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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ABSTRACT

This study was designed to evaluate the effects of enriching an essential amino acid (EAA) mixture with leucine on muscle protein metabolism in elderly and young individuals. Four (two elderly and two young) groups were studied before and following ingestion of 6.7 g of EAA. EAA were based on the composition of whey protein (26% Leu) or were enriched in leucine (41% Leu). A primed, continuous infusion of L-[ring-$^2$H$_5$]phenylalanine was used together with vastus lateralis muscle biopsies and leg arteriovenous blood samples for the determinations of fractional synthetic rate (FSR) and balance of muscle protein. FSR increased following amino acid ingestion in both the 26% (basal: 0.048 ± 0.005%·h$^{-1}$; post-EAA: 0.063 ± 0.007%·h$^{-1}$) and the 41% (basal: 0.036 ± 0.004%·h$^{-1}$; post-EAA: 0.051 ± 0.007%·h$^{-1}$) Leu young groups (P < 0.05). In contrast, in elderly, FSR did not increase following ingestion of 26% Leu EAA (basal: 0.044 ± 0.003%·h$^{-1}$; post-EAA: 0.049 ± 0.006%·h$^{-1}$; P > 0.05), but increased following ingestion of 41% Leu EAA (basal: 0.038 ± 0.007%·h$^{-1}$; post-EAA: 0.056 ± 0.008%·h$^{-1}$; P < 0.05). Similar to the FSR responses, the mean response of muscle phenylalanine net balance, a reflection of muscle protein balance, was improved (P < 0.05) in all groups with the exception of the 26% Leu elderly group. We conclude that increasing the proportion of leucine in a mixture of EAA can reverse an attenuated response of muscle protein synthesis in elderly but does not result in further stimulation of muscle protein synthesis in young subjects.

Keywords: nutrition, sarcopenia, muscle protein, stable isotopes
INTRODUCTION

Muscle protein metabolism alternates between periods of net catabolism in the postabsorptive state and net anabolism in the postprandial states, with the latter being primarily a result of changes in muscle protein synthesis (28). Ingestion of a protein-deficient meal does not stimulate muscle protein synthesis (38), since the availability of blood amino acids is not increased. Amino acids are known to be the key nutrient for the stimulation of muscle protein synthesis (27, 36). Among the blood amino acids, the essential amino acids (EAA) are primarily responsible for the regulation of muscle protein synthesis (34), and among the essential amino acids, leucine is recognized to have a particular role in the regulation of muscle protein synthesis (11).

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 (4E-BP1) (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, since 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 EAA 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 L-[ring-$^2$H$_5$]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). Leg lean mass and percent 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 EAA containing 1.7 g of leucine (26% Leu – percent of leucine found in whey protein), while subjects in the other group ingested 6.7 g of EAA containing 2.8 g of leucine (41% Leu). The latter EAA mixture was developed with the purpose of avoiding to substantially decrease the availability of the other EAA, while increasing the proportion of leucine (Table 2). Amino acids were dissolved in 250 ml of noncaloric/noncaffeinated soft drink. Subjects were determined to be healthy based on medical history, physical examination, resting electrocardiogram (ECG), and routine blood and urine tests. In the case of the elderly, the pretesting procedures also included estimation of leg vascular condition using the ankle/brachial index. Subjects were excluded from the study based on the presence of unstable metabolic medical condition, hypertension, ECG-documented heart abnormalities, 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 prior to 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 a 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. Latter 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$^{-1}$) 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 were used to calculate basal responses, and the samples taken between 0 and 210 were used to calculate the responses following the EAA ingestion (Fig. 1). The EAA were ingested at 0 mins as a bolus. The bolus also included L-[ring-$^2$H$_5$]phenylalanine (~ 9% of the unlabeled phenylalanine) with the purpose to maintain the isotopic enrichment of phenylalanine at a steady state during the post-EAA ingestion period.

Indocyanine green (ICG) dye was infused for ~ 20 minutes during each the postabsorptive and post-EAA periods at a constant rate (0.5 mg·min$^{-1}$) into the femoral
artery for the determination of leg blood flow. Blood samples were collected at least 10 minutes 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 minutes following 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 was 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 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 pre-weighed tubes containing 15% sulfosalicylic acid (SSA) and a known amount of internal standards (L-[U-13C0-15N]phenylalanine; L-[U-13C6]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 (t/T) and they were determined by gas chromatography-mass spectroscopy (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 based on this procedure 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 was weighed, and muscle protein was precipitated with 0.8 ml of 10% perchloroacetic acid. An internal standard solution (L-[U-13C9-15N]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 placing the tubes in a heating block (110°C) for 24 hours. 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 based on the weight of the blood sample, the amount of internal standard added, and the t/T for L-[U-13C9-15N]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 that 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 (Rd) and release from muscle proteins breakdown (Ra), since phenylalanine is not metabolized in muscle (35). These parameters were calculated by using the following equations:

\[ Rd = ((E_a x C_a) - (E_v x C_v)) \times BF / E_a \]

\[ Ra = Rd - NB \]

where \( E_a \) and \( E_v \) are the blood phenylalanine enrichments, expressed as t/T in the femoral artery and vein, respectively. Rd and Ra values during the basal and the post-EAA periods were averaged to calculate mean Rd and Ra responses during the respective period. The fractional synthetic rate (FSR; %/h) of mixed-muscle protein was calculated as follows (31):
\[
\text{FSR} = \frac{\Delta E_p}{E_b \times T} \times 60 \times 100
\]

where \( \Delta E_p \) defines the increment in the muscle protein-bound phenylalanine \( t/T \) between two biopsies, \( E_b \) 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 percent per hour.

**Statistical Analyses**

One-way analysis of variance (ANOVA) was used to compare subject characteristics across groups. Two-way (group x 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 ± 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 following the EAA ingestion for either group (\( P > 0.05 \)). For each subject, an average from the two blood flow values measured before and following the EAA ingestion was used to calculate study parameters in order to reduce variability. The mean leg blood flow in the elderly was \( 3.5 \pm 0.8 \text{ ml min}^{-1} \cdot 100 \text{ ml leg volume}^{-1} \) (26% Leu) and \( 3.4 \pm 0.4 \text{ ml min}^{-1} \cdot 100 \text{ ml leg volume}^{-1} \) (41% Leu), while in the young was \( 3.4 \pm 0.3 \text{ ml min}^{-1} \cdot 100 \text{ ml leg volume}^{-1} \).
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 compared to 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 to basal for 105 mins in the 26% Leu elderly group, 120 mins in the 41% Leu elderly group, 90 mins in the 26% Leu young group and 150 mins in the 41% Leu young group. With respect to the arterial blood phenylalanine concentration response, there was a significant time effect ($P < 0.05$), but there was no group effect. Arterial blood phenylalanine concentration increased significantly ($P < 0.05$) by 15 min and returned to a value that was not different ($P > 0.05$) than basal by 90 mins in all groups with the exception of the 41% Leu young group, where arterial blood phenylalanine concentration returned to basal by 75 mins.

Mean arterial $L$-[ring-$^2$H$_3$]phenylalanine enrichment of phenylalanine (tracer/tracee ratio) in the 26% Leu elderly group was 0.090 ± 0.004 for the period prior to and 0.095 ± 0.003 for the period following the EAA ingestion, while in the 41% Leu elderly group was 0.081 ± 0.005 prior to and 0.092 ± 0.005 following the EAA ingestion. In the young, the corresponding values for the 26% Leu group were 0.074 ± 0.004 for the
period prior to and $0.083 \pm 0.004$ for the period following the EAA ingestion, while for
the 41% Leu group were $0.089 \pm 0.007$ prior to and $0.091 \pm 0.007$ following the EAA
ingestion. The arterial blood phenylalanine enrichment during the course of the study in
the four groups is shown in Fig. 3.

**Blood Phenylalanine Rates of Disappearance (Rd) and Appearance (Ra)**

Blood phenylalanine Rd increased significantly ($P < 0.05$) following the EAA
ingestion in the 41% Leu elderly (basal: $28 \pm 3 \text{ nmol min}^{-1} \cdot 100 \text{ mL leg volume}^{-1}$; post-
EAA: $34 \pm 2 \text{ nmol min}^{-1} \cdot 100 \text{ mL leg volume}^{-1}$) and 26% Leu young (basal: $30 \pm 3$
nmol min$^{-1} \cdot 100 \text{ mL leg volume}^{-1}$; post-EAA: $36 \pm 3 \text{ nmol min}^{-1} \cdot 100 \text{ mL leg volume}^{-1}$)
groups, but not ($P > 0.05$) in the 26% Leu elderly (basal: $29 \pm 5 \text{ nmol min}^{-1} \cdot 100 \text{ mL leg
volume}^{-1}$; post-EAA: $30 \pm 3 \text{ nmol min}^{-1} \cdot 100 \text{ mL leg volume}^{-1}$) or the 41% Leu young
(basal: $27 \pm 6 \text{ nmol min}^{-1} \cdot 100 \text{ mL leg volume}^{-1}$; post-EAA: $29 \pm 6 \text{ nmol min}^{-1} \cdot 100 \text{ mL leg
volume}^{-1}$) groups. Blood phenylalanine Ra did not change significantly following the
EAA ingestion when compared to basal in any of the groups: 26% Leu elderly, basal: $43$
$\pm 6 \text{ nmol min}^{-1} \cdot 100 \text{ mL leg volume}^{-1}$; post-EAA: $42 \pm 6 \text{ nmol min}^{-1} \cdot 100 \text{ mL leg volume}^{-1}$,
41% Leu elderly, basal: $43 \pm 3 \text{ nmol min}^{-1} \cdot 100 \text{ mL leg volume}^{-1}$; post-EAA: $41 \pm 3$
nmol min$^{-1} \cdot 100 \text{ mL leg volume}^{-1}$; 26% Leu young, basal: $44 \pm 3 \text{ nmol min}^{-1} \cdot 100 \text{ mL leg
volume}^{-1}$; post-EAA: $43 \pm 4 \text{ nmol min}^{-1} \cdot 100 \text{ mL leg volume}^{-1}$; 41% Leu young, basal: $39$
$\pm 7 \text{ nmol min}^{-1} \cdot 100 \text{ mL leg volume}^{-1}$; post-EAA: $34 \pm 6 \text{ nmol min}^{-1} \cdot 100 \text{ mL leg volume}^{-1}$.

**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 ± 6 nmol·ml⁻¹; 41% Leu elderly: 75 ± 6 nmol·ml⁻¹; 26% Leu young: 68 ± 7 nmol·ml⁻¹; 41% Leu young: 76 ± 10 nmol·ml⁻¹. There was no significant group effect, but there was a significant time effect (P < 0.05) following the amino acids ingestion. As expected, muscle free phenylalanine concentration showed an increase at 60 mins after the EAA ingestion compared to 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 ± 5 nmol·ml⁻¹; 41% Leu elderly 69 ± 6 nmol·ml⁻¹; 26% Leu young 63 ± 8 nmol·ml⁻¹; 41% Leu young: 69 ± 11 nmol·ml⁻¹).

Plasma Insulin

Arterial plasma insulin concentrations in response to the ingestion of EAA 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 EAA, and remained significantly different from basal until 30 mins in the young groups and until 45 mins 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 mins vs 15 mins).

Muscle Protein Fractional Synthetic Rate (FSR)
Fig. 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 ($P > 0.05$). Following 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*

Fig. 6 depicts the leg phenylalanine net balance response in all four groups during the basal period and the period following 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 ingestion and remained significantly different from basal until 30 mins in the 26% Leu elderly and 41% Leu young groups, and until 45 mins in the 26% Leu young and 41% Leu elderly groups ($P < 0.05$).

Fig. 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 ($P > 0.05$). Following 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 following the ingestion of two different mixtures composed of ~7 grams of EAA: one mixture mimicked the distribution of the EAA in whey protein, while the other had higher leucine enrichment. The results suggest that the essential amino acid leucine has a unique role in the stimulation of muscle protein synthesis by EAA 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 establish that among all the plasma amino acids, the EAA are the most important for the stimulation of muscle protein synthesis (12, 36). Previous studies have shown that EAA 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 EAA are ingested (25), but the elderly have reduced muscle protein synthesis when small amounts of EAA 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 EAA in an amino acid supplement, since this decreases the availability of leucine.
It is long known that EAA 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 reflect blunted responsiveness to one or more of the ingested amino acids. However, the improved protein synthesis following the leucine-enriched EAA in the same age group can only be attributed to the increased leucine content in the EAA mixture, since the content of the rest of the EAA in the mixture was decreased. Although it is long known that the branched-chain amino acids (12) and more specifically leucine is unique among the amino acids in the stimulation of muscle protein synthesis (6, 14), only recently have the mechanisms of the regulation of muscle protein synthesis by leucine started to be understood. Based on this evidence, it can be speculated that in the present
study activation of S6K1 might have been implicated in the stimulation of muscle protein synthesis by higher plasma leucine concentration. Relative to that, it has been shown that S6K1 is regulated \textit{in vitro} by leucine and it requires higher levels of leucine concentration to be activated in aged rats (8). However, there is also evidence suggesting that high leucine concentrations increase protein synthesis without altering the activity of S6K1, but this response involves other mechanisms such as an enhanced binding of eIF4E to eIF4G (5).

Initiation of muscle protein synthesis by leucine may have not been optimally activated following ingestion of the 26\% Leu mixture in the elderly, since no significant change in muscle protein FSR was observed. The findings of the present study suggest that ingestion of extra leucine in an amount approximate to that provided in the 41\% Leu group may be required to activate the initiation of protein synthesis in the elderly. On the other hand, the young individuals showed improved muscle protein synthesis following the EAA ingestion regardless of the magnitude of changes in blood leucine concentration. It is possible that mechanisms associated with the activation of muscle protein synthesis by leucine in the young are either sufficiently active at basal leucine concentration or are highly sensitive to even small changes in blood leucine concentration. The findings of the present study indicating decreased sensitivity of muscle protein synthesis to leucine in the elderly are consistent with a recently reported study in rats (8). Extra leucine may have failed to further enhance the rate of muscle protein synthesis in the young because of corresponding decrease in the overall availability of the non-leucine EAA component of the 41\% Leu mixture. Support for this argument comes from one of our previously published reports, in which the same amount of leucine as in the 41\% Leu mixture in the
current study approximately doubled the net muscle protein synthesis in young when ingested as a component of 15 grams of EAA (25).

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 Ra, although recognizing that the accuracy of both the Rd and Ra values may be limited by the perturbation in the non-steady state in the concentration of muscle free phenylalanine after the amino acids 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. Based on the calculated phenylalanine Ra, inhibition of muscle protein degradation by leucine may be more effective at higher blood leucine concentrations in young, since among all groups the 41% Leu young group showed the larger decrease in phenylalanine Ra. Although not significant (P = 0.09) this decrease in phenylalanine Ra 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) as well as 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 grams) can acutely improve muscle protein retention and reverse a lack of stimulation of muscle protein synthesis following the ingestion of a small amount of EAA. 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 grams 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, since 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

The authors thank the nurses and the staff at the GCRC at UTMB in Galveston, TX, as well as Dan Creson, RN, Susan Minello, RN, MSN, ANP and Roxana Hirst, MS. We gratefully acknowledge Stephaine J. Blasé, Melissa Bailey, Christopher Danesi, Gaurang K. Jariwala, and Ming-Qian Zheng for skillful technical assistance.

GRANTS

The work was sponsored by National Institutes of Health Grant AR 49038 and Shriners Hospital Grant 8490. Studies were conducted on the General Clinical Research Center (GCRC) at the University of Texas Medical Branch at Galveston, funded by grant M01 RR 00073 from the National Center for Research Resources, NIH, USPHS. Support was also provided by AminoScience Laboratories, Ajinomoto Co., Inc.
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FIGURE LEGENDS

Figure 1. Experimental design of the study. Subjects ingested 6.7 g of essential amino acids (EAA) containing either 1.7 g (26% Leu) or 2.8 g (41% Leu) of leucine at 0 min. Mixed muscle protein fractional synthetic rate was determined from muscle biopsy samples collected before and after the EAA ingestion. Muscle phenylalanine net balance was determined from leg arteriovenous blood samples collected during the study. The experimental phase of the study started at ~ 07:30 am after an overnight fast.

Figure 2. Blood leucine concentration (nmol·ml⁻¹) in the basal state and following the ingestion of 6.7 g of essential amino acids containing either 1.7 g (26% Leu) or 2.8 g (41% Leu) of leucine at 0 min. Group effect for both 41% Leu groups compared to the 26% Leu groups (P < 0.05).

Figure 3. Mean arterial blood L-[ring-²H₅]phenylalanine enrichment of phenylalanine (tracer/tracee ratio) in the basal state and following the ingestion of 6.7 g of essential amino acids containing either 1.7 g (26% Leu) or 2.8 g (41% Leu) of leucine at 0 min.

Figure 4. Plasma insulin concentration (uIU·ml⁻¹) in the basal state and following the ingestion of 6.7 g of essential amino acids containing either 1.7 g (26% Leu) or 2.8 g (41% Leu) of leucine at 0 min.
**Figure 5.** Fractional synthetic rate (\%·hour^{-1}) of mixed muscle protein in the basal state (Basal) and following the ingestion of 6.7 g of essential amino acids (Post-EAA) containing either 1.7 g (26% Leu) or 2.8 g (41% Leu) of leucine. *Significantly different from the corresponding basal value (P < 0.05).

**Figure 6.** Leg phenylalanine net balance (nmol·min^{-1}·100 ml leg volume^{-1}) in the basal state and following the ingestion of 6.7 g of essential amino acids containing either 1.7 g (26% Leu) or 2.8 g (41% Leu) of leucine at 0 min.

**Figure 7.** Average responses for the leg phenylalanine net balance (nmol·min^{-1}·100 ml leg volume^{-1}) for the basal state (Basal) and following the ingestion of 6.7 g of essential amino acids (Post-EAA) containing either 1.7 g (26% Leu) or 2.8 g (41% Leu) of leucine. *Significantly different from the corresponding basal value (P < 0.05).
Table 1. Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Elderly 26% Leu (3F,7M)</th>
<th>Elderly 41% Leu (5F,5M)</th>
<th>Young 26% Leu (4F,4M)</th>
<th>Young 41% Leu (4F,4M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>66.7 ± 2.0</td>
<td>66.5 ± 2.2</td>
<td>30.6 ± 2.0*</td>
<td>28.8 ± 2.6*</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>81.7 ± 3.6</td>
<td>74.5 ± 4.7</td>
<td>70.1 ± 4.7</td>
<td>76.6 ± 7.7</td>
</tr>
<tr>
<td>Height, cm</td>
<td>171.5 ± 2.8</td>
<td>165.2 ± 3.1</td>
<td>170.2 ± 2.3</td>
<td>170.2 ± 3.7</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>31.2 ± 2.8</td>
<td>31.7 ± 2.2</td>
<td>25.2 ± 3.3</td>
<td>29.8 ± 2.0</td>
</tr>
<tr>
<td>Leg volume, L</td>
<td>10.3 ± 0.5</td>
<td>9.2 ± 0.7</td>
<td>10.0 ± 0.7</td>
<td>11.7 ± 1.0</td>
</tr>
<tr>
<td>LLM, kg</td>
<td>8.2 ± 0.5</td>
<td>7.8 ± 0.8</td>
<td>8.2 ± 0.6</td>
<td>8.5 ± 1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; F, females; M, males; LLM, leg lean mass;

26% (26% Leu) and 41% (41% Leu) leucine in 6.7 g of essential amino acids; *Significantly different from the elderly groups ($P < 0.05$).
Table 2. Composition of the essential amino acids mixtures (in grams).

<table>
<thead>
<tr>
<th></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>

26% Leu: 26% leucine; 41% Leu: 41% leucine; the 26% Leu mixture was based on the amounts of essential amino acids in 15 grams of whey protein.
Tracer Infusion

Essential Amino Acids (EAA)

Time (min)

Arteriovenous blood samples

Blood flow measurement

Muscle samples

L-[ring-$^3$H$_2$] phenylalanine

EAA
32% Leu Elderly
41% Leu Elderly
26% Leu Young
41% Leu Young
34% Leu Elderly
41% Leu Elderly
26% Leu Young
41% Leu Young

Time (min)

0 30 60 90 120 150 180 210

uIU/ml

-60 -30 0 30 60 90 120 150 180 210

-26% Leu Elderly
-41% Leu Elderly
26% Leu Young
41% Leu Young
0.000 0.025 0.050 0.075 0.100% / hour

Basal Post-EAA

26% Leu Elderly 41% Leu Elderly 26% Leu Young 41% Leu Young

* * *
nmol/min/100 ml Leg Volume

-20 -15 -10 -5 0 5 10

26% Leu Elderly
41% Leu Elderly
26% Leu Young
41% Leu Young

Basal  Post-EAA

*