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Amino Acids Stimulate Translation Initiation and Protein Synthesis through an Akt-Independent Pathway in Human Skeletal Muscle

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Studies *in vitro* as well as *in vivo* in rodents have suggested that amino acids (AA) not only serve as substrates for protein synthesis, but also as nutrient signals to enhance mRNA translation and protein synthesis in skeletal muscle. However, the physiological relevance of these findings to normal humans is uncertain. To examine whether AA regulate the protein synthetic apparatus in human skeletal muscle, we infused an AA mixture (10% Travesol) systemically into 10 young healthy male volunteers for 6 h. Forearm muscle protein synthesis and degradation (phenylalanine tracer method) and the phosphorylation of protein kinase B (or Akt), eukaryotic initiation factor 4E-binding protein 1, and ribosomal protein S6 kinase ($p70^{S6K}$) in vastus lateralis muscle were measured before and after AA infusion. We also examined whether AA affect urinary nitrogen excretion and whole body protein turnover.

Postabsorptively all subjects had negative forearm phenylalanine balances. AA infusion significantly improved the net phenylalanine balance at both 3 h ($P < 0.002$) and 6 h ($P < 0.02$). This improvement in phenylalanine balance was solely from increased protein synthesis ($P = 0.02$ at 3 h and $P < 0.003$ at 6 h), as protein degradation was not changed. AA also significantly decreased whole body phenylalanine flux ($P < 0.004$). AA did not activate Akt phosphorylation at Ser^{473}, but significantly increased the phosphorylation of both eukaryotic initiation factor 4E-binding protein 1 ($P < 0.04$) and $p70^{S6K}$ ($P < 0.001$). We conclude that AA act directly as nutrient signals to stimulate protein synthesis through Akt-independent activation of the protein synthetic apparatus in human skeletal muscle. (*J Clin Endocrinol Metab* 87: 5553–5558, 2002)

SKELETAL MUSCLE PROTEIN synthesis is closely regulated *in vivo*, and the phosphatidylinositol 3-kinase (PI3-knase)/mammalian target of rapamycin (mTOR) pathway has been implicated as having a pivotal role in this process. Within this pathway, protein kinase B (or Akt) (1), eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) (2), and ribosomal protein S6 kinase ($p70^{S6K}$) (3) are several key intermediates involved in the regulation of translation initiation and protein synthesis (4, 5). Activation of Akt, a Thr/Ser kinase downstream of PI3-kinase, promotes the phosphorylation and activation of mTOR (1, 6–8), which, in turn, phosphorylates 4E-BP1 and $p70^{S6K}$. Dephosphorylated 4E-BP1 represses mRNA translation initiation by binding to eIF4E. Phosphorylation of 4E-BP1 frees eIF4E, which can then associate with eIF4G to form the preinitiation complex and initiate protein synthesis. Phosphorylation of $p70^{S6K}$ increases the phosphorylation of ribosomal protein S6 (9) and facilitates the synthesis of some ribosomal proteins, initiation factors, and elongation factors that play important roles in protein synthesis (5, 10).

Amino acids (AA) have been shown to stimulate skeletal muscle protein synthesis in humans (11, 12). Recent evidence suggests that AA not only function as substrates for protein synthesis, but they also provide nutritional signals to activate translation initiation and protein synthesis (13–15). Many studies have demonstrated that AA, especially branched chain AA (BCAA) stimulate the phosphorylation of 4E-BP1 and $p70^{S6K}$ in various cell preparations and animal studies (15–28). It appears that AA signal to 4E-BP1 and $p70^{S6K}$ via mTOR activation, since the AA-stimulated translation initiation is abrogated by rapamycin, a specific inhibitor of mTOR (15–18, 29).

Most studies examining AA- or BCAA-stimulated translation initiation and protein synthesis, whether conducted *in vivo* in animals or *in vitro* in cells, used high concentrations of AA or BCAA, leaving uncertain the physiological significance of the above findings in human skeletal muscle. We previously reported that physiological concentrations of BCAA stimulate 4E-BP1 and $p70^{S6K}$ phosphorylation without increasing protein synthesis in human skeletal muscles (30). The major purpose of the current study was to examine whether mixed AA containing all essential AA at physiological concentrations regulate muscle protein synthesis and degradation and whether AA provide nutrient signals to regulate translation initiation in human skeletal muscle *in vivo*. We quantitated the effect of mixed AA on protein synthesis and degradation using the forearm phenylalanine tracer kinetic method (31) and their effects on Akt, 4E-BP1, and $p70^{S6K}$ phosphorylation in biopsied vastus lateralis muscle samples. The results showed that AA significantly stimulated forearm protein synthesis and improved forearm protein net balance, without affecting muscle protein degradation. The phosphorylation of both 4E-BP1 and $p70^{S6K}$, but not of Akt, was increased significantly after AA infusion. These findings suggest that AA stimulate protein synthesis

Abbreviations: AA, Amino acids; BCAA, branched chain amino acids; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; eIF4E, eukaryotic initiation factor 4E; mTOR, mammalian target of rapamycin; PI3-kinase, phosphatidylinositol 3-kinase; Ra, rate of appearance; Rd, rate of disappearance.

through an Akt-independent activation of translation initiation in human skeletal muscle.

Subjects and Methods

Subjects

Ten healthy young male volunteers were studied. Subjects ranged in age from 19–26 yr (22.6 ± 0.9 yr), with an average body mass index of 23.4 ± 0.8 kg/m^2. They had no history of major organ system disease and were not taking any medication. Informed written consent was obtained from each volunteer before the study. The study protocol was approved by the human investigation committee and the general clinical research center advisory committee at University of Virginia before subject recruitment.

Study protocol

Subjects were kept on a meat-free diet for 3 d and then were admitted to the University of Virginia General Clinical Research Center the evening before the study. After a 12-h overnight fast, a brachial artery and an ipsilateral, retrograde, median deep antecubital 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 L-[ring-2,6-^{3}H]phenylalanine was given for 8 h. After a 2-h tracer equilibration period, a mixed AA solution was infused systemically for the next 6 h. Figure 1 shows the overall study protocol. The mixed AA solution (Travesol, 10% in water; Travenol Laboratories, Deerfield, IL) was a mixture of various essential and nonessential AA (see Fig. 1 for detailed composition of this mixture) and was infused at a rate of 0.015 ml/min·kg body weight. Quadruplicate paired arterial and deep venous blood samples were obtained at 10-min intervals at 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 AA infusion for measurements of AA, 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 about the wrist to 200 mm Hg to exclude blood flow to the hand. Forearm blood flow was measured after each pair of blood samples using capacitance plethysmography. Urine samples were collected during the 12-h period before the infusion and during the 6-h period during the AA infusion for measurement of nitrogen and creatinine excretion. Just before beginning the systemic infusion of AA, the subject underwent a biopsy of vastus lateralis muscle, using a Bergstrom biopsy needle. Muscle 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 Akt, 4E-BP1, and p70^{S6K}. The detailed description of muscle biopsy procedure was reported previously (30).

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,

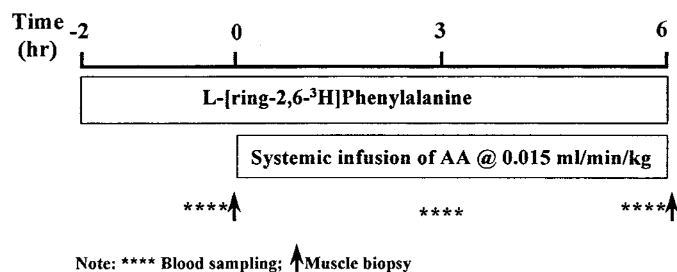

FIG. 1. Study protocol. AA were infused systemically for 6 h at a rate of 0.015 ml/kg·min, and each 100 ml infusate contained 480 mg histidine, 600 mg isoleucine, 730 mg leucine, 580 mg lysine, 400 mg methionine, 560 mg phenylalanine, 420 mg threonine, 180 mg tryptophan, 580 mg valine, 2070 mg alanine, 1150 mg arginine, 1030 mg glycine, 680 mg proline, 500 mg serine, and 40 mg tyrosine.

where [A] and [V] are arterial and venous substrate concentrations, and F is forearm blood flow in milliliters per minute per 100 ml forearm volume.

The forearm phenylalanine kinetics, determined using steady state isotope dilution equations, were calculated as previously described (30). In brief, phenylalanine is neither synthesized nor metabolized in muscle, and the balance of phenylalanine reflects the difference between its uptake for protein synthesis and its release from protein degradation. Simultaneous and paired sampling of forearm arterial and venous blood enables us to measure the concentrations and specific activities of phenylalanine and to calculate the uptake [rate of disappearance (Rd)] and the dilution [rate of appearance (Ra)] of [^{3}H]phenylalanine during steady state infusion of [^{3}H]phenylalanine, which can be used to estimate protein synthesis and degradation, respectively. Three formulas were used for calculations: 1) net balance = ([A] − [V]) × flow, 2) protein synthesis (Rd) = ([dpm_{artery} − dpm_{vein}] × flow)/SA_{vein}, and 3) muscle protein breakdown = synthesis − net balance.

Calculation of whole body phenylalanine fluxes

Whole body phenylalanine fluxes were estimated from the ratio of the tracer infusion rate to the arterial specific activity at steady state, both basally (−30 to 0 min) and at the end of AA infusion (330–360 min), as described previously (32).

Western immunoblotting technique

Pieces (~40 mg) of frozen vastus lateralis muscle tissue were weighed and powdered in frozen 25 mM Tris-HCl buffer [26 mM potassium fluoride and 5 mM EDTA (pH 7.5)], then disrupted by sonication using a microtip probe (0.5 sec on/0.5 sec off for 45 sec 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 (33). For Akt, one aliquot of the supernatant containing approximately 60 μg protein was diluted with an equal volume of sodium dodecyl sulfate sample buffer and electrophoresed on an 8% polyacrylamide gel. For 4E-BP1, one aliquot of the supernatant containing approximately 60 μg protein was diluted with an equal volume of sodium dodecyl sulfate sample buffer and electrophoresed on a 15% polyacrylamide gel. For p70^{S6K}, another aliquot of supernatant containing about 50 μg protein was diluted with an equal volume of sodium dodecyl sulfate sample buffer and electrophoresed on an 8% polyacrylamide gel. Proteins were then electrophoretically transferred to nitrocellulose membranes. After being blocked with 5% low fat milk in Tris-buffered saline plus Tween 20, membranes were incubated with either rabbit polyclonal Akt antibody or phospho-Akt (Ser^{473}) antibody (New England Biolabs, Inc., Beverly, MA) overnight at 4–8 C or rabbit anti-4E-BP1 or p70^{S6K} (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at room temperature. This was followed by a donkey antirabbit IgG coupled to horseradish peroxidase, and the blots were developed using an enhanced chemiluminescence Western blotting kit (Amersham Life Sciences, Piscataway, NJ).

Quantitation of Akt, 4E-BP1, and p70^{S6K} phosphorylation state

Autoradiographic films were scanned densitometrically (Molecular Dynamics, Inc., Sunnyvale, CA) and quantitated using ImageQuant 3.3. Figure 2 illustrates the Akt, 4E-BP1, and p70^{S6K} phosphorylation status on Western blots of biopsied muscle samples obtained during the basal period and at the end of AA infusion. For Akt, we intended to quantitate both the total and phospho-Akt (Ser^{473}) densities and calculate the ratios of phospho-specific Akt density to total Akt density if there was any phosphorylation of Akt at Ser^{473}. For both 4E-BP1 and p70^{S6K}, we exploited the different electrophoretic mobility properties of variously phosphorylated proteins to quantitate the phosphorylation status. When subjected to SDS-PAGE, the least phosphorylated portion (the α-form) migrates most rapidly, whereas the most phosphorylated portion (the γ-form) moves the slowest, with the modestly phosphorylated portion (the β-form) in between. The densities of all bands ($\alpha + \beta + \gamma$) were measured, and the fraction of protein migrating more slowly ($\beta + \gamma$) was determined as the appropriate ratios ($\beta + \gamma$/total). The β- and γ-forms

represent the more highly phosphorylated proteins, and the available data support a good correlation between electrophoretic mobility and biological activity for both 4E-BP1 (3, 34) and $p70^{S6K}$ (35). We also demonstrated a good correlation between the ratios of $\beta + \gamma$/total for both 4E-BP1 and $p70^{S6K}$ and the bulk protein synthesis rates *in vivo* in animal studies (data not shown).

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 an insulin ELISA (Diagnostic Systems Laboratories, Inc., Webster, TX). Blood oxygen content was measured spectrophotometrically using an OSM2 hemoximeter (Radiometer, Copenhagen, Denmark). Plasma AA concentrations were measured using an automated ion exchange chromatographic technique (D-500, Dionex, Sunnyvale, CA). Phenylalanine concentration and specific activity in arterial and venous blood were determined using an HPLC procedure as described previously (36).

Statistical analysis

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

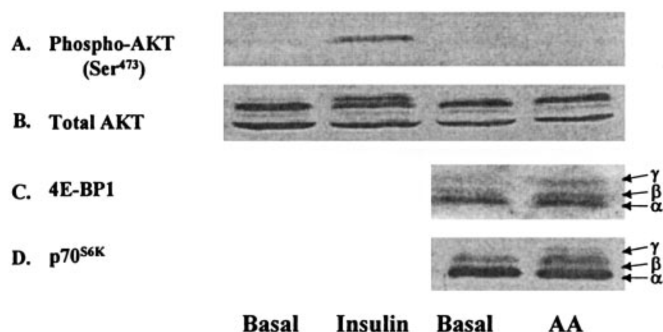

FIG. 2. Gel patterns of human skeletal muscle phospho-Akt (A), total Akt (B), 4E-BP1 (C), and $p70^{S6K}$ (D) on SDS-PAGE. For 4E-BP1 and $p70^{S6K}$, the α-band is the least phosphorylated portion and moves most rapidly. The β- and γ-bands are more phosphorylated and have slower electrophoretic mobility. Basal, Basal muscle biopsy sample; AA, muscle sample obtained after 6 h of AA infusion. The *two left lanes* are Akt gel patterns using muscle samples obtained from a young healthy human subject before and after systemic insulin infusion (20 mU/kg·min euglycemic clamp) for 2 h. As our positive control, insulin significantly stimulated the phosphorylation of Akt at Ser^{473}.

Results

Effects of AA infusion on forearm blood flow, and insulin and substrate levels

The postabsorptive forearm blood flow, and blood glucose, lactate, and insulin concentrations are shown in Table 1. AA infusion did not significantly alter arterial insulin concentrations, forearm blood flow, glucose balance, or oxygen balance, but increased forearm lactate release from −0.20 ± 0.11 to −0.51 ± 0.15 μmol/min·100 ml ($P < 0.02$). The basal venous phenylalanine concentrations were significantly higher than arterial phenylalanine concentrations, reflecting a net protein breakdown after an overnight fast (Table 1). This negative venous-arterial phenylalanine differential was either reversed (3 h) or abrogated (6 h) after AA infusion. Table 2 lists the concentrations of all AA measured at the basal period and at the end of 6-h infusion. Total AA concentrations were increased by 57 ± 3.5%, and total BCAA concentrations were increased by 109 ± 8.4% ($P < 0.00001$ for both) after 6-h AA infusion. The concentrations of asparagine, glutamic acid, and tyrosine were decreased by 13–21%, whereas the concentrations of all other AA were increased (by 9–227%) after AA infusion. The increment in AA concentrations was statistically significant for all AA except glutamine (9 ± 4.5%; $P < 0.07$).

Effects of AA infusion on whole body and forearm muscle protein metabolism

AA infusion slightly decreased urinary nitrogen excretion, although the difference was not statistically significant (6.72 ± 0.41 *vs.* 5.79 ± 0.35 g nitrogen/g creatinine, basal *vs.* AA infusion; $P = 0.09$; Fig. 3). However, whole body phenylalanine flux was dramatically decreased by AA infusion, from 0.54 ± 0.03 to 0.36 ± 0.04 μmol/min·kg ($P < 0.004$; Fig. 3).

Consistent with our previous reports (30, 37), all subjects had negative forearm protein balance after an overnight fast. AA infusion significantly improved forearm phenylalanine balance at 3 h, and the effect persisted at 6 h (Fig. 4). Protein synthesis was increased significantly at both 3 and 6 h. It appears that the improvement in protein balance at both 3 and 6 h can be accounted for solely by the increment in protein synthesis, as the rate of protein degradation (Ra) was not changed by AA infusion.

TABLE 1. Effects of AA infusion on forearm blood flow and substrate balances

	Basal	3 h	6 h
Blood flow (ml/min·100 ml)	4.31 ± 0.74	4.97 ± 0.86	5.17 ± 1.15
Glucose balance (μmol/min·100 ml)	0.60 ± 0.14	0.68 ± 0.17	1.05 ± 0.25
Lactate balance (μmol/min·100 ml)	−0.20 ± 0.11	−0.51 ± 0.12[a]	−0.51 ± 0.15[a]
Oxygen balance (μmol/min·100 ml)	7.3 ± 1.27	8.23 ± 1.73	8.85 ± 2.01
Arterial insulin (pmol/liter)	36 ± 3.6	41 ± 3.5	40 ± 4.7
Arterial phenylalanine (μmol/liter)	52.4 ± 3.6	106.0 ± 5.1[b]	118.5 ± 5.1[b]
Venous phenylalanine (μmol/liter)	58.8 ± 4.2[c]	101.9 ± 5.0[b,d]	116.7 ± 5.2[b]

[a] $P < 0.02$ *vs.* basal.
[b] $P < 0.0001$ *vs.* basal.
[c] $P = 0.01$ *vs.* arterial phenylalanine.
[d] $P < 0.005$ *vs.* arterial phenylalanine.

TABLE 2. Serum amino acid concentrations (μmol/liter) before and after amino acid infusion

	Basal	6 h	% Increase	*P*
α-Aminobutyrate	18 ± 0.9	32 ± 1.7	81 ± 11.1	<0.0002
Alanine	354 ± 38.7	669 ± 42.2	100 ± 16.3	<0.00001
Arginine	100 ± 3.4	237 ± 9.0	137 ± 9.6	<0.00001
Asparagine	39 ± 2.5	31 ± 3.0	−21 ± 4.5	<0.001
Aspartic acid	14 ± 1.1	16 ± 1.7	16 ± 4.8	<0.02
Citrulline	33 ± 1.0	44 ± 1.5	34 ± 4.6	<0.0002
Glutamic acid	199 ± 20	161 ± 14.3	−18 ± 1.8	<0.001
Glutamine	726 ± 57.8	788 ± 59.1	9 ± 4.5	<0.07
Glycine	225 ± 6.2	449 ± 18.2	99 ± 4.7	<0.00001
Histidine	80 ± 3.1	138 ± 5.2	73 ± 7.2	<0.00001
Isoleucine	60 ± 3.0	175 ± 5.6	199 ± 15.6	<0.00001
Leucine	109 ± 6.1	223 ± 8.9	108 ± 9.4	<0.00001
Lysine	134 ± 6.8	198 ± 8.5	49 ± 7.2	<0.00009
Methionine	26 ± 0.9	83 ± 3.8	227 ± 14.7	<0.00001
Ornithine	50 ± 2.5	80 ± 4.9	61 ± 8.4	<0.0002
Phenylalanine	50 ± 2.8	116 ± 5.1	134 ± 7.9	<0.00001
Serine	108 ± 4.7	190 ± 6.3	77 ± 6.8	<0.00001
Taurine	41 ± 1.7	45 ± 1.3	10 ± 3.8	<0.04
Threonine	106 ± 5.6	178 ± 8.6	70 ± 6.2	<0.00001
Tryptophan	52 ± 2.9	79 ± 4.5	54 ± 11.3	<0.0008
Tyrosine	49 ± 3.3	43 ± 3.2	−13 ± 2.8	<0.003
Valine	203 ± 8.7	372 ± 13.8	85 ± 6.9	<0.00001
Total BCAA	371 ± 17.0	770 ± 27.6	109 ± 8.4	<0.00001
Total AA	2777 ± 74.2	4348 ± 151.3	57 ± 3.5	<0.00001

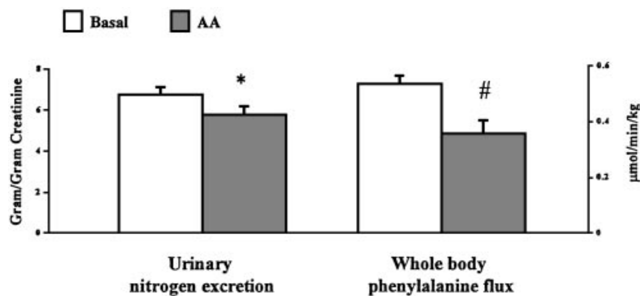

FIG. 3. Effects of AA infusion on urinary nitrogen excretion and whole body phenylalanine flux. AA infusion slightly decreased urinary nitrogen excretion, but this did not achieve statistical significance (*, $P = 0.09$), and significantly decreased whole body phenylalanine flux (#, $P < 0.004$).

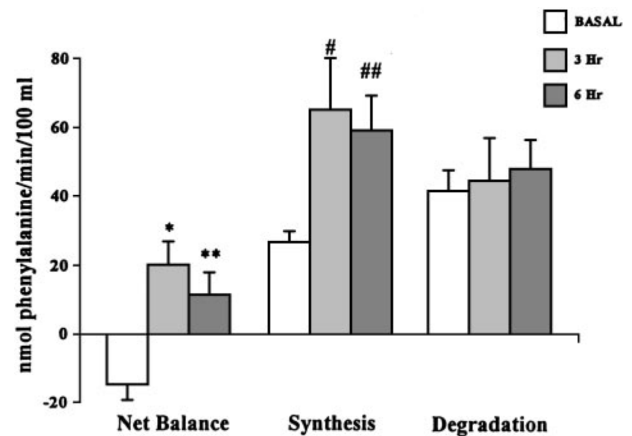

FIG. 4. Effects of AA infusion on forearm muscle protein metabolism. Postabsorptively all subjects had net release of phenylalanine from the forearm, resulting in negative protein balance. AA infusion significantly improved phenylalanine balance at both 3 h (*, $P < 0.002$) and 6 h (**, $P < 0.02$). This improvement is solely from increased forearm phenylalanine Rd [#, $P = 0.02$; ##, $P < 0.003$ (*vs.* basal)]. Forearm phenylalanine Ra was not changed by AA infusion.

Effects of AA infusion on Akt, 4E-BP1, and $p70^{S6K}$ phosphorylation

Phosphorylation of Akt at Thr^{308} and Ser^{473} is required for Akt kinase activity, and phosphorylation of Thr^{308} leads to the phosphorylation of Ser^{473} (8). AA infusion did not enhance Akt phosphorylation at Ser^{473} in our study subjects (Fig. 1, *two right lanes* in A and B).

To quantify the extent of phosphorylation of 4E-BP1 and $p70^{S6K}$, we measured the ratio of the intensity of the more slowly migrating species ($\beta + \gamma$) to that of the total integrated intensity ($\alpha + \beta + \gamma$). AA infusion significantly increased the $(\beta + \gamma)/(\alpha + \beta + \gamma)$ ratio of 4E-BP1 by decreasing the amount of rapidly migrating species (α) and increasing the density of the more slowly migrating forms ($\beta + \gamma$; 0.26 ± 0.04 *vs.* 0.32 ± 0.03; $P < 0.04$; Fig. 5), suggesting increases in 4E-BP1 phosphorylation and the amount of eIF4E available to initiate translation. For $p70^{S6K}$, the overall $p70^{S6K}$ kinase activity is dependent on the phosphorylation of at least seven Ser/Thr residues at three separate domains (9, 35). The uppermost bands (β and γ) represent the more highly phosphorylated forms of $p70^{S6K}$ and generally correspond to species with greater kinase activity. Similar to the effect on 4E-BP1, AA infusion significantly increased the ratios of $(\beta + \gamma)/(\alpha + \beta + \gamma)$ of $p70^{S6K}$ (0.161 ± 0.014 *vs.* 0.271 ± 0.019; $P < 0.001$; Fig. 5).

Discussion

Results from the current study demonstrated that moderate increments (within their physiological ranges) of circulating AA, similar to those seen postprandially (38), stimulated human skeletal muscle protein synthesis, improved protein balance, and enhanced the phosphorylation of both 4E-BP1 and $p70^{S6K}$. These findings indicate that changes in

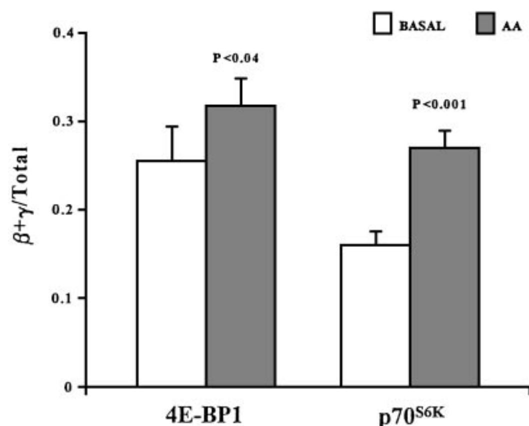

FIG. 5. Effects of AA infusion on 4E-BP1 and $p70^{S6K}$ phosphorylation. AA infusion significantly increased the phosphorylation of both 4E-BP1 and $p70^{S6K}$, evidenced by the increased ratios of $(\beta + \gamma)/(\alpha + \beta + \gamma)$ for both proteins.

AA concentrations within the physiological ranges were sufficient to stimulate protein synthesis in human skeletal muscle by providing a significant anabolic signal to activate mRNA translation initiation.

Inasmuch as AA have been shown to phosphorylate 4E-BP1 and $p70^{S6K}$ via an mTOR-dependent manner in a variety of *in vitro* cell studies and *in vivo* animal studies (15–18, 29), and mTOR is phosphorylated and thereby activated by Akt (1, 6–8), we must consider the possibility that AA activate mRNA translation initiation through the PI3-kinase/Akt signaling pathway. However, we believe that this is highly unlikely, and that AA must act downstream of Akt to signal the mRNA translation initiation. AA have been shown to provide positive signals for the maintenance of protein stores while inhibiting other actions of insulin at multiple levels, including insulin-mediated tyrosine phosphorylation of insulin receptor substate-1 and -2 and activation of PI3-kinase (15, 16, 39). In the current study AA infusion increased 4E-BP1 and $p70^{S6K}$ phosphorylation and protein synthesis, but did not increase Akt phosphorylation and did not affect glucose uptake by muscle. This is consistent with a recent report that leucine activates $p70^{S6K}$ through an Akt-independent mechanism (40).

The time course of AA action on protein synthesis remains unclear. A recent study (41) reported that human muscle protein synthesis responds rapidly (within 30–120 min) to increased availability of AA, but is then inhibited (after 120 min) despite continued AA availability. However, our results clearly indicate that protein synthesis was stimulated after 3 h, and this effect lasted for the entire 6 h of continuous AA infusion. There are several significant differences between these two studies, making direct comparisons difficult. Firstly, their study population was 10 yr older (33 ± 1 *vs.* 23.4 ± 0.8 yr), and their subjects' body weights were 6 kg heavier (80 ± 5 *vs.* 74 ± 4 kg). Younger and leaner subjects tend to engage in more physical activity and may respond better to anabolic stimuli, including AA. Secondly, in the study by Bohe and colleagues (41) the serum insulin levels increased approximately 3-fold within the first 30 min and remained elevated for more than 3 h. This insulinotropic effect was probably due to the large AA prime (54 mg/kg) and higher AA infusion rate (2.7 mg/kg·min) used in that study. We did not use a primed infusion, and the concentrations of blood glucose and insulin remained steady in our study population. We chose the infusion rate of 0.015 ml/kg·min (1.5 mg/kg·min) in the present study to avoid the potential confounding effect of AA-induced insulin secretion on the interpretation of results. Previous work has shown that iv infusion of mixed AA at 0.5, 1, and 2 mg/kg·min does not increase plasma insulin concentrations at 1, 2, and 3 h (42). Thirdly, different radiotracers and analytic techniques were used to assess the rate of protein synthesis in these two studies. In the current study, consistent with protein synthesis data, 4E-BP1 and $p70^{S6K}$ remained hyperphosphorylated after 6 h of AA infusion. Studies performed by others and us suggest that the effects of AA on protein synthesis and the phosphorylation of 4E-BP1 and/or $p70^{S6K}$ remain as long as the provocative stimulus exists. Infusion of mixed AA was able to improve protein balance and increase protein synthesis within 3 h (12), and infusion of leucine alone for 2 h caused a 4-fold increase in the phosphorylation of $p70^{S6K}$ (40) in postabsorptive humans. No *in vivo* human study has examined the acute effect (<2 h) of AA on the phosphorylation of 4E-BP1 and $p70^{S6K}$. In rats, oral administration of leucine stimulated phosphorylation of both proteins within 1 h (27).

We have previously reported that moderate elevation of plasma BCAA concentrations stimulates the phosphorylation of 4E-BP1 and $p70^{S6K}$ and decreases whole body phenylalanine flux and skeletal muscle protein degradation; however, skeletal muscle protein synthesis was not affected (30, 36, 43). These data suggest that BCAA activate mRNA translation initiation, but without the anticipated increase in protein synthesis. One possible explanation for this apparent discrepancy is that BCAA inhibit proteolysis and thereby decrease the arterial concentrations of other AA (36). The availability of AA from plasma has been shown to affect the rate of protein synthesis in muscle (11, 12). In the current study, mixed AA infusion doubled the total BCAA concentrations, whereas the phosphorylation of 4E-BP1 and $p70^{S6K}$ increased dramatically, and muscle protein synthetic rates were significantly stimulated, consistent with our data in laboratory rats reported previously (19). Taken together, we believe that mixed AA infusion not only provides substrates for protein synthesis, but it also directly provides nutrient signals to activate the protein synthetic apparatus.

It is of interest to note that the serum concentrations of BCAA were much lower in the current study than those we observed during the infusion of BCAA alone (36) (372 ± 13.8 *vs.* 662 ± 21, 223 ± 8.9 *vs.* 441 ± 14, and 175 ± 5.6 *vs.* 395 ± 16 μmol/liter for valine, leucine, and isoleucine, respectively). This occurred despite a slightly higher BCAA infusion rate in the current study (2.27 *vs.* 1.66 μmol/min·kg). Thus, in the current study clearance of infused BCAA was more than doubled compared with that seen during the infusion of BCAA alone. This probably arose from increased utilization of BCAA for protein synthesis in the current study. Despite this, we observed a similar increase in the extent of 4E-BP1 and $p70^{S6K}$ phosphorylation in the current study compared with that in our previous study using the 1.66 μmol BCAA/min·kg infusion rate (30). This suggests that

either BCAA can stimulate mRNA translation initiation at much lower concentrations and/or other AA may have also contributed in providing nutrient signals to activate this process in the current study.

In conclusion, mixed AA infusion stimulates protein synthesis, promotes positive protein balance, and enhances the phosphorylation of 4E-BP1 and $p70^{S6K}$ through an Akt-independent mechanism in human skeletal muscle. Our findings suggest that AA not only function as substrates for protein synthesis, but also play a major signaling role in mRNA translation in human skeletal muscle.

Acknowledgments

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