A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly

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Submitted 6 October 2005; accepted in final form 17 February 2006

Ingestion of an amino acid mixture containing extra leucine has the potential to affect muscle protein metabolism in several ways. In addition to providing leucine and other amino acids as precursors for protein synthesis, the extra leucine may stimulate specific intracellular pathways associated with muscle protein synthesis. Specifically, there is evidence implicating a leucine-mediated increase in plasma insulin, resulting in a regulation of the ribosomal protein S6 kinase (S6K1) and the eukaryotic initiation factor (eIF)4E-binding protein-1 (1), which are involved in the initiation of muscle protein synthesis. There is also evidence suggesting that plasma leucine can regulate muscle protein synthesis by insulin-independent mechanisms (2).

The importance of an improved response of skeletal muscle protein synthesis to the ingestion of amino acids is obvious for individuals across the age spectrum, and particularly the elderly, because skeletal muscle mass declines with advancing age (22). We have recently shown that elderly are less responsive than young individuals to the ingestion of a small bolus of EAA (20). Ingestion of extra leucine may be particularly important for the stimulation of skeletal muscle protein synthesis in the elderly, because evidence from animal studies indicates that skeletal muscle protein synthesis becomes less responsive to the stimulatory effects of leucine with aging (8). Additional evidence indicates that meals supplemented with leucine improve the postprandial muscle protein synthesis in old rats (29).

The purpose of this study was to determine the acute effects of two different EAA mixtures on skeletal muscle protein metabolism in elderly and young subjects, a mixture that is based on the distribution of EAAs in whey protein (26% leucine) and a similar mixture that is enriched in leucine (41% leucine). We determined muscle protein synthesis by calculating the incorporation rate of 1-[ring-2H5]phenylalanine in the skeletal mixed muscle protein pool and muscle protein retention by measuring the leg arteriovenous net balance of phenylalanine.

METHODS

Subjects. Elderly subjects were recruited through the Sealy Center on Aging Volunteers Registry of the University of Texas Medical Branch at Galveston. Young subjects were recruited through newspaper advertisements. Subject characteristics are presented in Table 1. Leg volume was determined using an anthropometric method (15, 18).

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http://www.ajpendo.org 0193-1849/06 $8.00 Copyright © 2006 the American Physiological Society

E381

First published February 28, 2006; doi:10.1152/ajpendo.00488.2005.
Leg lean mass and percentage of body fat were determined using dual-energy X-ray absorptiometry (DEXA). Subjects in the same age category were randomly assigned into two groups. Subjects in one group ingested 6.7 g of EAAs containing 1.7 g of leucine (26% Leu; percentage of leucine found in whey protein), whereas subjects in the other group ingested 6.7 g of EAAs containing 2.8 g of leucine (41% Leu). The latter EAA mixture was developed with the purpose of avoiding substantially decreasing the availability of the other EAA while increasing the proportion of leucine (Table 2). Amino acids were dissolved in 250 ml of a noncaloric/noncaffeinated soft drink.

Subjects were determined to be healthy on the basis of medical history, physical examination, resting electrocardiogram (ECG), and routine blood and urine tests. In the case of the elderly, the pretesting history, physical examination, resting electrocardiogram (ECG), and routine blood and urine tests. In the case of the elderly, the pretesting history, physical examination, resting electrocardiogram (ECG), and vascular disease. Elderly subjects were living independently with no limitations in ambulation. All subjects that qualified for the study were instructed to eat their usual diet for the week before the study and refrain from any type of organized physical exercise ≥2 days before the study. Subjects were informed about the purpose, procedures, and risks associated with the study, and written informed consent was obtained. The study protocol was approved by the Institutional Review Board and the General Clinical Research Center (GCRC) of the University of Texas Medical Branch at Galveston.

**Experimental protocol.** Subjects reported to the GCRC late in the afternoon the day before the experimental phase of the study. After a DEXA scan was performed, subjects were served dinner. Later in the evening, subjects were offered a light snack but were not allowed to have any food (except water) after 10:00 PM. In the morning (∼4:30 AM), an 18-gauge polyethylene catheter was inserted into an antecubital vein of each arm. One catheter was used for the infusion of L-[ring-\(^{2}H_{5}\)]phenylalanine (98% enriched), which was dissolved in normal saline the night before the infusion. The other catheter was used for the collection of blood samples that were used for the determination of leg blood flow. At ∼6:30 AM, 3-Fr, 8-cm polyethylene catheters (Cook, Bloomington, IN) were inserted in the femoral artery and vein of one leg under local anesthesia and used for leg arteriovenous blood sampling.

After the femoral catheters were inserted, background blood samples were drawn and were later used for determinations of blood L-[ring-\(^{2}H_{5}\)]phenylalanine enrichment and blood flow. The experimental phase of the study is depicted in Fig. 1. A primed (2.0 μmol/kg) constant (0.05 μmol·kg\(^{-1}\)·min\(^{-1}\)) infusion of L-[ring-\(^{2}H_{5}\)]phenylalanine was started at ∼7:30 AM. Blood and muscle samples were collected at selected time points during the postabsorptive and the post-EAA ingestion periods. The samples taken between −180 and 0 min were used to calculate basal responses, and the samples taken between 0 and 210 min were used to calculate the responses following the EAA ingestion (Fig. 1). The EAAs were ingested at 0 min as a bolus. The bolus also included L-[ring-\(^{2}H_{5}\)]phenylalanine (∼9% of the unlabeled phenylalanine) with the purpose of maintaining the isotopic enrichment of phenylalanine at a steady state during the post-EAA ingestion period.

Indocyanine green (ICG) dye was infused for ∼20 min during the postabsorptive and post-EAA periods at a constant rate (0.5 mg/min) into the femoral artery for the determination of leg blood flow. Blood samples were collected ∼10 min after the start of the ICG simultaneously from the femoral vein and a peripheral vein. Blood samples for the determination of blood phenylalanine enrichment and concentration, as well as blood leucine concentration, were drawn simultaneously from the femoral artery and vein catheters before the EAA ingestion and every 15 min after the EAA ingestion (Fig. 1). Plasma insulin concentrations were determined in the arterial blood at selected time points before and after the EAA ingestion.

Muscle samples were collected from biopsies taken from the lateral portion of vastus lateralis (∼15–20 cm above the knee) using a 5 mm Bergstrom biopsy needle (Depuy, Warsaw, IN). Approximately 50 mg of muscle tissue were obtained during each biopsy. After removing any visible fat and connective tissue, the muscle was rinsed with ice-cold saline to remove any blood, blotted dry, and immediately frozen in liquid nitrogen before being stored at −80°C.

**Analysis of samples.** The weight of each blood sample was determined by transferring the blood from the femoral artery and vein into preweighed tubes containing 15% sulfosalicylic acid and a known amount of internal standards (L-[U-\(^{13}C_{6}\),\(^{15}N\)]phenylalanine, L-[U-\(^{13}C_{6}\)]-leucine). After centrifugation, the supernatant was frozen and processed at a later time, as previously described (33). Phenylalanine and leucine isotopic enrichments were expressed as tracer-to-tracee ratio (UT), and they were determined by gas chromatography-mass spectrometry (GC-MS) using selected ion monitoring for mass-to-charge ratio (m/z) 336, 341, and 346 (phenylalanine) and 302 and 308 (leucine). Appropriate corrections for overlapping spectra and the natural distribution of stable isotopes were performed as previously described (30, 37). The coefficient of variation (CV) for the calculated blood amino acid concentrations was 4%. Leg blood flow was determined by spectrophotometrically measuring the ICG dye absorbance in serum from the femoral and peripheral veins at 805 nm (16, 17). These calculations provide leg plasma flow, which was then converted to leg blood flow by use of the hematocrit. The average CV for the determination of the leg blood flow was 5%. Plasma insulin was determined using an ELISA procedure (ALPCO Diagnostics, Windham, NH), with a CV of 1.8%.

About 20–25 mg of the muscle biopsy sample were weighed, and muscle protein was precipitated with 0.8 ml of 10% perchloroacetic acid. An internal standard solution (L-[U-\(^{13}C_{6}\),\(^{15}N\)]phenylalanine) was added for the determination of the muscle free phenylalanine concentration by the tracer dilution method, as previously described (33). The pellet resulting from centrifugation was dried, and the proteins were hydrolyzed in glass tubes by adding 6 N HCl and 4.7 mol/kg constant (0.05 mol/kg) alanine (Table 2).

**Table 2. Composition of the essential amino acid mixtures (in g)**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>26% Leu</th>
<th>41% Leu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>0.304</td>
<td>0.239</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.781</td>
<td>0.614</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.721</td>
<td>2.790</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.360</td>
<td>1.069</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.362</td>
<td>0.284</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.506</td>
<td>0.398</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.955</td>
<td>0.751</td>
</tr>
<tr>
<td>Valine</td>
<td>0.738</td>
<td>0.580</td>
</tr>
<tr>
<td>Total</td>
<td>6.726</td>
<td>6.726</td>
</tr>
</tbody>
</table>

The 26% Leu mixture was based on the amounts of essential amino acids in 15 g of whey protein.
Tracer Infusion

L-[ring-2H5] phenylalanine

Essential Amino Acids (EAA)

Time (min)
-240 -180 -120 -60 0 60 120 180 210

Arteriovenous blood samples

Blood flow measurement

Muscle samples

placing the tubes in a heating block (110°C) for 24 h. The hydrolysate was passed over a cation exchange column (AG 50W-8X 200–400 mesh H+ form cation resin; Bio-Rad Laboratories, Hercules, CA) and then processed the same way as the blood. Muscle protein-bound phenylalanine enrichment was determined on GC-MS using selected ion monitoring (m/z 234, 237, and 239) and the standard curve method (7, 26).

Calculations. The concentrations of phenylalanine and leucine in the blood were determined on the basis of the weight of the blood sample, the amount of internal standard added, and the t/T for L-[U-13C6]-phenylalanine and L-[U-13C6]leucine, respectively (4). The muscle free phenylalanine was determined similarly, and the intracellular concentration was calculated using the chloride method (3). The net balance (NB) of phenylalanine (NBphe) across the leg at each time point was calculated as follows:

\[ NB = (C_a - C_v) \times BF \]

where \( C_a \) and \( C_v \) are the phenylalanine concentrations in the femoral artery and vein, respectively, and BF is the leg blood flow. The NBphe was determined for the basal period and the post-EAA period by calculating the average NBphe during the respective period (19). At any given period where the intracellular free phenylalanine concentration remains constant, the rates of phenylalanine disappearance from the artery (Rd) and appearance to the vein (Ra) reflect the rates of incorporation of blood phenylalanine into muscle proteins (Ra) and release from muscle proteins breakdown (Rd), because phenylalanine is not metabolized in muscle (35). These parameters were calculated by using the following equations:

\[ R_a = \left( (E_a \times C_a) - (E_v \times C_v) \right) \times BF/E_a \]

\[ R_d = R_a - NB \]

where \( E_a \) and \( E_v \) are the blood phenylalanine enrichments, expressed as \( t/T \) in the femoral artery and vein, respectively. \( R_d \) and \( R_a \) values during the basal and the post-EAA periods were averaged to calculate mean \( R_d \) and \( R_a \) responses during the respective period. The fractional synthetic rate (FSR, %/h) of mixed-muscle protein was calculated as follows (31):

\[ FSR = \frac{\Delta E_p}{E_a \times T} \times 60 \times 100 \]

where \( \Delta E_p \) defines the increment in the muscle protein-bound phenylalanine \( t/T \) between two biopsies, \( E_a \) is the average arterial phenylalanine \( t/T \) during the isotopic steady state between the two biopsies, and \( T \) is the time interval (min) between the biopsies. The factors 60 and 100 are used to express the FSR values in percentage per hour.

Statistical analyses. One-way analysis of variance (ANOVA) was used to compare subject characteristics across groups. Two-way (group \( \times \) time) repeated-measures ANOVA was used to compare differences between and within groups. When appropriate, statistically significant \( F \) values were followed by Tukey’s tests. All data are expressed as means \( \pm \) SE, and a \( P \) value \( \leq 0.05 \) was considered statistically significant.

RESULTS

Blood flow. No differences were found in the blood flow measurements performed before and after the EAA ingestion for either group (\( P > 0.05 \)). For each subject, an average from the two blood flow values measured before and after the EAA ingestion was used to calculate study parameters to reduce variability. The mean leg blood flow in the elderly was 3.5 ± 0.8 ml·min⁻¹·100 ml leg volume⁻¹ (26% Leu) and 3.4 ± 0.4 ml·min⁻¹·100 ml leg volume⁻¹ (41% Leu), whereas in the young it was 3.4 ± 0.3 ml·min⁻¹·100 ml leg volume⁻¹ (26% Leu) and 2.7 ± 0.3 ml·min⁻¹·100 ml leg volume⁻¹ (41% Leu). There were no differences in the mean leg blood flow values between groups (\( P > 0.05 \)).

Arterial blood leucine and phenylalanine concentrations and phenylalanine enrichment. Mean arterial blood leucine response to the EAA ingestion in the four groups is presented in Fig. 2. There was a significant time effect for the leucine response (\( P < 0.05 \)), as well as a group effect, with the 41% Leu groups having a higher leucine concentration than the 26% Leu groups (\( P < 0.05 \)). Within groups, arterial blood leucine concentration increased immediately (15 min) and remained significantly (\( P < 0.05 \)) elevated compared with basal for 105 min in the 26% Leu elderly group, 120 min in the 41% Leu elderly group, 90 min in the 26% Leu young group, and 150
blood phenylalanine concentration returned to basal by 75 min. With the exception of the 41% Leu young group, where arterial concentrations were: 26% Leu elderly, 79 \pm 6 \text{ nmol/ml}; 41% Leu elderly, 75 \pm 6 \text{ nmol/ml}; 26% Leu young, 68 \pm 7 \text{ nmol/ml}; 41% Leu young, 76 \pm 10 \text{ nmol/ml}. There was no significant group effect, but there was a significant time effect (P \leq 0.05) after the amino acids ingestion. As expected, muscle free phenylalanine concentrations had returned to values that were not different (P > 0.05) from basal (26% Leu elderly, 88 \pm 5 \text{ nmol/ml}; 41% Leu elderly, 69 \pm 6 \text{ nmol/ml}; 26% Leu young, 63 \pm 8 \text{ nmol/ml}; 41% Leu young, 69 \pm 11 \text{ nmol/ml}).

**Muscle free phenylalanine concentration.** Muscle free phenylalanine concentrations were determined at the muscle biopsy sampling time points. Basal muscle free phenylalanine concentrations were: 26% Leu elderly, 79 \pm 6 \text{ nmol/ml}; 41% Leu elderly, 75 \pm 6 \text{ nmol/ml}; 26% Leu young, 68 \pm 7 \text{ nmol/ml}; 41% Leu young, 76 \pm 10 \text{ nmol/ml}. There was no significant group effect, but there was a significant time effect (P \leq 0.05) after the amino acids ingestion. As expected, muscle free phenylalanine concentration showed an increase at 60 min after the EAA ingestion compared with basal in all groups (values not shown). However, by the end of the study, muscle free phenylalanine concentrations had returned to values that were not different (P > 0.05) from basal (26% Leu elderly, 88 \pm 5 \text{ nmol/ml}; 41% Leu elderly, 69 \pm 6 \text{ nmol/ml}; 26% Leu young, 63 \pm 8 \text{ nmol/ml}; 41% Leu young, 69 \pm 11 \text{ nmol/ml}).

**Plasma insulin.** Arterial plasma insulin concentrations in response to the ingestion of EAAs containing either 26 or 41% leucine are shown for all groups in Fig. 4. There were no significant group effects, but there was a significant time effect (P < 0.05). Mean plasma insulin concentrations increased significantly (P < 0.05) in all groups in response to the EAAs and remained significantly different from basal until 30 min in the young groups and until 45 min in the elderly groups. Mean arterial plasma insulin values appeared to peak at a later time in the elderly groups than those in the young groups (i.e., 30 vs. 15 min).

**Muscle protein FSR.** Figure 5 depicts the muscle protein FSR for each group calculated for the basal and post-EAA periods. There were no differences between groups at the basal period (P > 0.05). After the EAA ingestion there was no significant increase in muscle protein FSR in the 26% Leu elderly group (P > 0.05). However, there was a significant increase in the 41% Leu elderly group (P < 0.05). Muscle protein FSR increased in both the 26 and the 41% Leu young groups in response to the EAA ingestion (P < 0.05).

**Leg phenylalanine net balance.** Figure 6 depicts the leg phenylalanine net balance response in all four groups during the basal period and the period after the EAA ingestion. There was no significant group effect (P > 0.05), but there was a significant time effect. Within each group, the leg phenylalanine net balance increased immediately after the EAA inges-
tion and remained significantly different from basal until 30 min in the 26% Leu elderly and 41% Leu young groups and until 45 min in the 26% Leu young and 41% Leu elderly groups (P < 0.05).

Figure 7 shows average values of the response of the leg phenylalanine net balance at the basal period and the period after the EAA ingestion for all four groups. There were no differences between groups in the basal period (P > 0.05). After the EAA ingestion there was a significant improvement in the mean leg phenylalanine net balance in all groups (P < 0.05), with the exception of the 26% Leu elderly group (P > 0.05). There were no significant differences between groups for the post-EAA period (P > 0.05).

DISCUSSION

We investigated muscle protein metabolism after the ingestion of two different mixtures composed of ~7 grams of EAA: one mixture mimicked the distribution of the EAA in whey protein, whereas the other had higher leucine content. The results suggest that the EAA leucine has a unique role in the stimulation of muscle protein synthesis by EAAs in elderly humans. Specifically, in the elderly, the leucine-enriched EAA mixture stimulated postprandial muscle protein synthesis and resulted in postprandial accretion of muscle proteins, reversing the lack of response following the whey protein-based EAA mixture. In contrast, in the young, both EAA mixtures stimulated muscle protein synthesis, and no unique advantage of extra leucine was evident.

It is now well established that among all the plasma amino acids, the EAAs are the most important for the stimulation of muscle protein synthesis (12, 36). Previous studies have shown that EAAs stimulate muscle protein synthesis in both elderly (25, 34) and young (25, 32) individuals. We have previously found that stimulation of muscle protein synthesis is not different between young and elderly when large amounts of EAAs are ingested (25), but the elderly have reduced muscle protein synthesis when small amounts of EAAs are ingested (20). The latter is evident in the present study, which further underscores the importance of the leucine content in the formulation of any amino acid supplement for the stimulation of muscle protein synthesis in the elderly. Leucine content becomes particularly important when decreasing the overall amount of EAAs in an amino acid supplement, since this decreases the availability of leucine.

It has long been known that EAAs stimulate insulin secretion (23), and among them, leucine appears to be one of the most potent stimuli (21). Following the EAA ingestion there was a transient, but significant, increase in the circulating insulin in all four groups (Fig. 4). An increase in plasma insulin concentration increases net muscle protein balance during hyperaminoacidemia (10, 13), and therefore, it would be expected to have played a role in the improved leg protein retention in the present study. However, the overall response was not different between the 26% Leu and 41% Leu groups, and therefore, the greater muscle protein retention in the elderly following the leucine-enriched EAA mixture cannot be attributed to differences in plasma insulin concentration. Therefore, the insulin data in the present study do not support an insulin-mediated but rather a direct effect of blood leucine on stimulating muscle protein synthesis in the elderly.

Because improvement in muscle protein balance following EAA ingestion (Fig. 7) was observed, together with the increase in muscle protein FSR (Fig. 5), the improved muscle protein balance can be attributed to the stimulation of muscle protein synthesis by the EAA mixtures. The lack of a response in muscle protein synthesis in the 26% Leu elderly group may
Support for this argument comes from one of our previously published reports (25), where the same amount of leucine as in the 41% Leu mixture in the present study approximately doubled the net muscle protein synthesis in the young when ingested as a component of 15 g of EAAs.

In addition to stimulation of muscle protein synthesis, inhibition of muscle protein breakdown may have contributed to the observed responses. Muscle protein breakdown can be estimated from the calculated value for $R_d$, although it is recognized that the accuracy of both the $R_d$ and $R_a$ values may be limited by the perturbation of the steady state in the concentration of muscle free phenylalanine after the amino acid ingestion. Nonetheless, an average value during the entire post-EAA period can provide an integrated response because the concentration of muscle free phenylalanine was not different from basal at the end of the study. On the basis of the calculated phenylalanine $R_a$, inhibition of muscle protein degradation by leucine may be more effective at higher blood leucine concentrations in the young, since among all groups the 41% Leu young group showed the largest decrease in phenylalanine $R_a$. Although not significant ($P = 0.09$), this decrease in phenylalanine $R_a$ is in line with previous data when blood leucine increased at similar levels (24).

Animal studies indicate that meals supplemented with leucine can, both acutely (9) and over a period of at least 10 days (29), beneficially affect muscle protein anabolism. The present study provides for the first time in vivo evidence in elderly humans that a relatively small bolus of ingested leucine (∼3 g) can acutely improve muscle protein retention and reverse a lack of stimulation of muscle protein synthesis following the ingestion of a small amount of EAAs. Whether these effects of leucine on muscle protein anabolism can be sustained over longer periods of time in conjunction with leucine-supplemented meals remains to be shown. It is important to note that the increase in plasma leucine concentration in the 26% Leu mixture in the present study was similar to that expected following consumption of a meal of average protein content (∼15 g of protein) and that the anabolic effect of extra leucine on muscle protein in the present study was observed in the elderly at less than double peak blood leucine concentration relative to the whey protein-based mixture. The present findings do not argue against greater stimulation of muscle protein synthesis by larger increases in peak blood leucine concentration in either elderly or young individuals. Any such hypothesis, however, should be evaluated in the context of blood availability of other amino acids, because limited supply of such amino acids may compromise the potential for muscle protein anabolism under conditions of stimulated muscle protein synthesis (12).

In conclusion, this study demonstrates for the first time in elderly humans that attenuated response of muscle protein synthesis following ingestion of small amounts of amino acids can be reversed by ingestion of extra leucine. The present data emphasize the important role of leucine in the formulation of any amino acid/protein supplement for reversing attenuated response of muscle protein synthesis to nutritional supplementation in the elderly.

**ACKNOWLEDGMENTS**

We thank the nurses and the staff at the General Clinical Research Center (GCRC) at University of Texas Medical Branch in Galveston, TX, as well as Dan Creson, Susan Minello, and Roxana Hirst. We gratefully acknowledge...
Stephaine J. Blase´, Melissa Bailey, Christopher Danesi, Gaurang K. Jariwala, and Ming-Qian Zheng for skillful technical assistance.

GRANTS
The work was sponsored by National Institute of Arthritis and Musculo-skeletal and Skin Diseases Grant AR-49038 and Shriners Hospital Grant 8490. Studies were conducted at the GCRC at the University of Texas Medical Branch at Galveston, funded by Grant M01-RR-00073 from the National Center for Research Resources, National Institutes of Health, United States Public Health Service. Support was also provided by AminoScience Laboratories, Ajinomoto.

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