly stimulated the phosphorylation of both eIF4E-BP1 and p70S6K. Although dexamethasone treatment did not change the basal phosphorylation status of either eIF4E-BP1 or p70S6K, it significantly blunted the muscle’s response to BCAA in phosphorylating p70S6K. Dexamethasone treatment produced a significantly more negative net muscle protein balance. These findings suggest that BCAA play a significant signaling role to increase mRNA translation, and glucocorticoid excess blunts this process.

Subjects and Methods

Subjects

Seven healthy volunteers (five men and two women) were studied. Subjects ranged in age from 20–31 yr (mean, 22.6 ± 1.5 yr), had an average body mass index of 25 ± 0.82 kg/m², and had no history of endocrine or other major organ system disease. No subject was taking any medication, and all female participants had a negative serum pregnancy test 1–2 days before the study. Informed written consent was obtained from each volunteer. The study protocol was approved by the human investigation committee and the General Clinical Research Center advisory committee at the University of Virginia.

Study protocol

Each subject was studied twice in random order. In each study subject was provided with a eucaloric flesh-free diet from the University of Virginia General Clinical Research Center metabolic kitchen for 3 days and then admitted the evening before the experiment. After a 12-h overnight fast, a brachial artery and an ipsilateral, retrograde, median cutaneous vein in the study arm were catheterized percutaneously. The patency of the catheters was maintained by a slow infusion of normal saline. Another catheter was placed into a contralateral arm vein, and a primed (45 µCi) continuous (0.5 µCi/min) infusion of 1-[ring-2,6-3H]phenylalanine was given for 8 h. After a 2-h tracer equilibration period, an infusion of BCAA was administered systemically for the next 6 h. The BCAA solution (Branchamin, 4% in water, Travelen Laboratories, Deerfield, IL) was an equimolar mixture of valine, leucine, and isoleucine and was infused at a rate of 1.66 µmol/m/kg after priming with 5.0 µmol/kg/min for 30 min. In previous studies we observed that systemic infusion of BCAA at this rate causes significant 3- to 7-fold elevations in the arterial concentration of valine (196 ± 11 to 662 ± 21 µmol/L; P < 0.005), leucine (111 ± 6 to 441 ± 14 µmol/L; P < 0.005), and isoleucine (54 ± 4 to 395 ± 16 µmol/L; P < 0.005), with near-plateau levels attained within 60 min (n = 10) (7). Quadruplicate, paired arterial and deep venous blood samples were obtained at 10-min intervals before the end of the tracer equilibration period (basal period at −30, −20, −10, and 0 min) and at the end of 3 h (150, 160, 170, and 180 min) and 6 h (330, 340, 350, and 360 min) of BCAA infusion for measurements of insulin, glucose, lactate, oxygen balance, phenylalanine balance, and phenylalanine kinetics. For 2 min before and during the withdrawal of each deep venous blood sample, a pediatric sphygmomanometer cuff was inflated around the wrist to 200 mm Hg to exclude blood flow to the hand. Forearm blood flow was measured after each pair of blood samples was taken using capacitance plethysmography. Urine samples were collected during the 12-h period before the infusion and the 6-h period during the infusion for measurement of nitrogen balance. Just before beginning the systemic infusion of BCAA, the subject underwent a biopsy of vastus lateralis muscle, using a Bergstrom biopsy needle. This biopsy was repeated in the opposite leg at the end of the study. Muscle tissues were immediately frozen and stored in liquid nitrogen for later analysis of eIF4E-BP1 and p70S6K (see Muscle biopsy below).

The second study paradigm is exactly the same as that described above, except that the subjects received dexamethasone (2 mg, orally, every 6 h) for 3 days before the study. Each subject was studied on the fourth day and continued to receive dexamethasone during the study. These two studies were separated by an 8-week washout period and were conducted in randomized order.

Muscle biopsy

Before the muscle biopsy, the anterior thigh was shaved and washed with iodine. The subject was prepped and draped in a sterile fashion. Anesthesia was obtained with approximately 10 cc 2% xylocaine in the overlying skin and muscle fascia of the vastus lateralis. Once adequate anesthesia was obtained, an incision was made in the skin and underlying tissue (~5 mm long × 2 cm deep) with a no. 11 scalpel needle. A Bergstrom muscle biopsy needle (od, 4 mm; Popper and Sons, New Hyde Park, NY) was then placed within the incision tract and advanced into the vastus lateralis muscle. Approximately 60 cc of suction were applied, and three muscle biopsies were rapidly obtained by rotating the needle clockwise. The biopsy needle was then removed, and the muscle specimen was quickly frozen and stored in liquid nitrogen until analyzed. To minimize bruising, firm pressure was maintained at the biopsy site for 10 min. The site was subsequently dressed, and 2–3 lb pressure were placed on the site for 30 min before wrapping with an Ace bandage. Subjects were instructed to avoid vigorous activity for 48 h.

Calculations of forearm phenylalanine kinetics

Net forearm balances for glucose, lactate, oxygen, and phenylalanine were calculated using the Fick principle: net balance = ([A] − [V]) × F, where [A] and [V] are arterial and venous substrate concentrations, and F is forearm blood flow in milliliters per min/100 mL forearm volume.

The forearm phenylalanine kinetics, determined using steady state isotope incorporation equations, were calculated as previously described (36–38). Phenylalanine is neither synthesized nor metabolized in muscle (39). The only metabolic fate of phenylalanine in muscle is to be incorporated into protein, whereas the only endogenous source of phenylalanine released into blood traversing muscle is protein. Phenylalanine is also
not concentrated by the muscle, and the intracellular and extracellular concentrations are nearly equal (1). Therefore, during steady state infusion of \[^{3}H\]phenylalanine, measurement of the rate of labeled phenylalanine by muscle [rate of disappearance (Rd)] and the dilution of its specific activity as it traverses the muscle [rate of appearance (Rq)] can be used to estimate protein synthesis and breakdown, respectively. The calculation is based on the measurement of the concentration and specific activity (SA) of systemically infused \[^{3}H\]labeled phenylalanine in both entering arterial and draining venous blood in the muscle bed under steady-state conditions, together with blood flow to the tissue. The balance of phenylalanine simply reflects the difference between its uptake for protein synthesis and its release from degraded protein. Therefore, net balance = ([A] – [V]) \times flow = synthesis – degradation. This can be better explained using the following algebraic formulations: net balance = ([A] – [V]) \times flow (Eq I); protein synthesis = ([dpm\text{artery} – dpm\text{vein}] \times flow)/SA\text{\textsubscript{artery}} (Eq II); muscle protein breakdown = synthesis – net balance (Eq III).

It is likely that this approach will underestimate rates of synthesis and degradation if phenylalanine derived from proteolysis reenters protein before mixing with the cellular pool through which it exits the cell (40, 41). Whole body phenylalanine fluxes at steady state both basally (90–120 min) and at the end of BCAA infusion (450–480 min) were estimated from the ratio of the tracer infusion rate to the arterial specific activity, as previously described (38).

Analysis of eIF4E-BP1 and p70\textsuperscript{S6K} phosphorylation

Pieces (20 mg) of frozen vastus lateralis muscle tissue were weighed and powdered in frozen 25 mmol/L Tris-HCl buffer (26 mmol/L potassium fluoride and 5 mmol/L ethylenediamine tetraacetate, pH 7.5), then disrupted by sonication using a microtip probe (0.5 s on/0.5 s off for 45 s total) at a 3.0 power setting on the Fisher XL2020 sonicator (Fisher Scientific, Pittsburgh, PA). The homogenate was centrifuged at 2000 rpm for 2 min, and the protein concentration was measured in the supernatant using the Bradford method (42). For eIF4E-BP1, one aliquot of supernatant (60 \mu g protein) was diluted with an equal volume of SDS sample buffer, and electrophoresed on a 15% polyacrylamide gel. For p70\textsuperscript{S6K}, another aliquot of the muscle homogenate supernatant (100 \mu g protein) was diluted with an equal volume of SDS sample buffer and run on an 8% SDS PAGE. Proteins on both gels were electrophoretically transferred to nitrocellulose membranes. After blocking with 5% low fat milk in Tris-buffered saline Tween, membranes were incubated with rabbit antirat eIF4E-BP1 (developed against intact recombinant rat eIF4E-BP1) or rabbit antirat p70\textsuperscript{S6K} (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at room temperature. This was followed by donkey antirabbit IgG coupled to horseradish peroxidase, and the blot was developed using an enhanced chemiluminescence Western blotting kit (Amersham Pharmacia Biotech, Piscataway, NJ). Autoradiography of the Western blot was carried out using the Molecular Dynamics, Inc., Piscataway, NJ) and quantitated using ImageQuant 3.3. The densities of all bands were measured, and the fraction of protein migrating more slowly (\beta\gamma) was determined as the appropriate ratio (\beta\gamma/total). Figure 1 illustrates the eIF4E-BP1 and p70\textsuperscript{S6K} phosphorylation status observed on Western blots of biopsied muscle obtained during the basal period and at the end of BCAA infusion in both study groups. For both eIF4E-BP1 and p70\textsuperscript{S6K}, the phosphorylation forms of two target proteins. To assess the effect of protein loading on the ratio of the several phosphorylated forms of both eIF4E-BP1 and p70\textsuperscript{S6K}, we measured the ratio of eIF4E-BP1 and p70\textsuperscript{S6K} (\beta\gamma/total) as a function of the amount of protein loaded on the gel. The amount of protein loaded ranged from 30–80 \mu g for eIF4E-BP1 and from 60–140 \mu g for p70\textsuperscript{S6K}, and we found that within these ranges the protein loaded in each lane does not affect the ratio between the various phosphorylation forms of two target proteins.

Analytic methods

Whole blood glucose and lactate concentrations were measured in duplicate using a combined glucose/lactate analyzer (YSI, Inc., Yellow Springs, OH). Plasma insulin concentrations were determined using a double antibody RIA technique. The blood oxygen content was measured spectrophotometrically using an OSM2 hemoximeter (Radiometer, Copenhagen, Denmark). The phenylalanine concentration and specific activity in arterial and venous blood were determined using a high pressure liquid chromatography procedure, described previously (7).

Statistical analysis

All data are presented as the mean ± SEM. Data for glucose, lactate, oxygen, and phenylalanine are averaged over the four time points in the basal (−30 to 0 min) and BCAA infusion (150–180 min and 330–360 min) periods for each subject. Stochastic comparisons between the basal and BCAA infusion periods and between control and dexamethasone-treated studies were made using a two-tailed paired t test.

Results

Effects of BCAA infusion and dexamethasone treatment on forearm blood flow, and insulin and substrate levels

Postabsorptive forearm blood flow and blood glucose, lactate, and insulin concentrations are shown in Fig. 2 and Table 1, both with and without dexamethasone treatment. BCAA infusion did not significantly alter forearm blood flow, glucose balance, oxygen balance, and arterial and venous insulin concentrations in either control or dexamethasone studies. It did increase forearm lactate release for both control and dexamethasone-treated subjects. Dexamethasone treatment produced a 4-fold increase in the basal insulin
TABLE 1. Effects of BCAA and dexamethasone on forearm blood flow and substrate balances

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal 3 h 6 h</td>
<td>Basal 3 h 6 h</td>
</tr>
<tr>
<td>Blood flow (mL/min-100 mL)</td>
<td>4.25 ± 0.5</td>
<td>5.0 ± 0.5</td>
</tr>
<tr>
<td>Glucose balance (µmol/min-100 mL)</td>
<td>1.06 ± 0.17</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Lactate balance (µmol/min-100 mL)</td>
<td>-0.15 ± 0.25</td>
<td>0.27 ± 0.2</td>
</tr>
<tr>
<td>Oxygen balance (µmol/min-100 mL)</td>
<td>9.54 ± 1.4</td>
<td>8.9 ± 1.4</td>
</tr>
<tr>
<td>Arterial insulin (pmol/L)</td>
<td>31.8 ± 8</td>
<td>126.6 ± 33.6d</td>
</tr>
<tr>
<td>Venous insulin (pmol/L)</td>
<td>25.7 ± 11.4</td>
<td>108.3 ± 35.4</td>
</tr>
<tr>
<td>Arterial phenylalanine (µmol/L)</td>
<td>46.95 ± 2.5</td>
<td>57.4 ± 2.6e</td>
</tr>
<tr>
<td>Venous phenylalanine (µmol/L)</td>
<td>51.67 ± 2.5</td>
<td>63.9 ± 2.9e</td>
</tr>
</tbody>
</table>

* P < 0.05 vs. control baseline.
* P < 0.03 vs. dexamethasone baseline.
* P < 0.01 vs. dexamethasone baseline.
* P < 0.04 vs. control baseline.
* P < 0.001 vs. control baseline.
* P < 0.001 vs. dexamethasone baseline.

A.

B.

![Fig. 3. Effects of BCAA infusion and dexamethasone treatment on urinary nitrogen excretion and whole body phenylalanine flux. A, BCAA infusion decreased urinary nitrogen excretion significantly in both control (#, P < 0.02) and dexamethasone-treated (##, P < 0.015) studies. Basal urinary nitrogen excretion was significantly higher in dexamethasone-treated studies (*, P = 0.02 vs. basal control). B, BCAA infusion decreased whole body phenylalanine flux significantly in both control (#, P < 0.02) and dexamethasone-treated (##, P < 0.007) studies.](image)

Concentration (from 31.8 ± 9.6 to 126.6 ± 33.6 pmol/L; P < 0.04), but had no effect on arterial blood flow (4.25 ± 0.5 to 5.0 ± 0.5 mL/min-100 mL). The arterial plasma glucose concentration rose from 4.70 ± 0.08 to 5.29 ± 0.17 mmol/L (P < 0.015), and the lactate concentration rose from 0.93 ± 0.19 to 1.46 ± 0.20 mmol/L (P = 0.0015). Despite higher basal insulin and glucose values, basal forearm glucose uptake was slightly decreased by dexamethasone treatment.

Effects of BCAA infusion and dexamethasone treatment on whole body and forearm muscle protein metabolism

BCAA infusion significantly decreased urinary nitrogen excretion (Fig. 3A), whole body phenylalanine flux (Fig. 3B), and plasma phenylalanine concentrations in both control and dexamethasone studies (Table 1). The net decrease in arterial phenylalanine levels was more pronounced in control studies (−9.10 ± 0.97 and −10.84 ± 1.44 µmol/L at 3 and 6 h) than in dexamethasone-treated studies (−3.69 ± 1.38 and −5.40 ± 1.82 µmol/L at 3 and 6 h; P = 0.006, by ANOVA). BCAA infusion significantly improved forearm phenylalanine balance in control subjects at 6 h (Fig. 4A), although, as we previously reported (7), it did not increase phenylalanine Rd.

Dexamethasone treatment significantly increased basal urinary nitrogen excretion (5.90 ± 0.44 vs. 9.51 ± 1.19 g nitrogen/g creatinine, control vs. dexamethasone, P = 0.02) without affecting basal arterial creatinine excretion (0.70 ± 0.09 vs. 0.86 ± 0.08 g, P = 0.262). It also significantly increased the basal plasma phenylalanine concentrations (46.9 ± 2.5 vs. 57.4 ± 2.6 µmol/L, control vs. dexamethasone, P < 0.001). The basal whole body phenylalanine flux was higher in dexamethasone-treated subjects (0.62 ± 0.07 µmol/min) than in control studies (0.48 ± 0.03 µmol/min), although the difference was not statistically significant (P = 0.09). Postabsorptive net release of phenylalanine from the forearm was significantly higher in dexamethasone-treated studies (−20.2 ± 3.1 vs. −32.7 ± 5.1 nmol/min-100 mL, control vs. dexamethasone, P < 0.02; Fig. 4A). Basal forearm phenylalanine Ra was not different (61.4 ± 12.3 vs. 58.8 ± 12.1 nmol/min-100 mL; Fig 4B), but Rd was lower in dexamethasone-treated studies (41.2 ± 11.2 vs. 26.9 ± 9.2 nmol/min-100 mL), although it was not statistically significant (P = 0.2; Fig. 4C). BCAA infusion did not change the balance in dexamethasone-treated subjects (Fig. 4A).

Effects of BCAA infusion and dexamethasone treatment on eIF4E-BP1 and p70S6K phosphorylation

To quantify the extent of phosphorylation of eIF4E-BP1, we measured the ratio of the intensity of the more slowly
migrating species (\(\beta + \gamma\)) to that of the total integrated intensity (\(\alpha + \beta + \gamma\)). The slowly migrating species represents the more phosphorylated forms of eIF4E-BP1, and an increase in the quantity of this form would correspond to an increase in phosphorylation of eIF4E-BP1 and a greater amount of eIF4E available to initiate translation. BCAA infusion significantly increased the \(\beta + \gamma/\alpha + \beta + \gamma\) ratio (i.e. the more phosphorylated portion) of eIF4E-BP1 in both control studies (0.81 ± 0.05 vs. 0.86 ± 0.03; \(P < 0.02\); dexamethasone-treated), whereas BCAA infusion significantly increased the \(\beta + \gamma/\alpha + \beta + \gamma\) ratio in both control and dexamethasone-treated studies (0.86 ± 0.03 vs. 0.91 ± 0.04; \(P = 0.01\)). The basal eIF4E-BP1 phosphorylation status was comparable in both control and dexamethasone-treated studies (0.85 ± 0.05 vs. 0.89 ± 0.02; Fig. 5A).

Similarly, to quantify the extent of phosphorylation of p70\(^{euk}\), we measured the ratio of the intensity of the more slowly migrating species (\(\beta\) and \(\gamma\)) to that of the total integrated intensity (\(\alpha + \beta + \gamma\); Fig. 5B). The overall p70\(^{euk}\) kinase activity is dependent on the phosphorylation of at least seven Ser/Thr residues at three separate domains (27, 43). The uppermost bands (\(\beta\) and \(\gamma\)) represent the more highly phosphorylated forms of p70\(^{euk}\) and generally correspond to species with greater kinase activity. BCAA infusion significantly increased the ratios of \(\beta + \gamma/\alpha + \beta + \gamma\) (i.e. the phosphorylated portions) of p70\(^{euk}\) in control studies (0.269 ± 0.027; \(P = 0.03\)), but not in dexamethasone-treated studies (0.196 ± 0.007; \(P = 0.3\)). The basal phosphorylation status of p70\(^{euk}\) was comparable in both control and dexamethasone-treated studies (0.185 ± 0.009 vs. 0.178 ± 0.011).

**Discussion**

By obtaining skeletal muscle biopsies before and after BCAA infusion and examining the phosphorylation status of both eIF4E-BP1 and p70\(^{euk}\) using quantitative Western blotting techniques, we found that BCAA stimulate the phosphorylation of these two key proteins involved in activating the mRNA translation apparatus. These results demonstrate that the cellular pathways that regulate translation initiation are, in fact, stimulated by BCAA in humans and suggest a potentially significant anabolic signaling role for BCAA in increasing mRNA translation and protein synthesis. This occurs with increments of circulating BCAA like those seen postprandially, suggesting that this is a normal physiological response. Inasmuch as insulin has been shown to stimulate eIF4E-BP1 and p70\(^{euk}\) phosphorylation in a variety of in vitro systems (9, 44, 45), the possibility must be considered that the BCAA are acting indirectly by stimulating insulin secretion,
which then promotes the phosphorylation of these proteins. This appears unlikely, as in the current study plasma insulin concentrations were not increased at either 3 or 6 h into the BCAA infusion. In addition, we observed that increments in plasma insulin from postabsorptive concentrations (~50 pmol/L) to midphysiological levels (~300 pmol/L) selectively promote phosphorylation of p70^{s6k}, but not eIF4E-BP1, in human skeletal muscle (46). Beyond that, infusion of low doses of insulin together with amino acids in the rat has no greater stimulatory effect on the phosphorylation of either eIF4E-BP1 or p70^{s6k} than is seen with giving amino acids alone (14). Experiments in laboratory rats also demonstrated that leucine administration enhances protein synthesis, increases the phosphorylation of eIF4E-BP1 and p70^{s6k}, and increases the availability of eIF4E for eIF4E:eIF4G complex formation independently of increases in plasma insulin (18, 19).

That amino acids have a direct signaling role to activate both eIF4E-BP1 and p70^{s6k} is well demonstrated in isolated cell systems where amino acid deficiency results in the reversible inactivation of p70^{s6k} and decreased phosphorylation of eIF4E-BP1 (9). Restoration of amino acids promptly restores the activity of translation toward normal. This occurs in the absence of any changes in hormonal milieu in the culture medium. Of all amino acids, BCAA, especially leucine, appear to have the bulk of the regulatory influence on protein synthesis. In cultured L6 myoblasts, leucine deprivation was associated with decreased protein synthesis, decreased phosphorylation of eIF4E-BP1 and p70^{s6k}, increased eIF4E-BP1:eIF4E complex, and decreased p70^{s6k}:eIF4E complex (17). In humans there is no circumstance when amino acid concentrations fall to negligible in the extracellular fluid as in these in vitro studies. Our current results underscore that changes in BCAA concentration within the physiological range should be sufficient to trigger an effect on the phosphorylation of eIF4E-BP1 and p70^{s6k}.

Like the BCAA, pharmacological concentrations of insulin can activate/phosphorylate p70^{s6k} and eIF4E-BP1 (47) via the phosphoinsitol-3-kinase/Akt pathway. However, the BCAA must act downstream of these early steps because they do not affect glucose uptake by muscle. Furthermore, in studies in isolated cells amino acid deficiency has no effect on the activity of early steps in the insulin signaling pathway, including receptor tyrosine phosphorylation, phosphoinositol 3-kinase and Akt/PKB activation, but results in rapid dephosphorylation of eIF4E-BP1 or p70^{s6k} (9) and renders them refractory to stimulation by insulin. This likely downstream site for BCAA action is of interest with regard to the blocking effect of dexamethasone as well. The results of several studies indicate that glucocorticoids induce insulin resistance by actions on proximal steps in the insulin signaling cascade (48, 49). Although we did find evidence for resistance to insulin-mediated glucose disposal in the current study, the action of dexamethasone on BCAA-induced phosphorylation of eIF4E-BP1 or p70^{s6k} suggests an action of glucocorticoids at a more distant site as well.

We do not know the time course for the effect of BCAA on the phosphorylation of eIF4E-BP1 or p70^{s6k}. In the current study we examined the changes in protein phosphorylation after 6 h of infusion. In previous studies in the rat we observed that a balanced amino acid mixture stimulated phosphorylation of both proteins within 3 h (14). Pharmacological doses of insulin produced these changes within 30–60 min (50). Current evidence would suggest that these proteins remain in a hyperphosphorylated state as long as the provocative stimulus remains.

The actions of infused BCAA to decrease plasma phenylalanine concentration, whole body phenylalanine flux, muscle net phenylalanine release, and urinary nitrogen excretion all indicate an anabolic action of these amino acids in healthy humans. These results are entirely consistent with our previously reported effect of BCAA on whole body and skeletal muscle protein metabolism (7, 8). Moreover, in both previous studies and the current study whole body and skeletal muscle Ra (a measure of protein degradation) declined (not a statistically significant change in the current study, but highly significant in two previous works) with BCAA infusion. The observation that muscle Rd (an index of protein synthesis) was not affected was fully anticipated based on our previous studies (7, 8). We (2) and others (1) have previously shown that the rate of protein synthesis in muscle is affected by amino acid availability from plasma. In the current study the decline in concentrations of multiple amino acids, other than the BCAA, may have blunted part of the anabolic response. We recently observed that infusion of a complete mixture of amino acids to rats increases the rate of protein synthesis, and this increase is accompanied by increased phosphorylation of eIF4E-BP1 and p70^{s6k} (14). A similar effect of feeding a high protein diet to rats has recently been reported (16). The present studies were designed to assess whether the BCAA initiate a signaling process in human muscle akin to the effect of amino acids (especially leucine) reported in cultured cells and more recently in animal tissues. That protein synthesis did not increase probably reflects the need for additional amino acids or other factors. We did not in the current study attempt to prevent the decline in the concentration of non-BCAA by infusing a balanced amino acid solution for several reasons. First, we were concerned that replacing all 15 other amino acids only to the basal concentration cannot be readily accomplished with available amino acid preparations. Invariably, some will be overreplaced and others underreplaced. Elevating circulating amino acids by overreplacement would obscure whether the BCAA and not another amino acid or mixture of amino acids was required to activate the translation initiation process. In addition, we could not anticipate the effect of dexamethasone, i.e. whether it would blunt the effect of BCAA to lower plasma amino acids. Infusing the same amounts of non-BCAA amino acids during the dexamethasone treatment would probably have yielded much higher amino acid levels, as dexamethasone raised the baseline post-absorptive amino acid concentrations and blunted the BCAA-induced decline in plasma amino acid concentrations.

With severe diabetes in the streptozotocin-treated rat there is a rapid decline in muscle protein synthesis secondary to a block in translation initiation (47, 51) and a rapid fall off in ribosomal number (52). Restitution of insulin rapidly reverses this translational block (47, 53). It is interesting that in insulin-withdrawn type 1 diabetes in humans (less severe than streptozotocin-treated rats), the concentrations of...
BCAA rise, and the rates of whole body protein synthesis (determined as nonoxidative leucine disposal) are not decreased despite the catabolic state (54, 55). This rise in BCAA may, if the same signaling pathway is activated during moderate insulin deficiency, serve to attenuate the protein wasting.

Dexamethasone treatment caused significant increases in urinary nitrogen excretion and more negative forearm phenylalanine net balance and an increase in whole body phenylalanine flux. These findings are in accord with those previous reported for forearm muscle by our laboratory (34) and the whole body flux findings from multiple studies (31, 56, 57). Glucocorticoid treatment in patients with rheumatoid arthritis reportedly decreases skeletal muscle protein synthesis (58). It is not clear mechanistically just how glucocorticoids exert their anabolic action, but effects on both protein synthesis and degradation have been reported. In experimental animals treated with glucocorticoids inhibition of protein synthesis is observed when measured in vitro (59) or in vivo (60, 61). Treatment of rats for 5 days with cortisone acetate reduced skeletal muscle protein synthesis by 56%, and this was accounted for by both a loss of tissue RNA and the development of a block in peptide chain initiation with decreased eIF2 activity (60, 61). Recent studies in experimental animals indicate that glucocorticoids can increase the activity of the proteasomal proteolytic pathway in skeletal muscle (62–64).

In the present study we observed that dexamethasone treatment induced significantly more negative skeletal muscle protein balance and whole body nitrogen balance without changing the basal phosphorylation status of eIF4E-BP1 and p70S6K. Dexamethasone, however, significantly blunted BCAA-induced phosphorylation of p70S6K in muscle. Data from other studies suggested that phosphorylation of p70S6K correlates strongly with increases in kinase activity (27), and it appears that p70S6K is required for maintaining the apparatus required for ongoing protein synthesis, rather than translation initiation. Microinjection of anti-p70S6K antibodies into fibroblasts inhibits serum-stimulated protein synthesis by about 90% (25). Our findings indicate that glucocorticoids exert their anabolic action partly through antagonizing the stimulatory effects of BCAA on the signal transduction pathway in maintaining protein synthesis.

A potential confounding factor in the present study is the 4-fold increase in arterial insulin concentrations induced by dexamethasone. Insulin itself can improve human skeletal muscle phenylalanine balance, principally by restraining proteolysis (36). We have shown that physiological hyperinsulinemia does not activate eIF4E-BP1 or protein synthesis (2, 46) and that dexamethasone can blunt the insulin-induced anticalcobic effect of hyperinsulinemia (34). Thus, in the current study despite the dual anabolic stimuli of increased insulin and BCAA infusion muscle protein balance became more negative in the dexamethasone-treated subjects. It is also of interest that even in the basal period the circulating concentrations of phenylalanine (Table 1) and of most amino acids (34) are elevated during dexamethasone treatment. Typically, increased concentrations of amino acids stimulate protein synthesis in muscle (2). That this does not occur during the baseline period in the current study may reflect an effect of dexamethasone to inhibit the protein synthesis usually provoked by generalized increase in plasma amino acids.

In conclusion, BCAA promote positive protein balance and stimulate phosphorylation of (and thereby activating) eIF4E-BP1 and p70S6K in human skeletal muscle. Thus, the BCAA have a direct signaling role in regulating normal skeletal muscle protein metabolism in humans. Dexamethasone does not change the basal phosphorylation status of eIF4E-BP1 and p70S6K, but significantly blunts BCAA-induced p70S6K phosphorylation and promotes negative whole body and skeletal muscle protein balance. Our findings suggest that BCAA play a major signaling role in mRNA translation in human skeletal muscle, and dexamethasone blunts this action.

References


